ATM Protein-dependent Phosphorylation of Rad50 Protein Regulates DNA Repair and Cell Cycle Control[^5]

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The Mre11/Rad50/NBN complex plays a central role in coordinating the cellular response to DNA double-strand breaks. The importance of Rad50 in that response is evident from the recent description of a patient with Rad50 deficiency characterized by chromosomal instability and defective ATM-dependent signaling. We report here that ATM (defective in ataxia-telangiectasia) phosphorylates Rad50 at a single site (Ser-635) that plays an important adaptor role in signaling for cell cycle control and DNA repair. Although a Rad50 phosphosite-specific mutant (S635G) supported normal activation of ATM in Rad50-deficient cells, it was defective in correcting DNA damage-induced signaling through the ATM-dependent substrate SMC1. This mutant also failed to correct radiosensitivity, DNA double-strand break repair, and an S-phase checkpoint defect in Rad50-deficient cells. This was not due to disruption of the Mre11/Rad50/NBN complex revealing for the first time that phosphorylation of Rad50 plays a key regulatory role as an adaptor for specific ATM-dependent downstream signaling through SMC1 for DNA repair and cell cycle checkpoint control in the maintenance of genome integrity.

The Mre11/Rad50/NBN (MRN)^[^2] complex is a conserved protein complex that plays a key role in the response to DNA damage (1, 2). Abrogation of members of the complex in mice causes embryonic lethality (3–5), and hypomorphic mutations in these genes give rise to ataxia-telangiectasia-related disorder (6), Nijmegen breakage syndrome (NBS/NBN) (7), and Rad50 deficiency (8). All three disorders are characterized by cellular radiosensitivity, cell cycle abnormalities, and a defective response to DNA damage (8–11). Although several families with ATLD and NBS have been identified, only a single patient with Rad50 deficiency has been described (8). This patient was initially diagnosed as probably having NBS because of overlap in features such as microcephaly, mental retardation, bird-like face, and short stature (12). However, more recently this patient was found to be compound heterozygous for mutations (3277C>T (R1093X) and 3939A>T (X1313Y) extX^[^6]66 in the RADS50 gene that gave rise to low levels of unstable Rad50 protein (8). The complex as a whole is required for recognizing damage in DNA, and specific functions have been attributed to individual members. A heterotetrameric Mre11-Rad50 complex forms a globular DNA binding head that bridges DNA free ends via extended Rad50 coiled coils and a hook region that facilitates this linkage (13, 14). On localization to DNA DSB, Mre11 employs its single-strand endonuclease activity for DNA end processing (5). However, other nucleases also participate in this process (15). NBN then tethers C-terminal Binding Protein Interacting Protein, which is required for efficient formation of replication protein A1-coated single-strand DNA adjacent to DNA DSB, and binds to Mre11-Rad50 to coordinate DNA DSB repair by homologous recombination (HR) (16). Despite a failure to observe a marked defect in repair of DNA DSB in ATLD-, NBS-, and Rad50-deficient cells, there is evidence for a role for MRN in non-homologous end-joining (NHEJ). Use of a chromatinized DSB repair assay in Xenopus laevis extracts, depleted of Mre11, revealed efficient and accurate NHEJ (17). On the other hand, Huang and Dyman (18) demonstrated that reconstitution of mammalian DNA DSB repair end-joining had a requirement for the MRN complex. This apparent contradiction has been resolved by a series of reports that provide evidence for a role for MRN in NHEJ. This complex plays a role in NHEJ during V(D)J recombination in developing immunocytes (19) and during isotype class switching (20). Silencing Mre11 reduced the efficiency of both the canonical and alternate pathways of NHEJ independent of ATM, mutated in the human genetic disorder ataxia-telangiectasia (A-T) (21, 22). More recently, Quennet et al. (23) have shown that C-terminal Bind-
ing Protein Interacting Protein and MRN promote NHEJ of DNA DSB in G1 phase.

Disorders in members of the MRN complex overlap in their clinical and cellular phenotype with A-T (24, 25). This syndrome is characterized by a defect in the ATM protein that responds to DNA DSB by signaling to the cell cycle checkpoints and the DNA repair machinery (26, 27). In its role as a sensor of DNA DSB, MRN recruits ATM to the sites of damage where it is fully activated in the presence of several other DNA damage response proteins (28–31). However, there is not an absolute requirement for the MRN complex for ATM activation as cells from patients with ATLD and NBS can activate ATM, albeit at a reduced efficiency (32). On the other hand, in vitro experiments point to an absolute requirement for MRN in ATM activation (33). In human cells, autophosphorylation on specific sites (Ser-367, -1893, -1981, and -2996) is an inherent part of the mechanism of activation (34–37). Mutations at these sites alter the functional activity of ATM. However, dependence on autophosphorylation for ATM activation is not observed in mutant mice (38). Furthermore, there is evidence that the mechanism of activation of ATM is also dependent upon the form of damage to the cell (39). Once activated, ATM phosphorylates a number of downstream substrates, including NBN (41–43). These studies show that ATM-dependent phosphorylation of NBN plays an adaptor role in downstream signaling as phosphosite mutants in this protein fail to correct the S-phase defect in NBS cells. On the other hand, there is evidence for and against the requirement for NBN phosphorylation for cell survival post-irradiation (40, 42–45).

There is also evidence that Mre11 is phosphorylated, observed as a partial mobility shift of Mre11 after exposure to high doses of radiation (46). More recently, Di Virgilio et al. (47) used Xenopus egg extracts to demonstrate that hyperphosphorylation of Mre11 inactivates the MRN complex by facilitating its dissociation from chromatin. This was achieved by analyzing the activity of different forms of Mre11 with all eight (S/T)Q ATM/ATR consensus sites mutated, but it was not possible to identify which specific sites were phosphorylated or which were responsible for MRN inactivation. On the other hand, Robison et al. (48) demonstrated that the majority of DNA damage-induced phosphorylated Mre11 was found in the chromatin-bound fraction, pointing to tighter binding after phosphorylation. Although ATM-dependent phosphorylation of the third member of the complex, Rad50, has been detected in large scale phosphoproteomic studies (49, 50), its functional significance has not been established. We report here that Rad50 phosphorylation on Ser-635 is required for ATM-dependent cell cycle control, DNA repair, and cell survival in response to DNA DSB.

### EXPERIMENTAL PROCEDURES

**Cell Lines and Tissue Culture**—Lymphoblastoid cell lines from normal (C2ABR), A-T patients (AT25ABR), and a Rad50-deficient patient (HA239) were grown in RPMI with 10% FCS and antibiotics. All cells were grown at 37 °C with 5% CO2.

**Plasmid Constructs and Transfections**—Full-length, wild-type FLAG-Rad50 was generated by ligation of an hRad50-FLAG PCR fragment into pcDNA3.1(+) vector (Invitrogen). Wild-type FLAG-Rad50 was mutated at Ser 635 using the QuikChange site-directed mutagenesis kit (Stratagene), and the resultant mutants were verified by sequencing. Transfection of Rad50-deficient fibroblasts with either empty vector or full-length wild-type or mutant Rad50 constructs was performed using Lipofectamine 2000 reagent (Invitrogen) as described by the manufacturer. Selection for resistant cells was initiated 48 h post-transfection with 200 μg/ml hygromycin B (Roche Applied Science).

**Cell Survival**—Clonogenic cell survival after exposure to radiation was carried out as described previously (8). Control and A-T non-transfected fibroblasts and Rad50-deficient fibroblasts transfected with Rad50 constructs were resuspended at 2 × 105 cells/ml in medium. Cells were irradiated with 1–5 Gy of gamma rays and plated out to determine survival by counting colonies. Survival was expressed as the percentage of irradiated/unirradiated cells. Error bars represent S.E. using data from three separate experiments.

**Charged Particle Irradiation**—Ion irradiation was done at the universal linear accelerator (GSI UNILAC). U2OS cells were transfected with either wild-type or mutant Rad50 constructs using the amaxa nucleofector 1 (Lonza, Switzerland) according to the manufacturer’s instructions. For the measurement of the Rad50 recruitment, U2OS were cultivated on bio-foil (18-mm diameter, 25-μm thickness), irradiated with 197Au (E_target: 4.55 MeV/n Linear energy transfer: 13,050 keV/μm) at the beamline microscope and analyzed as described (51). 35–40 nuclei were analyzed per plasmid. Low angle irradiation was performed on cells seeded on squared coverslips as described (52) using 197Au. All irradiations were done at a fluence of around 3 × 106 ions/cm2.

**Lysate Preparations, Immunoprecipitation, and Western Blotting**—Cell extracts were prepared by lysis in universal immunoprecipitation buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 25 mM sodium fluoride, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml aprotinin, 0.2% Triton X-100 and 0.3% Nonidet P-40). For immunoprecipitations, lysates were precleared with protein A beads and then incubated with the specific antibody for 4 h at 4 °C. Cell lysates and immunoprecipitates were fractionated on SDS-PAGE and transferred to nitrocellulose using Towbin’s buffer (25 mM Tris, 190 mM Glycine, 20% Methanol and 0.02% SDS) at 100 V for 1 h at 4 °C. Antibodies to SMC1, NBS1, KAP-1, and KAP-1 Ser(P)-15 (rabbit polyclonal) were purchased from Novus Biologicals; anti-FLAG and anti-β actin were from Sigma; anti-rabbit p53 and anti-mouse p53 Ser 15 were from Cell Signaling Technology; anti-mouse monoclonal antibodies to Rad50, SMC1 Ser-957, and NBS, Ser 343 were from Upstate Biotechnology; anti-rabbit Chk2 and Chk2 pT68 were from Abcam; anti-rabbit ATM Ser-1981 was from Rockland, and anti-ATM and Mre11 (mouse monoclonal antibodies) were from GeneTex. Anti-mouse and anti-rabbit secondary antibodies were
purchased from Millipore and Rockland, respectively. Immunoblottings and immunoprecipitations were carried out according to the manufacturers’ directions. Membranes were visualized using ECL (PerkinElmer Life Science). Rad50 anti-Ser-635-phosphospecific antibodies were generated by immunizing rabbits with the diphtheria toxoid-conjugated synthetic peptide DVCGpSQDFFES. Antibodies were purified from crude serum using the non-phosphorylated peptide immobilized on an Aminolink® Plus Immunoblotting kit in the first run-through followed by elution from an affinity column with the phosphorylated peptide. Purified antibody was separated into aliquots and stored at −80 °C.

**ATM Kinase Assay**—A series of 4 overlapping GST Rad50 constructs was generated that together spanned the full length of Rad50 (GST1 (aa 77–417), GST2 (aa 274–642), GST3 (aa 638–1021) and GST4 (aa 909–1312)). These fragments were generated by PCR and cloned in pGEX-5X-1. A short GST Rad50 containing Ser 635 (aa 588–666) was also prepared for substrate verification. The fusion proteins were expressed and purified according to standard procedures. Site-specific mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene). The resulting mutants were verified by sequencing. For kinase assays, cells were irradiated as indicated. ATM immunoprecipitations were prepared with ATM-5BA antibody (35) and protein G-Sepharose for 4 h at 4 °C. ATM protein kinase assays were prepared as described previously (35). Briefly, immune complexes were resuspended in 30 µl of kinase buffer (10 mM HEPES (pH 7.5), 50 mM β-glycerophosphate, 50 mM NaCl, 10 mM MgCl$_2$, 10 mM MnCl$_2$, 1 mM DTT and 5 µM ATP) containing 10 µCi of [γ-³²P]ATP and 1 µg of soluble GST-Rad50 fusion protein for 30 min at 30 °C. Kinase reactions were terminated, and samples were analyzed by SDS-PAGE followed by autoradiography. ATM kinase assays with purified components were performed as described previously (33).

**DNA Replication Labeling and DNA Fiber Spreads: S-phase Checkpoint**—Rad50-deficient cells transfected with Rad50 constructs were labeled consecutively with 15 µM chlorodeoxyuridine for 20 min, including a mock or 5 Gy treatment, and then further 20 min with 15 µM idodeoxyuridine. DNA fibers were prepared as described elsewhere (53). Briefly 2 µl of cells (1 × 10⁶ cells/ml) were mixed with 6 µl of lysis solution (0.5% SDS, 200 mM Tris-HCl (pH 7.4), and 50 mM EDTA), and after 10 min slides were tilted to 15° to spread the DNA fibers. Slides were air-dried both pre- and post-fixation in methanol/acetic acid for 5 min slides were tilted to 15° to spread the DNA fibers. Slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were captured using a Leica TCS confocal scanner equipped with an ArKr laser on the Leica DM IRBE inverted microscope (Lens: HCX PlanApo 63× oil/NAL.32).

**Chromatin Fractionation**—Cellular protein fractions were prepared as described previously (48). Briefly, cells were harvested and washed in ice-cold PBS. The nucleoplasmic fraction (NF) was obtained by incubation of cells for 10 min on ice in 0.5% Triton X-100 in cell lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Roche Diagnostics), 10 mM β-glycerophosphate and 1 mM sodium orthovanadate). The soluble fraction was collected by centrifugation at 13,000 × g for 10 min at 4 °C. The pellet, consisting of intact nuclei (verified microscopically), was washed with PBS and treated with

**G$_x$/M Checkpoint: Mitotic Phosphorylation of Histone H3-Ser(P)-10**—This assay was carried out as described previously (54). In short, control (NFF), A-T (AT5), and F239 Rad50-complemented cells were harvested by trypsinization at 1 and 2 h after exposure to 2 Gy irradiation and washed with PBS. Cells were fixed in suspension at a concentration of 10⁶ cells/ml in 2.0 ml of ice-cold 70% ethanol for 24 h at −20 °C. Cells were incubated in blocking solution (1% BSA in PBS) and with Alexa Fluor 488-conjugated phospho-H3 (S10) antibody (Cell Signaling Technology) at a 1:30 dilution in blocking solution for 3 h at room temperature. Cells were resuspended in 25 µg/ml propidium iodide and RNase A (0.1 mg/ml) in PBS for 30 min. Cellular fluorescence was measured using a BD Biosciences FACSCalibur flow cytometry and analyzed using BD Cell Quest Pro Version 5.1.1.

**Induced Chromosome Aberrations**—This assay was performed as described previously (8). Control and Rad50-deficient fibroblasts transfected with Rad50 constructs were irradiated with 1 Gy. For G$_x$/M phase, colcemid at a final concentration of 0.1 µg/ml was added immediately after irradiation, −1–2 h before harvesting. The cells were treated with 0.075 M KCl, fixed, and stained as described above. A total of 50 metaphases were analyzed for each sample.

**Immunofluorescence Microscopy**—Control and Rad50-deficient cells complemented with Rad50 constructs were seeded onto coverslips in 6-well plates for 24 h before treatment with 5 Gy and allowed to recover for 1 h. Cells were washed in PBS and then pre-extracted with Nonidet P-40 buffer (20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl$_2$, 0.5 mM DTT, 0.2% Nonidet P-40, 1 mM sodium orthovanadate and 1 mM sodium fluoride) for 30 min on ice. Cells were fixed in 4% formaldehyde on ice for 15 min and then blocked in PBS containing 5% bovine serum fraction V albumin (BSA) for 1 h at room temperature. Primary antibody incubations of either 53BP1 (Cell signaling, MA; 1:300), γH2AX (Upstate; 1:1000), Mre11 (Sigma; 1:1000), Rad50 (Sigma; 1:200), or Rad50 Ser(P)-635 (1:400) were performed in BSA blocking solution for 1 h at room temperature. After washing with PBS, cells were incubated with anti-rabbit Alexa Fluor 594 (Invitrogen) and anti-mouse Alexa Fluor 488 (Invitrogen) at a 1:1000 dilution for 1 h at room temperature. Nuclei were counterstained with DAPI for 10 min at room temperature. After three final washing steps, the coverslips were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were captured using a Leica TCS confocal scanner equipped with an ArKr laser on the Leica DM IRBE inverted microscope (Lens: HCX PlanApo 63× oil/NAL.32).
100 μg/ml RNase-free DNase1 (New England Biolabs) for 30 min at 37 °C. DNase-treated nuclei were collected by centrifugation at 16,000 × g for 10 min at 4 °C, and the supernatant was retained. The DNase pellet was washed with lysis buffer and incubated with buffer containing 500 mM NaCl for 10 min at 4 °C. This extract was clarified by centrifugation and pooled with the DNase-retained supernatant to constitute the chromatin-bound fraction. The NaCl concentration in this fraction was adjusted to 150 mM with the lysis buffer. The NF and chromatin-bound fraction were used for immunoprecipitation and Western blot analysis.

DNA End-joining—The NHEJ assay was carried out as described previously (55) in which the pEGFP-N1 vector was used as a reporter. pEGFP-N1 was digested with HindIII for 6 h to cleave between the promoter and the GFP coding region. The linearized plasmid was separated by agarose gel electrophoresis and then purified by a QiaHi gel extraction kit. The linearized plasmid was separated by agarose gel electrophoresis and then purified by a QiaHi gel extraction kit. The linearized plasmid was separated by agarose gel electrophoresis and then purified by a QiaHi gel extraction kit. The linearized plasmid was separated by agarose gel electrophoresis and then purified by a QiaHi gel extraction kit. The linearized plasmid was separated by agarose gel electrophoresis and then purified by a QiaHi gel extraction kit. The linearized plasmid was separated by agarose gel electrophoresis and then purified by a QiaHi gel extraction kit.

Flow Cytometry GFP Acquisition and Analysis—Data acquisition and analysis were performed on a MoFlo cell sorter. The excitation source was Innova 90C laser (Coherent Scientific Pty Ltd, Inc., in South Australia, Australia), tuned to give 488 nm at 200 milliwatts. GFP-emitted fluorescence was collected using 530/40 and 580/30 bandpass filters, and two-color fluorescence was collected to facilitate in the discrimination of autofluorescent non-GFP cells, from dim positive GFP cells. Software for data acquisition, analysis, and cell sorting was SummitTM version 4.3.02 (build 2451) (DakoCytomation).

Homologous Recombination Repair—Rad50-deficient cells containing an integrated form of the HR reporter pDR-GFP were generated as described previously (56). The cells were selected with 15 μg/ml puromycin (Sigma). To determine HR frequency, puromycin-resistant cells were transfected transiently with Rad50 and phCMV-I-Scel constructs (phCMV-I-Scel was a gift from Dr. Jac Nickoloff, Fort Collins, CO). These cells were assayed by flow cytometry to determine I-Scel-inducible HR as described previously (56).

GST Pulldown Assays—Rad50 GSTs were constructed and prepared as described under the “ATM kinase assay.” Control fibroblast cells were exposed to 5 Gy radiation and harvested 30 min later. Total cellular extracts were prepared as described under “Experimental Procedures.” Lysates were preclreated with glutathione S-transferase by constant mixing for 2 h at 4 °C. Pulldown assays were carried out as described previously (40). GST fusion protein complexes were resolved on SDS-PAGE and immunoblotted with Rad50 and SMC1 antibodies.

RESULTS

DNA Damage-induced Phosphorylation of Rad50 Is Not Required for ATM Activation—We initially confirmed the data from phosphoproteomic studies that ATM phosphorylates Rad50 in response to DNA damage using four GST fusion proteins that cover the full length of Rad50 in an in vitro ATM kinase assay (supplemental Fig. 1A). Immunoprecipitated ATM phosphorylated Rad50 only on fragment 2 (aa 274–642), and the activity increased in response to radiation. Comparison is made with a p53 peptide substrate. Fragment 2 contains 3 consensus sites for ATM phosphorylation, including S635Q, which was originally detected in proteomic studies (49, 50). Mutation of the Ser–635 site (S635G) abolished phosphorylation entirely, whereas activity was still evident with the wild-type fragment and the other two mutants (supplemental Fig. 1B), in agreement with previous studies that identified the Rad50 Ser–635 phosphorylated site. We subsequently looked for an interaction between ATM and Rad50 to determine whether these proteins exist in the same protein complex. The results in supplemental Fig. 1C, upper panel, demonstrate that Rad50 co-immunoprecipitates with ATM. A reverse immunoprecipitation with anti-Rad50 antibody confirmed interaction with ATM (supplemental Fig. 1C, lower panel). This association was constitutive and was not enhanced by exposure to radiation. AT25ABR cells, not expressing ATM protein, were used as a negative control (supplemental Fig. 1C). To ensure that this interaction was not mediated by the presence of DNA, immunoprecipitations were also carried out in the presence of ethidium bromide, which confirmed this interaction (supplemental Fig. 1D). Using a shorter GST fusion protein (Rad50 GST–(588–666)) containing only the S635Q site, no activity was observed in extracts from an A–T cell line (supplemental Fig. 1E). ATM kinase activity was also measured in extracts from a Rad50-deficient lymphoblastoid (HA239) cell line. The characteristics of this cell line, derived from a patient with microcephaly, growth retardation, chromosome instability, and radiosensitivity (12), have been reported (8). ATM kinase activity was much reduced in these cells (supplemental Fig. 1F). This is due to a reduced level of Rad50 protein as a consequence of inherited Rad50 germline mutations (R1093X and X1313Y extX*66) (8).

To investigate the phosphorylation of Rad50 at Ser–635 in vivo, a phosphospecific antibody was prepared against a phosphopeptide encompassing this site (DVGPpSDFES). The Rad50 Ser(P)–635 antibody reacted strongly with the phosphopeptide and did not cross-react with the unphosphorylated form of the peptide (results not shown). In response to DNA damage, Rad50 Ser(P)–635 phosphorylation was detected as early as 5 min post-irradiation, which increased up to 60 min in NFF control fibroblasts (Fig. 1A). A dose-dependent increase in phosphorylation was also observed (Fig. 1B). Phosphorylation was not detected in a fibroblast line from a patient with Rad50 deficiency or in extracts from an A–T cell line (AT4B1) (Fig. 1C). The specific requirement for ATM kinase activity in DNA damage-induced Rad50 phosphorylation was demonstrated by treating control cells with Ku–55933, a specific ATM inhibitor. These cells failed to show ATM Ser(P)–1981 autophosphorylation or Rad50 Ser–635 phosphorylation (Fig. 1D). Furthermore, a phospho-(S/T)Q-specific antibody that recognizes ATM consensus phosphorylation sites was previously used to detect Rad50 phosphorylation in response to doxorubicin treatment, and this was also shown to be defective in Atm–/– lymphoblasts (49).
To determine the functional significance of ATM-dependent phosphorylation of Rad50 in response to DNA damage, we generated full-length (wild-type) cDNA and a phospho-site mutant (S635G) Rad50 constructs. These constructs were transfected into the Rad50-deficient cell line to produce stable cell lines. A similar level of expression of wild-type and mutant Rad50 was observed in several isolated clones. Although the level of expression in these transfections was significantly higher than in vector-transfected cells, and expression was gradually lost over several months (results not shown). Co-immunoprecipitation of the wild-type and mutant complexes from FLAG-tagged

![Figure 1](image-url)

**Figure 1.** DNA damage-induced phosphorylation of Rad50 at a specific site and dependence on ATM. A phosphospecific site antibody was generated in rabbits against Rad50 peptide containing phosphorylated Ser-635. Rad50 immune complexes were probed with a variety of different antibodies. A, shown is the time course of Rad50-Ser-635 phosphorylation in control fibroblasts (NFF). These cells were exposed to 10 Gy of radiation and harvested at the time points indicated. Extracts were immunoprecipitated (IP) with Rad50 antibody or nonspecific antibody, resolved on 6% SDS-PAGE, and immunoblotted with both Rad50 Ser(P)-635 (pS635) phosphospecific and Rad50 antibodies. B, shown is the effect of increasing radiation dose on Rad50 Ser-635 phosphorylation. Control fibroblasts were exposed to the indicated doses of radiation and harvested after 1 h. Extracts were immunoblotted as in A. C, Rad50 Ser-635 phosphorylation is defective in A-T and Rad50-deficient cells. Control and Rad50-deficient and AT4B1 (A-T) fibroblasts were treated with 10 Gy, and extracts were harvested after 15 min. Rad50 immunoprecipitates were resolved on SDS-PAGE and immunoblotted with the indicated antibodies. D, inhibition of ATM kinase activity by the specific inhibitor KU-55933 abrogates DNA damage-induced Rad50 phosphorylation. Control fibroblasts were treated with 10 μM KU-55933 for 1 h, exposed to 5 Gy of radiation, and harvested after 15 min, and Rad50 was immunoprecipitated followed by immunoblotting with antibodies as described. E, expression of Rad50 phosphosite mutant (S635G) in Rad50-deficient cells (fibroblasts) does not destabilize the MRN complex. Rad50-deficient cells were transfected with either Rad50 wild-type (WT) or S635G mutant forms containing a FLAG epitope. Vector-only-transfected cells were used as a control. Stable cell lines were selected with hygromycin (200 μg/ml). Upper panel, cells were treated with 5 Gy of radiation and harvested after 15 min. This complex was immunoprecipitated with an anti-FLAG antibody followed by immunoblotting with anti-FLAG, Rad50, Mre11, and NBN antibodies. Lower panel, shown are steady state levels of Rad50, Mre11, and NBN in Rad50-deficient fibroblasts complemented with Rad50 constructs. F, upper panel, expression of the Rad50 phosphosite mutant in Rad50-deficient cells supports normal levels of ATM autophosphorylation after exposure to 5 Gy of radiation. Protein extracts were immunoblotted with Rad50, ATM, and ATM Ser(P)-1981 antibodies. Lower panel, ATM activation by Rad50 constructs using a low dose (1 Gy) of radiation. The same stably transfected cell lines were employed as for E.
Rad50-transfected cells demonstrated that the MRN complex is intact in these cells; thus, the Ser-635 mutation did not alter complex stability (Fig. 1E, upper panel). Input amounts are shown in Fig. 1E, lower panel. Because Rad50 is part of the DNA DSB sensing complex that recruits ATM to the break and subsequently participates in downstream signaling (25, 29), it was important to check initially for any effect of the Rad50 phosphorylation site mutant on ATM recruitment and autophosphorylation. The results in Fig. 1F, upper panel, show that the Rad50-deficient cell line is defective in ATM activation (Ser(P)-1981) after a radiation dose of 5 Gy, and introduction of wild-type Rad50 into these cells restored ATM activation to normal levels. This was also the case when the phospho-specific mutant S635G was transfected into Rad50-deficient cells, but these cells transfected with vector-only showed no ATM activation. We also demonstrated that ATM activation was not compromised in the mutant-transfected cells at a lower dose of radiation, 1 Gy (Fig. 1F, lower panel). Thus, phosphorylation of Rad50 Ser-635 does not appear to be necessary for ATM activation.

Lee and Paull (33) have shown that activation of ATM can be reconstituted in vitro in the presence of MRN and linear DNA. To investigate the effect on ATM activation further, we expressed a non-phosphorylatable mutant, S635A, as well as a phosphomimic mutant, S635D, of Rad50 in insect cells and purified MRN complexes containing these mutants (data not shown). Neither the Rad50 S635A nor the phosphomimic mutant S635D interfered with MRN complex formation (results not shown) and did not alter the efficiency of activation of ATM in vitro (Fig. 2A). The MRN complexes containing these Rad50 mutants also failed to exhibit any deficiencies in Mre11 exonuclease activity in vitro (data not shown), consistent with previous observations that ATM does not significantly alter the DNA binding or exonuclease activities of the MRN complex. In addition, mutation at the Ser-635 site (S635A) did not alter the ATPase activity of Rad50 (results not shown).

**Phosphorylated Rad50 Is Localized to Sites of DNA Damage**—Previous results have shown that when NBN is phosphorylated in response to DNA damage, it is retained at sites of DNA damage, where it functions to signal to the S-phase checkpoint (57, 58). However, the position is less clear for a second member of the complex, Mre11, which in one study becomes more tightly associated with chromatin after phosphorylation (48) and in a second hyperphosphorylation of Mre11 leads to dissociation of the MRN complex (47). Accordingly, we determined here the effect of DNA damage-induced phosphorylation of Rad50 on its association with chromatin. The results in Fig. 2B reveal that in response to radiation damage, approximately equal amounts of Rad50 Ser(P)-635 appear in the nucleoplasm and chromatin-bound fractions, indicating some dissociation of Rad50 from the sites of DNA damage post-irradiation. To visualize the dynamics of Rad50 wild type and the phosphomutant binding to chromatin, U2OS cells transfected with Rad50-GFP constructs were exposed to low energy-charged particle radiation. Images for the pre-irradiation and post-irradiation foci are shown (Fig. 2C, right-hand panel). As is evident from the results in Fig. 2C, left-hand panel, the recruitment times for both forms of Rad50 are indistinguishable with a lifetime of ~150 s, comparable with that reported for NBN and MDC1 (59, 60). A Rad50 phosphomimic mutant S635E also showed the same recruitment kinetics. These data are also supported by other live cell imaging experiments where in both cases (Rad50 wild type and S635G) the GFP-tagged Rad50 was visible on chromatin within 5 min (Fig. 2, D and E). To confirm that Rad50 wild type and S635G forms were localized to the sites of DNA DSB, we employed co-localization of Rad50-GFP with immunostaining for γH2AX as is evident in Fig. 2F at 1 h post-irradiation. Previous data have shown that the mediator protein 53BP1 promotes ATM kinase activity by facilitating productive interaction between ATM and the MRN complex (61). Furthermore, the interaction with MRN is through Rad50. Because Rad50 S635G mutant protein can be recruited to chromatin without affecting the activation of ATM, it was predicted that this mutant protein would not affect the recruitment of 53BP1 to sites of damage. As expected, in irradiated control cells 53BP1 foci were evident, and these co-localized with γH2AX at sites of damage (supplemental Fig. 2A). Introduction of both the wild type and the Rad50 S635G mutant into Rad50-deficient cells revealed the appearance of 53BP1 foci (supplemental Fig. 2, B and C). There appeared to be less 53BP1 foci in Rad50-deficient cells or when vector was introduced (supplemental Fig. 2D). We also demonstrated the appearance of MDC1 foci in Rad50 wild-type and mutant-transfected cells after exposure to radiation (results not shown). In addition, Rad50 was recruited to these foci in cells transfected with wild type and mutant forms but not in vector-only cells (Fig. 3, A–D). As expected, when we employed the Rad50 phospho-specific antibody, Rad50 wild-type-corrected cells showed foci formation in response to radiation, but the Rad50 S635G-transfected cells failed to do so (Fig. 3, E–H). Mre11 was also recruited to sites of DNA damage in Rad50 wild-type and mutant-corrected cells (supplemental Fig. 3, A–D).

**DNA Damage-induced Signaling through ATM to SMC1 Is Dependent on Rad50 Phosphorylation**—Once ATM is activated by autophosphorylation, it initiates a cascade of phosphorylation events that control DNA cell cycle checkpoint activation, DNA repair, and cell survival (24, 25, 62). Furthermore, there is a dependence on NBN for signaling to BRCA1, FANCDD, SMC1, and Chk2 (44, 63, 64). Because Rad50 is one of the ATM substrates and a member of the MRN complex, we determined whether the Rad50 phosphorylation site mutant was capable of correcting defective signaling in Rad50-deficient cells. Rad50-deficient cells transfected with vector only are defective in phosphorylation of p53, Kap1, SMC1, and Chk2 after 5 Gy of radiation (Fig. 4A). Introduction of wild-type Rad50 cDNA into these cells corrected defective radiation-induced phosphorylation of all four substrates (Fig. 4A). On the other hand, in Rad50 S635G cDNA-transfected cells, there was a complete failure to correct radiation-induced phosphorylation of SMC1 (Fig. 4A). Under these conditions the mutant largely restored radiation-induced phosphorylation of the other substrates. To discriminate further between the effects on downstream substrate...
phosphorylation, a lower radiation dose (1 Gy) was employed. The results in Fig. 4B again reveal a major defect in correction of SMC1 phosphorylation in Rad50 S635G-transfected cells. However, at the lower dose, radiation-induced phosphorylation of Kap1, p53, and Chk2 occurs at normal levels. The mutant allele (X1313Y extX*66) in Rad50-deficient cells is predicted to code for a protein with an extra 66 amino acids at the C terminus of Rad50 (8). To rule out that the defect in signaling through SMC1 was due to up-regulation of the germ line mutant form, immunoblotting was carried out on extracts from transfected cells. The results in Fig. 4C show that in the vector-transfected cells there is no evidence that Rad50 is up-regulated, ruling out any interfering effects from this form of the protein in the transfectants.

**FIGURE 2.** ATM activation and localization of phosphorylated Rad50 to sites of DNA damage. A, MRN complex members were expressed in baculovirus-transduced insect cells and purified as described previously (33). Dimeric ATM was purified from 293T cells transfected with FLAG and HA-tagged ATM. Two Rad50 phosphosite mutants were employed, MR(S635A)N and MR(S635D)N. ATM kinase activity was determined in the presence of ATM, wild-type or mutant MRN, linear DNA, GST-p53 substrate, and [γ-32P]ATP. B, shown is the association of Rad50 Ser(P)-635 with chromatin. Control fibroblasts were exposed to 5-Gy radiation (x-rays) and incubated for 15 min, and extracts were immunoprecipitated (IP) with Rad50 antibody. Immunoblotting was carried out with nucleoplasmic (NF) and chromatin-bound fractions for Rad50, Rad50 Ser(P)-635 (pS635), NBN, and Mre11. C, time-lapse imaging of Rad50-GFP wild-type and mutant forms in U2OS cells in response to ion irradiation. Left-hand panel, kinetics of Rad50 wild type, Rad50 S635E, and Rad50 S635G accumulation at sites of DNA damage induced by 197Au ions. The error bars represent a 95% confidence interval of the mean. 35–40 nuclei were analyzed/plasmid. Right-hand panel, shown are pre- and post-irradiation images of Rad50-GFP in transfected cells. Localization of Rad50 wild-type and Rad50 S635G before (−IR) and 5 min after irradiation with 197Au (+IR) is shown. D, shown is Rad50 wild type-transfected cells. E, Rad50 S635G-transfected cells are shown. F, localization of Rad50 wild-type and Rad50 S635G to sites of DNA damage is shown. U20S cells were transfected with Rad50-GFP constructs and subjected to charged particle irradiation, fixed 1 h post-irradiation, and stained with antibodies against γH2AX.
These data suggest that, as observed previously for NBN, phosphorylation of Rad50 also plays an adaptor role for signaling to downstream substrates. Because this appeared to be confined to SMC1, we investigated this relationship further. SMC1 co-immunoprecipitated with Rad50 in the presence of ethidium bromide (Fig. 4D), and this interaction was not enhanced by radiation (results not shown). The interaction between Rad50 and SMC1 was investigated in more detail employing a series of GST-Rad50 fusion proteins covering the whole molecule. The results in Fig. 4E reveal that Rad50 GST2 (274–642 aa) pulls down SMC1, and the radiation-induced phosphorylated form of SMC1 (Ser(P)-957) also associates with this region of Rad50. This is of interest because the ATM-dependent phosphorylation site on Rad50 (Ser(P)-635) is also

FIGURE 3. Recruitment of Rad50 to sites of DNA damage. Control and Rad50-deficient fibroblasts complemented with Rad50 were treated with 5 Gy of IR and allowed to recover for 1 h or were not irradiated. After permeabilization, cells were fixed in paraformaldehyde and immunostained with anti-mouse γH2AX (green) and anti-rabbit Rad50 antibody (red). IR induced nuclear Rad50 foci in control and in Rad50-deficient cells complemented with either Rad50WT or Rad50S635G. γH2AX and Rad50 foci co-localized to discrete nuclear foci. A, NFF control. B, Rad50WT. C, Rad50 S635G. D, Rad50-deficient transfected with vector only. IR-induced nuclear Rad50Ser-635 foci only in control and in Rad50-deficient cells complemented with Rad50WT. E, NFF control. F, Rad50WT. G, Rad50 S635G. H, Rad50-deficient transfected with vector only.
contained in this fragment. As observed previously for other ATM substrates, an ATM-GST fragment corresponding to the ATM kinase domain pulled down both Rad50 and SMC1 as well as the phosphorylated form of SMC1 (Fig. 4F). We then determined whether SMC1 could interact with the phosphosite mutant form of Rad50. The results in Fig. 4G demonstrate that SMC1 co-immunoprecipitated with wild-type and mutant Rad50 to approximately the same extent. Under these conditions, the phosphorylated form of SMC1 (Ser(P)-957) was also found to associate with wild-type Rad50 after radiation exposure (Fig. 4G). Previous data have shown that whereas SMC1 can interact with chromatin even in the undamaged state, only the phosphorylated form (SMC1 Ser(P)-957) avidly interacts with the DNA DSB-generated nuclear subcompartment after DNA damage (65). The results in Fig. 4H show that in Rad50 wild-type-transfected cells, SMC1 Ser(P)-957 is recruited to chromatin post-irradiation. These data point to the importance of Rad50 phosphorylation at the site of DNA damage for ATM-dependent phosphorylation of specific substrates and downstream signaling in this case through SMC1.

Rad50 Phosphorylation Mediates DNA Repair for Cell Survival in Response to DNA Damage—A well established characteristic of A-T cells is hypersensitivity to radiation (24). Increased sensitivity is also observed in NBS cells (7) and to an
intermediate extent in both ATLD and Rad50-deficient cells (6, 8). We determined whether Rad50 phosphorylation was important in cell survival after irradiation. As observed previously, Rad50-deficient cells were intermediate between control and A-T in their sensitivity to radiation (Fig. 5A, left panel). Wild-type Rad50 cDNA partially corrected this sensitivity, but the phospho-site S635G mutant was incapable of this. This was not due to reduced expression of the mutant protein (Fig. 5A, right panel). We also assayed for the capacity of the mutant Rad50 to affect radiation-induced chromosome aberrations. The Rad50-deficient cells were also characterized by intermediate (between A-T and control) levels of chromosome aberrations post-irradiation, and these were reduced to control levels by wild-type Rad50 but not by the S635G phospho-site mutant (Fig. 5B). The increased sensitivity to radiation, determined by cell survival and induced chromosome aberrations, suggested that the Rad50-deficient line would have a reduced capacity to repair radiation-induced DNA DSB. The MRN complex plays an important role in end-processing of DNA DSB in HR (15, 16). A series of recent reports has also shown that the MRN complex facilitates repair of DNA DSB by both the classical and alternative NHEJ pathways (20–22). Accordingly, we investigated whether there might be a defect in either of these DNA DSB repair mechanisms. For NHEJ, we employed a GFP-based assay that compares activity in circular and linearized EGFP-N1-transfected forms (55). Rad50 stably transfected cells were transiently transfected with EGFP-N1 before assaying GFP-positive cells by flow cytometry (supplemental Fig. 4, A–D). As observed for ATLD cells, Rad50-deficient cells (vector only) had an ~3-fold reduced level of NHEJ compared with control (Fig. 5C, left panel). Introduction of Rad50 wild-type cDNA into the Rad50-deficient cells restored NHEJ activity to a level comparable with that in control. On the other hand, the Rad50 S635G mutant caused only a small increase in NHEJ. In this experiment expression of mutant and wild-type Rad50 was comparable (Fig. 5C, right panel), and approximately the same number (60%) of cells was transfected in both cases (supplemental Fig. 4E).

For homology-directed repair we employed a GFP-recombination reporter together with the I-Sce1 endonuclease for the introduction of a DNA DSB (56). The pDR-GFP substrate was stably transfected into the Rad50-deficient cells before co-transfection of Rad50 wild type or Rad50 S635G together with an I-Sce1 construct, and the percentages of GFP-positive cells were determined by flow cytometry (supplemental Fig. 5A). Again in this case we monitored for efficiency of transfection (supplemental Fig. 5B). A low level of expression was observed in Rad50-deficient cells transfected with Rad50 wild-type without I-Sce1, comparable with that in untransfected cells (Fig. 5D). However, after introduction of a DNA DSB with I-Sce1, an ~5-fold increase in expression occurred after Rad50 wild-type transfection. Introduction of the Rad50 S635G mutant failed to increase GFP expression appreciably above basal levels (Fig. 5D), supporting a role for Rad50 phosphorylation in HR.

**Functional Role for Rad50 Phosphorylation**

Failure to inhibit DNA synthesis in response to radiation treatment to the same extent as controls is a hallmark of A-T, NBS, and ATLD cells (6, 7, 66). This is referred to as radioresistant DNA synthesis and is used as a measure of the S-phase checkpoint. This phenomenon has been reported previously in cells from the Rad50-deficient patient (8, 12). Radioresistant DNA synthesis is normally determined by incorporation of radioactive nucleotide into DNA and is subject to considerable variability. Previous observations suggest that both DNA chain initiation and elongation are resistant to damage introduced into DNA in A-T cells (67). To address this, we have developed a DNA replication assay to differentiate between chain elongation and new initiations with a view to increasing the sensitivity of the assay. The assay involves a short pulse with chlorodeoxyuridine to label existing chains followed by radiation exposure and a pulse with iododeoxyuridine to label new initiations. The extent of DNA synthesis is expressed as the percentage of new initiations to total elongations (Fig. 6A). Under these conditions, cells transfected with Rad50 wild type showed marked inhibition of DNA synthesis after radiation, whereas vector-only and Rad50 mutant (S635G)-transfected cells displayed radioresistant DNA synthesis (Fig. 6B), revealing that the S635G mutant was incapable of correcting the S-phase checkpoint defect in Rad50-deficient cells. Com-

![FIGURE 4. The mutant form of Rad50 (S635G) is defective in complementing DNA damage-induced SMC1 phosphorylation. A, shown is the effect of wild type and mutant Rad50 (S635G) on radiation-induced phosphorylation of ATM-dependent substrates. Rad50-deficient fibroblasts were transfected with Rad50 constructs as in Fig. 1E. Extracts were prepared from non-irradiated and irradiated (5 Gy) cells at 15 min post-irradiation. Proteins were separated on SDS-PAGE, and membranes were incubated with antibodies against ATM-downstream substrates and also phosphorylated forms of these substrates. Extracts from irradiated and unirradiated cells were blotted with antibodies to ATM substrate antibodies as indicated. B, shown is the effect of Rad50 constructs on radiation-induced phosphorylation of ATM-dependent substrates after exposure to 1-Gy radiation and incubation for 15 min. Experimental conditions and analysis were the same as used in A above but with lower dose radiation. C, investigation of the expression of the germ line mutant Rad50 (X1313Y extX*66) form. Rad50-deficient cells were transfected with vector, Rad50WT, and Rad50 mutant (S635G). Stable cell lines were established as described above, and extracts were prepared before separation on 4.2% SDS-PAGE and then analyzed by immunoblotting with specific antibodies against Rad50 and SMC1. Gels of the GST fusion proteins were stained with Coomassie stain (lower panel). Under these conditions GST alone had migrated off the gel. F, Rad50 regulates ATM-dependent SMC1 phosphorylation in response to DNA damage. A GST fusion protein corresponding to the kinase domain of ATM (GST12) was employed in pulldown experiments to look for interaction with Rad50, SMC1, and the phosphorylated form of SMC1 (SMC1 Ser(P)-957) with chronatin. Chromatin was fractionated from Rad50-deficient-transfected cells as described under “Experimental Procedures.” These fractions were resolved in 6% SDS-PAGE and immunoblotted with anti-SMC1 Ser(P)-957, SMC1, and Rad50 antibodies.](image-url)
Functional Role for Rad50 Phosphorylation

A

\[ \text{Dose (Gy)} \]

\[ \% \text{ Survival} \]

- Control
- Rad50defic
- AT4Bl
- Rad50defic+V
- Rad50defic+WT
- Rad50defic+MUT

B

| Cell Line   | Sb | Cb | Int | ICA/Metaphase |
|-------------|----|----|-----|---------------|
| Control     | 49 | 0  | 0   | 0.98 ± 0.07   |
| AT4Bl       | 166| 7  | 0   | 3.46 ± 0.11   |
| Rad50 defic | 122| 2  | 4   | 2.56 ± 0.10   |
| Rad50 WT    | 47 | 1  | 0   | 0.98 ± 0.09   |
| Rad50 S635G | 138| 1  | 2   | 2.82 ± 0.11   |
| Vector Only | 118| 1  | 1   | 2.40 ± 0.09   |

Sb: chromatid breaks, Cb: chromosome breaks, Int: interchanges. Fifty metaphases were analyzed for each sample after exposure to 1 Gy of radiation.

C

End-joining events (%)

NFF (Control)  Rad50 WT  Rad50 S635G  Vector Only

D

Frequency GFP positive cells (%)

POR-GFP  Rad50WT -1/Ser  Rad50WT +1/Ser  Rad50S635G -1/Ser  Rad50S635G +1/Ser  Vector +1/Ser
Functional Role for Rad50 Phosphorylation

DISCUSSION

This report reveals that the second member of the MRN complex, Rad50, is phosphorylated at a specific site (Ser-635) in response to DNA damage and that this phosphorylation is functionally important for DNA repair and cell cycle control. Phosphorylation of Rad50 on this site was first detected using tandem MS (69). This site was also identified in a large scale proteomic analysis of proteins phosphorylated in response to DNA damage (50). A computational approach developed by Linding et al. (49), NetworkIN, to assign in vitro substrate specificity identified Rad50 as a substrate for ATM. In that study they confirmed that Rad50 phosphorylation was ATM-dependent in fibroblasts from Atm−/− mice using a general anti-phospho-(S/T)Q antibody. We confirmed phosphorylation at the Ser-635 site using a phosphospecific antibody against this site but did not detect any other ATM-dependent phosphorylation sites on Rad50 using in vitro assays. Thus, it is now evident that all three members of the MRN complex are phosphorylated in an ATM-dependent manner in response to DNA DSB. This is best described for DNA damage-induced phosphorylation of NBN on Ser-278 and Ser-343 (40–43). Conflicting results on the importance of NBN phosphorylation for downstream signaling have been reported (33, 63, 70, 71). Although there is agreement on ATM-dependent phosphorylation of NBN, its functional importance in the DNA damage response is less clear. Consensus exists on the requirement for NBN S343 phosphorylation for activation of the S-phase checkpoint (40, 42, 43, 64). However, NBN phosphosite mutants were able to restore Chk2 signaling, focus formation, and radiosensitivity in NBS cells in some studies (63, 64), whereas other reports point to the importance of NBN phosphorylation in cell survival post-irradiation (40, 42, 43). We have shown in this report that the Rad50 S635G phosphosite mutant failed to correct the S-phase checkpoint defect in Rad50-deficient cells. Furthermore, mutation at this site also failed to correct radiosensitivity in these cells. Our data demonstrate that this is not due to the relocation of Rad50 S635G to sites of DNA damage but rather to a downstream effect. Failure to correct radiosensitivity by the Rad50 S635G mutant is consistent with a defective capacity by the mutant to restore normal levels of repair of DNA DSB in Rad50-deficient cells. The mutant was defective in restoring NHEJ and HR repair in Rad50-deficient cells, whereas wild-type Rad50 did so. Previous data show that the MRN complex plays a role in both NHEJ and HR of DNA DSB (20–22, 72, 73). The complex does so by recruiting and participating in the activation of ATM (32, 74). The results obtained here suggest that, in addition to the presence of members of the complex, post-translational control also contributes to ATM downstream signaling, DNA repair, cell cycle control, and cell survival post-irradiation. As described above, there is some evidence that NBN phosphorylation is important for at least some of these events.

Phosphorylation of the third member of the complex, Mre11, has also been described in phosphoproteomic and other studies (46, 50, 75). In vitro studies using Xenopus extracts and DNA free ends led to Mre11 phosphorylation at multiple sites (47). As observed for Rad50, they showed that DNA damage-induced phosphorylation of Mre11 is not required for ATM activation, but they demonstrated that abrogation of Mre11 dephosphorylation impaired ATM signaling. These data show that when Mre11 is hyperphosphorylated, complex inactivation occurs by disassembly from chromatin. MRN binding to DNA and DNA tethering is mediated primarily by phosphorylation at up to 8 S/T(Q) PIKK sites. Di Virgilio et al. (47) suggest a role for Mre11 phosphorylation in checkpoint inactivation. This is consistent with the data we reported here for Rad50.
Functional Role for Rad50 Phosphorylation

Ser(P)-635, where ~50% was present in the nucleoplasm, dissociated from the site of damage. However, an approximately equal amount remained tightly bound to chromatin, and this was evident at times up to 6 h post-irradiation. Continued association with chromatin is more in keeping with a role for phosphorylated members of the complex in participating in downstream signaling to substrates involved in the regulation of DNA repair and cell cycle control. The \textit{in vitro} experiments with \textit{Xenopus} extracts do not rule out a similar role for phosphorylated Mre11 to that for NBN and Rad50; timing may be all important.

The specific phosphorylation of Rad50 appears to mediate signaling to both the S-phase checkpoint and DNA repair to enhance cell survival post-irradiation. It seems likely that this signaling is mediated largely through SMC1. We have shown here that Rad50 interacts with SMC1 and a Rad50 phosphosite mutant (S635G) fails to correct radiation-induced phosphorylation of SMC1 in Rad50-deficient cells. Although there was some defect in signaling through other downstream substrates at a higher radiation dose, at a more physiological dose (1 Gy) phosphorylation of p53, Chk2, and Kap1 occurred at normal levels, but SMC1 phosphorylation was not restored by the Rad50 Ser-635 mutant. These data point to a very specific role for SMC1 in mediating signaling from the phosphorylated form of Rad50 to both the S-phase checkpoint and repair of DNA DSB. Yazdi \textit{et al.} (63) previously showed that phosphorylation of NBN by ATM is required for phosphorylation of SMC1 with NBN playing an adaptor role in an ATM/NBN/SMC1 pathway for S-phase checkpoint control. It seems likely that the phosphorylated form of Rad50 plays a similar adaptor role, but it functions not only in cell cycle control but also in DNA repair and cell survival. Previous data show that ATM-dependent phosphorylation of SMC1 is required not only for activation of the S-phase checkpoint but also for maintaining the integrity of the genome and cell survival (64, 76). Thus, a critical dependence on Rad50 phosphorylation for SMC1 phosphorylation can readily explain the DNA repair and cell cycle checkpoint defects described here. However, in this study we only investigated a few key ATM-dependent substrates, and as it has been reported that there may be as many as 700 ATM substrates (50), it is possible that Rad50 phosphorylation may play an adaptor role for other as yet undefined substrates.

Recent data combining structural and functional dissection of NBN reveal that it provides the key phosphoprotein sensor and recruitment scaffold for the MR complex (16, 77). By connecting the Forkhead associated phosphoprotein-binding site with the two BRCA1 C terminus domains, it recruits C-terminal Binding Protein Interacting Protein to DNA DSB for end processing and HR. These models suggest that NBN acts as a multimodal adaptor linking the MRN complex to ATM and several other proteins involved in the response to DNA DSB (78). Its function is attenuated by phosphorylation in controlling the S-phase checkpoint (44, 63, 64) in the down-regulation of DNA replication after UV exposure (79) and in mediating ATR-dependent replication protein A1 hyperphosphorylation during replication fork stalling (80). Although not demonstrated, it seems likely that NBN Ser(P)-343 leads to conformational change that alters the function or interaction of the sub-

![FIGURE 6. Role of Rad50 phosphorylation in the intra S-phase and G2/M checkpoints. A, shown is a schematic for radioresistant DNA synthesis, determined by DNA replication labeling on DNA spreads. Rad50-deficient cells transfected with Rad50 constructs were labeled with chlorodeoxyuridine (CldUrd) before radiation exposure (labeling of ongoing forks) followed by a pulse with iododeoxyuridine (IdUrd) to distinguish new initiations and ongoing DNA replication. For quantitation, initiations were counted on individual fields and expressed as the \% of total growing forks. B, Rad50 S635G mutant does not correct radiosensitive DNA synthesis in Rad50-deficient cells. Percentage of new DNA synthesis initiations at 0 or 5 Gy radiation in Rad50 WT, Rad50 S635G mutant, and vector-only-transfected Rad50-deficient cells (left-hand panel). Data for control (NFF) and A-T cells appear in the right-hand panel. 300–500 forks were counted for each sample. Cells were transfected as in Fig. 1E. Error bars represent S.D. n = 3. C, phosphorylation of IR-induced G2/M checkpoint is shown. Fibroblasts were treated with 2 Gy and incubated for either 1 or 2 h after IR. Cells were stained with histone H3-Ser(P)-10 (H3-pS10)-conjugated antibody and propidium iodide. Cellular fluorescence was measured using flow cytometry to analyze histone H3 phosphorylation and DNA content (propidium iodide staining). Control (NFF) cells and Rad50-deficient cells transfected with vector-only or complemented with Rad50 constructs exhibited a marked decrease in the number of G2 cells entering mitosis after IR, whereas ATM null (ATS) cells failed to show G2/M arrest after IR. The error bars represent the S.D. for three independent experiments.]

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units of MRN at the site of the DNA DSB. Structural studies suggest that each MRN subunit can exist in multiple distinct states, which might be expanded further by post-translational modification (78). In these models two Rad50 ATPase domains come together to form head-to-tail dimers and sandwich two molecules of ATP, which upon hydrolysis leads to monomerization and the formation of an open Mre11-Rad50 conformation. This change influences Mre11 nuclease activity and is dependent on NBN (81). On the other hand, ATM-dependent phosphorylation of Rad50 occurs at the other end of the molecule in the coiled-coil region close to the Zn²⁺-hook and away from the active site (78) (Fig. 6). The Zn²⁺-hook acts as a dimerization domain for either intramolecular or intermolecular linkage of Rad50 molecules for tethering DNA ends or DNA molecules (14). The position of the phosphorylation site on Rad50 would not be expected to directly influence its own ATPase activity or the activity of Mre11, which we showed to be the case in vitro. Interaction of the checkpoint mediator 53BP1 with the MRN complex has been shown to be through Rad50, but the site of interaction on Rad50 was not defined (61). However, this interaction involved the BRCA1 C terminus domains on 53BP1 that bind to (S/T)-phosphorylated sites. The phosphorylated Ser-635 site on Rad50 represents a good candidate for promoting this interaction. Only one other DNA damage-induced site of phosphorylation (Ser-690) has been reported for Rad50, and this is not an (S/T)Q consensus site. However, Lee et al. (61) did not show a dependence on Rad50 phosphorylation for binding to 53BP1 in vitro. Furthermore, we observed normal formation of 53BP1 foci in Rad50 phosphosite mutant-complemented cells, suggesting that another factor(s) is involved in the recognition and signaling from phosphorylated Rad50 in vivo. Thus, Rad50 phosphorylation in the context of the MRN complex appears to be the modifying event that determines pathway choice, in this case signaling through SMC1, as suggested by Williams et al. (78).

It is now evident that once the MRN complex recruits ATM to the DNA DSB, all three members of the complex are in turn phosphorylated by ATM. The presence of the intact complex is important for ATM activation and facilitating ATM-dependent signaling through a series of downstream substrates that are involved in mediating DNA repair, cell cycle checkpoint activation, maintaining the genome integrity, and cell survival. However, it is also evident that the fine-tuning of these events is achieved by specific phosphorylations on NBN, Mre11, and Rad50. It is also clear that these individual phosphorylations have different adaptor roles in signaling to the DNA repair machinery and cell cycle control. The identification of additional factors associating with the phosphorylated forms of MRN complex will assist in elucidating their roles in regulating these processes.

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