Identification of Carboxyl-terminal MCM3 Phosphorylation Sites Using Polyreactive Phosphospecific Antibodies*

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The functionally related ATM (ataxia telangiectasia-mutated) and ATR (ATM-Rad3-related) protein kinases are critical regulators of DNA damage responses in mammalian cells. ATM and ATR share highly overlapping substrate specificities and show a strong preference for the phosphorylation of Ser or Thr residues followed by Gln. In this report we used a polyreactive phosphospecific antibody (α-pDSQ) that recognizes a subset of phosphorylated Asp-Ser-Gln sequences to purify candidate ATM/ATR substrates. This led to the identification of phosphorylation sites in the carboxyl terminus of the minichromosome maintenance protein 3 (MCM3), a component of the hexameric MCM DNA helicase. We show that the α-DSQ antibody recognizes tandem DSQ phosphorylation sites (Ser-725 and Ser-732) in the carboxyl terminus of murine MCM3 (mMCM3) and that ATM phosphorylates both sites in vitro. ATM phosphorylated the carboxyl termini of mMCM3 and human MCM3 in vivo and the phosphorylated form of MCM3 retained association with the canonical MCM complex. Although DNA damage did not affect steady-state levels of chromatin-bound MCM3, the ATM-phosphorylated form of MCM3 was preferentially localized to the soluble, nucleoplasmic fraction. This finding suggests that the carboxyl terminus of chromatin-loaded MCM3 may be sequestered from ATM-dependent checkpoint signals. Finally, we show that ATM and ATR jointly contribute to UV light-induced MCM3 phosphorylation, but that ATM is the predominant UV-activated MCM3 kinase in vivo. The carboxyl-terminal ATM phosphorylation sites are conserved in vertebrate MCM3 orthologs suggesting that this motif may serve important regulatory functions in response to DNA damage. Our findings also suggest that DSQ motifs are common phosphoacceptor motifs for ATM family kinases.

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The ataxia telangiectasia-mutated (ATM) protein kinase is a broad regulator of cellular responses to DNA damage in mammals. Mutations in ATM cause ataxia telangiectasia (A-T), a syndrome characterized by progressive cerebellar degeneration, immune defects, and cancer susceptibility (1). ATM-deficient cells are hypersensitive to ionizing radiation (IR) and radiomimetic agents and exhibit cell cycle checkpoint abnormals and subtle DNA repair defects. These combined defects in DNA damage signaling and repair are responsible for the 100-fold increased cancer risk associated with A-T, and most likely contribute to the neuropathologic abnormalities associated with this disease.

ATM belongs to the extended family of highly conserved phosphoinositide 3-kinase-related kinases (2). Within the mammalian phosphoinositide 3-kinase-related kinase family, ATM is structurally and functionally most closely related to ATR (ATM-Rad3-related) (2). ATM and ATR exhibit similar substrate specificities in vitro, and display a strong preference for the phosphorylation of substrates on Ser/Thr-Gln ((S/T)-Q) motifs. Other (S/T)-Q-directed phosphoinositide 3-kinase-related kinases include the DNA-dependent protein kinase, which mediates DNA repair by non-homologous end joining (3); and SMG1, which is involved in both nonsense-mediated mRNA decay and genotoxic stress responses (4–6).

Thus far, at least 20 substrates for ATM have been identified (7). Outside of the minimal requirement of a Gln residue in the +1 position, a clear consensus phosphorylation site for ATM has not been identified. However, Kim et al. (8) showed that hydrophobic or acidic residues in the –1 position are preferred and used protein data base searches to identify a number of candidate ATM substrates. Most of the known ATM substrates, including p53 (9, 10), BRCA1 (11, 12), and CHK2 (13–16) are clearly implicated in DNA repair and/or cell cycle checkpoint regulation. Many of these factors are also substrates for ATR, although clear examples of substrate discrimination between these kinases exist (17). The current compendium of known ATM substrates suggests that the regulatory functions of ATM extend well beyond canonical DNA damage checkpoint and DNA repair pathways. Among the list of these non-canonical ATM substrates are chaperone proteins (valosin containing protein), translational regulators (4E-BP1), apoptosis modulators (BID), and transcriptional regulators such as E2F1, CREB, and the NEMO subunit of the IKK kinase that mediates activation of the NF-κB trancription factor (18–24).

Recent studies have suggested that the minichromosome maintenance (MCM) proteins represent a potentially impor-
tantal class of ATM and ATR substrates. Six MCM proteins, designated MCM2–7 assemble into a heterohexameric, ring-shaped structure that is believed to function as the replicative DNA helicase during S phase (25, 26). The MCM2–7 complex is loaded onto chromatin during G1 phase in an ORC, CDC6, and CDT1-dependent manner and, once loaded onto chromatin, MCM2–7 recruits CDC45 and additional components of the replication machinery (27, 28). MCM-dependent DNA unwinding is subsequently triggered, in part, by a series of phosphorylation events catalyzed by CDC7-DBF4 (29). Evidence of checkpoint-related functions for MCM comes from the observation that MCM7 is required for ATR-dependent checkpoint activation in S-phase and interacts with both RAD17 and the ATR cofactor, ATRIP (30, 31). In addition, ATM/ATR phosphorylation sites have been mapped in mammalian and Xenopus MCM2, and an additional ATM/ATR site has been mapped to Ser-535 of human MCM3 (31, 32). However, the functional consequences of DNA damage-dependent MCM2 and MCM3 phosphorylation are not known.

The identification and functional characterization of novel ATM substrates is a key to further understanding the physiologic functions of this kinase in mammalian cells. In previous studies we generated a phosphospecific antibody against Ser-121 of CREB, which is phosphorylated by ATM in response to genotoxic stress (21). We subsequently showed that this antibody exhibits cross-reactivity with an ATM phosphorylation site at Ser-120 in the SV40 large T antigen (LTag) (33). The mapped SQ phosphorylation site in LTag was found to contain an Asp residue in the −1 position, which was also present in the CREB peptide immunogen (33). This suggested that the α-phospho-CREB antibody may recognize a subset of ATM substrates harboring phosphorylated Asp-Ser-Gln (DSQ) motifs. In this report we describe the results of immunopurification and mass spectrometry experiments to identify ATM substrates using α-pDSQ antibodies. This analysis has identified a cluster of Ser-Gln phosphorylation sites in the carboxyl terminus of MCM3. We show that ATM directly phosphorylates the carboxyl terminus of MCM3 in vitro and in response to genotoxic stimuli and explore the effects of MCM3 phosphorylation on MCM complex integrity and chromatin binding. Our findings define the carboxyl termini of vertebrate MCM3 proteins as regulatory targets of ATM in response to genotoxic stimuli.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Antibodies**—HEK 293T cells, HeLa cells, U-2 OS cells, and SV40 large T antigen transformed ATM(+/+) and ATM(−/−) mouse embryo fibroblasts (MEFs) were maintained in Eagle’s minimum essential medium containing 5% fetal calf serum. K562 cells were maintained in RPMI medium containing 10% fetal calf serum and 10 mM HEPES (pH 7.2). UV irradiation was carried out using a UVCL-1000 UV cross-linker with a peak emission of 254 nm. Antibody suppliers included: Calbiochem (MCM3), NeoMarkers/Lab Vision (MCM7), BD Bioscience (MCM6), GeneTex (α-ATM), Santa Cruz Biotechnology (α-Myc 9E10), Upstate Biotechnology (α-tubulin, α-γH2AX), Cell Signaling (α-CREB), and Novus Biologicals (α-pCREB-121, referred to as α-pDSQ in this text). The ATM inhibitor, KU 55933 (34), was prepared in Me2SO and added to a final concentration of 10 μM 1 h prior to cell irradiation.

**Mass Spectrometry and Protein Analysis**—Approximately 2 × 10⁶ K562 cells were exposed to 20 Gy of γ-radiation (IR) and lysed 1 h later in lysis buffer (25 mM HEPES, pH 7.20, 100 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 0.2% Nonidet P-40) supplemented with protease and phosphatase inhibitors (50 mM β-glycerophosphate, 10 mM NaF, 20 mM microcystin, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 0.2 μM phenylmethysulfonyl fluoride). Approximately 4 mg of cell extract was then incubated with 15 μg of rabbit IgG or 15 μg of α-pDSQ antibody for 3 h. The immune complexes were isolated using Protein A-Sepharose beads (GE Healthcare) and, after extensive washing, the bound proteins were eluted by boiling in 2× SDS-PAGE sample loading buffer and electrophoresed on a 10% SDS-PAGE gel. The separated proteins were visualized by staining with colloidal Coomassie Blue stain (Novex) and bands that appeared to be induced in the IR-treated sample were excised and submitted for mass spectrometry sequencing (ProtTech, Pittsburgh, PA). Immunoblots were performed as described (21). Polyvinylidene difluoride membranes (Millipore) were blocked in Tris-buffered saline containing 0.2% Tween 20 and 5% dried milk and incubated overnight at 4°C with the indicated primary antibodies diluted in blocking solution. After washing, the blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse or goat anti-rabbit secondary antibodies (Jackson) and developed using SuperSignal chemiluminescent substrate (Pierce). Chromatin fractionation of HeLa cells was carried out essentially as described (35). HeLa cell pellets derived from ~2 × 10⁶ cells were extracted with CSK buffer (10 mM PIPES, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl₂, 1 mM dithiothreitol, 1 mM EGTA, 0.1% Triton X-100) with protease and phosphatase inhibitors (see above), and centrifuged at 3000 × g for 10 min. The supernatant was collected as the soluble protein fraction. The remaining cell pellets (chromatin) were washed once with CSK buffer and then boiled in 2× sample loading buffer prior to analysis by SDS-PAGE. Whole cell extracts were prepared using a high salt lysis buffer (25 mM HEPES (pH 7.40), 300 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 0.5% Nonidet P-40) containing protease and phosphatase inhibitors.

**DNA Constructs and Kinase Assays**—A murine MCM3 (mMCM3) cDNA clone was obtained from Open Biosystems. The open reading frame of mMCM3 was PCR amplified using Hi-Fi DNA polymerase (Roche Applied Science) and subcloned into pCMV-Myc (Clontech). A human MCM3 (hMCM3) cDNA clone was obtained in similar fashion and its open reading frame subcloned into pCMV-Myc. HEK 293T cells and HeLa cell transfections were carried out using calcium phosphate-DNA precipitation and FuGENE 6 (Roche Applied Science), respectively. ATM siRNA transfections were carried out as previously described (36). ATM kinase assays were performed as described using G-361 cells as a source of ATM for immunoprecipitation (21). A glutathione S-transferase-MCM3 fusion protein spanning amino acids 683–812 of mMCM3 was produced by subcloning a PCR-generated fragment into the EcoRI-NotI sites of pGEX-5X-2 (GE Healthcare). The follow-
ing oligonucleotide primers were used: 5′-GAATTCAAGGAGACACTGAGCAGAAAGCGAAAAGGGAGG-3′; 5′-GGCGGCGGATTAAGGAAGACGATGCGCTCGTCACTCTCCATTGAC-3′. Proteins produced in the BL21(DE3) strain of *Escherichia coli* were affinity purified using glutathione-agarose beads (GE Healthcare) using standard procedures. All mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene).

**RESULTS**

**Purification of Candidate ATM Substrates Using Phospho-DSQ Antibodies**—We have raised a polyreactive phosphospecific antibody (α-pDSQ) that recognizes phosphorylated DSQ motifs present in CREB (Ser-121) and SV40 LTag (Ser-120) (21, 33). In addition, this antibody detected several species present in extracts of irradiated K562 erythroleukemia cells (Fig. 1A), suggesting the antibody may detect additional ATM substrates. Consistent with this idea, immunostaining of IR-treated human U-2 OS cells revealed intense nuclear foci that were highly coincident with γ-H2AX, a phosphorylation target of ATM and marker of DNA double strand breaks (Fig. 1B) (37–39). The pDSQ nuclear foci were also observed in CREB-deficient MEFs, indicating they represent a protein distinct from CREB (data not shown). These findings suggested that the α-pDSQ antibodies cross-reacted with one or more DNA damage inducible ATM substrates in cellulo.

In an attempt to identify these candidate ATM substrates, we performed large scale α-pDSQ immunoprecipitations using extracts prepared from untreated or IR-exposed K562 cells. Coomassie Blue staining of the resulting SDS-PAGE gels led to the identification of at least six bands (P1–P6), with apparent molecular masses ranging from ~90 to 140 kDa, whose abundance was enhanced following IR exposure (Fig. 1C). We initially excised three of these bands (P1, P5, and P6) and sequenced derived tryptic peptides by mass spectrometry. Mass spectrometric analysis of the P1 band yielded a mixture of peptides in which MCM2, a component of the MCM DNA helicase, predominated (Fig. 1C). Similarly, the P5 band was comprised predominantly of MCM5 peptides, whereas P6 yielded primarily MCM7 and lesser amounts of MCM3 and MCM6. The presence of the MCM complex was further confirmed when the P2, P3, and P4 species were sequenced. P2 contained mixtures of MCM2, MCM6, and MCM3; P3 contained a mixture of MCM6 and MCM3; and P4 contained a mixture of MCM4, MCM3, and MCM6. The presence of multiple MCM components in the α-pDSQ immunoprecipitates strongly suggested that this antibody reacted with one or more MCM subunits in DNA-damaged cells.

In addition to the MCM complex, the α-pDSQ antibody purified a number of other proteins with known or potential links to the ATM pathway. Peptides derived from MRE11, RAD50, and NBS1 were identified in multiple bands. A complex of MRE11, RAD50, and NBS1 (MRN) is required for double strand break repair and is involved in the recruitment and activation of ATM at double strand breaks (40–45). In addition, NBS1 is a direct substrate for ATM in DNA-damaged cells.
The α-pDSQ antibody also identified UPF1, a substrate of the ATM-related kinase hSMG1 that is required for nonsense-mediated mRNA decay (4, 50); p116U5, which is implicated in RNA splicing (52), α-actinin 1, and the chaperone, HSP90 (Fig. 1C). p116U5 and α-actinin 1 contain DSQ sites and are thus candidates for direct recognition targets of the α-pDSQ antibody. The other purified proteins, including all three components of the MRN complex, lack DSQ sites and may be present as coimmunoprecipitating factors.

Identification of MCM3 as a Phosphorylation Target of ATM—We reasoned that the presence of a DSQ motif may distinguish proteins that are directly immunoprecipitated by the α-pDSQ antibody from those that are indirectly coimmunoprecipitated as part of a larger complex. Among components of the hexameric MCM complex, MCM3 contains conserved DSQ sites in the carboxyl terminus (Fig. 3A). To test whether mMCM3 was recognized by the α-pDSQ antibody we immunoprecipitated mMCM3 from control or IR-treated MEF extracts and then immunoblotted the immunoprecipitated proteins with α-pDSQ. This analysis revealed that the α-pDSQ antibody reacted with mMCM3 in an IR-inducible manner in wild-type MEFs, but not ATM-deficient MEFs (Fig. 2A). The α-pDSQ antibody also reacted with hMCM3 that had been immunoprecipitated from irradiated HEK 293T cells (data not shown). In HeLa cells, the phosphorylation of hMCM3 was strongly induced by IR and, UV light and, to a lesser extent, by hydroxyurea (HU) (Fig. 2, B and C). These data suggested that the MCM3 subunit of MCM complexes is a relevant target for the α-pDSQ antibody in rodent and human cell lines exposed to diverse DNA-damaging agents.

**mMCM3 Is Phosphorylated by ATM on Ser-725 and Ser-732 in Vivo**—The carboxyl terminus of mMCM3 contains SQ motifs at Ser-725, Ser-732, and Ser-738. The SER-725 and Ser-732 motifs in mMCM3 represent iterations of the sequence KTDDSQ and only one of these motifs is present in hMCM3. However, the hMCM3 protein does contain an ESQ site that is positionally conserved with the Ser-732 residue of mMCM3 (Fig. 3A). To test whether the carboxyl-terminal sites of mMCM3 are phosphorylated by ATM in vivo, we transfected HEK 293T cells with Myc epitope-tagged mMCM3 expression constructs containing Ala mutations at Ser-725 and Ser-732. The transfected cells were then exposed to IR or mock irradiated and cell extracts immunoblotted with α-pDSQ. These experiments demonstrated that mutation of Ser-725 and Ser-732 abolished α-pDSQ immunoreactivity (Fig. 3B). The residual immunoreactivity present in HEK 293T cells transfected with the Myc-mMCM3 725/732A mutant was attributable to endogenous hMCM3. These results strongly suggested that phosphorylated Ser-725 and Ser-732 of mMCM3 are recognized by the α-pDSQ antibody in vivo.

The ability of ATM to phosphorylate mMCM3 in vitro was also explored. We performed ATM immune complex kinase assays using a GST-mMCM3 fusion protein substrate spanning amino acids 683–812 of mMCM3. ATM strongly phosphorylated wild-type GST-mMCM3 in vitro, whereas Ala substitutions at Ser-725 or Ser-732 reduced phosphorylation by ~50% (Fig. 3C). Combined Ala substitutions at both sites inhibited phosphorylation by 80–90% and incorporation of a third Ala mutation at Ser-738 reduced the in vitro phosphorylation of GST-mMCM3 to near background levels, suggesting that Ser-738 is also an ATM phosphorylation site in vitro (Fig. 3C). Finally, the phosphorylation of GST-mMCM3 in vitro required a functional ATM kinase domain (Fig. 3D). Thus, ATM phosphorylates the carboxyl terminus of mMCM3 on three sites in vitro, and two of these residues, Ser-725 and Ser-732, are also phosphorylated by ATM in intact cells following DNA damage.

We also mapped the relevant α-pDSQ-reactive phosphorylation site in hMCM3, which contains a single DSQ phosphorylation site at Ser-728 (Fig. 3A). Mutation of Ser-728 abrogated reactivity with the α-pDSQ antibody, whereas mutation of a second SQ site at Ser-734 had no effect (Fig. 3E). This same experiment revealed that overexpression of the Myc-hMCM3728A mutant in HEK 293T cells suppressed endogenous hMCM3 phosphorylation on Ser-728 by ~30% (Fig. 3E). This suggests that the mutant MCM3 dominantly inhibits endogenous MCM3 phosphorylation; however, the abundance of endogenous MCM3 is most likely responsible for the lack of a stronger dominant-negative effect. The combined findings define the carboxyl termini of rodent and human MCM3 proteins as ATM phosphorylation targets in mammalian cells.
Phosphorylation of Nucleoplasmic Versus Chromatin-bound MCM3—The MCM complex is loaded onto chromatin during G1 phase as part of the preinitiation complex that mediates the recruitment of additional DNA replication factors, including CDC45 (27, 53). We were interested in determining whether the ATM-phosphorylated form of MCM3 harboring Ala substitutions at the indicated amino acid residues. The phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. The GST-mMCM3KD mutant contains Ala substitutions at Ser-725, Ser-732, and Ser-738, or Myc-MCM3 harboring Ala substitutions at the indicated amino acid residues. The phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. This finding suggests that MCM3 phosphorylation does not alter its chromatin binding characteristics under these conditions. Although steady-state levels of chromatin-bound MCM3 did not change in response to DNA damage, we observed that the phosphorylated form of MCM3 was preferentially localized to the nucleoplasmic fraction (Fig. 4A). Under the same conditions the ATM-phosphorylated form of CREB was associated with chromatin in HeLa cells. To substantiate these results we performed additional experiments in which the volume of the chromatin extract was overloaded 5-fold relative to the soluble fraction. Under conditions where the total level of MCM3 was higher in the chromatin sample, the phospho-mimetic Asp substitutions at the same residues. The cells were then exposed to IR (20 Gy, 2 h) or sham treated. Equal volumes of nucleoplasmic and chromatin extracts were prepared and immunoblotted with the indicated antibodies.

**FIGURE 3. Mapping of ATM phosphorylation sites in MCM3.** A, alignment of DSQ sites present in CREB, SV40 LT tag, and the carboxyl termini of murine and human MCM3. B, Ser-725 and Ser-732 of mMCM3 are required for recognition by the α-DSQ antibody. HEK 293T cells were transfected with empty vector or expression plasmids encoding wild-type Myc-tagged mMCM3 (WT) or Myc-mMCM3 containing Ala substitutions at Ser-725 and Ser-732. Cells were then mock treated or exposed to IR (10 Gy) and cell extracts sequentially immunoblotted with α-pDSQ and α-Myc antibodies. C, ATM phosphorylates mMCM3 in vitro. G-361 cell extracts were immunoprecipitated with α-ATM or IgG and the resulting immune complexes incubated with wild-type GST-mMCM3 or GST-mMCM3 harboring Ala substitutions at the indicated amino acid residues. The phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. D, relative phosphorylation of GST-mMCM3 by wild-type and kinase-dead ATM. HEK 293T cells were transfected with empty vector or plasmids encoding HA epitope-tagged wild-type ATM (WT) or kinase-dead ATM (KD). HA immunoprecipitates from the cell extracts were incubated with GST-mMCM3 in the presence of γ-[32P]ATP. The phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. E, human MCM3 (hMCM3) is phosphorylated on Ser-725 in response to DNA damage. HEK 293T cells were transfected with plasmids encoding Myc-hMCM3WT or the indicated Myc-hMCM3 point mutants. Cell extracts prepared from control or IR-treated cells were immunoblotted with α-pDSQ.

**FIGURE 4. Chromatin fractionation of phosphorylated MCM3.** A, HeLa cells were exposed to 20 Gy IR or mock irradiated and nucleoplastic and chromatin fractions prepared at the indicated times (see "Experimental Procedures"). The nucleoplasmic and chromatin fractions were then resolved by SDS-PAGE and immunoblotted with α-pDSQ and α-MCM3 antibodies. As a control, the chromatin fractionation patterns of total CREB and ATM-phosphorylated CREB were also monitored. In B, nucleoplasmic and chromatin fractions were prepared before or 1 h after exposure to 20 Gy IR and immunoblotted with α-MCM3 and α-pDSQ antibodies. A 5-fold volumetric excess of the chromatin fraction was loaded to examine whether phosphorylated MCM3 could be observed. C, chromatin fractionation pattern of mMCM3 phosphorylation site mutants. HeLa cells were transfected with empty vector or expression plasmids encoding wild-type Myc-mMCM3 (WT), Myc-mMCM3KD containing Ala substitutions at Ser-725, Ser-732, and Ser-738, or Myc-mMCM3 harboring phospho-mimetic Asp substitutions at the same residues. The cells were then exposed to IR (20 Gy, 2 h) or sham treated. Equal volumes of nucleoplasmic and chromatin extracts were prepared and immunoblotted with the indicated antibodies.

4A). This finding suggests that MCM3 phosphorylation does not alter its chromatin binding characteristics under these conditions. Although steady-state levels of chromatin-bound MCM3 did not change in response to DNA damage, we observed that the phosphorylated form of MCM3 was preferentially localized to the nucleoplasmic fraction (Fig. 4A). Under the same conditions the ATM-phosphorylated form of CREB was associated with chromatin in HeLa cells. To substantiate these results we performed additional experiments in which the volume of the chromatin extract was overloaded 5-fold relative to the soluble fraction. Under conditions where the total level of MCM3 was higher in the chromatin sample, the phospho-MCM3 signal was still restricted to the nucleoplasmic fraction (Fig. 4B). These findings suggest that phosphorylated MCM3 may be excluded from chromatin in HeLa cells or that chromatin-bound MCM3 is not accessible to the kinase domain of ATM.
We next tested whether mutation of the carboxyl-terminal ATM phosphorylation sites affected the chromatin fractionation pattern of MCM3. For these experiments we transfected HeLa cells with wild-type Myc-mMCM3, Myc-mMCM3<sup>3A</sup> harboring Ala mutations at Ser-725, Ser-732, and Ser-738, or Myc-mMCM3<sup>3D</sup> harboring Asp substitutions at the same residues. We then prepared nucleoplasmic and chromatin fractions before and after IR exposure and immunoblotted the fractions with α-Myc antibody. This experiment showed that the chromatin fractionation patterns of wild-type and mutant Myc-mMCM3 proteins were indistinguishable in the absence or presence of DNA damage (Fig. 4C, top panels). However, as observed above, the phosphorylated form of MCM3 was still largely restricted to the soluble fraction (Fig. 4C, middle panels). Thus, despite the fact that phosphorylated MCM3 is largely excluded from chromatin, the phosphorylation of the carboxyl-terminal sites does not appear to affect mMCM3 chromatin loading under these conditions.

We also examined the effect of IR-induced phosphorylation on MCM subunit composition. IR exposure did not alter the level of MCM6 or MCM7 that coimmunoprecipitated with MCM3 from HeLa extracts, suggesting that the MCM complex is not disassembled in response to DNA damage (supplemental Fig. 1A). Finally, we also examined the interaction between MCM3 and MCM3AP, an acetyltransferase that interacts with the carboxyl terminus of MCM3 in intact cells (56). Although we observed a robust interaction between MCM3 and MCM3AP, this association was not affected by IR under our experimental conditions (supplemental Fig. 1B).

**Genetic Requirements for MCM3 Phosphorylation in Response to DNA Replication Stress**—Previous work from our laboratory demonstrated that ATM is the predominant UV-activated kinase that directly phosphorylates CREB on Ser-121 in vivo (17). Although UV-induced CREB phosphorylation is also partially ATR dependent, this likely reflects a role for ATR in the full activation of ATM in response to this stimulus (17, 54). For this reason we wished to explore the mechanism of MCM3 phosphorylation by ATR/ATM and specifically the requirements for each kinase in the DNA replication-induced phosphorylation of MCM3. To this end, we measured hMCM3 Ser-728 phosphorylation in HEK 293T cells transfected with an ATR-specific siRNA or treated with the ATM-specific inhibitor KU-55933 prior to UV irradiation. UV light-induced hMCM3 phosphorylation was inhibited by ~70 and 50% in the ATR siRNA- and KU-55933-treated cells, respectively, whereas the combination of ATR siRNA and KU-55933 ablated UV-induced hMCM3 phosphorylation on Ser-728 (Fig. 5A). The inhibition of MCM3 phosphorylation by ATR siRNA and KU-55933 closely paralleled inhibition of CREB phosphorylation and ATM autophosphorylation. In all three cases the combination of ATR siRNA and KU-55933 inhibited UV light-induced substrate phosphorylation by more than 90%.

To further assess the importance of ATM for UV-induced MCM3 phosphorylation we performed experiments using ATM<sup>−/−</sup> MEFs. These cells exhibited a greater than 80% reduction in UV-induced MCM3 phosphorylation relative to ATM<sup>+/+</sup> MEFs (Fig. 5B). The residual MCM3 phosphorylation in ATM<sup>−/−</sup> MEFs is consistent with the idea that ATR directly phosphorylates a subset of MCM3 proteins in response to UV damage. ATM-deficient MEFs were also used to explore the ATM dependence of MCM3 phosphorylation in response to HU. The results of these experiments were similar to the findings obtained using UV light as a stimulus; in the absence of ATM the HU-induced phosphorylation of MCM3 was reduced by 80–90%, whereas IR-induced MCM3 phosphorylation was ablated (Fig. 5C). In the same cells, HU-induced phosphorylation of CREB was reduced to near background levels in the absence of ATM. Thus, ATM plays a major role in HU- and UV-induced MCM3 phosphorylation and is absolutely required for HU- and UV-induced phosphorylation of CREB, which is consistent with our earlier observations (17). The differential ATM dependence exhibited by CREB and MCM3 may reflect different accessibilities of these substrates to spatially restricted ATR complexes.
MCM3 Phosphorylation by ATM

DISCUSSION

In this study we have used a polyreactive phosphospecific antibody that recognizes a subset of phosphorylated DSQ motifs to show that the carboxyl terminus of MCM3 is phosphorylated by ATM in response to DNA damage. MCM3 is a component of the MCM2–7 hexamer that is a strong candidate for the replicative DNA helicase in eukaryotes (27). In addition to essential roles during DNA replication, the MCM complex has gained increasing recognition for its potential roles in cell cycle checkpoint regulation. MCM7 was identified in separate studies as an interacting partner of the RAD17 and ATRIP checkpoint regulators and was found to be required for the activation of DNA damage checkpoints during S phase (30, 31). MCM2 and MCM3 have also been implicated in ATM- and ATR-dependent checkpoint responses. MCM2 is a direct substrate for ATM and ATR in mammalian and Xenopus extracts (31, 32). An ATM phosphorylation site was previously mapped to Ser-535 of MCM3, which is distinct from the carboxyl-terminal sites identified in our study (31). The carboxyl-terminal region of mMCM3 contains three Ser-Gln motifs that are phosphorylated by ATM in vitro (Ser-725, Ser-732, and Ser-738), whereas hMCM3 contains two such residues (Ser-728 and Ser-734). The carboxyl-terminal 132-amino acid fragment of Xenopus laevis MCM3 contains eight SQ motifs, strongly suggesting that DNA damage-dependent regulation of the MCM3 carboxyl terminus has been conserved during vertebrate evolution.

The biochemical consequences of MCM subunit phosphorylation are presently not understood. In the specific example of the hMCM3 carboxyl terminus, we have provided evidence that its phosphorylation by ATM does not alter interaction with MCM6, MCM7, or MCM3AP (supplemental Fig. S1, A and B). We also failed to detect IR-dependent changes in MCM3 chromatin binding, nuclear localization, or stability (data not shown). We have, however, consistently observed that the phosphorylated form of MCM3 is preferentially localized to the nucleoplasmic fraction of HeLa cells, as assessed by immunoblotting with α-pDSQ antibodies (Fig. 4, A–C). However, the steady-state levels of chromatin-bound MCM3 do not appreciably change in response to IR and the chromatin fractionation pattern of MCM3 is not affected by either Ala or Asp mutations in the carboxyl-terminal sites (Fig. 4C). One interpretation of these results is that the carboxyl terminus of MCM3 is inaccessible to ATM when it is tightly associated with chromatin. In principle, this might reflect structural changes in the MCM holoenzyme, interactions of the carboxyl terminus of MCM3 with non-MCM proteins, or intramolecular folding events that bury the ATM phosphorylation epitope. Interestingly, preferential phosphorylation of nucleoplasmic versus chromatin-bound protein was recently described for the Ser-108 residue of MCM2, which is also a consensus ATM/ATR phosphorylation site (35). Thus, chromatin exclusion of ATM/ATR-phosphorylated MCM subunits may represent a general phenomenon in DNA-damaged mammalian cells.

Although the biochemical consequences of MCM3 phosphorylation are not established, potential roles in regulating MCM-dependent DNA replication seem most plausible. The MCM complex uncouples from DNA polymerase under conditions of DNA damage, and the resulting unwound, single strand DNA may provide a signal for checkpoint initiation (57). Although speculative, sequestration of the carboxyl-terminal phosphorylation sites may allow chromatin-loaded MCM3 to escape checkpoint-mediated inhibition, which might contribute to MCM-polymerase uncoupling. On the other hand, phosphorylation of nucleoplasmic MCM3 may prevent its chromatin loading under conditions of DNA damage. Clearly, the biochemical consequences of MCM3 carboxyl terminus phosphorylation merit further study.

Our experiments suggest that ATR and ATM jointly contribute to MCM3 carboxyl terminus phosphorylation in response to DNA replication stress, but that ATM is the predominant MCM3 kinase in asynchronously growing cells. The phosphorylation of MCM3 on a distinct site, Ser-535, was also found to be largely ATR independent, whereas replication stress-induced phosphorylation of MCM2 on Ser-108 exhibited strong ATR dependence (31). Thus, ATM and ATR apparently make differential contributions to MCM subunit phosphorylation in cells experiencing DNA replication stress. The MCM3 phosphorylation results should be considered in the context of recent reports that ATR functions upstream of ATM in UV light-damaged cells (17, 54). Thus, the contribution of ATR to UV-induced MCM3 carboxyl terminus phosphorylation may be partially attributed to its potentiation of ATM. However, the finding that neither KU-55933 nor ATM deficiency completely abolished UV-induced MCM3 phosphorylation suggests that ATR directly phosphorylates a subset of MCM3 complexes in cellulo.

The α-pDSQ antibody used here has now identified ATM phosphorylation sites in CREB, SV40 LTag, and MCM3 (21, 33). In all three cases immunoreactivity was mapped to a DSQ phosphorylation motif, suggesting that the antibody has general reactivity against phosphorylated DSQ sites. In addition, the α-pDSQ antibody detects DNA damage-induced nuclear foci that are independent of MCM3 or CREB expression (data not shown). Thus the antibody is likely to react with additional ATM substrates in vivo. In addition to CREB, LTag, and MCM3, at least four other substrates are known to be phosphorylated by ATM on DSQ motifs in vivo. These include FANCD2, ARTEMIS, E2F1, and 53BP1 (23, 33, 55, 58, 59). FANCD2 colocalizes with MRN at DNA damage-induced foci and is a strong candidate for recognition by α-pDSQ (51). Other candidate α-DSQ cross-reactive proteins include the splicing factor p116L5 and α-actinin 1. The potential phosphorylation of these proteins by ATM and related kinases is being explored. α-pDSQ antibody immunoprecipitation experiments, in conjunction with data base searches may also be useful for identifying new targets of ATM regulation in mammalian cells.

REFERENCES

1. Chun, H. H., and Gatti, R. A. (2004) DNA Repair 3, 1187–1196
2. Abraham, R. T. (2001) Genes Dev. 15, 2177–2196
3. Smith, G. C., and Jackson, S. P. (1999) Genes Dev. 13, 916–934
4. Yamashita, A., Ohnishi, T., Kashima, I., Taya, Y., and Ohno, S. (2001) Genes Dev. 15, 2215–2228
5. Denning, G., Jamieson, L., Maquat, L. E., Thompson, E. A., and Fields, A. P.
