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Inhibition of Cdc7/Dbf4 Kinase Activity Affects Specific Phosphorylation Sites on MCM2 in Cancer Cells

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Abstract The Cdc7/Dbf4 kinase is required for initiation of DNA replication and also plays a role in checkpoint function in response to replication stress. Exactly how Cdc7/Dbf4 mediates those activities remains to be elucidated. Cdc7/Dbf4 physically interacts with and phosphorylates the minichromosome maintenance complex (MCM), such as MCM2, MCM4 and MCM6. Cdc7/Dbf4 activity is required for association of Cdc45 followed by recruitment of DNA polymerase on the chromatin. Using high resolution mass spectrometry, we identified six phosphorylation sites on MCM2, two of them have not been described before. We provide evidence that Cdc7/Dbf4 mediates phosphorylation on serine 108 and serine 40 on human MCM2 in vitro and in vivo in cancer cells in the absence of DNA damage. Antibodies specific to pS108 or pS40 confirmed the sites and established useful read-outs for inhibition of Cdc7/Dbf4. This report demonstrates the utility of an in vitro to in vivo workflow utilizing immunoprecipitation and mass spectrometry to map phosphorylation sites on endogenous kinase substrates. The approach can be readily generalized to identify target modulation read-outs for other potential kinase cancer targets. J. Cell. Biochem. 104: 1075–1086, 2008.

Key words: kinase; phosphorylation site; mass spectrometry; cancer; target modulation

The serine/threonine kinase Cdc7 plays an important role in the initiation of DNA replication and has been implicated in S phase checkpoint signaling. The Cdc7 kinase forms a complex with Dbf4, its regulatory subunit also known as activator of S phase kinase (ASK), to generate an active Ser/Thr kinase, hence the synonym DBF4-dependent kinase (DDK). Cdc7/Dbf4 kinase activity is required for initiation of DNA replication and subsequent transition into S-phase of the cell cycle. A second activator protein of Cdc7 called Drf1 or ASKL1 has been identified in human cells, and appears to be involved in both S and M phase progression [Montagnoli et al., 2002; Yoshizawa-Sugata et al., 2005]. Cdc7 knock-out mice are embryonic lethal between E3.5 and E6.5 [Kim et al., 2002]. However the analysis of conditional Cdc7 as well as conditional Dbf4 knock-out ES cell lines revealed the essential roles of both proteins in mammalian cell proliferation and DNA synthesis [Kim et al., 2002, 2003].

DNA replication starts by the assembly of a pre-replication complex (pre-RC) onto origins marked by a six-member origin recognition complex (ORC) during G1 phase of the cell cycle. Binding of Cdc6 and Cdt1 facilitates the loading of the minichromosome maintanance (MCM) complex onto the ORC. The MCM2-7 heterohexamer complex is considered to be a good candidate to function as the helicase that unwinds DNA ahead of the replication fork during S-phase [Lei and Tye, 2001; Shechter et al., 2004]. Recently it was reported that a high molecular weight complex consisting of Cdc45, the GINS tetramer, and the MCM2-7 hexamer, has in vitro helicase activity and might function as an "unwindosome" at the

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Abbreviations used: MCM, minichromosome maintenance; ORC, origin of replication; MS, mass spectrometry.

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replication fork [Moyer et al., 2006; Pacek et al., 2006]. Interestingly, MCM proteins themselves are currently under evaluation as cancer biomarkers [Gonzalez et al., 2005]. In a mouse model, deregulated expression of MCM7 has been shown to actively contribute to tumor development, supporting the hypothesis that MCM proteins are not only proliferation markers, but also play a role in tumorigenesis [Honeycutt et al., 2006]. MCM proteins are expressed during all phases of the cell cycle and their expression is down regulated after exit of the cell cycle into quiescence, differentiation and senescence.

MCM proteins are the major physiological substrates of Cdc7. Among the six subunits that form the MCM2-7 complex, MCM2 appears to be the preferred substrate of Cdc7 in vitro [Lei et al., 1997; Kumagai et al., 1999; Masai and Arai, 2000; Sclafani, 2000]. Two-dimensional tryptic radio-labeled phosphopeptide-mapping analysis of MCM2 phosphorylated by Cdc7/Dbf4 revealed seven phosphorylation sites in vitro [Jiang et al., 1999]. Further, MCM2 can also be phosphorylated by another S-phase kinase, Cdk2/CycE, during DNA replication and by the ATM and ATM- and Rad3-related (ATR) checkpoint kinases in response to genotoxic stress [Cortez et al., 2004; Yoo et al., 2004; Montagnoli et al., 2006]. Recently, it has been reported that the N-terminal segments of MCM4 and MCM6 are phosphorylated by Cdc7 during S phase [Masai et al., 2006; Sheu and Stillman, 2006]. The presence of at least three Cdc7 targets in the MCM complex further emphasizes the importance of Cdc7 in initiation of DNA replication and may point to functional redundancy between phosphorylation events of different MCM proteins. To date the mechanism that relates Cdc7-mediated phosphorylation of the MCM complex to origin activation remains elusive.

In cancer cells Cdc7-depletion using siRNA was reported to induce apoptosis whereas treatment of normal fibroblasts resulted in p53-dependent cell cycle arrest [Montagnoli et al., 2004]. To begin to understand the downstream effects of Cdc7 in cancer, we embarked on a detailed analysis of the specific sites on MCM2 phosphorylated by the Cdc7/Dbf4 complex using peptide separation and tandem mass spectrometry. We began with an in vitro analysis in order to have enough peptides to yield a first pass "map" of putative specific phosphorylation sites. We then verified that these same sites are phosphorylated in vivo using RNAi mediated knockdown of endogenous Dbf4 in A549 lung cancer cells. The robust in vitro to in vivo workflow and analysis methodology is sufficiently general so that other kinase substrates of interest may be similarly mapped and validated.

Although progress is ongoing, phosphorylation site mapping using mass spectrometry continues to present a challenge due to the relative low-abundance of phosphorylated kinase substrate in the cell at a given time point, and the resulting high abundance of non-modified peptides [Arnott et al., 2003; Loyet et al., 2005]. Therefore many studies have focused on the enrichment of phosphopeptides using metal-chelation chromatography such as immobilized metal affinity chromatography (IMAC). [Posewitz and Tempst, 1999]. However these methods suffer from poor capacity due to non-specific binding of acidic peptides. Typically, only the most abundant phosphopeptides are captured, even in "model" proteins such as casein or ovalbumin. Other methods involving affinity tagging after beta elimination of the phosphate suffer from poor specificity of the chemical modification [McLachlin and Chait, 2003]. These methods are also difficult to translate to low-abundance endogenous cellular proteins. It is highly unlikely that any of these methods alone would be sufficient to analyze all of the phosphopeptides present in a sample. Indeed, a recent comprehensive study by Bodenmiller et al. [2007] compared three phosphopeptide enrichment strategies; IMAC, phosphoramidate chemistry (PAC) and titanium dioxide. The enriched phosphopeptides were detected by LC/MS/MS. As expected, each method isolated complementary segments of the phosphoproteome and it is possible that many phosphopeptides are still missing using affinity-based enrichment approaches.

More recently, a method that enriches phosphopeptides on the basis of charge differential between phosphorylated and unphosphorylated peptides provided a robust alternative to affinity enrichment strategies [Beausoleil et al., 2004]. Using strong cation exchange chromatography at low pH, phosphopeptides could be effectively fractionated followed by further separation on reverse phase LCMS. Using this approach, 2002 phosphorylation sites from 967 proteins were found from HeLa cell lysates.

This large scale approach allowed the automated identification of five phosphorylation sites on MCM2 in HeLa cells. However, because of the high throughput nature of the method, it is impossible to independently validate each specific site and to correlate the phosphorylation as a function of specific cellular perturbations.

Our aim was to develop a detailed and complete characterization of the phosphorylation sites on a single protein using mass spectrometry, followed by Western blotting confirmation of the sites found. Perturbation of the cell by siRNA would provide further evidence for the validity of the site. Therefore we chose to use a relatively new approach that is low throughput, but provides high sensitivity and high sequence coverage for low-abundance endogenous cellular proteins. The method employs offline reverse phase HPLC fractionation followed by MALDI-qTOF tandem mass spectrometry on each of the HPLC fractions [Krokhin et al., 2004]. Affinity-based enrichment of phosphopeptides is not required, thereby increasing the likelihood that no peptides are missed from the analysis. This is because all fractions are analyzed and contain greatly simplified mixtures. Nearly 75% sequence coverage of in vivo (endogeneous) full-length immunopurified MCM2 was obtained using the offline HPLC/MALDI-qTOF approach. Using this methodology, we report the identification of phosphorylation sites on MCM2 in vitro and in vivo that are specifically mediated by the Cdc7/Dbf4 kinase complex.

In summary, we identified six phosphorylation sites on MCM2 in vitro mediated by Cdc7/Dbf4, of which two have not been described before. Our findings are compared and discussed with respect to recent reports using complementary approaches [Cho et al., 2006; Montagnoli et al., 2006; Tsuji et al., 2006]. We further confirmed that phosphorylation of Ser108- and Ser40-MCM2 by Cdc7 occurs in cancer cells by RNAi depletion of Cdc7 and its cofactor Dbf4. The identification of Ser108 as a phosphorylation site is of particular interest because the same site was previously found to be phosphorylated by ATR in response to DNA damage. However, our findings demonstrate that in the absence of exogenous DNA damage, S108 on MCM2 can also be phosphorylated by the Cdc7 kinase.

RESULTS

Cdc7/Dbf4 Phosphorylation of MCM2 In Vitro

Due to the physiological role of MCM2 in the biology of the Cdc7/Dbf4 complex, we determined that a thorough characterization of MCM2 phosphorylation is required. Therefore, in order to identify the sites of phosphorylation on MCM2 by Cdc7/Dbf4 in cells, we began by first investigating the possible in vitro sites of phosphorylation on a MCM2 fragment (1–285) by recombinant Cdc7/Dbf4 heterodimer. Cdc7/Dbf4 was expressed in *E. coli* and enriched by a three column process to exceed 95% purity (supplementary Fig. 5). Previously in vitro kinase reactions revealed that 90% of the phosphorylation by Cdc7/Dbf4 occurred in the N-terminal region of murine MCM2 [Ishimi et al., 2001]. Therefore, we chose to focus on the N-terminal fragment of human MCM2 in vitro (Table IA). Six sites of phosphorylation were identified by peptide mass fingerprint and subsequently validated by MS/MS, Table IB.

Three of the six sites (S40, S53, S108) independently identified here have been confirmed recently to be phosphorylated by Cdc7 in HeLa cells using complementary methods [Montagnoli et al., 2006]. While S139, which has been reported to be a CK2 phosphorylation site by Montagnoli et al., has been confirmed to be a Cdc7 phosphorylation site in vitro and in vivo by Tsuji et al. [2006]. The two remaining in vitro phosphorylation sites S31 and S220 are novel sites and have not been reported by others so far. Of particular interest was the S108 site, which was found to be the site of phosphorylation by ATR in the presence of DNA damage [Cortez et al., 2004]. At the same time a high throughput characterization of HeLa cell nuclear phosphoproteins identified T106 as a phosphorylation site on MCM2, not S108 [Beausoleil et al., 2004]. In that study, the automated Sequest search engine was employed and the site was mapped directly by the

TABLE IA. Sequence of Recombinant Human MCM2(1–285)

MAESSESFTMASSPAQRRRGNDPLTSSPGR**S**SRRTDALT**S**SPGRDLPPFEDE**S**EGLLGTEGPLEEEEDGEELIGD
GMERDYRAIPELDAYEAEGLALDDEDVEELTA**S**QREAAERAMRQRDREAGRGLGRMRRGLLYD**S**DEEDEERPAR
KRRQVERATEDGEEDEEMIESIENLEDLKGHSVREWVSMAGPRLEIHHRFKNFLRTHVD

TABLE IB. Cdc7/Dbf4 Phosphorylation Sites on MCM2 N-Terminal Fragment In Vitro

Peptide	Location	Tryptic fragment found by MS	Calculated MW (Da)
1	S31/S40	SSRRTDALTSSPGR	1649.69
2	S53	DLPPFEDESEGLLGTEGPLEEEEDGEELIGDGMER	3927.63
3	S108	AIPELDAYEAEGLALDDEDVEELTASQREAAER	3697.65
4	S139	GLLYDSDEEDEERPAR	1972.8
5	S220	ERISDMCKENR	1459.62

software. It should be noted that in our hands, the S108 site found both in vitro and in vivo, was apparent after manual inspection of the raw MS/MS data. The large peptide appeared as a missed cleavage with molecular weight of 3697.65 Da. The MALDI MS/MS spectrum of this large peptide was obtained from one of the offline HPLC fractions, Figure 1A. The y9-H_3PO_4 fragment at 999.49 unambiguously places the phosphorylation site at S108 rather than T106. Further evidence to support the identification is the detection of y8 – H_3PO_4 at 928.48 Da, Figure 1B. Automated sequencing using the Mascot search engine, employing phosphorylation as a possible modification resulted in an error in the placement of the phosphorylation site. In that case, the proposed Mascot-generated sequences did not match to the raw data or more typically, were matched to peaks which were below the acceptable signal to noise threshold of $S/N > 2$. Therefore in the absence of manual inspection, the wrong assignment could be made. In the following paragraphs we will provide further evidence for the validity of S108 and S40 as true in vitro and in vivo Cdc7 phosphorylation sites.

Validation of MCM2 S108 and S40 Phosphorylation by Cdc7/Dbf4 In Vitro

In order to independently validate the S108 and S40 phosphorylation sites found by mass spectrometry, a series of Western Blot analyses was performed. First, mutant forms of MCM2 were generated with S108A, T106A, S12A and S40A. The mutant proteins were phosphorylated in vitro by Cdc7/Dbf4, followed by Western blotting analysis (Fig. 2). Probing with pSer-108 MCM2 antibodies yields a positive band for the wild type MCM2 and a T106A mutant form of MCM2. However, no band is visible when S108 is mutated to alanine, verifying that S108 is indeed a site of phosphorylation by Cdc7/Dbf4 and not the nearby T106 and that the pS108-MCM2 antibody specifically recognizes phosphorylation on S108 (Fig. 2A). It is important to point out, that the lower, faster migrating form of MCM2 in Western blots corresponds to the hyperphosphorylated form of MCM2, ascribed to the abnormal mobility of MCM2 in SDS–polyacrylamide gels [Todorov et al., 1994, 1995].

It can be noted that the S108A mutated version of MCM2 still runs lower after incubation with Cdc7/Dbf4, as seen in the lower panel of Figure 2A, providing additional evidence that S108 is not the only Cdc7 phosphorylation site on MCM2 in vitro, per Table IB. A similar experiment was performed to verify S40 as a Cdc7 phosphorylation site in vitro and to determine specificity of the pS40-antibody reagent (Fig. 2B). The pS40 rabbit polyclonal antibody, which was positively affinity purified on the phosphopeptide antigen and negatively purified using the unphosphorylated peptide, recognized wildtype MCM2 and the mutant form S12A MCM2 strongly if incubated with Cdc7 kinase. However, it reacted only very faintly with the mutant form S40A MCM2. All recombinant MCM2 versions that were not exposed to Cdc7 were also not recognized. Hence we can conclude that Cdc7/Dbf4 phosphorylates MCM2 on S40 and S108 in vitro and that both antibody reagents, pS108- and pS40 antibodies, recognize their respective phosphorylation sites and do not react with unphosphorylated forms of MCM2. The results of the Western blotting analysis therefore validate those obtained by mass spectrometry.

Cdc7/Dbf4 Phosphorylation of MCM2 in Cancer Cells

With the in vitro roadmap in hand, we sought to determine whether the S108 site was also phosphorylated in vivo. Furthermore, our aim was to verify that this site was phosphorylated in vivo specifically by the Cdc7/Dbf4 complex.

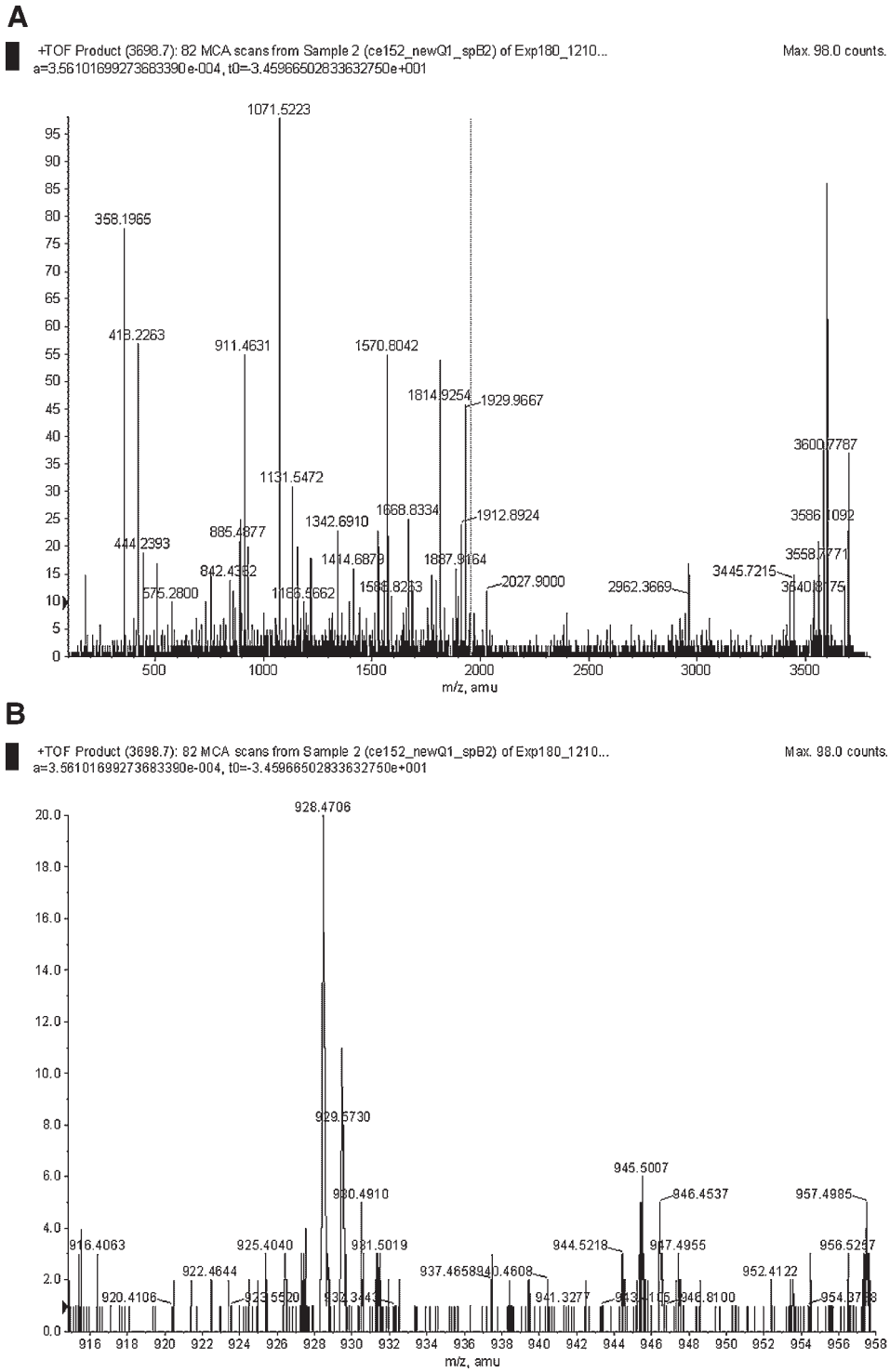


Fig. 1. Tandem-MS (MS/MS) fragmentation spectra of the peptide containing pS108, AIPELDAYEAEGLALDDEDVEELTA**S**QREAAER. The large peptide appeared as a missed cleavage with molecular weight of 3697.65 Da. **A**: The MALDI MS/MS spectrum of the peptide was obtained from one of the offline HPLC fractions. **B**: Expanded region containing the peak at 928.48 Da (y8 – H_3PO_4) used as further evidence to support the S108 site.

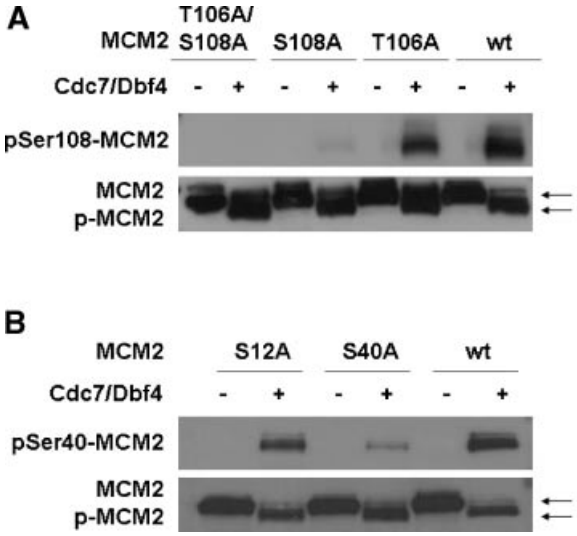


Fig. 2. Independent confirmation of the mass spectrometric finding that S108 and S40 is phosphorylated by Cdc7/Dbf4 in vitro. The blots utilize phosphospecific antibodies and mutated MCM2. Note: It is known that the lower, faster migrating form of MCM2 in Western blots corresponds to the hyper-phosphorylated form of MCM2, ascribed to the abnormal mobility of MCM2 in SDS–polyacrylamide gels [Todorov et al., 1994, 1995]. **A**: Mutant forms of MCM2 (S108A, T106A) were phosphorylated in vitro by Cdc7/Dbf4, followed by Western blotting analysis using pSer-108 MCM2 antibodies. **B**: Mutant forms of MCM2 (S12A, S40A) were phosphorylated in vitro by Cdc7/Dbf4, followed by Western blotting analysis using pSer-40 MCM2 antibodies.

Therefore, we used immunoprecipitation to pull down endogenous MCM2 from A549 cells and subjected the in-gel digested MCM2 protein to the same offline HPLC separation followed by spotting of the peptide fractions onto the chip for MALDI-MS. Using this approach, we could obtain nearly 75% sequence coverage of the endogenous full-length MCM2 based on the peptide mass fingerprint.

Examination of the peptide mass fingerprint for each fraction indicated the presence of the phosphorylated peptide 3 (Table IB), which was subsequently verified by MS/MS sequencing to be phosphorylated at S108 (Table II). However, this result alone was not sufficient to prove that the phosphorylation in cells was due to Cdc7/Dbf4 since the checkpoint kinases ATM and ATR may phosphorylate MCM2 on this site. Therefore, we used siRNA knockdown of Dbf4 to show that S108 is specifically phosphorylated by Cdc7/Dbf4 in cells. The Cdc7 kinase activity is regulated by Dbf4 during the cell cycle and Cdc7 is inactive in the absence of cofactor. We confirmed knock-down of Dbf4 mRNA by RT-PCR in cells that were harvested 48 h after siRNA transfection and found 71% inhibition of Dbf4 expression (Fig. 3A).

Although absolute quantification of the peptides by mass spectrometry is generally difficult in the absence of isotopic labeling, a semi-quantitative assessment could be made

TABLE II. Fragmentation of AIPELDAYEAEGLALDDEDVEELTASQREAAER (S108MCM2)

Fragment ions found	Found mass	Predicted mass	Comment
y10 + Pi:	1198.52	1198.54	Pi on either T or S
y10 – H_3PO_4	1100.53	1100.54	Pi on either T or S
y9 – H_3PO_4:	999.44	999.49	Pi on S
y8 + Pi:	1026.46	1026.44	Pi on S
y8 – H_3PO_4	928.48	928.46	Loss of Pi on S

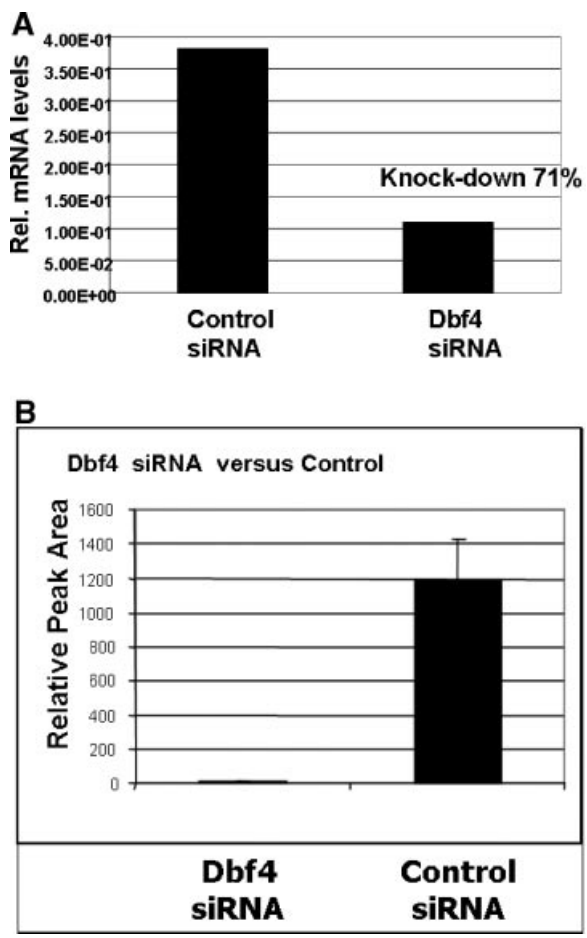
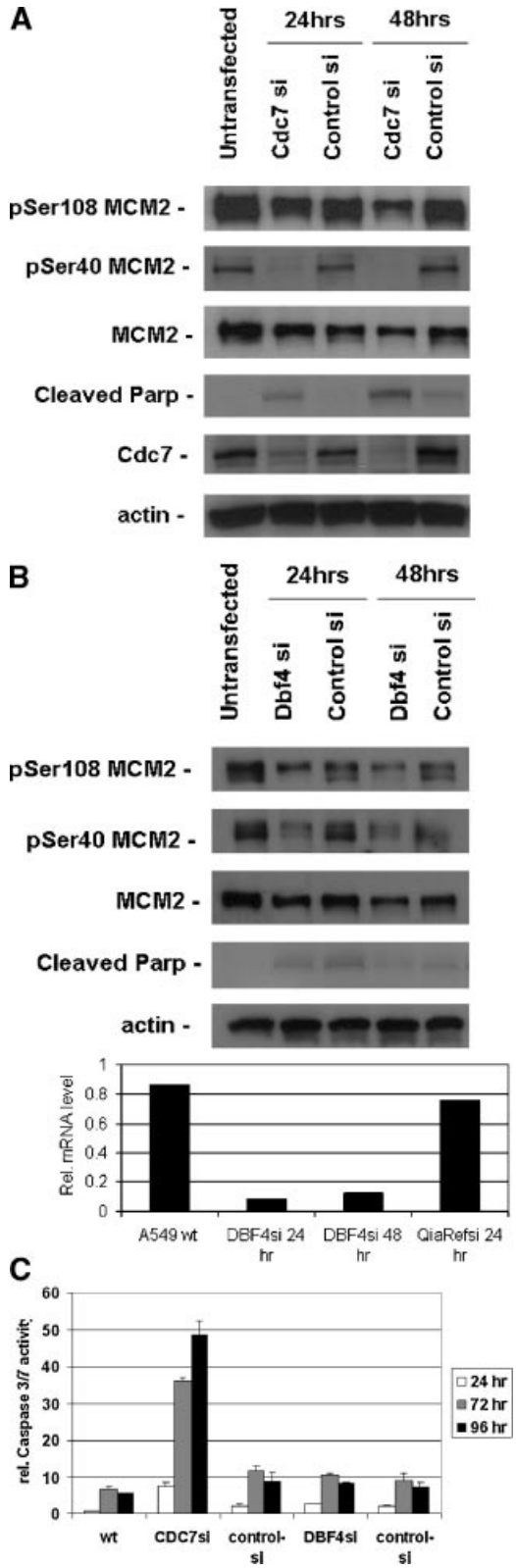


Fig. 3. Validation of MCM2 phosphorylation at S108 by Cdc7/Dbf4—siRNA knock-down of Dbf4. **A**: Confirmation of Dbf4 mRNA knock-down by RT-PCR. Expression is decreased by ~70%. **B**: Complete suppression (below the detectable limit) of the mass spectrometric signal from the peptide containing pS108 for the Dbf4 siRNA transfected cells compared to control siRNA. A small bar was added to the figure for illustrative purposes.

because of the complete absence of the pS108 peptide in the mass spectrum arising from cells where Dbf4 was knocked down by siRNA. Complete suppression of the phosphorylated peptide in the siRNA knockdown sample (below the detectable sensitivity limit) supports phosphorylation at this site by the Cdc7/Dbf4 complex in vivo (Fig. 3B). MS/MS fragmentation of the peptide found in the control siRNA sample again verified the in vivo site as pS108.



Depletion of Dbf4 or Cdc7 Reduces S108 and S40 Phosphorylation in A549 Cells

The "on/off" nature of the mass spectrometric identification of the pS108 peptide provides a reasonable semi-quantitative assessment of the siRNA knock-down of Dbf4 and its correlation to pS108 phosphorylation by the Cdc7/Dbf4 complex. However, to rule out any potential artifacts arising from the mass spectrometric analysis, we validated the result by independent methods. A549 cells were transfected with siRNA directed against Cdc7, Dbf4 or a negative control, and Western blotting was performed. Using the pS108 antibody previously validated, we detected at least two MCM2 bands at approximately 110 kDa in the control siRNA, or untransfected cells (Fig. 4A,B). However the cells treated with Cdc7 or Dbf4 siRNA showed a single band indicating that depletion of Cdc7 or Dbf4 results in a reduction of pS108 phosphorylation on MCM2 in A549 cells in the absence of exogenous DNA damage. Therefore, these data are in agreement with the mass spectrometric results. At the same time we observed reduced levels of phosphorylation of pS40 in cells transfected with Cdc7 or Dbf4 siRNA indicating that S40 as well as S108 are both MCM2 phosphorylation sites of the Cdc7/Dbf4 kinase complex. Interestingly, depletion of Cdc7 causes an increase of cleaved PARP in A549 cells 48 h after transfection indicating induction of apoptosis (Fig. 4A). On the other hand depletion of the cofactor Dbf4 does not induce PARP cleavage compared to control

Fig. 4. Confirmation of mass spectrometric results using Western blotting with phosphospecific antibodies and siRNA knockdown. Depletion of Dbf4 or Cdc7 reduces S108 and S40 phosphorylation in A549 cells. A549 cells were transfected with Cdc7si (**A**) or Dbf4si (**B**) and control siRNAs and harvested 24 and 48 h after transfection. Western blot analysis was performed. Knock-down of Dbf4 was confirmed by RT-PCR to be 91% and 86% at 24 and 48 h after transfection with Dbf4 siRNA oligonucleotides. **C**: Caspase 3/7 activity was measured in A549 cells treated with Cdc7si, Dbf4si or control siRNA oligonucleotides at 24, 72, and 96 h post-transfection. Values for Caspase 3/7 activity have been normalized by cell number. Shown is the average of triplicates with the corresponding standard deviation.

siRNA treated samples at the same time point (Fig. 4B). This finding is confirmed by an apoptosis assay measuring Caspase3/7 activity. Here depletion of Cdc7 but not of Dbf4 induces Caspase3/7 activity in A549 cancer cells significantly 72 and 96 h after transfection (Fig. 4C).

DISCUSSION

In this work we identified six phosphorylation sites of the Cdc7/Dbf4 kinase complex on MCM2 in vitro, S31, S40, S53, S108, S139, and S220. Two of them, S31 and S220, have not been reported by others before. Further we provide evidence that S40 and S108 are Cdc7/Dbf4 phosphorylation sites in vivo in cancer cells. Recently several reports have been published describing MCM2 phosphorylation sites. The authors explored different methods and used different cell lines. In Table III we summarize the published data regarding MCM2 phosphorylation (including this report) to facilitate the comparison of results and the discussion of potential reasons for discrepancies. Montagnoli et al. [2006] identified S40, S53 and S108 as Cdc7/Dbf4 phosphorylation sites in HeLa cells, which is in good agreement with our data. Contrary to our data they reported S139 to be phosphorylated by CK2. A recent study using nano-ESI [Tsuji et al., 2006] identified S27, S41 and S139 as major Cdc7 phosphorylation sites in vitro and in HeLa cells confirming S139 as a Cdc7 phosphorylation site. The authors mentioned that S53 and S108 phosphorylation sites had been detected in vivo but to a lesser extent, and were not confirmed by their in vitro experiment. On the other hand, we did not find the S27 and S41 sites described in that study. In yet another recent study, a completely different set of phosphorylated sites were found, where the only agreement with any previous work (including this present report) is S53 [Cho et al., 2006]. The authors identified three phosphorylation sites in the first 10 amino acids of MCM2, a fragment that was omitted by Montagnoli et al. but present in the studies performed by Tsuji et al. and us. However, that work was carried out completely with Mcm2 truncated protein and peptides in vitro and the discrepancies could be explained by the absence of secondary structures of the peptides.

In summary, in the three independent publications (including this report) on MCM2 phosphorylation by Cdc7/Dbf4 that investigated both in vitro and in vivo phosphorylation sites of human MCM2, the Cdc7 sites identified by at least two independent reports are S40, S53, S108, and S139 [Montagnoli et al., 2004, 2006; Tsuji et al., 2006]. Although there is agreement regarding some sites, each report had identified additional sites unique to their experimental design (see summary of MCM2 phosphorylation sites identified in Table III). Other possible reasons for the discrepancies between reports

TABLE III. Summary of Human MCM2 Phoshorylation Sites

MCM2 phosphorylation sites	Kinase	Method	References
S13, S27, S41	Cdk2/CycA Cdk1/CycB	MS—in vitro WB—in vivo (HeLa)	Montagnoli et al. [2006]
S139	CK2		
S40, S53, S108	Cdc7/Dbf4		
S5, S53 (major sites) S4, S7, T59 (minor sites)	Cdc7/Dbf4	Autoradiography—in vitro	Cho et al. [2006]
S27, S41, S139	Cdc7/Dbf4	MS—in vitro	Tsuji et al. [2006]
S27, S41, S139	Cdc7	MS and 2-D tryptic phosphopeptide map—in vivo (HeLa)	
S53, S108	N/D		
S31/S40, S53, S108, S139, S220	Cdc7/Dbf4	MS—in vitro	Charych et al. [this report]
S108, S40	Cdc7/Dbf4	MS and WB—in vivo (A549)	
S108	ATR	WB—in vivo (HCT116, HeLa)	Cortez et al. [2004]
S13, S26, S27, S40, S41, S53, T106, S108, Y137, S139	N/A	Literature and unpublished MS data	PhosphoSite www.phosphosite.org

could be differences in cell lines used, differences in detection sensitivity and percent of sequence coverage detected. Furthermore differences in the quality and purity of enzyme preparations used for in vitro kinase assays could contribute to different specific activities. Future studies are needed to determine the functional consequences of those phosphorylation sites in DNA replication and cell survival. Preliminary data presented here indicate that phosphorylation of MCM2 on S40 or S108 is not required for cell survival. We observed that depletion of the cofactor Dbf4 reduces MCM2 phosphorylation on those two sites without affecting cell viability of A549 lung cancer cells. On the other hand depletion of Cdc7, which also causes reduction of MCM2 phosphorylation on S40 and S108, results in induction of apoptosis. This was not observed after treatment of A549 cells with Dbf4 siRNA. One explanation could be that the knock-down achieved with Dbf4 siRNA (91% and 86% at 24 and 48 h) was not sufficient to induce apoptosis in A549 cells. On the other hand Cdc7 may have other roles in addition to those of the Cdc7/Dbf4 complex that contribute to cell viability. A second complex of Cdc7 with Drf1/ASK1L/DBF4B has been described in humans and *Xenopus* to play a role in cell cycle progression [Montagnoli et al., 2002; Yoshizawa-Sugata et al., 2005]. It will be important to identify the Cdc7/Drf1 specific substrates in human cells in order to discern the functional roles of the different Cdc7 complexes in cells.

Depletion of Cdc7 or Dbf4 results in the disappearance of the lower band of the MCM2 doublet recognized by the pS108 antibody in Western blot analysis. Data published by Cortez et al. [2004] showed that ATR phosphorylates MCM2 on this particular site in response to DNA damage. However they observed a reduction of S108 phosphorylation of ~50% in ATR-deficient cells in the absence of genotoxic agents indicating that another kinase is able to phosphorylate MCM2 on this specific site. The data presented in this report as well as data reported independently by others [Montagnoli et al., 2006] suggest that Cdc7/Dbf4 may be the "missing" kinase phosphorylating MCM2 on S108 in the absence of DNA damage checkpoint activation or ATR activity. It is intriguing to consider the reason why a DNA damage checkpoint kinase and a kinase involved in initiation of DNA replication would phosphorylate the same site. It is possible that the S108 phosphorylation by Cdc7/Dbf4 stabilizes the MCM complex on the chromatin. Such stabilization of the complex would allow recovery from replication arrest due to DNA damage checkpoint activation and enable the initiation of DNA replication. Further testing of this hypothesis and ongoing studies of how MCM2 phosphorylation can contribute to the functional effects observed by Cdc7 kinase inhibition in tumor cells will improve our understanding of this potential cancer target.

MATERIALS AND METHODS

Cell Lines

The human lung carcinoma cell line A549 and the colon carcinoma cell line HCT116 were purchased from American Type Culture Collection (Manassas, VA) and cells were cultured in RPMI supplemented with 10% FBS and 2 mM Glutamine.

Immunoblotting and Antibodies

For Western blot analysis cell extracts were prepared in NP-40 lysis buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris/Cl pH 7.5. Cells were harvested 24, 48, 72, or 96 h after transfection with siRNA oligonucleotides. We used rabbit cleaved PARP (Asp214) and rabbit β-actin at 1:1,000 dilutions from Cell Signaling Technology (Danvers, MA). To detect CDC7 we used the CDC7 kinase Ab-1 at 1:500 dilution from Neomarkers (Fremont, CA). Antibody phospho MCM2 (S108) from Bethyl Laboratories (Montgomery, TX) and the polyclonal rabbit anti-MCM2 from BD PharMingen were used at 0.2 μg/ml concentration. Rabbit polyclonal antibodies directed against phospho Ser40-MCM2 were generated in house and used at 0.1 μg/ml. Bleeds were affinity purified against the respective phosphopeptide after negative pre-absorbtion over a non-phospho peptide column and the antibodies were tested in ELISA and Western blot for specificity. As immunogen the following peptide was used: pS40-MCM2 (CTDALT(pS)SPGRD). Secondary antibody rabbit HRP was used at 1:10,000 dilution unless stated otherwise (Amersham Biosciences). The immunoblots were developed using ECL (Amersham Biosciences) and Kodak BioMax films.

RNA Interference

We used the following double-strand RNA oligonucleotides for our small interfering RNA (siRNA) experiments: Cdc7 siRNA 5′-agtaggacctgaagagaaa-3′ from Dharmacon Research (Boulder, CO); Dbf4 siRNA 5′-cagtatcaagttgttgatgat-3′ from Qiagen (Hilden, Germany). siRNAs were used according to manufacturer's recommendations and at final concentrations of 50 nM for tumor cells. As control siRNA oligonucleotides we used the recommended non-targeting control by Dharmacon and the AllStars negative control by Qiagen to match the chemistry of siRNAs used for the specific gene knock-down. The knock-down of Dbf4 is determined by real-time (RT) PCR reaction using Taqman primer sets directed against human ASK (Applied Biosystems) or by Western blot analysis for Cdc7. Expression of the HPRT gene is used for normalization of the RT-PCR results.

Apoptosis Assay

After transfection with siRNA oligonucleotides cells were subjected to Caspase-Glo 3/7 assay (Promega). For this homogenous apoptosis assay 2,000 cells in 100 μl per 96-well were plated in triplicates. One plate was harvested for each time point (1, 3 and 4 days after transfection). To each well 5 μl of Alamar blue reagent was added, the plate was incubated for 1 h at 37°C before reading the Fluorescence (OD 590 nm) on a Tecan plate reader. The Alamar-blue read-out is a measure of cell number and is used to normalize the Caspase 3/7 read-out, which is a measure of apoptosis. To each well an equal volume (105 μl) of Caspase-Glo 3/7 reagent (Promega) was added, the plate was incubated in the dark for 1 h and luminescence was read on the TriLux plate reader.

Expression and Purification of MCM2 and In Vitro Phosphorylation Reactions

His tagged human Cdc7/Dbf4 heterodimer was generated in *E. coli* and purified by a three column process consisting of IMAC Metal affinity chromatography followed by S200 size exclusion chromatography and MonoS ion exchange chromatography. The purity of the protein preparation exceeded 95%. Human MCM2 fragment 1–285 with N- terminal 6× His tag was cloned in pDest14 (Invitrogen Gateway vector) and expressed in *E. coli* BL21 (DE3) strain. Recombinant MCM2 was purified by Metal affinity chromatography. The purified MCM2 fragment was phosphorylated in vitro by purified Cdc7/Dbf4 complex for 1 h at room temperature in a buffer containing 50 mM HEPES pH 7.5/10 μM ATP/10 mM MgCl2. The reaction was stopped with the addition of 80 mM EDTA.

In Vitro Mutagenesis of MCM2

To generate the point mutations of the N-terminal fragment of human MCM2 the QuikChange Site-Directed Mutagenesis Kit from Stratagene was used according to manufacturer's instructions.

Primers for T106A-MCM2: 5′-GACGTAGAGGAGCTGGCCGCCAGTCAGAGGGAGGCAGC-3′ and its exact complement;
Primers for S108A-MCM2: 5′-GACGTAGAGGAGCTGACGGCCGCCCAGAGGGAGGCAGC-3′ and its exact complement;
Primers for T106A + S108A double mutant MCM2: 5′-GAGGACGTAGAGGAGCTGGCCGCCGCCCAGAGGGAGGCAGCAGAGC-3′ and its exact complement;
Primers for S12A-MCM2: 5′-CTTCACCATGGCAGCCAGCCCGGCCCAG-3′ and its exact complement;
Primers for S40A-MCM2: 5′-GATGCCCTCACCGCCAGCCCTGGCCG-3′ and its exact complement.

Immunoprecipitation of Endogenous MCM2 From A549 Cells

We incubated 1 mg of NP-40 cell lysate derived from A549 cells with either 5 μg MCM2 (BL248) antibody (Bethyl cat A300-191A) or 5 μg of Rabbit IgG (Jackson Immunoresearch cat 011-000-003) as control and 50 μl Protein G Sepharose 4 fast flow bead slurry (Amersham Biosciences) at 4°C over night. We washed the beads of each sample with 1 ml lysis buffer 6 times and resuspended the beads in 25 μl 5× sample buffer, boiled samples at 100°C for 5 min and separated the protein samples in a 8% Tris–glycine gel (Invitrogen).

Offline HPLC and Mass Spectrometry Analysis of Phosphopeptides

The in-vitro phosophorylation reaction mixture or immunoprecipitation elutions were separated by SDS-PAGE and stained overnight with Sypro

Ruby. The MCM2 band was identified and cut with a razor blade. The gel plug was destained 3× with 50% MeOH/20mM NH4HCO3 and dehydrated with 100% acetonitrile. In-gel tryptic digestion was carried out with 1,200 ng modified Trypsin (Sigma) at 37°C for 16 h. The tryptic peptides were separated offline by reversed phase HPLC using a Thermo Biobasic-C18 column, 100 mm × 1 mm. The separation conditions were as follows: Solvent A = Water + 0.1% TFA, Solvent B = acetonitrile + 0.1% TFA, flowing at 200 μl/min. Gradient was 5–60% B over 30 min. Twenty equal fractions were collected regardless of whether a UV peak was detected or not. The fractions were placed on a speedvac and solvent evaporated just to the point of near dryness. Peptides were re-dissolved in 10% ACN, 0.1% TFA and half the sample was spotted on a Ciphergen SEND ID protein chip (with hydrophobic surface and hydrophobic barrier). The chip was loaded onto a Ciphergen PCIII interface fitted onto an ABI Qstar XL. Resolution was tuned to greater than 10,000. MALDI-qTOF data was acquired manually by increasing the laser intensity from 30% to 70%. Typically 30–60 shots were acquired. MS/MS data were acquired similarly. For large molecular weight singly charged ions, the laser intensity was further increased up to 80% of maximum in order to obtain S/N > 500.

For siRNA knockdown experiments, MCM2 immunoprecipitations from A549 cells with and without Dbf4 knockdown were eluted and run on 1D SDS–PAGE. Using sypro ruby staining on a Typhoon fluorescence scanner, we could integrate the band intensity to quantify the MCM2 from the IP. No difference in protein amount was found between the siRNA cells and the control indicating that the same amount of total MCM2 was pulled down by the antibody from the cellular lysates regardless of siRNA knockdown of Dbf4. The MCM2 bands were digested and fractionated offline by reverse phase HPLC as before and the fractions containing the S108 peptide compared directly under the same conditions of laser power and number of shots. The total ion current in both samples was the same, indicating no bias due to sample processing.

Analysis of MS/MS Data to Validate Phosphorylation Sites

Putative phosphopeptides were identified by Mascot searching of the TOF data using phosphorylation, oxidation and pyro–glu as possible peptide modifiers. Potential phosphopeptides were validated by MS/MS fragmentation. For validated phosphopeptides, the site of phosphorylation was determined by manual inspection of the data compared to theoretical y and b ions containing the phosphorylation site. Protein Prospector was used to generate in silico fragmentation masses to match to the raw data. The site of phosphorylation was considered to be confirmed if a well-defined peak with S/N > 2 could be detected in the raw data.

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