The Xenopus Chk1 Protein Kinase Mediates a Caffeine-sensitive Pathway of Checkpoint Control in Cell-free Extracts

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Abstract. We have analyzed the role of the protein kinase Chk1 in checkpoint control by using cell-free extracts from Xenopus eggs. Recombinant Xenopus Chk1 (Xchk1) phosphorylates the mitotic inducer Cdc25 in vitro on multiple sites including Ser-287. The Xchk1-catalyzed phosphorylation of Cdc25 on Ser-287 is sufficient to confer the binding of 14-3-3 proteins. Egg extracts from which Xchk1 has been removed by immunodepletion are strongly but not totally compromised in their ability to undergo a cell cycle delay in response to the presence of unreplicated DNA. Cdc25 in Xchk1-depleted extracts remains bound to 14-3-3 due to the action of a distinct Ser-287-specific kinase in addition to Xchk1. Xchk1 is highly phosphorylated in the presence of unreplicated or damaged DNA, and this phosphorylation is abolished by caffeine, an agent which attenuates checkpoint control. The checkpoint response to unreplicated DNA in this system involves both caffeine-sensitive and caffeine-insensitive steps. Our results indicate that caffeine disrupts the checkpoint pathway containing Xchk1.

Key words: Cdc2 • Chk1 • 14-3-3 proteins • Cdc25 • caffeine

The entry into mitosis is controlled by regulatory proteins that ensure the proper segregation of replicated chromosomes to daughter cells. The integrity of chromosomal DNA is under constant surveillance during the cell cycle. In eukaryotic cells, when chromosomes become damaged or cannot be replicated completely, mitosis is prevented by checkpoint mechanisms until two intact copies of the genome can be produced (Hartwell and Weinert, 1989; Murray, 1995; Elledge, 1996). A key target of these checkpoint pathways is the Cdc2–cyclin B complex, also known as maturation or M phase–promoting factor (MPF).

In the presence of damaged or unreplicated DNA, Cdc2–cyclin B is kept inactive due to inhibitory phosphorylation of the Tyr-15 and Thr-14 residues of Cdc2 (Nurse, 1997). These phosphorylations are carried out collectively by the kinases Wee1 and Myt1 (Featherstone and Russell, 1991; Igarashi et al., 1991; Parker et al., 1992; Mueller et al., 1995a,b; Watanabe et al., 1995). At the onset of mitosis, the phosphatase Cdc25C removes these inhibitory phosphate groups and thereby activates Cdc2–cyclin B (Dunphy and Kumagai, 1991; Gautier et al., 1991; Strausfeld et al., 1991). The activity of Cdc25C is strictly regulated, being low during interphase and high at mitosis (Izumi et al., 1992; Kumagai and Dunphy, 1992). In Xenopus egg extracts, the activation of Cdc25C (hereafter referred to simply as Cdc25) at mitosis involves the stimulatory phosphorylation of its NH2-terminal regulatory domain by at least two kinases, including Cdc2–cyclin B itself and a Xenopus homologue of the Polo-like kinase called Plx1 (Hoffmann et al., 1993; Izumi and Maller, 1995; Kumagai and Dunphy, 1996).

In recent studies in humans and Xenopus, it has been shown that Cdc25 is negatively regulated during interphase by the binding of 14-3-3 proteins (Peng et al., 1997; Kumagai et al., 1998). The inactive form of Cdc25 found before mitosis is phosphorylated on Ser-216 and Ser-287 in humans and Xenopus, respectively. This phosphorylation, which occurs in a consensus 14-3-3 binding site, mediates the association between Cdc25 and 14-3-3 proteins. This phosphorylation-dependent interaction appears to suppress the activation and/or action of Cdc25. Mutants of

1. Abbreviations used in this paper: GST, glutathione-S-transferase; HBS, Heps-buffered saline; MPF, M phase–promoting factor; Xchk1, Xenopus Chk1.
Cdc25 that cannot be phosphorylated at this residue and thus cannot bind 14-3-3 proteins override the unreplicated/damaged DNA checkpoint(s), suggesting that Cdc25 is a target of checkpoint regulation (Peng et al., 1997; Kumagi et al., 1998).

In fission yeast, the protein kinase Chk1 is required for a cell cycle arrest in response to damaged DNA and also for the DNA replication checkpoint under certain circumstances (Walworth et al., 1993; al-Khodairy et al., 1994; Francesconi et al., 1997; Furnari et al., 1997). Chk1 acts downstream of several Rad gene products and is modified by phosphorylation upon DNA damage (Carr et al., 1995; Walworth and Bernard, 1996). Likewise, the kinase Rad53 from budding yeast is phosphorylated upon DNA damage in a manner that depends on several checkpoint control genes (Sanchez et al., 1996; Sun et al., 1996). In Drosophila, the genes encode a Chk1 homologue that appears to modulate cell cycle timing (Fogarty et al., 1997; Sibon et al., 1997). It has recently been shown that fission yeast Chk1 and its human homologue phosphorylate human Cdc25C on Ser-216, suggesting that Chk1 acts similarly to Chk1 in fission yeast Chk1 and its human homologue phosphorylate human Cdc25C on Ser-216, suggesting that Chk1 acts similarly to Chk1 in Drosophila Rad53 from budding yeast is phosphorylated upon DNA damage (Carr et al., 1995; Furnari et al., 1997). Chk1 acts similarly to Chk1 in Drosophila and yeast in that it acts similarly to Chk1 in response to damaged DNA and also for the DNA replication checkpoint under certain circumstances (Walworth et al., 1993; al-Khodairy et al., 1994; Francesconi et al., 1997; Furnari et al., 1997). Chk1 acts similarly to Chk1 in response to damaged DNA and also for the DNA replication checkpoint under certain circumstances (Walworth et al., 1993; al-Khodairy et al., 1994; Francesconi et al., 1997; Furnari et al., 1997).

**Production of Recombinant His6–Xchk1 and His6–Xchk1-N135A Proteins**

An NcoI site at the start codon of Xchk1 was created by PCR using Pfu DNA polymerase with the primers CATGCCATGGCAGTTCCGTTGTTGAAAG and GGCCCTGGAATTTAATTACCTTC. The resulting PCR fragment was digested with NcoI and EcoRV to produce a 400-bp fragment encoding the NH2-terminal region of Xchk1. This fragment and a 1.7-kb EcoRV-XhoI fragment encoding the remaining COOH-terminal portion of Xchk1 were ligated to pFastBacHTa that had been digested with NcoI and XhoI. The kinase-negative mutant of Xchk1 (N135A) was created by PCR with the primers GAGATATCAAGCCTGAGGCCT-GGCGCTGCTTTTAG and AAACAGCTATGACCATGATTACGCC. This PCR fragment was digested with EcoRV and XhoI and cloned into pFastBacHTa together with 400-bp NH2-terminal NcoI-EcoRV fragment encoding the remaining COOH-terminal portion of Xchk1. His6–Xchk1-N135A proteins were produced using the Bac-to-Bac system (GIBCO BRL, Gaithersburg, MD). Recombinant proteins were isolated using nickel agarose beads as described (Kumagi and Duhny, 1995).


The BamHI-HindIII fragment of pFastBacHTa encoding His6–Cdc25-S287A (Kumagi et al., 1998) was cloned into pGEX-Cdc25 (Kumagi and Duhny, 1992) that had been digested with BamHI and EcoRI. GST fusion protein constructs containing amino acids 254–316 of either wild-type Xenopus Cdc25 (GST–Cdc25[254–316]-WT) or its S287A mutant (GST–Cdc25[254–316]-S287A) were produced by PCR with Pfu DNA polymerase (Stratagene, La Jolla, CA) using the primers CTAGAGGGATCCATGGCAATTCTTCTGGGGAAAG and GGCCGTCGAATTCACGTCTCCTTTTCAC-TCTGAC and the appropriate DNA template. The PCR fragments were digested with BamHI and EcoRI and ligated into pGEX-Cdc25 that had been digested with BamHI and EcoRI. These GST fusion proteins were expressed in the E. coli strain BL21(DE3)pLysS and purified using glutathione agarose (Smith and Johnson, 1988).

**Materials and Methods**

**Cloning of a Xenopus Chk1 Homologue (Xchk1)**

Two degenerate oligonucleotides corresponding to sequences conserved between Schizosaccharomyces pombe Chk1 and Drosophila Grapes were designed: TTT(G/C)(G/A)TTAATIGAGCACCGAGTGG and A(G/A) TTATCGTCAAG(G/T)CGTTTGG. A polymerase chain reaction (PCR) using Xenopus oocyte cDNA as the template yielded a major 300-bp fragment in addition to a few minor fragments. DNA sequencing revealed that the 300-bp fragment encodes a segment of protein with significant homology to Chk1. The 300-bp fragment was radiolabeled with [3P] and used to screen a Xenopus oocyte cDNA library in the pAX-NMT vector to obtain a full-length cDNA clone (Mueller et al., 1995a). A 2.2-kb Apal-Xhol fragment encoding full-length Xchk1 was cloned into pBlueScript SK+ (pBS-Xchk1). A series of deletion clones was constructed by using the Erase-a-base kit (Promega Corp., Madison, WI). DNA sequencing of both strands was performed with an ABI model 373 automated sequencer (Perkin-Elmer Corp., Foster City, CA).

Egg Extracts and Isolation of the Nuclear Fraction

Xenopus egg extracts were prepared as described (Murray, 1991). Egg extracts used in in vitro experiments were prepared from unfertilized eggs.

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Kinase Assays
Xchk1 was incubated with full-length His6-Cdc25 or GST–Cdc25(254–316)-WT in 40 μl of kinase buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 1 mM DTT) containing 2 μCi [γ-³²P]ATP and 10 μM ATP.

Binding of 14-3-3-e Protein to Cdc25
Glutathione agarose beads (5 μl) containing either GST–Cdc25-WT, GST–Cdc25-S287A, or no protein were incubated with 0.3 μg His6-Xchk1, 0.3 μg His6-Xchk1-N135A, or neither protein in 50 μl of kinase buffer containing 1 mM cold ATP at 25°C for 30 min under agitation. Next, 1 μg Xenopus His6-14-3-3-e protein (Kumagai et al., 1998), 0.5% NP-40, and 1 mg/ml ovalbumin were added to the mixture, and the incubation continued for another 30 min at 4°C. Beads were washed four times with 10 mM Hepes-KOH, pH 7.5, containing 500 mM NaCl and 0.5% NP-40 and then washed two times with Hepes-buffered saline (HBS; 10 mM Hepes-KOH, pH 7.5, and 150 mM NaCl). Proteins were eluted from the beads with 5 mM glutathione in HBS.

Production of 35S-labeled Xchk1 Protein
35S-labeled Xchk1 and Xchk1–N135A proteins were synthesized using pBS-Xchk1 and pBS-Xchk1-N135A as templates in the TNT in vitro transcription/translation system (Promega Corp.) in the presence of pBS–Xchk1 and pBS–Xchk1-N135A as templates in the TNT in vitro
buffer containing 1 mM cold ATP at 23°C. Beads were washed four times with 10 mM Hepes-KOH, pH 7.5, containing 500 mM NaCl and 0.5% NP-40 and then washed two times with Hepes-buffered saline (HBS; 10 mM Hepes-KOH, pH 7.5, and 150 mM NaCl). Proteins were eluted from the beads with 5 mM glutathione in HBS.

Production of Antibodies
Polyclonal rabbit antibodies against the purified His6-Xchk1 protein from insect cells were produced at a commercial facility (Berkeley Antibody Tech, Inc., Piscataway, NJ). Antibodies against Xchk1 were described previously (Kumagai et al., 1998). Polyclonal rabbit antibodies against the purified His6–Xchk1 protein from insect cells were produced at a commercial facility (Berkeley Antibody Tech, Inc., Piscataway, NJ). Antibodies against Xchk1 were described previously (Kumagai et al., 1998).

Depletion of Xchk1 from Egg Extracts
M phase extract (170 μl) was incubated with 10 μg of affinity-purified Xchk1 antibodies or control rabbit IgG (Zymed Laboratories Inc., South San Francisco, CA) bound to 5 μl of Affiprep protein A beads. Reticulocyte lysates containing 35S-labeled proteins were added to egg extracts supplemented with 100 μg/ml of cycloheximide.

Results
Cloning of a Xenopus Chk1 Homologue (Xchk1)
To clone a Xenopus Chk1 homologue, we used degenerate PCR primers to amplify a segment of its complementary DNA (cDNA). The oligonucleotide primers were designed against homologous regions in the S. pombe Chk1 and Drosophila Grapes protein kinases. PCR amplification yielded an ~300-bp fragment that was used to isolate a full-length Xenopus oocyte cDNA that encodes a protein with strong homology to the Chk1 family of protein kinases (EMBL/GenBank/DDBJ accession number AF053120). The sequence data are available from GenBank/EMBL/DDBJ under accession number AF053120.
Xchk1 Phosphorylates Cdc25 on Ser-287 and Mediates 14-3-3 Binding

To study the biochemistry of Xchk1, we expressed a histidine-tagged version (His6–Xchk1) in baculovirus-infected Sf9 insect cells. We also produced a kinase-negative form of Xchk1 with a mutation in a catalytically essential residue (His6–Xchk1-N135A). His6–Xchk1 and His6–Xchk1-N135A could be purified to near homogeneity by chromatography on nickel agarose (Fig. 2 A). Recombinant His6–Xchk1 phosphorylates Xenopus Cdc25 efficiently in vitro (Fig. 2 B, lane 1). Thus, it appears that His6–Xchk1 isolated from baculovirus-infected Sf9 insect cells is catalytically active, even though these cells were not treated with replication inhibitors or DNA-damaging agents. Under these conditions, His6–Xchk1 also phosphorylates the S287A mutant of Cdc25 in which the critical serine in its 14-3-3 binding site has been changed to alanine (Fig. 2 B, lane 3). In contrast, the kinase-negative His6–Xchk1-N135A protein could not use either the wild-type or S287A form of Cdc25 as a substrate. To characterize the Xchk1-catalyzed phosphorylation of Cdc25 in greater detail, we isolated the 32P-labeled wild-type and S287A forms of Cdc25 and subjected them to tryptic phosphopeptide analysis (Fig. 2 C). These mapping studies indicated that His6–Xchk1 phosphorylates wild-type Cdc25 on multiple sites in vitro. One of these sites corresponds to Ser-287, because a single spot is missing from the tryptic phosphopeptide map of the 32P-labeled Cdc25-S287A mutant. In other experiments, we showed that His6–Xchk1 phosphorylates a GST fusion protein containing a small fragment of Cdc25 that includes the 14-3-3 binding site (GST–Cdc25[254-316]-WT) (Fig. 2 D, lane 1). In contrast, His6–Xchk1 did not phosphorylate a mutant form of this peptide containing alanine instead of serine at position 287 (GST–Cdc25[254-316]-S287A) (Fig. 2 D, lane 2).

Figure 2. Xchk1 phosphorylates Cdc25 on Ser-287 and mediates binding of 14-3-3. (A) Recombinant His6–Xchk1 (lane 1) and His6–Xchk1-N135A (lane 2) were purified from baculovirus-infected insect cells, subjected to SDS-PAGE, and then stained with Coomassie blue. (B) Xchk1 phosphorylates Cdc25 efficiently in vitro. His6–Xchk1 (lanes 1 and 3) and His6–Xchk1-N135A (lanes 2 and 4) were incubated with either His6–Cdc25-WT (lanes 1 and 2) or His6–Cdc25-S287A (lanes 3 and 4) in kinase buffer containing 10 μM [γ-32P]ATP. Reactions were terminated with gel sample buffer and subjected to SDS-PAGE and autoradiography. (C) Xchk1 phosphorylates Cdc25 at Ser-287 and other sites. His6–Cdc25-WT and His6–Cdc25-S287A that had been incubated with His6–Xchk1 in the presence of 10 μM [γ-32P]ATP were processed for tryptic phosphopeptide mapping. Arrows, position of the peptide containing Ser-287; dots, origins in the lower left corner of each map. (D) Specific phosphorylation by His6–Xchk1 of a fragment of Cdc25 containing the 14-3-3 binding site. GST–Cdc25[254-316]-WT (lane 1) or GST–Cdc25[254-316]-S287A (lane 2) were incubated with His6–Xchk1 in the presence of [γ-32P]ATP and subjected to SDS-PAGE and autoradiography. Arrow, position of the wild-type and mutant GST fusion proteins. (E) Phosphorylation of Cdc25 by Xchk1 creates a 14-3-3 binding site. Bacterially-expressed GST–Cdc25-WT (lanes 1–3) and GST–Cdc25-S287A (lane 4) bound to glutathione agarose were incubated with His6–Xchk1 (lanes 2 and 4), His6–Xchk1-N135A (lanes 3), or neither protein (lane 1) in kinase buffer containing 1 mM ATP at 23°C for 30 min. His6–14-3-3e was then added to the mixture, and the incubation was continued at 4°C for an additional 30 min. At the end of the incubation, proteins specifically bound to the beads were eluted with glutathione and subjected to SDS-PAGE and immunoblotting with either anti-Cdc25 antibodies (top) or anti-14-3-3e antibodies (bottom).
Previous studies have indicated that the phosphorylation of Cdc25C on Ser-216 and Ser-287 from humans and Xenopus, respectively, is necessary for the binding of 14-3-3 proteins (Peng et al., 1997; Kumagai et al., 1998). To ask whether the Xchk1-catalyzed phosphorylation of Xenopus Cdc25 can mediate 14-3-3 binding, we treated bacterially-expressed GST–Cdc25 and GST–Cdc25-S287A (both bound to glutathione agarose beads) with His6–Xchk1 and then added recombinant His6–14-3-3e protein to the reaction. After reisolating the beads and eluting bound proteins with glutathione, we found that His6–14-3-3e could associate specifically with GST–Cdc25 only if GST–Cdc25 had been phosphorylated previously by His6–Xchk1 (Fig. 2 E, lanes 1–3). In contrast, His6–14-3-3e did not bind specifically to the GST–Cdc25-S287A mutant that had been treated with His6–Xchk1 (Fig. 2 E, lane 4), even though this incubation led to substantial phosphorylation of GST–Cdc25-S287A on sites distinct from Ser-287 (Fig. 2, B and C). Treatment of GST–Cdc25 with the kinase-negative His6–Xchk1-N135A mutant did not lead to binding of His6–14-3-3e (Fig. 2 E, lane 3). In conclusion, the phosphorylation of Xenopus Cdc25 on Ser-287 by Xchk1 is sufficient for the binding of 14-3-3 proteins.

Immunodepletion of Xchk1 from Egg Extracts Reveals Xchk1-dependent and Xchk1-independent Checkpoint Control Pathways

To investigate the role of Xchk1 in checkpoint control, we set out to remove this protein from Xenopus egg extracts by immunodepletion. For this purpose, we used recombinant His6–Xchk1 to generate polyclonal antibodies that recognize a 54-kD polypeptide in immunoblots of Xenopus egg extracts (Fig. 3 A, lane 1). By using recombinant His6–Xchk1 as a standard, we estimate that endogenous Xchk1 is present at a concentration of 2 ng/μl (40 nM) in egg extracts. By comparison, the concentration of Cdc25C in these extracts is 10 ng/μl (140 nM) (Kumagai and Dunphy, 1992). By subjecting egg extracts to two rounds of immunodepletion with anti-Xchk1 antibodies bound to protein A beads, we were able to remove this protein quantitatively from egg extracts, as determined by immunoblotting with anti-Xchk1 antibodies (Fig. 3 A, lane 3). For comparison, no Xchk1 was removed by a mock immunodepletion with control antibodies (Fig. 3 A, lanes 1 and 2). Xchk1 could be restored to Xchk1-depleted extracts at close to the physiological level by the addition of recombinant His6–Xchk1 (Fig. 3 A, lane 4).

Next, we compared cell cycle progression and checkpoint function in Xchk1-depleted extracts and mock-depleted control extracts. Typically, egg extracts in which a point function in Xchk1-depleted extracts and mock-depleted extracts treated with aphidicolin were significantly delayed in mitotic entry in comparison with Xchk1-depleted extracts that had not been treated with aphidicolin (Fig. 3, B and C). Thus, it appears that the presence of unreplicated DNA can impede the entry into mitosis to some extent in Xchk1-depleted extracts but not nearly as efficiently as in mock-depleted extracts that contain Xchk1. Significantly, when His6–Xchk1 was added back to the Xchk1-depleted...

Figure 3. Immunodepletion of Xchk1 compromises checkpoint control in Xenopus egg extracts. (A) Immunodepletion of Xchk1 from Xenopus egg extracts. An M phase extract (lane 1) was treated with anti-Xchk1 antibodies (lanes 3 and 4) or control antibodies (lane 2) bound to protein A beads as described in Materials and Methods. In lane 4, His6–Xchk1 protein was added back to the Xchk1-depleted extract to a final concentration of 2 ng/μl. Samples (lanes 1–4) were processed for immunoblotting with anti-Xchk1 antibodies. (B) Effect of immunodepletion of Xchk1 on egg extracts lacking unreplicated DNA. M phase extracts were treated with anti-Xchk1 antibodies ( ) or control antibodies ( ). In one set of samples ( ), His6–Xchk1 was added back to a concentration of 2 ng/μl. The extracts were activated with calcium, and the timing of NEB (nuclear envelope breakdown) was monitored by microscopy. (C) Effect of immunodepletion of Xchk1 on egg extracts containing unreplicated DNA. Extracts that had been treated with either anti-Xchk1 antibodies ( , ) or control antibodies ( , ) were incubated with 1,000 sperm nuclei/μl and 100 μg/ml aphidicolin in the presence ( , ) or absence ( , ) of 5 mM caffeine. In one case ( ), 2 ng/μl His6–Xchk1 was added back to the Xchk1-depleted extract.
extracts, treatment with aphidicolin delayed mitotic entry as effectively as in mock-depleted extracts (Fig. 3 C). This observation indicates that the compromised checkpoint control in Xchk1-depleted extracts is due to the absence of Xchk1 and not to some non-specific effect of the immunodepletion procedure. Finally, Xchk1-depleted extracts lacking aphidicolin entered mitosis ~15 min earlier than mock-depleted extracts or Xchk1-depleted extracts containing exogenously added His6–Xchk1 (Fig. 3 B). Interestingly, Xchk1 immunoprecipitated with anti-Xchk1 antibodies from interphase egg extracts lacking aphidicolin phosphorylates His6–Cdc25 or GST–Cdc25(254–316) as efficiently as baculovirus-expressed His6–Xchk1 from Sf9 insect cells (data not shown). Thus, Xchk1 may contribute to mitotic timing to some extent in the apparent absence of a checkpoint response.

**Caffeine Disrupts the Checkpoint Control Pathway That Is Dependent upon Xchk1**

Caffeine has been reported to override the replication and damage checkpoints in various experimental systems, but its mechanism of action and molecular target(s) have been unknown (Schlegel and Pardee, 1986; Dasso and Newport, 1990; Kumagai and Dunphy, 1995; Poon et al., 1997). In *Xenopus* egg extracts containing unreplicated or damaged DNA, the inclusion of caffeine overrides the checkpoint-mediated delay of mitosis. As shown in Fig. 3 C, caffeine accelerates mitotic entry substantially in mock-depleted extracts containing aphidicolin. Nonetheless, these caffeine-treated extracts enter mitosis at a slower rate than extracts that lack unreplicated or damaged DNA (Fig. 3 C; see also Fig. 2 in Kumagai and Dunphy, 1995). However, the presence of caffeine did not affect mitotic timing in Xchk1-depleted extracts containing aphidicolin (Fig. 3 C). In particular, Xchk1-depleted extracts containing aphidicolin underwent nuclear envelope breakdown at the same time in the presence and absence of caffeine. Taken together, these results indicate that the checkpoint-mediated delay of mitotic entry involves collaborating Xchk1-dependent and Xchk1-independent pathways. The Xchk1-dependent pathway is sensitive to caffeine, whereas the Xchk1-independent pathway is unaffected by this agent.

**14-3-3 Remains Bound to Cdc25 in Xchk1-depleted Extracts**

Since in vitro phosphorylation of Cdc25 on Ser-287 by Xchk1 is sufficient for the binding of 14-3-3 proteins, we asked whether 14-3-3 could bind to Cdc25 in extracts lacking Xchk1 (Fig. 4 A). For this purpose, we subjected Xchk1-depleted and mock-depleted egg extracts to immunoprecipitation with antibodies against *Xenopus* 14-3-3e and then immunoblotted the resulting immunoprecipitates with either anti-Cdc25 antibodies or anti-14-3-3e antibodies (to monitor the recovery of 14-3-3e). In particular, M phase extracts were treated with anti-Xchk1 or control antibodies, driven into interphase by activation with calcium, and then subjected to immunoprecipitation with anti-14-3-3e antibodies. For this experiment, we chose to immunodeplete Xchk1 from M phase egg extracts because 14-3-3 proteins are not bound to Cdc25 at M phase (Peng et al., 1997; Kumagai et al., 1998). We observed that the amount of Cdc25 bound to 14-3-3e in Xchk1-depleted extracts that had been triggered to enter interphase was similar to that bound in either mock-depleted extracts or Xchk1-depleted extracts to which His6–Xchk1 had been added back (Fig. 4 A, lanes 1–3). With the same batch of egg extract, we confirmed that 14-3-3e binds to Cdc25 during interphase but not M phase and that the binding during interphase is similar in the presence and absence of unreplicated or damaged DNA (Fig. 4 A, lanes 4–7; Kumagai et al., 1998).
These findings suggest that a kinase distinct from Xchk1 can phosphorylate Ser-287 on Cdc25 in Xchk1-depleted extracts and thereby create a 14-3-3 binding site within Cdc25. To address this possibility more directly, we used the GST–Cdc25(254–316)-WT substrate to measure total Ser-287–specific kinase activity in both mock-depleted and Xchk1-depleted interphase extracts (Fig. 4 B). We observed that GST–Cdc25(254–316)-WT became phosphorylated quite efficiently in the Xchk1-depleted extract (Fig. 4 B, lane 3). The level of phosphorylation was ~80% of that observed in a mock-depleted extract, as quantitated with a phosphorimager (Fig. 4 B, lane 2). This assay appears to detect phosphorylation of Ser-287 specifically because the GST–Cdc25(254–316)-S287A mutant was not radiolabeled under these conditions (Fig. 4 B, lane 1).

**Exogenous Addition of Active Xchk1 Delays Mitosis in Egg Extracts in a Caffeine-resistant Manner**

As another approach to assess the role of Xchk1 in mitotic timing, we added extra Xchk1 protein exogenously to cycling *Xenopus* egg extracts (Fig. 5). For this experiment, we added active His6–Xchk1 or the catalytically inactive His6–Xchk1-N135A mutant to egg extracts at a final concentration of 10 ng/μl. Since the endogenous concentration of Xchk1 in these extracts is 2 ng/μl, this procedure leads to a sixfold increase in the level of Xchk1. We observed that the wild-type His6–Xchk1 protein elicited a substantial 60-min delay of mitosis (Fig. 5). In contrast, the kinase-negative His6–Xchk1-N135A protein had no effect on the timing of nuclear envelope breakdown (Fig. 5). Paraphenetically, kinase-negative His6–Xchk1-N135A also had no effect on the cell cycle delay in aphidicolin-treated extracts, indicating that this mutant protein does not act as a dominant-negative competitor of checkpoint regulatory factors under these conditions (data not shown).

As described above, Xchk1-mediated checkpoint control is sensitive to caffeine. Therefore, we asked whether the delay of mitosis elicited by exogenously added His6–Xchk1 would be affected by this agent. As shown in Fig. 5, the presence of caffeine did not reverse the inhibitory effect of exogenously added His6–Xchk1 on the timing of mitosis. In similar experiments, treatment with caffeine did accelerate the entry into mitosis in aphidicolin-treated extracts containing only endogenous Xchk1 (refer to Fig. 3 C). These results suggest that caffeine does not directly inhibit the kinase activity of Xchk1. To address this issue more directly, we assayed the kinase activity of His6–Xchk1 toward recombinant Cdc25 in the presence and absence of 5 mM of caffeine. These assays were performed in the presence of 1 mM ATP, which is the endogenous concentration of ATP in egg extracts. We observed that caffeine had no effect on either the phosphorylation of Cdc25 by Xchk1 or the in vitro autophosphorylation of Xchk1 (data not shown). Collectively, our results suggest that caffeine does not directly inhibit Xchk1 or its downstream effects but instead compromises some process upstream of Xchk1 that is required for proper checkpoint regulation.

**Caffeine Blocks the Checkpoint-dependent Phosphorylation of Xchk1**

To further explore the functional properties of Xchk1, we examined the modification of this protein in the presence and absence of unreplicated or damaged DNA. For these experiments, we first prepared 35S-labeled Xchk1 protein and added it to egg extracts containing either aphidicolin or UV-damaged sperm chromatin. We observed that a portion of the labeled Xchk1 protein underwent an obvious modification in extracts containing unreplicated or damaged DNA, as evidenced by a decrease of electrophoretic mobility in SDS gels (Fig. 6 A, top, lanes 2 and 4). Similar results were obtained by immunoblotting the endogenous Xchk1 in egg extracts with anti-Xchk1 antibodies (data not shown). This modification appears to be phosphorylation, since it is reversed by treatment with protein phosphatase 2A (Fig. 6 B). Typically, we observed two closely spaced phosphorylated bands with reduced electrophoretic mobility in comparison with the major 54-kD Xchk1 band that was found in extracts lacking damaged or unreplicated DNA (Fig. 6 A, top, lane 1). When the nuclear fraction was isolated from these extracts, a higher proportion of the nuclear Xchk1 protein appeared to be modified in comparison with Xchk1 in the whole egg extract (Fig. 6 A, bottom, lanes 2 and 4). Significantly, caffeine completely abolished the checkpoint-associated phosphorylation of Xchk1 in the presence of either unreplicated or damaged DNA (Fig 6 A, top and bottom, lanes 3 and 5). This observation also argues that caffeine modulates the regulatory pathway containing Xchk1. Since only a portion of Xchk1 is hyperphosphorylated in response to unreplicated or damaged DNA and this hyperphosphorylated form proved to be difficult to isolate by immunoprecipitation, it is unclear at this time whether this phosphorylation directly alters the kinase activity of Xchk1.

To characterize further the phosphorylation of Xchk1, we asked whether it might be due to autophosphorylation or to the action of a distinct kinase. For these studies, we added 32P-labeled Xchk1 or the kinase-negative Xchk1–N135A mutant to either Xchk1-depleted or mock-depleted egg extracts that contained either UV-damaged or undamaged sperm chromatin (Fig. 6 C). As expected, the labeled Xchk1 protein underwent phosphorylation (up-
Each extract was taken for SDS-PAGE and autoradiography (lanes 2 and 5) and subjected to SDS-PAGE and autoradiography. Similar results were obtained in extracts from which the total通过 a sucrose solution to isolate the nuclear fraction (lanes 4 and 8). Since this partially phosphorylated form of Xchk1–N135A was also found in Xchk1-depleted extracts, this phosphorylation is presumably due to the action of a distinct kinase rather than autophosphorylation. However, since production of the most highly phosphorylated form of Xchk1 does depend upon the presence of catalytically active Xchk1, a portion of the checkpoint-induced modification of Xchk1 may represent autophosphorylation.

Discussion

In this report, we have analyzed the biochemical properties of the Xenopus Chk1 protein kinase (Xchk1) and its role in checkpoint control in Xenopus egg extracts. Recombinant Xchk1 phosphorylates the mitotic regulatory phosphatase Cdc25 on the critical serine (residue 287) in its 14-3-3 binding motif as well as some additional undefined sites. The Xchk1-catalyzed phosphorylation of Cdc25 on Ser-287 confers upon Cdc25 the ability to bind 14-3-3 proteins. In particular, Cdc25 that has been phosphorylated by Xchk1 in vitro binds efficiently to a recombinant form of 14-3-3e, which is the major physiological partner of Cdc25 during interphase in Xenopus egg extracts (Kumagai et al., 1998). As described previously, the binding of 14-3-3 proteins correlates with negative regulation of the mitosis-inducing function of Cdc25C (Peng et al., 1997; Kumagai et al., 1998).

Various observations suggest that Xchk1 plays a significant role in the control of mitotic timing in Xenopus egg extracts. First, the addition of recombinant Xchk1 to egg extracts results in a strong inhibition of mitotic entry, whereas a kinase-negative form of Xchk1 has no effect. Secondly, immunodepletion of Xchk1 strongly compromises the ability of egg extracts to undergo a cell cycle arrest in response to the presence of unreplicated DNA. These two effects are consistent with a physiological role for Xchk1 in negatively regulating Cdc25. Finally, Xchk1 undergoes a marked phosphorylation in the presence of unreplicated or damaged DNA. This phosphorylation is abolished by caffeine under conditions in which this agent overrides checkpoint controls.

Overall, our results indicate that Xenopus egg extracts possess checkpoint regulatory molecules similar to those present in yeast and mammals (Carr, 1996; Nurse, 1997; Rhind et al., 1997; Saka et al., 1997; Sanchez et al., 1997; Weinert, 1997; Westphal, 1997). By analyzing Xchk1 and Cdc25 in egg extracts, we have learned about the complexity of the pathway that controls Cdc25 (Fig. 7). For example, Cdc25 can still associate with 14-3-3 proteins in extracts from which Xchk1 has been removed by immunodepletion, indicating that a kinase distinct from Xchk1 can phosphorylate Ser-287 of Cdc25. The identity of this kinase is presently unknown. A human kinase called C-TAK1 (Cdc twenty-five C-associated protein kinase 1) can phosphorylate Cdc25C on its 14-3-3 binding site in

Figure 6. Xchk1 is modified when the DNA replication and DNA damage checkpoints are activated. (A) 32P-labeled Xchk1 was incubated in interphase egg extracts containing 3,000 sperm nuclei/µl (lane 1), 3,000 UV-damaged sperm nuclei/µl (lanes 2 and 3), or 3,000 sperm nuclei/µl and 100 µg/ml aphidicolin (lanes 4 and 5) in the presence (lanes 3 and 5) or absence (lanes 1, 2, and 4) of 5 mM caffeine. After 100 min of incubation at 23°C, 2 µl of each extract was taken for SDS-PAGE and autoradiography (top). Alternatively, 50 µl of each extract was centrifuged through a sucrose solution to isolate the nuclear fraction (bottom), which was also subjected to SDS-PAGE and autoradiography. APH, aphidicolin. (B) 35S-labeled Xchk1 was immunoprecipitated from interphase extracts containing 3,000 sperm nuclei/µl and 100 µg/ml aphidicolin. The immunoprecipitates were incubated in phosphate buffer containing no addition (lane 1), protein phosphatase 2A (lane 2), or protein phosphatase 2A and 3 µM okadaic acid (lane 3). PP2A, protein phosphatase 2A; OA, okadaic acid. (C) 32P-labeled Xchk1 (lanes 1–4) and Xchk1–N135A (lanes 5–8) were incubated for 100 min in either control-depleted (lanes 1, 2, 3, and 6) or Xchk1-depleted (lanes 3, 4, 7, and 8) extracts in the presence of either 3,000 sperm nuclei/µl (lanes 1, 2, 3, and 7) or 3,000 UV-damaged sperm nuclei/µl (lanes 3, 4, 7, and 8). Nuclear fractions were isolated as described above and subjected to SDS-PAGE and autoradiography.

shift in SDS gels) in the extract containing damaged DNA but not in one containing undamaged DNA (Fig. 6 C). Similar results were obtained in extracts from which the endogenous Xchk1 protein had been removed by immu...
vitro, but the role of this enzyme in Cdc25C regulation in vivo has not been established (Peng et al., 1998). Similarly, it has been shown that the fission yeast kinase Cds1 overlaps functionally with Chk1 (Boddy et al., 1998). We have also found the pathway containing Xchk1, although it plays a major role in the checkpoint arrest in response to unreplicated/damaged DNA, does not appear to be fully responsible. In particular, Xchk1-depleted extracts containing unreplicated DNA, although strongly compromised in their checkpoint response, nonetheless enter mitosis at a significantly delayed rate relative to control extracts. This observation suggests that the checkpoint arrest involves both Xchk1-dependent and Xchk1-independent pathways, which appear to collaborate in the control of mitotic timing.

Caffeine has been a useful tool in discerning the existence of these collaborating pathways (Schlegel and Pardee, 1986; Dasso and Newport, 1990; Poon et al., 1997). Furthermore, our studies have provided the first molecular insight into how this simple compound disrupts checkpoint regulation. Caffeine overrules the unreplicated/damaged DNA checkpoint in Xenopus extracts, but nonetheless caffeine-treated extracts enter mitosis more slowly than control extracts in which a checkpoint has not been triggered. This observation could mean that caffeine is not fully effective at inhibiting its target(s) or that the checkpoint response involves multiple interacting pathways, not all of which are sensitive to caffeine. Our results favor the latter possibility because the attenuated cell cycle delay observed in Xchk1-depleted extracts containing unreplicated or damaged DNA is completely insensitive to caffeine. In contrast, extracts containing the normal amount of endogenous Xchk1 and unreplicated DNA enter mitosis at a considerably accelerated pace in the presence of caffeine. This observation, coupled with the fact that caffeine completely abolishes the checkpoint-dependent phosphorylation of Xchk1, argues that the Xchk1-dependent pathway in Xenopus egg extracts is caffeine sensitive. Caffeine appears not to directly inhibit Xchk1, since it does not block the phosphorylation of Cdc25 by recombinant Xchk1 in vitro at a physiological concentration of ATP. Also, caffeine does not override the delay of mitosis in egg extracts in response to the exogenous addition of active recombinant His6–Xchk1. Our results suggest that caffeine acts at some point upstream of Xchk1. This view is supported by the observation that the checkpoint-associated phosphorylation of Xchk1 is blocked entirely by caffeine.

Much remains to be learned about the biochemical details of how Xchk1 inhibits mitosis when unreplicated DNA is present. Earlier studies have indicated that mutation of the 14-3-3 binding site in Cdc25C strongly compromises G2 checkpoint control (Peng et al., 1997; Kumagai et al., 1998). For example, Xenopus egg extracts containing unreplicated or damaged DNA and the Cdc25–S287A mutant that lacks a 14-3-3 binding site enter mitosis at approximately the same time as control extracts in which a checkpoint has not been triggered (Kumagai et al., 1998). This observation indicates that the checkpoint response is strongly compromised when 14-3-3 proteins cannot bind to Cdc25, although it is not known whether checkpoint control is totally abolished under these circumstances. In Xchk1-depleted extracts, the checkpoint defect is less severe, most probably at least in part because another kinase in egg extracts can also phosphorylate Ser-287 of Cdc25.

Intriguingly, the amount of 14-3-3 bound to Cdc25 is the same in the absence and presence of unreplicated/damaged DNA (Kumagai et al., 1998). Furthermore, as shown here, 14-3-3 proteins remain bound to Cdc25 in Xchk1-depleted extracts, despite the fact that checkpoint control is clearly disrupted in such extracts. These observations might appear to be inconsistent with the concept that binding of 14-3-3 to Cdc25 causes extracts containing unreplicated/damaged DNA to arrest in interphase. The affinity of 14-3-3 for a target with a single binding site should allow dynamic binding and dissociation of these molecules (Yaffe et al., 1997). Thus, it will be critical to determine the rate of phosphorylation and dephosphorylation of Ser-287 on Cdc25 in the absence and presence of unreplicated/damaged DNA. On the other hand, it seems possible that, although binding of 14-3-3 to Cdc25 may be important for proper checkpoint control, this interaction may not be the whole explanation for checkpoint regulation. In this regard, it is intriguing that Chk1 phosphorylates Cdc25 in vitro at sites that apparently do not confer 14-3-3 binding. It will be important to determine whether these phosphorylations occur at sites on Cdc25 that are phosphorylated in vivo and, if so, to determine their role in the regulation of Cdc25. In addition to the possibility that Chk1 could regulate Cdc25 by phosphorylating sites distinct from Ser-287, Xchk1 could possess an additional target(s) besides Cdc25. For example, O’Connell et al. (1997) have proposed that Wee1 is a target of Chk1 in fission yeast.

Xchk1 undergoes a marked phosphorylation in the presence of either unreplicated or damaged DNA. This finding could mean that Xchk1 responds to both the replication and damage checkpoints in Xenopus egg extracts. However, it is possible that some of the DNA in aphidicolin-treated nuclei may undergo damage. Likewise, it is quite likely that sites of damage in UV-treated nuclei would impede the progression of replication forks. Nonetheless, the

![Figure 7](image-url)
phosphorylation of Xchk1 correlates well with the cell cycle delay that is imposed when unreplicated or damaged DNA is present. In principle, the checkpoint-dependent phosphorylation of Xchk1 could elicit increased kinase activity toward Cdc25, but technical difficulties in isolating Xchk1 in its fully phosphorylated form have thus far precluded an answer to this question. Alternatively, this phosphorylation could potentiate the action of Xchk1 by some other mechanism such as controlling its intracellular localization or protein–protein interactions. Finally, it is possible that the hyperphosphorylation of Xchk1 is not causally responsible for Xchk1-mediated cell cycle delay.

The checkpoint-specific phosphorylation of Xchk1 appears to involve both autophosphorylation and the action of a distinct kinase. The kinase-negative Xchk1–N135A mutant clearly becomes phosphorylated in Xchk1-depleted extracts containing unreplicated or damaged DNA, indicating that this phosphorylation cannot be due to either inter- or intramolecular autophosphorylation. However, the wild-type Xchk1 protein is more extensively phosphorylated in the presence of unreplicated or damaged DNA in both control and Xchk1-depleted extracts, suggesting that autophosphorylation may also be involved. The kinase(s) that phosphorylates Xchk1 is unknown, but studies in fission yeast have implicated the protein kinase Rad3 in the regulation of Chk1 (al-Khodairy et al., 1994; Bentley et al., 1996; Walworth and Bernards, 1996). Rad3 is a member of the DNA-dependent protein kinase family with homologues known as ATM and ATR in mammals (Paulovich and Hartwell, 1995; Keegan et al., 1996; Carr, 1997; Flagg et al., 1997; Westphal, 1997; Cliby et al., 1998). X. laevis homologues of ATM and ATR have not been described, but these proteins could potentially be involved either directly or indirectly in the checkpoint-associated phosphorylation of Xchk1.

In conclusion, we have shown that Xchk1 phosphorylates Ser-287 of Xenopus Cdc25, thereby allowing 14-3-3 proteins to associate with this activator of Cdc2. Xchk1 appears to play an important role in mitotic timing in egg extracts: Xchk1-depleted extracts are compromised in their checkpoint responsiveness and exogenous addition of activated Xchk1 can delay mitosis substantially even in the absence of unreplicated or damaged DNA. Nonetheless, Xchk1 does not account fully for the phosphorylation of Cdc25 on Ser-287, since binding of 14-3-3 to Cdc25 is not diminished in Xchk1-depleted extracts. The checkpoint response of egg extracts to unreplicated DNA appears to involve both Xchk1-dependent (caffeine-sensitive) and Xchk1-independent (caffeine-insensitive) pathways. The nature of the caffeine-insensitive, Xchk1-independent pathway remains to be determined. In principle, it could involve either the Ser-287–specific kinase that is distinct from Xchk1 or some other facet of Cdc2 regulation.

We are very grateful to W. Sullivan (University of California, Santa Cruz, CA) for providing the sequence of Drosophila Grapes. We thank our colleagues for comments on the manuscript.

This work was supported by a grant from the National Institutes of Health (GM-43974) and a postdoctoral fellowship from the Damon Runyon-Walter Winchell Cancer Research Fund to S.X. Wang. W.G. Dynunhy is an investigator of the Howard Hughes Medical Institute.

Received for publication 15 May 1998 and in revised form 7 July 1998.

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