Molecular Cell, Vol. 6, 839–849, October, 2000, Copyright ©2000 by Cell Press

Claspin, a Novel Protein Required for the Activation of Chk1 during a DNA Replication Checkpoint Response in *Xenopus* Egg Extracts

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Summary

We have identified Claspin, a novel protein that binds to *Xenopus* Chk1 (Xchk1). Binding of Claspin to Xchk1 is highly elevated in the presence of DNA templates that trigger a checkpoint arrest of the cell cycle in *Xenopus* egg extracts. Xchk1 becomes phosphorylated during a checkpoint response, and we demonstrate directly that this phosphorylation results in the activation of Xchk1. Immunodepletion of Claspin from egg extracts abolishes both the phosphorylation and activation of Xchk1. Furthermore, Claspin-depleted extracts are unable to arrest the cell cycle in response to DNA replication blocks. Taken together, these findings indicate that Claspin is an essential upstream regulator of Xchk1.

Introduction

The monitoring of damaged or incompletely replicated DNA by checkpoint mechanisms guarantees that eukaryotic cells maintain genomic integrity during cell division (Elledge, 1996). These pathways ensure that cell cycle progression is stalled until aberrant DNA structures or replication intermediates can be eliminated. An ultimate target of these control mechanisms is the Cdc2–cyclin B complex, also known as maturation or M phase promoting factor (MPF), the kinase that triggers the entry into mitosis. During interphase, the Cdc2 subunit of MPF is downregulated by inhibitory phosphorylations on its Thr-14 and Tyr-15 residues. A regulatory system containing two inhibitory kinases (Myt1 and Wee1) and a stimulatory phosphatase (Cdc25C) controls the activity of Cdc2 through reversible phosphorylation of these residues (Morgan, 1997). Checkpoint mechanisms prevent the activating dephosphorylation of Cdc2 at the G2/M transition unless two accurate copies of the genome are available for transmission to daughter cells.

Genetic studies in yeast have identified proteins that are involved in sensing damaged and/or incompletely replicated DNA and transmitting this information to effector molecules that interact directly with the cell cycle control machinery. In fission yeast, the sensor proteins are currently thought to include Rad1, Rad3, Rad9, Rad17, Rad26, Hus1, and Crb2/Rhp9. Effector proteins include the kinases Chk1 and Cds1 as well as 14-3-3 proteins such as Rad24 (O'Connell et al., 2000). Similar pathways are found in budding yeast, and homologs of many of these proteins have been identified in higher eukaryotes, including humans, *Xenopus*, *Drosophila*, and mice (Elledge, 1996; Longhese et al., 1998; O'Connell et al., 2000). At this time, the most well-established function of Chk1 in yeast and vertebrates is to mediate the binding of 14-3-3 proteins to Cdc25, which results in its cytoplasmic sequestration (Kumagai and Dunphy, 1999; Lopez-Girona et al., 1999; Yang et al., 1999; Zeng and Piwnica-Worms, 1999).

Phosphoinositide kinase relatives in fission yeast (Rad3), budding yeast (Mec1), and vertebrates (Atm and Atr) play an essential role in signaling the presence of damaged and/or unreplicated DNA to downstream regulators (Elledge, 1996; Longhese et al., 1998; O'Connell et al., 2000). For example, in fission yeast, Chk1 and Cds1 cannot function normally in the absence of Rad3 (Walworth and Bernards, 1996; Lindsay et al., 1998). Similarly, Mec1 is a critical regulator of Rad53, a Cds1 homolog in budding yeast (Sanchez et al., 1996). In vertebrates, Atm is an upstream regulator of Chk2/Cds1 (Matsuoka et al., 1998; Brown et al., 1999). Atr is essential for genomic stability and early embryonic viability in mice (Brown and Baltimore, 2000; de Klein et al., 2000).

Despite these insights, relatively little is known about how the Cds1 and Chk1 families respond to checkpoint signals. For example, in budding yeast, Rad53, a presumed target of Mec1, must bind to Rad9 to undergo phosphorylation and activation (de la Torre-Ruiz et al., 1998; Sun et al., 1998). Chk1 also becomes phosphorylated during checkpoint responses in various organisms (Walworth and Bernards, 1996; Sanchez et al., 1997; Kumagai et al., 1998). Although it is widely assumed that this phosphorylation leads to activation of Chk1, experimental proof of this possibility has not been provided. In fission yeast, both the phosphorylation of Chk1 and the ability of Chk1 to function in checkpoint control depend upon Crb2/Rhp9, a relative of budding yeast Rad9 (Saka et al., 1997; Willson et al., 1997). Genetic and two-hybrid experiments have established a close relationship between Chk1 and Crb2/Rhp9, but a direct interaction between these proteins has not been reported.

We have been using *Xenopus* egg extracts to investigate the regulation of Xchk1, the frog homolog of Chk1. As reported previously, Xchk1 becomes extensively phosphorylated in the presence of unreplicated or UV-damaged DNA (Kumagai et al., 1998). Furthermore, immunodepletion of Xchk1 from egg extracts greatly compromises, but does not completely abolish, the delay of mitotic entry that is triggered by unreplicated/UV-damaged DNA, indicating the existence of Xchk1-dependent and Xchk1-independent cell cycle arrest mechanisms.

In this report, we have developed a system in which the addition of synthetic oligonucleotides to *Xenopus* egg extracts results in the efficient phosphorylation of Xchk1. Using this system, we have been able to demonstrate that phosphorylation leads to the activation of Xchk1. Furthermore, we have discovered a novel protein, Claspin, that binds to Xchk1 in a checkpoint-dependent manner. Immunodepletion of Claspin from *Xenopus* egg extracts abolishes the activation of Xchk1 and,

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furthermore, greatly compromises the cell cycle delay induced by unreplicated DNA. Thus, Claspin is an essential upstream regulator of Chk1 in *Xenopus* and possibly other vertebrates, such as humans.

Results

Tautomycin Stabilizes the Phosphorylation of Xchk1 in *Xenopus* Egg Extracts

In the presence of incompletely replicated or UV-damaged chromosomal DNA, only a small portion of the Xchk1 in whole *Xenopus* egg extracts undergoes phosphorylation (Figure 1A, top; see also Kumagai et al., 1998). The phosphorylated form of Xchk1 is highly enriched in the nuclear fraction of the extracts (Kumagai et al., 1998). To study the properties of phosphorylated Xchk1 more readily, we developed a system in which Xchk1 could undergo efficient phosphorylation in whole egg extracts in the presence of synthetic oligonucleotides. The establishment of this system was facilitated by the use of a phosphatase inhibitor, tautomycin, that stabilizes the phosphorylation of Xchk1 in egg extracts. As described in Figure 1A, we added 3 μM tautomycin to egg extracts containing *Xenopus* sperm chromatin and aphidicolin, a DNA polymerase inhibitor that induces DNA replication blocks and thereby inhibits cell cycle progression. We observed that tautomycin caused a significant accumulation of phosphorylated Xchk1 (Figure 1A, bottom, lane 3). Likewise, treatment with tautomycin also resulted in an increase in the amount of phosphorylated Xchk1 that appeared in response to UV-damaged sperm chromatin (Figure 1A, bottom, lane 5). Significantly, tautomycin did not have any effect on the phosphorylation of Xchk1 in extracts lacking aphidicolin or UV-damaged DNA (Figure 1A, bottom, lanes 1 and 2). Furthermore, caffeine, an agent that inhibits the checkpoint-dependent phosphorylation of Xchk1 and overrides checkpoint controls in *Xenopus* egg extracts as well as other systems (see Kumagai et al., 1998), blocked the tautomycin-stimulated phosphorylation of Xchk1 in extracts containing aphidicolin or UV-damaged DNA (Figure 1A, bottom, lanes 4 and 6).

It is necessary to add tautomycin to egg extracts at least 30 min after the addition of *Xenopus* sperm chromatin and aphidicolin. In fact, the addition of tautomycin, sperm chromatin, and aphidicolin together at the start of the incubation actually prevents the phosphorylation of Xchk1 (data not shown). Removal of protein phosphatase 2A (PP2A) from egg extracts prevents the initiation of DNA replication but not elongation at existing replication forks (Lin et al., 1998). Tautomycin is an inhibitor of PP2A, among other phosphatases (see Discussion). Furthermore, a period of about 30 min is required for the formation of pre-replication complexes and initiation of replication in egg extracts (Coleman et al., 1996). Thus, premature addition of tautomycin most probably blocks the formation of DNA replication forks, which are required for activation of the DNA replication checkpoint (Li and Deshaies, 1993).

Phosphorylation of Xchk1 in the Presence of Synthetic Oligonucleotides

Previously, our laboratory has demonstrated that various defined DNA templates, including linearized plas-

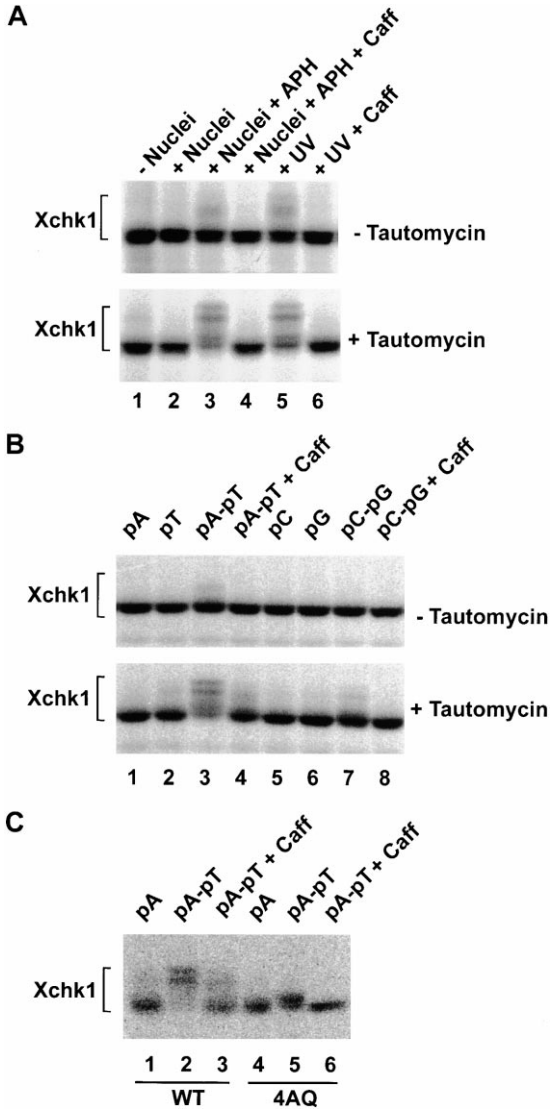

Figure 1. Phosphorylation of Xchk1 in Egg Extracts in the Presence of Synthetic Oligonucleotides

(A) Tautomycin stabilizes the phosphorylation of Xchk1. ^{35}S-labeled Xchk1 was incubated for 30 min in interphase egg extracts containing no sperm nuclei (lane 1), 3000 nuclei/μl (lane 2), 3000 nuclei/μl and 100 μg/ml aphidicolin (lanes 3 and 4), or 3000 UV-irradiated nuclei/μl (lanes 5 and 6) in the presence (lanes 4 and 6) or absence (lanes 1–3 and 5) of 5 mM caffeine. The extracts were divided, and the incubation was continued for 70 min in the absence (top) or presence (bottom) of 3 μM tautomycin. Aliquots (2 μl) were removed for SDS-PAGE and autoradiography.

(B) Effect of various oligonucleotides on the phosphorylation of Xchk1. ^{35}S-labeled Xchk1 was added to egg extracts containing 50 μg/ml poly(dA)$_{70}$ (lane 1), 50 μg/ml poly(dT)$_{70}$ (lane 2), 50 μg/ml poly(dA)$_{70}$-poly(dT)$_{70}$ (lanes 3 and 4), 50 μg/ml poly(dC)$_{70}$ (lane 5), 50 μg/ml poly(dG)$_{70}$ (lane 6), or 50 μg/ml poly(dC)$_{70}$-poly(dG)$_{70}$ (lanes 7 and 8) in the presence (lanes 4 and 8) or absence (lanes 1–3 and 5–7) of 5 mM caffeine. The extracts were incubated for 100 min in the absence (top) or presence (bottom) of 3 μM tautomycin. Aliquots (2 μl) were removed for SDS-PAGE and autoradiography. pA, pT, pC, and pG refer to poly(dA)$_{70}$, poly(dT)$_{70}$, poly(dC)$_{70}$, and poly(dG)$_{70}$, respectively.

(C) ^{35}S-labeled wild-type Xchk1 (lanes 1–3) and Xchk1-4AQ proteins (lanes 4–6) were incubated for 100 min in interphase extracts containing either poly(dA)$_{70}$ (lanes 1 and 4) or poly(dA)$_{70}$-poly(dT)$_{70}$ (lanes 2 and 3, 5, and 6) in the presence (lanes 3 and 6) or absence (lanes 1 and 2, 4 and 5) of 5 mM caffeine. All samples contained 3 μM tautomycin.

mids, double-stranded oligonucleotides, partially nicked M13 DNA, and poly(dT)$_{40}$, all trigger the phosphorylation of Xcds1 but not Xchk1 in *Xenopus* egg extracts (Guo and Dunphy, 2000). These templates either contain double-stranded DNA ends or undergo replication to a double-stranded form, in the case of M13 and poly(dT)$_{40}$, indicating that double-stranded DNA ends trigger the phosphorylation of Xcds1. Nonetheless, one intriguing aspect of these studies was that, even though M13 DNA and poly(dT)$_{40}$ must be replicated to a double-stranded form to induce phosphorylation of Xcds1, these templates did not elicit the phosphorylation of Xchk1. We considered the possibility that, since these templates are not incorporated into a nuclear structure, any phosphorylated Xchk1 that could potentially be generated as a result of their presence might be more susceptible to dephosphorylation in whole egg extracts.

To examine this possibility, we incubated a number of different DNA templates in *Xenopus* extracts in the presence or absence of the phosphatase inhibitor tautomycin. In one set of experiments, we first added various DNA homopolymers—poly(dA)$_{70}$, poly(dT)$_{70}$, poly(dC)$_{70}$, and poly(dG)$_{70}$—to extracts containing ^{35}S-labeled Xchk1 and subsequently examined the phosphorylation of Xchk1. As shown in Figure 1B, none of these individual homopolymers had any effect on the phosphorylation of Xchk1 in the absence of tautomycin. Poly(dT)$_{70}$ induced a modest phosphorylation of Xchk1 in the presence of tautomycin. However, if we added a preannealed mixture of poly(dA)$_{70}$-poly(dT)$_{70}$ to tautomycin-containing extracts, there was a pronounced phosphorylation of Xchk1 (Figure 1B, bottom, lane 3). Without tautomycin, this modification was significantly diminished (Figure 1B, top, lane 3). Interestingly, preannealed poly(dC)$_{70}$-poly(dG)$_{70}$ was significantly less effective than poly(dA)$_{70}$-poly(dT)$_{70}$ (Figure 1B, top and bottom, lane 7). We also found that there was a strict requirement with regard to the length of the homopolymers. In particular, a preannealed mixture of shorter homopolymers, such as poly(dA)$_{40}$-poly(dT)$_{40}$, was not able to induce the modification of Xchk1 efficiently (data not shown).

Further characterization indicated that the phosphorylation of Xchk1 in the presence of the annealed homopolymers and tautomycin has similar properties to the modification of Xchk1 that occurs in nuclei containing unreplicated or UV-damaged DNA. For example, this phosphorylation was strongly reduced by treatment with caffeine (Figure 1B, bottom, lanes 4 and 8; Figure 1C, lane 3). Furthermore, the phosphorylation that was induced by poly(dA)$_{70}$-poly(dT)$_{70}$ was largely abolished in the Xchk1-4AQ mutant (Figure 1C, lane 5). In this mutant, the phosphorylatable residues of Xchk1 in its four conserved SQ/TQ motifs (Thr-314, Ser-344, Ser-356, and Ser-365) have all been mutated to alanine. Finally, we also found that poly(dA)$_{70}$-poly(dT)$_{70}$, but not poly(dA)$_{70}$, induces a cell cycle arrest efficiently in egg extracts (data not shown).

Isolation of Claspin, an Xchk1-Binding Protein

As one approach to understand the regulation of Xchk1, we searched for proteins that bind to Xchk1. We incubated a recombinant form of Xchk1 (Xchk1-WT-GST-His6) in *Xenopus* egg extracts under various conditions and subsequently reisolated the protein by sequential chromatography on nickel and glutathione agarose, respectively. As shown in Figure 2 (lane 3), we identified a 215 kDa protein (see arrow) that had associated with Xchk1 in the presence of poly(dA)$_{70}$-poly(dT)$_{70}$. The binding of this protein was significantly reduced if either 5 mM caffeine was included in the incubation or if poly(dA)$_{70}$ was used as the template (Figure 2, lanes 2 and 4). The 215 kDa protein was not isolated from mock incubations containing no recombinant Xchk1 (Figure 2, lane 1). In these experiments, we did not observe any other polypeptide with an abundance comparable to that of the 215 kDa protein, but we cannot formally exclude the possibility that an additional protein is involved in the interaction with Xchk1.

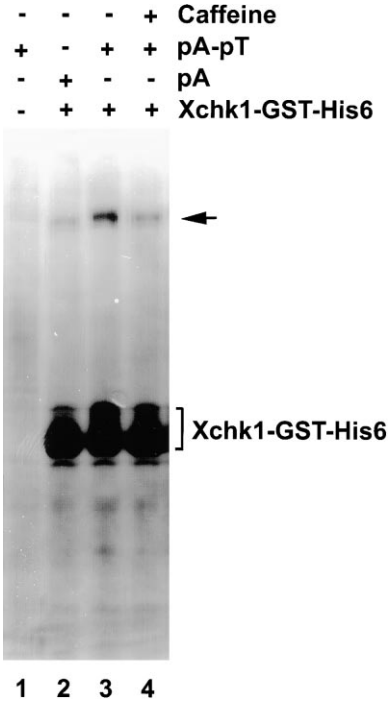

Figure 2. Isolation of an Xchk1-Binding Protein

Nickel agarose beads (10 μl) containing 5 μg of either Xchk1-WT-GST-His6 protein (lanes 2–4) or a control His6-GST protein (lane 1) were incubated in interphase egg extracts (100 μl) containing 3 μM tautomycin and either poly(dA)$_{70}$ (lane 2) or poly(dA)$_{70}$-poly(dT)$_{70}$ (lanes 1, 3, and 4) in the absence (lanes 1–3) or presence (lane 4) of 5 mM caffeine. The beads were reisolated and bound proteins were eluted with imidazole. The eluted proteins were purified further on glutathione agarose as described in Experimental Procedures. The samples were subjected to SDS-PAGE and silver staining. The control His6-GST protein was electrophoresed through the bottom of the gel in this experiment. Xchk1-WT-GST-His6 becomes only partially phosphorylated in the presence of poly(dA)$_{70}$-poly(dT)$_{70}$ in this case because it was added at a 25-fold molar excess over endogenous Xchk1.

We performed a large-scale purification to obtain a sufficient quantity of the 215 kDa protein for peptide sequencing analysis. We identified two peptide sequences that did not belong to a previously cloned protein. One combination of degenerate PCR primers based on these peptides was used to amplify a 450 bp DNA fragment, which in turn was employed to isolate a 4.8

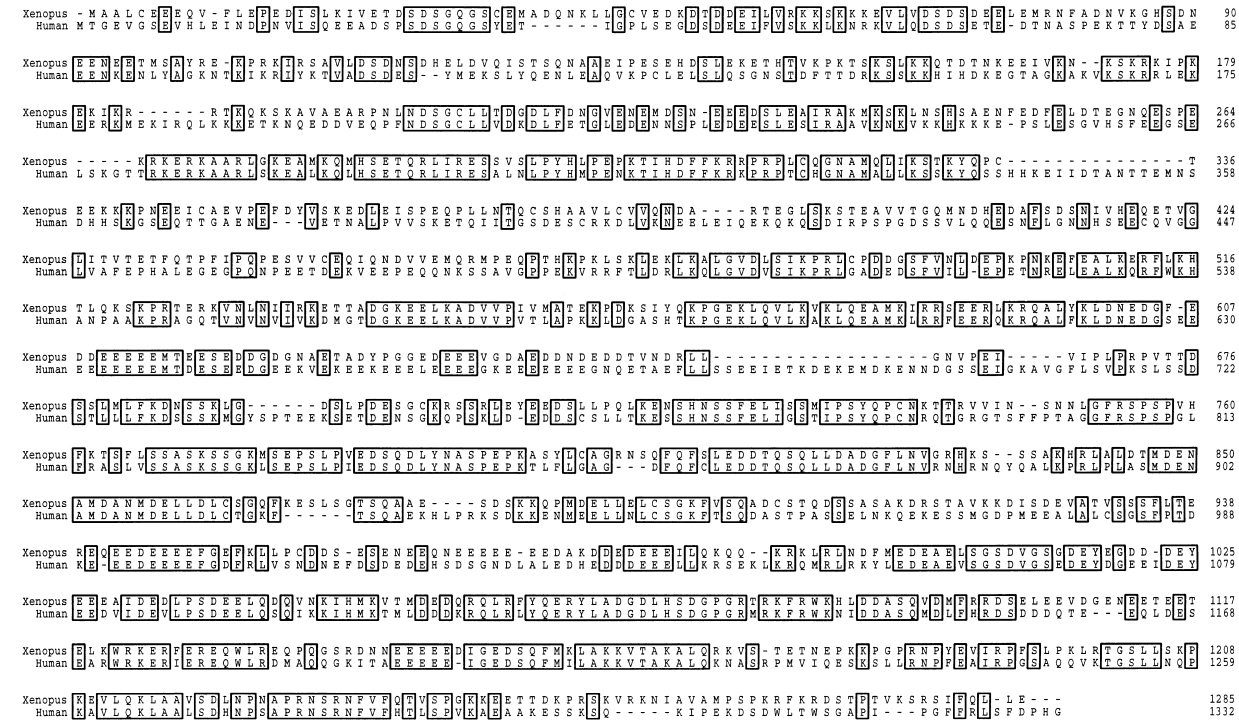

Figure 3. Amino Acid Sequences of *Xenopus* and Human Claspin

Sequences were aligned using the GCG PileUp program. Identical residues are boxed.

kb *Xenopus* oocyte cDNA (GenBank accession number AF297867). This cDNA encodes a 1285 amino acid polypeptide (calculated molecular mass, 145 kDa) that contains amino acid sequences (LAAVSDLNPNAPR and YLADGDLHSDGPGR) that are consistent with the peptide sequencing analysis. A BLAST search with the cDNA sequence indicated that it encodes a novel protein, which we have named Claspin.

To obtain further insight into the structure of Claspin, we identified its human homolog (Hu-Claspin) by using PCR primers based on sequences related to *Xenopus* Claspin in the human EST database. We isolated cDNA fragments from a human library and established the full-length cDNA sequence of Hu-Claspin (GenBank accession number AF297866). Conceptual translation of the cDNA encoding Hu-Claspin yielded a 1332 amino acid polypeptide that is 49% identical to *Xenopus* Claspin. The gene for Hu-Claspin is located at p34.1–34.3 on chromosome 1 according to the GenBank entry for its genomic sequence (AL139143). Although the *Xenopus* cDNA does not contain an in-frame termination codon upstream of the putative initiation codon, it most likely represents the full-length sequence. The human cDNA contains two in-frame stop codons upstream of an initiation codon that has a position almost identical to that of the putative start codon in *Xenopus* Claspin (Figure 3). Furthermore, endogenous Claspin in *Xenopus* egg extracts is just slightly smaller than recombinant His6-Claspin (see Figure 6A). It should be noted that *Xenopus* and human Claspin are quite acidic proteins (pI = 4.5), which may lead to anomalous migration during SDS-PAGE.

Claspin does not appear to possess any strictly defined sequence motifs that offer insight into its biochemical function. Both the *Xenopus* and human proteins have three conserved potential nuclear localization signals (amino acids 158–174, 312–316, and 1078–1084 in the *Xenopus* protein). Likewise, both proteins contain a relatively large number of SQ/TQ motifs (the *Xenopus* protein has eight SQ and four TQ motifs, while Hu-Claspin contains nine SQ and three TQ motifs). The serines and threonines in this type of motif are potential substrates for kinases such as ATM, ATR, and DNA-PK that are involved in checkpoint pathways (Kim et al., 1999). BLAST searches of the *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* databases did not reveal any obvious homolog of Claspin. However, a PSI-BLAST search identified a weak homology to the *Drosophila* CG1326 gene product (AAF47885), which is 28% identical to *Xenopus* Claspin.

Phosphorylation of Claspin Is Required for Binding to Xchk1

In order to characterize the interaction of Claspin with Xchk1, we prepared ^{35}S-labeled versions of full-length Claspin, its N-terminal domain (Claspin-N, amino acids 1–743), and a C-terminal fragment (Claspin-C, amino acids 776–1285). First, we added these labeled proteins to egg extracts in the presence of poly(dA)$_{70}$, poly(dA)$_{70}$-poly(dT)$_{70}$, or both poly(dA)$_{70}$-poly(dT)$_{70}$ and caffeine. We found that full-length Claspin became modified in the presence of poly(dA)$_{70}$-poly(dT)$_{70}$, but not poly(dA)$_{70}$, and that this modification was reversed by caffeine (Figure 4A, top, lanes 1–3). This modification appears to be restricted largely, if not exclusively, to the C-terminal domain of Claspin, since Claspin-C, but not Claspin-N,

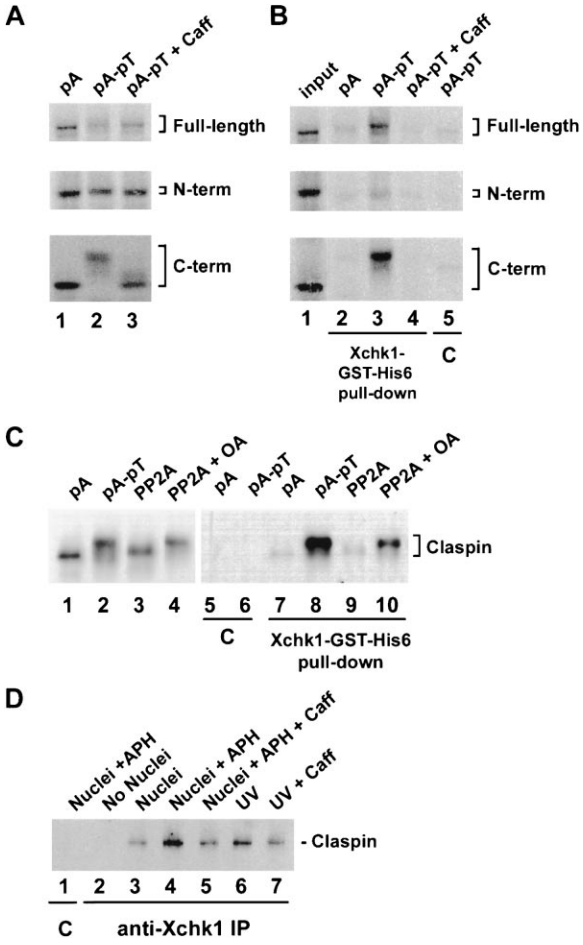

Figure 4. Phosphorylation of Claspin Is Required for Binding to Xchk1

(A) Modification of Claspin. ^{35}S-labeled full-length Claspin (top), Claspin-N (middle), and Claspin-C (bottom) were incubated for 100 min in egg extracts containing poly(dA)$_{70}$ (lane 1), poly(dA)$_{70}$-poly(dT)$_{70}$ (lane 2), or poly(dA)$_{70}$-poly(dT)$_{70}$ and 5 mM caffeine (lane 3). Aliquots were subjected to SDS-PAGE and autoradiography.

(B) Egg extracts containing ^{35}S-labeled full-length Claspin (top), Claspin-N (middle), and Claspin-C (bottom), and poly(dA)$_{70}$ (lane 2), poly(dA)$_{70}$-poly(dT)$_{70}$ (lanes 3 and 5), or poly(dA)$_{70}$-poly(dT)$_{70}$ and 5 mM caffeine (lane 4) were incubated in the presence of nickel agarose containing either Xchk1-WT-GST-His6 (lanes 2–4) or a control His6-GST protein (lane 5). The beads were isolated, washed, and subjected to SDS-PAGE and autoradiography. Lane 1 shows the input ^{35}S-labeled proteins.

(C) Phosphorylated Claspin associates with Xchk1. Claspin-GST-His6 was incubated in egg extracts containing either poly(dA)$_{70}$ (lanes 1, 5, and 7) or poly(dA)$_{70}$-poly(dT)$_{70}$ and 3 μM tautomycin (lanes 2–4, 6, and 8–10) and subsequently reisolated with nickel agarose. The GST-His6 tag was then removed with thrombin. Portions of some samples were incubated with PP2A in either the presence (lanes 4 and 10) or absence of 3 μM okadaic acid (lanes 3 and 9). The samples were subjected to SDS-PAGE directly (lanes 1–4) or assayed for binding to Xchk1 (lanes 5–10). For binding assays, samples were incubated at 4°C for 30 min with nickel agarose containing either Xchk1-WT-GST-His6 (lanes 7–10) or control His6-GST (lanes 5 and 6). After washing, the beads were subjected to SDS-PAGE. All samples were immunoblotted with anti-Claspin antibodies.

(D) Interaction of endogenous Claspin and Xchk1 in extracts containing unreplicated or UV-damaged *Xenopus* sperm chromatin. Interphase extracts containing 100 μg/ml cycloheximide and no sperm nuclei (lane 2), 3000 nuclei/μl (lane 3), 3000 nuclei/μl and 100 μg/ml aphidicolin (lanes 1, 4, and 5), or 3000 UV-damaged nuclei/μl (lanes 6 and 7) were incubated for 100 min at 23°C in the absence (lanes 1–4 and 6) or presence of 5 mM caffeine (lanes 5 and 7). Extracts were immunoprecipitated with control IgG (lane 1) or anti-Xchk1 antibodies (lanes 2–7). The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-Claspin antibodies.

underwent a substantial, caffeine-sensitive upshift in electrophoretic mobility in the presence of poly(dA)$_{70}$-poly(dT)$_{70}$ (Figure 4A, middle and bottom, lanes 1–3).

Next, we examined the interaction of full-length Claspin, Claspin-N, and Claspin-C with Xchk1. For this purpose, we incubated ^{35}S-labeled versions of these proteins in egg extracts containing Xchk1-WT-GST-His6 and subsequently reisolated the recombinant Xchk1 with nickel agarose. As shown in Figure 4B, both full-length Claspin and Claspin-C, but not Claspin-N, bound to Xchk1. Moreover, this interaction occurred in the presence of poly(dA)$_{70}$-poly(dT)$_{70}$, but not poly(dA)$_{70}$, and was sensitive to caffeine (Figure 4B, lanes 2–5).

To ask whether the modification of Claspin represented phosphorylation, we treated the upshifted form of Claspin with PP2A. For this experiment, we incubated recombinant Claspin-GST-His6 in egg extracts containing poly(dA)$_{70}$ or poly(dA)$_{70}$-poly(dT)$_{70}$. Subsequently, we reisolated recombinant Claspin with nickel agarose. Next, we treated the modified form of Claspin that had appeared in response to poly(dA)$_{70}$-poly(dT)$_{70}$ with PP2A in the absence or presence of okadaic acid, an inhibitor of PP2A (Figure 4C, lane 2). The modification of Claspin was reversed substantially by PP2A, and this reversal was sensitive to okadaic acid (Figure 4C, lanes 2–4).

To evaluate the importance of this phosphorylation for the binding of Claspin to Xchk1, we performed the following experiment. We removed the tags from Claspin with thrombin and then incubated the various modified forms of Claspin with Xchk1-WT-GST-His6 or, in some cases, a control His6-GST protein, both of which were bound to nickel agarose beads. After reisolating and washing the beads, we observed by immunoblotting with anti-Claspin antibodies that only the phosphorylated form of Claspin interacted specifically with Xchk1 (Figure 4C, lanes 8 and 10).

To explore further the physiological significance of the binding between Claspin and Xchk1, we examined this interaction in egg extracts containing incompletely replicated or UV-damaged nuclear DNA. To address this issue, we used demembranated *Xenopus* sperm chromatin that had been added to egg extracts to allow the formation of reconstituted nuclei (see Murray, 1991). To induce the formation of DNA replication blocks in the nuclear chromatin, the DNA polymerase inhibitor aphidicolin was added to these extracts (Dasso and Newport, 1990). Alternatively, UV-treated sperm chromatin can be used as a source of damaged DNA (Kumagai et al., 1998). As described in Figure 4D, we incubated egg extracts in the presence of no chromatin, chromatin and aphidicolin, or UV-damaged chromatin. In some cases, caffeine was also included in the incubation. Next, we performed immunoprecipitation with anti-Xchk1 or control antibodies and examined the immunoprecipitates by immunoblotting with anti-Claspin antibodies. The in-

teraction between endogenous Claspin and Xchk1 was increased in the presence of aphidicolin or UV-damaged DNA. Moreover, the binding between Claspin and Xchk1 was significantly reduced in the presence of caffeine.

Phosphorylation of Xchk1 Results in Its Activation

Chk1 undergoes phosphorylation during a checkpoint response in various experimental systems (Walworth and Bernards, 1996; Sanchez et al., 1997; Kumagai et al., 1998), but the functional consequence of this phosphorylation has not been established. In the case of *Xenopus* egg extracts, it had been difficult to isolate the phosphorylated form of Xchk1 efficiently to address this issue conclusively. Using the technical improvements reported here for the preparation of phosphorylated Xchk1, we investigated whether this modification affects the kinase activity of Xchk1. For this purpose, we added recombinant Xchk1-WT-GST-His6 to *Xenopus* egg extracts containing tautomycin and either poly(dA)$_{70}$ or poly(dA)$_{70}$-poly(dT)$_{70}$. After a 100 min incubation, we reisolated recombinant Xchk1 with glutathione agarose and assayed kinase activity toward Cdc25 with the model substrate GST-Cdc25(254–316)-WT.

As shown in Figure 5, the hyperphosphorylated form of Xchk1-WT-GST-His6 that was isolated from extracts containing poly(dA)$_{70}$-poly(dT)$_{70}$ was approximately 3-fold more active than the hypophosphorylated version of the protein from extracts that contained poly(dA)$_{70}$. This increase may be an underestimate of the maximum possible degree of activation because the recovery of hyperphosphorylated Xchk1 is consistently somewhat lower than that of the hypophosphorylated form. In parallel, we also examined a kinase-inactive mutant of Xchk1 (Xchk1-N135A-GST-His6) and a mutant (Xchk1-4AQ-GST-His6) that cannot undergo phosphorylation of the four conserved SQ/TQ motifs of Xchk1. The kinase-inactive mutant of Xchk1 that was isolated from either type of extract was not able to phosphorylate the Cdc25 substrate in these assays. Also, consistent with previous results (Kumagai et al., 1998), kinase-inactive Xchk1 became only partially phosphorylated in the presence of poly(dA)$_{70}$-poly(dT)$_{70}$, due to its incapacity for autophosphorylation. Finally, the Xchk1-4AQ mutant did not undergo phosphorylation or an increase in kinase activity in the presence of poly(dA)$_{70}$-poly(dT)$_{70}$, indicating that phosphorylation of the SQ/TQ domain is required for the activation of Xchk1.

In separate experiments, we also assayed the kinase activity of hyperphosphorylated Xchk1 that appeared in response to aphidicolin-induced replication blocks in nuclei that had formed around sperm chromatin in egg extracts. For this purpose, we added recombinant Xchk1-WT-GST-His6 to egg extracts containing 3 μM tautomycin and either no nuclei or 3000 nuclei/μl and 100 μg/ml aphidicolin. After reisolating the added Xchk1 with glutathione agarose, we found that Xchk1 was activated 3-fold when DNA replication blocks were present (data not shown). Thus, both incompletely replicated chromatin and poly(dA)$_{70}$-poly(dT)$_{70}$ elicit a similar activation of Xchk1.

Immunodepletion of Claspin Blocks Phosphorylation and Activation of Xchk1

In order to investigate the role of Claspin in the regulation of Xchk1, we immunodepleted endogenous Claspin

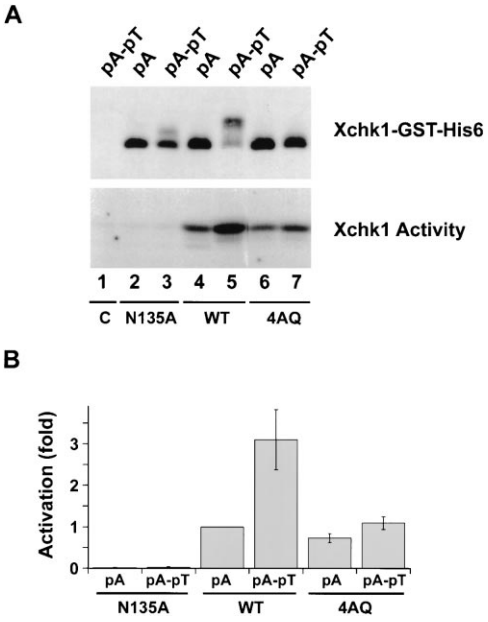

Figure 5. The Phosphorylated Form of Xchk1 Displays Increased Kinase Activity

(A) Xchk1-WT-GST-His6 (lanes 4 and 5), Xchk1-N135A-GST-His6 (lanes 2 and 3), Xchk1-4AQ-GST-His6 (lanes 6 and 7), and control His6-GST (lane 1) proteins were incubated for 100 min in egg extracts containing 3 μM tautomycin and either poly(dA)$_{70}$ (lanes 2, 4, and 6) or poly(dA)$_{70}$-poly(dT)$_{70}$ (lanes 1, 3, 5, and 7). Tagged proteins were isolated using glutathione agarose. The samples were assayed for kinase activity toward GST-Cdc25(254–316)-WT (bottom) or immunoblotted with anti-GST antibodies (top).

(B) Quantitation of the kinase activity of Xchk1 as measured in (A). The values are expressed relative to the activity of recombinant Xchk1 isolated from extracts containing poly(dA)$_{70}$. Results are the mean ± SD from three experiments.

from *Xenopus* egg extracts. Using polyclonal antibodies that were raised against an N-terminal fragment of the protein, we were able to remove Claspin from egg extracts (Figure 6A, lane 3). The level of Claspin was not diminished in a mock-depleted extract that was treated with control IgG (Figure 6A, lanes 1 and 2). We have estimated that the endogenous concentration of Claspin in egg extracts is approximately 35 μg/ml (240 nM). As shown by immunoblotting with anti-Claspin antibodies, the addition of 35 μg/ml His6-Claspin to Claspin-depleted extracts restored the protein to its normal level (Figure 6A, lane 4).

Next, we added recombinant Xchk1-WT-GST-His6 to the various extracts in the presence of either poly(dA)$_{70}$ or poly(dA)$_{70}$-poly(dT)$_{70}$. Subsequently, we reisolated recombinant Xchk1 from the extracts with glutathione agarose and examined its state of phosphorylation under each condition. As shown in Figure 6B (top, lanes 1–4), the checkpoint-dependent phosphorylation of Xchk1 in Claspin-depleted extracts was almost completely abolished in comparison with mock-depleted extracts. Significantly, the addition of recombinant His6-Claspin to Claspin-depleted extracts completely restored the phosphorylation of Xchk1 (Figure 6B, top, lanes 5 and 6), indicating that the defect in phosphorylation of Xchk1 in the depleted extracts is due to the absence of Claspin.

In parallel, we examined the kinase activity of recom-

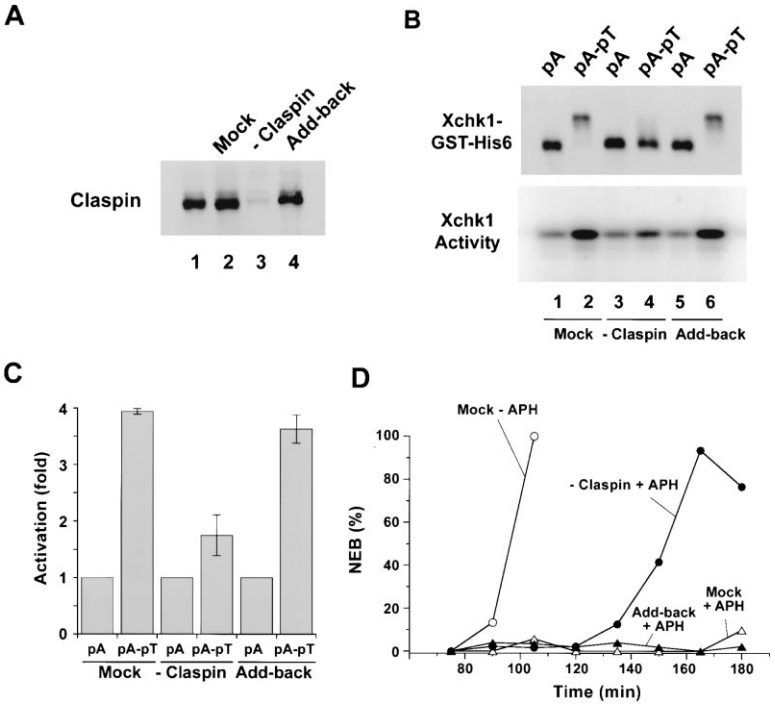

Figure 6. Claspin Is Required for the Phosphorylation and Activation of Xchk1 and for the Replication Checkpoint in *Xenopus* Egg Extracts

(A) Immunodepletion of Claspin. Extracts were treated with either anti-Claspin antibodies (lanes 3 and 4) or control IgG (lane 2) as described in Experimental Procedures. For lane 4, recombinant His6-Claspin was added to a Claspin-depleted extract at a final concentration of 35 μg/ml.

(B) Requirement for Claspin in the phosphorylation and activation of Xchk1. Xchk1-WT-GST-His6 was added to a mock-depleted extract (lanes 1 and 2), Claspin-depleted extract (lanes 3 and 4), or Claspin-depleted extract containing 35 μg/ml His6-Claspin (lanes 5 and 6). The extracts were incubated for 100 min in the presence of 3 μM tautomycin and either poly(dA)$_{70}$ (lanes 1, 3, and 5) or poly(dA)$_{70}$-poly(dT)$_{70}$ (lanes 2, 4, and 6). Tagged proteins were isolated using glutathione agarose. A portion of each sample was removed and processed for immunoblotting with anti-GST antibodies (top) or assayed for kinase activity as described in Figure 5A (bottom).

(C) Quantitation of the kinase activity of Xchk1 as measured in (B). Results are the mean ± SD from two experiments.

(D) Claspin is required for the DNA replication checkpoint. Extracts were treated with anti-Claspin antibodies (closed circles, closed triangles) or control antibodies (open circles, open triangles). In one case (closed triangles), His6-Claspin was added to a concentration of 35 μg/ml. The extracts were incubated with demembranated *Xenopus* sperm nuclei (1000/μl) in the absence (open circles) or presence of 100 μg/ml aphidicolin (closed triangles, closed circles, and open triangles). The timing of nuclear envelope breakdown (NEB) was monitored by microscopy.

binant Xchk1 that was isolated from the various extracts that underwent the immunodepletion procedure. Consistent with the results described above, recombinant Xchk1 was activated about 4-fold in extracts containing poly(dA)$_{70}$-poly(dT)$_{70}$ (Figure 6B, bottom; Figure 6C). This activation was strongly reduced in Claspin-depleted extracts, and was completely restored by the addition of recombinant His6-Claspin to these extracts. Taken together, these findings indicate that Claspin is required both for the checkpoint-dependent phosphorylation of Xchk1 and for the resulting increase in its kinase activity.

Claspin Is Necessary for the Delay of the Cell Cycle in Response to Incompletely Replicated DNA

In order to investigate the biological role of Claspin in the *Xenopus* system, we examined cell cycle progression in Claspin-depleted egg extracts containing incompletely replicated DNA. To address this question, we removed Claspin from egg extracts with anti-Claspin antibodies and then added aphidicolin and demembranated *Xenopus* sperm chromatin to induce the formation of DNA replication blocks. Finally, we examined the entry into mitosis by monitoring the timing of nuclear envelope breakdown (NEB). For comparison, we examined mock-depleted extracts that had been treated with a control IgG or Claspin-depleted extracts to which we added back recombinant His6-Claspin. As shown in Figure 6D, the Claspin-depleted extracts containing aphidicolin entered mitosis inappropriately (half-maximal NEB at 150 min), indicating that the DNA replication checkpoint had been compromised. By contrast, mock-depleted extracts arrested well in interphase in the presence of aphidicolin. Likewise, aphidicolin-treated, Claspin-depleted extracts that were supplemented with His6-Claspin remained in interphase, indicating that the defect in the extracts lacking Claspin is due to the absence of this protein. Immunodepletion of Claspin had no effect on chromosomal DNA replication in egg extracts, as measured by incorporation of [α-^{32}P]dATP (Coleman et al., 1996) (data not shown). Thus, the compromised cell cycle delay in aphidicolin-treated extracts lacking Claspin cannot be attributed to a defect in the formation of DNA replication blocks (see Li and Deshaies, 1993).

Significantly, the Claspin-depleted extracts containing aphidicolin entered mitosis more slowly than control extracts lacking aphidicolin. We observed a similar phenomenon in Xchk1-depleted extracts and therefore proposed that the aphidicolin-induced checkpoint in this system involves Xchk1-dependent and Xchk1-independent mechanisms (Kumagai et al., 1998). Overall, these findings indicate that removal of Claspin strongly compromises but does not completely eliminate the cell cycle delay that is triggered by DNA replication blocks. The response to aphidicolin is very similar in Xchk1-depleted extracts, which would be consistent with the interpretation that Claspin and Xchk1 act in the same pathway.

Discussion

In this report, we have searched for proteins that interact with Xchk1 in order to obtain insight into its function and regulation. Using *Xenopus* egg extracts, we were able to identify a large protein that binds to Xchk1 in the presence of DNA templates that induce the checkpoint-dependent phosphorylation of Xchk1. cDNA cloning

studies indicated that this Xchk1-associated protein is a novel polypeptide, which we have named Claspin. Functional characterization of Claspin has revealed that it is necessary for the phosphorylation and activation of Xchk1. Furthermore, Claspin is required for the proper operation of the DNA replication checkpoint. This observation is consistent with the earlier finding that Xchk1 plays an important role in the checkpoint monitoring the presence of incompletely replicated DNA (as well as UV-damaged DNA) in this system (Kumagai et al., 1998).

The identification and characterization of Claspin were facilitated by some technical improvements in how Xchk1 can be studied in *Xenopus* egg extracts. For example, we identified a phosphatase inhibitor, tautomycin, that causes accumulation of the phosphorylated form of Xchk1. Since tautomycin is a fairly broad inhibitor that acts upon protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), and other phosphatases (Hori et al., 1991), the relevant target for preventing the dephosphorylation of Xchk1 is unknown at this time. Nonetheless, the effect of tautomycin on Xchk1 appears to be quite specific for a number of reasons. This inhibitor does not affect the phosphorylation of Xchk1 in the absence of checkpoint-inducing DNA templates. Moreover, caffeine, an agent that blocks the checkpoint-dependent phosphorylation of Xchk1 and overrides checkpoint controls, also inhibits the tautomycin-stimulated modification of Xchk1.

We have also been able to identify synthetic oligonucleotides that trigger the phosphorylation of Xchk1 in *Xenopus* egg extracts. The most effective synthetic DNA template that we have found thus far is a preannealed mixture of the homopolymers poly(dA)$_{70}$ and poly(dT)$_{70}$. Poly(dA)$_{70}$ by itself has no effect on Xchk1, and poly(dT)$_{70}$ alone is much less effective than poly(dA)$_{70}$-poly(dT)$_{70}$. Likewise, poly(dC)$_{70}$-poly(dG)$_{70}$ is markedly inferior to poly(dA)$_{70}$-poly(dT)$_{70}$. The length of the homopolymers is also an important parameter. Interestingly, a mixture consisting of the shorter homopolymers poly(dA)$_{40}$ and poly(dT)$_{40}$ does not work well in comparison with poly(dA)$_{70}$-poly(dT)$_{70}$.

The molecular features of poly(dA)$_{70}$-poly(dT)$_{70}$ that trigger the downstream phosphorylation of Xchk1 remain to be identified. During the annealing reaction, the homopolymers would clearly form duplexes, most of which would contain 5′ and 3′ overhangs. In addition, more complex structures consisting of three or more oligonucleotides would be expected to form during this process. In principle, poly(dA)$_{70}$-poly(dT)$_{70}$ could be recognized directly by factors that control the phosphorylation of Xchk1. Alternatively, this template could be processed by DNA-metabolizing enzymes in the egg extracts. Our laboratory has previously shown that double-stranded DNA ends, which would mimic broken DNA generated by ionizing radiation, trigger the phosphorylation of Xcds1 but not Xchk1 (Guo and Dunphy, 2000). Thus, it is unlikely that double-stranded ends that either are initially present in the poly(dA)$_{70}$-poly(dT)$_{70}$ mixture or arise from replication of this template in egg extracts would serve as the signal that activates Xchk1. Nonetheless, various DNA replication enzymes, among other proteins, in egg extracts should interact well with poly(dA)$_{70}$-poly(dT)$_{70}$. In the case of chromosomal DNA, Xchk1 responds to inhibition of replication with aphidicolin, a drug that does not block the firing of origins but instead arrests replication near the priming stage (Mahbubani et al., 1997). Thus, some structure that either is originally present in poly(dA)$_{70}$-poly(dT)$_{70}$ or is generated from it by interaction with factors in egg extracts presumably resembles stalled replication forks in aphidicolin-treated nuclei.

Various observations indicate that Claspin is a critical upstream regulator of Xchk1. Xchk1 cannot undergo checkpoint-dependent phosphorylation and activation in the absence of Claspin. Furthermore, the DNA replication checkpoint in egg extracts, which is dependent upon Xchk1, becomes strongly compromised when Claspin is removed from the extracts. Claspin itself also becomes phosphorylated in the presence of DNA templates that induce the checkpoint-specific phosphorylation of Xchk1. Significantly, Claspin still becomes phosphorylated in Xchk1-depleted extracts (data not shown), suggesting that this modification occurs independently and, most probably, upstream of Xchk1 in this pathway. Phosphorylation of Claspin is necessary for its association with Xchk1, since dephosphorylation of Claspin in vitro with a protein phosphatase abolishes this binding. Taken together, these observations suggest the following model. In response to the appropriate DNA signal, Claspin becomes phosphorylated, binds to Xchk1, and thereby facilitates the phosphorylation/activation of Xchk1 by an upstream kinase.

The upstream kinase that directly phosphorylates and regulates Xchk1 has been identified as Xatr, the *Xenopus* homolog of Atr (Guo et al., 2000). Similarly, Liu et al. (2000) have provided evidence that Atr is a regulator of Chk1 in the human system. It is plausible that Claspin promotes the phosphorylation of Xchk1 by Xatr. One possibility is that Claspin could act as a scaffold or adaptor, as has been observed in other signal transduction systems (Pawson and Nash, 2000). Interestingly, Atm and Atr have similar substrate specificities toward a number of model peptide substrates (Kim et al., 1999), even though these kinases act in significantly distinct pathways in vertebrates. Claspin might serve to increase the fidelity of substrate recognition by presenting Chk1 to the appropriate regulatory kinase.

Genetic studies in budding and fission yeast have identified a group of proteins that act as upstream regulators of the Chk1 and Cds1 families. In fission yeast, for example, these proteins include Rad1, Rad3, Rad9, Rad17, Rad26, Hus1, and Crb2/Rhp9 (O'Connell et al., 2000). A similar group of proteins is found in budding yeast (Longhese et al., 1998). Interestingly, Claspin does not appear to be obviously homologous to any protein in fission or budding yeast. Nonetheless, Claspin could carry out a similar function as a yeast checkpoint protein. Alternatively, Claspin could execute a novel function that is required for the more complex and differently organized checkpoint pathways that exist in vertebrates.

Among the yeast checkpoint proteins, Claspin shares certain functional characteristics with budding yeast Rad9 and its fission yeast relative Crb2/Rhp9. In budding yeast, genetic studies have indicated that Rad9 is required for the phosphorylation of Rad53, a Cds1 homolog, in response to DNA damage (Longhese et al., 1998). In biochemical experiments, the phosphorylated form

of Rad9 interacts with Rad53, and this interaction is required for the phosphorylation and action of Rad53 (de la Torre-Ruiz et al., 1998; Sun et al., 1998). Likewise, Crb2/Rhp9 is necessary for both the phosphorylation and checkpoint function of Chk1 (Saka et al., 1997). Genetic and two-hybrid studies are consistent with an interaction between Chk1 and Crb2/Rhp9, but a complex containing these proteins has not been detected directly. Despite these similarities, there is not any statistically significant homology between the sequence of Claspin and that of either budding yeast Rad9 or fission yeast Crb2/Rhp9.

In conclusion, we have identified Claspin, a novel regulator of Chk1. Further study of Claspin may yield insights into the mechanisms by which effector kinases such as Chk1 are selectively regulated by distinct DNA signals from vertebrate genomes. In addition, it will be valuable to assess whether Claspin is essential for viability or genomic stability in mammals.

Experimental Procedures

Production of His6-GST, Xchk1-WT-GST-His6, and Xchk1-N135A-GST-His6 Proteins in Insect Cells

A KasI-NcoI fragment encoding glutathione S-transferase (GST) was created by PCR (polymerase chain reaction) using Pfu Turbo polymerase (Stratagene) with the appropriate primers and pGEX-2T as template. The fragment was digested with KasI and NcoI and cloned into pFastBacHTa (GIBCO–BRL) to yield a baculovirus vector with tandem six-histidine and GST tags (pFastBacHT-GST). pFastBac encoding C-terminally tagged Xchk1 (Xchk1-WT-GST-His6) was created by the following procedure. The coding sequence of Xchk1 was amplified in a PCR reaction, digested with RsrII and KasI, and cloned into pFastBacHT-GST. This plasmid was treated with BamHI and XbaI, and two annealed oligonucleotides encoding a six histidine tag were ligated into the vector to yield pFastBac-Xchk1-WT-GST-His6. pFastBac-Xchk1-N135A-GST-His6 and pFastBac-Xchk1-4AQ-GST-His6 were created by inserting the SacI-BstEII fragments of pBS-Xchk1-N135A (Kumagai et al., 1998) and pBS-Xchk1-4AQ, respectively, into pFastBac-Xchk1-WT-GST-His6. The preparation and characterization of the Xchk1-4AQ mutant are described elsewhere (Guo et al., 2000). The Xchk1-WT-GST-His6 protein contains a thrombin recognition sequence both between Xchk1 and GST and between GST and the six-histidine tag. Recombinant baculoviruses were produced using the Bac-to-Bac system (GIBCO–BRL). Recombinant proteins were isolated using nickel iminodiacetic acid (Ni-IDA) agarose as described (Kumagai and Dunphy, 1995).

Identification of an Xchk1-Binding Protein

Ni-IDA beads (10 μl) containing Xchk1-WT-GST-His6 protein (5 μg) were incubated for 100 min at 23°C in 100 μl of interphase egg extract containing 3 μM tautomycin, 100 μg/ml cycloheximide, and either 50 μg/ml poly(dA)$_{70}$ or 50 μg/ml poly(dA)$_{70}$-poly(dT)$_{70}$ in the presence or absence of 5 mM caffeine. The beads were isolated by centrifugation and washed four times in 10 mM HEPES-KOH [pH 7.5] containing 150 mM NaCl, 0.5% NP-40, 2.5 mM EGTA, and 20 mM β-glycerolphosphate. Bound proteins were eluted with 150 mM imidazole in the same buffer. The eluate was diluted to 500 μl and incubated with 10 μl Glutathione Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) for 30 min at 4°C. The beads were washed three times in the same buffer and then subjected to SDS-PAGE. For protein sequencing, 30 ml of interphase egg extract and 1.5 mg of Xchk1-WT-GST-His6 protein were used to isolate Claspin. Proteins were eluted from glutathione agarose with 0.1% SDS, concentrated with a Microsep 30K device (Filtron Technology), treated with dithiothreitol, alkylated, and subjected to SDS-PAGE. The gel was stained with SYPRO Red (Molecular Probes). Tryptic peptides from Claspin were separated on a Vydac C18 column and sequenced in the Caltech facility.

Cloning of *Xenopus* Claspin

Sets of degenerate oligonucleotides corresponding to the two peptide sequences [(L/D)AAVXDLNPNAPX and YLADGDLHSDGPGR; X denotes an ambiguous amino acid] obtained from Claspin were designed. A PCR reaction with GGIGC(A/G)TTIGG(A/G)TTIA(G/A)(G/A)TCI(G/C)(A/T)IACIGCIGC and GCIGA(T/C)GGIGA(T/C)(T/C)TICA(T/C)(T/A)(G/C)IGA(T/C)GGICCIGG, Ampli-Taq DNA polymerase (Perkin-Elmer), and *Xenopus* oocyte cDNA yielded a 450 bp fragment that encodes a segment of Claspin. The sequence of this fragment was used to design primers to isolate a 4.7 kb cDNA from a *Xenopus* oocyte library (Mueller et al., 1995) with the ClonCapture kit (Clontech). A 2.3 kb ApaI-XhoI fragment encoding the N-terminal half of the protein and a 2.4 kb XhoI-XhoI fragment encoding the C-terminal half of the protein were cloned into pBluescript SK− to yield pBS-Claspin-N and pBS-Claspin-C, respectively. The XhoI-XhoI fragment of pBS-Claspin-C was cloned into pBS-Claspin-N to yield a vector encoding full-length Claspin (pBS-Claspin). Nested deletions were sequenced at the Caltech DNA Sequencing Core Facility.

Cloning of Human Claspin

A BLAST analysis was conducted by using the sequence of *Xenopus* Claspin to search the human EST database. Several sequences with a strong homology to *Xenopus* Claspin sequence were found. Based on these sequences, two primers (CCACGGCTAGGTGCTGATGAA GATTCC and AACAGTGCTTGGCGCTTCTGGCG) were designed to isolate cDNAs by RACE (rapid amplification of cDNA ends) from a human fetal Marathon-Ready-cDNA library (Clontech).

Production of His6-Claspin, His6-Claspin-N, and Claspin-GST-His6 in Insect Cells

pFastBacHT-Claspin was created by cloning the NcoI-NheI and NheI-XhoI fragments of pBS-Claspin together into pFastBacHTa, thereby generating a baculovirus vector encoding a six-histidine-tagged, full-length Claspin. pFastBacHT-Claspin(1–464) encoding a six-histidine tagged N-terminal fragment of Claspin (amino acids 1–464) was created by amplifying the 1.4 kb NcoI-EcoRI fragment from pBS-Claspin-N by PCR and cloning it into pFastBacHTa that had been digested with NcoI and EcoRI. A baculovirus vector encoding Claspin with GST and six histidine tags at the C-terminal end (pFastBac-Claspin-GST-His6) was prepared as follows. pBS-Claspin was used in a PCR reaction with the appropriate primers to introduce an SpeI site at the termination codon of Claspin. The PCR product was digested with NheI and SpeI and ligated to the appropriate pFastBac fragment. Baculovirus-expressed His6-Claspin, His6-Claspin(1–464), and Claspin-GST-His6 were produced as described above.

Production of Anti-Claspin Antibodies

Polyclonal rabbit antibodies against the purified His6-Claspin(1–464) protein were produced at a commercial facility and affinity-purified as described (Kumagai et al., 1998).

Production of ^{35}S-Labeled Proteins

^{35}S-labeled full-length Claspin, Claspin-N (amino acids 1–743), and Claspin-C (amino acids 776–1285) were synthesized using pBS-Claspin, pBS-Claspin-N, and pBS-Claspin-C, respectively, as templates in the TNT in vitro transcription/translation system (Promega) in the presence of [^{35}S]Translabel (ICN Biomedicals). ^{35}S-labeled-Xchk1 proteins were synthesized as described (Kumagai et al., 1998).

In Vitro Claspin-Xchk1 Binding Assays

Claspin-GST-His6 protein (5 μg) bound to 10 μl of Ni-IDA beads was incubated for 100 min in interphase extracts (100 μl) containing 100 μg/ml cycloheximide, 3 μM tautomycin, and 50 μg/ml of either poly(dA)$_{70}$ or poly(dA)$_{70}$-poly(dT)$_{70}$. The beads were isolated by centrifugation and washed twice with buffer A (10 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 0.1% CHAPS, 2.5 mM EGTA, and 20 mM β-glycerolphosphate), once with HEPES-buffered saline (HBS; 10 mM HEPES-KOH [pH 7.5], 150 mM NaCl), and eluted with 150 mM imidazole in HBS. Eluted proteins were treated with 0.06 U thrombin in HBS containing 2.5 mM $CaCl_2$ for 2 hr at 4°C during dialysis against

HBS to remove imidazole. Digestion was stopped by adding 1 mM PMSF and 5 mM EGTA. Undigested protein and thrombin were removed by incubating with 10 μl Ni-IDA agarose and 5 μl aminobenzamidine agarose for 30 min at 4°C. In some cases, recombinant Claspin from extracts containing both poly(dA)$_{70}$-poly(dT)$_{70}$ and tautomycin was treated with 0.5 U protein phosphatase 2A (Upstate Biotechnology) in the presence or absence of 3 μM okadaic acid for 30 min at 23°C. The various preparations of Claspin were then incubated with either His6-GST or Xchk1-WT-GST-His6 bound to Ni-IDA beads in buffer A containing 1 mg/ml ovalbumin for 30 min at 4°C. The beads were washed three times in buffer A, and bound proteins were analyzed by immunoblotting with anti-Claspin antibodies.

Isolation and Assay of Xchk1 from Egg Extracts

Xchk1-WT-GST-His6, Xchk1-N135A-GST-His6, and Xchk1-4AQ-GST-His6 proteins (final concentration, 6 μg/ml) were incubated for 100 min in egg extracts (100 μl) containing 100 μg/ml cycloheximide, 3 μM tautomycin, and 50 μg/ml of either poly(dA)$_{70}$ or poly(dA)$_{70}$-poly(dT)$_{70}$. Extracts were diluted to 300 μl with buffer A and centrifuged in a 1 ml Sephadex G-25 column that had been pre-equilibrated with the same buffer to remove endogenous glutathione in the extracts. Glutathione agarose (10 μl) was incubated with the flowthrough fraction for 30 min at 4°C. The glutathione beads were washed three times with buffer A, once with HBS, and incubated in kinase assays with GST-Cdc25(254–316)-WT as the substrate as described (Kumagai et al., 1998).

Depletion of Claspin from Egg Extracts

Interphase egg extracts (170 μl) that had been activated for 15 min at 23°C by the addition of $CaCl_2$ were mixed with 30 μg of either affinity-purified anti-Claspin antibodies or control rabbit IgG bound to 30 μl of Affiprep-protein A beads (Bio-Rad) and incubated while rocking at 4°C for 1 hr. At the end of incubation, the beads were removed by centrifugation and the immunodepletion procedure was repeated.

Acknowledgments

We are grateful to members of Dunphy laboratory for discussion and comments. We also thank Felicia Rusnak and Gary Hathaway in the Caltech Protein/Peptide Micro Analytical Laboratory for peptide fragmentation and peptide sequencing analysis. This work was supported by a grant from the NIH. W. G. D. is an investigator in the Howard Hughes Medical Institute.

Received July 7, 2000; revised August 25, 2000.

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GenBank Accession Numbers

The accession numbers for the sequences reported in this article are AF297867 and AF297866.