Degradation of APC^{cdc20} and APC^{cdh1} substrates during the second meiotic division in mouse eggs

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Accepted 29 September 2004
Journal of Cell Science 117, 6289-6296 Published by The Company of Biologists 2004
doi:10.1242/jcs.01567

Summary

Metaphase II-arrested mouse eggs are stimulated to complete meiosis by sperm-induced Ca^{2+} spiking. The Ca^{2+} signal causes activation of the E3 ligase anaphase-promoting complex/cyclosome (APC), leading to the destruction of key proteins necessary for meiotic exit. We show, using western blots of mouse eggs, the presence of both APC activators cdc20 and cdh1, which target D-box and D-box/KEN-box substrates, respectively, for proteolysis. We decided to examine the temporal activation of APC^{cdc20} and APC^{cdh1} by coupling APC substrates to GFP and examining their destruction in real-time following release from second meiotic division arrest. D-box substrates were degraded quickly after the initiation of sperm-induced Ca^{2+} spiking, such that their degradation was complete by the time of second polar body extrusion. By contrast, KEN-box-containing substrates were degraded when CDK1 activity was low, during the period between polar body extrusion and pronucleus formation. This observation of apparent APC^{cdh1} activity in meiosis II based on destruction of exogenous GFP-coupled substrates was then confirmed by observing destruction of endogenous APC^{cdh1} substrates. These data are consistent with a model of initial APC^{cdc20} activation on sperm-induced activation, followed by APC^{cdh1} activation after second polar body extrusion. Interestingly, therefore, we propose that mammalian eggs undergo meiosis II with both APC^{cdc20} and APC^{cdh1}, whereas eggs of other species so far described have APC^{cdc20} activity only.

Key words: Calcium, Calcium signalling, Cell cycle proteins, Fertilization, Mammals, Meiosis, Metaphase, Mice, Oocytes, Signal transduction, Ubiquitin-protein ligases

Introduction

Destruction of key cell cycle proteins is coordinated by the activity of the anaphase-promoting complex/cyclosome (APC/C) (Morgan, 1999; Peters, 2002; Zachariae and Nasmyth, 1999). This E3 ligase complex degrades its substrates by ligating them with ubiquitin, thereby earmarking them for immediate proteolysis through the 26S proteasome. Its substrate specificity changes with the cell cycle, such that, for example, cyclin A is ubiquitinated during prometaphase (den Elzen and Pines, 2001; Geley et al., 2001), cyclin B1 and securin during the metaphase-anaphase transition (Clute and Pines, 1999; Hagting et al., 2002) and aurora A and cdc20/fizzy/p55cdc/slp1 during late mitosis and G1 (Hagting et al., 2002; Littlepage and Ruderman, 2002). Thus coordinated destruction of cell cycle proteins is achieved by making them APC/C substrates at specific points in the cell cycle.

The precise mechanisms that underlie why substrates are degraded at each specific phase of the cell cycle are being resolved. One key element is the association of the APC/C with one of two WD-40 repeat-containing proteins: Cdc20/fizzy/p55cdc/slp1 or cdh1/fizzy-related/hct1/srw1/ ste9 (Peters, 2002; Pfleger et al., 2001; Visintin et al., 1997). Substrates of APC^{cdc20}, including cyclin B1 and securin, contain a RXXL destruction box (D box) motif that is essential for ubiquitin ligation. Thus D-box mutants of cyclin B1 and securin are stable during metaphase (Clute and Pines, 1999; Hagting et al., 2002), while the D-box motif is portable and confers degradation on otherwise stable proteins (Glotzer et al., 1991). During exit from mitosis both cyclin B and securin destruction are driven by APC^{cdc20} activity. At this time cdh1 is inactivated by phosphorylation through M-phase (maturation)-promoting factor (MPF) (Blanco et al., 2000; Jaspersen et al., 1999; Zachariae et al., 1998). However, during late mitosis, MPF activity declines as its regulatory cyclin B subunit is degraded by APC^{cdc20}, and at this time APC^{cdh1} activity can be observed. APC^{cdh1} ubiquitinates substrates that have either a D-box or a KEN-box motif (Pfleger and Kirschner, 2000; Zur and Brandeis, 2002). Like the D box, the KEN box is portable. One of the main functions of APC^{cdh1} is thought to be in maintaining low mitotic cyclin levels during G1; it also functions in making a G1/S checkpoint possible (Sudo et al., 2001) and regulates S-phase entry (Bashir et al., 2004; Wei et al., 2004).

Cdc20, but not cdh1, appears to be the mediator of meiotic exit in eggs of species so far described. This has been most directly shown in *Xenopus* (Lorca et al., 1998) where cdh1 protein is absent in frog eggs and is not expressed until the mid-blastula transition; and as such inhibition of cdc20 blocks the normal process of egg activation. In *Drosophila*, cdh1 is absent during the early syncytial stages of embryogenesis, functioning

only after cellularisation (Raff et al., 2002; Sigrist and Lehner, 1997). And finally in *C. elegans* cdh1 RNAi embryos can develop into adults (Fay et al., 2002), but cdc20 RNAi embryos arrest at metaphase I (Kitagawa et al., 2002).

In mouse eggs the first reductional meiotic division follows a mid-cycle gonadotropin surge, while the second equational division follows a sperm-triggered Ca^{2+} signal. Our understanding of these processes in mouse, as a mammalian model system, lags behind *Xenopus* and other higher eukaryotes. This reflects the fact that mouse eggs are not available in the quantity necessary to facilitate many biochemical analyses and usually require specialized techniques to microinject, such as Piezo-driven or negative-capacitance-driven injection procedures. However mouse eggs are optically clear and are able to translate efficiently exogenous cRNA. Therefore we took advantage of our ability to microinject metaphase II (MII) eggs to examine the role of APC^{cdc20} and APC^{cdh1} during exit from MII arrest. We examined the destruction of GFP-coupled substrates of APC^{cdc20} and APC^{cdh1} in single eggs in real-time during the second meiotic division. In so doing we found that D-box-containing substrates of APC^{cdc20} are degraded quickly at fertilization. KEN-box substrates of APC^{cdh1} were also degraded during meiosis II exit, but degradation was confined to a period following extrusion of the second polar body. We conclude that both APC^{cdc20} and APC^{cdh1} play a role in mouse meiosis, in contrast to reports in other higher eukaryotes.

Materials and Methods

Gamete handling

All chemicals were from Sigma-Aldrich (UK) unless stated otherwise, and of tissue culture or embryo-tested grade where appropriate. In the present studies outbred MFI mice (Harlan, UK) were used. Ovulated eggs were collected from the ampulla oviductal region, 12-13 hours after intraperitoneal injection of 5 IU human chorionic gonadotrophin in mice that had been primed with 7.5 IU pregnant mares' serum gonadotrophin 44-52 hours previously. Spermatozoa were collected from the caudal region of ex-breeding MF1 mice (Harlan) and capacitated for at least 2 hours in medium T6 at a concentration of 1-2×10^5 sperm/ml.

Preparation of cRNA

Full length human cdc20 was amplified by PCR from testis cDNA and cloned into a modified pRN3 vector, designed to produce mRNA transcript C-terminally coupled to GFP, with maximal stability conferred by the presence of 5′ globin UTR upstream and both 3′UTR and a poly(A)-encoding tract downstream of the gene (Levasseur and McDougall, 2000). Cyclin B1, securin and $securin^{dm}$ were cloned as described previously (Herbert et al., 2003; Nixon et al., 2002) using the same vector. cRNA was synthesised using T3 mMESSAGE and mMACHINE (Ambion, UK), and dissolved in nuclease-free water to a concentration of 1 μg/μl prior to microinjection. The KEN box of cdc20 was mutated to AAA ($cdc20^{km}$) using a GeneEditor in vitro mutagenesis kit (Promega, UK) and standard cloning procedures.

Egg microinjection and imaging

Microinjection of cRNA constructs and of Fura2 dextran were made into MII eggs as described previously (Jones and Nixon, 2000). A bolus injection of 0.1-0.3% total egg volume was made and we estimate that between 0.3-1 pg of cRNA was injected, dependent on the size of injection and the dilution made with Fura2. In vitro fertilization was performed on the stage on an inverted microscope fitted for epi-fluorescence (Nixon et al., 2002). Imaging of eggs for intracellular Ca^{2+} and bright-field imaging was performed with a Xe lamp and CCD camera.

Western blotting

MII eggs were probed for cdc20 and cdh1 protein using one anti-cdc20 polyclonal antibody (sc8358; Santa Cruz, CA, USA) and two monoclonal anti-cdh1 antibodies, AR38 (Cell Cycle Control Laboratory; Cancer Research UK) and ab3242 (AbCam, UK). SDS-PAGE was performed on 10% polyacrylamide gels and primary antibodies were detected using ECL techniques according to the manufacturer's instructions (Amersham, UK).

Kinase assay

H1 kinase assays were performed on groups of four eggs at specific times during egg activation. Eggs were lysed in kinase buffer (80 mM B glycerophosphate, 25 mM Hepes, 5 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM 4-(2-aminoethyl)benzenesulphonylfluoride hydrochloride (AEBSF), 1 mM benzamidine, 100 μM $NaVO_4$, 5 mM NaF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 10 μg/ml aprotinin, pH 7.2). Lysed eggs were then incubated for 30 minutes at 30°C with 0.1 mM ATP, 0.5 mCi/ml [γ-^{32}P]ATP (Amersham, UK), 10 μM cAMP protein kinase inhibitor, in kinase buffer containing 0.15 mg/ml histone H1 (type III-S, from calf thymus). Reactions were terminated by introduction of sample buffer and incubation at 95°C for 5 minutes. Proteins were separated by SDS-PAGE and enzyme activity was visualized using a Fujifilm BAS-1500 Bioimaging analyzer (Fuji).

Results

Cdc20 and Cdh1 protein present in mouse eggs

In this study we were interested in examining how the APC/C is regulated in mouse eggs as they undergo the second meiotic division. This is interesting given the likely signal transduction pathway employed at fertilization, in which a sperm-triggered Ca^{2+} signal leads to APC/C-mediated destruction of cell cycle factors that are responsible for egg arrest (Jones, 2004; Tunquist and Maller, 2003).

Both of the two main APC/C activators, cdc20 and cdh1, were present in mouse eggs at MII (Fig. 1). We also observed these proteins in oocytes at all stages of the first meiotic division (not shown). The fact that mouse eggs contain cdc20 is not surprising, given the probable role of APC^{cdc20} in destruction of cyclin B1/securin during both the first and second meiotic divisions in mouse (Herbert et al., 2003; Hyslop et al., 2004; Madgwick et al., 2004). By contrast, we were surprised to find cdh1, given its apparent absence in eggs and embryos of other species so far described (see Introduction). Two different anti-cdh1 antibodies were used to verify the presence of cdh1 in MII eggs, one of which (AR38) has been used previously to probe for mouse cdh1 (Listovsky et al., 2004).

Degradation of APC^{cdc20} substrates begins minutes after the initiation of Ca^{2+} spiking

The data above show that cdh1 is present in mouse eggs, but does not address whether APC^{cdh1} is actually responsible for any protein degradation during meiosis II. This is what we set out to

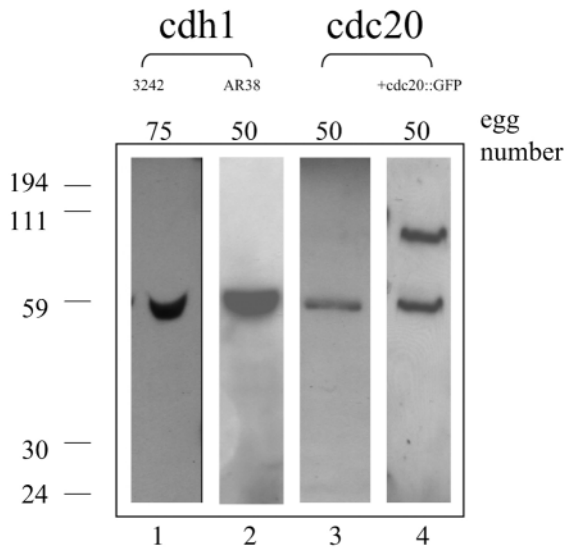


Fig. 1. Cdc20 and cdh1 protein in mouse eggs. Western blots of MII eggs. Anti-cdh1 (lane 1, antibody ab3242; lane 2, AR38) and anti-cdc20 (lanes 3 and 4, sc8358). In lane 4, germinal-vesicle (GV) stage oocytes were microinjected with cdc20::GFP cRNA and allowed to mature to MII. The upper band in lane 4 is at the predicted molecular mass of cdc20::GFP. The number of eggs used for each lane is as stated; and the migration of protein standards (kDa) are marked.

examine. Our approach was to simultaneously microinject MII mouse eggs with fura2-dextran and cRNA for substrates of both APC^{cdc20} and APC^{cdh1} that had been C-terminally coupled to EGFP. We would then be able to follow intracellular Ca^{2+} changes triggered by the fertilizing sperm and the effect of fertilization on the levels of expressed GFP fusion proteins. In these experiments eggs were inseminated with low numbers of sperm, so that extrusion of the second polar body (at 0.6-3 hours after the first Ca^{2+} spike) and pronucleus formation (at 4-6 hours) could be observed with some accuracy (Fig. 2).

Securin and cyclin B1 degradation should begin at a similar time given that both are APC^{cdc20} substrates. Therefore eggs were microinjected with either cyclin B1::GFP or securin::GFP and then inseminated. Their profile of degradation was compared relative to the sperm Ca^{2+} signal and to the morphological events of activation. The results showed that securin was degraded with identical dynamics to cyclin B1 (Fig. 3). Securin::GFP was quickly degraded when eggs were inseminated (Fig. 3A), and the process appeared complete before second polar body formation. When eggs were simultaneously imaged for GFP levels and Ca^{2+} changes, it was calculated that securin (Fig. 3B, n=16) and cyclin B1 (Fig. 3C, n=16) degradation began at the same time, just a few minutes after the first Ca^{2+} spike, 12±5 versus 13±5 minutes, respectively (mean ± s.d., not significantly different, P=0.47, t-test). Both cyclin B1 and securin were degraded by 80-90%, such that their minimum intracellular concentration was associated with the time of second polar body extrusion.

Degradation of APC^{cdh1} substrates begins at second polar body extrusion

The above data suggest that the Ca^{2+} signal at fertilization rapidly switches on APC^{cdc20} activity so that cyclin B1 and securin are degraded fully by the time of second polar body formation. Both cyclin B1 and securin are also substrates of APC^{cdh1}, therefore, it is possible that APC^{cdh1} activity is also responsible for their degradation at this time. However, if there is similar control of meiotic and mitotic cdh1 then the high CDK1 activity present in MII eggs, which does not decline until about 30 minutes after the start of egg activation (Fig. 4), should phosphorylate cdh1 and in so doing prevent any cdh1 association with the APC/C.

Any potential role of APC^{cdh1} in degradation of cyclin B1 and securin in the time before second polar body formation can be assessed directly by removing the D box of securin and measuring its degradation during fertilization. Wild-type securin contains both a D box and a KEN box. Therefore mutation of the D box (RXXL to AXXA) generates a securin construct ($securin^{dm}$; securin D-box mutant) that is a substrate of APC^{cdh1} only. In mitotic somatic cells this construct generates a so called 'cut' phenotype, in which disjunction of sister chromatids is prevented but cytokinesis is not (Hagting et al., 2002; Zur and Brandeis, 2001). Recently we reported that eggs expressing $securin^{dm}$, which were parthenogenetically activated with Sr^{2+}, went on to extrude a second polar body, but failed to separate their sister chromatids (Madgwick et al., 2004), consistent with studies in mitotic cells. In this study we fertilized $securin^{dm}$-expressing eggs and we also observed the normal morphological events of meiosis II, second polar body extrusion and pronucleus formation. By monitoring the $securin^{dm}$::GFP levels in these eggs, it was found that $securin^{dm}$ was degraded following fertilization consistent with APC^{cdh1} activity (Fig. 5, n=12). However, the

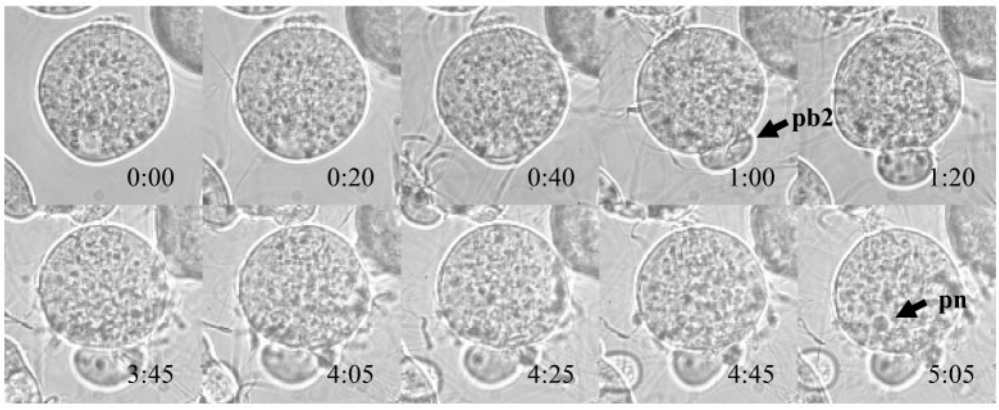


Fig. 2. Egg progression through meiosis II. By inseminating eggs with low numbers of sperm we were able to monitor accurately both second polar body formation (pb2), which occurred within 3 hours of insemination, and pronucleus (pn) formation, at about 4-6 hours. Sperm were added at time 00:00 (hours:minutes). Sample images at 20-minute intervals are shown but images were captured every 15 seconds. Scale bar: 20 μm.

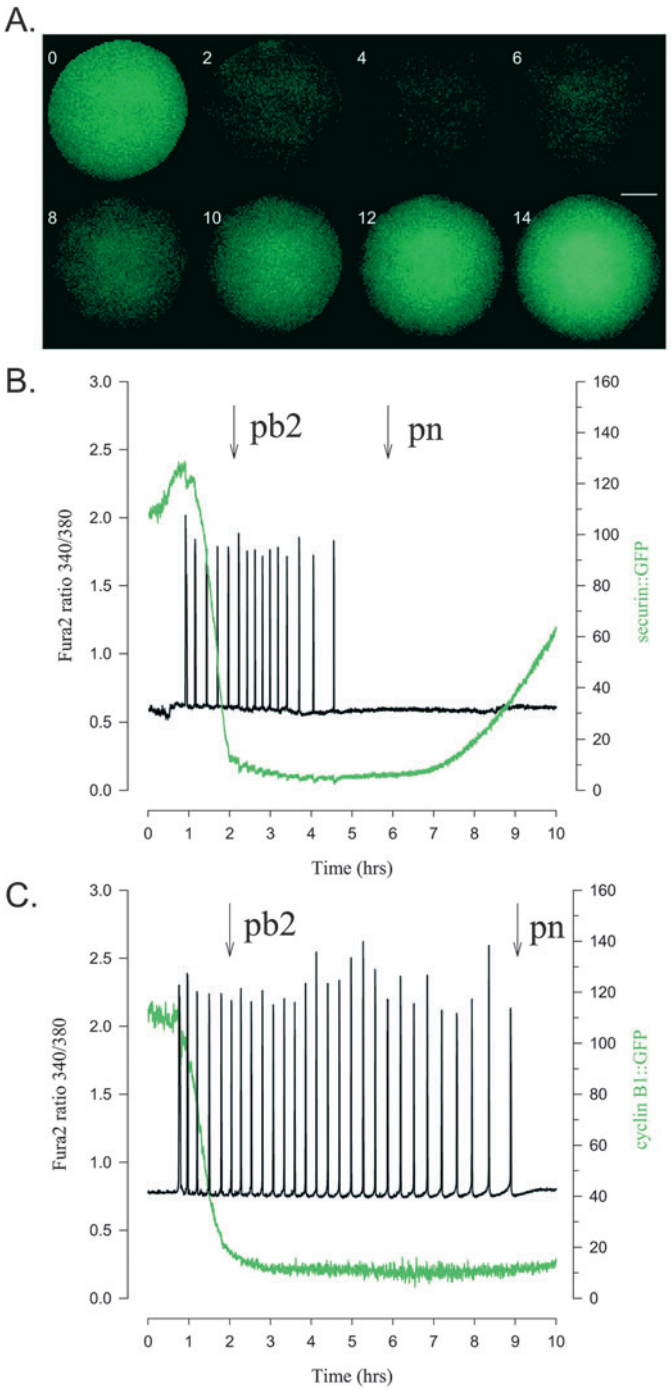


Fig. 3. Degradation of securin and cyclin B1 is initiated simultaneously. Eggs were microinjected with the Ca^{2+} reporter fura2-dextran and cRNA to either securin::GFP (A,B) or cyclin B1::GFP (C). Sperm were added at the beginning of recording and following gamete fusion a series of Ca^{2+} spikes were observed that were responsible for initiating exit from MII arrest. Degradation of cyclin B1 and securin occurred at the same time: approximately 10-20 minutes after the initiation of Ca^{2+} spiking. (A) Representative securin::GFP images captured from an inseminated egg every 2 hours at the times indicated (in hours); time is relative to the addition of sperm, and in this egg polar body extrusion occurred at 2.3 hours and pronucleus formation at 6.5 hours. Scale bar: 20 μm. (B,C) Sperm induced a series of Ca^{2+} spikes that lasted for several hours (black trace), simultaneous imaging of eggs for GFP showed that degradation of both GFP constructs were complete by second polar body formation. Levels increased again as the pronuclei formed in the 1-cell embryo. A single representative recording from 16 eggs is shown for both constructs. GFP levels are represented as the mean egg average fluorescence (in arbitrary units) from a region-of-interest defined on the Metafluor program.

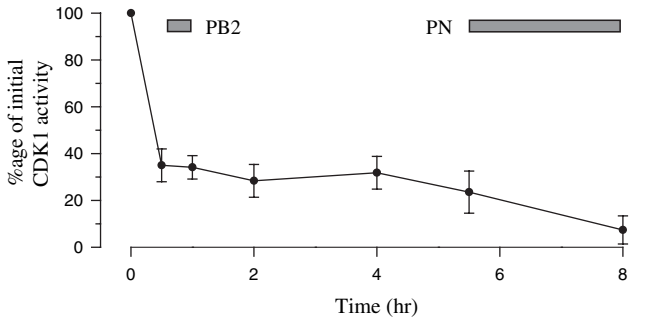


Fig. 4. CDK1 activity declines rapidly with polar body extrusion during meiosis II. Small groups of eggs were assayed, at the times indicated, for CDK1 activity by the ability of CDK1 to phosphorylate histone H1 in an in vitro assay. Eggs sampled at 1 hour had extruded their second polar body and eggs sampled at 8 hours had pronuclei. The shaded bars indicate that the polar body (pb2) and pronucleus (pn) formation must have occurred during this time. CDK1 activity declined rapidly in activating eggs, such that the largest decrease occurred in eggs that had extruded their pb2. Values are means ± standard deviation (n=5, separate experiments) in H1 kinase activity normalised with respect to non-activated eggs.

timing of degradation was greatly delayed relative to the wild-type securin carrying the intact D box. Securin^{dm} degradation began 80±29 minutes after the first Ca^{2+} spike, this time is similar to when the second polar body is extruded (85±29 minutes). Degradation continued until pronuclei formed in the one-cell embryo. Although the timing of securin degradation was delayed considerably by the mutation of its D box, the actual rate of securin^{dm} degradation, as calculated by the decrease in GFP signal, was unaffected. Loss of securin^{dm} signal proceeded at 65±20 arbitrary units/hour; while that of wild-type securin was 70±25 arbitrary units/hour (not statistically significantly different).

The above findings indicate that the removal a functional D box from securin uncovers a KEN-box-mediated degradation pathway, which is active following second polar body extrusion. Such timing in the activation of APC^{cdh1} would be consistent with cdh1 being inactive at metaphase and suggests that meiotic cdh1 may be controlled similar to mitotic cdh1. Phosphorylation changes in cdh1 can be detected on western blots; phosphorylated cdh1 is retarded on gels. Therefore cdh1 protein was compared between eggs and oocytes at metaphase, both MI and MII, and in activated eggs at the pronucleate stage. A cdh1 bandshift was observed in pronucleate stage eggs as compared with those at metaphase, consistent with phosphorylation of cdh1 at the MII stage (Fig. 6).

Cdc20 is degraded in meiosis II

To confirm that APC^{cdh1} was indeed active during meiosis II the degradation profile of cdc20, a well-characterized APC^{cdh1}

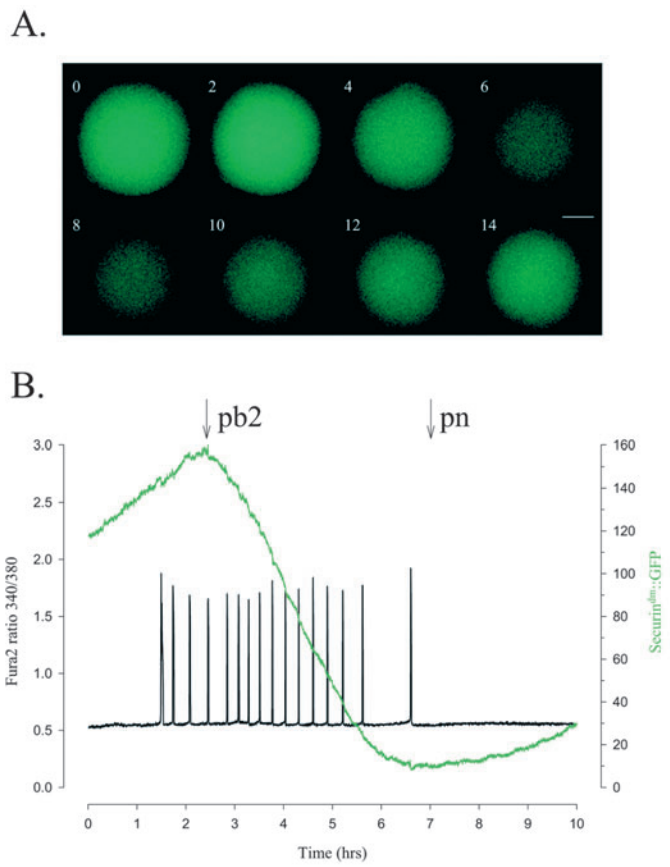


Fig. 5. Delay in securin degradation by removal of its D box. Mouse eggs expressing securin^{dm} were inseminated and egg activation monitored. The destruction of securin was significantly delayed by removal of its D box. (A) Representative securin^{dm}::GFP images captured from an inseminated egg every 2 hours at the times indicated (in hours). In this egg, polar body extrusion occurred at 2.8 hours and pronucleus formation at 5.6 hours. Scale bar: 20 μm. (B) The securin^{dm}::GFP profile in a fertilized egg. Ca^{2+} spiking, initiated at 1.5 hours, induced second polar body extrusion at 2.3 hours but during this time securin^{dm} was stable. At the time of polar body extrusion degradation of securin^{dm} began, and this continued until pronucleus formation at 7 hours.

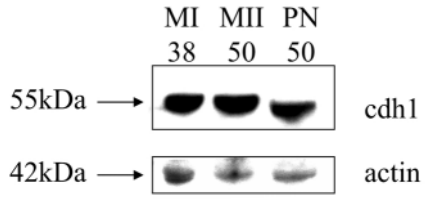


Fig. 6. CDH1 during egg activation. Western blots of cdh1 were consistent with the reported bandshift in cdh1 following dephosphorylation. Mouse oocytes at MI and MII are compared with activated eggs at the pronuleate stage (PN). An actin loading control is as indicated. The number of eggs used for each lane is indicated above.

substrate, was examined. Cdc20 has no D box and its only known mechanism of degradation in late mitosis is through APC^{cdh1}, a process that has been widely reported and extensively examined in many other cell types (Kallio et al., 2002; Pfleger and Kirschner, 2000; Raff et al., 2002; Zur and Brandeis, 2002).

Cdc20 was coupled to GFP at its C terminus and cRNA expressed in mouse eggs to act as a reporter for APC^{cdh1} activity (Fig. 1). In agreement with the temporal pattern of securin^{dm} degradation, cdc20::GFP was degraded when inseminated eggs had extruded their second polar body (Fig. 7A, n=7). The KEN box of cdc20 was mutated to AAA (cdc20^{km}) to confirm the need of the KEN box in degradation, and by inference APC^{cdh1} activity. The KEN-box mutation rendered this protein completely stable in eggs following fertilization (Fig. 7B, n=10), there being no inflexion in the synthesis rate of the cdc20^{km}::GFP construct following polar body extrusion. Although there was a defined period of cdc20 degradation after polar body extrusion (Fig. 7A) that was blocked by removal of the KEN box, the extent of cdc20::GFP degradation was not as great as that for securin. These data suggest that cdc20::GFP may be a poor substrate for APC^{cdh1}, an assumption that has also recently been made in HeLa cells, in which fluorescently labelled cdc20 was poorly degraded by APC^{cdh1} (Lindon and Pines, 2004). Therefore, although the above data are clear in that there is a period of cdc20::GFP degradation, we decided to confirm that APC^{cdh1} is effective at degrading endogenous cdc20. Mouse eggs were activated for different periods of time: for 0 hours, as a control; for 2 hours, at which point second polar body extrusion had already occurred; for 6 hours, when pronuclei had formed. In keeping with a role for APC^{cdh1} during meiosis II, we observed cdc20 destruction in eggs that had extruded their second polar body and those that had pronuclei (Fig. 7C).

Discussion

In the present study we found that the second meiotic division of mouse eggs was associated with a period of destruction in proteins that contained either a D- or KEN-box motif, consistent with activity of both APC^{cdc20} and APC^{cdh1} at fertilization. Following insemination, a sperm-triggered series of Ca^{2+} spikes quickly induced destruction of both securin and cyclin B1 in eggs. Their destruction was complete by the time CDK1 activity had declined and the second polar body had been extruded. Since cyclin B1 and securin both contain a D box, this degradation could potentially have been due to APC^{cdc20} and/or APC^{cdh1}. However, in MII eggs we observed that cdh1 protein was phosphorylated, which in mitotic cells prevents it binding the APC/C. The inability of cdh1 to activate the APC/C at MII was confirmed directly by using substrates of APC^{cdh1} that were not recognized by APC^{cdc20}. Thus we used cdc20 itself and a D-box-mutated form of securin that both contain a KEN box, to generate GFP-fusion proteins that were substrates of APC^{cdh1}. Both of these constructs were initially stable in fertilized eggs, but their degradation began abruptly 1-2 hours after sperm-triggered Ca^{2+} spiking, at the time of polar body extrusion. Their degradation continued until meiosis II was complete and pronuclei formed in the 1-cell embryo. Therefore there was no immediate activation of APC^{cdh1} at fertilization, instead there appeared to be a switch from APC^{cdc20} to APC^{cdh1} activity at the time of second polar body extrusion (this is depicted in Fig. 8). All substrates of the APC/C appeared stable after pronucleus formation.

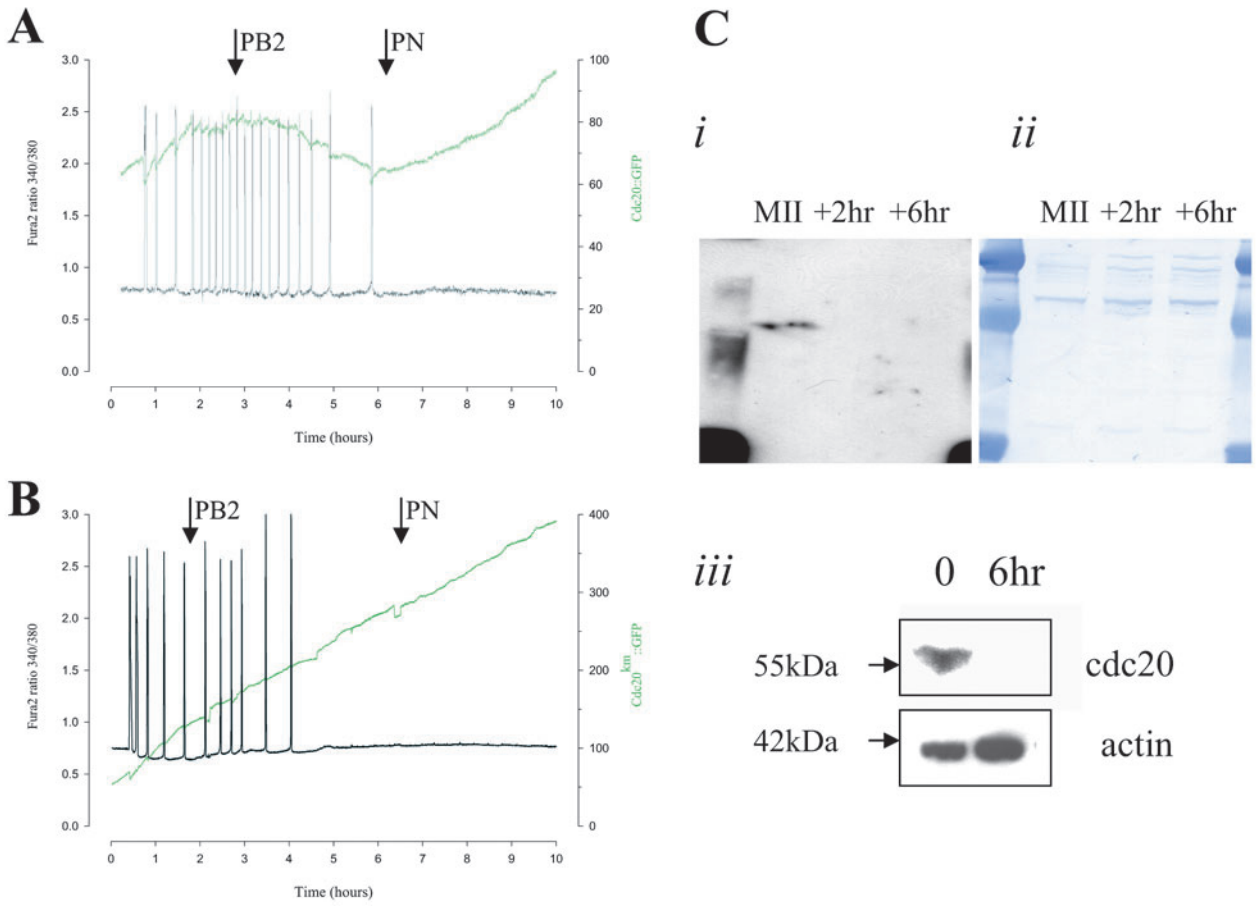
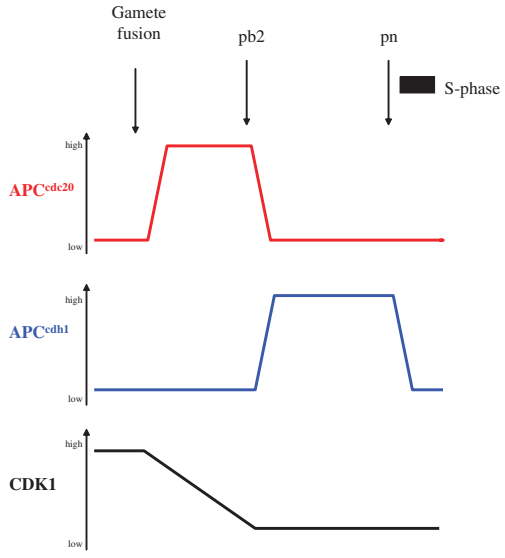


Fig. 7. Meiosis II degradation of cdc20 is KEN-box dependent. (A) Wild-type cdc20 coupled to GFP was expressed in MII eggs that were subsequently inseminated. Following sperm-triggered Ca^{2+} spiking cdc20 degradation was observed around the time of second polar body extrusion and continued until pronuclei formed in the 1-cell embryo. (B) Degradation of cdc20 was observed to be dependent on the presence of a KEN box, since its removal rendered cdc20 stable. Thus despite fertilization of these eggs, as observed by a series of Ca^{2+} spikes, $cdc20^{km}$:: GFP levels continued to rise throughout the completion of meiosis II. Recordings are representative of 8 eggs for both constructs. (C) Endogenous cdc20 was observed to be degraded at the two time points sampled: 2 hours (+2hr) and 6 hours (+6hr) after addition of Sr^{2+}-containing medium to induce parthenogenetic activation. Polar bodies were extruded after about 1 hour, and pronuclei were visible at around 5 hours. (*i*) western blot; (*ii*) Coomassie Blue stained membrane; (*iii*) eggs at time 0 hours and 6 hours probed for cdc20 and actin as a loading control. All lanes were loaded with 50 eggs.



Activation of APC^{cdc20} by Ca^{2+}

Sperm trigger a long-lasting series of Ca^{2+} spikes by releasing into the egg a sperm-specific member of the phospholipase C (PLC) family, PLCζ (Saunders et al., 2002). This PLC raises egg $InsP_3$ levels, so resulting in a train of Ca^{2+} spikes (Jones and Nixon, 2000). Ca^{2+} spiking, in a process mediated by a calmodulin-dependent protein kinase II (Markoulaki et al., 2003; Markoulaki et al., 2004), is the necessary and sufficient trigger for resumption of meiosis following a MII arrest

Fig. 8. Model of APC^{cdc20} and APC^{cdh1} activation during meiosis II. Unfertilized MII eggs, which have high CDK1 levels, have low APC^{cdc20} and APC^{cdh1} activity. A sperm-derived Ca^{2+} signal initiates activation of APC^{cdc20} at gamete fusion, which is responsible for cyclin B1/securin degradation and an associated loss in CDK1 activity. Degradation of these APC^{cdc20} substrates permits second polar body formation and at this time APC^{cdh1} activity appears. APC^{cdh1} is responsible for degradation of KEN-box substrates such as cdc20. APC^{cdh1} activity drops when pronuclei form and the zygote enters S phase of the first embryonic cell cycle.

(Hyslop et al., 2004). D-box dependent proteolysis of cyclin B1 (Nixon et al., 2002) and securin (present data) begin at the same time; just a few minutes after the first Ca^{2+} spike. We interpret this to mean that activation of the E3 ligase APC^{cdc20} occurs shortly after spiking is initiated in the same way that APC^{cdc20} has shown to be turned on, and essential, during frog egg activation (Lorca et al., 1998). However, mouse eggs also express RFLP4, an E3 ligase whose substrates include cyclin B1 (Suzumori et al., 2003) that may fulfil the same function as APC^{cdc20}; indeed novel meiosis variants of cdc20 have been described in yeast (Cooper et al., 2000) and *Drosophila* (Chu et al., 2001). The present data do not rule out a function for RFLP4 in mouse meiosis. However, we show here that mouse eggs do contain cdc20, and previously we have demonstrated that Ca^{2+}-mediated cell cycle resumption and cyclin B1 degradation can be blocked by induction of a spindle checkpoint (Jones et al., 1995; Nixon et al., 2002). This would support a role for APC^{cdc20} since activation of the spindle checkpoint is a potent repressor of APC^{cdc20} activity (Yu, 2002). This effect is mediated by mad2, one of the spindle checkpoint proteins, which binds specifically and directly to cdc20 (Luo et al., 2000). Therefore, it is likely that RFLP4 may have a specific role during meiosis I, or alternatively contributes to securin/cyclin B1 degradation during meiosis II, once the APC/C has been activated.

In further support of APC^{cdc20} having a role during meiosis II is the observation that both D-box- and KEN-box-containing substrates are targeted for proteolysis when CDK1 activity drops at second polar body extrusion. This implies a switch from APC^{cdc20} to APC^{cdh1} activity at this time. This switch does not occur in frog eggs that lack cdh1 protein, but the timing of this switch would be consistent with that reported from mitotic studies in mammalian cells (Hagting et al., 2002; Zur and Brandeis, 2002) and the negative regulation of cdh1 by MPF.

At this time it is unknown how Ca^{2+} activation of calmodulin-dependent protein kinase II leads to a switching on of APC^{cdc20}. Historically the term 'cytostatic factor' has been used to describe the activity in eggs which is responsible for MII arrest. Cytostatic factor activity is likely to constitute a repression of APC^{cdc20} activity. Indeed, some candidates that have cytostatic factor activity are inhibitors of $APC^{cdc20.}$ These include Emi1, which binds cdc20 and in so doing prevents cdc20 binding the APC/C (Reimann and Jackson, 2002), and various components of the spindle checkpoint (Schwab et al., 2001; Tunquist et al., 2003).

Why some eggs have cdh1 and others do not

Further studies are needed to determine both the function and regulation of APC^{cdh1} during mouse meiosis II, and in addition, since we have observed its presence at all stages of oocyte maturation, its role in the first meiotic division. It is possible that mouse APC^{cdh1} is switched off during meiosis I, since by analogy in budding yeast a meiosis-specific inhibitor of cdh1 prevents APC^{cdh1} activity during the first meiotic division (Bolte et al., 2002). The present work points to a role of CDK1 activity in regulating APC^{cdh1} during meiosis II, but nothing is known about the role of the phosphatase cdc14, which is necessary to activate cdh1, in mouse eggs. Thus future studies should address how cdh1 is regulated by CDK1/cdc14 during the two meiotic divisions, which temporally are separated by just a few hours. In the mitotic division, cdh1 activity appears readily dispensable, such that cells can still exit mitosis in the absence of cdh1. Therefore it is possible that cdh1 can be dispensed with for the process of completing meiosis II. However, APC^{cdh1} has been found to have specific functions later in the cell cycle at G1, it is responsible for making a G1-S checkpoint permissible (Sudo et al., 2001) and preventing premature entry into S phase (Bashir et al., 2004; Wei et al., 2004). APC^{cdh1} appears also to have functions outside the cell cycle, such as in post-mitotic neurons where it regulates the growth of axons (Konishi et al., 2004) and in some studies its presence has been found to be associated with the onset of cell differentiation (Blanco et al., 2000). It will therefore be interesting to determine what role maternal cdh1 plays in embryos up until the activation of the zygotic genome.

Most cell divisions, including those that occur in early mouse embryos and mammalian mitotic divisions have associated gap phases to their cell cycle and so there would be a need for cdh1 in G1 for regulating entry to S phase. However eggs of some species, exemplified by *Xenopus*, *C. elegans* and *Drosophila*, appear to have dispensed with gap phases. Based on the present finding of APC^{cdc20} and APC^{cdh1} activity during meiosis II in mouse eggs it is probably not the case that the embryonic cell cycle is fundamentally different to the cell cycle in adult cells. Rather, the lack of cdh1 activity in eggs and embryos of some species probably reflects the requirement to speed up the cell cycle in order to reach a motile stage and avoid predation.

We thank Dr Julian Gannon and Professor Tim Hunt, Cell Cycle Control Laboratory, Cancer Research UK, for the donation of AR38 cdh1 antibody. Drs Junyong Huang, Alex McDougall and Professor Michael Whitaker for critical reading of the manuscript. This work was supported by the Wellcome Trust in the form of a project grant (069236/Z/02/Z) and equipment grant (065354/Z/01/Z) to K.T.J.

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