The role of RPA2 phosphorylation in homologous recombination in response to replication arrest

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Introduction

DNA double-strand breaks (DSBs) may arise spontaneously, e.g. during DNA replication or following exposure to DNA-damaging agents (1), such as ionizing radiation (IR) or chemotherapeutic drugs. Homologous recombination (HR) is a major pathway in the repair of DSBs, especially those arising from stalled/collapsed replication forks (2–4). Defective HR may lead to genetic exchanges that result in genomic instability. In addition, cells deficient in HR are sensitive to IR and some chemotherapeutic drugs, especially S phase cells because HR preferentially repairs DSBs in this phase of the cell cycle.

The molecular mechanism of HR can be subdivided further, depending on the nature of the DNA structure. Two-ended DSBs can be caused directly by IR; however, only one-ended DSBs or no DSBs are produced as a result of damage or discontinuities during interrupted replication (5,6). The HR mechanism required for repairing two-ended DSBs has been extensively studied in the past. In general, it is believed that HR is triggered when a two-ended DSB is processed to a 3' single-strand DNA (ssDNA) tail via resection. Once the ssDNA is generated, it is rapidly bound by the ssDNA-binding protein replication protein A (RPA) that in turn is displaced by Rad51. The resultant Rad51 filament facilitates DNA strand invasion and exchanges steps. Although the mechanisms required for HR after DNA replication stalling have not yet been defined in mammalian cells, it appears that eukaryotic cells have evolved a mechanism similar to that described in bacteria for re-establishment of replication forks after their progression has been impeded by lesions in the template. It has been suggested that similar to RuvABC complex in Escherichia coli, the endonuclease Mus81 in mammalian cells contributes to the generation of one-ended DSBs by cleavage of stalled DNA replication forks, which subsequently promotes HR (7–9). In some instances, replication may be restarted downstream of the unrepaired parental strand lesion, leaving a ‘daughter strand gap’ (DSG)—a ssDNA lesion in which no DSB-end is involved. It has been suggested that DSG repair in E.coli requires the RecFOR complex, which initiates with RecA, an equivalent of human Rad51, loading at ssDNA region (10). Studies in mammalian cells have shown the existence of DSGs during stalled DNA replication (11–14), but it is not clear how this process is regulated. Therefore, the detailed molecular mechanisms of the HR that occur in response to replication arrest in mammalian cells is much less understood compared with the HR process stimulated by two-ended DSBs.

RPA is a heterotrimer composed of three tightly associated subunits, RPA1, RPA2 and RPA3. The major ssDNA-binding subunit is RPA1. However, the other two subunits also participate in the interactions with ssDNA (15,16). RPA is required for almost all aspects of cellular DNA metabolism, including DNA replication, DNA damage checkpoint activation, DNA repair and recombination. During DNA replication, RPA binds to ssDNA and is required for the initiation as well as elongation processes (17,18). In addition to its role in DNA replication, RPA is also critical for cell cycle checkpoint activation: RPA-coated ssDNA recruits the ataxia-telangiectasia and Rad3-related (ATR)–ATR interacting protein complex, which facilitates S phase checkpoint signaling under the condition of replication arrest (19,20). RPA is also essential for multiple DNA repair pathways, including HR-mediated repair. RPA is thought to facilitate two-ended DSBs stimulated HR by removing secondary structures in ssDNA that may prevent the formation of the Rad51 filament (21,22) or by actively regulating protein–protein interactions (23). However, the role of RPA in HR after replication block remains unknown.

RPA becomes phosphorylated in cells when bound to ssDNA. Although no precise phosphorylation site has been identified in RPA1, nine potential phosphorylation sites have been suggested within the unstructured N-terminal domain of RPA2 (24–29). A novel DNA damage-induced phosphorylation site at Thr98 in RPA2 was also recently identified (26). RPA2 is phosphorylated in both cell cycle and DNA damage-dependent manner. S phase-dependent phosphorylation of RPA2 is mediated by the cyclin-dependent kinase2 kinase. Cyclin-dependent kinase modifies RPA2 at serine residues 23 and 29 in mitotic cells. DNA damage-induced RPA2 phosphorylation, so-called RPA2 hyperphosphorylation includes modification of threonine 21, serines 4, 8 and 33 as well as at least one phosphoserine in residues 11–13. These events are carried out by the phosphatidylinositol 3-OH-kinase-related kinase family, which includes ataxia-telangiectasia mutated, ATR and DNA-dependent protein kinase (17). A recent study suggests that cyclin-dependent kinase is also required for DNA damage-induced RPA2 hyperphosphorylation (27). However, the question remains as to whether and how RPA2 hyperphosphorylation regulates the function of RPA in DNA replication, cell cycle checkpoint and DNA repair.

Abbreviations: ATR, ataxia-telangiectasia mutated and Rad3-related; BrdU, bromodeoxyuridine; DSB, DNA double-strand break; DSG, daughter strand gap; GFP, green fluorescent protein; HR, homologous recombination; HU, hydroxyurea; IR, ionizing radiation; ssDNA, single-strand DNA; RPA, replication protein A; Thy, thymidine; shRNA, small hairpin RNA.

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Earlier studies suggested that hyperphosphorylation of RPA2 may not be essential for RPA function in vitro or in vivo (18) since deletion of the N-terminus of RPA32 does not affect ssDNA-binding activity, heterotrimer complex formation or the ability to support DNA replication in vitro. However, recent evidence suggests a role for RPA2 hyperphosphorylation in DNA metabolism. It has been suggested that RPA2 phosphorylation facilitates mitotic exit in response to mitotic DNA damage (30). Several studies indicated that RPA2 hyperphosphorylation negatively regulates the role of RPA in DNA replication both in vitro and in vivo (17) although the mechanism underlying the inhibition of replication by RPA2 hyperphosphorylation is controversial. Despite the negative regulation of DNA replication, RPA2 hyperphosphorylation is likely to be involved in DNA repair, particularly HR-mediated repair due to the following reasons. First, RPA2 phosphorylation stimulates repair of chromosomal DNA damage caused either by camptothecin or bleomycin treatment (27). Second, cells expressing a RPA2 mimetic that has persistent phosphorylation exhibit normal checkpoint activation and re-enter the cell cycle normally after recovery but display a pronounced defect in the repair of DNA breaks (31). Since HR-mediated repair is a major mechanism to repair DNA damage caused by stalled/collapsed DNA replication forks, these results suggest a role for RPA2 phosphorylation in HR. Third, hyperphosphorylated RPA2 was found to directly interact with Rad51 in vitro (32) and phosphorylation of RPA2 increased the affinity between RPA and Rad51. Fourth, the observation that hyperphosphorylated RPA2 occurs predominantly in late S and G2 phases (27), where HR is a major repair mechanism, suggests a role for RPA hyperphosphorylation in HR. Therefore, we tested the biological function of RPA2 phosphorylation in HR that occurs in response to DNA damage, particularly after replication arrest. Here, we demonstrate that hyperphosphorylated RPA2 colocalizes with ssDNA and Rad51 in response to hydroxyurea (HU)-induced DNA replication fork stalling. Cells expressing phosphorylation-defective RPA2 exhibit a defect in Rad51 foci formation and a decreased frequency of HR in response to replication arrest but have no obvious defect on IR-induced Rad51 recruitment and I-Sce-I-induced HR. In addition, an increased frequency of chromosomal abnormalities after treatment with HU, but not upon exposure to IR, was observed in cells expressing phosphorylation-defective RPA2. Therefore, we propose that RPA2 hyperphosphorylation is required for HR, which plays a critical role in the maintenance of genomic stability and cell survival in response to replication arrest.

Materials and methods

Plasmids, cell lines, infections and transfections

MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA). Retroviral vectors carrying a HA-tagged wild-type RPA2 or phosphorylation mutant RPA2, RPA2-(7x-A)-S4A/S8A/S11A/S12A/S13A/T21A/S33A, have been described previously (33). RPA2 small hairpin RNA (shRNA) was generated based on lentiviral pLKO.1-puro vector (Sigma, St Louis, MO). Lentivirus transfer vector in which RNA polymerase III U6 promoter drives the expression of small hairpin RNAs complementary to RPA2 3'-untranslated region of messenger RNA target sequence was produced based on previously published sequence (34). In brief, the oligonucleotides encoding RPA2 were cloned into the AgeI and EcoRI sites of the pLKO.1-puro vector. All the DNA plasmid transfections were performed using Lipofectamine according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA).

Generation of cell lines expressing wild-type RPA2 or hyperphosphorylation mutant RPA2

HA-tagged wild-type RPA2 (RPA2-WT) or RPA2-(7x-A)-mutant form (RPA2-A) were introduced into MCF7 cells by three rounds of retroviral infection. Silencing of endogenous RPA2 in these cells was accomplished by two rounds of lentiviral infection using pLKO.1 vector expressing the sequence targeting RPA2 (RPA2 shRNA). The cells were irradiated with X-rays or treated with HU for Rad51 foci analysis or chromosome aberration and survival assay.

Immunofluorescence analysis

The cells were treated with 0.5% Triton 100 cytoskeletal buffer and fixed with 4% formaldehyde. The fixed cells were permeabilized using 0.1% Triton X-100 phosphate-buffered saline for 15 min, followed by blocking with 10% fetal bovine serum and then incubated with primary antibodies. The bound antibodies were revealed by goat anti-mouse IgG Alexa Fluor 594 and chicken anti-rabbit IgG Alexa Fluor 488. The slides were viewed at ×1000 magnification on an Olympus fluorescence microscope (BX40 with Magna-Fire CCD camera). The protocol for ssDNA detection was conducted according to a protocol described previously (35).

Western blotting analysis

Proteins from whole cell lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The primary antibodies used for this analysis are anti-HA tag antibody (Covance, Princeton, NJ; 1:1000 dilution), rabbit polyclonal antibody phosphor RPA32 Ser2/ Ser8 (Bethyl, Montgomery, TX; BL647, 1: 1000 dilution), monoclonal antibody RPA2 (EMD Bioscience, Brookfield, WI; Ab-2, 1:100 dilution) and polyclonal antibodies RPA2 (Abcam, Cambridge, MA; 1:1000 dilution). Secondary antibodies used were goat anti-mouse IgG-horsradish peroxidase conjugated and goat anti-rabbit IgG horseradish peroxidase conjugated (Pierce, Rockford, IL), both at 1:5000 dilutions.

Comet assay

The Neutral Comet Assay was performed using the Comet Assay kit from Trevigen (Gaithersburg, MD) following manufacturer’s instructions. The lyses occurred at 4°C for 30 min. Comets were analyzed using CometScore software (TriTek, Sumerduck, VA).

Homologous recombination assays

The HR analysis induced by I-Sce-I expression has been described previously (36). In brief, the HR was measured in MCF7-pDr- green fluorescent protein (GFP) cells with RPA2-WT or RPA2-A expression where the endogenous RPA2 was depleted by shRPA2. The HR analysis upon replication arrest was conducted according to the protocol described previously (36). Briefly, the cells were treated for 24 h with 2 μM thymidine (Thy). The cells were subsequently washed in phosphate-buffered saline and incubated in fresh medium for 3 h and then the cells were treated with 2 mM HU for 24 h. All cells were left for 24 h to recover before being subjected to flow cytometric analysis for GFP-positive cells. For each analysis, at least 1 000 000 cells were processed.

Flocrescence in situ hybridization for chromosome aberration analysis

Flocrescence in situ hybridization was performed using pan-telomeric and pan-centromeric peptide nucleic acid probes. Telomere (C,T)43-specific and centromere (16-mer repeat DNA)-specific probes directly labeled with Cy3 and fluorescein isothiocyanate fluorescent dyes, respectively, were obtained from Applied Biosystems (Foster City, CA). The cells were radiated (2 Gy) or treated with HU (1 mM, 6 h) and processed with fluorescence in situ hybridization analysis 24 h later. The cells were incubated with colcemid (0.1 μg/ml, Sigma) for 2.5 h before fixation onto slides. The slides were treated with 1 mg/ml pepsin/0.01 N HCl for 10 min at 37°C, after fixed in 4% formaldehyde, dehydrated in ethanol series (70, 90 and 100%), stained with a telomeric and centromeric probe mixture, denatured 3 min at 80°C and hybridized in a moist chamber for 2 h at reverse transcription, then dehydrated again and sealed in anti-fading 4,6-diamidino-2-phenylindole mounting (Vector laboratories Burlington, CA).

Clonogenic survival assays

Cells expressing RPA2-WT or RPA2-A where endogenous RPA2 has been deleted were used in a colony formation assay to measure cell survival after HU or IR treatment. The cells were harvested, filtered with cell strainers (BD Falcon®) to achieve single-cell suspension and plated in triplicates into P60 dishes at various cell densities, with a target number of surviving colonies at 50–100 per dish. Treatment with HU or IR was carried out 18 h after cell plating. After a 24 h exposure to HU at 0.1, 0.5, 1, 2 and 10 mM of concentrations or IR at 2, 4 and 6 Gy of doses, cells were rinsed twice with phosphate-buffered saline and allowed to grow in fresh medium for 14–21 days. Visible colonies were identified with methanol fixation and 0.35% methylene blue staining. The number of colonies (>50 cells) per dish was counted, and the surviving fractions were calculated as the ratio of the plating efficiencies of treated cells to untreated cells.

Results

Hyperphosphorylated RPA2 predominately occur in response to replication arrest

To test whether hyperphosphorylation of RPA2 is critical for the DNA damage response, we first analyzed RPA2 hyperphosphorylation profiles in response to various DNA-damaging agents, including the
replication inhibitors HU and Thy and the DSB inducer IR. HU inhibits ribonucleotide reductase and leads to DNA synthesis arrest. Thy disrupts DNA synthesis by altering the balance between thymidine triphosphate and deoxycytidine triphosphate through the allosteric inhibition of ribonucleotide reductase. Both, HU and Thy, inhibit the DNA elongation step of replication. While Thy does not generate detectable DSBs (37), HU causes DSBs that are obvious after ~12 h of continuous treatment as assessed by pulsed-field gel electrophoresis (36). In contrast to the replication inhibitors HU and Thy, IR can directly cause DNA DSBs. In agreement with previous studies (33), exposure to HU and Thy significantly increased RPA2 hyperphosphorylation (Figure 1A, first panel), as shown by using an antibody specific for phosphorylated RPA2. However, this hyperphosphorylation can only be detected after exposure to a high dose of IR (Figure 1B, first panel) (39,40). We also detected the amount of DSBs in the cells treated with replication inhibitors as well as IR using the comet assay under neutral conditions, which allows detection of DSBs but not ssDNA breaks. No obvious DSBs were detected after 6 h or 24 h of continuous treatment with HU or Thy. In contrast, IR causes enormous DSBs at the doses utilized (Figure 1C). Therefore, our observation is consistent with the previous report that RPA2 hyperphosphorylation is more prevalent when replication forks are stalled rather than when DSBs are generated (33). Since it has been shown that at high doses of IR DNA replication chain elongation is blocked (41), the observation that only higher doses of IR cause RPA2 hyperphosphorylation may be caused by the blockage of DNA replication forks. Alternatively, since it has been suggested that ssDNA resection is restricted to S and G2/M phase cells and RPA2 hyperphosphorylation induced by stalled/collapsed DNA replication is ATR dependent (33), a second explanation is that higher doses of IR can cause more ssDNA, which can activate ATR and subsequently RPA2 hyperphosphorylation. Together, the results shown in Figure 1 and previous reports (33) suggest that RPA2 hyperphosphorylation is more prevalent when DNA replication forks are stalled.

Here, we need to note that our observation and others (33) are not inconsistent with a recent publication showing that rapid hyperphosphorylation of RPA2 are observed in response to IR which is ataxia-telangiectasia mutated and DNA-dependent protein kinase dependent. This form of IR-induced RPA2 hyperphosphorylation was only observed in mitotic cells but not in interphase cells (42). Thus, hyperphosphorylation of RPA2 can take place in specific mitotic cells when DSBs are generated, a process that is dependent on ataxia-telangiectasia mutated and DNA-dependent protein kinase (42) but primarily upon stalling of replication forks, an ATR-dependent process (33).

Hyperphosphorylated RPA2 colocalizes at the ssDNA region that occurs in response to stalled/collapsed DNA replication forks

Arrest of replication fork progression should lead to the accumulation of ssDNA. We tested if hyperphosphorylated RPA2 is localized at ssDNA regions during replication block. We first examined whether HU induces ssDNA by immunofluorescence staining. ssDNA was detected by using an assay that permits identification of sites of ssDNA >1 kb in situ. This assay is based on the observation that the nucleotide base analogue bromodeoxyuridine (BrdU) is recognized by an anti-BrdU antibody when incorporated into ssDNA but not dsDNA (35). The approach has been successfully utilized in different laboratories (35,43,44). We found that HU treatment significantly increased the percentage of cells with ssDNA in vivo, indicating that ssDNA accumulated in response to replication arrest (Figure 2A). To determine if hyperphosphorylated RPA colocalized with ssDNA, cells treated with HU were costained with BrdU and phospho-specific RPA2 antibody. An extensive colocalization of hyperphosphorylated RPA2 with ssDNA foci was observed (Figure 2B) indicating that hyperphosphorylated RPA2 associates with ssDNA regions after a replication block. Given the requirement of DNA binding for RPA2 hyperphosphorylation, we reasoned that phosphorylated RPA2 foci occur later than RPA2 recruitment. Therefore, we examined the recruitment of RPA2 and hyperphosphorylated RPA2 at various time points after HU treatment by immunofluorescence staining using anti-RPA2 and specific anti-phosphorylated RPA2 antibodies. We found that RPA2 recruitment starts to increase as early as 1 h following HU treatment. In contrast, HU-induced phosphorylated RPA2 foci were not observed until 2 h after HU treatment (Figure 2C). Representative RPA2 and hyperphosphorylated RPA2 foci in response to HU are shown in Figure 2C. Moreover, RPA2 foci and phosphorylated RPA2 foci show nearly complete colocalization (Figure 2C). This result indicates that RPA2 is recruited to ssDNA and then is further hyperphosphorylated, which is consistent with previous observations that RPA2 can only be hyperphosphorylated after binding to DNA or chromatin (33,45,46).

Hyperphosphorylated RPA2 is required for Rad51 recruitment following HU treatment

The accumulated ssDNA during stalled/collapsed DNA replication forks could lead to DSBs. The increased DSBs induced by HU treatment were detected by pulsed-field gel electrophoresis (36). Consistent with this report, an increase in cells with γ-H2AX foci after HU treatment has also been observed (47). DSBs and other lesions associated with DNA replication are repaired by HR in mammalian cells (37,48). A key protein in HR-mediated repair is RAD51, and cells deficient in RAD51 accumulate DSBs after replication or at stalled replication forks (49). Since it has been demonstrated that hyperphosphorylated RPA2 directly interacts with Rad51 (32), we next determined if hyperphosphorylated RPA2 colocalized with Rad51 in response to replication arrest. Hyperphosphorylated RPA2 was observed to extensively colocalize with Rad51 in response to HU treatment (Figure 3A), suggesting the association of hyperphosphorylated RPA2 with Rad51 in vivo in stalled/collapsed DNA replication response pathways. To further study the role of phosphorylated RPA2 in Rad51 recruitment, we used an RPA2 mutant construct in which seven DNA damage-associated phosphorylation sites (33) were

Fig. 1. Hyperphosphorylation of RPA2 predominately occurs in response to replication arrest. (A) Whole cell lysates were isolated from MCF7 cells that were mock treated or exposed to replication inhibitor, HU (2 mM, 4 h) or Thy (2 mM, 48 h) and immunoblotted with an antibody specific to phosphorylated RPA2 (RPA2-p48, first panel) or RPA2 (Abcam, second panel). The same blot membrane from Figure 1A was subsequently reprobed with β-actin for loading control. (B) Whole cell lysates were isolated from MCF7 cells with or without IR treatment (8, 20 or 40 Gy, 6 h after) and then immunoblotted with antibodies against phosphorylated RPA2, RPA2 or β-actin. (C) The neutral comet assay of genomic DNA of MCF7 cells treated with replication inhibitor or IR. The doses used for HU and Thy are same as Figure 1A. The number of cells in each treatment group was 150. Results were expressed as Olive moment. The amount of breaks is correlated with the tail moment values (38).
Fig. 2. Hyperphosphorylated RPA2 colocalizes at the ssDNA region in response to stalled/collapsed DNA replication forks. (A) HU treatment results in ssDNA accumulation. The protocol for ssDNA detection has been described in a previous publication (35). In brief, the cells were grown in the presence of bromodeoxyuridine (BrdU, 10 μg/ml; Invitrogen) for 24 h. Cells with BrdU labeling were treated with 2 mM of HU or left untreated and 6 h later, the cells were fixed. After fixation, the cells will be blocked and stained with anti-BrdU mouse monoclonal antibody clone B44 (BD Biosciences, San Jose, CA) antibody. Then, the samples are incubated with an Alexa Fluor 488-conjugated goat anti-mouse antibody. Cells were scored positive when 10 nuclear foci were visible. Representative phosphorylated RPA2 with ssDNA. MCF7 cells are cultured 24 h in the presence of BrdU. Cells with BrdU labeling were treated with 2 mM of HU and 6 h later, the cells were fixed. The fixed cells were stained by RPA2-p-S4/8 and anti-BrdU antibody. Representative phosphorylated RPA2 foci and ssDNA foci in response to HU were shown in Figure 2B. (C) The appearance of phosphorylated RPA2 foci occurs later than RPA2 foci after HU treatment. Cells in exponential growth phase were treated with HU (2 mM), fixed at indicated time points and costained with anti-RPA2 (Ab-2) and anti-phosphorylated RPA2 antibody (RPA2-p-S4/8). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue).

Notably, RPA2 hyperphosphorylation has no obvious effect on the fraction of cells with Rad51-positive foci in untreated cells (Figure 3D, T-test, P > 0.05). This finding is consistent with our observation that no obvious RPA2 hyperphosphorylation was observed in cells without any treatment (Figure 1A and B). Our finding further supports the previous study suggesting that the repair mechanism of spontaneous HR is different to that induced by replication inhibitors (50). Collectively, the data in Figure 3 demonstrate that hyperphosphorylated RPA2 accumulates in ssDNA regions and is important for the formation of Rad51 foci after replication block.
replication duplicates each chromosome during S phase to create a source of genomic instability. In cells without DNA damage, DNA failure to restart stalled/collapsed replication forks is a potential aberration. RPA2 hyperphosphorylation prevents HU-induced chromosomal hyperphosphorylation in HR triggered by DNA replication arrest. Together, these findings strongly support a key role for RPA2 inhibition compared with the targeted DSB induced with I-Sce-I (50).

The frequency of the HR induced by HU is lower than that stimulated by I-Sce-I expression due to the genome-wide effect of replication block but not essential for IR induced Rad51 foci formation. After 18 h treatment with 2 mM HU or 8 h after 8 Gy IR, cells depleted of endogenous RPA2 expressing RPA2-WT or RPA2-A were fixed and processed for immunocytochemistry with an anti-Rad51 antibody. Cells were scored positive when at least 10 nuclear foci were visible. Data shown are averages from three independent experiments (T-test, P < 0.05 for HU treatment; P > 0.05 for IR treatment).

Most importantly, cells expressing RPA2-A exhibited a higher rate of radial structures (Figure 5C), such as tri-radial and quadri-radial aberrations (Figure 5D), generated by the increased frequency of chromatid and chromosome breaks after exposure to HU (Figure 5A and B) compared with cells expressing RPA2-A is shown in Figure 5B.

RPA2 hyperphosphorylation prevents HU-induced chromosomal aberrations

Failure to restart stalled/collapsed replication forks is a potential source of genomic instability. In cells without DNA damage, DNA replication duplicates each chromosome during S phase to create a sister chromatid. If the chromosomal DNA replication is blocked, the ssDNA generated due to replication arrest could be processed into DSBs (36). In normal cells, the DSBs are repaired by HR since only one-end DSBs are created, which is not an appropriate substrate for non-homologous end joining. However, if DSBs are not repaired by HR, these persistent DSBs will result in chromatid breaks or chromosome breaks. The deficient HR and the lack of HU-induced Rad51 focus formation in cells expressing RPA2-A indicates a defective response to stalled/collapsed DNA replication forks, which could lead to genomic instability. Therefore, we examined whether a deficiency in RPA2 hyperphosphorylation results in the accumulation of chromosomal abnormalities in response to HU using fluorescence in situ hybridization. We observed that cells expressing RPA2-A displayed higher frequencies of chromatid and chromosome breaks after exposure to HU (Figure 5A and B) compared with cells expressing RPA2-WT. The representative metaphases spread after HU treatment in cells expressing RPA2-A is shown in Figure 5B.
Hyperphosphorylation of RPA2 is specifically required for HR in response to replication arrest

Fig. 4. RPA2 hyperphosphorylation is required for the HR stimulated by HU but not for HR induced by I-Sce-I. (A) RPA2 hyperphosphorylation is specifically critical for HR after replication arrest. The frequencies of HR were calculated in cells expressing RPA2-WT or RPA2-A after HU treatment by flow cytometry. Results are means from three independent experiments with standard deviation. (B) Cells expressing phosphorylation-defective RPA2 show similar HR frequency induced by I-Sce-I expression in comparison to cells with wild-type RPA2. The HR frequency was measured by flow cytometric analyses (see Materials and Methods) in cells expressing RPA2-WT or RPA2-A. Results are means from three independent experiments, with standard deviation. (C) Immunoblot analysis of RPA2 hyperphosphorylation expression in MCF7 cells with I-Sce-I plasmid transfection or HU and Thy. Cell lysates were harvested 24 h after I-Sce-I transfection or after HU and Thy treatment. The doses used for HU and Thy are same as Figure 1A. Proteins were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and visualized by immunoblot with anti-RPA2 p4/8 antibody or anti-RPA2 antibody (Abcam). (D) Foci of phosphorylated RPA2 in cells with I-Sce-I transfection or HU treatment. The cells were fixed 24 h after I-Sce-I transfection or 4 h after HU treatment. Fixed cells were stained by a specific anti-RPA2 phosphorylation antibody and anti-RPA2 p4/8 antibody. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue).

Similar frequencies of chromatid and chromosome breaks after exposure to IR (Figure 5E). Thus, Figure 5 indicates that RPA2 hyperphosphorylation is specifically critical for HR arrest, which perfectly fits its role in Rad51 foci formation and HR induced by HU (Figures 2 and 3).

RPA2 hyperphosphorylation is required for resistance to HU but not IR

The HR-mediated repair is required to restart stalled or collapsed replication forks under replication stress conditions. The role of hyperphosphorylated RPA2 in Rad51 recruitment and HR in response to HU indicates that RPA2 hyperphosphorylation plays a role in cell survival after repair arrest. To test this hypothesis, we determined the effect of RPA2 hyperphosphorylation on survival following HU treatment or IR exposure. As shown in Figure 6A, cells expressing RPA2-A produced a phenotype of greater sensitivity to HU by clonogenic cell survival (Figure 6A). In contrast, the cells expressing hyperphosphorylation mutant RPA2 showed a similar sensitivity to IR (Figure 6B). Thus, Figure 6 suggests that RPA2 hyperphosphorylation is required for cell survival under conditions of replication arrest, which is correlated with its role in Rad51 recruitment and HR stimulated by HU treatment (Figure 3). It should be noted that although hyperphosphorylation of RPA2 plays a role in inhibition of DNA replication after activation of S phase checkpoint during replication stress, the defects of HR in cells expressing phosphorylation defective RPA2 may explain the sensitivity to HU (Figure 6A) since it has been indicated that S phase checkpoint is not required for cell viability under replication stress conditions (58).

Discussion

The activation of oncogenes and inactivation of tumor suppressor genes frequently results from cancer-promoting mutations. These genetic alterations are often mediated by impaired DSB repair. RPA is an essential factor for DSB repair and cell cycle checkpoint activation. In the present report, we demonstrate that hyperphosphorylated RPA2 colocalizes with ssDNA and Rad51 and is essential for Rad51 recruitment and HR in response to replication arrest. In addition, we also demonstrate that RPA2 hyperphosphorylation is critical for maintenance of genomic stability and cell viability after replication stalling. These findings provide direct evidence that RPA2 hyperphosphorylation facilitates HR and is important for maintenance of genomic stability and cell survival under conditions of replication stress in addition to its role on mitotic exit in response to mitotic DNA damage (30).

Hyperphosphorylation of RPA2 is a central factor in response to replication arrest

Given the complexity of DNA replication, S phase is a vulnerable period for the genome in the cell-division cycle. During replication...
arrest, long regions of RPA-coated ssDNA at replication forks are necessary for ATR checkpoint signaling. Interestingly, RPA2 is also a direct substrate of ATR (33). The evidence suggests a regulated process in which ssDNA-bound RPA activates the ATR kinase, which in turn induces RPA hyperphosphorylation. In addition, it has been documented that hyperphosphorylation of RPA2 prevents RPA from participating in DNA replication in response to DNA damage. In the present study, we show that RPA2 hyperphosphorylation is essential for HR after stalled or collapsed DNA replication forks (Figure 4). Therefore, hyperphosphorylated RPA2 appears to serve as a central regulator in response to replication arrest, which is evidenced by the increased chromosome instability and cell toxicity observed in cells lacking RPA2 hyperphosphorylation (Figures 5 and 6). Therefore, a possible scenario under conditions of replication stress is that ssDNA bound by RPA activates ATR and subsequently RPA2 hyperphosphorylation induced by ATR promotes HR via facilitation of Rad51 recruitment (supplementary Figure S2 is available at Carcinogenesis Online).

Interestingly, a recent study suggests that dephosphorylation of RPA2 is also important to DNA repair process after replication arrest, and persistent RPA2 phosphorylation leads to the increased DSBs (31). The complexity of DNA DSB repair processing requires that the individual reaction steps are ordered. The HR-mediated repair process in a highly organized process. RPA2 hyperphosphorylation

**Fig. 5.** RPA2 hyperphosphorylation is required for maintenance of genomic stability. (A) Frequencies of HU-induced chromosome aberrations are indicated. The data shown is from one of two independent experiments. Chid and Chme represent chromatid and chromosome, respectively. Fifty metaphases for each sample were analyzed. (B) Representative metaphase spreads in cells treated with 1 mM HU for 6 h and incubated in fresh medium for 24 h. Fluorescence in situ hybridization using telomeric and centromeric probes reveals the red/pink and green color. Chromatid-type aberrations indicated in the white arrow. Chromosome-type aberrations indicated in the yellow arrow. (C) Frequencies of HU-induced radial structures are indicated. Fifty metaphases for each sample were analyzed. The data shown is from one of two typical independent experiments. (D) A typical radial structure induced by HU from cells expressing RPA2-A. Y-shaped tri-radial chromosomes and star-shaped quadri-radial chromosomes were indicated by arrowheads. (E) Frequencies of IR-induced chromosome aberration are indicated. Fifty metaphases for each sample were analyzed. The data shown is from one of two typical independent experiments.

**Fig. 6.** RPA2 hyperphosphorylation is critical for cell survival in response to HU but not IR. Clonogenic survival in MCF7 cells expressing RPA2-WT or RPA2-A after HU treatment (A) or exposure to IR (B). The cells expressing RPA2-WT or RPA2-A were treated with HU or IR at the indicated doses. The plotted points are on the averages of three to four experiments with standard deviations.

arrest, long regions of RPA-coated ssDNA at replication forks are necessary for ATR checkpoint signaling. Interestingly, RPA2 is also a direct substrate of ATR (33). The evidence suggests a regulated process in which ssDNA-bound RPA activates the ATR kinase, which in turn induces RPA hyperphosphorylation. In addition, it has been documented that hyperphosphorylation of RPA2 prevents RPA from participating in DNA replication in response to DNA damage. In the present study, we show that RPA2 hyperphosphorylation is essential for HR after stalled or collapsed DNA replication forks (Figure 4). Therefore, hyperphosphorylated RPA2 appears to serve as a central regulator in response to replication arrest, which is evidenced by the increased chromosome instability and cell toxicity observed in cells lacking RPA2 hyperphosphorylation (Figures 5 and 6). Therefore, a possible scenario under conditions of replication stress is that ssDNA bound by RPA activates ATR and subsequently RPA2 hyperphosphorylation induced by ATR promotes HR via facilitation of Rad51 recruitment (supplementary Figure S2 is available at Carcinogenesis Online).

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and dephosphorylation could be an important mechanism to regulate the assembly and/or disassembly of protein complexes at the site of stalled/collapsed DNA lesions in a sequential order. Therefore, HR-mediated repair in response to stalled/collapsed DNA replication forks is a strictly regulated process in which both phosphorylation and dephosphorylation of RPA is required. Defects in either part of the regulation process results in genomic instability.

Hyperphosphorylated RPA2 may be a potential indicator for sensitivity to replication arrest agents

We have shown that defective RPA2 hyperphosphorylation results in the enhanced sensitivity to HU, which is an approved therapeutic agent for chronic myelogenous leukemia. HU is commonly used for other myeloproliferative disorders and also used occasionally for metastatic melanoma and other cancers. Given that hyperphosphorylated RPA2 can also be induced by topoisomerase I inhibitor camptothecin, cross-linking agents cisplatin and other chemotherapeutic drugs (50,64), our studies should have significant clinical implications. A recent study has suggested that RPA2 hyperphosphorylation confers resistance to cisplatin and other chemotherapy drugs (65). Therefore, the evaluation of RPA2 hyperphosphorylation can be potentially used as an indicator for the sensitivity/resistance to these chemotherapeutic agents. The understanding of the molecular mechanism by which RPA2 phosphorylation promotes HR should eventually lead to the development of a novel class of anticancer drugs targeting RPA2 hyperphosphorylation.

Supplementary material

Supplementary Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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References


