CDC7 kinase phosphorylates serine residues adjacent to acidic amino acids in the minichromosome maintenance 2 protein

Won-Ho Cho*†, Young-Joo Lee*†, Soo-Im Kong*, Jerard Hurwitz‡§, and Joon-Kyu Lee*§

*Department of Biology Education, Seoul National University, Seoul 151-742, Korea; and ‡Molecular Biology Program, Memorial Sloan–Kettering Cancer Center, New York, NY 10021

Contributed by Jerard Hurwitz, June 14, 2006

Cdc7 is an essential kinase required for the initiation of eukaryotic DNA replication. Previous studies in many species showed that the minichromosome maintenance complex is a major physiological target of this kinase. In this study, we have mapped the sites in human Mcm2 protein that are phosphorylated by Cdc7. The *in vitro* phosphorylation of several Mcm2 truncated proteins and peptides revealed that Mcm2 contains two major (^{5}S and ^{53}S) and at least three minor phosphorylation sites (^{4}S, ^{7}S, and ^{59}T) located at the N-terminal region. Alanine substitution experiments with Mcm2 peptides showed that the phosphorylation of ^{5}S and ^{53}S by Cdc7 required the presence of an acidic amino acid adjacent to a serine residue. Furthermore, although Cdc7 was unable to phosphorylate a Mcm2 peptide (spanning amino acids 19–30 and containing ^{26}S and ^{27}S), it phosphorylated ^{26}S efficiently when this peptide contained a chemically synthesized phospho-^{27}S modification. Hence, additional Cdc7 phosphorylation sites could be generated in Mcm2 by its prior phosphorylation by a cyclin-dependent kinase. This finding may explain why the sequential action of cyclin-dependent and Cdc7 kinases is essential for the initiation of DNA replication.

replication | prereplicative complex | origin activation

Cdc7 plays an essential role in the initiation of eukaryotic DNA replication (1–3). Cdc7 encodes a serine/threonine kinase that is highly conserved from yeast to human (4). The activity of this kinase fluctuates during the cell cycle and depends completely on the regulatory subunit, Dbf4. Dbf4 accumulates during the S and G_2 phases of the cell cycle and is degraded rapidly during the G_1 phase by the anaphase promoting complex (5, 6). In *Saccharomyces cerevisiae*, Dbf4 binds to chromatin at the G_1/S transition and remains on chromatin during the S phase (7). In the *Xenopus* egg extract cell-free system, Cdc7 was found to bind to chromatin during S phase, and this association required the minichromosome maintenance (MCM) complex (8).

Both genetic and biochemical studies in *S. cerevisiae* indicate that Cdc7 kinase activity is required throughout S phase to activate replication origins on chromosomes (9). Cdc7 kinase acts directly on individual replication origins, presumably by phosphorylating components of the prereplicative complex (pre-RC), which may lead to the remodeling of the pre-RC for the unwinding of replication origins and subsequent recruitment of the replication fork machinery. Although the mechanism-of-origin activation by Cdc7 kinase remains unclear, the MCM complex appears to be the primary physiological target of this kinase activity (2).

The MCM complex is composed of six distinct subunits (Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7), which are all related structurally and highly conserved in eukaryotes (10). All six proteins are essential for the initiation of DNA replication and contain sequence motifs required for DNA helicase activity. In keeping with this notion, the MCM subcomplex containing Mcm4, Mcm6, and Mcm7 possesses DNA helicase activity *in vitro*, although the six-subunit complex is devoid of this activity (11–13), suggesting that additional factors are required to activate the helicase of the complete complex. The MCM complex containing all six subunits is a component of the pre-RC. The location of this complex shifts from origin regions to distal regions during S phase (14). Furthermore, studies with mcm degron mutants showed that all of the Mcm proteins are also required for the progression of the replication fork (15). These results suggest that the MCM complex is likely to be the replicative helicase required for both initiation and elongation stages in DNA replication.

Because the unwinding of replication origins is a major consequence of origin activation by S phase-promoting kinases, the MCM complex is an attractive cellular target of the Cdc7 kinase. The Mcm proteins interact genetically and physically with Cdc7 and are good substrates of this kinase *in vitro* and *in vivo* (16–18). The *Schizosaccharomyces pombe* MCM complex containing all six subunits is also phosphorylated efficiently by this kinase in the presence of Mcm10 *in vitro* (19). Cells containing a mutant allele of *MCM5*, *bob1*, can bypass the requirements for Cdc7 and Dbf4 in *S. cerevisiae* (20). These results strongly suggest that the MCM complex is the physiological target of Cdc7 kinase for DNA replication. However, studies on the cellular role of Cdc7 kinase and its phosphorylated products are limited, partly because there is no information about the substrate sites phosphorylated by this kinase.

To define the substrate specificity of Cdc7 kinase, we have identified the sites phosphorylated in the human Mcm2 protein and the sequence required to support this reaction. By using various Mcm2 peptides, we detected several serine residues located at the N terminus of Mcm2 that were phosphorylated by this kinase. Based on the sequences at these sites and alanine substitutions within these regions, glutamic acid adjacent to a serine residue was identified as a minimal requirement that supports Cdc7 kinase activity. The substrate specificity reported in this study should aid in the identification of new cellular targets of Cdc7 kinase, as well as contribute to a better definition of the physiological role of this kinase.

Results

Cdc7 Kinase Phsophorylates the N Terminus of Human Mcm 2 Proteins. Previous studies showed that human Cdc7 kinase phosphorylated the mouse Mcm2 protein efficiently, and that the N-terminal region of ≈62 aa contained major phosphorylation sites (21). Because the sequence of mouse Mcm2 is 95% identical to the human protein, we considered it likely that the N terminus

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Conflict of interest: No conflicts declared.

Abbreviations: MCM, minichromosome maintenance; pre-RC, prereplicative complex; CK2, casein kinase II; CDK, cyclin-dependent kinase.

†W.-H.C. and Y.-J.L. contributed equally to this work.

§To whom correspondence may be addressed. E-mail: joonlee@snu.ac.kr or j-hurwitz@mail.mskcc.org.

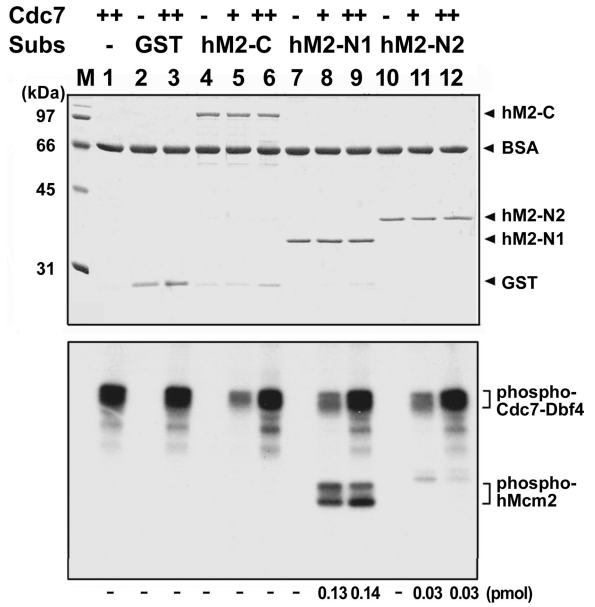

Fig. 1. Phosphorylation of the N terminus at a region of human Mcm2 protein by Cdc7 kinase. GST-tagged Mcm2 truncated proteins (0.2 μg, each equivalent to ≈5 pmol of hM2-N1 or hM2-N2, and 2 pmol of hM2-C) were incubated with indicated amounts of Cdc7 kinase (+, 5 ng; ++, 15 ng) in the kinase assay reaction, as described in *Materials and Methods*, at 30°C for 30 min. Mixtures were then 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 incorporation, determined by PhosphoImager analysis. The symbol – at the bottom of the autoradiogram denotes incorporation of <0.01 pmol. hM2-N1, GST fusion protein containing the amino acid 1–70 region of human Mcm2 protein; hM2-N2, amino acids 98–180; hM2-C, amino acids 175–904.

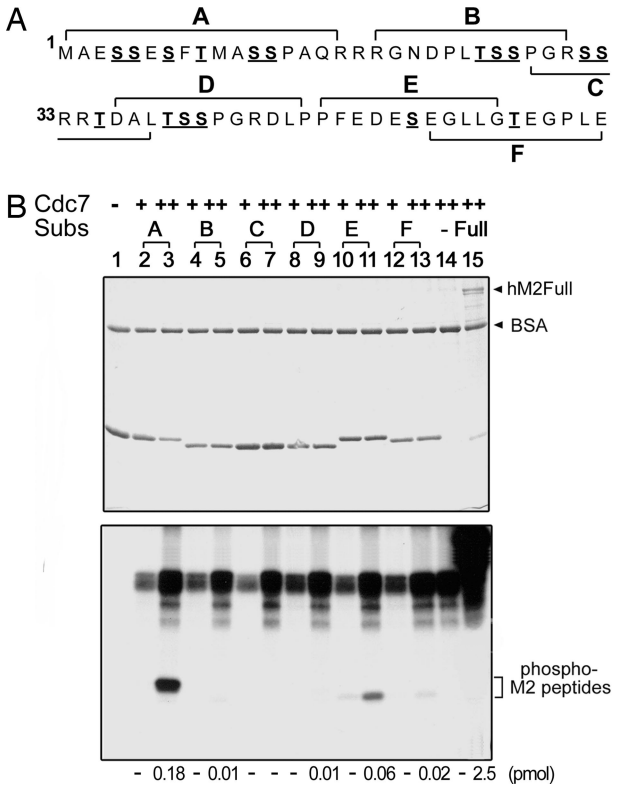

Fig. 2. Phosphorylation of GST-tagged Mcm2 N-terminal peptides. (*A*) Amino acid sequence of the Mcm2 N terminus and peptide regions fused to GST. (*B*) GST-tagged Mcm2 N-terminal peptides (A–F, 1 μg, ≈30 pmol) or full-length Mcm2 protein (Full, 0.3 μg, 3 pmol) were phosphorylated with Cdc7 kinase (+, 5 ng; ++, 15 ng) at 30°C for 30 min. The mixtures were subjected to SDS/PAGE followed by staining with Coomassie blue (*Upper*) and autoradiography (*Lower*).

of the human Mcm2 protein contained the major Cdc7-mediated phosphorylation sites. To confirm this, truncated human Mcm2 proteins (hM2-N1, amino acids 1–70; hM2-N2, amino acids 98–180; hM2-C, amino acids 175–904) were prepared as GST-fusion proteins, and their phosphorylation by the Cdc7 kinase was examined. To ensure that no other eukaryotic protein kinase contributed to the phosphorylation of the Mcm2 proteins or peptides, both the human Cdc7–Dbf4 kinase complex and Mcm2 protein were purified by using *Escherichia coli* expression systems. As shown in Fig. 1, lanes 8 and 9, the N-terminal region of human Mcm2 protein (amino acids 1–70, hM2-N1) was phosphorylated significantly by Cdc7 kinase, whereas the protein fragment containing amino acids 98–190 (hM2-N2, lanes 11 and 12) was only weakly phosphorylated. No detectable phosphorylation was observed with hM2-C (lanes 4 and 5), which contained amino acids 175–904. These results suggest that the major sites phosphorylated in human Mcm2 are located at the N terminus within amino acids 1–70. It should be noted that substantial autophosphorylation of the Cdc7–Dbf4 complex occurred as reported (16, 22).

Because the short N-terminal region of Mcm2 (amino acids 1–70) contains multiple serine (S) and threonine (T) residues, we divided this region into several GST-fused oligopeptides and examined their phosphorylation (Fig. 2*A*). Although the level of phosphorylation of the peptides was low (varying between 1% and 0.1%) compared to that observed with full-length Mcm2 protein (Fig. 2*B*, lane 15), N-terminal peptides containing amino acids 1–17 (M2A) and 48–58 (M2E) were phosphorylated significantly by Cdc7 kinase (Fig. 2*B*, lanes 2, 3 and 10, 11, respectively). The M2F peptide (amino acids 54–64) was only weakly phosphorylated by the kinase (Fig. 2*B*, lanes 12 and 13), whereas all other peptides (M2B, M2C, and M2D) were not phosphorylated. These results suggest that peptides M2A and M2E included significant phosphorylation sites, whereas peptide M2F contained minor phosphorylation sites. Because peptides M2E and M2F each include only one residue that could be phosphorylated, ^{53}S appears to be a major phosphorylation site in Mcm2 protein, whereas ^{59}T appears to be a minor phosphorylation site. Because peptide M2A was phosphorylated most effectively by Cdc7 kinase, it is likely that this peptide contains either the most efficient Cdc7 phosphorylation site or multiple phosphorylation sites. To determine the sites phosphorylated among the six serine and one threonine residues present in this peptide, alanine-substituted peptides were prepared and examined as substrates (Fig. 3). Only a low level of phosphorylation was observed with the peptide containing alanine residues in place of all three serine residues, ^{4}S, ^{5}S, and ^{7}S (Fig. 3*B*, lanes 9 and 10), suggesting that the phosphorylation sites in this peptide resided in ^{4}S, ^{5}S, or ^{7}S. The replacement of individual amino acid residue, ^{4}S, ^{5}S, or ^{7}S with alanine (peptide A′mt1, A′mt2, or A′mt3, respectively) reduced phosphorylation of the peptides to a level ≈85%, 56%, or 67% of that observed with the unsubstituted hM2A′ peptide (Fig. 3*B*, lanes 3–8). The peptide containing serine only at position ^{5}S (A′mt8) was phosphorylated relatively well (≈37% compared to the wild-type protein; Fig. 3*C*, lanes 5 and 6), whereas the peptide containing serine only at position ^{4}S (A′mt5) showed only limited phosphorylation (≈16% compared to the wild-type peptide). These results suggested that ^{5}S is a major phosphorylation site in this peptide, and both ^{4}S and ^{7}S are also

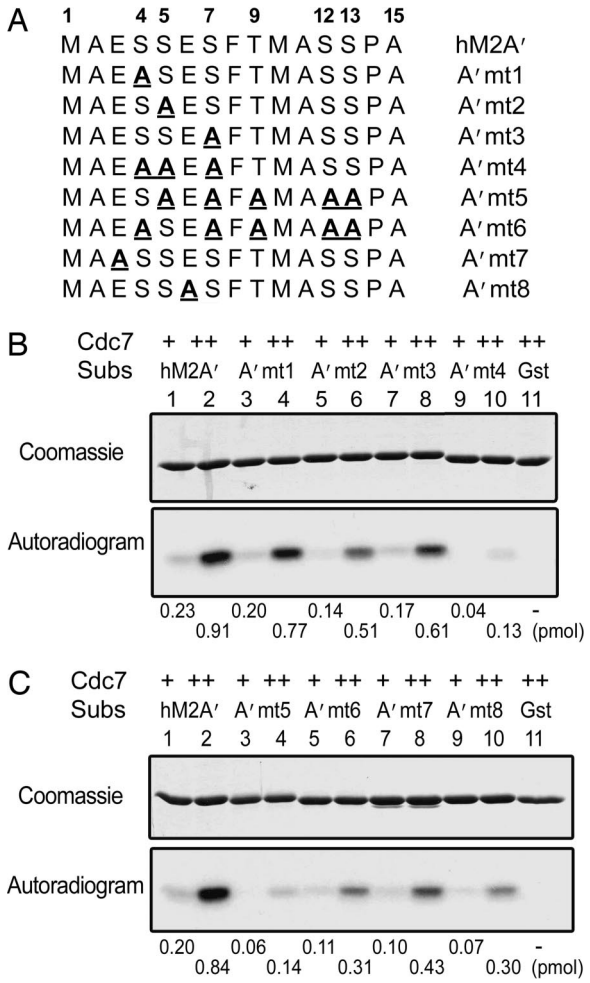

Fig. 3. Identification of phosphorylation sites in the A peptide region by alanine substitution analysis. (*A*) The amino acid sequences of several alanine-substituted peptides of the A region that were fused to GST protein. (*B* and *C*) GST-tagged Mcm2 N-terminal peptides (3 μg, 90 pmol) were incubated with Cdc7 kinase (+, 5 ng; ++, 15 ng) at 30°C for 15 min. The mixtures were subjected to SDS/PAGE followed by staining with Coomassie blue and autoradiography.

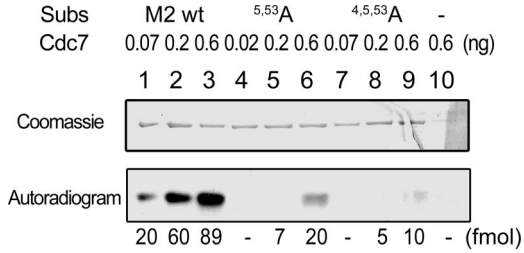

Fig. 4. The ^{5}S and ^{53}S residues of Mcm2 protein are major phosphorylation sites. The full-length and alanine-substituted Mcm2 proteins, as indicated, were expressed and purified by using the *E. coli* expression system. Mcm2 proteins (0.15 μg, 1.5 pmol) were incubated with indicated amounts of Cdc7 kinase at 30°C for 15 min. The mixtures were then subjected to SDS/PAGE followed by staining with Coomassie blue and autoradiography. The symbol – at the bottom of the autoradiogram denotes the incorporation of <1 fmol.

phosphorylated, although the levels of phosphorylation are relatively low. Taken together, there are two major (^{5}S and ^{53}S) and three minor phosphorylation sites (^{4}S, ^{7}S, and ^{59}T) located at the N terminus of the Mcm2 protein.

To confirm these phosphorylation sites, full-length Mcm2 proteins containing alanine substitutions of some of the phosphorylation sites were isolated. As shown in Fig. 4, substitution of ^{5}S and ^{53}S with alanine residues markedly reduced phosphorylation of the mutated Mcm2 protein (lanes 4–6) compared with that observed with the wild-type protein (lanes 1–3). The additional replacement of ^{4}S with alanine, which yielded the Mcm2 mutant protein containing alanines in place of ^{4}S, ^{5}S, and ^{53}S, further reduced the phosphorylation of this protein marginally (lanes 7–9). These findings are in good agreement with our previous mapping results, suggesting that the major sites in Mcm2 phosphorylated by Cdc7 kinase are located at positions ^{5}S and ^{53}S, whereas ^{4}S is only a minor site.

The Presence of an Acidic Amino Acid Adjacent to a Serine Residue Is Essential for Cdc7 Kinase Activity. Comparison of the amino acid sequence surrounding all phosphorylation sites revealed the presence of an acidic amino acid adjacent to either a serine or threonine residue in each case. This relationship was also noted in other studies carried out on the phosphorylation of Mcm2 by Cdc7 kinase with proteins isolated from *S. pombe* (W.-H.C. and J.-K.L., unpublished observation). To examine whether the presence of acidic amino acid adjacent to a serine residue influenced Cdc7 kinase activity, various alanine-substituted M2A peptides were prepared. Replacement of glutamic acid (E) with alanine at position 3 of the M2A peptide (^{3}E) or at position ^{6}E significantly reduced the phosphorylation of the peptides (Fig. 3*C*, lanes 7–10), suggesting that the presence of a glutamic acid adjacent to a phosphorylation site stimulated Cdc7 kinase activity. Because the M2A peptide contains three phosphorylation sites at positions 4, 5, and 7, the influence of glutamic acid on the phosphorylation of each of these sites would be difficult to evaluate. Therefore, we prepared alanine-substituted peptides of the M2E region (residues 48–58 in Fig. 2*A*), which contained only one internal serine (^{53}S) surrounded by a number of glutamic acid residues and examined their phosphorylation by Cdc7 kinase. Alanine replacement of individual amino acids other than glutamic acid residues did not significantly reduce the level of phosphorylation of the substituted peptides. On the other hand, alanine substitution of the glutamic acid residues (^{52}E or ^{54}E) adjacent to ^{53}S reduced phosphorylation to levels observed with the peptide in which ^{53}S was changed to alanine (data not presented). These results indicate that glutamic acid adjacent to a serine residue is critical for the phosphorylation of the peptide.

Although amino acids other than glutamic acid did not appear to be critical for the phosphorylation by Cdc7 kinase, in some cases, their replacement with alanine increased the level of peptide phosphorylation (data not presented). To reduce such effects, they were converted to alanine in peptide Mcm2E (Fig. 5*A*, hM2E′). This change permitted a more specific examination of the sequence bordering the serine residue required to support its phosphorylation. As shown in Fig. 5*B*, substitution of alanine for the glutamic acid residue following ^{53}S (lanes 9 and 10), or the movement of the glutamic acid from residues 54 to 55 (lanes 3 and 4), reduced the phosphorylation of the peptide substantially. On the other hand, replacement of the glutamic acid residue before ^{53}S with alanine (Fig. 5*B*, lanes 7 and 8) or the movement of glutamic acid from residues 52 to 51 (Fig. 5*B*, lanes 5 and 6) did not significantly affect phosphorylation. These results indicate that the presence of a serine residue followed by glutamic acid is a minimal requirement for Cdc7 phosphorylation.

We also noted that the replacement of ^{52}E with ^{52}D did not influence the phosphorylation reaction (Fig. 5*C*, lanes 5 and 6). However, changing ^{54}E to ^{54}D significantly reduced phosphorylation (lanes 7 and 8), and alterations of both ^{52}E and ^{54}E to ^{52}D and ^{54}D partially restored phosphorylation (Fig. 5*C*, lanes 9 and

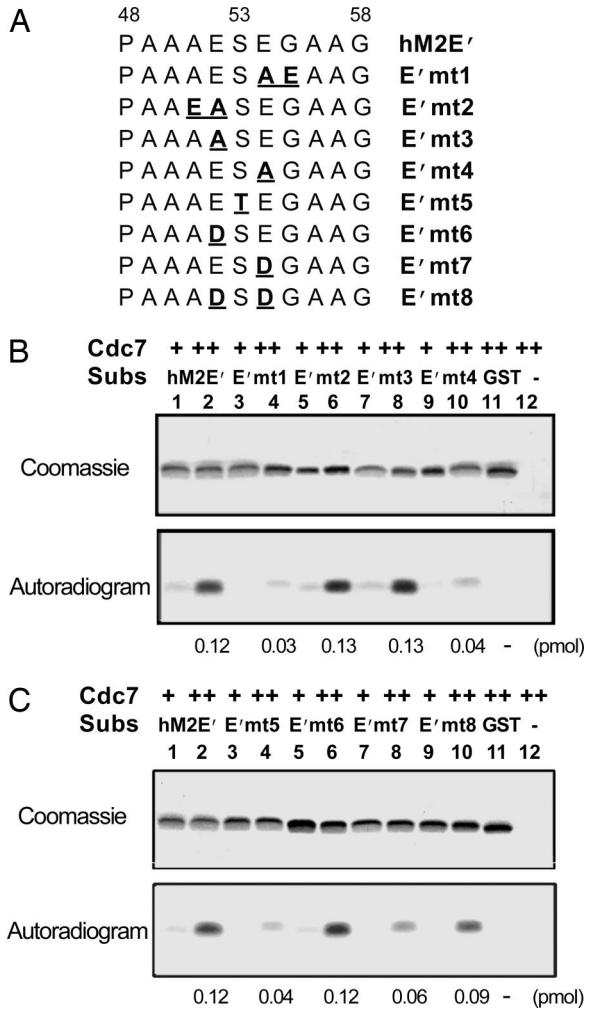

Fig. 5. Influence of the acidic amino acids bordering phosphorylation sites on Cdc7 kinase activity. (*A*) Amino acid sequences of the modified GST-tagged hM2E peptides. (*B* and *C*) GST-tagged Mcm2 N-terminal peptides (1 μg, 30 pmol) were incubated with Cdc7 kinase (+, 1 ng; ++, 3 ng) at 30°C for 20 min and mixtures subjected to SDS/PAGE followed by staining with Coomassie blue and autoradiography.

10). These findings suggest that glutamic acid appears to be the most effective acidic amino acid for phosphorylation, although aspartic acid can also support phosphorylation. Replacement of serine with threonine was also tested; as shown in Fig. 5*C*, lanes 3 and 4, phosphorylation of this peptide was reduced substantially by this change.

Generation of Additional Cdc7 Phosphorylation Sites by the Action of Cyclin-Dependent Kinase (CDK). The above findings suggest that the presence of an acidic amino acid adjacent to a serine residue is essential for Cdc7 kinase activity. As shown in Fig. 2*A*, the N terminus of Mcm2 includes several regions with consecutive serine or threonine residues (^{4}S^{5}S, ^{12}S^{13}S, ^{25}T^{26}S^{27}S, ^{31}S^{32}S, and ^{39}T^{40}S^{41}S). If the presence of a negatively charged amino acid adjacent to the site phosphorylated were important for Cdc7 kinase activity, phosphorylation of a serine or threonine residue in these repeats could produce additional sites for Cdc7 kinase activity. Indeed, many of the serine residues within these repeats contained putative Cdc7 as well as CDK phosphorylation sites. Two such sites, particularly those around serine residues ^{27}S and ^{41}S, match the consensus sequences (S/T-P-X-K/R) required for CDK phosphorylation and were identified as residues phosphorylated by CycA-CDK2 (23). In support of this notion, the prior *in vitro* phosphorylation of Mcm2 with CycE-CDK2 followed by addition of the Cdk inhibitor p27 increased the phosphorylation of Mcm2 by Cdc7≈1.6- to 2-fold (data not presented), suggesting that the phosphorylation of Mcm2 by CycE-CDK2 might generate additional Cdc7 phosphorylation sites. More definitive evidence for this was obtained by examining the phosphorylation of the hM2B peptides containing either a serine or a phosphorylated serine residue at the CDK phosphorylation site, ^{27}S (hM2B or ^{27}pS-hM2B in Fig. 6*A*). In the presence of large amounts of peptide (5 μg, 3.8 nmol), the derivative containing phosphoserine was phosphorylated much more efficiently by Cdc7 kinase (Fig. 6*B*, lanes 7–9) than the peptide with serine moiety (lanes 2–4). These results suggest that the presence of a phosphorylated serine residue can play the same role as an acidic amino acid in supporting phosphorylation by Cdc7 kinase.

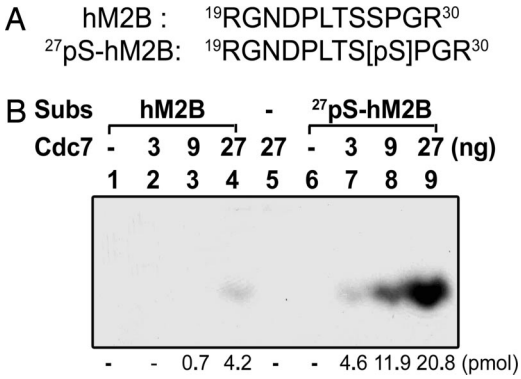

Fig. 6. The generation of additional Cdc7 phosphorylation sites by prior phosphorylation. (*A*) The amino acid sequences of two synthetic peptides with or without the phosphor-serine at the CDK phosphorylation site. (*B*) The two hM2B oligopeptides were synthesized chemically (Peptron). These oligopeptides (5 μg, 3.8 nmol) were incubated with the indicated levels of Cdc7 kinase at 30°C for 20 min. Reaction mixtures were then subjected to 18% Tricine-SDS/PAGE followed by autoradiography. The symbol –, located at the bottom of the autoradiogram, indicates that incorporation was <0.1 pmol.

Discussion

In this report, both the phosphorylation sites in Mcm2 and the amino acid sequence required for Cdc7 kinase activity were determined. *In vitro* phosphorylation of both truncated Mcm2 proteins and alanine-substituted Mcm2 peptides revealed two major (^{5}S and ^{53}S) and three minor (^{4}S, ^{7}S, and ^{59}T) phosphorylation sites located at the N terminus of Mcm2. Recently, the sites in Mcm2 phosphorylated *in vitro* by Cdc7 kinase were mapped by mass spectrometry (23). This study, which used the N-terminal region (amino acids 10–294) of Mcm2, identified two sites, located at ^{40}S and ^{53}S. Because amino acid residues 1–9 were omitted, the three phosphorylation sites reported here (^{4}S, ^{5}S, and ^{7}S) were not identified. Although both the previous and our studies identified ^{53}S as a major phosphorylation site, we did not detect ^{40}S (which is located next to ^{41}S) as a major Cdc7 phosphorylation site. In our work, the phosphorylation of ^{40}S by Cdc7 kinase was observed only in peptides after the downstream ^{41}S was phosphorylated by CDK (data not presented). In the experiments carried out by Montagnoli *et al.* (23), Cdc7 kinase was isolated from baculovirus infected insect cells, whereas our kinase was isolated by using an *E. coli* expression system. We suggest that the Cdc7 kinase preparation used by Montagnoli *et al.* (23) may have included trace levels of CDK, which could account for the differences noted above.

All major and minor phosphorylation sites found in human Mcm2 contained an acidic amino acid adjacent to the phosphorylated serine residue, and similar results were obtained with the

S. pombe Mcm2 protein and Hsk1-Dfp1 kinase (*S. pombe* Cdc7 kinase complex; data not presented). From studies with a number of alanine-substituted peptides, we noted that an acidic amino acid adjacent to a serine residue is essential for the Cdc7 phosphorylation. Furthermore, the presence of an acidic amino acid downstream of the serine phosphorylated was more important than an upstream acidic residue (Fig. 5). Serine rather than a threonine appears to be a more-preferred phosphorylation target, a result consistent with previous observations that phosphoserine is the major Cdc7 kinase phosphorylation product found with Mcm2 (3).

The amino acid sequence required for Cdc7 phosphorylation is similar in many ways to that observed for casein kinase II (CK2). CK2 is an essential kinase required for cell viability that plays many roles in tRNA and rRNA synthesis, apoptosis, cell survival, and transformation. To date, >300 proteins are known substrates of this kinase. CK2 phosphorylation sites contain multiple acidic residues that surround the site phosphorylated (24). Although the presence of an acidic amino acid at the third position ($n + 3$) downstream of the amino acid phosphorylated is critical for CK2 phosphorylation (minimal consensus sequence of S/T-x-x-E/D/pS), an acidic amino acid located at position $n + 1$ is also important, and phosphorylation of serine is preferred over threonine. The presence of a phosphorylated serine residue (in place of E/D) downstream of the amino acid phosphorylated also supports CK2 activity, analogous to the results described here with Cdc7 kinase. The similar substrate specificity of these two kinases might be because of their structural similarities. Indeed, the catalytic subunit of human Cdc7 kinase and the catalytic α' subunit of CK2 show 30.2% sequence identity and 46.6% similarity. A major difference in the substrate specificity of these two kinases is the location of the critical acidic amino acid, which is positioned $n + 1$ and $n + 3$ (from their target sites) for Cdc7 and CK2, respectively. Our findings, however, were obtained from studies with only a few phosphorylation sites in the Mcm2 protein, and for that reason, we cannot rule out the possibility that the sequence requirements for other substrates might differ. Analyses of additional phosphorylation sites in multiple proteins will be required to define a more reliable consensus sequence for Cdc7.

We also noted that additional Cdc7 kinase phosphorylation sites were generated after phosphorylation of Mcm2 by CDK. Although it was reported previously that *in vitro* CDK stimulated the phosphorylation of Mcm2 by Cdc7 kinase (16), the mechanism contributing to this effect was not defined. Our findings suggest that a phosphorylated serine residue located downstream of a serine mimics an acidic amino acid and support the phosphorylation by Cdc7 kinase (Fig. 6). The N terminus of Mcm2 protein contains consecutive serine or threonine residues that can be phosphorylated by CDK. Therefore, phosphorylation of Mcm2 by CDK generates at least two additional phosphorylation sites (^{26}S and ^{40}S) for Cdc7 kinase. Previous *in vivo* studies in *S. cerevisiae* also suggested that the sequential action of CDK and Cdc7 kinase is essential for the initiation of DNA replication (25). During S phase, Cdc7 kinase supports DNA replication only after CDK activation. Although there are many possible reasons why the sequential action of CDK and Cdc7 is required for the initiation of DNA replication, the generation of additional Cdc7 phosphorylation sites might contribute importantly to this temporal order.

All of the phosphorylation sites mapped in Mcm2 protein are located within the N-terminal 60 amino acids. These include two major Cdc7 phosphorylation sites (^{5}S and ^{53}S), three minor phosphorylation sites (^{4}S, ^{7}S, and ^{59}T), and at least two additional phosphorylation sites that can be generated by CDK phosphorylation (^{26}S and ^{40}S). Based on studies with the mouse Mcm2 protein, the N-terminal region of Mcm2 contains a histone H3-binding domain as well as nuclear localization signals. However, deletion of the N-terminal 62-aa region had no effect on either the nuclear localization or histone-binding activity of mouse Mcm2. N-terminal-deleted Mcm2 protein can also form a complex with Mcm4/6/7 and inhibit its DNA helicase activity, suggesting that the N-terminal region of Mcm2 with its Cdc7 phosphorylation sites is dispensable for its inclusion in the MCM complex and nuclear localization (21). These results are consistent with the fact that Cdc7 activity is required at later stages of the initiation reaction after pre-RC assembly. It would be of interest to examine whether phosphorylation of the MCM complex affects its interactions with other initiation proteins, including Cdc45. Because the N-terminal region of Mcm2 is not required for the formation of the MCM complex, this portion of the protein may be exposed and located on the outside of the complex. For this reason, conformational changes caused by Cdc7 phosphorylation may affect the interactions between the MCM complex and other components of the pre-RC, the Sed^{5}, Psf^{1}, Psf^{2}, and Psf^{3} (GINS) complex, or Cdc45.

Although the importance of Cdc7 kinase is well established, studies on its cellular role and the consequences of its phosphorylation of target protein are limited, partly because of the lack of information about its phosphorylation sites in target protein. Cdc7 kinase also influences other cellular transactions, including checkpoint control and mutagenesis. Thus, it is reasonable to assume there are multiple *in vivo* targets of Cdc7 kinase. Although these substrates may depend on their interactions with the regulatory subunit, Dbf4, the results presented here, augmented with more information about Cdc7 kinase phosphorylation sites, should help to identify new cellular targets of this kinase and its physiological role.

Materials and Methods

Preparation of Cdc7 Kinase. Human Cdc7 and Dbf4 proteins were expressed and purified from *E. coli* cells. The cDNAs encoding full-length Cdc7 and Dbf4 proteins were obtained by PCR and subcloned into pET30a plasmids (Novagen) under the influence of a T7 promoter. A myc epitope tag was added to the N terminus of Cdc7, and a 6-histidine (His)-tag was added at the N terminus of Dbf4 to aid in their detection and purification. For the coexpression of these two proteins, the DNA fragment including the Dbf4 gene from the ribosome-binding site to the stop codon in pET30a-Dbf4 was subcloned into the downstream region of the Cdc7 gene in the pET30a-Cdc7 plasmid. After expression of both proteins in BL21(DE3) codon plus (Stratagene) cells, the 6-His-tagged protein was purified by Ni-NTA (Qiagen) column chromatography by using the manufacturer's protocol, except that buffer T (20 mM Tris·HCl, pH 7.5/0.05% Nonidet P-40/10% glycerol) containing 0.3 M NaCl and 15–150 mM imidazole was used during the purification. The eluted fractions were diluted with buffer T to 0.1 M NaCl, loaded onto a 1-ml HiTrap Q column (Amersham Pharmacia) and eluted with a 15-ml linear gradient from 0.1 to 0.5 M NaCl in buffer T. Fractions containing the Cdc7–Dbf4 complex, peaking at 0.3 M NaCl, were pooled and used in this study. This procedure typically yielded ≈150 μg of protein from 1.5 liters of culture.

Preparation of Mcm2 Proteins and Peptides. For the preparation of various full-length human Mcm2 proteins, cDNA fragments encoding either the wild-type or mutated Mcm2 protein were cloned into the pET28a (Novagen) plasmid, and the C-terminal 6-His-tagged proteins were purified by Ni-NTA chromatography followed by HiTrap Q column chromatography. GST-fusion systems were used for the preparation of truncated Mcm proteins and small peptides. Various cDNA fragments encoding truncated human Mcm2 or peptides were cloned into the pGEX5X-1 plasmid as GST-fusion proteins. After expression in BL21(DE3) pLysS cells, GST-fusion proteins were purified by glutathione Sepharose 4B column chromatography by

using the manufacturer's protocol, except that buffer T containing 0.3 M NaCl was used during the purification. Two oligopeptides (hM2B and ^{27}pS-hM2B) were chemically synthesized (Peptron, Daejeon, Korea), dissolved in DMSO, and used as substrates in the kinase assay as described below.

Preparation of CDK. Baculoviruses expressing human CycE and 6 His-tagged Cdk2 were obtained from B. S. Yang (Korea Institute of Science and Technology, Seoul, Korea). Sf9 cells were coinfected with baculoviruses expressing CycE and Cdk2 at a multiplicity of infection of 10. After incubation at 27°C for 2 days, the CycE–Cdk2 complex was purified by Ni-column chromatography by using the manufacturer's protocol, except that buffer T containing 0.1 M NaCl was used during the purification.

Kinase Assay. Reaction mixtures (15 μl) contained 25 mM Hepes–NaOH (pH 7.5), 75 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), 1 mM NaF, 0.1 mM sodium vanadate, and indicated levels of substrate and kinase. After incubation at 30°C for the indicated times, reaction mixtures were analyzed by SDS/11% PAGE followed by autoradiography. The level of phosphorylation was quantitated by phosphorimager (Fuji) analyses.

This study was supported by the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (Grant A030116, to J.-K.L.).

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