Identification of Mcm2 Phosphorylation Sites by S-phase-regulating Kinases*

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Minichromosome maintenance 2–7 proteins play a pivotal role in replication of the genome in eukaryotic organisms. Upon entry into S-phase several subunits of the MCM hexameric complex are phosphorylated. It is thought that phosphorylation activates the intrinsic MCM DNA helicase activity, thus allowing formation of active replication forks. Cdc7, Cdk2, and ataxia telangiectasia and Rad3-related kinases regulate S-phase entry and S-phase progression and are known to phosphorylate the Mcm2 subunit. In this work, by in vitro kinase reactions and mass spectrometry analysis of the products, we have mapped phosphorylation sites in the N terminus of Mcm2 by Cdc7, Cdk2, Cdk1, and CK2. We found that Cdc7 phosphorylates Mcm2 in at least three different sites, one of which corresponds to a site also reported to be phosphorylated by ataxia telangiectasia and Rad3-related. Three serine/proline sites were identified for Cdk2 and Cdk1, and a unique site was phosphorylated by CK2. We raised specific anti-phosphopeptide antibodies and found that all the sites identified in vitro are also phosphorylated in cells. Importantly, although all the Cdc7-dependent Mcm2 phospho sites fluctuate during the cell cycle with kinetics similar to Cdc7 kinase activity and Cdc7 protein levels, phosphorylation of Mcm2 in the putative cyclin-dependent kinase (Cdk) consensus sites is constant during the cell cycle. Furthermore, our analysis indicates that the majority of the Mcm2 isoforms phosphorylated by Cdc7 are not stably associated with chromatin. This study forms the basis for understanding how MCM functions are regulated by multiple kinases within the cell cycle and in response to external perturbations.

In eukaryotic organisms during the G1 phase of the cell cycle a multiprotein complex known as the pre-replicative complex is formed around origin DNA. This contains the origin recognition complex, Cdc6, Cdt1, and minichromosome maintenance (MCM) proteins. Once in S-phase several components of the pre-replicative complex are phosphorylated by at least two kinases, a cyclin-dependent kinase (CDK) and the Cdc7 kinase. This leads to the unwinding of double-stranded DNA and to the loading of several proteins such as Cdc45 and the GINS complex, which, together with DNA polymerases, participate in the semi-conservative synthesis of new DNA strands during chain elongation (1–5). The MCM complex appears to be a crucial target of the S-phase-promoting kinases, and multiple subunits become phosphorylated at the time of origin activation (reviewed in Ref. 6). Once initiation has occurred, the Mcm2–7 complex travels together with replicating enzymes on DNA, being required for replication fork progression possibly by acting as the replicative helicase (4, 7, 8). Several lines of evidence indicate that various subunits of Mcm2–7 complex are subject to multiple phosphorylations. In particular, phosphorylation of the Mcm2 protein, generally detected as altered mobility in SDS-PAGE (9, 10), appears to occur in a cell cycle-dependent manner, being detected as cells enter S-phase until mitosis. Although Mcm2 is a good substrate for both CDKs and Cdc7 kinases, it is unclear whether these kinases act independently or cooperate in MCM phosphorylation. In budding yeast it was suggested that the only cyclin-dependent kinase, Cdk1, must act before Cdc7/Dbf4 (11), whereas in a Xenopus in vitro DNA replication system only chromatin that was exposed in a sequential manner to Cdc7 first, followed by Cdk2 kinase, can efficiently replicate. Reversing the order of the kinases appeared to be detrimental to DNA replication in this system (12). Finally, in vitro phosphorylation assays using purified human recombinant proteins have indicated that pre-phosphorylation of MCMs with CDKs can stimulate Cdc7 activity, suggesting that Cdk phosphorylation may be a prerequisite for Cdc7 function at origins (9).

Recently, the checkpoint kinase ataxia telangiectasia and Rad3-related was shown to interact with Mcm7 through its regulatory subunit ATRIP and to phosphorylate Mcm2 specifically at Ser-108, suggesting that MCM is a target of the S-phase checkpoint pathway (14, 15). This is consistent with the finding that Ser-108 Mcm2 phosphorylation is increased in cells challenged with DNA-damaging agents. However, because basal levels of Mcm2 Ser-108 phosphorylation are detected in the absence of damage or in cells in which ATR activity is impaired, it is likely that a different kinase exists that is able to phosphorylate Mcm2 at the same site (14, 15). Finally, Mcm2 contains a canonical consensus site for casein kinase 2 (CK2), a kinase with pleiotropic functions that has also been implicated in regulating DNA replication proteins (16, 17). At present it is not known whether all these kinases indeed phosphorylate Mcm2 in cells, what is the temporal sequence of different phosphorylation events, and, ultimately, how phosphorylation at different sites contributes to MCM function. To begin to address these questions, we set out to map specific phosphosites in the human Mcm2 protein. By generating specific immunological reagents we found that the Mcm2 N-terminal tail is phosphorylated in at least seven different sites and that the observed cell cycle-dependent phosphorylation of Mcm2 occurs almost exclusively at Cdc7-dependent sites. We also found that Cdc7 kinase can phosphorylate Mcm2 in the same site that is also recognized by ATR and that Cdc7 and Cdk can phosphorylate adjacent serines.

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The abbreviations used are: CDK, cyclin-dependent kinase; DTT, dithiothreitol; CK2, casein kinase 2; Pipes, 1,4-piperazinediethanesulfonic acid; MS, mass spectrometry; ESI, electrospray ionization; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; NHDF, human dermal fibroblast; HA, hemagglutinin; HU, hydroxyurea; FACs, fluorescence-activated cell sorter; ATR, Ataxia telangiectasia and Rad3-related.
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EXPERIMENTAL PROCEDURES

Recombinant Proteins and Synthetic Peptides—Recombinant human N-terminal Mcm2 protein, corresponding to residues 10–294, was produced as described in Ref. 18. Mcm2-HA for cell expression was obtained by cloning the full-length Mcm2 into the mammalian expression vector pCDNA-HA. Point mutations described under “Results” were generated by oligonucleotide-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene). All mutants were sequenced in their entirety. Cdc7/Dfb4 kinase was obtained as described in Ref. 18. Cdk2/A, Cdk1/B were produced as described elsewhere (19), and CK2 was purchased from Calbiochem. Synthetic biotinylated peptides with sequence RTDALTSSPGRDLPPFG were synthesized in-house.

N-terminally and C-terminally phosphorylated or phosphorylated in residues Ser-40, Ser-41, or Ser-40 and Ser-41 were custom synthesized (Tufts University). Mcm2 biotinylated peptides 49–64 and 36–44 were synthesized in-house.

Kinase and Phosphatase Assays—For in vitro kinase assays 6 μM N-terminal recombinant Mcm2 (wild type or mutants) was incubated for 30 min at 37 °C in the presence of 50 nM Tris, pH 7.5, 10 mM MgCl2, 2 mM DTT, 100 μM ATP (kinase buffer), and 10 nM Cdc7/Dfb4 or Cdk2/cyclin E or CK2. For kinase assay after immunoprecipitation, the beads were equilibrated in kinase buffer and incubated in the same buffer containing 6 μM Mcm2 for 30 min at 37 °C.

For liquid chromatography/MS analysis, enzyme substrate ratio was decreased to 1:10, and the reaction was performed for 2 h in 50 mM Hepes, pH 7.9, 10 mM MgCl2, 2 mM DTT. Peptide phosphorylation in Fig. 2E was performed with 50 μM peptide as substrate, 4 nM enzyme, 10 μM ATP traced with radiolabeled [γ-32P]ATP in 40-μl reaction in a 96-well plate. Dowex resin (Supelco) was used to capture residual ATP before counting radioactivity. For Dot Blot analysis 50 μM peptide substrate were incubated for 2 h at 37 °C in the presence of 50 mM Tris, pH 7.5, 10 mM MgCl2, 2 mM DTT, 500 μM ATP, and 50 nM Cdc7/Dfb4 or Cdk2/CycA. For the phosphatase experiment, 15 μg of HeLa extract prepared without phosphatase inhibitors was incubated with 100 units of λ-phosphatase (Calbiochem) for 30 min at 30 °C.

Liquid Chromatography/MS Analysis—20 μg of Mcm2-(10–294) protein was analyzed before or after phosphorylation with the different kinases. The chromatographic separations were performed on a 1100 Agilent instrument using a Vydac C-4 column (2.1-mm i.d., 25-cm length, particle size 5 μ, pore size 300 Å). After column equilibration by 10% aqueous acetonitrile containing 0.05% trifluoroacetic acid, an eluent program was performed with a linear gradient from 10 to 75% ACN containing 0.05% trifluoroacetic acid in 35 min. The flow rate was set to 0.2 ml/min. The eluate from the column was sent directly to the MS instrument. Positive ion ESI mass spectra were obtained using 1946 single quadrupole mass spectrometer (Agilent) with an orthogonal ESI source. The needle voltage was set at 3000 V. The nebulizer as well as drying gas (nitrogen) were maintained at 30 psi and a flow rate of 10 liters/min, respectively. The mass range was set to m/z 300–2000. The resulting final ESI mass spectrum of intact protein samples was deconvoluted automatically using Agilent chemstation deconvolution software. For peptide analysis the chromatography separation was performed on a Vydac C-18 column (2.1-mm i.d., 25-cm length, particle size 5 μ, pore size 300 Å) with an elution gradient from 5 to 75% ACN containing 0.05% trifluoroacetic acid in 60 min.

In-gel Tryptic Digestion—Protein digestion was performed with trypsin by using the Digest Pro system (Intavis, Koeln, Germany) following the standard protocol. The elution mixture was then dried in a speed vacuum and redissolved in 10 μl of aqueous 0.1% trifluoroacetic acid.

Sample desalting was performed by ZipTip C18 (Millipore) with fractionated elution of 10 μl of 10% acetonitrile, 0.1% trifluoroacetic acid in water, followed by 10 μl of 30% acetonitrile, 0.1% trifluoroacetic acid in water and finally 10 μl of 60% acetonitrile, 0.1% trifluoroacetic acid in water. 0.5 μl of each sample was used for MALDI-MS analysis, and the remaining material was dried down and redissolved in 0.1% formic acid, 50% acetonitrile for nano-ESI MS/MS analysis. Additional digestion with AspN on ZipTip eluates was performed, redissolving dried ZipTip elute in 10 μl of NH4HCO3 50 mM and adding 0.1 μg of AspN protease and incubating at 37 °C. The digestion was monitored by MALDI MS, mixing 0.5 μl of digestion mixture with 0.5 μl of matrix and analyzing it in reflector mode.

MALDI-MS—Samples for matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analysis were prepared by spotting 0.5 μl of peptide mixture with 0.5 μl of α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50:50 acetonitrile:water containing 0.1% trifluoroacetic acid) and analyzed on a Voyager DE-PRO (Applied Biosystems). All spectra were collected in reflector mode using four peptides of known mass as external calibration standards.

Nano-ESI-MS/MS—Tandem mass spectrometry was performed on a hybrid quadrupole-time of flight instrument (Q-ToF2; Micromass, Manchester, UK) equipped with a Z-spray source and calibrated by injecting a solution of Glu-Fibrinopeptide (Sigma-Aldrich) (0.5 pmol/μl) at 0.5 μl/min, applying 3.2 kV to the spraying capillary, and a collision energy of 29 V.

For phosphosite assignment by MS/MS, the desalted samples were dried down in a speed vacuum, redissolved in a 1:1 acetonitrile/0.1% formic acid mixture, and loaded directly into nanoflow probe tips (Micromass). Each fraction was subjected to MS analysis over the 100–2000 m/z scan range; MS/MS analyses were performed by manually selecting the phosphorylated peptides and fragmenting them under user-defined parameters of collision energy.

Antibodies—The anti-pSer-13, -pSer-40/41, -pSer-108, and –pSer-139 Mcm2-specific antibodies were generated in collaboration with Zymed Laboratories Inc. by immunizing rabbits with the phosphopeptides MAS-pS-PAQRRR, APLT-pS-pS-PGR, EELTA-pS-QRE, and LLYD-pS-DEEDE, respectively. The antibodies were then purified from serum with two rounds of affinity chromatography using both phospho- and nonphosphopeptide affinity columns. The phospho-Ser-53 Mcm2-specific antibody was generated in collaboration with BIOSOURCE by injecting the rabbits with the phosphopeptide FEDE-pS-EGLLG. In this case crude antisera was used in the experiments. Antibodies against pSer-41 and pSer-27 Mcm2 were purchased from Bethyl. Antibody to total Mcm2 was from BD Biosciences; anti-Cyclin A and Cyclin B were from Santa Cruz. For Cdc7 Western blots an antibody from Neomarker was used. Immunoprecipitation of HA-Mcm2 was performed using anti-HA affinity matrix (Roche Applied Science). Cdc7 immunoprecipitations were performed with the monoclonal antibody 12A10 that was developed together with ARETA (www.Areta.com), while an anti-ATR antibody from Santa Cruz (N19) was used for ATR immunoprecipitation. For peptide competition experiments, the anti-phospho Mcm2 antibodies were preincubated for 1 h at room temperature with the corresponding peptides before adding to the membranes. The peptides used were MAS-pS-PAQRRR, FEDE-pS-EGLLG, EELTA-pS-QRE, LLYD-pS-DEEDE (and the corresponding unphosphorylated peptides) for anti-pSer-13, pSer-53, pSer-108 and –pSer-139 antibodies, respectively.

Synthetic peptides with sequence RTDALTSSPGRLPFGG were synthesized in-house using the standard protocol. The elution mixture was then dried in a speed vacuum and redissolved in 10 μl of saline for injection. Synthetic peptides with sequence RTDALTSSPGRLPPFG were synthesized in-house using the standard protocol. The elution mixture was then dried in a speed vacuum and redissolved in 10 μl of saline for injection.
Cell Synchronization and Protein Preparations—HeLa cells were grown in modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. Human dermal fibroblasts (NHDF) were grown in fibroblast basal medium (Promocell) supplemented with 10% fetal bovine serum and growth factors. NHDF were synchronized in G0 by culturing the cells in medium without serum for 48 h. Thymidine synchronization was monitored by flow cytometry using FACScan (BD Biosciences). For total protein extraction, cells were lysed in SDS buffer (100 mM NaCl, 300 mM sucrose, 1 mM MgCl2, 1 mM DTT, 1 mM EGTA, 0.1% Triton X-100, protease and phosphatase inhibitors) following the manufacturer’s instructions. Small inter-nuclei binding experiments, cells were lysed in CSK buffer (10 mM Pipes, pH 6.8, 5% SDS, 1 mM DTT) and sonicated. For chromatin binding experiments, cells were lysed in CSK buffer (10 mM Pipes, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl2, 1 mM DTT, 1 mM EGTA, 0.1% Triton X-100), protease (Roche Applied Science) and phosphatase inhibitors (Sigma). In the indicated cases, cells were incubated in CSK buffer for 10 min on ice before fixation. For competition experiments the antibodies were preincubated with the corresponding peptides as previously indicated before adding to the coverslips.

Transfections—HeLa cells were transfected using FuGENE (Roche Applied Science) following the manufacturer’s instructions. Small interference RNA experiments were as previously described (10).

RESULTS

Identification of Mcm2 Phosphosites in Vitro—To address the regulation of McMs by phosphorylation, we began to identify phosphorylated sites on the Mcm2 protein. Annotation of the human Mcm2 amino acid sequence has been recently updated in its N terminus, and here we refer to its new Swiss Prot accession number, P49736. We and others have previously shown that an Mcm2 N-terminal fragment, corresponding to amino acids 10–294, is a good substrate for both Cdc7 and CDKs (9, 18, 20). We extended these observations and also found that Cdk2 kinase, which has been proposed to modulate DNA replication by phosphorylating multiple substrates (17), efficiently uses the Mcm2 N-terminal fragment as a substrate.

Incubation of Mcm2-(10–294) with recombinant Cdk2/A, Cdc7/Dbf4, or CK2 in the presence of radiolabeled ATP resulted in a highly labeled Mcm2-(10–294) (Fig. 1A) after SDS-PAGE separation and autoradiography. LC/MS analysis of Mcm2-(10–294) after incubation with the kinases of interest and cold ATP clearly showed the presence of several Mcm2-(10–294) species differing by 80 Da and phosphorylated in up to three sites in the reactions performed either with Cdk2 or Cdc7. Similar results were obtained using Cdk1/CyclB kinase (data not shown). In contrast, only one phosphate group was added by CK2 (Fig. 1B).

For all three samples, phosphorylated Mcm2-(10–294) was digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF mass spectrometry. Comparison of spectra obtained before and after phosphatase treatment allowed us to determine that Cdk2/CycA and Cdk1/CyclB kinase complexes showed a completely superimposable pattern with three phosphorylated peptides (regions 10–17, 20–30, 34–44). Nano-ESI MS/MS analysis on the selected peptides identified Ser-13, Ser-27, and Ser-41 as phosphorylated residues (supplemental Fig. S1 and data not shown).

Two of three of these sites, Ser-27 and Ser-41, correspond to canonical S/TPX/R/K CDK consensus sites (21), whereas just the partial consensus sequence S/TP was observed in Ser-13. Using the same procedure we found that the peptide 134–149 was stoichiometrically phosphorylated (MW 1973.84) by Ck2, and again by nano-ESI MS/MS we confirmed that Ck2 can phosphorylate Mcm2 at serine 139 (data not shown) in a context that corresponds to the canonical casein kinase II phosphorylation motif (17).
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Mutation of serine 139 into alanine completely abolished CK2 phosphorylation, clearly indicating that Ser-139 is the only amino acid that can be modified by the kinase in the Mcm2-(10–294) fragment (supplementary Fig. S2A).

Upon tryptic digestion of Mcm2-(10–294) phosphorylated by Cdc7, three different phosphopeptides were detected (34–44, 45–79, and 83–115). The phosphorylated form of peptide 83–115 after partial digestion with AspN endoprotease could be analyzed by nano-ESI MS/MS, and phosphorylation on Ser-108 was clearly detected (Fig. 2A).

The nano-ESI MS/MS analysis of the other two peptides was not successful due to the large molecular weight of peptide 45–79 and to the poor ionization properties and low stoichiometry of peptide 36–44 after Cdc7 phosphorylation. Therefore, the corresponding synthetic peptides (36–44 and 49–64) were produced, and upon in vitro phosphorylation with Cdc7/Dbf4, pSer-53 (Fig. 2B) and pSer-40 (Fig. 2C) were identified as specific phosphoamino acids. Mcm2 Ser-40, Mcm2 Ser-53, and Mcm2 Ser-108 are the first specific sites reported for Cdc7 phosphorylation, clearly indicating that Ser-139 is the only amino acid that can be modified by the kinase in the Mcm2-(10–294) fragment (supplementary Fig. S2A).

Intriguingly, Ser-108 is the same residue that was found phosphorylated by ATR kinase (14) by two separate groups. We then independently confirmed that immunoprecipitated ATR phosphorylates Mcm2 N terminus at Ser-108 and that Ser-108 is the only residue phosphorylated by this kinase in this Mcm2 fragment (supplementary Fig. S2B).

We also noticed that Cdc7 phosphorylation on Ser-40 immediately precedes Ser-41, which in the previous experiment was found phosphorylated by Cdk2. If surrounding negative charges are needed for better substrate recognition by Cdc7, then the introduction of a phosphate group in position +1 (Ser-41) may facilitate phosphorylation by Cdc7 on Ser-40. This observation prompted us to further investigate the relationships between Cdk2 and Cdc7 in the phosphorylation of a Mcm2 peptide spanning this region (amino acids 36–44). As expected, both kinases were able to independently phosphorylate the peptide, and MS/MS analysis confirmed that only Ser-40 was phosphorylated by Cdc7 (Fig. 2C) and only Ser-41 was phosphorylated by Cdk2 (supplementary Fig. S1). We then tested whether the same peptide could be phosphorylated on both residues. Peptide 36–44 was first incubated with Cdk2, and products of the reaction were isolated by high performance liquid chromatography as a single phosphorylated peptide on Ser-41. This was then incubated with Cdc7 kinase, and MALDI analysis indicated that a second phosphate group was transferred. MS/MS analysis confirmed that both Ser-40 and Ser-41 were modified (Fig. 2D).

Thus, under these experimental conditions Cdc7 and Cdk2 phosphorylation of this peptide are not mutually exclusive. By MALDI-MS we observed that pre-phosphorylation of Mcm2 peptide 36–44 on Ser-41 by Cdk2 appears to increase the efficiency of Cdc7 phosphorylation on Ser-40 (data not shown). To further explore this phenomenon we compared the efficiency of Cdc7 kinase in the phosphorylation of two synthetic peptides spanning this region, either unphosphorylated or monophosphorylated in Ser-41. Indeed, we found that Cdc7 activity was increased ~3-fold when the phosphorylated pSer-41 peptide was used as substrate (Fig. 2E). This result supports the hypothesis that the negatively charged residues may facilitate phosphorylation by Cdc7. Alignment of N-terminal tails of Mcm2 proteins from human, mouse, and Xenopus indicates that the serines phosphorylated by Cdc7 and CK2 are highly conserved, whereas only two of three sites identified for Cdk phosphorylation are found in all three species (Fig. 3).

Characterization of Anti-phospho-Mcm2 Antibodies—With the goal of verifying whether the phosphosites that were mapped in Mcm2 protein in vitro with purified kinases were also phosphorylated in vivo, we raised rabbit polyclonal antibodies against synthetic phosphopeptides.
spanning Ser-13, Ser-53, Ser-108, and Ser-139. We also immunized animals with a peptide carrying double-phosphorylated Ser-40 and Ser-41. Specific anti-pSer-13, -pSer-108, and -pSer-139 antibodies were then affinity purified on antigen columns. Anti-pSer-27 and anti-pSer-41 antibodies were purchased from a commercial source.

To check the specificity of the immunological reagents, purified antibodies were used as probes in Western blot experiments against recombinant Mcm2 mock treated or phosphorylated with the relevant kinase. We found that anti-pSer-13 antibody specifically recognizes Mcm2 phosphorylated by Cdk1/B and Cdk2/E. Recombinant N-terminal human Mcm2 was incubated in the absence or in the presence of recombinant Cdk2/cyclin E, Cdk1/cyclin B, or Cdc7/Dbf4 expressed and purified from insect cells and ATP. After phosphorylation, proteins were subjected to Western blot analysis using anti-pSer-13 antibody. B, anti-pSer-139 antibody specifically recognizes Mcm2 phosphorylated by Cdk2. Recombinant Mcm2 was subjected to kinase reaction in the absence or the presence of Cdc7/Dbf4 or casein kinase 2 and analyzed by Western blot using anti-pSer-139 antibody. C, anti-pSer-53 and anti-pSer-108 antibodies specifically recognize Mcm2 phosphorylated by Cdc7/Dbf4. Recombinant N-terminal human Mcm2 was incubated in an in vitro kinase assay in the absence or the presence of Cdc7/Dbf4. D, anti-pSer-40/41 antibody specifically recognizes double-phosphorylated Mcm2 (34–49) peptide. Unphosphorylated or synthetically modified phosphopeptides in Ser-40, Ser-41, and Ser-40 plus Ser-41 were incubated in a kinase assay with mock or Cdc7/Dbf4 or Cdk2/cyclin E and captured on streptavidin-coated plates. A Dot Blot was performed with the anti-pSer-40/41 antibody. E, anti-pSer-41 recognizes both Ser-41 mono-phosphorylated and Ser-40/41 double-phosphorylated peptides. Dot Blot was performed as in panel D but with anti-pSer-41 antibody. F, HeLa cells were transfected with empty vector or with wild type, S40A-mutated, or S41A-mutated HA-tagged Mcm2. Cell lysates were immunoprecipitated with HA affinity matrix and immunoprecipitated material analyzed by Western blot with the indicated antibodies.

Ser-41 Phosphorylation Is Not Required for Cdc7-dependent Phosphorylation at Ser-53 and Ser-108 in Vivo—To achieve more insight into the relationship among the different Mcm2 phosphosites, we transfected plasmids expressing HA-Mcm2 fusion proteins, either wild type or carrying S40A or S41A substitution under the control of the cytomegalovirus promoter, into HeLa cells. HA-tagged Mcm2 proteins were immunoprecipitated with anti-HA antibodies and probed with anti-phospho-Mcm2 antibodies. We found that S40A substitution pre-
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Identification of Endogenous Mcm2 Phosphosites in Vivo—When whole HeLa cell extracts were blotted on membranes we found that all the antibodies generated against Mcm2 phosphopeptides recognized one or more bands migrating with an apparent molecular weight compatible with Mcm2 protein (Fig. 5A). These bands co-migrated with the bands recognized by an anti-Mcm2 antibody, and treatment of the extracts with λ-phosphatase abolished the immunological reactions, strongly suggesting that they correspond to phosphorylated Mcm2 (Fig. 5A).

To further confirm the specificity of the anti-phospho-Mcm2 antibodies and to begin the studies of the phosphorylation status of endogenous Mcm2 protein, we found that Ser-40/41, Ser-53, and Ser-108 Mcm2 phosphorylation appear to fluctuate during the cell cycle with kinetics overlapping with Cdc7 kinase activity. Ser-40/41 phosphorylation peaked in late S-phase and G2, Ser-53 phosphorylation was observed throughout S-phase and G2/M, and levels of Ser-108 phosphorylation appeared to be maximal in middle S-phase. On the contrary and very surprisingly, phosphorylation levels at the sites Ser-13, Ser-27, and Ser-41, putative CDK-dependent sites, appear to be constant throughout the experiment. Therefore, as phosphorylation of Ser-41 is unchanged, we conclude that the fluctuation observed with the anti-pSer-40/41 antibody is caused by fluctuation in the levels of Ser-40 phosphorylation. Finally, levels of Ser-139 phosphorylation were decreased in serum-starved cells and failed to reach steady state levels during the course of the experiment, indicating that Ser-139 phosphorylation is not primarily under cell cycle control.

To further examine phosphorylation of Mcm2 in the cell cycle, cells were arrested in early S-phase by a thymidine block (Fig. 7). Upon release, cells synchronously progressed through S-phase and by 12 h they had completed mitotic division. A second round of DNA replication began to rise concomitantly with CycA protein levels, as well as the binding to its regulatory subunits Dbf4 and Drf1 (18, 22), is a mechanism that can contribute to the overall regulation of Cdc7 kinase activity during the cell cycle.

When we blotted the same protein samples with anti-phospho-Mcm2 antibodies, we found that Ser-40/41, Ser-53, and Ser-108 Mcm2 phosphorylation appear to fluctuate during the cell cycle with kinetics overlapping with Cdc7 kinase activity. Ser-40/41 phosphorylation peaked in late S-phase and G2, Ser-53 phosphorylation was observed throughout S-phase and G2/M, and levels of Ser-108 phosphorylation appeared to be maximal in middle S-phase. On the contrary and very surprisingly, phosphorylation levels at the sites Ser-13, Ser-27, and Ser-41, putative CDK-dependent sites, appear to be constant throughout the experiment. Therefore, as phosphorylation of Ser-41 is unchanged, we conclude that the fluctuation observed with the anti-pSer-40/41 antibody is caused by fluctuation in the levels of Ser-40 phosphorylation. Finally, levels of Ser-139 phosphorylation were decreased in serum-starved cells and failed to reach steady state levels during the course of the experiment, indicating that Ser-139 phosphorylation is not primarily under cell cycle control.

Cdc7 Depletion Affects Mcm2 Phosphorylation at Specific Sites Only—We have previously shown that the inhibition of Cdc7 kinase by small interference RNA affects migration of the Mcm2 protein in polyacrylamide gel (10); it is therefore likely that one or more Cdc7-dependent phosphorylation events on Mcm2 can account for the alteration of Mcm2 electrophoretic mobility. We therefore asked how many and which phosphosites in the N-terminal tail of Mcm2 were indeed dependent on Cdc7 activity in cells. For this purpose HeLa cells were transfected with either luciferase or Cdc7-specific small interference RNAs. Cells were collected 24, 48, and 72 h post-transfection, and protein samples were prepared and analyzed by Western blot. We observed Ser-53 were also increased. On the contrary, Ser-13, Ser-41, and Ser-139 were not affected (Fig. 5B). These findings also indicate that phosphorylation of multiple sites in the Mcm2 N-terminal tail is not co-regulated and may involve multiple kinases.

Cdc7 Levels and Mcm2 Phosphorylation during the Cell Cycle—We next examined levels of Mcm2 phosphorylation at the different sites during the cell cycle. In the first experiment, primary normal fibroblasts were arrested by serum deprivation and stimulated to re-enter the cell cycle by the addition of complete medium. Samples were taken for protein (Fig. 6, A and B) and FACS analysis (Fig. 6C). DNA content analysis indicated that cells began to enter S-phase ~12.5 h post-stimulation and by 20 h they had mostly duplicated their DNA. Consistently, CycA levels began to rise at 12.5 h, and CycB levels peaked ~20 h post-stimulation (Fig. 6A). In this experiment Cdc7 kinase activity began to rise concomitantly with CycA protein levels. Very interestingly, we also noticed that Cdc7 protein levels were decreased in serum-starved cells and increased within S-phase (Fig. 6, A and B). Accumulation of Cdc7 protein mirrored accumulation of Cdc7 mRNA (data not shown). These observations suggest that control of Cdc7 abundance, as well as the binding to its regulatory subunits Dbf4 and Drf1 (18, 22), is a mechanism that can contribute to the overall regulation of Cdc7 kinase activity during the cell cycle.

In the previous experiment, little if any variation in overall levels of Ser-13, Ser-27, and Ser-41 was observed. Unlike serum deprivation, thymidine block did not affect phosphorylation at Ser-139.

Cdc7 Depletion Affects Mcm2 Phosphorylation at Specific Sites Only—We have previously shown that the inhibition of Cdc7 kinase by small interference RNA affects migration of the Mcm2 protein in polyacrylamide gel (10); it is therefore likely that one or more Cdc7-dependent phosphorylation events on Mcm2 can account for the alteration of Mcm2 electrophoretic mobility. We therefore asked how many and which phosphosites in the N-terminal tail of Mcm2 were indeed dependent on Cdc7 activity in cells. For this purpose HeLa cells were transfected with either luciferase or Cdc7-specific small interference RNAs. Cells were collected 24, 48, and 72 h post-transfection, and protein samples were prepared and analyzed by Western blot. We observed Ser-53 were also increased. On the contrary, Ser-13, Ser-41, and Ser-139 were not affected (Fig. 5B). These findings also indicate that phosphorylation of multiple sites in the Mcm2 N-terminal tail is not co-regulated and may involve multiple kinases.
that Ser-40/41 and Ser-53 phosphorylation is strongly decreased when Cdc7 kinase is down-regulated. In addition, ~50% reduction in the Ser-108 signal was detected after 48 h. Mcm2 phosphorylation at Ser-13, Ser-41, and Ser-139 was not affected by Cdc7 depletion (Fig. 8). Again, as phosphorylation of Ser-41 is unchanged by Cdc7 depletion, we concluded that the decrease observed with the anti-pSer-40/41 antibody is caused by decrease in the levels of Ser-40 phosphorylation. These data show that phosphorylation of Ser-40, Ser-53, and partially Ser-108 requires Cdc7 activity and strongly suggest that Cdc7 directly phospho-
rylates Mcm2 at these sites \textit{in vivo}.

**Chromatin Binding Properties of Phosphorylated Mcm2 Isoforms—**

Human MCM proteins are normally confined in the nuclear compartment; in particular Mcm2 protein, a subunit of the MCM complex, is always nuclear but its binding to DNA is cell cycle regulated, being loaded at the exit from mitosis to G1 (23) and released during DNA replication. Hydroxyurea, which prevents the completion of DNA replication and induces Mcm2 hyperphosphorylation at Ser-40/41, Ser-53, and Ser-108 (Fig. 5B) also prevents release of Mcm2 from the chromatin (24, 25).

Using a biochemical fractionation approach we asked whether specific Mcm2 phosphoisoforms could be detected on the chromatin. The analysis was performed either in normally growing cells or in cells homogenously arrested in S-phase by HU.

In non-synchronized cells we observed that Mcm2 phosphorylated at Ser-13 and/or Ser-27 and/or Ser-41 and/or Ser-53 was found in both soluble and chromatin-enriched fractions, whereas Mcm2 phosphorylated at Ser-40/41 and/or Ser-108 was only detectable in the soluble, non-chromatin-bound fractions. Phospho-Ser-139 Mcm2 also was found mainly in the soluble fraction. Following HU treatment some pSer-40/41 and pSer-108 Mcm2 were detected in the chromatin-associated fractions (Fig. 9A).

To understand whether a relationship between cell cycle chromatin binding and cell cycle-dependent phosphorylation exists, we performed single cell analysis by immunofluorescence. HeLa cells were grown on glass coverslips, and they were either mock or HU treated for 24 h. Treatment with low concentrations of non-ionic detergents before fixation allows discrimination between a chromatin-engaged and a free Mcm2 form (25–28). Indeed, when anti-Mcm2 antibodies were used as a probe, all cells showed a strong nuclear staining; however, upon Triton X-100 treatment, 56% of the cells retained Mcm2 on chromatin. Blockade of S-phase by HU increased to 90% the number of Mcm2-positive cells after detergent extraction (Fig. 9B).

When we used anti-pSer-41 (Fig. 9B) and anti-pSer-13 (data not shown) antibodies, a pattern identical to anti-Mcm2 was observed. Instead, when the staining was performed with either anti-Ser-40/41 or anti-Ser-108 antibodies, only 55 and 60%, respectively, of the cells were stained. Competition with corresponding phosphopeptides completely blocked the immunological reaction, whereas competition with unphosphorylated peptides did not affect the signal, thus also confirming the specificity of the anti-phospho-Mcm2 antibodies in immunofluorescence (supplemental Fig. S3).
These observations together with the previous cell cycle analysis by Western blot suggests that phosphorylation at Ser-41 occurs in all cells, that it is therefore independent from the phase of the cell cycle, and it is also independent from binding to chromatin. On the contrary, Ser-40/41 and Ser-108 phosphorylation occurs only in post-G1 cells. Consistent with this idea, arrest in S-phase by HU caused a marked increase of positive cells. Very interestingly, in unchallenged cells Mcm2 phosphorylated at both Ser-40/41 and Ser-108 is fully released by non-ionic detergents; however, some phosphorylated Ser-40/41 and Ser-108 Mcm2 is retained on chromatin in every single HU-treated cell.

DISCUSSION

In this work we have used a biochemical approach to identify phosphorylation sites in the Mcm2 N-terminal region. After in vitro phosphorylation with three different kinases and MS analysis of the substrate, distinct phosphosites were identified for Cdc7, Cdk1/2, and CK2. Production and characterization of anti-phosphopeptide antibodies allowed us to test whether these sites are also phosphorylated in vivo. We found that all the Mcm2 phosphosites identified were indeed also phosphorylated in intact cells. Thus the in vitro to in vivo approach used for mapping phosphosites in Mcm2 N-terminal tail proved to be particularly successful and can be extended to identify and validate phosphorylated sites in other known phosphoproteins. Our analysis was performed using an N-terminal truncation of Mcm2 protein, and therefore it is possible that by using a full-length Mcm2 protein or a hexameric Mcm2–7 complex other sites phosphorylated by these kinases could be identified. Furthermore, while prolonged incubation of Mcm2-(10–294) with CDKs and CK2 kinases did not change the phosphorylation pattern, an increased amount of Cdc7/Dbf4 together with an overnight incubation led to incorporation of up to five phosphate groups (data not shown), suggesting that other Cdc7 sites may exist.

An important result of this work is the identification of the first Cdc7-specific phosphorylation sites on any given protein substrate. Cdc7 kinase appears to be able to phosphorylate peptides with different sequences that contain negatively charged residues. However, to date, no clear indication of a Cdc7 consensus site can be drawn. Identification of an increased number of Cdc7-dependent phosphorylation sites, possibly in other protein substrates, may help to define whether a Cdc7 phosphorylation consensus sequence exists. Very intriguingly, in our analysis we found that Cdc7 kinase can phosphorylate Ser-108, a residue that is in a context of a classical phosphatidylinositol 3-kinase-like kinase consensus site and that had previously been shown to be phosphorylated in vitro by the ATR kinase both in human and Xenopus (14,
In cells we found that although Cdc7 depletion caused a strong decrease in the levels of Ser-40/41 and Ser-53 Mcm2 phosphorylation, it only caused a mild change in the levels of phospho-Ser-108, consistent with either more than one kinase phosphorylating the same site or with differential accessibility of Mcm2 phosphosites to cellular phosphatases. We have recently found that Cdc7 kinase is not only required for the initiation of DNA replication at origins but is also involved, as ATR kinase, in controlling MCM helicases at stalled replication forks, thus contributing to S-phase checkpoint signaling. \(^4\) Therefore, the capability of Cdc7 and ATR of phosphorylating the same Mcm2 site may reflect an overlapping function in the regulation of replication fork machinery.

One key question is how Cdc7 and Cdk2 kinases may cooperate in origin firing and in the phosphorylation of Mcm2. Previous work has suggested that Cdk2 phosphorylation of Mcm2 stimulates phosphorylation by Cdc7 (9). Importantly, Cdc7-dependent phosphorylation was primarily measured as an increased amount of Mcm2 with altered mobility in SDS-PAGE. Our analysis indicates that in vitro Cdc7 phosphorylation can occur independently of CDK phosphorylation at all the sites we have mapped. Cdc7 phosphorylation of a peptide spanning Ser-40 and Ser-41 was stimulated if the same peptide were preincubated with Cdk2 kinase or synthetically modified with a phosphate group in Ser-41. On the other hand, phosphorylation of Ser-40 completely prevents Cdk2 from phosphorylating Ser-41. Because in vivo phosphorylation at Ser-53 and Ser-108 occurs normally in the S41A mutant, we concluded that if cooperation between CDKs and Cdc7 exists, it is limited to Ser-40 phosphorylation while the other Cdc7-dependent phosphosites do not require previous priming by CDKs. Altogether, our results and previous results prompt us to speculate that a double phosphorylation event with phosphorylation at Ser-40, which is Cdc7 dependent, and phosphorylation at Ser-41, which is Cdc7 independent, may be necessary to cause a conformational change in the protein responsible for the classical mobility shift of the Mcm2 protein observed in SDS-PAGE and often used as marker of Cdc7 activity.

Importantly, cell cycle analysis indicated that in cells all serine/proline sites, Ser-13, Ser-27, and Ser-41, are phosphorylated throughout the cell cycle, thus raising the obvious questions of whether CDKs are the only kinases responsible for targeting Mcm2 at these sites and whether this phosphorylation is relevant for MCM function during origin activation and/or replication fork progression. Future work will address these issues. On the contrary, phosphorylation at Cdc7 sites Ser-40 and Ser-53 and the Cdc7/ATR site Ser-108 fluctuates with a kinetics overlapping with Cdc7 kinase activity, suggesting that they are possibly relevant for Mcm2 function during the S-phase of the cell cycle. In the same synchronization experiments we found that levels of Cdc7 catalytic polypeptide also fluctuate in the cell cycle, being induced at entry into S-phase and falling at the exit of mitosis and correlating with mRNA levels (data not shown). This finding challenges the current thinking that human Cdc7 protein is constant during the cell cycle and that its activity is regulated solely by the binding of the cyclin-like subunits Dbf4 and Drf1 (18, 22, 29). Preliminary stability experiments indicate that in cycling cells Cdc7 has an approximate half-life of 8 h (data not shown); however, it is possible that upon exit from mitosis an active mechanism that targets Cdc7 for degradation may exist.

In this work we have also identified Ser-139 as an in vivo phosphorylated site in Mcm2. Although Ser-139 is phosphorylated by CK2 in vitro, we do not yet have evidence that CK2 kinase is responsible for this phosphorylation in cells. Levels of Ser-139 are decreased by serum starvation, are not under cell cycle regulation, and are not increased by HU or Etoposide treatment, also suggesting that checkpoint signaling does not play a role in Ser-139 phosphorylation. Further work will be required to establish whether CK2 is the major kinase responsible for Ser-139 phosphorylation in vivo and the relevance of Ser-139 phosphorylation for Mcm2 function.

Finally, chromatin binding experiments performed by biochemical fractionation or by immunofluorescence after removal of soluble proteins indicate that while phosphorylated Mcm2 at Ser-41 (potential CDK site) and Ser-53 (Cdc7 site) are found equally in the soluble and chromatin-enriched fractions, Mcm2 phosphorylated in Ser-40/41 and

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\(^4\) P. Tenca and C. Santocanale, unpublished data.
in Ser-108 have a very similar pattern and are mainly found in the soluble fractions. HU treatment, which both delays replication fork progression and prevents late origin firing (13), allows detection of chromatin-bound pSer-108 and pSer-40/41 Mcm2; under these conditions we have recently found that Cdc7 is a fully active kinase.4 This observation raises the question of whether Cdc7 and ATR phosphorylation at Ser-40/41 and Ser-108 occurs on MCM complexes that are located at late origins or at replication forks. It definitely also occurs on soluble MCMs that are most likely not engaged in DNA replication. One tempting hypothesis is that Mcm2 phosphorylation at Ser-40/41 and Ser-108 could represent a dual mechanism to favor checkpoint signaling from stalled replication forks and at the same time to prevent new origin firing within S-phase, possibly by preventing reassembly of chromatin-bound complexes from soluble MCM complexes.

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