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The Mre11 Complex Mediates the S-Phase Checkpoint through an Interaction with Replication Protein A▽¶

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The Mre11/Rad50/Nbs1 complex (MRN) plays an essential role in the S-phase checkpoint. Cells derived from patients with Nijmegen breakage syndrome and ataxia telangiectasia-like disorder undergo radioresistant DNA synthesis (RDS), failing to suppress DNA replication in response to ionizing radiation (IR). How MRN affects DNA replication to control the S-phase checkpoint, however, remains unclear. We demonstrate that MRN directly interacts with replication protein A (RPA) in unperturbed cells and that the interaction is regulated by cyclin-dependent kinases. We also show that this interaction is needed for MRN to correctly localize to replication centers. Abolishing the interaction of Mre11 with RPA leads to pronounced RDS without affecting phosphorylation of Nbs1 or SMC1 following IR. Moreover, MRN is recruited to sites at or adjacent to replication origins by RPA and acts there to inhibit new origin firing upon IR. These studies suggest a direct role of MRN at origin-proximal sites to control DNA replication initiation in response to DNA damage, thereby providing an important mechanism underlying the intra-S-phase checkpoint in mammalian cells.

The Mre11/Rad50/Nbs1 complex (MRN) participates in multiple pathways to maintain genome stability (1, 10, 55). In humans, hypomorphic mutations in *NBS1* and *MRE11* lead to Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia-like disorder (ATLD), respectively (7, 41, 54, 64). Both NBS and ATLD patients show developmental defects, immunodeficiency, and a high incidence of cancer, phenotypically similar to the ATM-deficient disorder ataxia-telangiectasia (AT) (53, 60). In addition, cells from NBS and ATLD patients are sensitive to radiation, exhibit genome instability, and have a defective S-phase checkpoint.

The phenotypic resemblance of *NBS*, *ATLD*, and *AT* cells implies that MRN and ATM participate in similar biological pathways. This is supported by recent findings that MRN acts both upstream and downstream of ATM to mediate the damage response when double-strand breaks (DSBs) are generated (32). MRN migrates to DSB sites immediately after damage in an ATM-independent manner (44, 47) and is required for ATM activation, especially in response to low doses of ionizing radiation (IR) (6, 8, 25, 35, 63). MRN is also a direct substrate of ATM. Multiple ATM phosphorylation sites on Nbs1 have been identified, and these phosphorylation events play important roles in mediating the intra-S and G_2/M checkpoints, although the mechanism by which Nbs1 phosphorylation regulates these pathways is not well defined (8, 22, 36, 69, 75).

Mre11 is the core of the MRN complex and interacts with both Rad50 and Nbs1 (24, 34, 62). Mre11 carries 3′ to 5′ exonuclease activity and single-strand endonuclease activity and acts on various types of DSB ends and hairpins (49, 61). These nuclease activities are believed to be involved in processing of DNA ends for DSB repair (18, 37, 50). In multiple organisms, it has been shown that MRN is essential for DNA repair by homologous recombination (27, 59, 70).

The radioresistant DNA synthesis (RDS) phenotype of *NBS* and *ATLD* cells suggests an important role of MRN in the S-phase checkpoint (53, 54). ATM activates the S-phase checkpoint, and two distinct pathways, ATM-Chk2-Cdc25A-Cdk2 and ATM-MRN-SMC1, have been suggested to mediate the S-phase checkpoint downstream of ATM (16). The ATM-Chk2 pathway modulates Cdc25A stability by Chk2-mediated phosphorylation and ultimately regulates Cdc45 chromatin loading (15, 16). In *NBS* and *ATLD* cells, Chk2 activation, Cdc25A degradation, and Cdc45 chromatin loading are indistinguishable from those of normal cells after IR, suggesting that the ATM-Chk2-Cdc25A-Cdk2 pathway is intact and that the RDS phenotype observed in *NBS* and *ATLD* cells is due to a defect independent from the regulation of Cdc45 chromatin loading (16). SMC1 has been shown to be phosphorylated in the ATM-Nbs1 pathway in response to IR in an ATM- and Nbs1-dependent manner (28, 71). The IR-induced phosphorylation of SMC1 is important for mediating the S-phase checkpoint; thus, SMC1 was proposed as a downstream effector of the ATM-Nbs1 pathway. However, the details of the mechanisms by which SMC1 inhibits DNA synthesis are not clear.

A connection of MRN to DNA replication was illustrated by its colocalization with proliferating cell nuclear antigen (PCNA) and BrdU incorporation sites in the S phase (45). Chromatin immunoprecipitation (ChIP) also revealed that MRN binds to replication-origin-proximal sites in mammalian cells in a manner similar to that seen with E2F family members

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(38). However, it remains unclear how MRN is recruited to replication centers and origin-proximal sites.

An association of replication protein A (RPA) and MRN after replication fork blockage induced by hydroxyurea has been previously described (52), but the biological significance of this interaction has not been elucidated. We independently detected this interaction and found that, in fact, a subset of nuclear MRN and RPA form stable complexes through a direct interaction in unperturbed cells. This interaction increases at the G_1-to-S transition with a peak in S phase and is regulated by cyclin-dependent kinase (CDK)-mediated phosphorylation. We also demonstrated that through this interaction, MRN is recruited to replication centers and origin-proximal sites in S phase. Importantly, the Mre11 mutants specifically defective in RPA binding exhibit a profound RDS phenotype, although phosphorylation of Nbs1 and SMC1 remains unaffected. These studies suggest that recruitment of MRN to replication-proximal sites by RPA is critical for MRN to suppress DNA replication initiation in response to IR, thereby suggesting a new mechanism of the ATM-Nbs1 pathway to mediate the S-phase checkpoint.

MATERIALS AND METHODS

Cell culture, cell synchronization, retroviral infections, and short hairpin RNA (shRNA). HeLa, IMR90, U2OS, and 293T cells were cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum. The Nbs1-deficient (GM07166) or Mre11-deficient (ATLD) cell lines immortalized by human telomerase reverse transcriptase (hTERT) (43) were cultured as previously described (51). T98G cells were synchronized by culturing in Dulbecco modified Eagle medium supplemented with 0.1% fetal bovine serum for 48 h and then releasing by adding 10% fetal bovine serum.

Different Nbs1 and Mre11 alleles were introduced into the GM07166, ATLD, or U2OS cell line by infection of retroviruses followed by selection of puromycin- or G418-resistant cells. Silencing of endogenous Mre11 in U2OS cells complemented by shRNA-resistant wild-type Mre11, Mre11-NAAIRS, or Mre11-DD alleles was accomplished by two rounds of retroviral infection using pMKO vector (39) expressing two different Mre11 shRNA target sequences, GATGA GAACTCTTGGTTTAAC and GAGTATAGATTTAGCAGAACA.

MRN complex purification. Nuclear extracts were prepared from 5g of BJAB cells and were fractionated by using phosphocellulose resin P11 followed by DEAE-Sephacel resin as previously described (19). Columns were loaded with 0.1 M KCl in BC buffer (20 mM Tris [pH 7.3], 0.2 mM EDTA) and eluted with 0.75 M KCl (BC buffer). Eluted fractions from DEAE were pooled, dialyzed, and applied to a Superose 6 (Pharmacia) column in BC buffer with 0.1 M KCl.

Plasmids and mutagenesis. Full-length Mre11 and Nbs1, fragments of Mre11 and Nbs1, and Mre11 internal-deletion alleles were subcloned into pcDNA3β, a mammalian expression vector containing the sequence encoding the myc epitope (9). Glutathione *S*-transferase–Mre11 (GST-Mre11) (amino acids 360 to 708) and GST-Nbs1 (amino acids 476 to 754) were constructed by cloning PCR products into pGEX4T-1 (Pharmacia). His-tagged RPA1/3/2, RPA1/3, and RPA3/2 were made by cloning PCR products generated by using p11d-tRDS RPA (23) (a generous gift from Marc Wold) as a template with pET28b (Novagen). Mre11 and Nbs1 mutations were generated by site-directed mutagenesis (Stratagene).

Immunoprecipitation, in vitro binding, and immunofluorescence. Immunoprecipitation (IP) and Western blot analysis were performed as previously described (9). IP in the presence of protein phosphatase was conducted by incubating cell lysates prepared from each 10-cm dish with 400 U of lambda phosphatase (NEB) for 30 min at room temperature followed by immunoprecipitation at 4°C.

Flag-Nbs1, Flag-Mre11, and Flag-Mre11 mutants were expressed in insect cells and purified using anti-Flag M2 agarose (Sigma) followed by elution with 3xFlag peptide (sigma). His-tagged RPA was purified from *Escherichia coli* on nickel-nitrilotriacetic acid resin (QIAGEN), and GST-Mre11 (amino acids 360 to 708) was purified using glutathione Sepharose (GE). In vitro binding was performed with NETN buffer (20 mM Tris [pH 8.0], 1 mM EDTA, 150 mM NaCl, 0.5% NP-40).

For immunostaining of S-phase foci, IMR90 cells were fixed with a modified Streck Tissue fixative for 30 min at room temperature and then permeabilized for 15 min at room temperature as previously described (47). After blocking with 5% goat serum in phosphate-buffered saline (PBS), cells were stained with primary antibody at 4°C overnight followed by staining for 1 h at room temperature with either fluorescein isothiocyanate-conjugated anti-mouse or rhodamine-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories). For ATLD fibroblast cells, fixation and immunostaining were performed 5 h after release from a double thymidine (2 mM) block. To detect damage foci, asynchronized cells were fixed by 70% methanol and 30% acetone at −20°C for 15 min followed by immunostaining analysis as previously described (68).

Antibodies. The polyclonal antibodies against Nbs1 (D29), and Mre11 (D27) and the monoclonal antibody against Nbs1 (EE15) were described previously (67, 69). Antiserum to GST was raised in rabbits by use of purified GST. The other antibodies used were purchased from Oncogene (ATM, Mre11, Nbs1, RPA1, and RPA2), Upstate (Rad50), Cell Signaling Technology (pT68-Chk2 and pS343Nbs1), Novus (pS1981-ATM), Bethyl (pS966-SMC1), and Santa Cruz Biotechnology (Chk2 and Ku70).

ChIP. ChIP was performed as previously described (48) with the following modifications. T98G and its derivative cell lines were synchronized by serum starvation in the presence of 0.1% fetal bovine serum (FBS) for 48 h and released into the cell cycle by the addition of 10% FBS into the culture medium. At specified time points after release, cells were cross-linked with 5 mM dimethyl-3,3′-dithiobispropionimidate-2HCl (DTBP) in PBS for 30 min on ice (21) followed by 1% formaldehyde in PBS at 37°C for 10 min. Chromatin was sheared to obtain DNA fragments approximately 1 kb in length. IP was performed using anti-Myc antibody at 4°C overnight. IP with rabbit anti-mouse immunoglobulin G (IgG) antibody was used as a negative control. After washing, the immunoprecipitates were eluted, incubated at 65°C overnight to reverse the cross-links, and then analyzed by PCR using primers specific to B2-lamin, heat shock protein 70 (HSP70), and c-*myc* loci (2).

Radioresistant DNA synthesis. Inhibition of DNA synthesis following IR was performed as previously described (46, 54). Cells were grown in the presence of 20 nCi/ml (U2OS) or 50 nCi/ml (ATLD and GM07166) [^{14}C]thymidine for 24 h. After removal of [^{14}C]thymidine, cells were either mock treated or treated with 10 Gy of IR, allowed to recover for 1 h, and then labeled with 2.5 μCi/ml [^{3}H]thymidine for 15 min (U2OS) (46) or 2 h (ATLD and GM07166) (54). Cells were spotted onto Whatman glass microfiber filters and washed with trichloroacetic acid and 100% ethanol. The filters were allowed to dry, and radioactivity was measured using a liquid scintillation counter and a dual energy program.

Sensitivity to ionizing radiation. ATLD cells complemented with vector, Mre11, Mre11-NAAIRS, or Mre11-DD were plated in triplicate at 3.5×10^5 cells per dish. Cells were treated with 0, 2, 5, or 10 Gy of IR. Seven days later, cells were stained with trypan blue and counted.

RESULTS

A subset of nuclear MRNs and RPAs form stable complexes in unperturbed cells. When nuclear extracts were fractionated using columns (Fig. 1A), we found a small percentage of RPA was consistently copurified with MRN. While most MRNs bound to the phosphocellulose P11 column, only 10% to 20% of RPA species were retained on the P11 column and the rest were present in the flow-through volume (Fig. 1B, left panel). Further purification showed that most P11-bound MRN and RPA species were retained on a DEAE column (Fig. 1B, right panel). Protein complexes eluted from the DEAE column were separated according to molecular weight by Superose 6 gel filtration (Fig. 1C, left panel). Most input RPA was coeluted with MRN as large protein complexes. Notably, RPA was present only in the fractions containing MRNs of relatively higher molecular weight and there was a substantial amount of MRN that was eluted in the absence of RPA. Coeluted RPA and MRN were truly associated with each other, as demonstrated by coimmunoprecipitation (Fig. 1C, right panel). These studies suggest that a subset of MRNs and RPAs form stable complexes in unperturbed cells. The large size of the complexes

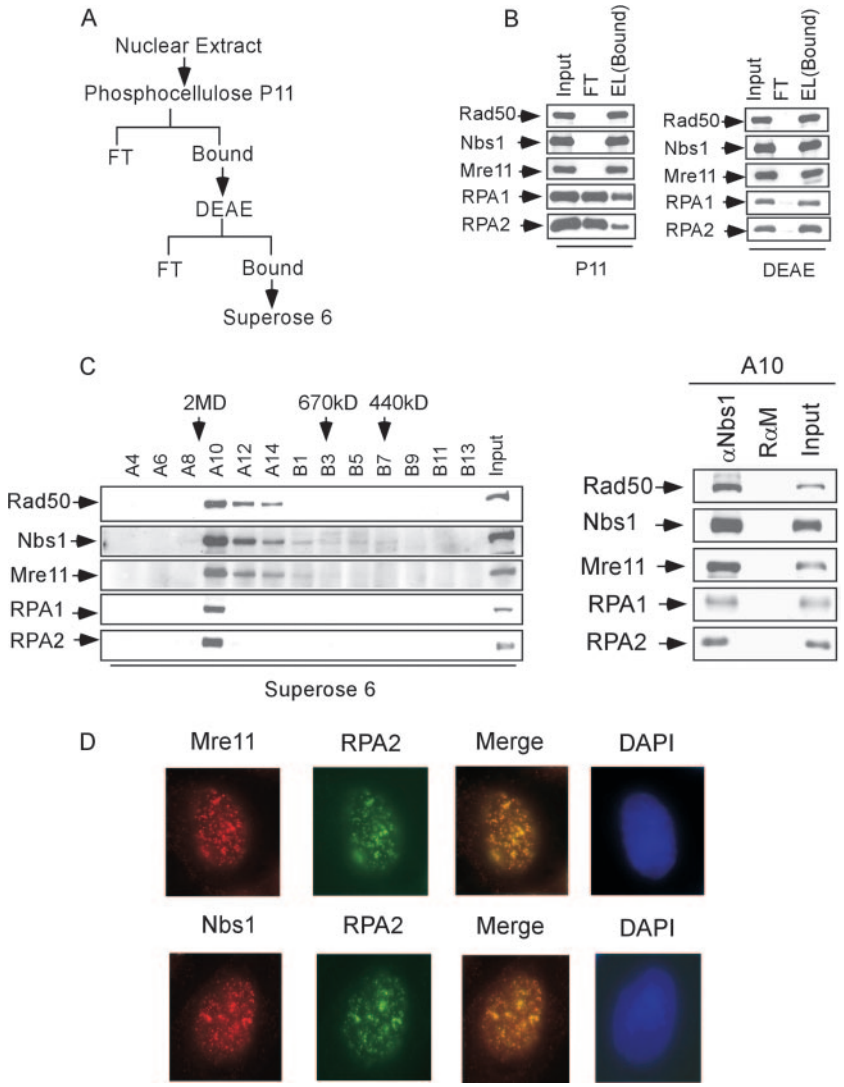

FIG. 1. The MRN complex interacts with RPA and colocalizes with RPA at replication centers. (A) Purification scheme. BJAB cell nuclear extract was fractionated using a phosphocellulose (P11) column. After washing, P11-bound proteins were eluted with 0.75 M KCl in BC buffer. A DEAE-Sephacel column was used subsequently and was eluted with 0.75 M KCl, which was followed by fractionation on a Superose 6 gel filtration column. FT, flowthrough. (B) The presence of MRN and RPA in the flowthrough (FT; 0.1 M KCl) and/or in the elution (EL; 0.75 M KCl) of P11 and DEAE columns was monitored by Western blot analysis using antibodies as indicated. (C) The eluted material collected further from the DEAE column was fractionated on a Superose 6 gel filtration column. Western blot analysis was performed on alternate fractions to monitor the elution of MRN and RPA (left panel). Affinity-purified and protein A bead-coupled polyclonal antibody D29 (anti-Nbs1) was used to perform immunoprecipitation using the A10 fraction from the Superose 6 column (right panel). The immunoprecipitates were immunoblotted using the indicated antibodies. Rabbit anti-mouse IgG antibody (RαM) was used as a negative control. (D) Primary fibroblast IMR90 cells were extracted and immunostained with the specified antibodies as indicated. Merged images of Mre11 or Nbs1 with RPA2 immunostaining are shown (Merge). DAPI (4′,6′-diamidino-2-phenylindole) was used for nuclear staining.

also suggests there may be other proteins associated with MRN/RPA. We estimate the amount of RPA associated with MRN to be approximately 5% to 10% in nuclear extracts purified from asynchronized cells. The interaction of MRN and RPA in unperturbed cells was observed by coimmunoprecipitation in multiple cell lines, including HeLa, U2OS, T98G, and BJAB cells (see Fig. S1A to C in the supplemental material; data not shown). To exclude the possibility that the interactions were mediated by DNA, coimmunoprecipitation was performed in the presence of different concentrations of ethidium bromide (see Fig. S1D in the supplemental material). Although DNA-protein interactions can be effectively inhibited by ethidium bromide (30), the interaction of MRN and RPA was largely unaffected by the addition of ethidium bromide.

The idea of an interaction of MRN with RPA in unperturbed cells is further supported by the colocalization of Nbs1 and Mre11 with RPA in S-phase foci, which represent centers of DNA replication (11). As described previously, Nbs1 and Mre11 form S-phase-specific foci in PCNA-positive and BrdU-incorporating cells (45). These S-phase-specific foci are visualized after detergent extraction followed by immunostaining

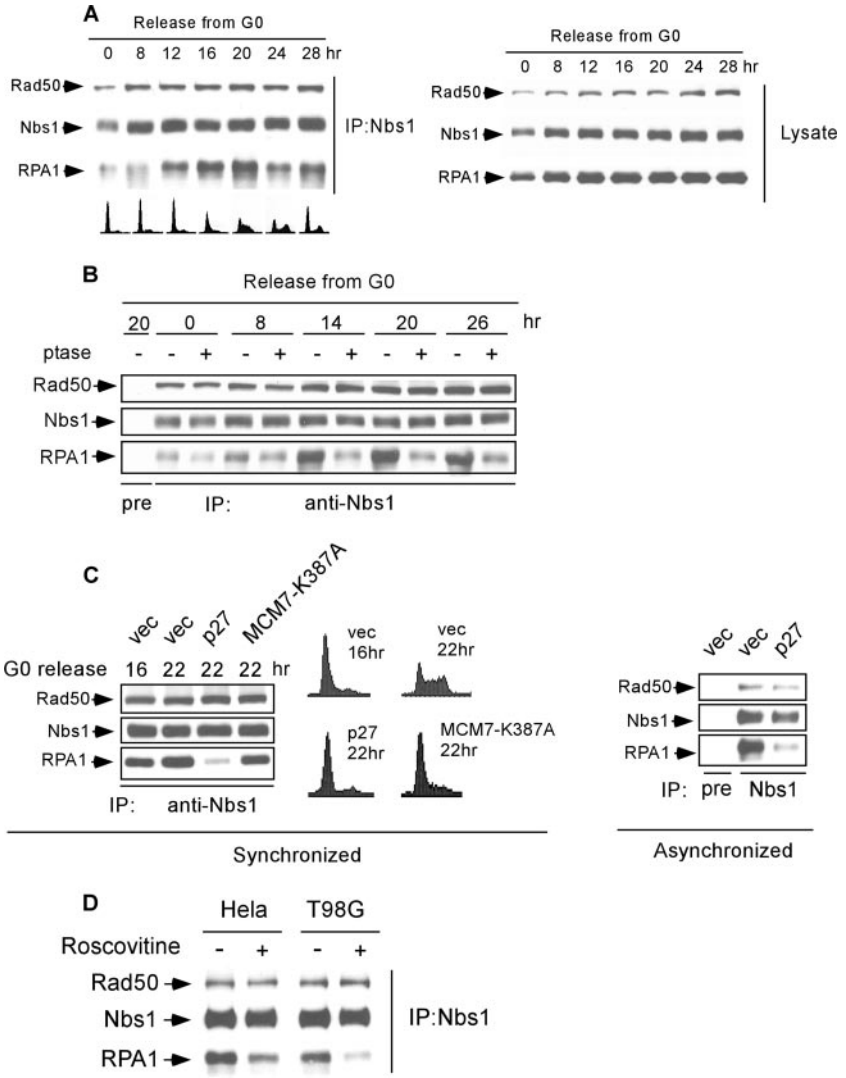

FIG. 2. The interaction of MRN and RPA is cell cycle regulated and is dependent on CDK activities. (A) T98G cells were arrested in G_0 by serum starvation (0.1% FBS) and lysed at indicated time points after serum addition (10% FBS). Cell cycle profiles are shown. Association of MRN and RPA was examined by anti-Nbs1 immunoprecipitation and Western blotting with the indicated antibodies. Protein levels of MRN and RPA during the cell cycle were demonstrated by Western blot analysis using cell lysates generated from the same number of cells. A 5% volume of the cell lysate was used in the lysate lanes. (B) T98G cells were released from serum starvation and lysed at indicated time points. Anti-Nbs1 IP was performed after incubation with (+) or without (−) lambda phosphatase (ptase). IP with preimmune serum (pre) was used as a negative control. The immunoprecipitates were examined by Western blotting with indicated antibodies. (C) At 24 h after arrest in G_0 by serum starvation, T98G cells were infected with Ad encoding green fluorescent protein (vec), p27 (p27), or MCM7-K387A. After another 24 h, cells were released from serum starvation and lysed at the indicated time after releasing. Anti-Nbs1 IP was performed, and the association of MRN and RPA was examined by Western blotting using the indicated antibodies. Cell cycle profiles are shown. Asynchronized T98G cells were also infected with Ad-vec or Ad-p27. At 36 h after infection, anti-Nbs1 immunoprecipitation was performed. Pre, preimmune serum. The MCM7-K387A allele carries a mutation at the conserved ATPase motif, which is essential for DNA helicase activity of MCM proteins (72, 73). (D) T98G cells were synchronized in G_0 by serum starvation (0.1% FBS) and then released into aphidicolin (1 μg/ml)-containing medium (10% FBS). Twenty-four hours later, cells were treated with or without roscovitine (50 μM, 3 h). A synchronized HeLa cells were arrested at the G_1-to-S transition by aphidicolin (1 μg/ml, 14 h) and treated with roscovitine (50 μM) for 3 h or left untreated. The interaction of RPA and MRN was examined by anti-Nbs1 IP followed by Western blot analysis using the indicated antibodies.

using specific antibodies. Consistent with previous findings (45), Mre11 and Nbs1 formed S-phase foci in all S-phase cells during an unperturbed cell cycle (data not shown). These Mre11 and Nbs1 S-phase foci were colocalized with those of RPA1 and RPA2 in multiple cell lines, including IMR90, WI38, and U2OS cells (Fig. 1D and data not shown).

The observation that MRN and RPA were colocalized in S-phase-specific foci prompted us to examine whether the interaction of MRN and RPA was cell cycle regulated. T98G cells were synchronized by serum starvation, and at different time points after release, the interaction of MRN with RPA was determined. Although the association was detected throughout the cell cycle, the interaction increased when cells were at the G_1-to-S transition, with a peak in the S phase (Fig.

2A). Similar results were obtained when HeLa cells were synchronized by release from nocodazole-mediated mitotic arrest (data not shown). Increased association of MRN/RPA right before the onset of S phase and the colocalization of MRN and RPA S-phase foci suggest a potential role of MRN/RPA complexes in regulating DNA replication and/or sensing and repairing DSBs at replication forks.

CDK activity is important for regulating the interaction of MRN and RPA. Since the interaction of MRN and RPA is regulated during the cell cycle, we investigated whether the interaction is phosphorylation dependent. Coimmunoprecipitation of MRN and RPA was performed using cell lysates prepared from different stages of the cell cycle with or without phosphatase treatment (Fig. 2B). The increased association of MRN and RPA at the G_1-to-S transition and in S phase was abolished after phosphatase treatment, suggesting that cell-cycle-regulated phosphorylation is required for stimulating the interaction and maintaining the increased MRN/RPA interaction. Since the interaction started to increase at the G_1-to-S transition, we examined whether CDKs that are required for replication initiation are involved in regulating the MRN/RPA interaction. T98G cells were infected with adenovirus (Ad) encoding p27 or green fluorescent protein while arrested in G_0 by serum starvation, and 22 h after release from G_0, coimmunoprecipitation was performed. Inhibition of CDK activity by p27 prevented cells from entering the S phase and also inhibited the interaction of MRN and RPA (Fig. 2C, left panel). The loss of the interaction of MRN and RPA was not due to a failure of replicating DNA, since overexpression of an MCM7 helicase mutant (MCM-K387A), which suppressed DNA replication (72, 73; A. Lee and X. Wu, unpublished data), did not significantly impair the interaction of MRN and RPA. Aphidicolin and thymidine treatment, which arrested cells at the G_1-to-S transition, also did not prevent the association (data not shown). These data support the idea that inhibition of the interaction by p27 is due to an inhibition of CDK activities. These data also suggest that active DNA replication is not necessarily required for stimulating the interaction of MRN and RPA; thus, the increased association of MRN and RPA in the S phase is likely not triggered by spontaneous DNA damage generated during DNA replication.

Overexpression of p27 in asynchronized cells also inhibited the association of MRN and RPA (Fig. 2C, right panel), suggesting that CDK activity is required to mediate the interaction of MRN and RPA not only for cells exiting from G_0 but also for normal cycling cells. To more directly examine whether CDKs are involved in regulating the MRN/RPA interaction, T98G cells were released from G_0 and arrested at the G_1/S boundary by aphidicolin when the interaction reached relatively high levels. Inhibition of CDK activity by the presence of roscovitine significantly reduced the interaction of MRN with RPA (Fig. 2D). Similar results were obtained when HeLa cells were used. These data suggest that CDK activity is not only important for stimulating the cell-cycle-dependent association of MRN and RPA but is also required for maintaining the increased association.

A specific site at the C terminus of Mre11 directly interacts with RPA1. Obtaining MRN mutants specifically defective in RPA binding would help to clarify the biological role(s) of the interaction of MRN and RPA. To do this, a series of Myc-tagged Mre11 fragments was expressed in 293T cells and immunoprecipitated. Only the carboxy-terminal fragment $Mre11^{493–708}$ interacted with endogenous RPA1 and RPA2 (Fig. 3A). This fragment also interacted with endogenous Nbs1 and Rad50. However, the $Mre11^{338–546}$ fragment bound to Rad50 but did not interact with RPA. These data suggest that either Mre11 or Nbs1, but not Rad50, mediates the binding of MRN with RPA. Consistently, the Nbs1 carboxy-terminal region required for Mre11 association interacted with RPA (data not shown).

To examine whether the interaction of MRN and RPA is direct, full-length Nbs1 and Mre11 were expressed in insect cells and purified. Mre11, but not Nbs1, bound to RPA isolated from bacteria (Fig. 3B, first through third lanes). In vitro analysis also showed that the entire RPA heterotrimer, or a complex consisting of RPA1 and RPA3, bound to GST-$Mre11^{360–708}$ whereas a complex consisting only of RPA2 and RPA3 did not (Fig. 3C). These data suggest that Mre11 and RPA1 mediate a direct interaction between the MRN complex and RPA.

To further narrow down the RPA-binding region on Mre11, a series of Mre11 deletion alleles was analyzed. Among them, two internal deletion alleles (lacking residues 521 to 543 and 543 to 569) failed to coimmunoprecipitate RPA, whereas another internal deletion allele (lacking residues 494 to 521) still associated with RPA (Fig. 3D, bottom left panel). This result defined the RPA-binding domain on Mre11 as being between amino acids 521 and 569. To identify the precise amino acid sequence required for RPA binding to Mre11, the six-amino-acid sequence NAAIRS was used to replace the most conserved sequences within this region in vertebrates (Fig. 3D). The NAAIRS peptide is a flexible linker sequence that would minimize the conformational changes in the mutant protein (65). When expressed in cells, the Mre11-p540 NAAIRS mutant (changing amino acids 540 to 545) failed to bind RPA, whereas the other two Mre11-NAAIRS mutants (p522 and p529) still associated with RPA (Fig. 3D, bottom left panel). Within the sequence replaced by the p540 NAAIRS, two acidic residues at positions 543 and 544 of the human protein are conserved in several eukaryotic species (Fig. 3D). Mutation of these two aspartic acid residues to alanine residues (Mre11-DD→AA) completely abolished the binding of Mre11 to RPA (Fig. 3D, bottom right panel). The interaction of the NAAIRS and DD mutants with Nbs1 and Rad50 was not affected (Fig. 3D and data not shown). Introducing p540 NAAIRS or the DD mutation into full-length Mre11 also abolished the in vitro direct binding of Mre11 with RPA (Fig. 3B). These data suggest that residues D543 and D544 in Mre11 are required for Mre11 to directly bind to RPA.

The interaction of MRN and RPA is required for correct localization of MRN to replication centers. Colocalization of MRN and RPA in S-phase foci (Fig. 1D) suggests a possible role of the MRN/RPA complex in S phase. To determine whether MRN recruitment to S-phase foci is dependent upon its interaction with RPA, we examined Mre11 colocalization with RPA in S-phase foci when the binding between Mre11 and RPA was disrupted. Mre11-deficient ATLD cells immortalized by hTERT (43) were complemented with vector, wild-type Mre11, or the RPA binding mutants Mre11-NAAIRS and Mre11-DD. Immunostaining without extraction showed that more than 80% of ATLD cells were reconstituted (data not

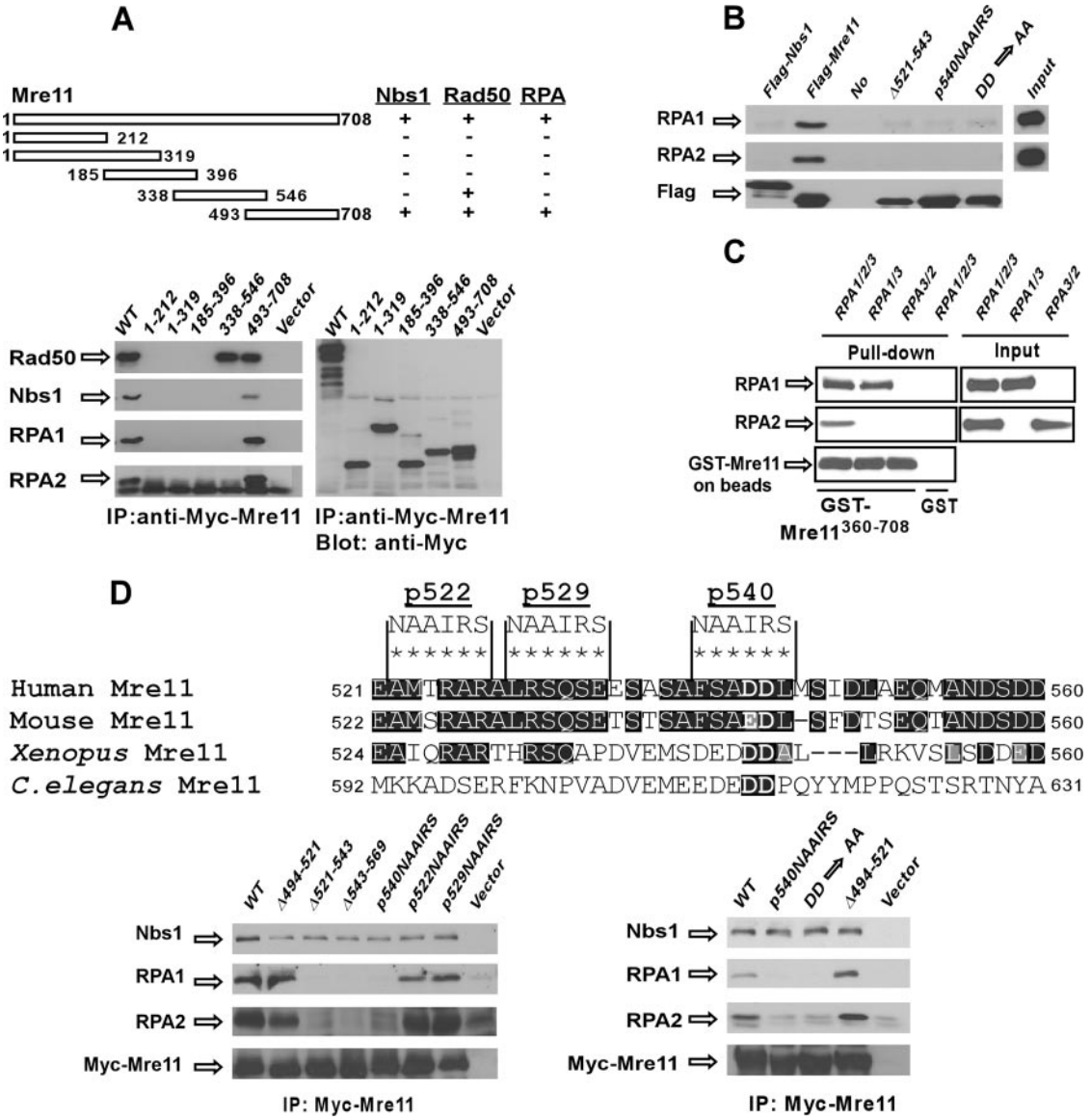

FIG. 3. The direct interaction between MRN and RPA requires residues D543 and D544 at the C terminus of Mre11. (A) Fragments of Mre11 corresponding to the indicated positions of the human Mre11 protein were transiently transfected into 293T cells and immunoprecipitated using an anti-Myc antibody (9E10). The immunoprecipitates were immunoblotted as indicated. The schematic representation of the fragments indicates whether each retains (+) or loses (−) its ability to interact with RPA, Nbs1, and Rad50. (B) Anti-Flag immunoprecipitation was performed after incubating RPA purified from *E. coli* with Flag-Nbs1, -Mre11, -Mre11-Δ521–543, -Mre11-p540NAAIRS, or -Mre11-DD that was expressed and purified from baculovirus-producing insect cells. Immunoprecipitates were immunoblotted with anti-RPA1, -RPA2, and -Flag antibodies. Input, total input of RPA; No, RPA alone with no Mre11 or Nbs1 protein added. (C) GST-Mre11$^{360–708}$ or GST was expressed in *E. coli* and purified with glutathione Sepharose beads. GST-Mre11$^{360–708}$ or GST on beads was incubated with imidazole-eluted His-tagged RPA complexes purified from *E. coli*. After the mixture was washed, RPA1 and RPA2 retained on the GST-Mre11$^{360–708}$ beads were detected by Western blotting. Input, 50% of the total input of various RPA complexes. (D) The amino acid sequence from positions 521 to 560 of the human Mre11 protein was aligned with Mre11 from other indicated species. Black boxes indicate conserved residues, and gray boxes indicate similar residues. The positions of the three NAAIRS mutations that were introduced into Mre11 are indicated above the sequence at the positions at which each mutation occurred. The two aspartic acid residues represented in white bold characters are essential for the interaction between the human Mre11 protein and RPA. Three Myc-tagged Mre11-NAAIRS mutants (p522 [changing amino acids 522 to 527], p529 [changing amino acids 529 to 534], and p540 [changing amino acids 540 to 545]), wild-type (WT) Mre11, and empty vector were transiently transfected into 293T cells and immunoprecipitated using an anti-Myc antibody (9E10) followed by immunoblotting as indicated (left panel). Similar experiments were performed using the Myc-tagged Mre11-DD to -AA mutant (aspartic acid residues 543 and 544 mutated to alanine residues), p540NAAIRS, and wild-type Mre11 (right panel). *C. elegans*, *Caenorhabditis elegans*.

shown) and that the Mre11 wild type as well as the DD and NAAIRS mutants were all correctly located in the nucleus (see Fig. S2A in the supplemental material). Mre11, Mre11-DD, and Mre11-NAAIRS, as well as Rad50 and Nbs1, were also restored to similar protein levels in the reconstituted ATLD cells (Fig. 4A, top row). To enrich the S-phase population, cells were synchronized by a double thymidine block. Five hours after release from thymidine block, approximately 20% of the

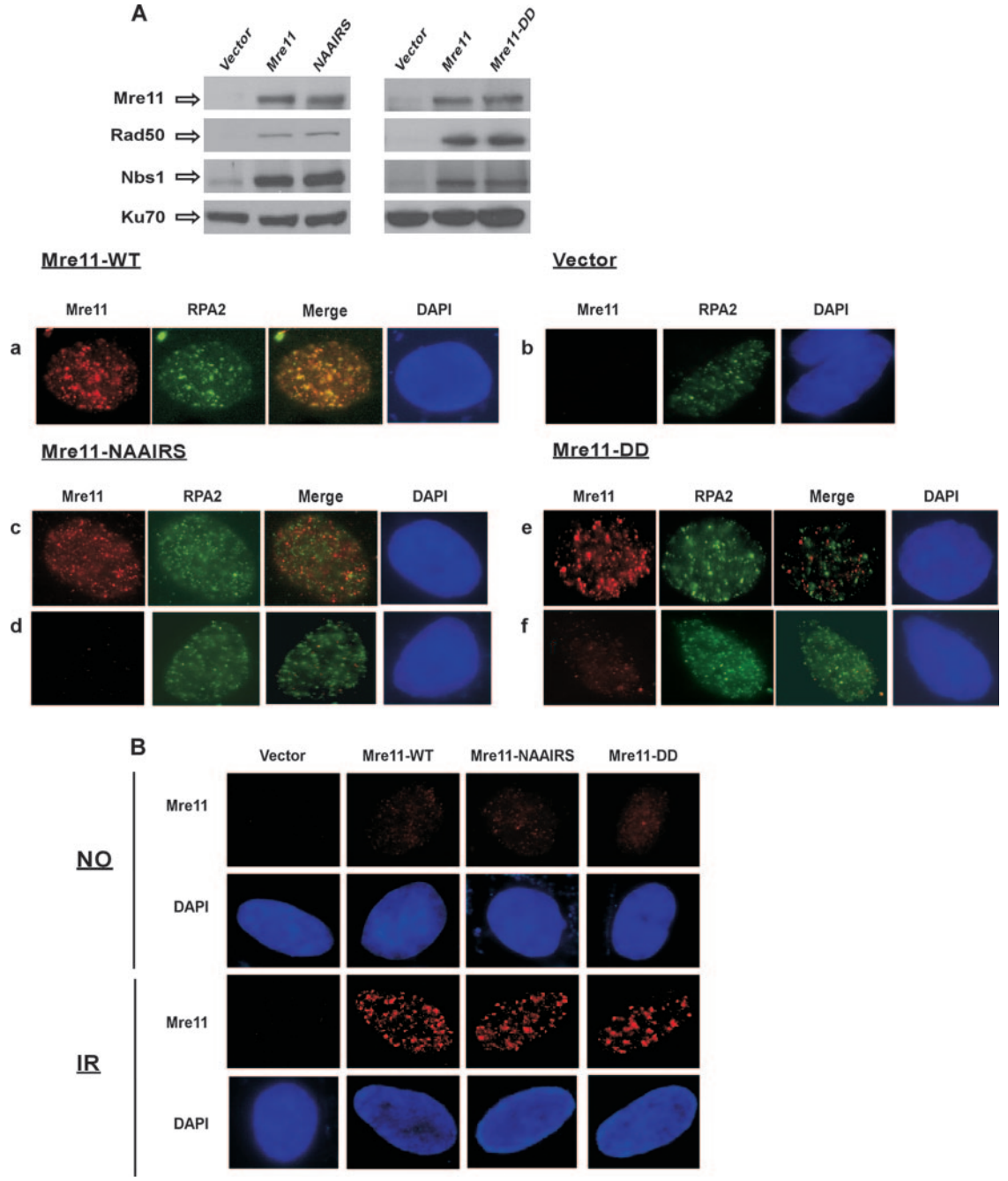

FIG. 4. Mre11 mutants defective in RPA binding fail to colocalize with RPA S-phase foci in ATLD-reconstituted cells. (A) ATLD cells immortalized by the expression of hTERT were retrovirally inoculated with vector only or with the indicated alleles of Mre11. Whole-cell lysates were immunoblotted as indicated. ATLD cells reconstituted with the indicated alleles of Mre11 were enriched for the S phase by a double thymidine block. At 5 h after release from the second thymidine block, cells were extracted and immunostained with the specified antibodies. Panels c and e show examples of bright staining of Mre11 foci in the Mre11-NAAIRS and Mre11-DD mutants, whereas panels d and f show examples of faint staining as indicated in Table 1. DAPI was used for nuclear staining. Merge, merged pictures of Mre11 and RPA2 staining; WT, wild type. (B) ATLD cells reconstituted with the indicated alleles of Mre11 were mock treated (NO) or treated with IR (20 Gy). Eight hours later, cells were fixed with methanol-acetone and immunostained with an antibody to Mre11. DAPI was used for nuclear staining.

cells from each ATLD cell line were in the S phase, as judged by RPA and PCNA S-phase foci (Table 1 and data not shown). At this time point, cells were extracted and immunostained with antibodies specific to Mre11 and RPA2 (Fig. 4A). While the Mre11-NAAIRS and the Mre11-DD proteins formed S-phase foci, there were two major differences when these foci were compared with wild-type Mre11 foci. First, a higher percentage of S-phase cells (as judged by RPA foci) contained faint-staining Mre11 foci (Fig. 4A, panels d and f versus panel a, and Table 1) in the Mre11-NAAIRS and the Mre11-DD cell

TABLE 1. Percentages of wild-type and mutant Mre11 proteins in S-phase foci

Protein or vehicle	% of S-phase cells (as judged by RPA foci)	% of S-phase cells:		
		With bright Mre11 foci	With faint Mre11 foci	That were not reconstituted
Mre11-WT[a]	19.2	69.9	12.5	17.6
Mre11-NAAIRS	17.8	26.5	62.7	10.8
Mre11-DD	18.9	30.1	51.9	18.0
Vector	19.6	NA[b]	NA	NA

[a] WT, wild type.
[b] NA, not applicable.

lines. Second, the S-phase foci of Mre11-NAAIRS and Mre11-DD, both faint and bright, were not colocalized with RPA, unlike the foci of wild-type Mre11 (Fig. 4A, panels c to f versus panel a). These results suggest that the interaction between Mre11 and RPA is required for MRN to correctly localize to replication centers in S phase. The remaining S-phase foci of the Mre11 RPA-binding mutants are apparently not at sites of DNA replication, as defined by the presence of RPA, PCNA, and BrdU incorporation (Fig. 4A; see Fig. S2B in the supplemental material; data not shown). Considering the multiple activities of MRN, including its repair function in S phase, MRN is possibly anchored to chromatin by other mechanisms when it loses its ability to associate with RPA.

While the association of Mre11 with RPA is required for MRN to be properly localized to replication centers, the interaction does not seem to be needed for MRN to be recruited to IR-induced foci. Using a nonextraction method (7), we detected approximately the same percentage of ATLD cells complemented with wild-type Mre11, Mre11-NAAIRS, or Mre11-DD that formed Mre11 damage foci after IR (Fig. 4B and data not shown).

The interaction of MRN with RPA is required for mediating the S-phase checkpoint. RPA plays an essential role in DNA replication (5). The observation that RPA-binding mutants of Mre11 specifically lose their interaction with DNA replication centers suggests that the association between MRN and RPA may be required for replication control or replication-related events. This idea is also supported by the cell-cycle-regulated association of MRN and RPA. Since ATLD cells reconstituted with Mre11, Mre11-NAAIRS, Mre11-DD, or vector grew at approximately the same rate (data not shown), the interaction of MRN with RPA does not appear to affect normal DNA replication under nonstressed conditions. We further examined whether the interaction of MRN with RPA might be important for mediation of the intra-S-phase checkpoint response by suppressing DNA replication after IR. The RDS phenotype of ATLD cells was corrected when wild-type Mre11 was expressed in these cells. Strikingly, ATLD cells complemented with Mre11-NAAIRS and Mre11-DD mutants failed to suppress the RDS phenotype (Fig. 5A, left panel). The down-regulation of DNA synthesis upon IR was also compromised in U2OS cells expressing Myc-Mre11-NAAIRS or Mre11-DD but not wild-type Mre11 when the expression of endogenous Mre11 was silenced (Fig. 5A, middle and right panels). Since RDS in the ATLD cells expressing Mre11-NAAIRS and Mre11-DD mutants reached levels similar to those seen with vector-complemented ATLD cells, these data suggest that the interaction of MRN and RPA is required in a major pathway for MRN to mediate the S-phase checkpoint.

MRN is involved in activating ATM in response to IR, but the dependence of ATM activation on MRN is limited only to low doses of IR (25, 35, 63). ATM autophosphorylation at Ser1981 and Chk2 phosphorylation at T68 was compromised in ATLD cells following IR at a dose of 2 Gy but not at a dose of 10 Gy compared with the results seen with ATLD cell lines reconstituted with wild-type Mre11 (Fig. 5B and data not shown). The Mre11 mutants defective in RPA binding (Mre11-DD and Mre-NAAIRS) exhibited ATM activation similar to that seen with wild-type Mre11 at both low and high doses of IR (Fig. 5B and data not shown). These results suggest that MRN does not require its association with RPA to sense DSBs and activate the S-phase checkpoint. Therefore, the defective S-phase checkpoint observed with the Mre11 RPA-binding mutants is not attributable to a failure of ATM activation and is more likely caused by an impaired function downstream of ATM.

Since the interaction of MRN and RPA is important for suppression of DNA replication following IR, we examined whether this interaction is regulated by DNA damage. Multiple cell lines, including U2OS, HeLa, and T98G, were lysed before and after IR (10 Gy), and the interaction was assayed by coimmunoprecipitation. The interaction of MRN and RPA remained at similar levels before and after IR (Fig. 5C and data not shown). To minimize the possibility that the interaction is regulated in the presence of chromatin, we performed coimmunoprecipitation after extraction of cell lysate pellets with high levels of salt and detergent; the interaction of RPA and MRN in untreated cells and after IR were similar (see Fig. S3 in the supplemental material). Since both Nbs1 and Mre11 are phosphorylated after IR, these data also suggest that IR-induced phosphorylation of Nbs1 and Mre11 is not involved in regulating the interaction between MRN and RPA.

The MRN/RPA complex is likely a downstream effector in mediating the S-phase checkpoint. ATM-mediated phosphorylation of Nbs1 at S343 plays a critical role in down-regulating DNA replication following IR. Although loss of the interaction between MRN and RPA led to an RDS phenotype, IR-induced phosphorylation of Nbs1 at S343 in ATLD cells expressing wild-type Mre11 or the RPA-binding mutants (Mre11-DD and -NAAIRS) was at similar levels (Fig. 5D, left panel). Furthermore, in similarity to the results seen with wild-type Mre11, both Mre11-DD and Mre11-NAAIRS mutants bound to phosphorylated Nbs1 following IR (Fig. 5D, right panel). These data suggest that association with RPA is not required for Nbs1 to be phosphorylated at S343 by ATM. Since the S-phase checkpoint is impaired when the interaction of MRN and RPA is disrupted, even though Nbs1 is perfectly phosphorylated by ATM at S343 in the MRN mutant complexes, Nbs1 phosphorylation must occur prior to the step that requires the MRN-RPA interaction for mediation of the S-phase checkpoint. These data are consistent with a scenario in which MRN/RPA is a downstream effector in the ATM-Nbs1 branch of the S-phase checkpoint pathway such that the interaction between MRN and RPA is required for ATM-phosphorylated Nbs1 to suppress DNA replication.

According to previous studies, SMC1 is a downstream effector

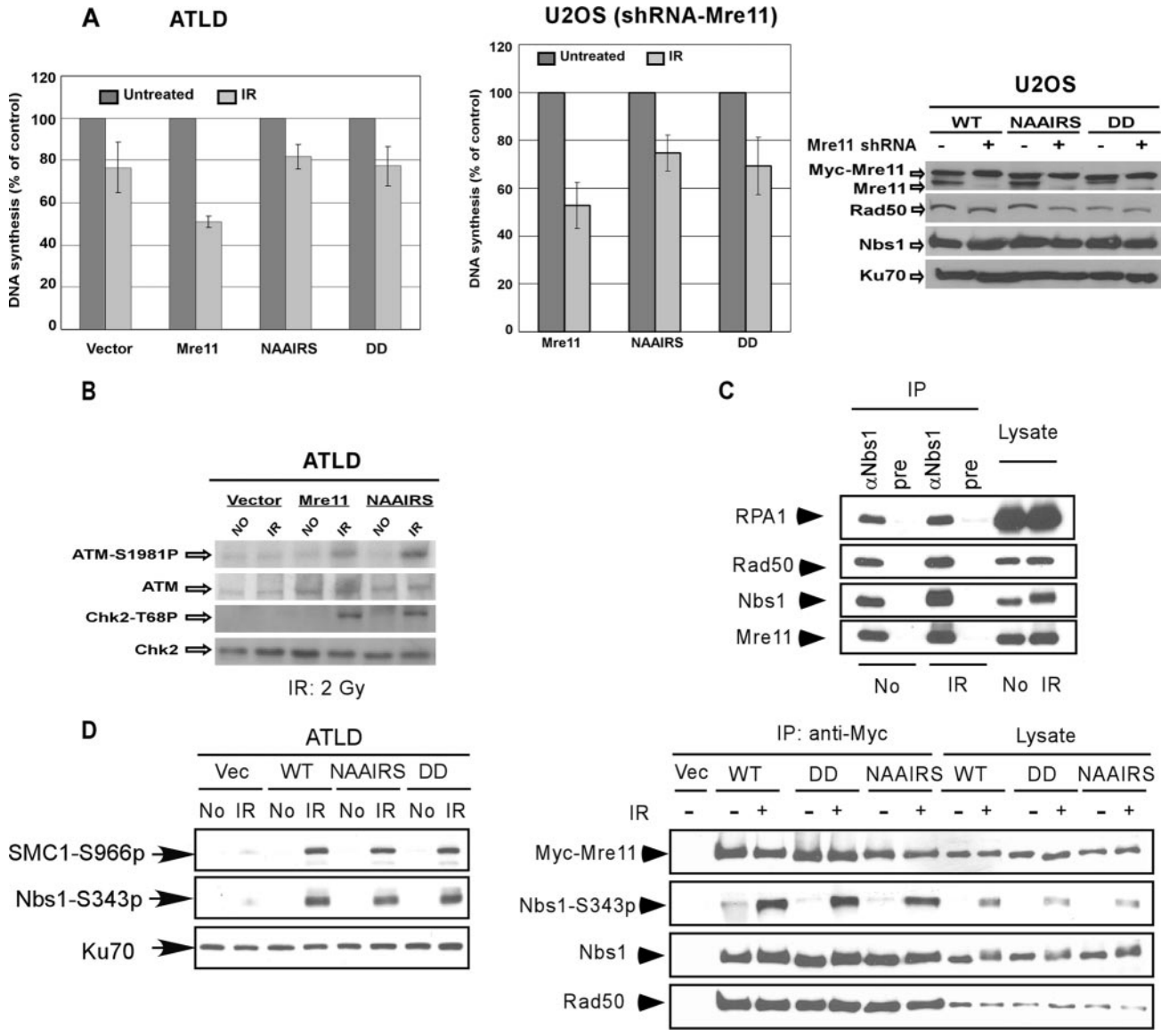

FIG. 5. Disruption of the interaction between MRN and RPA results in RDS without affecting ATM-mediated checkpoint signaling. (A) The rate of DNA synthesis following IR (10 Gy) was determined in ATLD cells immortalized by the expression of hTERT and complemented by vector, wild-type (WT) Mre11, Mre11-NAAIRS, or Mre11-DD (left panel). Similar experiments were performed using U2OS cells expressing vector or the indicated Myc-tagged Mre11 alleles containing several silent mutations at the two shRNA targeting sequences, while the endogenous Mre11 was silenced by shRNAs (middle panel). The rate of DNA synthesis was normalized to the appropriate mock-treated control (Untreated). Standard deviation values were derived from the results at least three independent experiments. The expression of Mre11 alleles and the silencing of endogenous Mre11 in U2OS cells are shown by the results of Western blotting of cell lysates (right panel). (B) ATLD cells reconstituted with vector-, Mre11-, or Mre11-NAAIRS were either mock treated (NO) or treated with IR (2 Gy 30 min later). The whole-cell lysates were immunoblotted with antibodies specifically recognizing phosphorylated ATM at S1981 (ATM-S1981P), phosphorylated Chk2 at T68 (Chk2-T68P), ATM, and Chk2. (C) Nbs1 IP was performed after IR (10 Gy 1 h later) or mock treatment (No). Preimmune serum was used as a negative control for IP. Western blotting was performed using the indicated antibodies. (D) ATLD cells complemented with vector, wild-type Mre11, Mre11-NAAIRS, or Mre11-DD were treated with IR (10 Gy) or left untreated and lysed 1 h after IR (left panel). Phosphorylation of SMC1 at S966 and Nbs1 at S343 was examined by using phosphospecific antibodies. Ku70 was used as a loading control. U2OS cells expressing the Myc-Mre11 wild type (WT), Myc-Mre11-DD (DD), or Myc-Mre11-NAAIRS (NAAIRS) with endogenous Mre11 silenced by shRNAs were treated with IR (10 Gy 1 h later) or mock treated (right panel). Anti-Myc immunoprecipitation was performed. U2OS cells infected with vector (Vec) were used as a negative control. Immunoprecipitates and cell lysates were immunoblotted with the indicated antibodies. SMC1-S966p and Nbs1-S343p, antibodies that specifically recognize phosphorylated SMC1 at S966 and Nbs1 at S343.

in the ATM/Nbs1 pathway for the S-phase checkpoint (28, 29, 71). IR-induced SMC1 phosphorylation is Nbs1 dependent and is required for mediating the S-phase checkpoint. We showed that although ATM-directed phosphorylation of SMC1 at S966 was impaired in Mre11-deficient cells, it was normal when Mre11-DD and Mre11-NAAIRS mutants were expressed (Fig. 5D, left panel). These data suggest that the interaction between MRN and RPA is not required for IR-induced SMC1 phosphorylation.

The MRN/RPA complex is situated downstream of ATM-dependent phosphorylation of SMC1, or it acts in a separate pathway to mediate the S-phase checkpoint.

MRN is tethered to replication-proximal sites via its interaction with RPA and acts to suppress origin firing upon IR to mediate the S-phase checkpoint. Our studies have shown that the interaction of MRN with RPA is required for the inhibition of DNA synthesis following IR. Previous studies have shown that IR-induced down-regulation of replication is mainly caused by blocking new origin firing whereas fork movement is minimally affected (31, 33, 42). However, the nature of the mechanisms that mediate the inhibition of origin firing during the intra-S-phase checkpoint in mammalian cells is still not clear. We investigated whether the MRN complex and the interaction of MRN with RPA would contribute to the prevention of origin firing after IR. We used a DNA fiber-labeling assay to quantitatively assess replication origin firing before and after IR. ATLD cells complemented with wild-type Mre11, Mre11-NAAIRS, Mre11-DD, or vector were pulse labeled with CldU, a modified nucleotide precursor, and subsequently treated with IR or left untreated followed by pulse labeling with IdU, another nucleotide precursor (Fig. 6A). After immunostaining of chromatin fibers with antibodies recognizing CldU and IdU, DNA that is being actively synthesized during the labeling time is visualized as labeled tracks, which could be interpreted as (i) newly fired origins, (ii) ongoing forks, or (iii) terminating forks. We counted origins fired during IdU labeling in comparison to the total number of forks. As shown in Fig. 6A, the percentage of newly fired origins after IR in the wild-type Mre11-reconstituted ATLD cell line was greatly reduced, suggesting that new origin firing was inhibited following IR. However, the IR-mediated inhibition of new origin firing was severely compromised in the vector and the Mre11-NAAIRSi-expressing and Mre11-DD-expressing ATLD cell lines. These data suggest that Mre11, as well as the interaction of Mre11 with RPA, is required for preventing new origin firing following IR.

A connection of MRN with replication is elucidated by its association with sites near replication origins in the S phase (38). To further explore the mechanism of how MRN prevents new origin firing by way of its interaction with RPA, we determined whether Mre11 binding to RPA is essential for MRN to associate with known replication origins. One possible scenario is that MRN is recruited to origins by RPA and subsequently acts to suppress new origin firing when the intra-S-phase checkpoint is activated. The Myc-tagged Mre11 wild type and the Mre11 mutants (NAAIRRS and DD), as well as Myc-tagged RPA2, were stably expressed at levels similar to those seen with endogenous proteins in T98G cells after retroviral infection (see Fig. S4 in the supplemental material). ChIP was performed 8 h (G_1 phase) or 20 h (S phase) after release of T98G cells from serum starvation. Primers specific to sites of DNA replication initiation, such as those in the B2-lamin gene (2, 4), the HSP70 gene (2, 56), and the c-*myc* gene (38, 58), were used to detect bound DNA. Wild-type Myc-Mre11 bound to the origin-proximal sites specifically in S-phase cells in a manner similar to that seen with Myc-RPA2 (Fig. 6B and data not shown). However, binding of the Mre11 mutants (NAAIRS and DD) defective in RPA binding at origin-proximal sites was dramatically reduced (Fig. 6C). These results suggest that Mre11 largely depends on RPA to associate with sites near DNA replication origins. Since MRN acts to inhibit DNA replication only after IR, we examined whether Mre11 origin binding was altered after IR. ChIP analysis showed that the results with respect to association of Mre11 with origin-proximal sites at HSP70, c-*myc*, and B2-lamin were similar before and after IR (Fig. 6D), as is consistent with the observation that the interaction of MRN and RPA was not altered by IR (Fig. 5C). These data suggest that IR-induced damage signaling does not modulate RPA-mediated recruitment of MRN to replication-proximal sites. The inhibition of replication initiation likely occurs after ATM-phosphorylated MRN is recruited to the origin-proximal sites by RPA (see the model in Fig. 7B and the Discussion).

The RPA-binding mutants of Mre11 exhibit minor radiation sensitivity. Both MRN and RPA are required for DNA repair (40, 66); thus, we investigated whether their interaction might also be needed for damage repair in addition to its role in inhibiting DNA replication after IR. We examined the ability of the reconstituted ATLD cells to survive following IR (Fig. 7A). The cells expressing Mre11-NAAIRS or Mre11-DD were slightly more sensitive to IR than Mre11-expressing cells but much less sensitive than vector-complemented cells. This demonstrates that cell survival following IR is dependent on Mre11 but only minimally dependent on the interaction between Mre11 and RPA. Our results suggest that Mre11, but not the interaction of MRN with RPA, plays a critical role in the DNA repair processes that allow a cell to survive after IR treatment. This result is also consistent with the observation that MRN forms damage foci in a manner independent of its interaction with RPA (Fig. 4B). The slight increase of radiation sensitivity in the RPA-binding mutants of Mre11 is likely caused by the loss of the intra-S-phase checkpoint.

DISCUSSION

MRN plays a critical role in the S-phase checkpoint, but the nature of the underlying mechanism is not clear. Our study demonstrated that MRN is recruited to replication-proximal sites via a direct interaction with RPA and acts there to inhibit DNA replication initiation in response to IR. Since loss of the interaction between Mre11 and RPA leads to a profound RDS phenotype at levels similar to those caused by Mre11 or Nbs1 deficiency, our studies suggest that the recruitment of MRN by RPA to replication-proximal sites is a major mechanism in the ATM-Nbs1 pathway to regulate the S-phase checkpoint.

The interaction of MRN and RPA is cell cycle regulated. A previous study showed that MRN forms S-phase-specific foci colocalized with sites of BrdU incorporation (45). Our studies further demonstrated that MRN also colocalizes with RPA in the S phase, and importantly, that the interaction of RPA with Mre11 is critical for the recruitment of MRN to the replication centers, revealing an important mechanism for MRN for localization to S-phase-specific foci.

The interaction of MRN with RPA appears to be regulated by CDKs. p27 overexpression or roscovitine treatment leads to a decrease in the association between MRN and RPA. Phosphatase treatment abolishes cell-cycle-dependent MRN-RPA association, suggesting that CDK activities are not only required for stimulating but are also important for maintaining

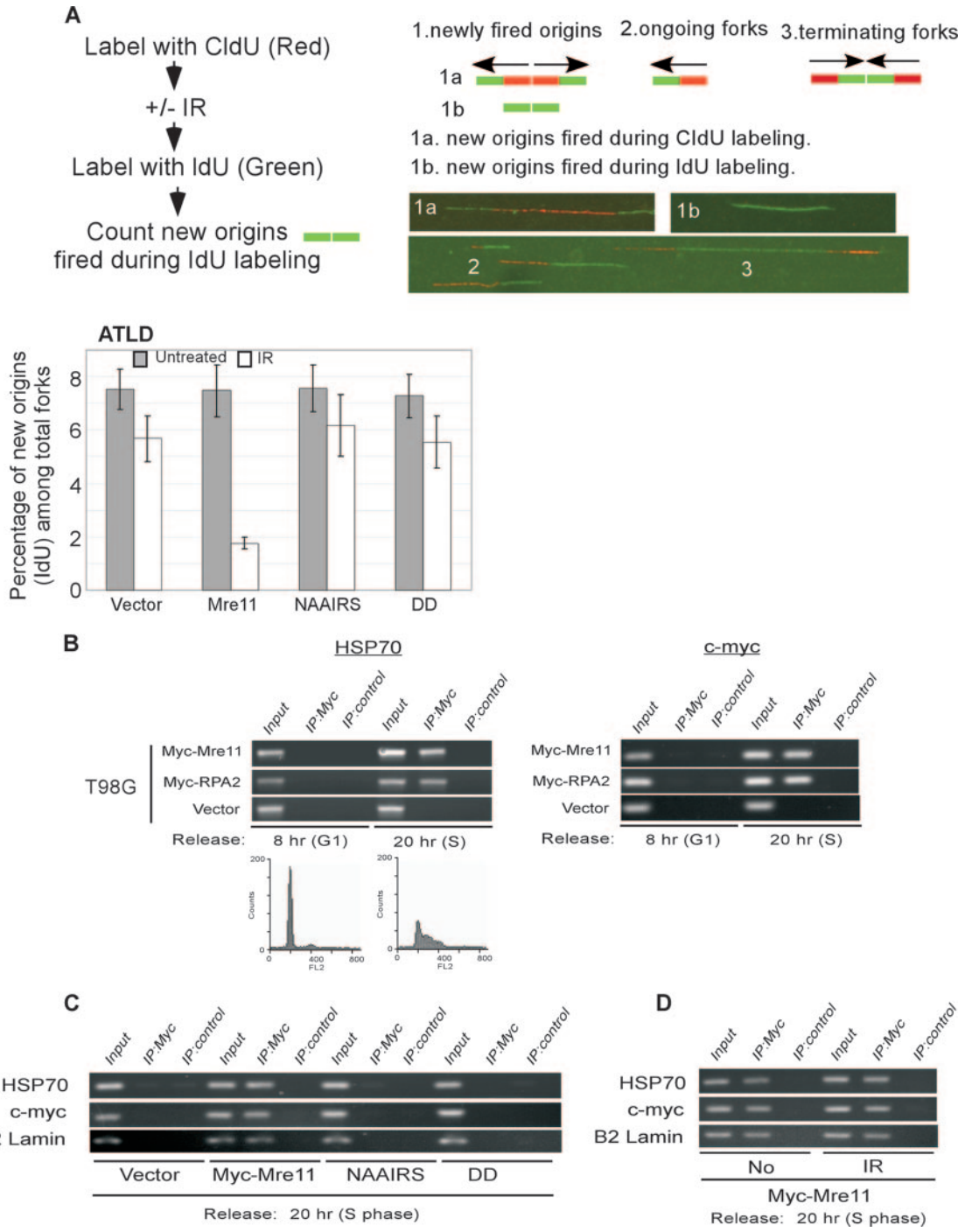

FIG. 6. The recruitment of Mre11 to origin-proximal sites by RPA is required for suppressing replication initiation following IR. (A) Outline of the protocol used to examine new origin firing after IR treatment. ATLD cells expressing wild-type Mre11, Mre11-NAAIRS, Mre11-DD, or vector were labeled with CIdU (10 μM) for 20 min, treated with IR (10 Gy) or left untreated, and labeled again with IdU (15 μM) for 40 min. Examples of labeled replication tracks are shown. The percentage of fired origins after IR treatment was determined for each cell line by normalizing the number of IdU tracks (green) to the number of all labeled tracks counted (>100 in each experiment). The average and standard deviation values were derived from the results of three independent experiments. (B) T98G cells expressing Myc-Mre11 or Myc-RPA2 were synchronized by serum starvation and released into the cell cycle by the addition of 10% FBS into the culture medium. At 8 h (G_1 enriched) and 20 h (S enriched) following release, ChIP analysis was performed using anti-Myc antibody conjugated on protein A agarose (IP:Myc) and a rabbit anti-mouse IgG antibody was used as a negative control (IP:control). PCR of the immunoprecipitates was performed using primers specific to HSP70 and c-*myc* to detect coimmunoprecipitated DNA. Input, 0.1% of the total isolated chromatin. The cell cycle profile was monitored by flow cytometry analysis. (C) T98G cells expressing vector, Myc-tagged Mre11, Mre11-NAAIRS, or Mre11-DD were synchronized by serum starvation. At 20 h after release, when cells entered the S phase, ChIP analysis was performed as described above. PCR of the immunoprecipitates was performed with primers specific to the HSP70, c-*myc*, and B2-lamin origins. (D) At 20 h after release from serum starvation, T98G cells expressing Myc-tagged Mre11 were mock treated (No) or treated with IR (10 Gy 1 h later). ChIP analysis was performed as described above. PCR of the immunoprecipitates was performed using primers specific to the HSP70, c-*myc*, and B2-lamin origins.

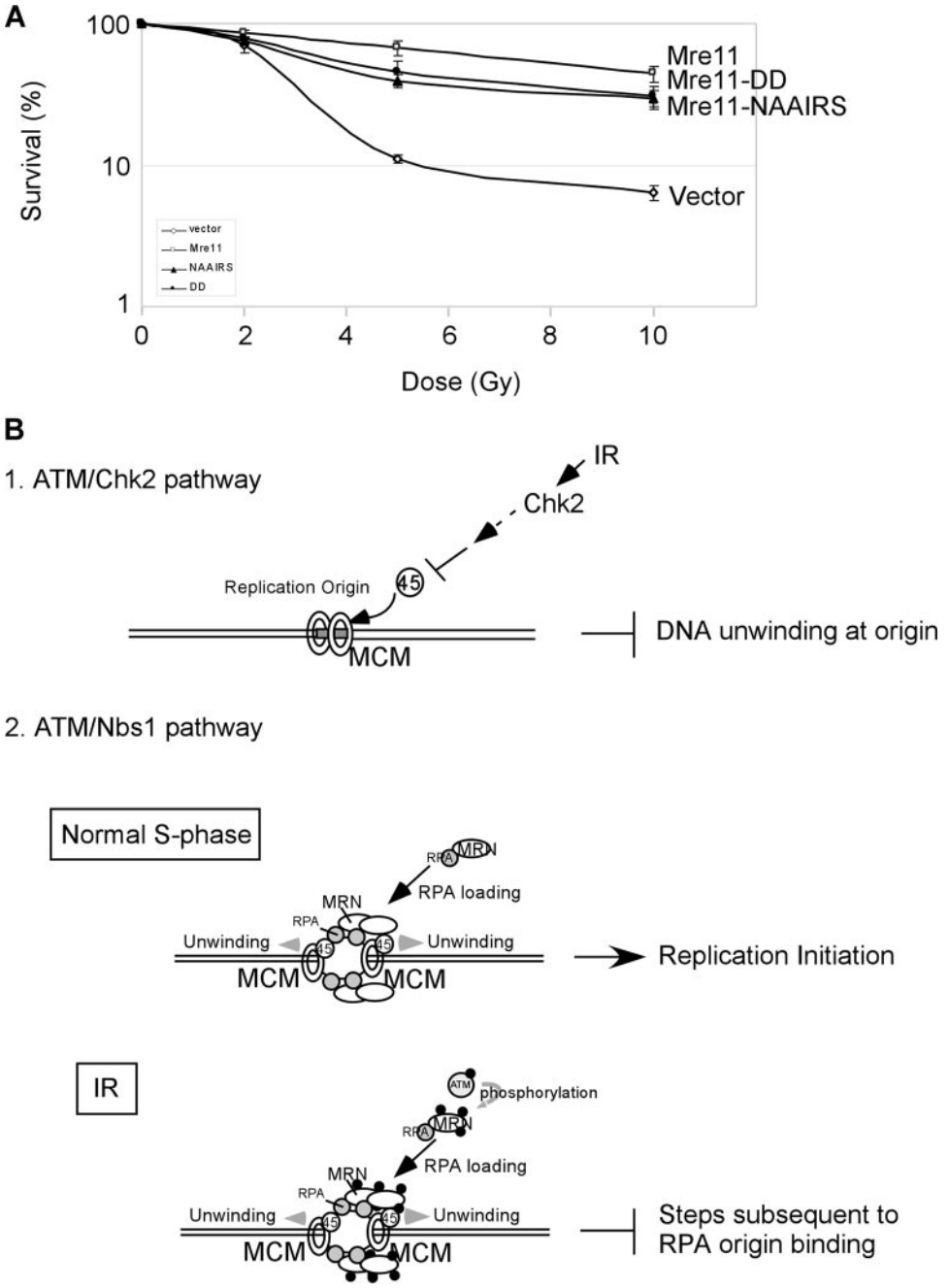

FIG. 7. Radiation sensitivity of RPA-binding mutants of Mre11 and a schematic model of MRN/RPA in the control of S-phase checkpoint. (A) ATLD cells reconstituted with wild-type Mre11, Mre11-NAAIRS, Mre11-DD, or vector were exposed to the indicated doses of IR. Surviving cells were counted 7 days later following trypan blue staining. Error bars represent standard deviations from the average results for three plates for each cell line at each dose of IR. (B) Two distinct pathways (ATM/Chk2 and ATM/Nbs1) are involved in the regulation of the S-phase checkpoint in mammalian cells. In the ATM/Chk2 pathway, Cdc45 chromatin loading is suppressed when ATM is activated, leading to an inhibition of DNA unwinding at origins. In the ATM/Nbs1 pathway, we propose that MRN acts at replication-proximal sites to suppress replication initiation. MRN is recruited by RPA to origins when ssDNA is generated after origin unwinding, and IR does not affect the recruitment process. While MRN at origins in a complex with RPA does not influence replication initiation in an unperturbed cell cycle (normal S phase), ATM-phosphorylated MRN, when recruited by RPA to origins following IR, suppresses replication initiation at steps subsequent to RPA origin binding. The ATM-Chk2 and ATM-Nbs1 pathways target different steps of replication initiation and work together to mediate the S-phase checkpoint. Filled small circles (gray) represent the phosphorylation on ATM and MRN after IR. Open circles containing the number 45 represent Cdc45.

the interaction between MRN and RPA. Active DNA replication is seemingly not the cause for inducing the MRN/RPA interaction, since overexpression of a dominant-negative MCM7 helicase mutant, unlike overexpression of p27, does not lead to an inhibition of the interaction between MRN and RPA, although DNA replication is significantly suppressed. These results disfavor the idea that MRN and RPA association is induced by spontaneous DNA damage occurring during rep-

lication in the S phase. Increased association of MRN and RPA at the G_1-to-S transition and in the S phase is most likely due to a rise in CDK activities. Although MRN and RPA association is regulated during the cell cycle in a phosphorylation-dependent manner, the association remained at similar levels before and after IR. These data suggest that DSBs generated after IR- and ATM-mediated phosphorylation events, including Nbs1 phosphorylation, do not play a major role in regulating the interaction between MRN and RPA.

The interaction of RPA and MRN is direct and mediated by a specific site in Mre11. Since a direct interaction of MRN and RPA can be achieved by using purified Mre11 and RPA expressed in insect cells and bacteria, respectively, it seems that CDK-mediated phosphorylation events stimulate the interaction but are not absolutely required for initiation of the interaction. Interestingly, two DD residues at the RPA-binding site are critical for Mre11 to bind RPA, which is analogous to the requirement of DD/EE residues in conserved motifs in Nbs1, ATRIP, and Ku80 to mediate the interaction of Nbs1/ATM, ATRIP/ATR, and Ku80/DNA-PK (see Fig. S5 in the supplemental material) (14). This raises the possibility that a general mechanism may be involved in mediating protein-protein interactions for DNA damage responses. The nature of the exact mechanism by which CDKs regulate the interaction between MRN and RPA is not clear. The increased association of MRN and RPA could be caused by direct phosphorylation of MRN, RPA, or both by CDKs. Previous studies have shown that RPA is phosphorylated by CDKs at multiple sites during the cell cycle (12, 17, 20). In vivo mass spectrometry analysis also identified phosphorylation of Nbs1 at CDK conserved sites in unperturbed cells (reference 69 and data not shown). CDK activity is important for activation of homologous recombination in the S phase when DSBs are generated (3, 13, 26). Our findings indicating that CDK activity is also required for stimulation of the interaction between MRN and RPA to mediate S-phase checkpoint control suggest an interesting mechanism to couple S-phase checkpoint control with DNA repair.

The interaction of MRN and RPA is essential for mediating the S-phase checkpoint. The ATM/Chk2 and ATM/Nbs1 pathways are two parallel pathways required to mediate the intra-S-phase checkpoint (16). After ATM activation, the ATM/Chk2 pathway inhibits Cdc45 chromatin loading (15), thus preventing MCM-mediated DNA unwinding at origins (Fig. 7B). What is the mechanism by which the ATM/Nbs1 complex inhibits replication after DNA damage? Our studies suggest that when ATM-phosphorylated MRN is recruited to origin-proximal sites by RPA, it acts there to prevent replication initiation.

At the onset of replication initiation, DNA at origins is unwound by MCM proteins, forming single-stranded DNA (ssDNA) that is subsequently bound by RPA (57) and probably at the same time MRN is recruited by RPA to replication origins. This idea is supported by the ChIP analysis results showing that MRN binds to replication origin-proximal sites in a manner similar to that seen with RPA, while Mre11 mutants defective in RPA binding fail to do so.

The ATLD cell line expressing Mre11 mutants defective in RPA binding do not show obvious growth defects, suggesting that the presence of MRN at replication origin-proximal sites in a complex with RPA does not affect DNA replication per se in a normal cell cycle. However, loss of this interaction causes a severe defect in down-regulation of DNA replication following IR. Since MRN/RPA complexes associate with origin-proximal sites similarly before and after IR, damage-induced inhibition of replication initiation likely occurs after RPA origin loading. One possible scenario is that ATM phosphorylates MRN after IR and that it is only when phosphorylated MRN is recruited by RPA to origins that replication initiation is prevented (Fig. 7B).

The phosphorylation of Nbs1 at S343 is critical for the S-phase checkpoint (36, 75). Our studies showed that in the Mre11 mutant cell lines defective in RPA binding, IR-induced Nbs1 phosphorylation at S343 was not affected. Mre11-DD and Mre11-NAAIRS form complexes with phosphorylated Nbs1 (S343) in a manner similar to that seen with wild-type Mre11, but those mutant cell lines exhibit a profound RDS phenotype. These data support the idea that MRN/RPA complexes are a downstream effector for mediation of the S-phase checkpoint after Nbs1 S343 is phosphorylated by ATM; this is consistent with the observation that the association of MRN with RPA is not required for ATM activation. In the ATM/Nbs1 branch of the S-phase checkpoint pathway, SMC1 is downstream of Nbs1 (28, 71). SMC1 phosphorylation is Nbs1 dependent and is important for mediating the S-phase checkpoint. Although IR-induced SMC1 phosphorylation depends on MRN, disruption of the interaction of MRN and RPA does not influence this ATM-mediated SMC1 phosphorylation. This again suggests that the MRN/RPA complex is a downstream effector situated downstream of ATM-directed SMC1 phosphorylation events for mediation of replication inhibition following IR.

It is still unclear what step in DNA replication following RPA loading is disrupted by ATM-mediated phosphorylation of MRN at origin-proximal sites. Given the critical role of RPA in replication initiation, MRN may directly inhibit RPA replication activity to inhibit replication initiation in response to IR. For instance, RPA is required for recruiting DNA polymerase α/primase and is also needed for replication factor C to load PCNA onto primed origins to initiate DNA replication (74). A conformational change of MRN/RPA may be induced by ATM-directed phosphorylation of MRN following IR, which in turn would lead to an inhibition of RPA function in replication initiation. Alternatively, phosphorylation of MRN may interfere with the association of MRN/RPA with other replication factors at origins, causing the suppression of replication initiation. Nevertheless, our results suggest a direct communication of MRN with the cellular replication machinery at replication-proximal sites to mediate the intra-S-phase checkpoint. While the ATM/Chk2 pathway inhibits Cdc45 origin binding before DNA unwinding, the ATM/Nbs1 pathway likely prevents replication initiation after phosphorylated MRN is recruited by RPA to replication origins when RPA binds to ssDNA generated through MCM-mediated DNA unwinding (Fig. 7B). Since inactivation of either of the two pathways (ATM/Chk2 and ATM/Nbs1) leads to a partial RDS phenotype, both of these mechanisms are required for sufficient inactivation of replication initiation following IR.

The MRN/RPA complex and DSB repair. DSB repair via the homologous recombination pathway requires both MRN and RPA complexes (10, 66), which raises a question as to whether

the interaction of MRN/RPA is also involved in mediating the repair process. The radiation survival assay demonstrated that Mre11 deficiency causes severe radiation sensitivity whereas the RPA-binding mutants of Mre11 exhibited only a minor defect. This suggests that the MRN/RPA complex is not involved in the major pathways mediated by MRN for repair of DSBs after IR, although a minor role cannot be excluded. In this aspect, DSB recognition by MRN seemingly does not require its association with RPA, since ATM activation that requires MRN at DSBs is not dependent on the interaction of MRN with RPA. The Mre11 mutants defective in RPA binding also migrate to DSBs to form damage foci similar to those seen with wild-type Mre11. Partial radiation sensitivity of Mre11 mutants defective in RPA binding is likely caused by a severe defect in the S-phase checkpoint, leading to inefficient repair of DSBs generated by IR in the S phase.

MRN forms multiple protein complexes to mediate various cellular functions. Forming MRN/RPA complexes is essential for the S-phase checkpoint. Upon ATM activation, MRN is phosphorylated, conceivably in different protein complexes. Phosphorylated MRN at DSB sites may stimulate the repair process, while phosphorylated MRN/RPA that is recruited to replication-proximal sites acts to prevent origin firing. Through S-phase checkpoint activation, ATM-mediated phosphorylation of MRN may provide an important mechanism to link DNA repair with replication and thus ensure the delay of DNA replication until the completion of DNA repair.

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