Replication Protein A and the Mre11/Rad50/Nbs1 Complex Co-localize and Interact at Sites of Stalled Replication Forks

Jacob G. Robison, James Elliott, Kathleen Dixon and Gregory G. Oakley*

Department of Environmental Health University of Cincinnati College of Medicine
Cincinnati, OH

*Corresponding author. Mailing address: Department of Environmental Health, University of Cincinnati, 3223 Eden Avenue, Cincinnati OH 45267. Phone: (513) 558-5265. Fax: (513) 558-3709. Email: Greg.Oakley@uc.edu

Running Title: RPA and MRN complex interaction

The abbreviations used are: λ.PPase, lambda phosphatase; CIP, calf intestinal phosphatase; DSB, double-strand break; dsDNA, double-stranded DNA; EtBr, ethidium bromide; HU, hydroxyurea; MRN, Mre11/Rad50/Nbs1; RPA, replication protein A; ssDNA, single-stranded DNA; UV, ultraviolet light
SUMMARY

In response to replicative stress, cells relocate and activate DNA repair and cell cycle arrest proteins such as replication protein A (RPA; a three subunit protein complex required for DNA replication and DNA repair) and the MRN complex (consisting of Mre11, Rad50 and Nbs1; involved in DNA double-strand break repair). There is increasing evidence that both of these complexes play a central role in DNA damage recognition, activation of cell cycle checkpoints and DNA repair pathways. Here we demonstrate that RPA and the MRN complex co-localize to discrete foci and interact in response to DNA replication fork blockage induced by hydroxyurea (HU) or ultraviolet light (UV). Members of both RPA and the MRN complexes become phosphorylated during S-phase and in response to replication fork blockage. Analysis of RPA and Mre11 in fractionated lysates (cytoplasmic/nucleoplasmic, chromatin-bound, and nuclear matrix fractions) showed increased hyperphosphorylated-RPA and phosphorylated-Mre11 in the chromatin-bound fractions. HU and UV treatment also led to co-localization of hyperphosphorylated-RPA and Mre11 to discrete detergent-resistant nuclear foci. An interaction between RPA and Mre11 was demonstrated by co-immunoprecipitation of both protein complexes with anti-Mre11, anti-Rad50, anti-NBS1 or anti-RPA antibodies. Phosphatase treatment with calf intestinal phosphatase (CIP) or lambda phosphatase (λPPase) not only de-phosphorylated RPA and Mre11, but also abrogated the ability of RPA and the MRN complex to co-immunoprecipitate. Together, these data demonstrate that RPA and the MRN complex co-localize and interact following HU- or UV-induced replication stress, and suggest that protein phosphorylation may play a role in this interaction.
INTRODUCTION

Stalled replication forks threaten DNA replication fidelity and genomic integrity. Stalled forks occur following deficiencies in replication substrates, inhibition of replication machinery, or blocking of the replication machinery by DNA damage or DNA-protein complexes. Replication fork stalling, with subsequent replication stress, is also thought to occur during normal DNA replication, particularly at DNA sequences that are prone to form secondary structures (e.g. tRNA genes) or in regions where transcription complexes may collide. Failure to resolve this replication stress may result in the collapse of stalled forks and genomic instability. In order to prevent such instability, replication stress triggers the activation of a DNA damage response. This response involves the recruitment and activation of proteins involved in DNA repair and cell cycle regulation. This DNA damage response uses proteins to detect damage, signal the site of damage, and transduce and amplify the signal in order to activate needed effector proteins. Many proteins involved in these three aspects accumulate and form large nuclear foci following DNA damage. Examples of such proteins include γH2AX, 53BP1, ATM, ATR, BRCA1, Werner’s protein, the MRN complex, and RPA. The functions of these foci are not fully understood, but they may represent sites of fork reactivation, protein activation, DNA repair and/or non-repairable damage.

Replication Protein A (RPA), the major single-stranded DNA (ssDNA) binding protein in eukaryotic cells, accumulates along stretches of ssDNA generated by stalled replication forks and/or DNA damage. It has been suggested that the RPA/DNA complex created by the accumulation of RPA at these sites may signal the presence of damage and activate the DNA damage response. In support of this, Dart et al. have shown that recruitment of ATR to nuclear
foci following replication fork stalling is dependent on RPA. Additionally, it has been shown that RPA promotes DNA binding and activation of ATR/ATRIP in vitro. RPA is also required to recruit and activate Rad17 complexes for checkpoint signaling in vivo. Further evidence that RPA acts as a common intermediate for signaling stalled replication forks/DNA damage is demonstrated by the RPA-dependent binding of Cut5 to chromatin following DNA damage and its subsequent recruitment of ATR and DNA polymerase α to chromatin. In that report, Parrilla-Castellar and Karnitz put forth a model suggesting that ssDNA generated at a stalled replication fork is coated with RPA, leading to Cut5 chromatin association. Cut5 then facilitates the chromatin association of ATR and DNA Polα, which, in turn, leads to the loading of the 9-1-1 complex. These data have given credence to the hypothesis that RPA-coated ssDNA acts as a common intermediate for signaling stalled replication forks and/or DNA damage, and subsequent recruitment and activation of DNA damage response proteins. It is likely that RPA plays a dual role in the damage response network: that of a sensor of damage and also as an effector. Replication protein A is an essential component of most, if not all, DNA repair processes.

Petrini and Stracker have postulated that, similar to RPA, the MRN complex acts as a sensor of DNA damage needed to activate the DNA damage response. Recent data suggests that the MRN complex functions as a damage sensor upstream of ATM/ATR activation, in addition to acting as an effector downstream of ATM/ATR in double strand break repair and cell cycle checkpoints. Although it is known that the MRN complex binds to DNA ends, there is no clearly defined mechanism by which the MRN complex recognizes other types of DNA damage.
Since both RPA and the MRN complexes are believed to be involved in the sensing and signaling of DNA damage, we postulated that these two complexes might interact at sites of stalled replication forks and DNA damage. To test this hypothesis, we investigated the interaction of these proteins in response to hydroxyurea- (HU) and ultraviolet light- (UV) induced replication stress and DNA damage. Following treatment with these agents, RPA and Mre11 became phosphorylated and co-localized to discrete chromatin-bound nuclear foci. RPA and the MRN complex also co-immunoprecipitated together, suggesting that these proteins interact, either directly or indirectly. The interaction between RPA and the MRN complex was abrogated by phosphatase treatment. Taken together, our data suggest that following replication stress induced by HU or UV, RPA and the MRN complex co-localized and interacted at sites of stalled replication forks, and that the protein interaction may be mediated, at least in part, by protein phosphorylation.

**EXPERIMENTAL PROCEDURES**

*Cell lines and treatments:* HeLa cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and maintained at 37°C and 5% CO₂ in Dulbecco’s Modified Eagles Medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin-streptomycin (Gibco). For UV exposure, the growth medium was removed (and held at 37°C) and cells were washed with phosphate buffered saline (PBS). The PBS was replaced with minimum essential medium (MEM; Gibco) without phenol red and cells were treated with 30 J/m² UVC (for asynchronous cells) or 20 J/m² (for cells synchronized in S-phase) using a low-pressure mercury lamp (Mineralight lamp; model UVG-11; UVP, Inc., San Gabriel, CA) with a maximal output at 254 nm. Following UV exposure, the
MEM was removed and replaced with the original growth medium, and cells were allowed to recover for 8 h at 37°C before harvesting. For hydroxyurea (HU; Sigma-Aldrich, St. Louis, MO) treatment, cells were incubated in growth medium containing HU (2 mM) for 3 h before harvesting.

*Western Immunoblots:* Cell lysates and immunoprecipitates were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore Corp., Bedford, MA). Membranes were probed using the following primary antibodies: anti-Mre11 (Novus Biological, Littleton, CO; 1:20,000), anti-Mre11 (GeneTex, San Antonio, Texas; 1:10,000), anti-RPA-p34 (Neomarkers, Freemont, CA; 1:5000) and anti-RPA-p34-S^p^4-S^p^8 (Bethyl Laboratories, Inc., Montgomery, TX; 1:10,000). Secondary antibodies were horseradish peroxidase-linked anti-rabbit and anti-mouse (Amersham Biosciences, Buckinghamshire, England; 1:3000), and bound antibodies were visualized using chemiluminescent detection.

*Cell Synchronization:* Cells were synchronized in S-phase and G1-phase of the cell cycle as previously described 21. To synchronize in S-phase, cells were incubated in growth medium containing aphidocolin (final concentration of 1 µM; Sigma-Aldrich) for 15 h. Following incubation, the aphidocolin-containing medium was removed, cells were washed with PBS, and then incubated in fresh medium without aphidocolin for an additional 2 h at 37°C. For nocodazole synchronization in G1- or S-phase of the cell cycle, cells were incubated in medium containing nocodazole (0.3 µM final concentration; Sigma-Aldrich) for 17 h. The mitotic cells, which become detached from the culture dish as they enter M-phase, were collected and pelleted at 500 x g. Mitotic cells were released from nocodazole treatment by incubating in fresh
medium. We have shown previously that HeLa cells enter G1-phase about 5 h after release and enter S-phase about 12 h after release. For the experiments reported here, cells were treated with HU from 4.5 h to 7.5 h after release (for G1-phase) or from 12 h to 15 h after release (for S-phase) and then harvested.

**Subcellular Fractionation:** The cellular protein fractionation protocol was performed as previously described with slight modifications. Briefly, S-phase synchronized HeLa cells were treated with either HU, UV, or mock-treated. The free cytoplasmic/nucleoplasmic fraction was prepared by allowing cells to lyse 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% NP-40; 5 µl/mL pepstatin; 5 µg/mL leupeptin; 5 µg/mL aprotonin; 10 mM NaF; 10 mM β-glycerophosphate; 1 mM Na3VO4; 1 mM PMSF). The insoluble fraction was pelleted by centrifugation at 13,000 x g for 10 min at 4°C and the supernatant (free cytoplasmic/nucleoplasmic; fraction FCN) was collected. The pellet was washed with PBS, and treated with 100 µg/mL DNase I in cell lysis buffer at 37°C for 15 min, followed by addition of ammonium sulfate (250 mM final concentration) and further incubation at room temperature for 10 min. The insoluble fraction was pelleted by centrifugation at 13,000 x g for 10 min at 4°C and the supernatant was collected and designated as the chromatin-bound fraction (fraction CB). The remaining insoluble material was washed with PBS, suspended in SDS buffer (2% SDS; 20 mM Tris-HCl, pH 7.5; 1 M β-mercaptoethanol; 10% glycerol), and designated the nuclear matrix fraction (fraction NM). The presence of glucose-3-phosphate dehydrogenase, Histone H1 and Lamin A/C within the free nucleoplasmic/cytoplasmic fraction, chromatin-bound fraction and nuclear matrix fraction respectively verified the validity of this fractionation method.
Immunofluorescence: Cells were grown on 18 mm or 12 mm coverslips (Becton Dickinson Labware, Bedford, MA) for 20-24 h prior to treatment. Cells were treated with 30 J/m² UVC (asynchronous cells) and allowed to recover for 8 h or with 2 mM HU for 3 h. After treatment, cells were washed with PBS, then washed with PBS containing 0.5% Triton X-100, and fixed for 5 min with PBS containing 3% paraformaldehyde (Fisher Scientific, Hampton, NH). Cells were then blocked for 1 h in PBS containing 15% FBS. Primary antibody dilutions used are as follows: anti-RPA-p34-S⁴-S⁸ 1:2000 (Bethyl Laboratories, Inc.), anti-RPA 1:1000 (Neomarkers), anti-Mre11 1:500 (Novus Biological), anti-Mre11 1:500 (Genetex), anti-γH2AX 1:300 (Upstate Cell Signaling Solutions, Waltham, MA) and anti-Wrn 1:300 (Novus Biological). Secondary antibody dilutions are as follows: anti-rabbit Alexa Fluor 488 1:250 and anti-mouse Alexa Fluor 568 1:250 (Molecular Probes, Eugene, OR). Images were captured with a Nikon inverted fluorescent microscope with attached CCD camera at 100 X magnification and processed using Photoshop 7.0 (Adobe) software.

Co-immunoprecipitation assays: For co-immunoprecipitation reactions, 50 µL of protein G-agarose beads (Invitrogen, Carlsbad, CA) were incubated with 3.0-5.0 µg of anti-Mre11 (Novus Biological), anti-RPA-p70 (Bethyl Laboratories, Inc.), or normal rabbit IgG (Oncogene, San Diego, CA) antibodies in PBS for 1 h at room temperature with end-over-end mixing. Following the addition of approximately 1000 µg of cell lysate, the immunoprecipitation reactions were incubated for 20-24 h at 4°C with end-over-end mixing. The immunoprecipitates were separated from the supernatant by centrifugation and washed with PBS containing 0.05% NP-40. Proteins
were extracted from the agarose beads by resuspending in 1 X Laemmli gel-loading buffer and separated on 12% SDS-polyacrylamide gels.

When antibodies cross-linked to the agarose beads were used, 50 µL of protein G-agarose beads (Invitrogen) were incubated with 3.0 – 5.0 µg of anti-Mre11 (Novus Biological) for 1 h at room temperature with end-over-end mixing. The beads were washed twice with 200 mM triethanolamine (Sigma-Aldrich) and then incubated in 20 mM dimethylpimelimidate (DMP; Sigma-Aldrich) in 200 mM triethanolamine for 30 min at room temperature. The DMP solution was replaced with 50 mM Tris-HCl, pH 7.5 for an additional 15 min. The Tris-HCl was removed and the beads were suspended in PBS and stored at 4°C until addition of cell lysate.

Phosphatase Treatment: For phosphatase treatments of cellular lysates, 10 X NEBuffer 3 (New England BioLabs, Beverly, MA; 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol) was added to approximately 1000 µg total protein to give a final concentration of 1 X NEBuffer. 200 U of calf intestinal alkaline phosphatase (CIP) was added to the mixture and then incubated for 2 h at 37°C. For phosphatase treatment of immunoprecipitates, samples were prepared by washing immunoprecipitates with PBS to remove non-specifically bound proteins and remaining medium. For CIP treatment, the pellets were resuspended in 1X NEBuffer 3 with the addition of 50 U CIP. The reaction mixtures were incubated at 37°C for 2 h before collection of the Mre11-associated pellet and supernatant. For lambda phosphatase (λPPase) treatment, the pellets were resuspended in 1X λPPase buffer (New England Biolabs) with 2 mM MnCl₂ or 1X λPPase buffer with 1 mM Na₂VO₄ and 10 mM NaF (known inhibitors of λPPase). 400 U of λPPase was added to each reaction tube and then incubated at 30°C for 30 min.
RESULTS

Replication stress induces hyperphosphorylation of RPA and phosphorylation of Mre11. Both RPA-p34 and Mre11 are phosphorylated in a DNA damage-dependent and a DNA replication-dependent manner\textsuperscript{23,24}. To verify that phosphorylation of RPA-p34 and Mre11 occurred after exposure of HeLa cells to 2 mM HU for 3 h and 20 J/m\textsuperscript{2} or 30 J/m\textsuperscript{2} UV, whole cell lysates from treated cells were visualized for RPA-p34 and Mre11 via western blotting. Similar to previous reports, these treatments resulted in the hyperphosphorylation of the RPA-p34 subunit and phosphorylation of Mre11 (Figure 1A, lanes 2-3, 5-6; Figure 1B, lanes 2-3, 8-9). When cells were synchronized in S-phase prior to treatment with HU or UV, the proportion of hyperphosphorylated-RPA and phosphorylated-Mre11 increased (Figure 1A, lanes 5-6; Figure 1B, lanes 8-9). This increase in RPA and Mre11 phosphorylation in treated S-phase cells is most likely due to the replication stress induced by these agents. An antibody that recognizes phosphorylated serine 4 and serine 8 of RPA-p34 specifically recognized only the DNA damage-dependent hyperphosphorylated form of RPA-p34 (Figure 1B, lanes 4-6, 10-12). The phosphorylation of RPA and Mre11 suggests that these proteins are involved in the cellular response to replication stress.

Phosphorylated RPA and Mre11 are more tightly bound to the chromatin. It has been previously reported that following DNA damage, RPA becomes re-distributed within the nucleus and is found predominantly bound to the chromatin\textsuperscript{22}. Mre11, which has increased chromatin binding in S-phase, does not show a re-distribution within the nucleus following DNA damage in any stage of the cell cycle\textsuperscript{6,25}. In order to investigate the nuclear re-distribution of the
phosphorylated isoforms of RPA and Mre11 following replication fork stalling and DNA damage, cellular proteins of S-phase synchronized cells were separated into three fractions: (1) free cytoplasmic/nucleoplasmic, (2) chromatin-bound, and (3) nuclear matrix fractions. To confirm the separation of the three fractions, we verified the presence of glucose-3-phosphate dehydrogenase (a cytosolic protein), histone H1 and lamin A/C (a nuclear matrix protein) within the free nucleoplasmic/cytoplasmic fractions, chromatin-bound fractions and nuclear matrix fractions, respectively (data not shown). In mock-treated cells, approximately 60% of the total RPA was in the free cytoplasmic/nucleoplasmic fraction; whereas Mre11 was present in all three fractions, with the chromatin-bound fraction containing the largest percent (Figure 2A, lanes 2-3; Figure 2B). Following HU or UV treatment, there was a redistribution of RPA to the chromatin-bound fraction (Figure 2A, lanes 6-8, 10-12; Figure 2B), with the percent of total RPA changing from 40% chromatin-bound in mock-treated cells to approximately 85% in HU or UV treated cells. As previously reported 22, the damage-induced hyperphosphorylated isoform of RPA was found predominantly in the chromatin-bound fraction. While the distribution of Mre11 between the different fractions did not change after damage, the majority of the damage-induced phosphorylated form of Mre11 was found in the chromatin-bound fraction (Figure 2A, lanes 7, 11; Figure 2B).

RPA and Mre11 co-localize to discrete nuclear foci following HU or UV treatment. Following the induction of DNA damage, many proteins associated with DNA damage signaling, DNA repair and cell cycle checkpoints become localized to sites of damage and form nuclear “foci” 4. In order to visualize these foci and to determine if Mre11 and RPA were present in these “repair foci,” we used immunofluorescent staining. Pre-extraction of cells with a detergent-containing
buffer removes the nucleoplasmic and cytoplasmic proteins, leaving behind the chromatin-bound and matrix-associated proteins \(^6\). In mock-treated cells stained with antibodies against RPA and Mre11, there was a diffuse nuclear staining. Following HU or UV treatment, there was a redistribution of RPA and Mre11 to discrete foci that co-localized (Figure 3A, Panels D, H and L). Although studies have shown that both RPA and the MRN complex are able to form nuclear foci following DNA damage by various genotoxic agents \(^6;7;11;26-29\), no previous reports have indicated that RPA and Mre11 foci co-localize. We also looked for co-localization of Mre11 with hyperphosphorylated-RPA using the RPA-p34-S\(^{\gamma P}4\)-S\(^{\gamma P}8\) phospho-specific antibody. Under mock-treatment conditions, there is no staining with the phospho-specific RPA antibody as expected (Figure 3B, Panel B). Following treatment with HU or UV, similar to staining with the antibody that recognizes all isoforms of RPA, phosphorylated-RPA aggregated into nuclear foci that co-localized with Mre11 (Figure 3B, Panels D, H and L).

In addition to co-localization of RPA and hyperphosphorylated-RPA with Mre11, and similar to previous reports, these foci also showed limited co-localization with phosphorylated histone H2AX (\(\gamma H2AX\)) \(^6;29-31\) and with Werner’s protein (Wrn) (data not shown) \(^5;26\). \(\gamma H2AX\) foci formation is a marker of double-strand breaks (DSBs) and has also been reported to form following replication stress and at replication forks \(^6;8;32\). Wrn protein is a RecQ-class DNA helicase that localizes to sites of stalled replication where it is involved in the resolution and prevention of aberrant recombination events \(^5\), and is able to directly interact with RPA \(^5;33\) and the Mre11 complex \(^34\). Co-localization of hyperphosphorylated-RPA and the MRN complex with \(\gamma H2AX\) and Wrn, along with previous reports \(^5;6\) indicate that these foci are at sites of stalled
replication forks, which at the time points investigated may have progressed to the point of collapse and generation of DSBs.

**RPA and the MRN complex interact.** The observation of co-localization of RPA and the MRN complex raised the possibility that these proteins may directly interact. We performed co-immunoprecipitation experiments using whole cell lysates of S-phase-synchronized HeLa cells from HU, UV or mock-treated cells to probe for such RPA/MRN complex interactions. Rabbit anti-Mre11, anti-Rad50 or anti-Nbs1 antibodies were able to immunoprecipitate RPA, and rabbit anti-RPA-p70 antibodies were able to immunoprecipitate Mre11 (Figure 4A, lanes 7-12; Figure 4B, lanes 3-5; Figure 4C). Normal rabbit IgG did not immunoprecipitate RPA or Mre11 (Figure 4A, lanes 4-6; Figure 4B, lane 6), demonstrating the co-immunoprecipitation of RPA and Mre11 was not due to non-specific antibody binding.

RPA is a very abundant protein in the cell. Therefore we considered the possibility that the apparent co-immunoprecipitation of RPA with Mre11 using anti-Mre11 antibodies could be non-specific and simply due to RPA’s abundance. In addition, the co-immunoprecipitation of Mre11 with RPA using anti-RPA-p34 antibodies was barely detectable and co-immunoprecipitations using anti-RPA-p70 antibodies was present, but at very low levels. To demonstrate that the co-immunoprecipitation of RPA and MRN was specific, we carried out the following experiment. Cell lysates were incubated with anti-Mre11 antibodies cross-linked to protein-G agarose beads, bound protein was eluted with sodium citrate (pH 3.0), neutralized and then immunoprecipitated again with anti-RPA-p70, anti-Mre11 or anti-IgG antibody coated agarose beads. The sequential immunoprecipitations removed the excess RPA from the lysate, allowing for a more
stoichiometrically equal amount of RPA and MRN complex to interact in the second round. The ability of anti-RPA-p70 antibodies to immunoprecipitate Mre11 was increased under these conditions (Figure 4B, lanes 3 and 4), confirming the specificity of the interaction.

In addition to co-immunoprecipitation of RPA and MRN in lysates from damaged cells, co-immunoprecipitation was also observed in lysates from mock-treated cells (Figure 4A, lane 7; Figure 4B, lane 3). This suggested that the interaction might not be dependent on DNA damage. It has been shown that the MRN complex is required for the resolution of breaks that occur spontaneously during DNA replication 24, so we considered the possibility that the co-immunoprecipitation in the mock-treated samples might be an S-phase phenomenon due to normal replication. To investigate this possibility, immunoprecipitation reactions were done using whole cell lysates from mock-treated and HU-treated cells synchronized in G1-phase or S-phase of the cell cycle. The amount of RPA that co-immunoprecipitated with Mre11 in mock-treated cells in S-phase of the cell cycle was six-times more than mock-treated G1-phase cells as measured by densitometry (Figure 4D, lanes 1 and 3). Following HU treatment, the amount of immunoprecipitated RPA increased eight-fold when comparing S-phase to G1-phase. (Figure 4D, lanes 2 and 4). This suggests that the interaction between RPA and the MRN complex, while present in both G1- and S-phase of the cell cycle, is increased when cells are in S-phase, and increased to a greater extent following damage in S-phase. The increase in interaction in mock-treated cells in S-phase may represent interactions that occur at spontaneously stalled replication forks during normal DNA replication.
We also considered the possibility of an indirect interaction between RPA and the MRN complex mediated by independent binding of both protein complexes to DNA. To test this possibility, we pre-treated lysates with ethidium bromide (EtBr) or DNase I, or treated immunoprecipitates with DNase I. EtBr is a DNA intercalator known to disrupt DNA-protein interactions. Pretreatment of lysates with 50 µg/mL EtBr did not alter the ability of anti-Mre11 antibodies to immunoprecipitate RPA (Figure 4E, lane 9). DNase I treatment of lysates or immunoprecipitates, using the same conditions as the sub-cellular fractionation protocol, did not affect the RPA/MRN complex interaction as well (Figure 4E, lanes 3 and 6 respectively), suggesting this interaction is not indirect via DNA-protein interactions.

**Phosphatase treatment abrogates the RPA/MRN complex interaction.** The Nbs1 protein of the MRN complex contains two domains associated with protein-protein interactions, the forkhead-associated domain (FHA) and the BRCA1 carboxy-terminal domain (BRCT). FHA and BRCT domains are both known to mediate protein-protein interactions in a phosphorylation-dependent manner, which is demonstrated in the interaction of Nbs1 with γH2AX and BRCA1 with BACH1. Since increased RPA/MRN complex interactions occur under conditions of increased RPA-p34 phosphorylation, specifically RPA hyperphosphorylation, we wanted to investigate if protein phosphorylation played a part in the RPA/MRN complex interaction. To address this question, whole cell lysates from HU-treated cells were incubated for 2 h at 37°C with or without calf intestinal phosphatase (CIP) before incubation with anti-Mre11 antibody coated agarose beads. Pre-treatment with CIP abrogated the ability of anti-Mre11 antibodies to immunoprecipitate RPA (Figure 5A, lane 3), while the 2 h incubation at 37°C had no effect (Figure 5B, lane 2). To verify that the CIP treatment resulted in protein de-
phosphorylation, the supernatant from the CIP treated sample was analyzed for RPA-p34. Loss of the hyper-phosphorylated form of RPA-p34 and retention of non-phosphorylated RPA (Figure 5B, lane 4) indicated that protein de-phosphorylation had occurred.

To continue investigating the effect of phosphatase activity on the RPA/MRN complex interaction, we immunoprecipitated proteins from whole cell lysates of HU-treated cells with anti-Mre11 antibody coated beads. The resulting immunoprecipitate treated with CIP demonstrated a loss of RPA in the Mre11-associated pellet and appearance of non-phosphorylated RPA in the supernatant (Figure 5B, lanes 2 and 3).

To verify that the abrogation of the RPA/MRN interaction was dependent upon phosphatase activity and not just the physical presence of the phosphatase enzymes, we used another phosphatase, lambda phosphatase (λPPase), and specific phosphatase inhibitors. Immunoprecipitates from HU-treated lysates immunoprecipitated with anti-Mre11 antibodies were treated with lambda phosphatase (λPPase) with (Figure 5C, lanes 5 and 6) and without the presence of specific λPPase inhibitors (sodium orthovanadate and sodium fluoride; Figure 5C, lanes 3 and 4). The addition of the λPPase inhibitors preserved the RPA/MRN complex interaction (and the presence of phosphorylated-RPA) with the anti-Mre11 antibody coated beads (Figure 5C, lanes 5 and 6), suggesting that the abrogation of the RPA/MRN complex interaction was indeed dependent upon phosphatase activity. Together, these data suggest that the RPA/MRN complex interaction may be mediated by protein phosphorylation. These data, however do not address whether the abrogation of the RPA/MRN complex interaction is due to de-phosphorylation of RPA, the MRN complex or some other protein.
DISCUSSION

The results presented here offer insight into the role of RPA and the MRN complex in the replicative stress-induced DNA damage response. Upon treatment with HU or UV, RPA becomes hyperphosphorylated and Mre11 is phosphorylated. The relative proportion of the phosphorylated isoforms of RPA and Mre11 were increased when cells were synchronized in S-phase of the cell cycle prior to treatment. The phosphorylated isoforms of RPA and Mre11 were chromatin-bound; most likely at sites of stalled and/or collapsed replication forks.

While redistribution of phosphorylated RPA to chromatin-bound fractions has been previously reported, this may seem to be in contradiction to reports that show phosphorylated RPA has a decreased affinity for double-stranded DNA (dsDNA). Two possible explanations may account for this apparent discrepancy. First, stalled replication forks lead to the generation of large regions of ssDNA. While phosphorylated RPA has decreased affinity for dsDNA, it shows no difference in ssDNA binding activity. Alternatively, following replication fork stalling and/or DNA damage, RPA may have increased interactions with a protein that is itself associated with the chromatin (possibly the MRN complex or some other, as yet, unidentified protein). Either scenario could explain the increased chromatin-association of phosphorylated RPA we observed following HU or UV treatment.

Consistent with a previous report showing constitutive chromatin association of the MRN complex, we saw similar amounts of Mre11 in the chromatin-bound fraction in mock-treated, HU and UV treated samples. This is also consistent with a report that shows increased chromatin-association of the MRN complex in S-phase as compared to G1 or G2/M, but no change in S-phase chromatin-association with or without damage. Interestingly, the damage-
dependent phosphorylated Mre11 is predominantly contained within the chromatin-bound Mre11 pool. These data suggest that phosphorylated Mre11 may have increased DNA binding affinity or that Mre11 may have to be chromatin-bound to be phosphorylated.

While studies have shown that both RPA and MRN form nuclear foci following treatment with a variety of genotoxic agents, no one has previously reported that these foci co-localize. Mre11 foci formation following UV-induced damage has been reported to occur only in xeroderma pigmentosum variant (XPV) cells, and not in ‘normal’ cells. We observed UV-induced Mre11 foci in both HeLa and a normal-telomerase transformed cell line (data not shown) 8 h following UV irradiation. It has recently been reported that the large T-antigen in SV40 transformed cell lines disturbs the formation of Mre11 nuclear foci. While previous studies that reported Mre11 foci only in XPV cell lines investigated similar time points and doses of UV, their use of SV40 transformed cells may explain the discrepancies with our results.

Both RPA and the MRN complex are known to bind DNA and interact with numerous other proteins. However, our data demonstrate that DNA binding is not required for the RPA/MRN complex interaction. In addition, we observed that protein phosphorylation enhanced RPA/MRN complex interaction, similar to the reported phosphorylation-enhanced BRAC1/BACH1 interaction.

Our data, as well as previous reports, have led to our proposed model of events associated with stalled replication forks (Figure 6). RPA and the MRN complex are normally associated with replication forks during S-phase. Stalled replication forks generate stretches of ssDNA
that become coated with RPA, leading to the recruitment and activation of ATR/ATRIP\textsuperscript{12;13}, and possibly ATM\textsuperscript{12}. The close proximity of these proteins at stalled forks would allow for ATR/ATM phosphorylation of RPA and the MRN complex. This enhances recruitment of additional MRN complex and phospho-dependent RPA/MRN interaction. Phosphorylation most likely has functional consequences in addition to altering protein interactions. For example, phosphorylation of Mre11 is thought to increase its nuclease activity\textsuperscript{24}, which could increase DNA processing necessary for resolution of the stalled fork. Phosphorylation of RPA is known to decrease its unwinding or destabilization activity of dsDNA but does not effect ssDNA binding ability\textsuperscript{41;42}. This may help prevent further migration of the replication fork and provide the opportunity for further protein recruitment. The recruited proteins, RPA/ATR/ATRIP/Mre11/Rad50/Nbs1 (and possibly ATM), may then initiate DNA repair and act as a scaffold to activate other proteins such as Rad17, Rad9-Rad1-Hus1 or Wrn\textsuperscript{5;14}. Possible outcomes may include cell cycle arrest, resolution of Holliday junctions and continuation of replication, non-homologous end joining (NHEJ)-mediated repair of subsequent DSBs, or recombination-like repair of subsequent DSBs. This model suggests that RPA and the MRN complex work together at sites of stalled or collapsed replication forks, and that this cooperative interaction occurs via MRN’s ability to interact with RPA in a phosphorylation-dependent manner.

In this study, a single time point for each DNA-damaging agent was selected based on peak incidence of RPA-p34 hyperphosphorylation (data not shown). Future work needs to investigate additional time points to determine the exact role of RPA and MRN in the replication stress-induced damage response, and if additional proteins mediate the RPA/MRN complex interaction.
ACKNOWLEDGEMENTS

We thank Dr. John Bissler for critical review of the manuscript. This work was supported by grants R01-NS34782, P30-ES06096 and T32-ES07250 from the National Institutes of Health.

REFERENCES

1. Rothstein, R., Michel, B., and Gangloff, S. (2000) *Genes Dev.* **14**, 1-10
2. Bierne, H. and Michel, B. (1994) *Mol.Microbiol.* **13**, 17-23
3. Michel, B., Ehrlich, S. D., and Uzest, M. (1997) *EMBO J.* **16**, 430-438
4. Rouse, J. and Jackson, S. P. (2002) *Science* **297**, 547-551
5. Constantinou, A., Tarsounas, M., Karow, J. K., Brosh, R. M., Bohr, V. A., Hickson, I. D., and West, S. C. (2000) *EMBO Rep.* **1**, 80-84
6. Mirzoeva, O. K. and Petrini, J. H. (2003) *Mol.Cancer Res.* **1**, 207-218
7. Maser, R. S., Monsen, K. J., Nelms, B. E., and Petrini, J. H. (1997) *Mol.Cell Biol* **17**, 6087-6096
8. Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J., and Qin, J. (2000) *Genes Dev.* **14**, 927-939
9. Mirzoeva, O. K. and Petrini, J. H. (2001) *Mol.Cell Biol* **21**, 281-288
10. Wold, M. S. (1997) *Annu.Rev.Biochem.* **66**, 61-92
11. Raderschall, E., Golub, E. I., and Haaf, T. (1999) *Proc.Natl.Acad.Sci.U.S.A* **96**, 1921-1926
12. Dart, D. A., Adams, K. E., Akerman, I., and Lakin, N. D. (2004) *J.Biol Chem.* **279**, 16433-16440
13. Zou, L. and Elledge, S. J. (2003) *Science* **300**, 1542-1548
14. Zou, L., Liu, D., and Elledge, S. J. (2003) *Proc. Natl. Acad. Sci. U.S.A* **100**, 13827-13832

15. Parrilla-Castellar, E. R. and Karnitz, L. M. (2003) *J. Biol. Chem.* **278**, 45507-45511

16. Petrini, J. H. and Stracker, T. H. (2003) *Trends Cell Biol* **13**, 458-462

17. Petrini, J. H., Huwiler, K. G., and Weaver, D. T. (1991) *Proc. Natl. Acad. Sci. U.S.A* **88**, 7615-7619

18. Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L., and Shiloh, Y. (2003) *EMBO J.* **22**, 5612-5621

19. Carson, C. T., Schwartz, R. A., Stracker, T. H., Lilley, C. E., Lee, D. V., and Weitzman, M. D. (2003) *EMBO J.* **22**, 6610-6620

20. Mochan, T. A., Venere, M., DiTullio, R. A., Jr., and Halazonetis, T. D. (2003) *Cancer Res.* **63**, 8586-8591

21. Oakley, G. G., Loberg, L. I., Yao, J., Risinger, M. A., Yunker, R. L., Zernik-Kobak, M., Khanna, K. K., Lavin, M. F., Carty, M. P., and Dixon, K. (2001) *Mol. Biol. Cell* **12**, 1199-1213

22. Liu, J. S., Kuo, S. R., Yin, X., Beerman, T. A., and Melendy, T. (2001) *Biochemistry* **40**, 14661-14668

23. Carty, M. P., Zernik-Kobak, M., McGrath, S., and Dixon, K. (1994) *EMBO J.* **13**, 2114-2123

24. Costanzo, V., Robertson, K., Bibikova, M., Kim, E., Grieco, D., Gottesman, M., Carroll, D., and Gautier, J. (2001) *Mol. Cell* **8**, 137-147

25. Zhao, S., Renthal, W., and Lee, E. Y. (2002) *Nucleic Acids Res.* **30**, 4815-4822

26. Sakamoto, S., Nishikawa, K., Heo, S. J., Goto, M., Furuichi, Y., and Shimamoto, A. (2001) *Genes Cells* **6**, 421-430
27. Golub, E. I., Gupta, R. C., Haaf, T., Wold, M. S., and Radding, C. M. (1998) *Nucleic Acids Res.* **26**, 5388-5393

28. Vassin, V. M., Wold, M. S., and Borowiec, J. A. (2004) *Mol. Cell Biol* **24**, 1930-1943

29. Limoli, C. L., Giedzinski, E., Bonner, W. M., and Cleaver, J. E. (2002) *Proc. Natl. Acad. Sci. U.S.A* **99**, 233-238

30. Kobayashi, J., Tauchi, H., Sakamoto, S., Nakamura, A., Morishima, K., Matsuura, S., Kobayashi, T., Tamai, K., Tanimoto, K., and Komatsu, K. (2002) *Curr. Biol* **12**, 1846-1851

31. Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgesner, C. U., Gellert, M., and Bonner, W. M. (2000) *Curr. Biol* **10**, 886-895

32. Ward, I. M. and Chen, J. (2001) *J. Biol. Chem.* **276**, 47759-47762

33. Shen, J. and Loeb, L. A. (2001) *Mech. Ageing Dev.* **122**, 921-944

34. Cheng, W. H., Von Kobbe, C., Opresko, P. L., Arthur, L. M., Komatsu, K., Seidman, M. M., Carney, J. P., and Bohr, V. A. (2004) *J. Biol. Chem.* **279**, 21169-21176

35. Schroter, H., Maier, G., Ponstingl, H., and Nordheim, A. (1985) *EMBO J.* **4**, 3867-3872

36. Lai, J. S. and Herr, W. (1992) *Proc. Natl. Acad. Sci. U.S.A* **89**, 6958-6962

37. Varon, R., Vissinga, C., Platzer, M., Ceresaletti, K. M., Chrzanowska, K. H., Saar, K., Beckmann, G., Seemanova, E., Cooper, P. R., Nowak, N. J., Stumm, M., Weemaes, C. M., Gatti, R. A., Wilson, R. K., Digweed, M., Rosenthal, A., Sperling, K., Concannon, P., and Reis, A. (1998) *Cell* **93**, 467-476

38. Bork, P., Hofmann, K., Bucher, P., Neuwald, A. F., Altschul, S. F., and Koonin, E. V. (1997) *FASEB J.* **11**, 68-76

39. Durocher, D., Henckel, J., Fersht, A. R., and Jackson, S. P. (1999) *Mol. Cell* **4**, 387-394

40. Yu, X., Chini, C. C., He, M., Mer, G., and Chen, J. (2003) *Science* **302**, 639-642
41. Oakley, G. G., Patrick, S. M., Yao, J., Carty, M. P., Turchi, J. J., and Dixon, K. (2003)

*Biochemistry* **42**, 3255-3264

42. Binz, S. K., Lao, Y., Lowry, D. F., and Wold, M. S. (2003) *J. Biol. Chem.* **278**, 35584-35591

43. Limoli, C. L., Giedzinski, E., Morgan, W. F., and Cleaver, J. E. (2000)

*Proc. Natl. Acad. Sci. U.S.A* **97**, 7939-7946

44. Digweed, M., Demuth, I., Rothe, S., Scholz, R., Jordan, A., Grotzinger, C., Schindler, D.,
Grompe, M., and Sperling, K. (2002) *Oncogene* **21**, 4873-4878

45. Canman, C. E. and Lim, D. S. (1998) *Oncogene* **17**, 3301-3308

46. Maser, R. S., Mirzoeva, O. K., Wells, J., Olivares, H., Williams, B. R., Zinkel, R. A.,
Farnham, P. J., and Petrini, J. H. (2001) *Mol. Cell Biol* **21**, 6006-6016
FIGURE LEGENDS

Fig. 1. Phosphorylation of RPA and Mre11 following HU and UV treatment. **Asynchronous:** Asynchronous, sub-confluent HeLa cells were treated with 2 mM HU for 3 h; or 30 J/m² UV with 8 h recovery. **S-Phase:** HeLa cells synchronized with aphidicolin (1 µM, 15 h) and allowed to enter S-phase were treated with 2 mM HU for 3 h; or 20 J/m² UV with 8 h recovery. Whole cell lysates prepared from these cells were separated on a 12% SDS-PAGE gel. **A,** Mre11 was visualized with a polyclonal antibody against Mre11. Lanes 1 and 4, no treatment; lanes 2 and 6, UV treated; lanes 3 and 5, HU treated. **B,** RPA-p34 was visualized using a monoclonal antibody against RPA-p34 (lanes 1-3, 7-9) or a polyclonal antibody specific for RPA-p34 phosphorylated on serine 4 and serine 8 (anti-RPA-S⁴-S⁸, lanes 4-6 and 10-12). At least five different forms of the p34 subunit of RPA can be visualized; form 1 is the fastest migrating band and is unphosphorylated, while form 5 is the slowest migrating band and is the DNA damage-induced hyperphosphorylated form.

Fig. 2. Phosphorylated RPA and Mre11 are chromatin-bound. HeLa cells synchronized with aphidicolin (1 µM, 15 h) and allowed to enter S-phase were treated with 2 mM HU for 3 h, 20 J/m² UV with 8 h recovery, or mock-treated. **A,** Whole cell lysates (WC; lanes 1, 5 and 9) and fractionated extracts were prepared as described in “Experimental Procedures.” Three different fractions were obtained: free cytoplasmic/nucleoplasmic (Fraction FCN, lanes 2, 6 and 10), chromatin-bound (Fraction CB, lanes 3, 7 and 11) and nuclear matrix fractions (Fraction NM, lanes 4, 8 and 12). Whole cell extracts and cell fractions were separated on a 12% SDS-PAGE gel and visualized with anti-Mre11 and anti-RPA antibodies. **B,** Densitometry measurements of the western blot depicted in panel A. The numbers reported are: percent of total Mre11 or RPA-
p34 protein for each treatment group in the indicated lane, the ratio of the phosphorylated to non-phosphorylated form of Mre11 (form 2/form 1) for each lane, or the ratio of the hyper-phosphorylated to non-phosphorylated form of RPA-p34 (form 5/form 1) for each lane.

Fig. 3. **RPA and Mre11 co-localize to discrete nuclear foci following HU and UV treatment.**
Asynchronous HeLa cells were treated with 2 mM HU for 3 h, 30 J/m^2 UV and allowed 8 h to recover or mock-treated. Following extraction of cytoplasmic and nucleoplasmic proteins with PBS containing 0.5% Triton X-100, cells were fixed in paraformaldehyde, incubated in primary and secondary antibodies, and visualized by fluorescent microscopy. **A**, RPA-p34 and Mre11 co-localize to HU- and UV- induced nuclear foci. Cells were stained with anti-Mre11 antibodies (Green, Panels B, F and J) and anti-RPA-p34 antibodies (Red, Panels C, G and K). Panels A, E and I are the DAPI-stained nuclei, and Panels D, H and L are the merged images of the anti-Mre11 and anti-RPA stained cells. **B**, Hyperphosphorylated-RPA-p34 and Mre11 co-localize to HU- and UV- induced nuclear foci. Cells were stained with damage-induced hyperphosphorylation specific anti-RPA-p34-S^4^S^8^ antibodies (Green, Panels B, F and I) and anti-Mre11 antibodies (Red, Panels C, G and K). Panels A, E and I are the DAPI-stained nuclei, and Panels D, H and L are the merged images of the anti-RPA-p34-S^4^S^8^ and anti-Mre11 stained cells.

Fig. 4. **Co-immunoprecipitation of RPA and Mre11.** **A**, HeLa cells synchronized with aphidicolin (1 µM, 15 h) and allowed to enter S-phase were treated with 2 mM HU for 3 h, 20 J/m^2 UV and allowed 8 h to recover, or mock-treated. Whole cell lysates were incubated with agarose beads coated with non-specific anti-rabbit IgG (lanes 4-6), anti-Mre11 (lanes 7-9) or
anti-RPA-p70 (lanes 10-12) antibodies for 20-24 h. 10% of whole cell lysate volumes used for IP reactions were included as loading controls (Input, lanes 1-3). Proteins from the immunoprecipitates were detected by western blotting using anti-RPA-p34 and anti-Mre11 antibodies. **B**, S-phase synchronized HeLa cells were either treated with 2 mM HU for 3 h (lanes 2, 4-6) or mock-treated (lanes 1 and 3). Whole cell lysates were incubated with agarose beads cross-linked with anti-Mre11 antibodies as the primary IP. The proteins were eluted from the beads using sodium citrate and then incubated with anti-RPA-p70 (lanes 3 and 4), anti-Mre11 (lane 5) or anti-IgG (lane 6) antibody coated agarose beads for the secondary IP. The proteins were eluted from the second set of antibody-coated beads with Laemmli loading buffer and separated on a 12% SDS-PAGE gel. Proteins were visualized by western blotting using anti-RPA-p34 and anti-Mre11 antibodies. **C**, S-phase synchronized HeLa cells were treated with 2 mM HU for 3 h. Whole cell lysates were incubated with agarose beads coated with anti-Mre11, anti-Rad50 or anti-Nbs1 antibodies. The immunoprecipitated proteins were eluted with Laemmli loading buffer and subjected to western blotting using anti-RPA-p34 antibodies. **D**, HeLa cells were synchronized in M-phase of the cell cycle with nocodazole and allowed time to enter G1-phase (lanes 1 and 2) or S-phase of the cell cycle with aphidicolin (lanes 3 and 4) as described in “Experimental Procedures.” Cells were either mock-treated or treated with 2 mM HU for 3 h before harvesting and whole cell lysate formation. Lysates were incubated with anti-Mre11 antibody coated agarose beads and eluted off using Laemmli gel loading buffer. The immunoprecipitated proteins were subjected to western blotting using anti-RPA-p34 and anti-Mre11 antibodies. **E**, S-phase synchronized HeLa cells were treated with 2 mM HU for 3 h. Whole cell lysates were either treated with 100 µg/ml DNase I for 20 min at 37°C (lane 3) or 50 µg/ml ethidium bromide (EtBr) on ice for 30 min (lane 9). Pre-treated lysates, lysates without
DNase or EtBr incubated under similar conditions (lanes 2 and 8), as well as lysates with no previous incubation were immunoprecipitated with anti-Mre11 antibody coated agarose beads. The two IP reactions with non-treated lysates were washed with PBS and incubated for 20 min at 37°C with or without DNase I in cell lysis buffer (100 µg/ml, lanes 5 and 6 respectively). Proteins from all the immunoprecipitation reactions were eluted from the beads with Laemmli gel loading buffer and separated on 12% SDS gels. The immunoprecipitated proteins were subjected to western blotting using anti-RPA-p34 antibodies.

Fig. 5. **Phosphatase treatment disrupts the RPA/MRN complex interaction.**

**A**, Lysates from S-phase synchronized HeLa cells subjected to 2 mM HU were either treated with calf intestinal phosphatase (CIP) prior to incubation with anti-Mre11 antibody coated agarose beads (lane 3) or mock treated (lane 2). 10% of the whole cell lysate was used as a loading control (lane 1) and 10% of the supernatant (S) from the IP of the CIP treated lysate was loaded to verify protein dephosphorylation (lane 4).

**B**, Whole cell lysates from S-phase synchronized HeLa cells treated with 2 mM HU for 3 h were immunoprecipitated with anti-Mre11 antibody coated agarose beads. The immunoprecipitates were incubated with (lane 2) or without CIP (lane 1) and the proteins associated with the anti-Mre11 pellet (P) and the supernatant (S) (lane 3) were analyzed by western blotting with anti-RPA-p34 antibodies.

**C**, S-phase synchronized HeLa cells treated with 2 mM HU were incubated with anti-Mre11 antibody coated agarose beads. Immunoprecipitate pellets were washed with PBS and resuspended in phosphatase buffer with (lanes 3-6) or without λ phosphatase (lanes 1 and 2) or specific phosphatase inhibitors (lanes 5 and 6) as indicated. Samples were incubated at 30°C for 30 min and the immunoprecipitate pellets (P) and supernatants (S) separated by centrifugation and subjected to western blotting.
using anti-RPA-p34 antibodies. Whole cell lysate (10% of total protein used in IP reaction) was included as a loading control (Input, lane 1).

Fig. 6. Model of the interaction of RPA and the MRN complex in response to stalled replication forks (see “Discussion” for details).
### Figure 1

#### A.

|          | Asynchronous | S-Phase |
|----------|--------------|---------|
| HU       | –            | –       |
| UV       | –            | +       |

**Mre11**

- 1
- 2
- 3
- 4
- 5
- 6

- Phosphorylated Mre11

#### B.

|          | Anti-RPA     | Anti-RPA-S\(^{\text{p}}\)4-S\(^{\text{s}}\)8 |
|----------|--------------|---------------------------------------------|
| HU       | –            | –                                           |
| UV       | –            | +                                           |

**RPA-p34**

- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10
- 11
- 12

- Hyperphosphorylated RPA-p34
Figure 2

A.

| Fraction | WC | FCN | CB | NM |
|----------|----|-----|----|----|
| Mock-Treated | | | | |
| HU | | | | |
| UV | | | | |

B.

| Lane | Mock-Treated | HU | UV |
|------|--------------|----|----|
| 2    | 27           | 61 | 61 |
| 3    | 59           | 39 | 39 |
| 4    | 14           | 0  | 0  |
| 6    | 26           | 19 | 13 |
| 7    | 59           | 81 | 87 |
| 8    | 15           | 0  | 0  |
| 10   | 26           | 13 | 1.4|
| 11   | 67           | 87 | 9.2|
| 12   | 67           | 0  | 0  |
Figure 3

A.  

Control

+HU

+UV

B.  

Control

+HU

+UV
Figure 4

A. Input (10%)  |  IP-IgG  |  IP-Mre11  |  IP-RPA-p70

| HU | UV | HU | UV | HU | UV |
|---|---|---|---|---|---|
| - | - | + | + | - | - |

Mre11

RPA

B. Input (10%)  |  1st IP-Mre11  |  2nd IP

| HU | UV | HU | UV | 1st IP-Mre11 | 2nd IP |
|---|---|---|---|-------------|--------|
| - | - | + | + | + | + |

Mre11

RPA

C. IP-Mre11  |  IP-Rad50  |  IP-Nbs1

| HU | HU | HU |
|---|---|---|
| + | + | + |

Mre11

RPA

D. G1-Phase  |  S-Phase

| HU | HU |
|---|---|
| - | + |

Mre11

RPA
Figure 4

E.

| Input | IP-Mre11 | Input | IP-Mre11 | Input | IP-Mre11 |
|-------|----------|-------|----------|-------|----------|
| HU    | +        | +     | +        | +     | +        |
| DNase | -        | -     | +        | -     | +        |
| EtBr  | -        | -     | -        | -     | -        |

RPA

1 2 3 4 5 6 7 8 9
Figure 5

A.

| Input  | IP-Mre11 |
|--------|----------|
| CIP    | -        |
| -      | -        |
| +      | +        |
| +      | CIP      |

Mre11

RPA

B.

| Input  | IP-Mre11 |
|--------|----------|
| CIP    | +        |
| -      | P        |
| +      | P        |

RPA

C.

| Input  | IP-Mre11 |
|--------|----------|
| λ Phosphatase PPase Inhibitors | - |
| -      | -        |
| +      | +        |

RPA

Downloaded from http://www.jbc.org/ on March 20, 2020
Normal Replication Fork

Recruitment and activation of ATR/ATRIP

Phosphorylation of RPA and the MRN complex

Recruitment of additional MRN complex and other factors (e.g. Rad17, Rad9-Rad1-Hus1 complex, Wrn…)

Phosphorylation-dependent interaction of RPA and the MRN complex

Checkpoint activation

Resolution of Holliday junctions and continued replication

NHEJ-mediated repair of subsequent DSBs

Recombination-like repair of subsequent DSBs (Rad51-independent)
Replication protein A and the Mre11/Rad50/Nbs1 complex Co-localize and interact at sites of stalled replication forks

Jacob G. Robison, James Elliott, Kathleen Dixon and Gregory G. Oakley

J. Biol. Chem. published online June 4, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404750200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts