Regulation of Cdc2/Cyclin B Activation in *Xenopus* Egg Extracts via Inhibitory Phosphorylation of Cdc25C Phosphatase by Ca^{2+} /Calmodulin-dependent Kinase II

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Activation of Cdc2/cyclin B kinase and entry into mitosis requires dephosphorylation of inhibitory sites on Cdc2 by Cdc25 phosphatase. In vertebrates, Cdc25C is inhibited by phosphorylation at a single site targeted by the checkpoint kinases Chk1 and Cds1/Chk2 in response to DNA damage or replication arrest. In *Xenopus* early embryos, the inhibitory site on Cdc25C (S287) is also phosphorylated by a distinct protein kinase that may determine the intrinsic timing of the cell cycle. We show that S287-kinase activity is repressed in extracts of unfertilized *Xenopus* eggs arrested in M phase but is rapidly stimulated upon release into interphase by addition of Ca^{2+} , which mimics fertilization. S287-kinase activity is not dependent on cyclin B degradation or inactivation of Cdc2/cyclin B kinase, indicating a direct mechanism of activation by Ca^{2+} . Indeed, inhibitor studies identify the predominant S287-kinase as Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). CaMKII phosphorylates Cdc25C efficiently on S287 in vitro and, like Chk1, is inhibited by 7-hydroxystaurosporine (UCN-01) and debromohymenialdisine, compounds that abrogate G2 arrest in somatic cells. CaMKII delays Cdc2/cyclin B activation via phosphorylation of Cdc25C at S287 in egg extracts, indicating that this pathway regulates the timing of mitosis during the early embryonic cell cycle.

INTRODUCTION

Our understanding of how cell cycle phase transitions are regulated has been greatly assisted by studies of the embryonic cell cycle and the use of cell-free systems made from *Xenopus laevis* eggs. Freshly laid *Xenopus* eggs are kept arrested in metaphase of meiosis II due to an activity known as cytostatic factor (CSF), which maintains a high activity of Cdc2/cyclin B protein kinase, the M phase-promoting factor (MPF) (Masui and Markert, 1971). Fertilization of an egg by a sperm triggers an intracellular wave of Ca^{2+} ions (Busa and Nuccitelli, 1985; Kubota *et al.*, 1987) and release from CSF arrest. This switches on the ubiquitin-conjugating complex known as the anaphase-promoting complex/cyclosome (APC/C) (King *et al.*, 1995; Sudakin *et al.*, 1995) that targets proteins such as cyclin B (Glutzer *et al.*, 1991; King *et al.*, 1995) for degradation by the proteasome. Consequently, Cdc2 kinase is inactivated and exit from M phase occurs. CSF-arrested egg extracts made by including Ca^{2+} -chelators during preparation contain active Cdc2/cyclin B and can assemble mitotic spindles around added chromatin (Murray, 1991). Addition of exogenous Ca^{2+} to these extracts (Lohka and Maller, 1985; Murray *et al.*, 1989) inactivates CSF, triggers cyclin B degradation, and causes exit from M phase in vitro. The inactivation of CSF by Ca^{2+} requires the activation of the multifunctional Ca^{2+} /calmodulin-dependent

protein kinase II (CaMKII) (Lorca *et al.*, 1993; Morin *et al.*, 1994), which presumably phosphorylates proteins involved in the regulation of the APC/C (Reimann and Jackson, 2002).

After fertilization, there is a prolonged interphase of 75–90 min during which cyclin B is gradually synthesized before entry into the first mitosis (Murray *et al.*, 1989). The mechanism controlling entry into mitosis can be studied in interphase egg extracts in which MPF activation is initiated by the addition of cyclin B in the form of either mRNA or purified protein (Minshull *et al.*, 1989; Murray and Kirschner, 1989; Solomon *et al.*, 1990). In addition to a requirement for a threshold level of cyclin B, the timing of MPF activation is determined by the phosphorylation state of the complex. Phosphorylation of T161 on Cdc2 is essential for activity, but the kinase is kept inactive during interphase by dominant inhibitory phosphorylation at T14 and Y15, catalyzed by Myt1 and Wee1 kinases. The final activation step is the removal of these inhibitory phosphates by the dual-specificity protein phosphatase Cdc25 (Dunphy, 1994).

Cdc25 is itself highly regulated by reversible phosphorylation. In mitosis, Cdc25C is activated by phosphorylation at multiple sites (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992) catalyzed by the polo-like kinase Plx1 (Kumagai and Dunphy, 1996) and by Cdc2/cyclin B itself (Hoffmann *et al.*, 1993; Izumi and Maller, 1993), the latter creating a feedback mechanism that promotes the rapid activation of Cdc2/cyclin B during entry into mitosis. During interphase and in response to checkpoint stimuli, Cdc25C is phosphorylated on an inhibitory serine residue (S287 in *Xenopus*, S216 in human), which promotes the binding of a 14-3-3 protein

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(Peng *et al.*, 1997; Kumagai *et al.*, 1998b). Mutation of this serine to a nonphosphorylatable alanine shows that this phosphorylation site is critical for determining the length of interphase and for restraining mitotic initiation in response to checkpoint stimuli in both *Xenopus* egg extracts (Kumagai *et al.*, 1998b) and mammalian cells (Peng *et al.*, 1997). Enzymes that catalyze the phosphorylation of S287/S216 include the checkpoint kinases Chk1 and Cds1/Chk2, which in vertebrates are activated in response to replicating DNA and double-stranded DNA breaks, respectively (Sanchez *et al.*, 1997; Kumagai *et al.*, 1998a; Guo and Dunphy, 2000; Michael *et al.*, 2000). Phosphorylation by Chk1 or Cds1/Chk2 down-regulates Cdc25C by inhibiting its catalytic activity (Blasina *et al.*, 1999; Furnari *et al.*, 1999). In addition, 14-3-3 protein binding to the phosphorylated site has the dual roles of preventing the nuclear translocation of Cdc25C (Dalal *et al.*, 1999; Kumagai and Dunphy, 1999; Lopez-Girona *et al.*, 1999) and inhibiting the dephosphorylation of the site by a type-2A protein phosphatase (Hutchins *et al.*, 2002).

DNA damage checkpoints, however, do not normally operate in the early embryonic cell cycles in *Xenopus* without high concentrations of exogenous DNA (Dasso and Newport, 1990). Furthermore, removal of both Chk1 and Cds1/Chk2 from *Xenopus* egg extracts has been reported to reduce S287-kinase activity by only 30% (Guo and Dunphy, 2000), indicating the existence of additional protein kinase(s) that keep S287 phosphorylated during interphase and thereby determine the normal timing of entry into mitosis. In this article, we show that S287 phosphorylation is repressed in M-phase (CSF-arrested) *Xenopus* egg extracts and is activated upon release into interphase by the addition of Ca^{2+} . Kinase activity toward S287 of Cdc25C in interphase extracts is Ca^{2+} dependent and attributable to CaMKII. Phosphorylation of S287 is catalyzed efficiently by purified CaMKII and is inhibited by compounds that also inhibit Chk1 and overcome G2 arrest. Activation of CaMKII by Ca^{2+} delays Cdc2 activation by inhibiting Cdc25C via phosphorylation of S287. Together, these data identify a novel pathway by which the timing of mitosis is regulated by CaMKII via inhibitory phosphorylation of Cdc25C.

MATERIALS AND METHODS

Proteins and General Reagents

All chemicals were from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. Full-length *Xenopus* Cdc25C was polymerase chain reaction-amplified from the cDNA clone (Izumi *et al.*, 1992) with the addition of an N-terminal His₆-tag and cloned into the pFastBac1 vector (Invitrogen, Carlsbad, CA). His₆-Cdc25C protein was expressed in baculovirus-infected insect cells and purified by metal-affinity chromatography. Glutathione S-transferase (GST)-tagged Cdc25C(271-316) protein and variants were expressed in *Escherichia coli* (Hutchins *et al.*, 2002). GST-hChk1 was produced by baculovirus expression as described previously (Hutchins *et al.*, 2000). Recombinant CaMKII enzyme (New England Biolabs, Beverly, MA) was activated by prior incubation with 2 mM CaCl_2 , 1.2 μM calmodulin and 100 μM ATP in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , 2 mM dithiothreitol at 30°C for 10 min. CaMKII(281-309) peptide, CaMK IINtide, and debromohymenialdisine (DBH) were from Calbiochem (San Diego, CA). 1,2-bis(*o*-Amino-5-bromophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (Br_2BAPTA) was from Molecular Probes (Eugene, OR). Tautomycin was from BIOMOL Research Laboratories (Plymouth Meeting, PA). 7-Hydroxystaurosporine (UCN-01) was a gift from Dr. R.J. Schultz (Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD); a 10 mM stock solution was prepared in dimethyl sulfoxide. Poly(dT)₇₀ and poly(dA)₇₀ oligonucleotides synthesized and purified by MWG Biotech were annealed as described previously (Guo and Dunphy, 2000).

Peptide Synthesis and Anti-phospho-S287 Antibody Production

The SPS peptide (RLYRSPSMPEKLDRLK) derived from residues 281–294 of *Xenopus* Cdc25C, the variant peptides APS and SPA, the phosphopeptides

S*PS and SPS* (where S* is pSer), and the destruction box (D-box) peptide (RRALGDVTKVSE) were synthesized and purified by Dr. G. Bloomberg (University of Bristol, Bristol, United Kingdom). The AMARA peptide (AMARAASAAALARRR) was synthesized by Chiron (Victoria, Australia). To generate the α -pS287 antibody, the SPS* peptide was coupled to bovine serum albumin and used to raise a rabbit polyclonal antiserum by a commercial facility (Moravian Biotechnology, Brno, Czech Republic). The antiserum was then affinity purified on a column containing immobilized SPS* phosphopeptide.

Xenopus Egg Extracts

Interphase extracts of *X. laevis* eggs prepared as 10,000 $\times$ *g* supernatants by crushing eggs in the absence of calcium chelators (Murray, 1991) were supplemented with 5% (vol/vol) glycerol, snap-frozen in 100- μl aliquots, and stored in liquid nitrogen. CSF-arrested egg extracts were prepared with the addition of EGTA to buffers (Murray, 1991) and were used without freezing. In experiments where mitosis was induced in interphase egg extract, 2 μM *Arbacia punctulata* cyclin B Δ 90 protein (Glotzer *et al.*, 1991) was added to egg extract containing 10 $\mu\text{g}/\text{ml}$ cycloheximide in a total volume of 10 μl . Samples of extract (1 μl) were then taken at various times for measurement of Cdc2/cyclin B kinase activity by using histone H1 as substrate (Clarke, 1995). Where kinase activity was measured in CSF extract, samples (2 μl) were frozen in liquid nitrogen for assay later.

Western Blotting

Proteins were separated on 12% polyacrylamide gels and transferred to nitrocellulose. Blots were blocked with phosphate-buffered saline plus 0.1% (vol/vol) Tween 20 (PBST) containing 5% (wt/vol) milk powder (PBSTM) for 30 min at room temperature and probed for 60 min with primary antibodies α -pS287 (rabbit) or α -PSTAIR (mouse monoclonal; Sigma-Aldrich), diluted 1:1000 in PBSTM. After washing extensively in PBST, the blots were probed by horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG (Amersham Biosciences, Piscataway, NJ) (1/1000 dilution in PBSTM, 60 min), extensively washed with PBST, and developed by chemiluminescence.

Phosphorylation of the S287 Residue of Cdc25C in Xenopus Egg Extracts

GST-Cdc25C(271-316) protein (100 $\mu\text{g}/\text{ml}$) was added to interphase *Xenopus* egg extract diluted 1:10 in buffer AM (20 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol, 10 mM MgCl_2) plus 1 mM ATP, 1 mg/ml bovine serum albumin, 1 mM benzamide, and 0.1 mM phenylmethylsulfonyl fluoride. Samples of extract (1 μl) were taken at the desired time, added to 15 μl of 2 $\times$ SDS-PAGE sample buffer, and analyzed by Western blotting with the α -pS287 antibody.

Protein Kinase Assays

GST-Cdc25C(271-316) protein was phosphorylated in a reaction containing 1 pmol of kinase, in buffer AM plus 100 μM [γ -³²P]ATP (approx. 7.4 kBq/nmol), at room temperature for 15 min. Full-length His₆-Cdc25C protein was first dephosphorylated by protein phosphatase 2A (PP2A; Upstate Biotechnology, Lake Placid, NY) for 60 min at 30°C, and then 1 μM okadaic acid (Calbiochem) was added to inhibit the phosphatase before incubation with CaMKII for a further 60 min. Phosphorylated proteins were separated on SDS-12% PAGE gels and analyzed by autoradiography or Western blotting. Peptides (100 μM) were phosphorylated under the same conditions, except where specified, and analyzed as described previously (Hutchins *et al.*, 2000). Peptide kinase assays were carried out in duplicate. In experiments examining the effect of UCN-01 and DBH, reactions also contained 10% (vol/vol) dimethyl sulfoxide.

In Vitro Expression of Xenopus Cdc25C, Cds1, Chk1 Δ KD, and Cyclin B1

Full-length *Xenopus* Cdc25C cDNA was subcloned into the pcDNA3.1 vector (Invitrogen). The S287A (SPA) mutant of this clone was generated using the QuikChange site-directed mutagenesis kit from Stratagene. Full-length *Xenopus* Cds1 (*Xcds1*) cDNA was amplified from a *Xenopus* oocyte cDNA library (Nicolás *et al.*, 1997) by PCR, using the primers 5'-CATATG ATGATG TCTCGT GATACT AAAAC-3' and 5'-CTCGAG TTATCT TTTTGC TCTCTT TTTCG-3', and cloned into the pGEM-T Easy vector (Promega). After sequencing, the *Xcds1* cDNA was subcloned into pcDNA3.1. *Xenopus* cyclin B1 cDNA was also subcloned into pcDNA3.1. A cDNA clone encoding amino acids 260–474 (i.e., the C-terminal regulatory domain) of *Xenopus* Chk1 (*Xchk1 Δ KD*) in pET28a was a gift from Dr. W. M. Michael (Harvard University, USA). These plasmids were used to express ³⁵S-labeled proteins using the TnT reticulocyte lysate in vitro transcription/translation system (Promega) in the presence of L-[³⁵S]methionine (Amersham Biosciences) according to the manufacturer's protocol.

Figure 1. Specific recognition of the phospho-S287 residue within *Xenopus* Cdc25C. (A) Location of the S287 residue within full-length Cdc25C, the 271-316 fragment, and peptide. (B) Dot blot of 1 nmol of each peptide (or 1 μ l of buffer), showing specific recognition of phospho-S287 by the α -pS287 antibody. (C) Western blot of GST-Cdc25C(271-316) SPS, APS, and SPA proteins incubated with Chk1 with or without Mg.ATP, as indicated, showing specific recognition of phospho-S287 by α -pS287.

RESULTS

Specific Antibody Recognition of S287 Phosphorylation of Cdc25C

To investigate the phosphorylation status of the S287 residue of *Xenopus* Cdc25C, we raised a polyclonal antiserum against a phosphopeptide derived from this region containing phosphoserine (pSer) at position 287 (Figure 1A) and affinity purified the antibody (α -pS287) on a column containing immobilized phosphopeptide. The purified antibody recognized peptides derived from *Xenopus* Cdc25C in which the residue corresponding to serine-287 was phosphorylated, but did not recognize the unphosphorylated peptide or a peptide in which S285 was phosphorylated (Figure 1B). α -pS287 also recognized pS287 within a GST-tagged fragment of Cdc25C, corresponding to residues 271–316 of the N-terminal regulatory domain [GST-Cdc25C(271-316), SPS protein], which had been phosphorylated on S287 by Chk1 (Figure 1C). Mutation of S287 to nonphosphorylatable alanine (SPA protein) prevented recognition by the antibody, whereas a protein in which S285 is mutated to alanine (APS protein) that is equally well phosphorylated at S287 by Chk1 is recognized. The antibody is therefore specific for the phosphorylated S287 site in *Xenopus* Cdc25C.

Figure 2. Phosphorylation status of the Cdc25C(S287) residue changes during the cell cycle. A CSF-arrested (M-phase) egg extract containing GST-Cdc25C(271-316) SPS protein was induced to cycle by the addition of 0.8 mM CaCl_2 . Top, Cdc2 (histone H1 kinase) activity profile; bottom, α -pS287 Western blot showing phosphorylation status of S287 in endogenous Cdc25C (end.) and exogenous GST-Cdc25C(271-316) (exo.).

Cell Cycle Changes in S287-Kinase Activity in *Xenopus* Egg Extracts

To investigate changes in S287-phosphorylation status between M phase and interphase, a CSF-arrested egg extract was incubated with addition of either buffer (which maintained CSF arrest) or 0.8 mM Ca^{2+} , which induced release into interphase assessed by the decline of Cdc2/cyclin B protein kinase activity (Figure 2). Extracts released from CSF arrest synthesized cyclin B, reactivated Cdc2/cyclin B kinase, and entered mitosis after a 105- to 120-min incubation. Samples were taken at different times for analysis by Western blotting with α -pS287. Although the CSF-arrested extract displayed a very low level of S287-phosphorylation, the extent of S287-phosphorylation of endogenous Cdc25C after Ca^{2+} addition was greatly increased until 75–90 min, when phosphorylation declined before reactivation of Cdc2/cyclin B and entry into mitosis at 105–120 min (Figure 2B, top). This analysis was complicated, however, by the shifting of endogenous Cdc25C to a number of more rapidly migrating forms, consistent with dephosphorylation of mitotic sites (Peng *et al.*, 1997; Kumagai *et al.*, 1998b). To analyze S287 kinase activity more readily, the extracts were supple-

mented with GST-Cdc25C(271-316) (Figure 2, bottom), which was strongly phosphorylated at S287 after Ca^{2+} -induced CSF release. Phosphorylation of S287 declined after 75 min, reaching a nadir at 120 min, corresponding to the peak of Cdc2/cyclin B activity, and then was rephosphorylated as Cdc2/cyclin B activity dropped in the second interphase. This oscillation in S287 phosphorylation suggests that S287-kinase activity is stimulated upon Ca^{2+} -induced release from M-phase exit, is inhibited before the first mitosis and is then reactivated during the second interphase.

S287 Phosphorylation during Mitotic Exit Is Independent of Cyclin B Degradation and Cdc2 Inactivation

The observation that S287 was phosphorylated upon Ca^{2+} -induced CSF release led us to address whether activation of S287-kinase is a consequence of the degradation of cyclin B and loss of Cdc2/cyclin B activity. GST-Cdc25C(271-316) protein was first incubated in a CSF-arrested egg extract (supplemented with ^{35}S -labeled *Xenopus* cyclin B1) for 10 min and then 0.8 mM Ca^{2+} was added to induce M-phase exit, and samples of extract were taken at different times to analyze Cdc2/cyclin B activity, S287 phosphorylation status, and cyclin B1 stability (Figure 3). CSF release caused a steady reduction in histone H1 kinase activity, and there was a corresponding degradation of ^{35}S -cyclin B1 that became noticeable 10 min after addition of Ca^{2+} (20-min incubation in total). However, S287 phosphorylation occurred much more rapidly after Ca^{2+} addition, reaching a level within 1 min that persisted for at least the preceding 40 min.

The activity of the APC/C can be inhibited in a competitive manner by a small protein or peptide containing a D-box motif (Holloway *et al.*, 1993; Peter *et al.*, 2001; Reimann and Jackson, 2002). To determine whether the degradation of cyclin B and inactivation of Cdc2 kinase is required for the phosphorylation of S287, we added a D-box peptide to a CSF extract before the addition of CaCl_2 . When this peptide was present in the extract, the inactivation of Cdc2 and degradation of cyclin B1 induced by Ca^{2+} were blocked (Holloway *et al.*, 1993), but S287 still became phosphorylated (Figure 4). Therefore, inactivation of Cdc2/cyclin B is not required for S287 phosphorylation to occur during mitotic exit, suggesting that activation of S287 phosphorylation is induced independently by Ca^{2+} addition.

S287 Phosphorylation in Interphase Egg Extract Requires CaMKII

One explanation for the rapid phosphorylation of S287 in an egg extract after Ca^{2+} addition could be that the protein kinase that catalyzes the phosphorylation of S287 is directly activated by Ca^{2+} . To test this possibility, we analyzed the effects of manipulating Ca^{2+} levels on kinase activity toward S287 by using GST-Cdc25C(271-316) as a substrate. In a dilute egg extract prepared by crushing eggs in the absence of exogenous Ca^{2+} or Ca^{2+} chelators, the basal level of S287 phosphorylation was almost completely inhibited by the Ca^{2+} -chelating agent Br_2BAPTA , demonstrating that the basal kinase activity is dependent on endogenous Ca^{2+} (Figure 5A). The extent of this activity was somewhat different between extracts, which probably reflects variability in the amount of free Ca^{2+} released from intracellular stores during extract preparation. S287 kinase activity was strongly stimulated by addition of Ca^{2+} . By assessing S287 phosphorylation in an extract with low levels of basal S287 kinase activity, we established that the S287-kinase is very sensitive to Ca^{2+} , its activity being stimulated in the nanomolar to micromolar range (Figure 5B).

Figure 3. Effect of CaCl_2 on CSF-release and on S287 phosphorylation. A CSF-arrested egg extract, supplemented with GST-Cdc25C(271-316) SPS protein and ^{35}S -cyclin B1 was treated with either 0.8 mM CaCl_2 or buffer, as indicated, immediately after the 10-min time point (indicated by arrow). Top, Cdc2 (histone H1 kinase) activity time course; middle, stability of ^{35}S -cyclin B1 analyzed by SDS-PAGE and autoradiography; and bottom, Cdc25C (S287) phosphorylation status analyzed by Western blotting.

A prime candidate for the Ca^{2+} -stimulated S287-kinase in the egg extract is the multifunctional Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), which is present in *Xenopus* oocytes, eggs, and embryonic tissues (Schulman and Greengard, 1978; Stevens *et al.*, 2001; Matsumoto and Maller, 2002). CaMKII is kept inactive in the absence of

Figure 4. CaCl₂ induces Cdc25C(S287) phosphorylation independently of cyclin B degradation. A CSF-arrested egg extract, supplemented with GST-Cdc25C(271-316) SPS protein and ³⁵S-cyclin B1, was treated with either 2 mM D-box peptide (DBP) or buffer at zero time, and either 0.8 mM CaCl₂ or buffer at 10 min, as indicated. Top, Cdc2 (histone H1 kinase) activity time course. Bottom, Cdc25C(S287) phosphorylation status analyzed by Western blotting, and ³⁵S-cyclin B1 stability analyzed by SDS-PAGE and autoradiography.

Ca²⁺/calmodulin by an autoinhibitory domain, which interacts with the ATP-binding and protein substrate-binding sites, blocking the active site of the enzyme (Hudmon and Schulman, 2002). Peptides derived from the autoinhibitory domain of CaMKII specifically inhibit the kinase (Colbran *et al.*, 1988; Payne *et al.*, 1988), allowing the role of this enzyme to be examined in vivo and in cell extracts (Lorca *et al.*, 1993; Matsumoto and Maller, 2002). As shown in Figure 5C, addition of one such peptide, CaMKII(281-309), completely blocked Ca²⁺-stimulated S287-kinase activity in diluted egg extract, demonstrating that this kinase activity is dependent on CaMKII. The majority of S287 kinase activity in a concentrated interphase extract (prepared without exogenous Ca²⁺) was also strongly inhibited by both Br₂BAPTA (Figure 5D) and the CaMKII(281-309) peptide (Figure 5E). An unrelated peptide derived from a CaMKII inhibitor protein (CaMK IIN) that specifically inhibits CaMKII and not closely related Ca²⁺-stimulated kinases such as CaMKI and CaMKIV (Chang *et al.*, 1998) also completely inhibited S287 kinase activity in interphase egg extracts (Figure 5E), showing that CaMKII is required for the phosphorylation of S287 of Cdc25C during interphase.

Figure 5. Characterization of S287-kinase activity in dilute (1:10) (A-C) and concentrated (D and E) interphase egg extract. GST-Cdc25C(271-316) SPS protein was added to the extracts with the following additions: 1 mM Br₂BAPTA or 0.8 mM CaCl₂ (A), the indicated concentration of added CaCl₂ (B), 0.8 mM CaCl₂ or 0.4 mM CaMKII(281-309) peptide (C), 1 mM Br₂BAPTA (D), and 0.4 mM CaMKII(281-309) peptide or CaMK IINtide (E). Samples were removed after 1, 2, and 5 min (A, C, D, and E) or 5 min (B), and S287 phosphorylation was analyzed by Western blotting with α-pS287 antibody.

CaMKII Phosphorylates Cdc25C on S287

Peptide substitution and degenerate peptide library studies have established the minimal sequence motif for phosphorylation by CaMKII as being Φ-X-R-(NB)-X-S/T*-Φ, where Φ is a hydrophobic residue, NB is a nonbasic residue, and X is

Figure 6. Phosphorylation of Cdc25C by CaMKII and Chk1 in vitro. (A) Phosphorylation of recombinant full-length His₆-Cdc25C (100 ng) by CaMKII after treatment with protein phosphatase 2A (PP2A) and then okadaic acid, as indicated. Top, α -pS287 Western blot; bottom, ³²P autoradiograph. (B) Phosphorylation of GST-Cdc25C(271-316) SPS, APS, or SPA protein (1 μ g) by Chk1 or CaMKII (1 pmol). Phosphorylation of GST-Cdc25C(271-316) SPS, APS, or SPA protein (1 μ g) by Chk1 or CaMKII (1 pmol). Top, ³²P autoradiograph; bottom, Coomassie-stained SDS-PAGE gel. (C) Time course of phosphorylation of synthetic peptides (as described in the text) by Chk1 (top) or CaMKII (bottom).

any amino acid (Songyang *et al.*, 1996; White *et al.*, 1998). The amino acid sequence surrounding the S287 residue of *Xenopus* Cdc25C (...RSRLYRSPS²⁸⁷MPEK...) shows a perfect match with the motif for CaMKII as well as that for Chk1 (Hutchins *et al.*, 2000), suggesting that CaMKII targets this residue directly like Chk1.

To test whether S287 is phosphorylated by CaMKII, we produced full-length recombinant *Xenopus* Cdc25C in baculovirus-infected insect cells. The purified protein was already fully phosphorylated on S287, but after dephosphorylation by protein phosphatase-2A, recombinant CaMKII catalyzed the phosphorylation of that site (Figure 6A). CaMKII, like Chk1, also phosphorylated GST-Cdc25C(271-316) determined by incorporation of ³²P-phosphate (Figure 6B). Both SPS and APS variants were phosphorylated equally well by CaMKII, whereas Chk1 preferred the APS protein in which serine 285 is mutated. The SPA protein was not phosphorylated by either kinase, showing their specificity for serine 287.

To compare the rates of S287 phosphorylation by CaMKII and Chk1, we used an in vitro peptide kinase assay (Glass *et al.*, 1978; Hutchins *et al.*, 2000). Identical amounts of purified CaMKII and Chk1 catalyzed phosphorylation of the Cdc25C SPS peptide (RLYRSPSMPEKLDRLK) at very similar rates (Figure 6C). The APS peptide (RLYRAPSMPEKLDRLK) was also phosphorylated well by both kinases. However, the AMARA peptide (AMARAASAAALARRR) that is a minimal substrate for several members of this family of protein kinases (Dale *et al.*, 1995) was a very poor substrate for CaMKII, although it was a good substrate for Chk1, indicating that there are some differences between the substrate specificities of these two kinases (Figure 6C). The SPS peptide kinase activity of CaMKII was absolutely dependent on the presence of Ca²⁺/calmodulin and was completely blocked by the CaMKII(281-309) peptide, whereas Chk1 was not stimulated by Ca²⁺/calmodulin and was only slightly inhibited by this peptide (our unpublished data). Together, these data show that CaMKII can directly phosphorylate the S287 residue of Cdc25C in vitro and is very likely to account for the Ca²⁺-stimulated S287 kinase activity in egg extracts.

Inhibitor Sensitivity of CaMKII and Chk1

Chk1 is inhibited by UCN-01 and DBH, compounds that abrogate G2 checkpoints in mammalian cells (Wang *et al.*, 1996; Busby *et al.*, 2000; Graves *et al.*, 2000; Curman *et al.*, 2001) and overcome DNA damage-induced inhibition of Cdc2/cyclin B activation in *Xenopus* egg extracts (our unpublished data). UCN-01 strongly inhibited CaMKII in peptide kinase assays, being effective at an even lower IC₅₀ value (2.9 nM) than with Chk1 (7.7 nM) (Figure 7A). CaMKII was also inhibited by DBH with an IC₅₀ value of 16.3 μ M, compared with 8.6 μ M for Chk1 (Figure 7B). In egg extracts, Ca²⁺-stimulated S287 kinase activity was partially inhibited by UCN-01 at 1 μ M and blocked by 10 μ M, whereas DBH inhibited at 1 mM (Figure 7C). Thus, although these compounds do not distinguish between Chk1 and CaMKII in egg extracts, the results are consistent with the predominant Ca²⁺-activated S287 kinase activity being CaMKII. It is likely that higher concentrations are required for inhibition in extracts than with purified enzymes due to binding to other targets and a higher, competing ATP concentration (1 mM).

Ca²⁺-induced Inhibition of Cdc2/Cyclin B Activation via Targeting of S287 on Cdc25C by CaMKII

To determine whether CaMKII plays a role in the timing of Cdc2/cyclin B kinase activation and entry into mitosis, we

Figure 7. Inhibition of CaMKII, Chk1, and S287 kinase activity in egg extracts by UCN-01 and DBH. Sensitivity of purified Chk1 and CaMKII, assayed using a Cdc25C-peptide substrate, to UCN-01 (A) and DBH (B). (C) Inhibition of S287-kinase activity toward GST-Cdc25C SPS-protein in dilute (1:10) interphase egg extract to UCN-01 and DBH. Samples were removed after 5-min incubation, and S287 phosphorylation was analyzed by Western blotting with α -pS287 antibody.

used interphase *Xenopus* egg extracts containing cycloheximide, which prevents synthesis of endogenous cyclins. Cdc2 kinase activity and migration on SDS-PAGE were monitored after addition of recombinant cyclin B (cyclin B Δ 90) that lacks the D-box sequence by which it is targeted for degradation (Solomon *et al.*, 1990; Glotzer *et al.*, 1991). Consistent with previous results (Solomon *et al.*, 1990; Clarke *et al.*, 1992), cyclin B Δ 90 caused the transient upshift of Cdc2 on SDS-PAGE after 15-min incubation due to phosphorylation at T14 and Y15 (Figure 8). After 30-min incubation, Cdc2 became activated, and there was a corresponding increase in mobility on SDS-PAGE due to dephosphorylation of T14 and Y15 by Cdc25C. Phosphorylation at T161 caused the active form of Cdc2 to migrate slightly faster than the non-phosphorylated form. As expected, addition of vanadate ions, which inhibit Cdc25C, prevented activation of Cdc2/cyclin B Δ 90 and caused accumulation of the upshifted, T14/Y15-phosphorylated form of Cdc2 when cyclin B Δ 90 was present.

When added simultaneously with the cyclin, 0.8 mM Ca^{2+} blocked Cdc2/cyclin B Δ 90 activation and caused accumulation of the slower migrating form of Cdc2 phosphorylated on T14/Y15. The block caused by Ca^{2+} (but not vanadate) was overcome by addition of the CaMKII(281-309) peptide with corresponding dephosphorylation of T14/Y15 (Figure 8). Similarly, inhibition of Cdc2/cyclin B Δ 90 activation by Ca^{2+} was relieved by UCN-01 (our unpublished data). Thus, Ca^{2+} acts via CaMKII to inhibit the dephosphorylation of T14/Y15 on Cdc2 by Cdc25C, thereby blocking Cdc2/cyclin B Δ 90 activation. The inhibitory effect of Ca^{2+} was partially reversed by addition of full-length Cdc25C in which serine 287 was mutated to alanine (SPA), but not wild-type (SPS) Cdc25C (Figure 9). CaMKII therefore inhibits Cdc2/cyclin B activation through the inhibition of Cdc25C by phosphorylation at S287.

Given that the protein kinases Chk1 and Cds1/Chk2 have already been identified as targeting S287 of Cdc25C, the formal possibility remained that CaMKII activated by Ca^{2+} increased S287 phosphorylation in extracts by acting through these kinases. Both Chk1 (Walworth and Bernards, 1996; Kumagai and Dunphy, 2000; Michael *et al.*, 2000) and Chk2/Cds1 (Matsuoka *et al.*, 1998; Guo and Dunphy, 2000) undergo a characteristic phosphorylation and retardation in mobility on SDS-PAGE, in response to the activation of DNA structure checkpoints. As shown previously (Guo and Dunphy, 2000; Kumagai and Dunphy, 2000), oligonucleotides that generate a checkpoint signal in *Xenopus* egg extracts caused a retardation in the mobility of Chk1 and Cds1 (Figure 10). In contrast, 0.8 mM Ca^{2+} , which also produced phosphorylation of Cdc25C on S287 (Figure 5) and delayed Cdc2/cyclin B activation (Figure 8), failed to induce phosphorylation of Chk1 or Cds1. Thus, CaMKII does not act via inhibition of Chk1 or Cds1, indicating that these kinases function in distinct pathways that converge on the phosphorylation and inhibition of Cdc25C.

DISCUSSION

The timing of mitosis is controlled by the abundance of cyclin B and changes in the phosphorylation status of the Cdc2/cyclin B complex, determined by the activities of protein kinases and phosphatases toward the Cdc2 and cyclin B subunits. These posttranslational mechanisms permit control by positive and negative feedback pathways that are regulated by extracellular hormonal signals, for instance during oocyte maturation; by checkpoints activated by DNA

Figure 8. Inhibition of Cdc2/cyclin B activation by Ca^{2+} . Top, time course of Cdc2 (histone H1 kinase) activation initiated by addition of 2 μM cyclin B $\Delta 90$ ($\Delta 90$) to interphase egg extract, with further additions of 0.8 mM CaCl_2 (Ca), 0.4 mM CaMKII(281-309) peptide (pep), and 1 mM Na vanadate (van), as indicated. Bottom, analysis of the phosphorylation status of Cdc2 in samples of extract taken at the indicated time points, analyzed by SDS-PAGE and Western blotting with a α -PSTAIR antibody. The major band is p34^{cdc2}, which shifts up when phosphorylated on the inhibitory sites T14 and Y15, and shifts down when it is phosphorylated on T161 and activated. The lower weak reactive band is p33^{cdk2}, which does not form a complex with cyclin B $\Delta 90$ (Solomon *et al.*, 1990; Clarke *et al.*, 1992).

Figure 9. Rescue of the inhibition of the Ca^{2+} -induced inhibition of Cdc2/cyclin B activation by a Cdc25C mutant lacking the inhibitory phosphorylation site. (A) Time course of Cdc2 (histone H1 kinase) activation initiated by addition of 2 μM cyclin B $\Delta 90$ ($\Delta 90$) to interphase egg extract in the presence of 0.8 mM CaCl_2 with addition of in vitro translated Cdc25C S287A (SPA) or wild-type Cdc25C (SPS). Control incubations contained reticulocyte lysate (1/10th total volume) lacking recombinant Cdc25C.

damage, replication arrest, and probably other stress signals; and by intrinsic timing mechanisms that determine the period of cell cycle oscillations in an early embryo such as that of *Xenopus* (Murray, 1992). A critical event in the activation

Figure 10. Activation of Chk1 and Cds1/Chk2 in egg extract by DNA oligonucleotides, but not by CaCl_2 . Autoradiographs show the migration on SDS-PAGE of ³⁵S-labeled, in vitro-translated Xcds1 or Xchk1 Δ KD incubated for the indicated time in interphase egg extract containing 50 ng/ μl poly(dA)₇₀:poly(dT)₇₀ DNA oligonucleotide duplex (DNA oligo) or 0.8 mM CaCl_2 . Incubations with Xchk1 Δ KD also contained 3 μM tautomycin (Kumagai and Dunphy, 2000).

of Cdc2/cyclin B is the dephosphorylation of inhibitory sites on Cdc2 by Cdc25 phosphatase. Cdc25C is activated in mitosis by phosphorylation at multiple sites, but phosphorylation at a single site (S287 in *Xenopus* Cdc25C) restrains its activation during interphase. This study describes changes in the phosphorylation status of S287 of Cdc25C in *Xenopus* egg extracts and identifies the major kinase acting on this site in interphase extracts as CaMKII. Phosphorylation and inhibition of Cdc25C by CaMKII inhibits Cdc2/cyclin B activation during the first interphase and may play a role in the intrinsic timing of the cell cycle in the early embryo.

Dual Control of Cdc25C Inhibition and Cyclin B Degradation by CaMKII

Release of *Xenopus* eggs from CSF arrest in meiosis II into interphase upon fertilization involves an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and activation of CaMKII. In CSF-arrested egg extract prepared in the presence of the Ca^{2+} chelator EGTA, cyclin B is stable, but addition of Ca^{2+} triggers CaMKII activation (Matsumoto and Maller, 2002), activation of APC/C (King *et al.*, 1995; Sudakin *et al.*, 1995), and cyclin B polyubiquitination and degradation (Glotzer *et al.*, 1991), resulting in inactivation of Cdc2 kinase (MPF) (Lorca *et al.*, 1993). Although the mechanism by which CaMKII activates the APC/C is not clear, it may involve the inactivation of Emi1, which suppresses the APC/C activator Cdc20 during CSF arrest (Reimann and Jackson, 2002). In these experiments, a high concentration of CaCl_2 (typically 0.4–0.8 mM) was added, but the Ca^{2+} -buffering capacity of concentrated extracts is such that the free $[\text{Ca}^{2+}]$ is only in the micromolar range. This free $[\text{Ca}^{2+}]$ is sufficient to activate CaMKII, but not general proteases activated by Ca^{2+} (Lorca *et al.*, 1991, 1993; Lindsay *et al.*, 1995; Matsumoto and Maller, 2002), and is comparable to that generated in vivo after fertilization of the egg (Busa and Nuccitelli, 1985; Kubota *et al.*, 1987; Lindsay *et al.*, 1995).

We have found that activation of CaMKII under these conditions also causes phosphorylation of Cdc25C at the inhibitory site, S287. Indeed, CaMKII is the predominant kinase acting on this site in interphase egg extracts. Together with dephosphorylation of the activating phosphorylation sites on Cdc25C (Kumagai and Dunphy, 1992) and activation of the opposing Myt1 and Wee1 kinases (which target the inhibitory T14 and Y15 residues on Cdc2) (Mueller *et al.*, 1995a,b) on exit from M phase, this mechanism would prevent premature reactivation of Cdc2/cyclin B kinase as cyclin B is synthesized and associates with Cdc2 during the subsequent interphase. Thus, both cyclin B degradation and inhibition of Cdc25C are coupled by activation of CaMKII upon release from CSF arrest by Ca^{2+} (Figure 11A).

Inhibition of Cdc2/cyclin B Activation by CaMKII-mediated S287 Phosphorylation

The phosphorylation of S287 of Cdc25C upon release into interphase is maintained in cycling egg extracts until just before Cdc2/cyclin B activation and entry into mitosis. Phosphorylation of S287 is very low during mitosis and is reactivated in the subsequent interphase, albeit less strongly than after the initial addition of Ca^{2+} . This oscillation in phosphorylation status of S287 in extracts closely matches the situation in vivo in fertilized *Xenopus* embryos (Duckworth *et al.*, 2002). Phosphorylation of S287 in extracts persists for 75–90 min after release from CSF arrest, even after $[\text{Ca}^{2+}]$ is likely to have dropped to basal levels due to uptake into organelles and CaMKII activity has declined (Matsumoto and Maller, 2002). It is probable that the stability of S287

Figure 11. Models for the role of CaMKII in the control of Cdc25C. (A) Dual regulation of APC/C activation and Cdc25C inhibition in *Xenopus* eggs upon release from arrest in M phase. Fertilization induces an increase in $[\text{Ca}^{2+}]_i$ and activates CaMKII. This causes the activation of the APC/C by overcoming the inhibition of the Cdc20 subunit by Emi1. Cyclin B is then polyubiquitinated and targeted for degradation by the proteasome. CaMKII also promotes the S287-phosphorylation and inhibition of Cdc25C, thereby inhibiting reactivation of Cdc2/cyclin B during the after interphase. (B) Phosphorylation of S287 on Cdc25C by multiple protein kinases integrates inhibitory signals that delay mitotic initiation.

phosphorylation is due to the binding of a 14-3-3 protein to the phosphorylated site, inhibiting its dephosphorylation by protein phosphatase 2A (Hutchins *et al.*, 2002). The mechanism determining the precise timing of S287 dephosphorylation before mitosis therefore remains unclear, but it may involve inhibition of 14-3-3 binding activity as well as inactivation of CaMKII.

Addition of Ca^{2+} to interphase extracts delays Cdc2/cyclin B activation, and this inhibition is overcome by Cdc25C in which the S287 phosphorylation site is abolished, indicating that S287 phosphorylation by CaMKII is required for the effect of Ca^{2+} . Inhibition of Cdc2/cyclin B activation and entry into mitosis by CaMKII in egg extracts is consistent with the induction of G2 arrest in cultured mammalian cells (Planas-Silva and Means, 1992) and in the fission yeast *Schizosaccharomyces pombe* (Rasmussen and Rasmussen, 1994) by overexpression of constitutively active forms of mammalian CaMKII. A similar response is produced by overexpression in *S. pombe* of the CaMKII homolog Cmk2, an oxidative stress response kinase (Alemany *et al.*, 2002; Sánchez-Piris *et al.*, 2002). Inhibition of Cdc25C by CaMKII may provide a mechanism to restrain entry into mitosis in vertebrate cells when Ca^{2+} is elevated by extracellular signals or under stress conditions (Berridge *et al.*, 1998).

Additional Roles for Ca^{2+} and CaMKII during the Cell Cycle in *Xenopus*

The observation of oscillations in $[\text{Ca}^{2+}]_i$ and CaMKII activity in dividing *Xenopus* embryos (Grandin and Charbonneau, 1991; Kubota *et al.*, 1993; Keating *et al.*, 1994) and cycling egg extracts (Tokmakov *et al.*, 2001; Matsumoto and Maller, 2002) is consistent with functions for CaMKII during the early embryonic cell cycles. As well as restraining mitotic entry at elevated Ca^{2+} concentrations, CaMKII may have other roles in the control of Cdc2/cyclin B activity. Studies in *Xenopus* have previously found that chelation of free Ca^{2+} by Br_2BAPTA or addition of a CaMKII-inhibitor peptide slightly delays entry into mitosis in cycling egg extracts, suggesting a positive function for Ca^{2+} and CaMKII in Cdc2/cyclin B activation. Ca^{2+} may be required for cyclin B synthesis in these extracts, but there also seems to be an additional role that is independent of protein synthesis (Lindsay *et al.*, 1995). Consistent with this conclusion, we found that a CaMKII inhibitor peptide (Figure 8) or Br_2BAPTA (our unpublished data) reduced the level of Cdc2/cyclin B activity attained after addition of cyclin B $\Delta 90$ to a cycloheximide-treated egg extract. In addition to phosphorylation of S287, purified CaMKII has been reported to phosphorylate Cdc25C on multiple sites in vitro, causing a modest activation of the enzyme (Izumi and Maller, 1995; Patel *et al.*, 1999). Although these sites are clearly not phosphorylated in *Xenopus* egg extracts during interphase (Izumi and Maller, 1995), it is possible that CaMKII might also play a positive role in maintaining Cdc25C activity during mitosis when phosphatase activity toward activating phosphorylation sites on Cdc25C is suppressed (Clarke *et al.*, 1993). CaMKII also plays a role in the inactivation of Cdc2/cyclin B in prophase extracts that cycle through mitosis, in part, through inhibition of cyclin B degradation (Lindsay *et al.*, 1995).

Targeting of S287 by Multiple Kinase Pathways

Although CaMKII is the predominant S287-kinase during the first postfertilization interphase, other kinases may play this role at other stages of development. For example, during oogenesis, protein kinase A (PKA) is active and has been

recently shown to phosphorylate S287 on Cdc25C (Duckworth *et al.*, 2002), keeping Cdc25C inhibited and the oocyte arrested in G2. The action of the hormone progesterone inhibits production of cAMP, relieving the inhibitory action of PKA on Cdc25C and allowing the oocyte to progress through meiotic maturation. PKA activity oscillates in cycling *Xenopus* egg extracts and has been shown to inhibit Cdc2/cyclin B activation (Grieco *et al.*, 1994; Grieco *et al.*, 1996). It is therefore possible that PKA also contributes to the control of Cdc25C through phosphorylation of S287 during embryonic development (Duckworth *et al.*, 2002).

In response to DNA damage or replication arrest, the S287 site of *Xenopus* Cdc25C is phosphorylated by Chk1 and Cds1/Chk2 (Kumagai *et al.*, 1998a; Guo and Dunphy, 2000) in a pathway that is conserved from vertebrates to yeast (Walworth, 2000). Chk1, Cds1/Chk2, and CaMKII all belong to the evolutionarily conserved "CaMK" subfamily of protein Ser/Thr kinases (Hanks and Quinn, 1990; Manning *et al.*, 2002). This subfamily, which also includes CaMKI, AMP-activated protein kinase and yeast SNF1, has closely related catalytic domains but distinct regulatory domains that respond to different cellular stresses. These kinases share the substrate recognition motif $\Phi\text{-X-X-X-X-S/T}^*$ (where Φ is a hydrophobic and β is a basic residue) (Dale *et al.*, 1995; Songyang *et al.*, 1996; White *et al.*, 1998; Hutchins *et al.*, 2000; O'Neill *et al.*, 2002), due to conservation of the residues lining their catalytic clefts that interact favorably with amino acid side chains surrounding the target sites in their substrates (Goldberg *et al.*, 1996; Chen *et al.*, 2000). An important physiological consequence of the shared specificities of these kinases may be that the cell can integrate diverse signaling pathways at the same key target. In the case of vertebrate Cdc25C, the S287/S216 site conforms perfectly to this recognition motif and may be targeted for phosphorylation by different kinases in response to cellular stresses as diverse as DNA damage, DNA replication arrest, and elevated $[\text{Ca}^{2+}]_i$. Additionally, extracellular signals that elevate $[\text{Ca}^{2+}]_i$ or [cAMP], may cause phosphorylation of this site (Figure 11B). Chk1, Cds1/Chk2, and possibly other members of this kinase family can also target mammalian Cdc25A and B, which regulate cyclin-dependent kinase activation in G1 and G2 (Sanchez *et al.*, 1997; Mailand *et al.*, 2000; Falck *et al.*, 2001).

Sensitivities of Checkpoint Kinases to Inhibitors

In mammalian cells, UCN-01 overcomes a G2 checkpoint (Wang *et al.*, 1996; Hirose *et al.*, 2001) and an S-phase checkpoint (Feijoo *et al.*, 2001). Because UCN-01 potently inhibits Chk1, whereas Chk2 is less sensitive (Busby *et al.*, 2000; Graves *et al.*, 2000), this inhibitor has been used to assign a role for Chk1 in these checkpoints. However, our studies have shown that CaMKII is also highly sensitive to UCN-01 (Figure 7B), as are a number of other protein kinases (Davies *et al.*, 2000). We have also found that DBH, which inhibits both Chk1 and Cds1/Chk2 and overcomes G2 arrest (Curman *et al.*, 2001), inhibits CaMKII with similar potency. Although the kinases inhibited by UCN-01 and DBH may target the same substrates, such as Cdc25 phosphatases, caution must therefore be exercised when using these compounds to assign roles for particular protein kinases.

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