Inactivation of the checkpoint kinase Cds1 is dependent on cyclin B-Cdc2 kinase activation at the meiotic G_2/M-phase transition in *Xenopus* oocytes

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Accepted 30 May 2001
Journal of Cell Science 114, 3397-3406 (2001) © The Company of Biologists Ltd

SUMMARY

Checkpoint controls ensure chromosomal integrity through the cell cycle. Chk1 and Cds1/Chk2 are effector kinases in the G_2-phase checkpoint activated by damaged or unreplicated DNA, and they prevent entry into M-phase through inhibition of cyclin B-Cdc2 kinase activation. However, little is known about how the effector kinases are regulated when the checkpoint is attenuated. Recent studies indicate that Chk1 is also involved in the physiological G_2-phase arrest of immature *Xenopus* oocytes via direct phosphorylation and inhibition of Cdc25C, the activator of cyclin B-Cdc2 kinase. Bearing in mind the overlapping functions of Chk1 and Cds1, here we have studied the involvement of *Xenopus* Cds1 (XCds1) in the G_2/M-phase transition of immature oocytes and the regulation of its activity during this period. Protein levels of XCds1 remained constant throughout oocyte maturation and early embryonic development. The levels of XCds1 kinase activity were high in immature oocytes and decreased at the meiotic G_2/M-phase transition. Consistently, when overexpressed in immature oocytes, wild-type, but not kinase-deficient, XCds1 significantly delayed entry into M-phase after progesterone treatment. The inactivation of XCds1 depended on the activation of cyclin B-Cdc2 kinase, but not MAP kinase. Although XCds1 was not directly inactivated by cyclin B-Cdc2 kinase in vitro, XCds1 was inactivated by overexpression of cyclin B, which induces the activation of cyclin B-Cdc2 kinase without progesterone. Thus, the present study is the first indication of Cds1 activity in cells that are physiologically arrested at G_2-phase, and of its downregulation at entry into M-phase.

Key words: Cds1, Checkpoint kinase, Cyclin B-Cdc2 kinase, Meiosis reinitiation, *Xenopus* oocyte

INTRODUCTION

Eukaryotic cells use cell cycle checkpoints to guarantee the accurate transmission of genetic information from a cell to its daughters (Hartwell and Weinert, 1989; Elledge, 1996). During the ordinary cell cycle, the integrity of chromosomal DNA is under constant surveillance. In particular, entry into mitosis is controlled by the monitoring of damaged or incompletely replicated DNA. An ultimate target in the G_2 checkpoint control is cyclin B-Cdc2 kinase, which governs the entry into M-phase (Nurse, 1990; Morgan, 1995), while the surveillance system is composed of checkpoint sensors, effector kinases and their targets (O'Connell et al., 2000; Zhou and Elledge, 2000). Rad3 in fission yeast, Mec1 in budding yeast and ATR/ATM in vertebrates, all of which belong to a larger subfamily of protein kinases with a phosphoinositide kinase (PIK)-related domain, are involved in sensing damaged DNA and/or stalled replication complexes. Chk1 and Cds1 (fission yeast and frog)/Rad53 (budding yeast)/Chk2 (mammals) are effector kinases that receive signals from the checkpoint sensors. Both Chk1 and Cds1 phosphorylate and suppress the function of Cdc25 (Russell, 1998), which activates Cdc2 by the dephosphorylation of Thr14 and Tyr15 (see Morgan, 1995). Both kinases are also thought to target Wee1, an inhibitory kinase for Cdc2 (see Morgan, 1995), by direct phosphorylation (Murakami and Nurse, 2000). In contrast to the substantial data on how Chk1 and Cds1 are phosphorylated/activated and on how they function in response to checkpoint signaling, little is known about how they are regulated when checkpoint is inactivated or adapted. The only reports on this issue are that in fission yeast, the Cdc2-dependent phosphorylation of Crb2/Rhp9, which resembles human BRCA1 (Scully and Livingston, 2000), is thought to be required for inactivation of Chk1 during recovery from the DNA damage checkpoint (Esashi and Yanagida, 1999), and that in budding yeast, Cdc5, a homolog of polo-like kinase (Glover et al., 1998; Nigg, 1998), is required for adaptation to the DNA damage checkpoint (Toczyski et al., 1997).

Fully grown immature oocytes of *Xenopus* are arrested naturally at prophase of the first meiotic cell cycle (prophase-I), which corresponds to the late G_2-phase (Ferrell, 1999). The release from the arrest, meiosis reinitiation, is equivalent to the G_2/M-phase transition, and is induced by exposure of oocytes to progesterone (Masui and Clarke, 1979). After oocyte maturation, fertilization initiates early cleavage cycles. Completion of the 12th cleavage marks the midblastula

transition (MBT), when zygotic transcription begins and the so-called somatic cell-type cell cycle is established (Newport and Kirschner, 1982). Despite the expression of *Xenopus* Chk1 protein (XChk1) at approximately constant levels throughout oocyte maturation and early embryogenesis until the neurula stage (Nakajo et al., 1999; Kappas et al., 2000), *Xenopus* embryos lack checkpoint controls for damaged and unreplicated DNA prior to the MBT (Dasso and Newport, 1990; Clute and Masui, 1997; see Kappas et al., 2000). However, checkpoints can be created in cell-free extracts derived from *Xenopus* eggs (Dasso and Newport, 1990; Kumagai et al., 1998). When the extracts are supplemented with a high number of sperm nuclei, XChk1 and *Xenopus* Cds1 (XCds1) are phosphorylated and activated in response to unreplicated or damaged DNA, leading to a delay in the entry into M-phase (Kumagai et al., 1998; Guo and Dunphy, 2000; Guo et al., 2000; Kumagai and Dunphy, 2000). These studies indicate that while the Chk1 and Cds1 pathways are functional even prior to the MBT, the DNA damage/replication checkpoints are not operational in the intact pre-MBT embryos (see also Kappas et al., 2000). Nonetheless, XChk1 is involved in the G_2-phase arrest of immature oocytes, even though the G_2-phase arrest of immature oocytes is formally distinct from the checkpoint arrest that depends on damaged or unreplicated DNA. Furthermore, in immature oocytes there is no obvious phosphorylation of Chk1, as is usually seen in other cell types in response to the activated G_2 checkpoint (Nakajo et al., 1999). However, it is as yet unclear whether XChk1 and XCds1 are regulated when the checkpoints created in egg extracts are attenuated (adapted or recovered) or when progesterone releases the G_2-phase arrest in immature oocytes.

In immature *Xenopus* oocytes, XChk1 appears to directly phosphorylate Cdc25C at Ser287 and inhibit its activity (Nakajo et al., 1999), as seen in the activated G_2 checkpoint (Russell, 1998). However, it has been suggested that other Ser287-specific kinases, distinct from XChk1, could be involved in G_2-phase arrest of immature oocytes (Nakajo et al., 1999). Therefore, bearing in mind the overlapping functions of Cds1 and Chk1 in the DNA damage and replication checkpoint controls, we have studied the involvement of XCds1 in the arrest of immature *Xenopus* oocytes. Our results show that XCds1 is active in G_2-phase-arrested oocytes, but during meiosis reinitiation following progesterone treatment it is downregulated in response to the activation of cyclin B-Cdc2 kinase, but not MAP kinase activation. This is the first indication of the downregulation of Cds1 activity at entry into M-phase, regardless of the presence or absence of the activated G_2-phase checkpoint.

MATERIALS AND METHODS

Preparation of oocytes and embryos

Xenopus oocytes were prepared as described (Iwabuchi et al., 2000). To induce maturation, oocytes were treated with 5 μg/ml progesterone in MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM Hepes-NaOH, pH 7.5) or MBS (88 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.4) (Peng, 1991) and cultured at 22°C. Meiosis reinitiation was monitored by the appearance of a white spot at the animal pole of the oocyte, an indication of germinal vesicle breakdown (GVBD). In some experiments, oocytes were incubated overnight in the presence of 250 μM olomoucine (Research Biochemicals International), or for 3 hours in the presence of 50 μM U0126 (Promega), before progesterone addition. *Xenopus* embryos were prepared as described (Gotoh et al., 1998). Some embryos were incubated in the presence of 30 mM hydroxyurea (Sigma) or 100 μg/ml aphidicolin (Wako, Japan). Stock solutions were prepared as 100 mM olomoucine in DMSO, 10 mM U0126 in DMSO, 1 M hydroxyurea in distilled water, and 10 mg/ml aphidicolin in DMSO.

Preparation of oocyte and egg extracts

Oocytes were washed with extraction buffer (EB: 100 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, 20 mM Hepes-KOH, pH 7.5), and then transferred to a centrifuge tube containing EB plus 50 μg/ml cytochalasin B. The oocytes were centrifuged (1,000 ***g***, 30 seconds, 4°C), and then excess EB was removed. After the oocytes were crushed by centrifugation (15,000 ***g***, 30 minutes, 4°C), their cytoplasmic extracts were recovered. The extracts were further centrifuged (15,000 ***g***, 15 minutes, 4°C), and the clear supernatants were recovered and immediately frozen in liquid nitrogen. *Xenopus* egg extracts were prepared as described (Nishiyama et al., 2000).

Construction of recombinant plasmids

To clone the coding sequence of *Xenopus cds1* into pBluescript-RN3 vector (Lemaire et al., 1995), a *Bam*HI site at the start codon and a *Not*I site at the stop codon were created by PCR using Pfu DNA polymerase (Stratagene) with *Xenopus cds1* cDNA (GenBank accession number AF326574) and the primers 5′-CGCGGATCC-ATGATGTCTCGTGATACTAAAACAGAGTCG-3′ and 5′-GGAA-GAATGCGGCCGCTTATCTTTTTGCTCTCTTTTCGGCTGATG-3′. The resulting PCR fragment was digested with *Bam*HI and *Not*I, and then it was ligated to pBluescript-RN3 that had been digested with *Bgl*II and *Not*I. A kinase-deficient XCds1 mutant was made by mutagenesis of wild-type *Xenopus cds1* using QuikChange Site-Directed Mutagenesis Kit (Stratagene). The pBluescript-RN3-Xcds1-WT construct was mutagenized using 5′-GGGGTGATACATCGT-GCACTGAAGCCTGAAAATGTGC-3′ as a primer (in the sense strand), where the underlined codon, residue 319, now encodes Ala rather than Asp, generating pBluescript-RN3-Xcds1-KD. To clone the coding sequence of *Xenopus cds1* into pTrcHisB vector (Invitrogen), a *Bam*HI site at the start codon and an *Eco*RI site downstream of the stop codon were created by subcloning the PCR fragment into another vector and digesting the vector with *Bam*HI and *Eco*RI. The *Bam*HI-*Eco*RI fragment encoding Xcds1 was ligated to pTrcHisB that had been digested with *Bgl*II and *Eco*RI. To obtain pGEX-Xcdc25C(254-316)-WT construct, a DNA encoding amino acids 254-316 of wild-type *Xenopus* Cdc25C was prepared by PCR using Pfu DNA polymerase with wild-type *Xenopus cdc25C* cDNA (gift from Dr N. Sagata) as a template and the primers 5′-CGCGGATCCATG-GCAATTCTTCTGTCGGGACC-3′ and 5′-GGAATTCTCAACG-TCTCCTTTTCACTCTGACCG-3′. The resulting PCR product was digested with *Bam*HI and *Eco*RI and ligated into pGEX-4T-1 vector (Amersham Pharmacia Biotech) that had been digested with *Bam*HI and *Eco*RI. To obtain the S287A mutant of GST-XCdc25C(254-316)-WT, the pGEX-Xcdc25C(254-316)-WT construct was mutagenized using 5′- CCTTTACCGCTCACCTGCGATGCCAGAGAAACTTG-3′ as a primer (in the sense strand), where the underlined codon, residue 287, now encodes Ala rather than Ser, generating pGEX-XCdc25C(254-316)-S287A construct. To obtain pBluescript-RN3-*Xenopus* cyclin B1-myc/His6, full-length *Xenopus cyclin B1* cDNA (gift from Dr T. Hunt) whose C terminus end was fused to myc and His6 genes, was cloned to pBluescript-RN3 vector (gift from Dr M. Iwabuchi).

Production of anti-XCds1 antibodies

The pTrcHisB-Xcds1 construct was introduced into the *E. coli* strain BL21. Expression of His6-XCds1 was induced by growing the cells at 37°C for 4 hours in the presence of 0.5 mM isopropyl-β-D-

thiogalactoside (IPTG). Insoluble His6-XCds1 was subjected to SDS-PAGE and eluted from the gels using Model 422 Electro-eluter (Bio-Rad Laboratories). XCds1 antiserum was raised in a rabbit using gel-purified His6-XCds1 as the antigen. For affinity purification of immune rabbit serum, His6-Cds1 was subjected to SDS-PAGE and transferred to the Immobilon membrane (Millipore). Blots were stained with 0.1% Ponceau S in 0.1% acetic acid and the band of His6-Cds1 was excised. The His6-Cds1 strips were neutralized in TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.5), blocked in 5% skimmed milk in TBS for 30 minutes, and incubated with 50% serum in TBS overnight at 4°C or for 1-2 hours at room temperature. The strips were washed with TBS, and the protein-bound antibodies were eluted by incubating the strips with 0.1 M glycine-HCl, pH 2.5, for three 3-minute periods. The eluates were neutralized by the addition of one twentieth volume of 1 M Tris. Another XCds1 antiserum was raised in rabbits against a synthetic peptide of the C terminus of XCds1 (CSEILPTSAEKRAKR) using the services of Sawady Technology (Tokyo). Whole serum was affinity-purified against the peptide antigen coupled to sulfolink agarose beads (Pierce).

Production of GST-XCdc25C(254-316)-WT and GST-XCdc25C(254-316)-S287A

The pGEX-Xcdc25C(254-316)-WT or -S287A constructs were introduced into the *E. coli* strain BL21. Expression of GST-XCdc25C(254-316) was induced by growing the cells at 37°C for 3 hours in the presence of 0.5 mM IPTG. GST-XCdc25C(254-316) was purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech). The resulting eluate was dialyzed against kinase buffer (20 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 40 mM Hepes-KOH, pH 7.3), concentrated to approximately 10 mg/ml using a Centricon 30 (Amicon) and stored in portions at –80°C.

Immunoprecipitation and kinase assay for XCds1

For immunoprecipitation, oocyte extracts (2 μl) were diluted in 300 μl of immunoprecipitation buffer (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 50 mM NaF, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Nonidet P40 (NP-40), 20 mM Hepes-NaOH, pH 7.3). The diluted extracts were incubated with 20 μl of Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) and 2 μg of control rabbit IgG (Zymed) for 30 minutes at 4°C with constant rotation. After removal of the beads by centrifugation, 20 μl of Protein A-Sepharose CL-4B and 2 μg of affinity-purified XCds1 antibodies was added to the supernatant. After a 2-hour incubation with constant rotation at 4°C, the beads were washed four times with beads wash buffer (5 mM NaF, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.5 mM PMSF, 0.1% NP-40, 50 mM Tris-HCl, pH 7.5). The immunoprecipitates were dissolved in SDS-sample buffer, and boiled for 3 minutes, followed by separation with SDS-PAGE. For measuring XCds1 kinase activity, XCds1 immunoprecipitates were washed four times with bead wash buffer, twice more with kinase buffer and incubated with 30 μl of kinase buffer containing 10 μM ATP, 5 μCi [γ-^{32}P]ATP and 5 μg GST-XCdc25C(254-316)-WT as a substrate (Guo and Dunphy, 2000). After incubation at 22°C for 30 minutes, the reactions were stopped by the addition of SDS-sample buffer, boiled for 3 minutes, and separated by SDS-PAGE. Phosphorylated forms of GST-XCdc25C(254-316)-WT were detected on gels by autoradiography and quantitated by Cherenkov counting, or by immunoblots with anti-phospho human Cdc25C (Ser-216) antibody. This antibody was able to recognize phosphorylation of Ser-287 on *Xenopus* Cdc25C as well, but not phosphorylations by cyclin B-Cdc2 kinase (data not shown).

Treatment of XCds1 with cyclin B-Cdc2 kinase

To phosphorylate XCds1 with cyclin B-Cdc2 kinase, XCds1 immnoprecipitates were mixed with 30 μl of kinase buffer containing 10 μM ATP and 5 μCi [γ-^{32}P]ATP with or without active cyclin B-Cdc2 kinase purified from starfish oocytes (Okumura et al., 1996), and then incubated at 22°C for 30 minutes. The reactions were stopped by the addition of SDS-sample buffer, subjected to SDS-PAGE, and autoradiographed on X-ray film. For XCds1 kinase assay, XCds1 immnoprecipitates were mixed with 30 μl of kinase buffer containing 600 μM ATP with or without starfish cyclin B-Cdc2 kinase. After incubation at 22°C for 30 minutes, the reaction mixture was added with 5 μg of GST-XCdc25C(254-316)-WT and incubated at 22°C for 30 minutes. The reaction was stopped by the addition of SDS-sample buffer, and subjected to western blot analysis.

Histone H1 kinase assay

Oocyte extracts (1 μl) were diluted in βGP-EB (80 mM β-glycerophosphate, 5 mM $MgCl_2$, 20 mM EGTA, 20 mM Hepes-KOH, pH 7.4) to a final volume of 10 μl. The diluted extracts were mixed with 20 μl of reaction buffer (80 mM β-glycerophosphate, 20 mM $MgCl_2$, 0.6 μg/ml leupeptin, 0.6 μg/ml aprotinin, 600 μM ATP) containing 12 μg histone H1 and 2 μCi [γ-^{32}P]ATP, and incubated for 30 minutes at 22°C. The reactions were stopped by the addition of SDS-sample buffer, boiled for 3 minutes, and subjected to SDS-PAGE, and autoradiographed on X-ray film.

Microinjection of mRNA into oocytes

The pBluescript-RN3-Xcds1-WT, -Xcds1-KD, -LacZ or -*Xenopus* cyclin B1-myc/His6 constructs were cut singly and in vitro transcribed into 5′-capped mRNAs by using the mCAP RNA Capping Kit (Stratagene). The in vitro synthesized mRNA was purified by Sephadex G-50 (Amersham Pharmacia Biotech) and dissolved in distilled water. Microinjection of mRNA into immature *Xenopus* oocyte was performed using Nanoject (Drummond Scientific Co.). Except for *Xenopus* cyclin B1 mRNA, the oocytes injected with mRNA were cultured overnight to overexpress the encoded protein, and then treated with progesterone.

Western blot analysis

Oocytes, eggs and embryos were homogenized with ten volumes of βGP-EB. The homogenates were centrifuged (10,000 ***g***, 10 minutes, 4°C) and their cytoplasmic extracts were recovered. The extracts were mixed with SDS-sample buffer, and boiled for 3 minutes. The samples were subjected to SDS-PAGE and blotted onto an Immobilon membrane according to Towbin et al. (Towbin et al., 1979). After blocking with TBS containing 5% skimmed milk for 30 minutes, the membrane was incubated with primary antibodies overnight at 4°C or for 1-2 hours at room temperature. The primary antibodies used in this study were anti-XCds1, anti-XChk1 (gift from Dr N. Sagata), anti-MAP kinase (Upstate), anti-β-galactosidase (Promega), and anti-phospho human Cdc25C (Ser-216) (New England Biolabs). The membrane was then incubated with alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies for 1-2 hours at room temperature. Signals were visualized by the BCIP/NBT phosphatase substrate system (KPL) or the ECL system (Amersham Pharmacia Biotech).

RESULTS

Developmental expression of XCds1

In order to examine the expression of XCds1 in *Xenopus* oocytes and embryos, we raised polyclonal anti-XCds1 antibodies that were capable of detecting 1 pg of His6-XCds1 on western blots (data not shown). Detection of His6-XCds1 was blocked by preincubation of the antibodies with His6-XCds1 (Fig. 1A). On western blots of *Xenopus* egg extracts, the antibodies recognized a protein of approximately 60 kDa (Fig. 1B), which comigrated with XCds1 overexpressed in *Xenopus* oocytes (see Fig. 4A). A protein of the same size was

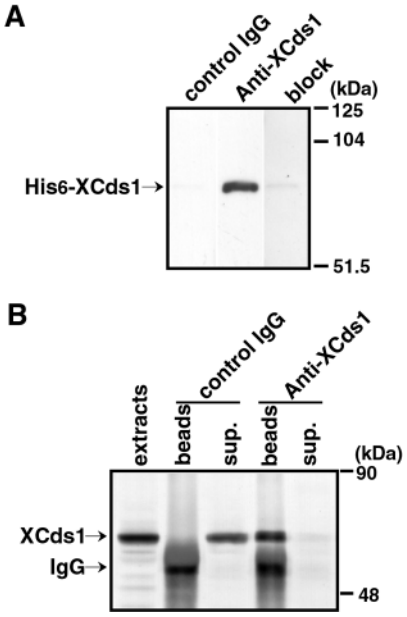

Fig. 1. Specificity of anti-XCds1 antibody. (A) His6-XCds1 (1 ng/lane) was subjected to western blot analysis with control rabbit IgG, purified anti-XCds1 antibody, or anti-XCds1 antibody preabsorbed to His6-XCds1 (block). (B) *Xenopus* egg extracts were immunoprecipitated with control rabbit IgG or anti-XCds1 antibody. Western blot analysis confirmed that XCds1 protein was recovered in the immunoprecipitates with anti-XCds1 antibody (beads) but undetectable in the supernatant (sup).

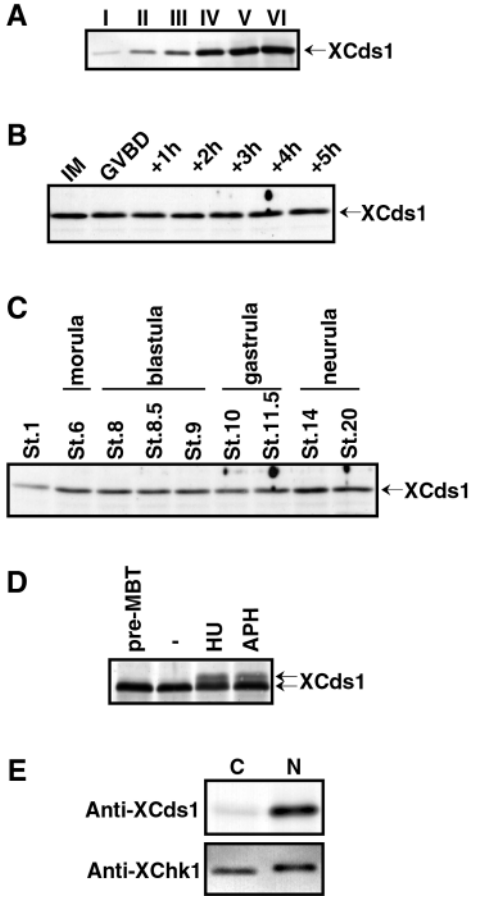

Fig. 2. Developmental expression and localization of XCds1. (A) Expression of XCds1 during oogenesis from stage I to VI. (B) Expression of XCds1 during oocyte maturation. Immature oocytes (IM) were treated with progesterone to induce maturation and GVBD occurred at about 2 hours. Metaphase arrest of meiosis II occurred at about 4 hours after GVBD. (C) Expression of XCds1 during embryogenesis. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). (D) Embryos were untreated (–) or treated with hydroxyurea (HU) or aphidicolin (APH) as early as stage 8 to inhibit DNA replication and then sampled at stage 9, post-MBT. Note that mobility shift of XCds1 (upper arrow) was detectable in hydroxyurea- or aphidicolin-treated post-MBT embryos, while it was undetectable in untreated post-MBT embryos (–), as in stage-6 untreated embryos (pre-MBT). Oocytes or embryos were subjected to western blot analysis with anti-XCds1 antibody. Each lane was loaded with proteins equivalent to one oocyte or embryo. (E) Nuclear localization of XCds1 in immature *Xenopus* oocytes. An immature oocyte was dissected into cytoplasmic (C) and nuclear (N) fractions. Proteins were resolved by SDS-PAGE and visualized by western blot with anti-XCds1 (top) or anti-XChk1 (bottom) antibodies. Proteins equivalent to one quarter of the cytoplasmic or nuclear fraction were analyzed.

also recognized by western blots in the immunoprecipitates of egg extracts with the antibodies (Fig. 1B). These observations indicate that the antibodies recognize and immunoprecipitate the XCds1 protein in *Xenopus* egg extracts.

We first examined the expression pattern of XCds1 during oogenesis from small stage I to full-grown stage VI oocytes (Dumont, 1972), during oocyte maturation, and during early embryogenesis. XCds1 was already present in stage I oocytes and showed a progressive increase in its protein levels during oogenesis to stage VI (Fig. 2A). After treatment of stage VI oocytes with progesterone to reinitiate meiosis, XCds1 protein levels remained constant during oocyte maturation (Fig. 2B). After fertilization of mature eggs, XCds1 was detectable at the same levels through the cleavage, blastula, gastrula, and neurula stages (Fig. 2C). Thus, the developmental expression pattern of XCds1 was similar to that of XChk1 reported previously (Nakajo et al., 1999; Kappas et al., 2000). In addition, no significant changes were detectable in the electrophoretic mobility of XCds1 in SDS-PAGE (10% acrylamide) of samples taken during oogenesis, oocyte maturation (see below, however, Figs 3A and 6C in SDS-PAGE with 8% acrylamide) and early embryogenesis, in contrast to the remarkable retardation of its mobility in samples from late blastulae in response to the activation of the replication checkpoint by hydroxyurea or aphidicolin treatment (Fig. 2D).

Nuclear localization of XCds1 in G_2-phase-arrested oocytes

Immature *Xenopus* oocytes contain a large nucleus, known as the germinal vesicle (GV). The GV can be easily isolated surgically from its surrounding cytoplasm, allowing the preparation of cytoplasmic and nuclear fractions from oocytes (Evans and Kay, 1991). Western blots of each fraction with anti-XCds1 antibodies indicated that XCds1 was almost entirely nuclear in immature oocytes (Fig. 2E). In contrast, XChk1 was both cytoplasmic and nuclear (Fig. 2E) (see also Oe et al., 2001). It is presumed that XCds1 could function in

the nucleus while XChk1 could function in both cytoplasm and nucleus, suggesting non-redundancy of both proteins.

XCds1 is inactivated at G_2/M-phase transition after progesterone treatment

We examined whether endogenous XCds1 is active in immature oocytes and whether its kinase activity changes during meiosis reinitiation. For this purpose, XCds1 was immunoprecipitated from oocyte extracts that were prepared at different times after progesterone treatment, and the immunoprecipitates were assayed for kinase activity using a GST fusion protein containing a fragment of Cdc25C that includes the 14-3-3 binding site (GST-XCdc25C[254-316]-WT) as a substrate. XCds1 recovered from immature oocytes highly phosphorylated GST-XCdc25C(254-316)-WT, but not its mutant form containing Ala instead of Ser at position 287 (GST-XCdc25C[254-316]-S287A) (Fig. 3A,B), indicating that the phosphorylation occurs on Ser-287 in the Cdc25C fragment. After the addition of progesterone into immature oocytes, GVBD started at 2 hours, and 100% GVBD was reached at 3.5 hours (Fig. 3B) (in this batch, GVBD occurred earlier than usual; see Fig. 4C). XCds1 kinase activity decreased in parallel with the progression of GVBD and reached background levels shortly after 100% of the oocytes had undergone GVBD, while XCds1 protein levels remained constant (Fig. 3A,B). A slight but significant retardation in the electrophoretic mobility of XCds1 was detected along with the decrease in XCds1 kinase activity (Fig. 3A; see also Fig. 6C), unlike the remarkable shift to a slower mobility seen in response to the activated DNA replication checkpoints after the MBT (compare with Fig. 2D). The small shift-up of XCds1 at GVBD was not cancelled after treatment with alkaline- or λ-phosphatase (data not shown). These observations indicate that endogenous XCds1 is active in G_2-phase-arrested oocytes but is inactivated through some modification at the meiotic G_2/M-phase transition after progesterone treatment.

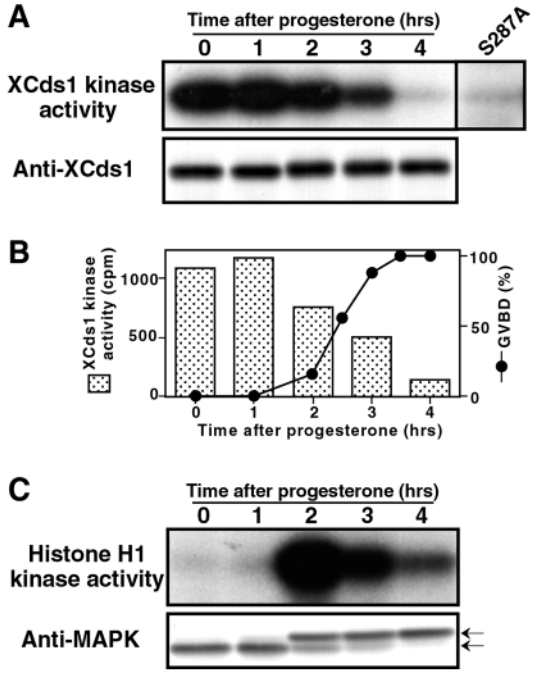

Fig. 3. XCds1 is inactivated at meiotic G_2/M-phase transition. (A) XCds1 was immunoprecipitated with anti-XCds1 antibody from oocyte extracts prepared at different times during meiosis reinitiation after progesterone treatment. The immunoprecipitates were assayed for kinase activity using GST-XCdc25C(254-316)-WT as a substrate, whose phosphorylation was detected by autoradiography (top). Note that GST-XCdc25C(254-316)-S287A was not phosphorylated by XCds1 immunoprecipitated from immature oocyte extracts (S287A). Levels of XCds1 were confirmed by western blot with anti-XCds1 antibody (bottom). (B) Changes in XCds1 kinase activity shown in A were measured and compared with the occurrence of GVBD. (C) The same extracts as in A were subjected to total histone H1 kinase assay (top) and western blot analysis with anti-MAP kinase antibody (bottom). The decrease in H1 kinase activity at 3 hours may reflect the fact that a significant number of oocytes had already passed through metaphase-I, which is observed within 1 hour after GVBD (Ohsumi et al., 1994).

Overexpression of wild-type XCds1 delays entry into M-phase after progesterone treatment

To address whether the downregulation of XCds1 is implicated in the release from the G_2-phase arrest of immature *Xenopus* oocytes, we injected immature oocytes with 10 ng of mRNA for wild-type XCds1 (XCds1-WT), a kinase-deficient XCds1 mutant (XCds1-KD) or LacZ (as a control for mRNA injection). Immunoblots with anti-XCds1 antibody revealed that XCds1-WT and XCds1-KD were expressed at similar levels, which were approximately tenfold higher than those of endogenous XCds1 (Fig. 4A). The electrophoretic mobility of overexpressed XCds1-WT was partially retarded compared to that of overexpressed XCds1-KD, indicating that this modification was dependent on the presence of catalytically active XCds1. After progesterone treatment, oocytes that had been injected with either XCds1-KD or LacZ mRNA underwent GVBD essentially with the same kinetics as uninjected oocytes (Fig. 4B). In contrast, GVBD in oocytes injected with XCds1-WT mRNA occurred about 2 hours later than that in control oocytes (Fig. 4B). Thus, an increased amount of XCds1-WT, but not of XCds1-KD, could significantly delay the meiotic G_2/M-phase transition. At present we do not know the reason why overexpression of XCds1-KD did not exert a dominant-negative effect (i.e. acceleration of GVBD), and accordingly it is unclear whether XCds1 is involved in the G_2-phase arrest of immature oocytes. But the downregulation of XCds1 activity might be implicated in the progression of the meiotic G_2/M-phase transition.

Unlike endogenous XCds1, overexpressed XCds1 was both cytoplasmic and nuclear (Fig. 4C). After the overexpression of XCds1-WT, the GV was removed from immature oocytes (Ford and Gurdon, 1977), and then XCds1 kinase activity was compared between enucleated and nucleated oocytes. Although significant XCds1 kinase activity was detected in the enucleated oocytes, its level was less than that in the nucleated oocytes and was as low as that in the control (uninjected and nucleated) immature oocytes (Fig. 4D). Considering the volume ratio of the GV to the cytoplasm (approx. 3%), these observations support the theory that relative XCds1 activity is much less in the cytoplasm of overexpressed oocytes than in the GV of overexpressed or uninjected oocytes. It is plausible that an increased activity of XCds1 in the GV might be a major cause for delaying GVBD after progesterone treatment, although cytoplasmic XCds1 may also be involved in the delay.

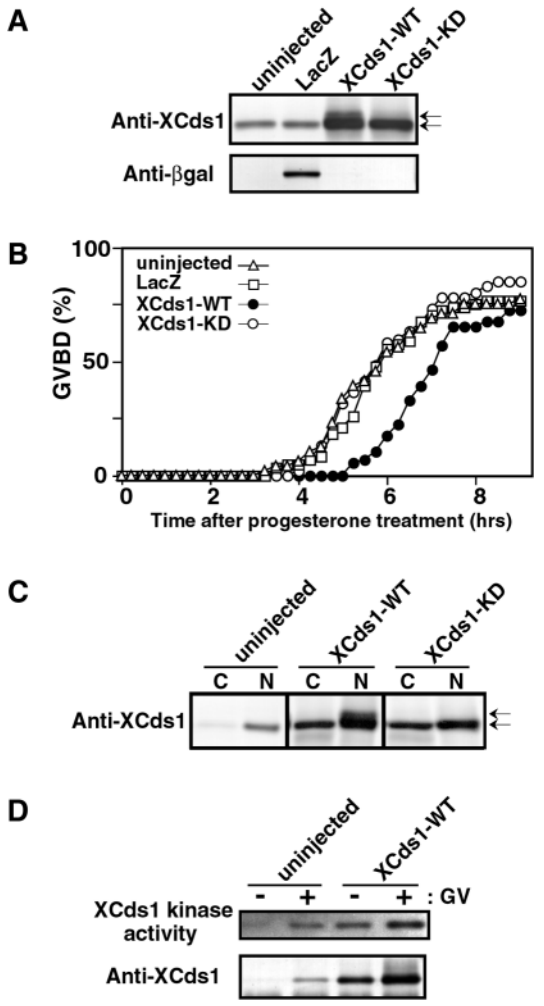

Fig. 4. Overexpression of XCds1 delays meiosis reinitiation. (A) Immature oocytes were uninjected or injected with 10 ng of mRNA for XCds1-WT, XCds1-KD or LacZ and cultured for 12 hours. Western blots show the expression levels of endogenous XCds1 (uninjected and LacZ), introduced XCds1-WT or -KD proteins in addition to endogenous XCds1, and β-galactosidase (βgal) derived from control LacZ mRNA. (B) 12 hours after the injection, immature oocytes of A were treated with progesterone to induce maturation and then scored for the percentage of GVBD at the indicated times. Oocytes were uninjected (open triangles), or injected with mRNA for XCds1-WT (solid circles), XCds1-KD (open circles) or control LacZ (open squares). (C) XCds1-overexpressed immature oocytes were dissected into cytoplasmic (C) and nuclear (N) fractions, followed by western blot analysis with anti-XCds1 antibody. (D) Before or after overexpression of XCds1-WT, the GV was removed from some of the immature oocytes. Then, XCds1 was recovered with immunoprecipitation from enucleated (–) or nucleated (+) oocytes (100 each), and was assayed for kinase activity using GST-XCdc25C(254-316)-WT as a substrate (top). Phosphorylation at Ser287 was detected by immunoblots with anti-phospho human Cdc25C (Ser-216) antibody. Immunoblots with anti-XCds1 indicate protein amounts of XCds1 (bottom).

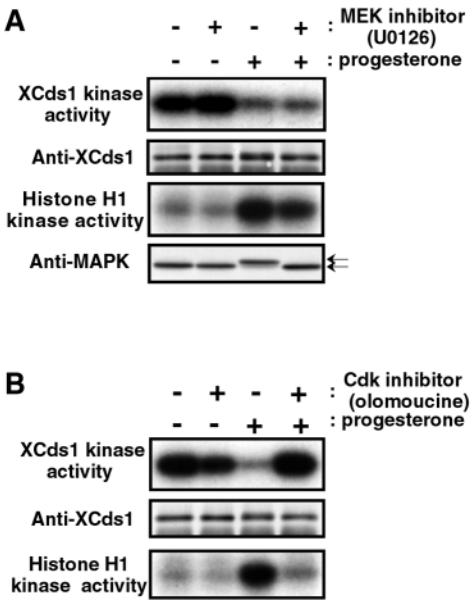

Fig. 5. XCds1 inactivation depends on the activation of cyclin B-Cdc2 kinase but not MAP kinase. (A) Immature oocytes were incubated for 3 hours in the presence of U0126, a MEK inhibitor, and then added with progesterone. Oocytes were collected when GVBD occurred in progesterone-treated oocytes. (B) Immature oocytes were incubated overnight in the presence of olomoucine, a Cdk inhibitor, and then incubated with progesterone. Olomoucine and progesterone-treated oocytes with intact GV were collected when 100% of the control oocytes, which were treated with progesterone alone, had undergone GVBD. XCds1 was immunoprecipitated with anti-XCds1 antibody and assayed for its kinase activity using GST-XCdc25C(254-316)-WT as a substrate (top). Western blot analysis confirmed that equivalent amounts of XCds1 were recovered in the immunoprecipitates with anti-XCds1 antibody (A, middle upper; B, middle). The same extracts were subjected to total histone H1 kinase assay (A, middle lower; B, bottom) and western blot analysis with anti-MAP kinase antibody (A, bottom).

XCds1 inactivation is dependent on cyclin B-Cdc2 kinase activation

To understand how XCds1 is inactivated after progesterone treatment, we compared the timing of XCds1 inactivation with the activation of cyclin B-Cdc2 kinase and MAP kinase, since both kinases are concurrently activated shortly before progesterone-treated oocytes undergo GVBD (Gotoh et al., 1991). The activity of cyclin B-Cdc2 kinase was measured by total histone H1 kinase activity, and the MAP kinase activation was estimated by its electrophoretic retardation due to its phosphorylation. The inactivation of XCds1 coincided with the activation of both kinases (Fig. 3C, compare with Fig. 3A,B). To distinguish the effects of each kinase, we first examined whether XCds1 inactivation was dependent on MAP kinase activation. For this purpose, immature oocytes were incubated for 3 hours in the presence of U0126, a MEK inhibitor, before progesterone addition (see Gross et al., 2000). GVBD in U0126-treated oocytes occurred 1-2 hours later than that in control oocytes treated with progesterone alone, and approximately 80% of U0126-treated oocytes underwent GVBD. Immunoblots for MAP kinase showed that U0126 treatment completely suppressed the electrophoretic retardation of MAP kinase in oocytes which underwent GVBD (Fig. 5A, bottom), whereas the levels of histone H1 kinase activity in the U0126-treated oocytes were almost as high as those in progesterone-treated control oocytes (Fig. 5A, middle lower).

These observations confirm the previous report by Gross et al. (Gross et al., 2000), indicating that after progesterone treatment, cyclin B-Cdc2 kinase is activated even if the activation of MAP kinase is prevented by U0126 treatment. Under these conditions, the level of XCds1 kinase activity in the inhibitor-treated oocytes that had undergone GVBD was as low as that in the control oocytes (Fig. 5A, top and midde upper). Thus, XCds1 was inactivated even in the absence of MAP kinase activation after meiosis reinitiation, indicating that XCds1 inactivation occurs independently of MAP kinase activity.

We next examined whether XCds1 inactivation depends on cyclin B-Cdc2 kinase activation. For this purpose, immature oocytes were incubated for 12 hours in the presence of olomoucine, an Cdk inhibitor (Meijer, 1996), before progesterone was added. The olomoucine-treated oocytes did not undergo GVBD in the following 12 hours. When 100% GVBD was reached in control oocytes treated with progesterone alone, the olomoucine and progesterone-treated oocytes were recovered and their histone H1 kinase activities were compared. The levels of histone H1 kinase activity in the olomoucine-treated oocytes remained just as low as those in untreated immature oocytes, indicating that cyclin B-Cdc2 kinase activation after progesterone treatment is completely prevented by olomoucine (Fig. 5B, bottom). Under these conditions, the levels of XCds1 kinase activity in the inhibitor-treated oocytes were almost as high as in immature oocytes (Fig. 5B, top and middle). Thus, XCds1 was not inactivated after progesterone treatment in the absence of cyclin B-Cdc2 kinase activation, suggesting that XCds1 inactivation depends on cyclin B-Cdc2 kinase activation.

Since XCds1 contains in its kinase domain a consensus sequence, 359TP, for phosphorylation by cyclin B-Cdc2 kinase, it is plausible that XCds1 could be directly inactivated via its phosphorylation by cyclin B-Cdc2 kinase. To examine this possibility, XCds1 that was immunoprecipitated from extracts of immature *Xenopus* oocytes was incubated in vitro with active cyclin B-Cdc2 kinase purified from starfish oocytes (Okumura et al., 1996). As shown in Fig. 6A, XCds1 was indeed phosphorylated in vitro by cyclin B-Cdc2 kinase sufficiently within 30 minutes in the present condition. However, the phosphorylation did not cause the inactivation of XCds1 (Fig. 6B).

Finally, we verified that cyclin B-Cdc2 kinase activation could induce XCds1 inactivation in *Xenopus* oocytes. To this end, immature oocytes were injected with 10 ng of mRNA for *Xenopus* cyclin B1 or control LacZ. Overexpression of cyclin B1, but not that of β-galactosidase (the *LacZ* gene products), caused cyclin B-Cdc2 kinase activation and GVBD without progesterone treatment (Fig. 6C, bottom), as reported previously (Freeman et al., 1991). The levels of XCds1 kinase activity in the cyclin B1-overexpressing oocytes that had undergone GVBD were almost as low as those in progesterone-treated oocytes (Fig. 6C, top and middle), indicating that cyclin B-Cdc2 kinase activation leads to XCds1 inactivation. Taken together, these observations support the theory that XCds1 is indirectly inactivated by cyclin B-Cdc2 kinase during meiosis reinitiation in *Xenopus* oocytes.

An indirect effect of cyclin B-Cdc2 kinase could possibly be mediated by polo-like kinase, Plx1, because at meiosis reinititation in *Xenopus* oocytes Plx1 is concurrently activated with cyclin B-Cdc2 kinase, and a positive feedback regulation is

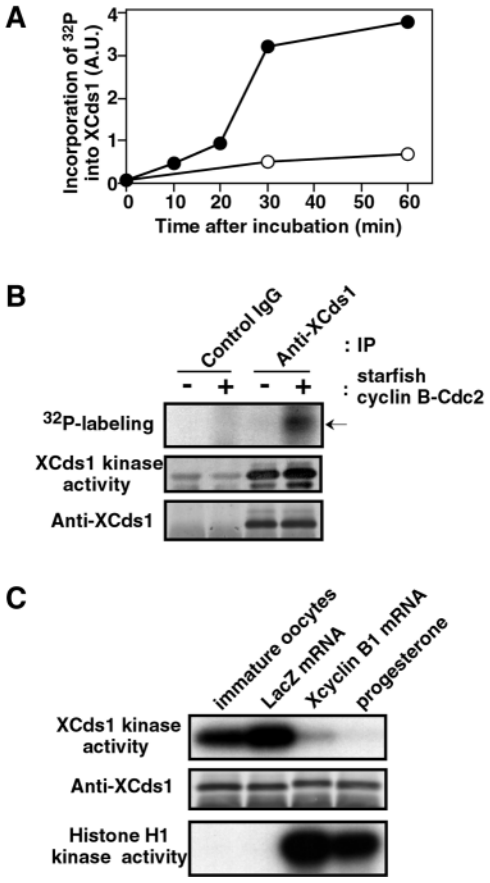

Fig. 6. XCds1 is indirectly inactivated by cyclin B-Cdc2 kinase. (A) Extracts prepared from immature oocytes were immunoprecipitated with control rabbit IgG (open circles) or anti-XCds1 antibody (closed circles). The immunoprecipitates were incubated with starfish cyclin B-Cdc2 kinase. Phosphorylation was measured as a function of incubation time. (B) After 30 minutes incubation with cyclin B-Cdc2 kinase in A, phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography (top). The arrow indicates the position corresponding to XCds1. Western blot analysis confirmed that equivalent amounts of XCds1 were recovered in the immunoprecipitates with anti-XCds1 antibody (bottom). The immunoprecipitates were subjected to XCds1 kinase assay, using GST-XCdc25C(254-316)-WT as a substrate (middle). Phosphorylation at Ser-287 on XCdc25C was visualized by western blot with anti-phospho human Cdc25C (Ser-216) antibody. (C) Immature oocytes were injected with 10 ng of mRNA for LacZ or *Xenopus* cyclin B1. Cyclin B1 mRNA-injected oocytes and progesterone-treated oocytes were collected when they underwent GVBD. XCds1 was immunoprecipitated with anti-XCds1 antibody and assayed for its kinase activity (top). Western blot analysis confirmed that equivalent amounts of XCds1 were recovered in the immunoprecipitates with anti-XCds1 antibody (middle). The same extracts were subjected to total histone H1 kinase assay (bottom).

reported between both kinases (Abrieu et al., 1998; Qian et al., 1998). However, recombinant mouse Plk (gift from Dr N. Watanabe), which is able to phosphorylate α-casein as a control, neither phosphorylated XCds1 recovered from immature *Xenopus* oocytes nor affected its kinase activity (data not shown), excluding the direct effect of Plx1 on XCds1 inactivation.

DISCUSSION

In the present study, we demonstrated that XCds1 is expressed as an active kinase in G_2-phase-arrested immature oocytes of *Xenopus* in the apparent absence of damaged or unreplicated DNA and that XCds1 is inactivated at the meiotic G_2/M-phase transition by a mechanism that is dependent on the activation of cyclin B-Cdc2 kinase, but not of MAP kinase.

Cds1 activity in G_2-phase

Originally, both Chk1 and Cds1 were identified as effector kinases that respond to the G_2 checkpoint activated by damaged or unreplicated DNA (reviewed by Elledge, 1996; Murakami and Nurse, 2000; O'Connell et al., 2000; Zhou and Elledge, 2000). Accordingly, unless the G_2 checkpoint is activated, both Chk1 and Cds1 should be nonessential, as seen in fission yeast (Walworth et al., 1993; Murakami and Okayama, 1995). In mice, nonetheless, targeted gene disruption of *Chk1* or *ATR* results in embryonic lethality as early as the blastocyst stage, or causes a severe proliferation defect in embryonic stem (ES) cells, indicating that ATR-Chk1 is essential in early embryonic development (Brown and Baltimore, 2000; Liu et al., 2000; Takai et al., 2000). Similarly, *Grapes*, a *Drosophila* homolog of *Chk1*, has an essential developmental function during the late stage of syncytial blastoderm divisions and for the MBT that marks the transition from maternal to zygotic control of embryogenesis, since loss of *Grapes* fails to terminate syncytial division after mitosis 13 leading to embryonic degeneration (Fogarty et al., 1997; Sibon et al., 1997; Yu et al., 2000). It has been suggested that during early embryonic development Chk1/Grapes might be required to sense incomplete DNA replication and prevent premature entry into mitosis. It remains unclear, however, whether the embryonic lethality is due to the failure of a cell cycle checkpoint function that requires Chk1 (see Liu et al., 2000). In fact, it has not yet been verified that Chk1 is phosphorylated and activated when it is required in the embryo, as is ordinarily seen at the G_2 checkpoint in response to damaged or incompletely replicated DNA. Rather, in normal human fibroblasts (MJ90) Chk1 is expressed from S- to M-phase without obvious phosphorylation, and is an active kinase at this time in the absence of DNA damage (Kaneko et al., 1999). Furthermore, in immature *Xenopus* oocytes, which apparently lack damaged or unreplicated DNA, Chk1 is involved in the G_2-phase arrest through a form that is apparently distinct from that seen at the activated G_2 checkpoint after the MBT (Nakajo et al., 1999; Oe et al., 2001; compare with Kappas et al., 2000). Together, these observations make it plausible that Chk1 might be implicated in both the G_2-phase control that is dependent on the activated G_2 checkpoint and in one that is independent of a G_2 checkpoint.

In contrast to Chk1, there has been no report that implicates Cds1 in the control of G_2-phase when the G_2 checkpoint is not activated. In this context, our present study provides the first example that Cds1 is active in G_2-phase in the apparent absence of the G_2 checkpoint activation, because the electrophoretic mobility of endogenous XCds1 in immature oocytes was clearly different from that seen at the activated G_2 checkpoint after the MBT (see Fig. 2D). At present, however, we do not know how XCds1 becomes an active kinase in the absence of the G_2 checkpoint activation, although ataxia telangiectasia mutated (ATM), which normally directly phosphorylates and activates Cds1 upon the activation of G_2 checkpoint (Matsuoka et al., 2000; Melchionna et al., 2000), is localized with XCds1 to the GV of immature *Xenopus* oocytes (Robertson et al., 1999). Alternatively, Cds1 might have an intrinsic or basal kinase activity, which would be independent of the activated G_2 checkpoint. Presumably, when the G_2 checkpoint is activated in response to DNA damage or incomplete DNA replication, Cds1 might be hyperphosphorylated and hyperactivated.

Downregulation of Cds1 activity

Our present study is the first demonstration of the downregulation of Cds1 activity at the entry into M-phase. The results indicate that the inactivation of XCds1 depends on the activation of cyclin B-Cdc2 kinase (Fig. 5, Fig. 6). Conversely, it is well understood that the ultimate function of Cds1 is to prevent the activation of cyclin B-Cdc2 kinase (Elledge, 1996; Murakami and Nurse, 2000; O'Connell et al., 2000; Zhou and Elledge, 2000). In accordance with the latter function, overexpression of wild-type XCds1 in immature oocytes caused the delay in the occurrence of M-phase (Fig. 4B). The apparent contradiction between these observations would be reconciled, however, if the intracellular localization of the cyclin B-Cdc2 complex and XCds1 in immature *Xenopus* oocytes were taken into consideration. Nuclear XCds1 (see Fig. 2E) might be involved in preventing the activation of the small amounts of inactive cyclin B-Cdc2 kinase that enter the germinal vesicle from the cytoplasm, where the majority of cyclin B-Cdc2 kinase is found in immature oocytes (Gautier and Maller, 1991). Progesterone stimulation leads to the initial activation of cyclin B-Cdc2 kinase that occurs in the cytoplasm (see Oe et al., 2001). Once activated, cyclin B-Cdc2 kinase may accumulate in the nucleus just before GVBD (Pines, 1999; see also Ookata et al., 1992 in starfish oocytes), and the increase in the amount of active nuclear cyclin B-Cdc2 kinase could cause the inactivation of nuclear XCds1. Thus, once the activity of XCds1 begins to decrease, the activation of cyclin B-Cdc2 kinase might be enhanced through a feedback control that results in abrogation of the XCds1 activity (Fig. 7).

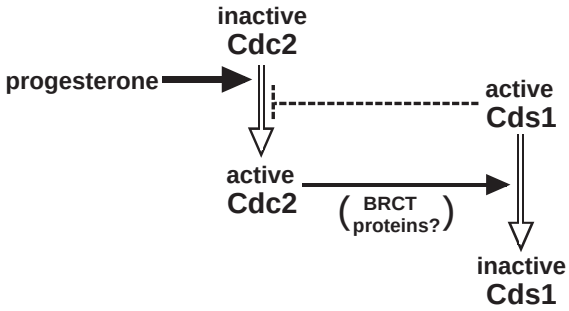

Fig. 7. Model for mechanism of XCds1 inactivation at meiotic G_2/M-phase transition in *Xenopus* oocytes. Although it remains unclear whether XCds1 is actually involved in G_2-phase arrest of immature oocytes (dashed line), progesterone might overcome or bypass its effect to induce the initial activation of cyclin B-Cdc2 kinase, leading to the downregulation of XCds1. The inactivation of XCds1 would presumably be accomplished through phosphorylation of *Xenopus* functional homolog of BRCT protein by cyclin B-Cdc2 kinase.

How XCds1 is inactivated by cyclin B-Cdc2 kinase? A hint is provided by the mechanism of the Cdc2 kinase-dependent downregulation of Chk1 that is seen at the recovery from DNA damage checkpoint in fission yeast. Phosphorylation of Chk1 upon DNA damage in fission yeast requires Crb2, which contains a domain homologous to the C terminus of BRCA1, termed BRCT protein (Saka et al., 1997; O'Connell et al., 2000; Zhou and Elledge, 2000). BRCT protein is thought to play a role in modulating protein-protein interactions, thereby helping the recruitment of downstream effector kinases (Chk1 in fission yeast) to sensor kinases (Rad3 in fission yeast) (O'Connell et al., 2000; Zhou and Elledge, 2000). Cells having a mutant form of Crb2 that cannot be phosphorylated at Thr215 in a consensus phosphorylation site by Cdc2 kinase can enter the arrested state in response to DNA damage but fail to reenter the cell cycle after DNA damage is repaired (Esashi and Yanagida, 1999). Thus, phosphorylation of Crb2 at Thr215 by Cdc2 kinase might shut down the recruitment of Chk1 to Rad3, leading to the inactivation of Chk1. In the checkpoint response of *Xenopus* egg extracts, Claspin is required for the phosphorylation and activation of XChk1 by binding to it, indicating that Claspin is functionally similar to BRCT proteins (Kumagai and Dunphy, 2000). The functions of both human Cds1 and budding yeast Rad53 are modulated through BRCT proteins, BRCA1 and Rad9, respectively, in the DNA damage response (Sun et al., 1998; Lee et al., 2000). Taken together, it is plausible that the Cdc2-dependent downregulation of XCds1 would also be accomplished through the phosphorylation of a functional *Xenopus* homolog of BRCT protein, or possibly Claspin, by cyclin B-Cdc2 kinase (Fig. 7).

In conclusion, Cds1 has so far been studied only in the context of the checkpoint response that is activated by damaged or unreplicated DNA. By contrast, the present study demonstrates that Cds1 is also active in immature *Xenopus* oocytes that are physiologically arrested at G_2-phase, apparently in the absence of checkpoint activation, and that Cds1 is inactivated at entry into M-phase by an indirect mechanism that depends on the activation of cyclin B-Cdc2 kinase. The implication is that Cds1 might be involved in the negative regulation of the G_2/M-phase transition through two mechanisms, one that is dependent upon the activated G_2 checkpoint and the other that is independent of it. In both, the cyclin B-Cdc2 kinase-dependent inactivation of Cds1 might enhance the activation of cyclin B-Cdc2 kinase for entry into M-phase.

We thank Drs Noriyuki Sagata, Tim Hunt, Nobumoto Watanabe, Mari Iwabuchi and Eiichi Okumura for reagents, Kazunori Tachibana and Takayuki Okano-Uchida for discussion, and Manfred J. Lohka for critical reading of the manuscript. This study is supported by grants from Ministry of Education, Science, Sports and Culture, Japan, the CREST Research Project of Japan Science and Technology Corporation and the Human Frontier Science Program to T.K.

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