The Cdc23 (Mcm10) protein is required for the phosphorylation of minichromosome maintenance complex by the Dfp1-Hsk1 kinase

Joon-Kyu Lee*†, Yeon-Soo Seo†, and Jerard Hurwitz*‡

*Program in Molecular Biology, Memorial Sloan–Kettering Cancer Center, New York, NY 10021; and †Department of Biological Science, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea

Contributed by Jerard Hurwitz, December 4, 2002

Previous studies in *Saccharomyces cerevisiae* have defined an essential role for the Dbf4-Cdc7 kinase complex in the initiation of DNA replication presumably by phosphorylation of target proteins, such as the minichromosome maintenance (Mcm) complex. We have examined the phosphorylation of the Mcm complex by the Dfp1-Hsk1 kinase, the *Schizosaccharomyces pombe* homologue of Dbf4-Cdc7. *In vitro*, the purified Dfp1-Hsk1 kinase efficiently phosphorylated Mcm2p. In contrast, Mcm2p, present in the six-subunit Mcm complex, was a poor substrate of this kinase and required Cdc23p (homologue of Mcm10p) for efficient phosphorylation. In the presence of Cdc23p, Dfp1-Hsk1 phosphorylated the Mcm2p and Mcm4p subunits of the Mcm complex. Cdc23p interacted with both the Mcm complex and Dfp1-Hsk1 by selectively binding to the Mcm4/6/7 subunits and Dfp1p, respectively. The N terminus of Cdc23p was found to interact directly with Dfp1-Hsk1 and was essential for phosphorylation of the Mcm complex. Truncated derivatives of Cdc23p that complemented the temperature-sensitive phenotype of *cdc23* mutant cells also stimulated the phosphorylation of Mcm complex, implying that this activity might be a critical role of Cdc23p *in vivo*. These results suggest that Cdc23p participates in the activation of prereplicative complex by recruiting the Dfp1-Hsk1 kinase and stimulating the phosphorylation of the Mcm complex.

DNA replication | scaffold protein

The initiation of eukaryotic DNA replication is a multistep process that commences with the binding of the origin recognition complex (Orc) to origins. During the G_1 phase of the cell cycle, components of the prereplicative complex (pre-RC), such as Cdc6/Cdc18, Cdt1, and the minichromosome maintenance (Mcm) complex, are recruited sequentially to origin DNA in an Orc-dependent manner. Upon entering S phase, replication is initiated by the activation of pre-RC, which leads to the binding of Cdc45, subsequent unwinding of replication origins, and the recruitment of the replication fork machinery. Two S phase-promoting kinases, the S-phase cyclin-dependent protein kinase (Cdk) and Dbf4-Cdc7 kinase, are essential for the activation of the pre-RC in budding yeast. Although *in vivo* targets of the S-phase Cdk are unclear, the Mcm complex appears to be a major target of the Cdc7 kinase (1–4).

The Mcm complex is composed of six subunits (Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7), which are all structurally related and highly conserved in eukaryotes (5, 6). All six proteins are essential for the assembly of the pre-RC and DNA replication. Although the role of this complex in DNA replication is not fully understood, *in vivo* and *in vitro* studies suggest that the Mcm proteins may play a role as a replicative helicase (1). Biochemical analysis of the Mcm complex showed that the human and *Schizosaccharomyces pombe* Mcm4/6/7 complex contains DNA helicase activity *in vitro* (7, 8). *In vivo* cross-linking and chromatin immunoprecipitation experiments performed in *Saccharomyces cerevisiae* showed that the localization of the Mcm proteins shifted from origin regions to inter-origin regions during S phase (9). Furthermore, studies in *S. cerevisiae* with *mcm* degron mutants showed that the Mcm proteins are also required for the progression of the replication fork (10). These observations suggest that the Mcm complex is the replicative helicase.

Cdc7, a serine/threonine kinase conserved from yeast to humans (reviewed in ref. 11), is activated by the regulatory protein Dbf4. Although the level of Cdc7p is constant throughout the cell cycle, the activity of this kinase peaks at the G_1/S transition, concomitant with the cellular level of Dbf4p. In *S. cerevisiae*, Dbf4p binds to chromatin at the G_1/S transition and remains attached to chromatin during S phase (12). In the *Xenopus* cell free DNA replication system, Cdc7p was found to bind to chromatin during the G_1 and S phase and this association required the Mcm complex (13). Mcm proteins interact with Dbf4p-Cdc7p and Mcm2p is a good substrate of the kinase *in vivo* and *in vitro* (12, 14). Cells containing an allele of *MCM5*, *mcm5-bob1*, bypass the requirements for *CDC7* and *DBF4* (15), suggesting that the Mcm complex is the major target of the Cdc7 kinase.

MCM10 was initially identified in *S. cerevisiae* as a gene required for chromosomal DNA replication and stable plasmid maintenance (16, 17). Homologues of this gene have been identified in other organisms, including *S. pombe, Xenopus*, and human (18–20). Studies in *S. cerevisiae* showed that *MCM10* is essential for the initiation of replication and interacts genetically with many genes involved in the initiation and elongation steps of DNA replication. These included *MCM*, *CDC45*, *ORC*, *DNA2*, and genes encoding DNA polymerase ε and δ. Physical interaction of Mcm10p with the Mcm complex and Orc proteins have also been demonstrated (16, 18). In *S. cerevisiae*, Mcm10p was reported to be a component of the pre-RC and required for the association of the Mcm complex with origin DNA. In the *Xenopus* replication system, the binding of Mcm10p onto chromatin required the presence of chromatin-bound Mcm complex and Mcm10p was required for loading Cdc45p and origin unwinding (20).

Although the six-subunit Mcm complex appears to be a major target of the Cdc7 kinase, there is little information available about the phosphorylation of the Mcm complex by this kinase. To investigate the role of the Cdc7 kinase and the biological consequences of its phosphorylation of the Mcm complex, we have reconstituted the Dfp1-Hsk1 kinase complex, the *S. pombe* homologue of Dbf4-Cdc7 kinase, and examined its phosphorylation of the Mcm complex *in vitro*. Although the purified Dfp1-Hsk1 kinase phosphorylated Mcm2p efficiently, phosphorylation of the six-subunit Mcm complex was less effective. We identified Cdc23p as an additional factor required for the efficient phosphorylation of the Mcm complex. We present data suggesting that Cdc23p may play an important role in the

Abbreviations: HA, hemagglutinin; Mcm, minichromosome maintenance; Orc, origin recognition complex; pre-RC, prereplicative complex; ts, temperature sensitive; IVT, *in vitro* transcription and translation.

‡To whom correspondence should be addressed. E-mail: j-hurwitz@mskmail.mskcc.org.

 www.pnas.org/cgi/doi/10.1073/pnas.0237384100

activation of the pre-RC by recruiting Dfp1-Hsk1 kinase into the pre-RC through its interaction with the regulatory subunit Dfp1, leading to the stimulation of the phosphorylation of Mcm complex by this kinase.

Materials and Methods

Purification of Dfp1-Hsk1 Kinase Complex, Cdc23p, and Mcm Complex. Baculoviruses expressing the *S. pombe* Dfp1 or Hsk1 protein were prepared from cDNAs encoding full-length proteins that were subcloned into the plasmid pFastBac1 (Life Technologies, Rockville, MD). Two FLAG and three hemagglutinin (HA) epitope tags were added at the N terminus of Dfp1 and C terminus of Hsk1 proteins, respectively, to facilitate their detection and purification. For the purification of Dfp1-Hsk1 kinase complex, *Sf9* cells (2×10^6 cells per ml, 300 ml) were infected with baculoviruses expressing these two proteins and incubated at 27°C for 60 h. Cells were harvested, washed with ice-cold PBS, and resuspended with 20 ml of buffer H (25 mM Hepes-NaOH, pH 7.5/5 mM $MgCl_2$/1 mM EGTA/1 mM DTT/0.05% Nonidet P-40/10% glycerol) containing 0.15 M sodium glutamate, 50 mM β-glycerophosphate, 15 mM *p*-nitrophenyl phosphate, 0.1 mM sodium vanadate, 1 mM PMSF, 1 mM benzamidine·HCl, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 5 μg/ml aprotinin, and 0.5% Triton X-100. After incubation on ice for 20 min, the supernatant solution was collected by centrifugation at 35,000 $\times$ *g* at 4°C for 30 min, mixed with 1 ml of anti-FLAG M2 Ab agarose (Sigma) beads, and incubated at 4°C each time for 3 h with rocking. The beads were collected, washed with 15 ml of buffer H four times, and eluted three times by incubation at 4°C for 30 min with an equal bead volume of buffer H containing 0.2 mg/ml FLAG peptide. This procedure yielded about 1 mg of Dfp1-Hsk1 kinase complex.

For the preparation of full-length or truncated Cdc23 proteins, cDNA fragments encoding full-length Cdc23p (amino acids 1–593) or truncated cdc23p (see Fig. 7 for derivatives prepared) were cloned into pET28-a plasmids (Novagen). N-terminal six histidine-tagged proteins were purified by Ni-NTA (Qiagen, Chatsworth, CA) column chromatography by using the manufacturer's protocol except that buffer T (30 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/0.05% Nonidet P-40/10% glycerol/15–150 mM imidazole) containing 0.3 M NaCl was used during the purification, which yielded ≈6 mg of Cdc23p from 6 g (wet weight) of *Escherichia coli*. For further purification of full-length Cdc23p, a portion of the imidazole eluate was diluted with buffer T lacking NaCl to 0.2 M NaCl, loaded onto a MonoS PC 1.6/5 column (Amersham Pharmacia), and eluted with a 3-ml linear gradient from 0.2 to 0.6 M NaCl in buffer T. Cdc23p, peaking at 0.3 M NaCl, was pooled and used in this study.

Mcm proteins were expressed and purified from *Sf9* cells as described (8).

Kinase Assay. Reaction mixtures (15 μl) contained 25 mM Hepes-NaOH (pH 7.5), 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT, 0.1 mg/ml BSA, 0.1 mM [γ-^{32}P]ATP (1.5×10^4 cpm/pmol), 0.1 mM sodium vanadate, 1 μM okadaic acid, and indicated levels of substrates and kinase. After incubation at 30°C as indicated, reaction products were analyzed by SDS-9% PAGE followed by autoradiography. The level of phosphorylation was quantitated by PhosphorImager (Fuji) analysis.

Immunoprecipitation Assays. Indicated amounts of purified proteins were mixed in buffer H (0.15 ml) containing 0.2 M sodium glutamate and 0.2 mg/ml BSA and incubated at 4°C for 1 h. Antibodies against Mcm4 or HA adsorbed to protein A agarose beads or anti-FLAG M2 agarose beads (10 μl) were added and mixtures were incubated at 4°C for 30 min. The beads were washed three times with 1 ml of buffer H containing 0.2 M sodium glutamate and 0.2 mg/ml BSA and then once with buffer H containing 0.2 M sodium glutamate. Proteins bound to the beads were analyzed by SDS/9% PAGE followed by Western blot analysis or autoradiography [for *in vitro* transcribed and translated (IVT) proteins].

Complementation of *cdc23* Temperature-Sensitive (ts) Phenotype by Truncated Cdc23 Proteins. To examine the complementation of the *cdc23* ts phenotype (18), cDNA fragments encoding truncated Cdc23 proteins were subcloned into the *S. pombe* expression vector pREP1 containing the nmt1 promoter. After transformation of these plasmids into the *S. pombe* strain, h$^-$ *ade6-M216 leu1–32 ura4-D18 his3-D1 cdc23-M36*, complementation of the ts phenotype was examined by cell growth in minimal medium plates lacking thiamine (induced condition) at 36.5°C for 4 days.

Results

Purification and Biochemical Properties of the Dfp1-Hsk1 Kinase Complex. To examine the biochemical properties of Dfp1-Hsk1 kinase, the complex was reconstituted by using the baculovirus expression system and purified. This procedure yielded the Dfp1-Hsk1 complex that was ≈90% pure (Fig. 1*A*), which was free of contamination by other kinases such as cyclin-dependent protein kinase. Western blot analysis showed that both regulatory and catalytic subunits migrated to the same position in SDS/PAGE (data not presented), as reported (14). This kinase preparation phosphorylated Mcm2p at a maximum rate of about 1.5 pmol/min per pmol of kinase (Fig. 1 *B* and *C*). It should be noted that the phosphorylated form of Mcm2p migrated faster than unphosphorylated Mcm2p on SDS/PAGE (see Fig. 4). Phosphorylation of Mcm2p resulted in the formation of at least three distinct faster migrating bands on SDS/PAGE (detected by Coomassie staining and autoradiography), suggesting the presence of multiple phosphorylation sites in Mcm2p. These results were consistent with the level of ^{32}P incorporated into the Mcm2p (2.7 pmol/pmol of Mcm2p) at the highest level of kinase added (Fig. 1*C*).

The rate of phosphorylation of Mcm2p decreased sharply after 10 min of incubation (Fig. 1*D*). This marked decline was observed in all reactions regardless of the level of kinase added and the amount of substrate available for phosphorylation (20% of the substrate was phosphorylated in the presence of 12.5 ng of kinase, Fig. 1*D*). These findings suggested that Dfp1-Hsk1 kinase activity was inactivated significantly under the condition used. To determine whether this was caused by the intrinsic instability of the kinase, we examined the phosphorylation of Mcm2p after preincubation of the kinase in the absence of Mcm2p and ATP. Under these conditions, 85% of the input kinase activity was retained after preincubation for 1 h (Fig. 2, compare lane 2 with lanes 7–10) suggesting that the marked decrease in the rate of Mcm2p phosphorylation was not caused by the instability of the kinase. When the Dfp1-Hsk1 kinase was preincubated in the presence of ATP and absence of Mcm2p, the kinase activity decreased significantly concomitant with the increase in the level of autophosphorylation of both kinase subunits (Fig. 2, compare lane 2 with lanes 3–6). The level of ^{32}P incorporation indicated that Dfp1p was phosphorylated more effectively than the catalytic subunit (≈2-fold). The maximum level of ^{32}P incorporated into the kinase complex under these reaction conditions was ≈8 pmol/pmol of complex. Thus the activity of Dfp1-Hsk1 kinase is negatively regulated by autophosphorylation.

Phosphorylation of Mcm Complex by Dfp1-Hsk1 and the Influence of Pre-RC Proteins on the Reaction. In contrast to the efficient phosphorylation of Mcm2p by Dfp1-Hsk1, the kinase complex hardly phosphorylated the six-subunit Mcm complex *in vitro* (Fig. 3, lane 2; see Fig. 5*B*). We surmised that interactions between pre-RC components and the Mcm complex or Dfp1-

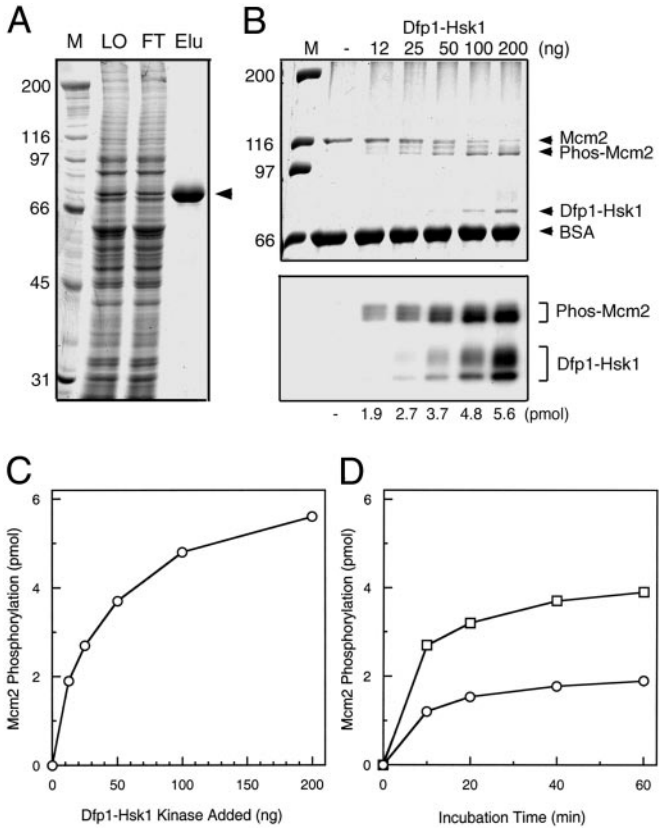

Fig. 1. Purification and characterization of the Dfp1-Hsk1 kinase complex. (*A*) *S. pombe* Dfp1-Hsk1 kinase was purified from baculovirus-infected Sf9 cells by using anti-FLAG Ab agarose column chromatography. Aliquots of fractions obtained at each purification step were analyzed by 9% SDS/PAGE separation and stained by Coomassie blue. Lanes: M, molecular mass markers; LO (load on), 20 μg of the total extract; FT, 20 μg of anti-FLAG Ab column flow-through fraction; Elu, 2.5 μg of the anti-FLAG Ab column eluate. The arrow indicates the position of Dfp1 and Hsk1 proteins. (*B*) Phosphorylation of Mcm2p by the Dfp1-Hsk1 kinase. Indicated amounts of the Dfp1-Hsk1 kinase were incubated with 2 pmol of Mcm2p at 30°C for 30 min in the presence of [γ-^{32}P]ATP. These mixtures were subjected to SDS/PAGE followed by staining with Coomassie blue (*Upper*) and autoradiography (*Lower*). The numbers at the bottom of the autoradiogram indicate the ^{32}P incorporated into Mcm2p, determined by PhosphorImager analysis. (*C*) Phosphorylation of Mcm2p as a function of the level of the kinase complex. Increasing levels of the Dfp1-Hsk1 kinase were incubated with 2 pmol of Mcm2p and the extent of phosphorylation was determined by PhosphorImager analysis of the SDS/PAGE gel. (*D*) Rate of Mcm2p phosphorylation. Mcm2p (2 pmol) was incubated with Dfp1-Hsk1 kinase (○, 12.5 ng of kinase; □, 50 ng of kinase) for the indicated time, and the extent of phosphorylation was determined by PhosphorImager analysis of an SDS/PAGE gel.

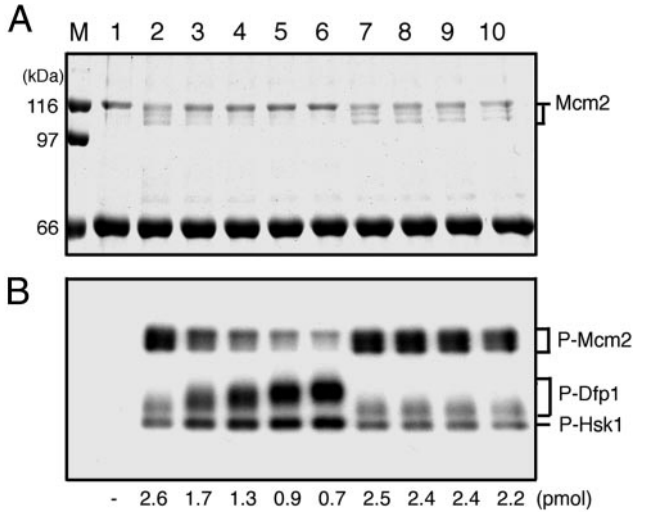

Fig. 2. Autophosphorylation of the Dfp1-Hsk1 kinase complex inhibits its kinase activity. Dfp1-Hsk1 kinase (25 ng) was preincubated in 10 μl of the kinase reaction mixture at 30°C in the presence (lanes 3–6) or absence (lanes 7–10) of [γ-^{32}P]ATP (0.1 mM) for 10 (lanes 3 and 7), 20 (lanes 4 and 8), 40 (lanes 5 and 9), or 60 min (lanes 6 and 10). Mixtures were then cooled on ice, supplemented with Mcm2p (2 pmol) and additional [γ-^{32}P]ATP (to a final concentration of 0.2 mM), adjusted to 20 μl, and then incubated at 30°C for 10 min. Phosphorylation of Mcm2p was analyzed by 9% SDS/PAGE separation, and the dried gel was subjected to PhosphorImager analysis. Lane M, molecular mass markers; lane 1, reaction carried out in the absence of kinase; lane 2, reaction carried out without preincubation. All other lanes were as described above. (*A*) Coomassie blue-stained SDS/PAGE gel. (*B*) Autoradiogram of SDS/PAGE gel shown in *A*. The level of ^{32}P incorporated into Mcm2p is shown at the bottom of the gel. The positions of phosphorylated proteins are indicated.

Hsk1 kinase might influence the phosphorylation of the Mcm complex, and we examined the effect of Orc, Cdc18p, Cdt1p, Cdc23p, and Sna41p (*S. pombe* homologue of Cdc45p) on this reaction. As shown in Fig. 3, Cdc23p addition markedly stimulated the phosphorylation of the Mcm complex (compare lane 2 with lane 6). The addition of Cdc18p or Cdt1p also increased the phosphorylation of the Mcm complex (Fig. 3, lanes 4 and 5) but to a lower extent than Cdc23p. The stimulatory effects of Cdc18p and Cdt1p were not increased further by higher levels of these proteins (data not shown). We detected phosphorylation of the Orc4p subunit in Orc and Cdc23p (Fig. 3, lane 3, and see Fig. 4), suggesting that these proteins are also substrates of the Dfp1-Hsk1 kinase.

The role of Cdc23p in activating the phosphorylation of the Mcm complex by Dfp1-Hsk1 kinase was examined in detail. Increasing concentrations of Cdc23p (up to 100 ng, 1.5 pmol) resulted in an increased phosphorylation of the Mcm complex (Fig. 4, lane 6), suggesting that near stoichiometric levels of Cdc23p were required for maximal stimulation of Mcm phosphorylation. At this stoichiometric level, Mcm2p was converted almost quantitatively to the faster migrating form upon SDS/PAGE analysis, and the migration of Mcm4p was also retarded significantly, judged by Coomassie blue staining and Western blot analysis. These findings indicate that Mcm2p and Mcm4p are major targets of the Dfp1-Hsk1 kinase. Phosphorylation of Cdc23p by the Dfp1-Hsk1 kinase was observed as well. The influence of this modification on the biological role of Cdc23p remains to be determined.

Cdc23p stimulated the phosphorylation of Mcm2p as well, particularly at low kinase levels (Fig. 5*A*). At high levels of the kinase complex, Mcm2p phosphorylation was stimulated only ≈1.5-fold by Cdc23p. In contrast, in the absence of Cdc23p the extent of phosphorylation of the six-subunit Mcm complex was low even when high levels of kinase were added (Fig. 5*B*). These results suggest that in the absence of Cdc23p interactions between the kinase and Mcm complex might be weak and/or that phosphorylation sites of Mcm2p and Mcm4p within the six-subunit complex are inaccessible to the kinase.

Interactions Among Cdc23p, the Mcm Complex, and Dfp1-Hsk1 Kinase. The influence of Cdc23p on the interactions between the Mcm complex and the kinase was examined. For this purpose, these proteins were mixed in various combinations and subjected to immunoprecipitation and Western blot analyses (Fig. 6*A*). Whereas Cdc23p interacted stably with the Mcm complex and Dfp1-Hsk1 kinase (Fig. 6*A*, lanes 2 and 3), stable interaction between the Mcm complex and Dfp1-Hsk1 kinase was not

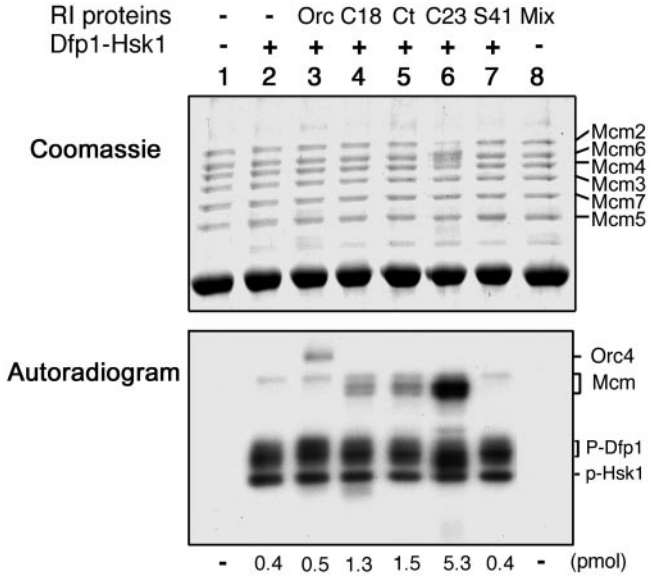

Fig. 3. Influence of replication initiation proteins on Mcm complex phosphorylation by the Dfp1-Hsk1 kinase. Reaction mixtures (20 μl) containing the six-subunit Mcm complex (550 ng, 1 pmol) and Dfp1-Hsk1 kinase (80 ng) were incubated in the absence or presence of the indicated proteins. Lane 1, reaction without kinase; lane 2, reaction with kinase in the absence of any additional protein; lanes 3–7, reactions carried out in the presence of 0.2 pmol of the Orc protein, 1 pmol of either Cdc18p (C18), Cdt1p (Ct), Cdc23p, or Sna41p (S41), respectively; lane 8, reaction carried out in the presence of a mixture of all of these proteins but lacking the kinase complex. The levels of ^{32}P incorporated into the Mcm complex are indicated below the autoradiogram.

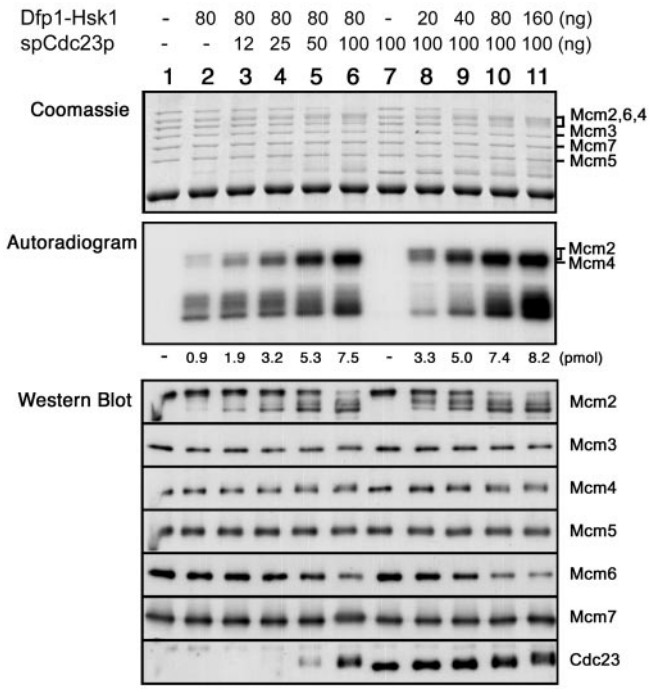

Fig. 4. Effect of increasing levels of Cdc23p on the Mcm complex phosphorylation by the Dfp1-Hsk1 kinase. Phosphorylation reactions were performed with the Mcm complex (1 pmol) and indicated amounts of Cdc23p and Dfp1-Hsk1 kinase. Aliquots of the reaction mixtures were subjected to Western blot analysis probed with polyclonal antibodies specific for the each subunit of the Mcm complex and Cdc23p. The remaining mixture was used for autoradiography and quantitation of the level of phosphorylation.

observed (Fig. 6*A*, lane 4). However, when the three protein preparations were combined, a complex containing all three components was detected (Fig. 6*A*, lanes 5 and 6), suggesting that Cdc23p mediates the stable interaction between the Mcm and kinase complexes. Coimmunoprecipitation experiments using Mcm subcomplexes showed that Cdc23p interacted with the Mcm complex through the Mcm4/6/7 subassembly (Fig. 6*B*). However, at lower salt conditions (<150 mM sodium glutamate), Cdc23p also coimmunoprecipitated with Mcm3p/5p and Mcm2p (data not shown), suggesting weak interactions between these proteins. In addition, Cdc23p interacted stably with Dfp1-Hsk1 kinase through its selective binding of Dfp1p (Fig. 6*C*).

Generation of Cdc23p-Truncated Proteins and Their Properties. Truncated derivatives of Cdc23p were examined for their ability to interact with the Mcm complex and the Dfp1-Hsk1 kinase, stimulate phosphorylation of the Mcm complex by this kinase, and complement the ts phenotype of *cdc23* mutant cells (Fig. 7). The C terminus of Cdc23p appeared to be essential for its interaction with the Mcm complex because all C-terminal truncated proteins generated (CD1–CD4) failed to interact with the Mcm complex under the conditions used. However, the C-terminal domain alone (ND4) was not sufficient for the interaction with the Mcm complex, whereas the ND2 protein supported stable interaction. On the other hand, all C-terminal truncated Cdc23p derivatives (CD1–CD4) interacted with the kinase as efficiently as full-length Cdc23p, suggesting that kinase interacting motifs exist within the N-terminal 220-aa region. However, ND2, in which the N-terminal 211 aa were deleted, also interacted with the kinase and the further deletion of 84 aa from the N terminus (ND3) abrogated kinase binding activity. These results suggest that a region between amino acids 211 and 295 of Cdc23p also contains a Dfp1-Hsk1 interacting site.

Derivatives CD3 and ND3 did not stimulate Mcm complex phosphorylation, whereas CD2, ND1, and D9 were active. These results suggest that the region responsible for the stimulation of Mcm complex phosphorylation is located between amino acids 96 and 423. All truncated derivatives that stimulated phosphorylation also interacted stably with the Dfp1-Hsk1 kinase, suggesting that this interaction is essential for the increase in Mcm complex phosphorylation. Stable interaction with the Mcm complex, on the other hand, did not appear critical for the stimulation of the phosphorylation. Transient interactions between Cdc23p derivatives and the Mcm complex, which were not detected by coimmunoprecipitation, might be sufficient to stimulate Mcm complex phosphorylation.

Plasmids expressing Cdc23 or the truncated derivatives were

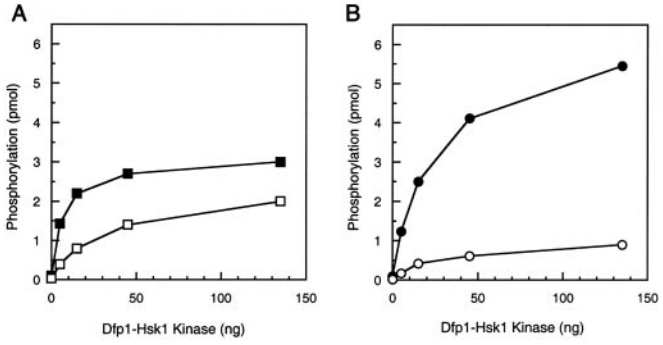

Fig. 5. Influence of increasing levels of the kinase complex in the presence or absence of Cdc23p on the phosphorylation of Mcm2p and the Mcm complex. Kinase assays were carried out with 1 pmol of Mcm2p (*A*) or the Mcm complex (*B*) in the absence (○ and □) or presence (● and ■) of 1 pmol of Cdc23p.

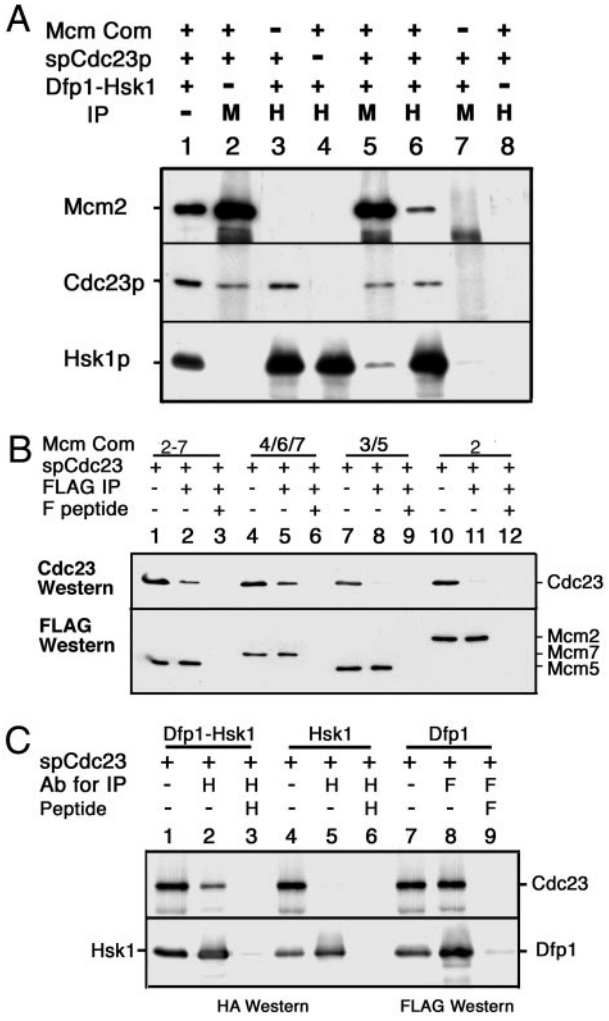

Fig. 6. Interactions among Cdc23p, the Mcm complex, and Dfp1-Hsk1 kinase. (*A*) Various combinations of the Mcm complex (0.2 pmol), Cdc23p (0.3 pmol), and Dfp1-Hsk1 kinase (0.3 pmol) were mixed, as indicated, in buffer H (150 μl) containing 0.2 M sodium glutamate and 0.2 mg/ml BSA and incubated at 4°C for 30 min. Immunoprecipitations were carried out by using polyclonal antibodies against Mcm4p (M) or a mAb against the HA epitope (H). Western blot analysis was performed by using polyclonal antibodies against Mcm2p or Cdc23p and a mAb against the HA epitope (12CA5) to detect HA-tagged Hsk1p. Lane 1 contained 10% of the input material used for immunoprecipitations. (*B*) Interaction of Cdc23p with the Mcm complex and Mcm subcomplexes. Cdc23p (0.3 pmol) was mixed with 0.3 pmol of the six-subunit Mcm complex (lanes 1–3), the Mcm4/6/7 complex (lanes 4–6), the Mcm 3/5 complex (lanes 7–9), or Mcm2p (lanes 10–12). Immunoprecipitations were performed with anti-FLAG M2 agarose beads (Sigma) in the absence or presence of 0.2 mg/ml FLAG peptide. Lanes 1, 4, 7, and 10 contained 20% of input material used in the immunoprecipitation steps. (*C*) Interaction of Cdc23p with Dfp1p and Hsk1p. Cdc23p was prepared by IVT. To obviate interactions mediated by DNA, all IVT reaction mixtures were incubated with DNase I (0.5 mg/ml) at 30°C for 10 min. Cdc23p (5 μl of the IVT product) was mixed with 0.5 pmol of the Dfp1-Hsk1 complex, Dfp1p, or Hsk1p and subjected to immunoprecipitation using anti-HA (H) or anti-FLAG (F) antibody in the absence or presence of HA peptide (1 mg/ml) or FLAG peptide (0.2 mg/ml), as indicated. Cdc23p was detected by autoradiography, and Western blot analysis was carried out by using an antibody specific for the HA or FLAG epitope to detect Hsk1p or Dfp1p, respectively.

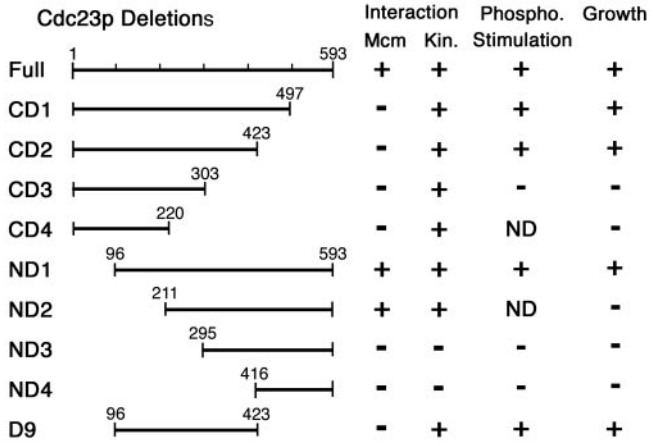

Fig. 7. Generation and biological properties of truncated Cdc23p derivatives. To examine the physical interactions of these Cdc23p derivatives with the proteins indicated, IVT-prepared Cdc23 proteins (5 μl) were mixed with 0.5 pmol of purified Mcm complex or Dfp1-Hsk1 complex in 150 μl of buffer H containing 0.2 M sodium glutamate. Immunoprecipitation experiments were performed by using anti-FLAG Ab (for interaction with the Mcm complex) or anti-HA Ab (for interaction with the Dfp1-Hsk1 kinase) in the absence or presence of competing peptides. To obviate interactions mediated by DNA, IVT Cdc23p preparations were treated with DNase I (500 units/ml) at 30°C for 10 min. The influence of Cdc23p and truncated proteins on the phosphorylation of the Mcm complex by the kinase complex was carried out with preparations expressed and purified from bacterial cells. Kinase assays were carried out by using 1 pmol of the Mcm complex, 80 ng of Dfp1-Hsk1 kinase, and three different levels of the truncated Cdc23p derivatives, ranging from 0.2 to 5 pmol in each case. These results are summarized under the heading Phospho. Stimulation. For complementation assays, Cdc23 truncated proteins were expressed in the *cdc23* ts mutant strain by using the *nmt1* promoter (pREP1) and the complementation of the ts phenotype was determined by growth of cells at 36.5°C (under heading Growth). + indicates that the Cdc23 derivative interacted with the Mcm complex or the kinase, stimulated the kinase-catalyzed phosphorylation of the Mcm complex, and supported cell growth at the restrictive temperature. − indicates no interaction, phosphorylation, and growth. ND, not done.

examined for their ability to support growth of the *cdc23-M36* mutant cells at the nonpermissive temperature. As shown in Fig. 7, Cdc23p derivatives, CD1, CD2, or ND1 complemented the defects of cdc23-M36 cells, and D9 was the smallest Cdc23p derivative that supported the growth of mutant cells. All of these Cdc23 derivatives also stimulated the phosphorylation of the Mcm complex. These results suggest that the stimulation of Mcm phosphorylation by the kinase complex might contribute to the essential role of the Cdc23p required for cell viability and DNA replication.

Discussion

Purified *S. pombe* Dfp1-Hsk1 kinase possesses biochemical properties that are similar to the Cdc7-Dbf4 kinase complex isolated from *S. cerevisiae* (12) and humans (21). *In vitro*, the fission yeast kinase efficiently phosphorylated Mcm2p and autophosphorylated both Hsk1p and Dfp1p subunits. Autophosphorylation of the kinase complex reduced its ability to phosphorylate Mcm2p, suggesting that its activity might be negatively regulated by autophosphorylation *in vivo*. Although this kinase activity is regulated during the mitotic cell cycle by the level of the Dbf4 subunit (12), it is possible that the kinase activity is also negatively regulated by autophosphorylation because substantial amounts of Dbf4 accumulate during S to G_2. In *S. pombe,* Dfp1p has been shown to be a phosphoprotein between S and G_2 (22). The intrinsic autophosphorylation activity of the complex might be at least partly responsible for the phosphorylation state of Dfp1p.

Although previous studies have shown that Mcm10p is essential for the initiation of replication, its biochemical properties and role in replication are obscure. Here, we demonstrated that

Cdc23p interacts directly with both the Mcm complex and the Dfp1-Hsk1 kinase. The N-terminal domain of Cdc23p interacted with the Dfp1 subunit of the kinase and the C terminus of Cdc23p was essential for stable interaction with the Mcm complex. We also demonstrated that Cdc23p is essential for efficient Mcm complex phosphorylation by the Dfp1-Hsk1 kinase *in vitro*, suggesting an important role for Cdc23p in the activation of pre-RC. Although the timing of the assembly of Cdc23p in the pre-RC is not clear in *S. pombe*, Cdc23p appeared to act as a bridge supporting formation of a stable complex between the Mcm complex and Dfp1-Hsk1. The direct interaction between Cdc23p and Dfp1-Hsk1 kinase suggests that Cdc23p may help to recruit the Dfp1-Hsk1 kinase to the pre-RC at the G_1/S phase *in vivo*. Cdc23p not only interacted with the Mcm complex and the Dfp1-Hsk1 kinase but also interacted with the Orc complex in coimmunoprecipitation experiments with purified proteins (Y. Jiang, J.-K.L., and J.H., unpublished observations). Mcm10p in human cells has also been reported to interact with the Orc (18).

Among the truncated Cdc23p derivatives examined, all proteins that stimulated Mcm complex phosphorylation also supported growth of mutant cells at the nonpermissive temperature (Fig. 7). Similarly, all of these active derivatives interacted with the Dfp1-Hsk1 complex but not all of these interacted with the Mcm complex. However, it is possible that the truncated derivatives CD1, CD2, and D9 might interact stably with the Mcm complex under conditions used in the kinase assays. The latter reactions were carried out at lower ionic strength than those used in the immunoprecipitation experiments (0.05 and 0.2 M sodium glutamate, respectively).

In solution, purified Cdc23p (a 67-kDa protein based on its amino acid content) appears to be a monomer with a highly extended structure. This protein was eluted from a Superdex 200 gel filtration column close to Ferritin (440 kDa) and sedimented slightly slower than BSA (66 kDa) on glycerol gradient sedimentation (data not shown). The Stokes radius and sedimentation coefficient of Cdc23p are 64 Å and 3.9 s, respectively, leading to a calculated frictional ratio of 2.39 (23), which suggests that Cdc23p is a highly asymmetric structure. This property, together with its known ability to interact with other replication initiation factors, suggest that Cdc23p may act as a scaffold protein for the assembly of the pre-RC.

Although the Mcm4/6/7 subcomplex was shown to be a processive DNA helicase (24), to date, demonstrable helicase activity has not been observed with the six-subunit Mcm complex (7, 8). The phosphorylation of this complex might lead to its remodeling and activate the DNA helicase activity. However, we have not detected activation of the DNA helicase activity of the Mcm complex or alterations of its structure after phosphorylation by the Dfp1-Hsk1 complex in the presence or absence of Cdc23p. Additional factors might be important for the activation of helicase activity.

Current models of origin activation, derived from studies in *S. cerevisiae*, indicate that Mcm10p is a component of the pre-RC required for the recruitment of the Mcm complex to origins (16). In *Xenopus*, the converse appears to be true, i.e., the Mcm complex is required to recruit Mcm10p to chromatin (20). In both systems, however, the essential role of Cdc7-Dbf4 occurs after both Mcm10p and the Mcm complex are part of the pre-RC. Thus, our findings that Cdc23p markedly stimulates phosphorylation of the Mcm complex by the Hsk1-Dfp1 kinase are likely to apply to all eukaryotic systems.

We are indebted to Dr. Z.-Q. Pan for critical review of this paper. These studies were supported by National Institutes of Health Grant GM38559 (to J.H.) and by a grant from the Creative Research Initiatives Program of the Korean Ministry of Science and Technology (to Y.-S.S.). J.H. is a Professor of the American Cancer Society.

1. Tye, B. K. (1999) *Annu. Rev. Biochem.* **68,** 649–686.
2. Bell, S. P. & Dutta, A. (2002) *Annu. Rev. Biochem.* **71,** 333–374.
3. Stillman, B. (1994) *J. Biol. Chem.* **269,** 7047–7050.
4. Diffley, J. F. (1996) *Genes Dev.* **10,** 2819–2830.
5. Adachi, Y., Usukura, J. & Yanagida, M. (1997) *Genes Cells* **2,** 467–479.
6. Koonin, E. V. (1993) *Nucleic Acids Res.* **21,** 2541–2547.
7. Ishimi, Y. (1997) *J. Biol. Chem.* **272,** 24508–24513.
8. Lee, J.-K. & Hurwitz, J. (2000) *J. Biol. Chem.* **275,** 18871–18878.
9. Aparicio, O. M., Weinstein, D. M. & Bell, S. P. (1997) *Cell* **91,** 59–69.
10. Labib, K., Tiercero, J. A. & Diffley, J. F. (2000) *Science* **288,** 1643–1647.
11. Sclafani, R. A. (2000) *J. Cell Sci.* **113,** 2111–2117.
12. Weinrich, M. & Stillman, B. (1999) *EMBO J.* **18,** 5334–5346.
13. Walter, J. C. (2000) *J. Biol. Chem.* **275,** 39773–39778.
14. Brown, G. & Kelly, T. J. (1998) *J. Biol. Chem.* **34,** 22083–22090.
15. Hardy, C. F., Dryga, O., Seematter, S., Pahl, P. M. & Sclafani, R. A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3151–3155.
16. Merchant, A. M., Kawasaki, Y., Chen, Y., Lei, M. & Tye, B. K. (1997) *Mol. Cell. Biol.* **17,** 3261–3271.
17. Soloman, N. A., Wright, M. B., Chang, S., Buckley, A. M., Dumas, L. B. & Gaber, R. F. (1992) *Yeast* **8,** 273–289.
18. Aves, S. J., Tongue, N., Foster, A. J. & Hart, E. A. (1998) *Curr. Genet.* **34,** 164–171.
19. Izumi, M., Yanagi, K., Mizuna, T., Yokoi, M., Kawasaki, Y., Moon, K. Y. Hurwitz, J., Yatagai, F. & Hanaoka, F. (2000) *Nucleic Acids Res.* **28,** 4769–4777.
20. Wohlschlegel, J. A., Dhar, S. K., Prokhorova, T. A., Dutta, A. & Walter, J. C. (2002) *Mol. Cell* **9,** 233–240.
21. Masai, H., Matsui E., You, Z., Ishimi, Y., Tamai, K. & Arai, K. (2000) *J. Biol. Chem.* **275,** 29042–29052.
22. Brown, G. & Kelly, T. J. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 8443–8448.
23. Siegel, L. M. & Monty, K. J. (1966) *Biochim. Biophys. Acta* **112,** 346–362.
24. Lee, J. K. & Hurwitz, J. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 54–59.