The activity of human replication protein A (RPA) in DNA replication and repair is regulated by phosphorylation of the middle RPA2 subunit. It has previously been shown that up to nine different N-terminal residues are modified in vivo and in response to genotoxic stress. Using a novel antibody against phospho-Ser29, a moiety formed by cyclin-Cdk, we observed that RPA2 was phosphorylated during mitosis in nonstressed cells. Robust phosphorylation of Ser29 was also seen in interphase cells following treatment with the DNA-damaging agent camptothecin, a rare example of stress stimulating the modification of a repair factor by cyclin-Cdk. RPA2 phosphorylation is regulated both in cis and trans. cis-Phosphorylation follows a preferred pathway. (That is, the initial modification of Ser33 by ATR stimulates subsequent phosphorylation of Cdk sites Ser23 and Ser29). These events then facilitate modification of Thr21 and extreme N-terminal sites Ser4 and Ser8, probably by DNA-PK. Our data also indicate that the phosphorylation of one RPA molecule can influence the phosphorylation of other RPA molecules in trans. Cells in which endogenous RPA2 was “replaced” with a double S23A/S29A-RPA2 mutant were seen to have an abnormal cell cycle distribution both in normal and in stressed cells. Such cells also showed aberrant DNA damage-dependent RPA foci and had persistent staining of γH2AX following DNA damage. Our data indicate that RPA phosphorylation facilitates chromosomal DNA repair. We postulate that the RPA phosphorylation pattern provides a means to regulate the DNA repair pathway utilized.

Replication protein A (RPA) is a heterotrimeric single-stranded DNA-binding factor that is critical for the “three Rs” of eukaryotic DNA enzymology: DNA replication, DNA recombination, and DNA repair (1, 2). For DNA replication, the study of cellular and viral model systems demonstrates that RPA is needed both for origin denaturation and replication elongation, in the latter case to facilitate the switch from DNA polymerase α to DNA polymerase δ during Okazaki fragment synthesis (3). RPA acts in homologous recombination (HR) to stimulate DNA annealing using physical interactions with Rad52 (4–7) and in HR-mediated DNA repair, probably employing specific interactions with BRCA2 (8, 9). RPA is a required factor in both the nucleotide excision (10, 11) and mismatch repair pathways (12, 13) and in somatic hypermutation (14). Because of these many roles, it is of significant interest to understand the mechanisms that regulate RPA activity.

Of the ~70-kDa (RPA1), 30-kDa (RPA2), and 14-kDa (RPA3) subunits, human RPA is subject to extensive phosphorylation on RPA2 (2) and at one RPA1 site (15). The N-terminal 33 residues of RPA2 undergo both cell cycle- and stress-dependent phosphorylation on approximately nine sites (Fig. 1A), which are thought to exist in an unstructured conformation (16, 17). Ser23 and Ser29 are constitutively modified during mitosis by cyclin B-Cdk1 (18, 19) and have been suggested to be partially modified beginning at the G1/S boundary by the cyclin A-Cdk2 complex (18, 20, 21). These two residues may also undergo heightened phosphorylation in response to UV irradiation (22). The Thr21 and Ser33 residues are consensus sites for phosphatidylinositol 3-kinase-like kinase (PIKK) family members (ATM, ATR, and DNA-PK) that signal the presence of DNA damage and replication stress. Under DNA damage conditions, the phosphorylation of Thr21 is apparently catalyzed by ATM, DNA-PK, and probably ATR (22, 23). Olson et al. (24) have concluded that Ser33 is modified by ATR. The remaining sites (Ser4, Ser8, Ser11, Ser12, and Ser13) are phosphorylated in response to genotoxic stress, although the responsible kinase(s) in vivo has not yet been identified. However, all can be modified by DNA-PK in vitro (22). Others have also shown the involvement of DNA-PK in supporting RPA2 hyperphosphorylation (25, 26).

RPA activity in vivo is regulated by phosphorylation. RPA containing RPA2 mutations that mimic hyperphosphorylation selectively prevent the association of RPA with replication centers but not repair foci (24, 27). Similarly, others have found that wild type RPA2: FACS, fluorescence-activated cell sorting; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; DNA-PKcs, DNA-PK catalytic subunit; DAPI, 4′,6-diamidino-2-phenylindole.
ATR-dependent phosphorylation of RPA inhibits DNA synthesis following UV irradiation (24). These effects are probably mediated by RPA phosphorylation regulating its association with other factors. It has been found that treatment of cells with camptothecin (CPT) led to dissociation of RPA-DNA-PK complexes, an event presumably mediated by RPA phosphorylation (25). A test of the mitotic RPA species (mentioned above) found that it had reduced affinity for ATM and DNA polymerase α, as compared with nonphosphorylated RPA (19). The Wold laboratory similarly observed that RPA phosphorylation reduced its interaction with DNA polymerase α and SV40 T antigen but increased association with p53 (2).

Along with being a substrate for ATR, RPA is also instrumental in signaling the presence of replication stress and is an essential component of a pathway that activates the kinase (28, 29). The primary activation pathway during DNA replication stress apparently involves binding of RPA to ssDNA formed after DNA polymerase stalling and continued movement of the replicative helicase. This persistent RPA-ssDNA intermediate supports binding of the ATR-ATRIP complex and, in combination with other factors, leads to activation of the ATR kinase (30), which then phosphorylates RPA2 at Ser33. ATR is also regulated across the cell cycle with the processing of double strand breaks (DSBs) to RPA-ssDNA intermediates (that allow kinase activation) occurring only in the S and G2 phases when Cdk kinase activity is significant (31). Interestingly, a test of human cell extracts indicated that RPA phosphorylated by Cdk stimulates modification by DNA-PK (32). Such data indicate that RPA phosphorylation events catalyzed by Cdk, PIKK, and other kinases may be interdependent.

Because of the importance of RPA phosphorylation, we employed phospho-specific antibodies, including a novel antibody recognizing the Ser29 cyclin-Cdk site, to examine the modification pattern of RPA2 at five of the N-terminal sites. We find that, under conditions of genotoxic stress, the actions of cyclin-Cdk and PIKK in causing RPA hyperphosphorylation are synergistic, and both are necessary to cause subsequent modification of extreme N-terminal RPA2 residues. Cells expressing RPA2 mutated at the two cyclin-Cdk sites had more intense RPA2 staining and increased persistence of γH2AX foci following genotoxic stress. These data indicate that RPA phosphorylation stimulates DNA repair.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—U2-OS cells were cultured in McCoy’s medium containing 10% fetal bovine serum. Cell lines positive (MO59K) or null (MO59J) for the DNA-PK catalytic subunit were cultured in Dulbecco’s modified Eagle’s medium/F-12 (1:1, v/v) containing 10% fetal bovine serum. As required, cells were treated for 1 h with 2 μM CPT (Sigma) or 75 μg/ml bleomycin (Ble; Calbiochem). For synchronization experiments, cells were arrested in prometaphase by treatment with 100 ng/ml nocodazole (Sigma) for 14–16 h, and the mitotic cells were isolated by shake-off. To release cells from the mitotic block, nocodazole-arrested cells were washed in phosphate-buffered saline (PBS), released in medium devoid of nocodazole, and then collected at various times postrelease. CPT or Ble treatment occurred 1 h prior to harvest.

When testing kinase activity against mitotic RPA, cells were incubated with 100 ng/ml nocodazole and 200 μM roscovitine (cyclin-Cdk inhibitor; Calbiochem) for 16 h. To examine the role of cyclin-Cdk in phosphorylation of RPA during genotoxic stress, cells were first treated with 50 or 200 μM roscovitine for 45 min, followed by treatment with 2 μM CPT (in the continued presence of roscovitine) for 1 h. In order to inhibit ERK activation, cells were pretreated with U0126 (a kind gift from Dr. Paolo Mignotti) at 10 μM for 1 h. Genotoxic stress was induced by treatment with 2 μM CPT for 1 h in the continued presence of the inhibitor.

Transient transfections were performed using Effectene (Qiagen). Kit reagents were used at one-third of the recommended quantity, and cells were incubated with these reagents for 12 h followed by a change of medium. Cells were collected for analysis 48 h post-transfection.

**λ-Phosphatase Treatment**—For phosphatase treatment, cells were lysed in a λ-protein phosphatase buffer (New England Biolabs) containing 1% Triton X-100, 2 mM MnCl₂, and protease inhibitor mixture tablet (Roche Applied Science). Cell lysates (20 μg of protein) were then incubated with 400 units of λ-protein phosphatase for 30 min at 30 °C or mock-treated in the presence of protein phosphatase inhibitors (0.5 mM Na₃VO₄, 10 mM β-glycerophosphate, and 50 mM NaF). Lysates were examined by Western blotting.

**RPA2 Mutagenesis and Retrovirus Expression**—The Myc-tagged RPA2 expression vector was previously described (27). This vector was used to generate the Ser to Ala or Asp phosphorylation site mutations (Fig. 1B) using the QuikChange site-directed mutagenesis kit (Stratagene). Top strand primers were as follows: S23A (5′-CTA CAC GCA GGC CCC GGG GT-3′); S23D (5′-TAC ACG CAG GAC CCC GGG GGC T-3′); S29A (5′-GGG GGG CTG TGG AGC ACC CGC ACC TTC CT-3′); S29D (5′-GGG GGG CTG TGG AGA TCC CGC ACC TTC TC-3′); S23A (5′-GGG GGG CTG TGG AGC ACC CGC ACC TTC TC-3′); S23D (5′-GGG GGG CTG TGG AGA TCC CGC ACC TTC TC-3′); T21A (5′-CCG GCC GCC CCT GCC ACC GCC CGG G-3′). The S23A-RPA2 was used as a template to generate the S23A/S29A double mutant, whereas S23D-RPA2 was the template to prepare the S23D/S29D double mutant. Similarly, we used the S33A-RPA2 mutant as the template to prepare the T21A/S33A double mutant.

To analyze the effect of Cdk site mutation on mitotic RPA2 phosphorylation and hyperphosphorylation during genotoxic stress, the Myc-tagged versions of the Cdk site mutants were transiently transfected into U2-OS cells (see Fig. 2C). For all other analyses, untagged versions of RPA2 were generated. In order to do so, the mutated version of RPA2 was amplified by PCR using primers (forward, 5′-TGC AGA TAT CCA GCA CAG TGG CGG CCG CTC GAG-3′; reverse, 5′-AAT GGA TCC TTA TTC TGC ATC TGT GGA TTA AAA ATG GTC ATC ATC C-3′) that contain NotI and BamHI sites in the forward and reverse primers, respectively. PCR products were then subcloned into the NotI and BamHI sites in the pRetroOFF retroviral vector (Clontech). The mutants tested are shown (Fig. 1B). Production of retroviruses containing the RPA2 expression cassette was performed in Phoenix cells (obtained from the laboratory of J. A. Borowiec, manuscript in preparation.)
from G. Nolan; Stanford University) using the Phoenix Retroviral Helper dependent protocol (available on the World Wide Web).

U2-OS cells at 40% confluence were infected with the desired retrovirus for 48 h in the presence of 2 \( \mu \)g/ml doxycycline (to inhibit ectopic untagged RPA2 expression). Clones resistant to puromycin (1 \( \mu \)g/ml) were isolated and assayed for expression of the ectopic RPA2. This was done by Western blot analysis, after silencing of endogenous RPA2 (see below), both in the presence and absence of doxycycline. Clones that showed strong doxycycline-regulated induction of RPA2 were selected and amplified for further analysis.

**RPA2 Replacement Strategy and Silencing**—For replacement of endogenous RPA2, retrovirally infected U2-OS clones were first grown for 48 h in medium lacking doxycycline to allow ectopic RPA2 expression (Fig. 1C). The endogenous RPA2 was then down-modulated using an siRNA molecule (top strand sequence, 5’-AAC CUA GUU UCA CAA UCU GUU-3’) targeting the 3’-untranslated region of the RPA2 mRNA (27). Silencing was achieved using Hiperfect (Qiagen) as per the manufacturer’s instructions, with cells tested 72 h post-transfection. Representative levels of RPA2 following down-modulation and ectopic induction by Western blot are shown (Fig. 1D), demonstrating the efficiency of the silencing and replacement procedure. A parallel investigation using immunofluorescence microscopy demonstrates that the “replaced” cells each have similar levels of ectopic RPA2 expression (supplemental Fig. 1).

**Immunoblotting and Antibodies**—For Western analysis, cells were directly lysed in SDS-PAGE sample buffer, and the lysate proteins were separated by SDS-PAGE. Proteins were immobilized onto Protran nitrocellulose membranes (0.2-m pore size). The antibodies used in this study were against c-Myc (Bethyl Biolabs), general RPA2 (NeoMarkers), Thr(P)\(^{21}\) (Abcam), and Ser(P)\(^33\)/Ser(P)\(^8\)-, Ser(P)\(^33\), and Ser(P)\(^{29}\)-RPA2 antibodies (Bethyl Laboratories). The Ser(P)\(^{29}\) RPA2 antibody was custom-synthesized and affinity-purified by Bethyl using a “CSPGGFgpSPAPSPQ” phosphopeptide (where pS represents phosphoserine). For developing Western blots, Western wash buffer (PBS containing Tween 20 (0.3%, v/v), 5 mM sodium fluoride, and 0.1 mM sodium orthovanadate) was used. All phospho-specific antibodies were incubated in Western wash buffer containing nonfat dry milk (0.5%, w/v) and bovine serum albumin (0.5%, w/v). The secondary antibodies and nonphospho-specific primary antibodies were incubated in the Western wash buffer containing 0.2% (w/v) nonfat dry milk. Detection was carried out using enhanced chemiluminescence (Amer sham Biosciences).

**Immunofluorescence Microscopy**—For visualization of RPA2 foci, cells were split onto coverslips 48 h postsilencing. Cells were then either mock- or CPT-treated 24 h later. For treatment, cells were incubated with 2 \( \mu \)M CPT for 1 h, followed by washing with PBS and extraction with 0.5% (v/v) Triton X-100 in CSK buffer (10 mM Hepes-KOH, pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl\(_2\)) for 2 min on ice. Cells were then fixed with 4% (w/v) paraformaldehyde either immediately (0 h) or 8 h post-CPT treatment. γH2AX foci were generated by treatment of replaced cells with either 5 \( \mu \)M CPT (70 h postsilencing) or 30 \( \mu \)g/ml Ble (56 h postsilencing), in both cases for 2.5 h. Cells were then washed twice with PBS and extracted and fixed either immediately (0 h) or at 8 h (CPT) or 15 h (Ble) postwash. Coverslips were then stained with primary and secondary antibodies. Quantitation of foci intensity was performed using IPLab software (RPA2; BD Biosciences) or ImageJ (γH2AX; National Institutes of Health). In order to observe RPA2 expression patterns in wt-RPA2 versus S23A/S29A-RPA2 clones, cells were directly fixed and stained with a monoclonal RPA2 antibody.

**Flow Cytometry**—Trypsinized cells were washed with PBS and fixed by dropwise addition into a 10× volume of ice-cold 70% ethanol. Following an overnight incubation at 4 °C, cells were pelleted and stained with 0.5 ml of a solution containing PBS, 0.02% (v/v) propidium iodide (Sigma), 0.1% (v/v) Triton X-100, and 200 \( \mu \)g/ml RNase A, for 15 min at 37 °C followed by cell sorting. FACS was performed on a BD Bioscience flow cytometer using Cell Quest software. Cell cycle analysis was performed using Mod-Fit software.

**RESULTS**

**Phosphorylation of the RPA2 Ser\(^{29}\) Cyclin-Cdk Site in Vivo**—Characterization of the effect of genotoxic stress on the human RPA phosphorylation pattern has been aided by the recent development of phospho-specific antibodies directed against the various phosphorylation sites. At the time of this study, antibodies were commercially available against Thr(P)\(^{21}\), Ser(P)\(^{33}\), and doubly phosphorylated Ser(P)\(^4\)/Ser(P)\(^8\) (Fig. 1A). To add to this collection, we developed an antibody recognizing Ser(P)\(^{29}\), a cyclin-Cdk site reported to be modified constitutively during mitosis.

Like the anti-Ser(P)\(^{33}\) and -Thr(P)\(^{21}\) RPA2 antibodies, the Ser(P)\(^{29}\) antibody was observed to show little reactivity tolysates prepared from control U2-OS cells (Fig. 2A). To induce genotoxic stress, cells were treated with CPT, which indirectly causes damage (e.g. DSBs) through the collision of DNA replication forks with trapped topoisomerase I-DNA complexes (33). Incubation of cells with CPT caused the appearance of a hyperphosphorylated wt-RPA2 species (lane 1), which was also significantly reactive to each of the phospho-specific antibodies (lanes 2, 5, and 8; marked H). Pretreatment of the lysates with λ-phosphatase caused the disappearance of the hyperphosphorylated RPA2 species (using a general RPA2 antibody) and a loss of reactivity by each of the phospho-specific antibodies. For the Ser(P)\(^{33}\) and Thr(P)\(^{21}\) antibodies, these data confirm previous reports showing high specificity of these antibodies (e.g. see Ref. 24). The Ser(P)\(^4\)/Ser(P)\(^8\) antibody was previously demonstrated by our laboratory to lose reactivity of hyperphosphorylated RPA2 following phosphatase treatment (27).

To further verify the specificity of the novel Ser(P)\(^{29}\) antibody, we employed stable cell lines that allow inducible expression of an *untagged* version of wild type or mutant RPA2. Expression of this ectopic RPA2 is coupled with RNA interference-mediated knockdown of endogenous RPA2 by targeting a 3’-untranslated region sequence contained only in the endogenous RPA2 message. In this manner, the endogenous RPA2 is replaced with an ectopic version. Note that previous studies have found that the ectopic RPA2 is incorporated into heterotrimeric RPA, with the level of RPA2 apparently regulating the
Sequential RPA Phosphorylation

FIGURE 1. hRPA2 phosphorylation sites and replacement strategy. A, the phosphorylation sites in the N-terminal region of RPA2 are indicated with underlines. The consensus sites for putative kinases are shown (below the sequence), as are the residues for which phospho-specific antibodies are available (above). 8, RPA2 mutants tested in these studies. Of the nine different RPA2 mutants, the Ser23 and Ser29 aspartate mutants were only tested in the context of Myc-RPA2. The Ser23 and Ser29 alanine mutants were examined using both Myc-tagged and untagged RPA2, whereas the Thr21 and Ser29 mutants were only tested using untagged-RPA2. B, schema for RPA2 replacement and induction of genotoxic stress. To replace RPA2, individual stable cell clones were first induced to express the ectopic RPA2 variant by removal of doxycycline, then subjected to genotoxic stress conditions, such as CPT treatment prior to harvest. C, induction profile of a representative stable cell clone expressing ectopic RPA2. A representative S23A-RPA2 U2-OS clone was incubated for 48 h in the presence (lane 1) or absence (lane 2) of doxycycline, followed by silencing for 72 h. Postsilencing, cell lysates were analyzed by Western blot, by probing with an antibody against general RPA2. Nonsilenced U2-OS cells were used as a control to compare endogenous and ectopic RPA2 expression (lane 3). For a protein loading control, a portion of the Ponceau S-stained transfer membrane is shown. pS4pS8, Ser(P)23; pT21, Tyr(P)21; pS29, Ser(P)29; pS33, Ser(P)33.

Using clonal U2-OS cells, the expression of either wt-RPA2 or a double S23A/S29A-RPA2 mutant was induced, and the endogenous RPA2 protein levels were then down-modulated by treatment with a specific siRNA molecule. Lysates were prepared from cells experiencing genotoxic stress or mitotic cells. Incubation of cells with CPT again caused the appearance of a hyperphosphorylated wt-RPA2 species that was also recognized by the anti-Ser(P)29 antibody (Fig. 2B, lane 1). The S23A/S29A mutation reduced the amount of hyperphosphorylated RPA2 and reactivity to the Ser(P)29 probe (lane 2). Examining mitotic RPA2, although the wild-type subunit generated a strong Ser(P)29 signal, the S23A/S29A mutation nearly completely caused the loss of the mitotic RPA2 species and the Ser(P)29 signal (lanes 3 and 4).

We tested Ser29 modification across the cell cycle. U2-OS cells were arrested in mitosis with nocodazole, and aliquots of the arrested cells were then released into the cell cycle for 0.5 h (early and late G1 phases), 16 h (S phase), or 20 h (a mixture of S and G2 cells) (Fig. 2C). Lysates from these mitotic cell cultures (0 h) were subjected to Western blot analysis using antibodies directed to Ser(P)29-RPA2 or total RPA2. In mitotic cells, the RPA2 pool was present in three bands, a basal (nonphosphorylated) form and two slower migrating species. Of the three mitotic RPA species, the Ser(P)29 antibody recognized the slower migrating forms. When cells were released into the cell cycle, we did not observe any significant reactivity of RPA2 to the Ser(P)29 antibody in G1, S, or G2 cells. Along with verifying the usefulness of these antibodies, these data indicate that Ser29 is phosphorylated both during mitosis and in interphase only in response to genotoxic stress.

Effect of Cyclin-Cdk Site Mutation on Formation of Mitotic and Hyperphosphorylated RPA2—Expression of an S23A/S29A-RPA2 mutant in vivo was previously found to prevent RPA2 phosphorylation (20) but did not reveal the relative importance of each site. We therefore examined the relative importance of the Ser23 and Ser29 sites in supporting mitotic RPA phosphorylation in vivo. To address these questions, we first tested Myc-tagged RPA2 molecules with one or both sites mutated to alanine (to prevent phosphorylation). A previous test of Myc-tagged RPA2 indicated that the subunit is efficiently incorporated into heterotrimeric RPA (27).

U2-OS cells were transiently transfected with S23A-RPA2, S29A-RPA2, doubly mutated S23A/S29A-RPA2, or the control wt-RPA2. Following transfection, lysates were prepared from nocodazole-arrested mitotic cells and probed by Western analysis for the Myc tag (Fig. 2D). Similar to the endogenous RPA2 in mitotic cells (see Fig. 2C), the Myc-tagged wt-RPA2 had three bands corresponding to the basal RPA2 (marked B), mitotic RPA2 (+p2), and a form that migrated between these two species (+p1) (lane 1). The S23A and S29A single mutants resulted in a loss of the slowest migrating species (lanes 2 and 3), and the double S23A/S29A mutant gave rise to only a single band that migrated identically to nonphosphorylated RPA2 (lane 4).

It is important to note that the migration of RPA2 in SDS-PAGE is governed by the RPA phosphorylation state (22) (i.e., the addition of a single phosphate residue causes a small reduction in RPA2 mobility, and two phosphates provide a greater
Sequential RPA Phosphorylation

reduction, up until the addition of approximately five or more phosphates, which cause RPA2 to migrate in the hyperphosphorylated position. Thus, the observed changes in the RPA2 migration pattern are consistent with the +2p species being phosphorylated at two sites, Ser23 and Ser29. The S23A and S29A single mutants each contain one phosphate at the non-mutated Cdk site. The co-migration of the S23A/S29A mutant with basal RPA2 indicates a lack of phosphorylation of this species in mitotic cells.

To provide additional evidence for these conclusions, we also tested constructs in which the same sites were mutated to aspartate (to mimic phosphorylation). The negative charge on aspartate reduces RPA2 mobility similar to that of a phosphate residue (e.g. see Ref. 27). The S23D and S29D single mutants each generated two mitotic bands that co-migrated with the upper two species seen for wt-RPA2 (Fig. 2D, lanes 5 and 6). The S29D mutant showed a greater amount of phosphorylation (i.e. the +2p species) compared with the S29A mutant (i.e. the +1p species), suggesting that the negative charge on the Asp29 residue facilitates subsequent phosphorylation of Ser23. The double S23D/S29D mutant gave rise to only a single band that migrated similarly to the wt-RPA2 +2p species (lane 7). These data demonstrate that both Ser23 and Ser29 are modified during mitosis. The lack of detection of any mitotic S23A/S29A-RPA2 species migrating more slowly than basal RPA2 and the presence of only a single S23D/S29D-RPA2 form argue that Ser23 and Ser29 are probably the only residues significantly phosphorylated in mitotic RPA2 (i.e. if other positions were modified in mitotic cells, these modifications would be expected to cause a mobility shift in either S23A/S29A- or S23D/S29D-RPA2).

Cyclin-Cdk Complexes Phosphorylate Ser29 during Mitosis and under Conditions of Genotoxic Stress—Ser23 and Ser29 are known Cdk sites, with both cyclin A-Cdk2 and cyclin B-Cdk1 competent to phosphorylate Ser23 and Ser29 in vitro (20, 32, 36–39). We used the anti-Ser(P)29 antibody to further test if Cdk kinases also phosphorylate endogenous RPA2 protein in vivo during mitosis and following genotoxic stress. We tested roscovitine, a highly selective inhibitor of Cdk1 and Cdk2 (40). Because Ser(P)29 is only formed in non-stressed cells during mitosis, we merely enriched for mitotic cells by an overnight treatment with nocodazole, yielding a robust

FIGURE 2. Modification of Ser(P)29 on RPA2. A, extracts prepared from CPT-treated cells (2 µM for 1 h) were subjected to λ-phosphatase treatment or mock-treated in the presence of phosphatase inhibitors. Lysates were examined by Western blotting for Ser(P)29 (pS29), Thr(P)21 (pT21) and general RPA2. Western blots probed with the anti-Thr(P)21 antibody were later stripped and probed with a general RPA2 antibody. The Ponceau stain loading control is therefore identical for these two samples (indicated by an asterisk). B, cloned U2-OS cells were induced to express untagged wt- or S23A/S29A-RPA2, using the strategy shown in Fig. 1C. Replaced cells were mock-treated, incubated with CPT (2 µM for 1 h), or incubated with nocodazole (100 ng/ml for 12 h). For cells treated with nocodazole, loosely attached cells were shaken off to isolate mitotic cells. Subsequent to all treatments, cells lysates were prepared and subjected to Western analysis using either an anti-Ser(P)29 or general RPA2 antibody. As a control, lysates were also probed with an anti-β-actin antibody. C, U2-OS cells were arrested with nocodazole and then released and harvested at different time points postrelease. Cells collected at the various time points were analyzed by FACS, and the cycle-cycle distribution is indicated at the bottom. Lysates were prepared from each cell sample and analyzed by Western using anti-Ser(P)29-RPA2 and general RPA2 antibodies, as indicated. For both panels, H refers to hyperphosphorylated RPA2, M indicates mitotic RPA2, and B designates basal (i.e. nonphosphorylated) RPA2. D, mutation of Ser23 and Ser29 alters formation of mitotic RPA. U2-OS cells were individually transfected with Myc-tagged wt-RPA2 or the RPA2 mutants S23A, S29A, S23A/S29A, S23D, S29D, and S23D/S29D, as indicated. Post-transfection (48 h), cells were nocodazole-treated for 17 h. Mitotic cells were collected by shake off, and lysates prepared and subjected to Western analysis using an antibody against the Myc epitope. The wt-RPA2 showed three distinct bands: B (basal), +1p, and +2p, the last migrating similarly to mitotic RPA2. Our data indicate that these species represent nonphosphorylated, monophosphorylated, and diphosphorylated RPA2, respectively.
phosphorylation state. These data suggest that inhibition of Ser\textsuperscript{23} and Ser\textsuperscript{29} phosphorylation by roscovitine reduces subsequent modification events (e.g. at Ser\textsuperscript{4} and Ser\textsuperscript{8}), precluding conversion of Ser(P)\textsuperscript{23,29}-RPA2 to the hyperphosphorylated state. This point is examined more rigorously below.

Analysis of the RPA2 N-terminal region for phosphorylation motifs (i.e. using Scansite) (41) indicated that Ser\textsuperscript{23} and Ser\textsuperscript{29} are also potential sites for extracellular signal-regulated kinases (ERK1/2) (data not shown). Although normally associated with mitogen activation, signaling through the ERK pathway has been found to be induced in response to DNA damage (reviewed in Ref. 42). To test the possibility that ERK1/2 also phosphorylates Ser\textsuperscript{29} under conditions of genotoxic stress, we employed the MEK inhibitor U0126. The addition of U0126 to CPT-treated U2-OS cells greatly reduced the level of ERK phosphorylation by MEK (Fig. 3C). However, U0126 did not have significant effects on RPA2 hyperphosphorylation or on the level of Ser(P)\textsuperscript{29}-RPA2. We therefore conclude that Ser\textsuperscript{29} phosphorylation in response to DNA damage is catalyzed by a cyclin-Cdk complex. Although this event probably occurs early in the DNA damage signaling pathway, it nevertheless represents a rare example of genotoxic stress stimulating phosphorylation of a DNA repair protein by cyclin-Cdk.

**Lack of DNA-PK Reduces RPA Phosphorylation at All Detectable RPA2 Sites**—We examined the role of DNA-PK in the phosphorylation of various RPA2 residues. We employed two paired cell lines that are either wild-type (M059K) or null (M059J) for expression of the DNA-PK catalytic subunit (DNA-PK\textsubscript{cs}) (43). Following mock treatment or incubation with CPT, lysates from these lines were prepared, and the phosphorylation status of the different RPA2 sites was examined by Western analysis. Very little RPA2 phosphorylation was seen in either of the mock-treated cell lines, with the exception of Ser(P)\textsuperscript{29} migrating at an intermediate position, probably arising from mitotic cells (Fig. 3D, lanes 1 and 2). Following exposure to CPT, cells expressing DNA-PK\textsubscript{cs} showed significant RPA2 hyperphosphorylation and formation of Ser(P)\textsuperscript{33,29}, Thr(P)\textsuperscript{21}, and Ser(P)\textsuperscript{4/8} (lane 3). In contrast, cells lacking DNA-PK\textsubscript{cs} were severely deficient in the phosphorylation of Ser(P)\textsuperscript{33,29} and Thr(P)\textsuperscript{21} (lane 4). Loss of the Ser(P)\textsuperscript{29} and Ser(P)\textsuperscript{29} signals was also noted but only in the hyperphosphorylated position. For both modifications, the presence of DNA-PK\textsubscript{cs} did not have any notable effects on the Ser(P)\textsuperscript{33} and Ser(P)\textsuperscript{29} signals in the intermediate position. Combined with past studies examining RPA phosphorylation by DNA-PK (22, 23), these data are consistent with the proposal that DNA-PK is a primary but not sole kinase responsible for phosphorylating RPA2. Although normally associated with mitogen activation, signaling through the ERK pathway has been found to be induced in response to DNA damage (reviewed in Ref. 42). To test the possibility that ERK1/2 also phosphorylates Ser\textsuperscript{29} under conditions of genotoxic stress, we employed the MEK inhibitor U0126. The addition of U0126 to CPT-treated U2-OS cells greatly reduced the level of ERK phosphorylation by MEK (Fig. 3C). However, U0126 did not have significant effects on RPA2 hyperphosphorylation or on the level of Ser(P)\textsuperscript{29}-RPA2. We therefore conclude that Ser\textsuperscript{29} phosphorylation in response to DNA damage is catalyzed by a cyclin-Cdk complex. Although this event probably occurs early in the DNA damage signaling pathway, it nevertheless represents a rare example of genotoxic stress stimulating phosphorylation of a DNA repair protein by cyclin-Cdk.

**Sequential RPA Phosphorylation**—To comprehensively examine the interplay between RPA2 phosphorylation events, we tested the effect of various RPA2 mutations. The mutations were located in either the Cdk sites (Ser\textsuperscript{23} and Ser\textsuperscript{29}) or the PIKK sites (Thr\textsuperscript{21} and Ser\textsuperscript{33}), with the RPA2 mutants inductively expressed from stable U2-OS cell clones (see Fig. 1C). Following “replacement” of the endogenous RPA2, cells were treated with CPT (2 \mu M for 1 h). The lysates were separated on gels that allowed clear separation of the different phosphorylation species and analyzed by Western blot. Control experiments indicated that, in the absence of CPT, replacement per se does not cause RPA hyperphosphorylation (Fig. 4A).
We first examined the relative importance of the cyclin-Cdk and PIKK sites in supporting the formation of hyperphosphorylated RPA2 in response to CPT. Cells expressing RPA2 with double mutations in the cyclin-Cdk (S23A/S29A) or PIKK (T21A/S33A) sites were analyzed (Fig. 4B). Although either double mutant inhibited formation of Ser(P)^33-RPA2 and RPA2 hyperphosphorylation, the mutation of the two PIKK sites caused a greater loss of the Ser(P)^33 signal (lane 2). Regarding the Ser^33 site, it was seen that the double Cdk site mutant caused a significant reduction in the level of Ser^33 in hyperphosphorylated RPA2 (H) while not inhibiting the Ser(P)^33 signal migrating near basal RPA2 (L; lane 3). Mutation of both PIKK sites resulted in a significant diminution of Ser(P)^29. The level of Ser(P)^33 in cells expressing the T21A/S33A-RPA2 mutant and the amount of Ser(P)^29 in S23A/S29A-RPA2 cells was low, demonstrating the efficiency of the silencing procedure. Note also that the Ser(P)^33/Ser(P)^8 signal seen for wt-RPA2 is significantly greater than a simple addition of the signals seen for each double mutant protein. These data reveal that the two kinase pathways (Cdk and PIKK) have a synergistic action on RPA2 hyperphosphorylation.

To further dissect the role of individual amino acids in supporting RPA phosphorylation, we studied single mutants for each class of sites. The effect of Cdk site mutations was examined. In response to CPT, the control wt-RPA2 showed robust phosphorylation of Ser^33, Ser^29, Thr^21, and Ser^4/Ser^8 (Fig. 4C, lane 1). The vast bulk of the phosphorylated RPA2 migrated in the hyperphosphorylated form. The S23A/S29A double mutation had significant inhibitory effects on Ser^33/Ser^8 and Thr^21 modification and on the generation of hyperphosphorylated RPA2 (Fig. 4C, lane 2). The level of Ser(P)^33 migrating at the position of hyperphosphorylated RPA2 was also strongly reduced in intensity, although the Ser(P)^33-positive species that migrated slightly above basal RPA2 (marked L) persisted, similar to the effect seen above (Fig. 4B, lane 3). In other studies, we have found that HU treatment caused the formation of a Ser^33-positive RPA2 species that migrates only slightly above nonphosphorylated RPA2, indicating that Ser^33 is the first, or one of the first, RPA2 residues to be modified in response to genotoxic stress (supplemental Fig. 2). Because RPA2 migration on SDS-PAGE is governed by the RPA phosphorylation state (22) (see above), the slight reduction in the L form of Ser(P)^33-RPA2 relative to basal nonphosphorylated indicates that Ser^33 is probably the only residue modified on this RPA2 species. Logically, the fact that Ser(P)^33 is present in RPA2 of a low phosphorylation state also demonstrates that Ser^33 can be one of the first residues modified in response to CPT treatment (also see “Discussion”). Overall, these data suggest that the Cdk site double mutation does not greatly affect initial formation of the Ser(P)^33-RPA2 but rather prevents addi-
tional phosphorylation events, including those at Ser\(^4\), Ser\(^8\), and Thr\(^{21}\), leading to RPA2 hyperphosphorylation.

We tested the possibility that phosphorylation of the two Cdk sites stimulates RPA2 hyperphosphorylation. Cells were replaced with wt-RPA2, the S23A/S29A variant, or an RPA2 mutant containing two aspartate substitutions at the Cdk phosphorylation sites (S23D/S29D-RPA2). Concerning the S23D/S29D mutant, it has been previously found that aspartate (or glutamate) residues often mimic phosphorylated serines/threonines. We successfully used an aspartate-substituted RPA2 as a surrogate “phosphoprotein” in an earlier examination of RPA phosphorylation (27). Following replacement, the cells were examined under control conditions or after exposure to CPT (2 \(\mu\)M for 1 h). Testing the RPA2 status by Western blot, we found that the RPA2 variants from nonstressed lysates had a single major band migrating in the basal RPA2 position (Fig. 4D). As seen above (see Fig. 2D), S23D/S29D-RPA2 migrated on SDS-PAGE with reduced mobility (Fig. 4D, lane 2). Under conditions of genotoxic stress, wt-RPA2 but not S23A/S29A-RPA2 showed the appearance of the hyperphosphorylated form (H; Fig. 4D, lanes 4 and 6, respectively), as seen above. Importantly, the S23D/S29D mutant also showed the appearance of a hyperphosphorylated form (Fig. 4D, lane 5). Thus, a variant that mimics RPA2 phosphorylated by Cdk facilitates further phosphorylation events seen after genotoxic stress. These data provide further support for the conclusion that RPA2 phosphorylation events are interrelated.

Of the S23A- and S29A-RPA2 single mutants, S23A had significantly stronger effects on the formation of Ser\(^{P}\)\(^{23}\), Thr\(^{P}\)\(^{21}\), and Ser\(^{P}\)\(^{4}\)/Ser\(^{P}\)\(^{8}\) (Fig. 4C, lane 4), with the reduction in modification of these sites comparable to that seen with the double mutant. The S23A mutation also reduced the amount of Ser\(^{P}\)\(^{29}\), indicating that Cdk-mediated phosphorylation of Ser\(^{23}\) stimulates co-modification of Ser\(^{29}\). The S29A mutation had only weak effects on the various RPA2 modifications (lane 3), again attesting to the lack of effect of RPA2 mutations on antibody recognition (see also below). Similar to the Cdk double mutant, the S23A single mutant showed a significant Ser\(^{P}\)\(^{23}\) signal migrating at the “L” position. These data indicate that the inability to phosphorylate the two Cdk sites, primarily Ser\(^{23}\), strongly inhibits modification of all other detectable residues, including the distal Ser\(^4\) and Ser\(^8\) sites.

Considering the importance of the two PIKK sites in response to CPT, the T21A/S33A double mutation strongly inhibited modification of Ser\(^{P}\)\(^{29}\), both at the hyperphosphorylated and intermediate positions (Fig. 4E, lane 4; see also Fig. 4B, lane 2). In addition, the mutation also abolished detectable formation of Ser\(^{P}\)\(^{4}\)/Ser\(^{P}\)\(^{8}\) and hyperphosphorylated RPA2. To determine the relative importance of the two amino acid changes in causing reduction of Ser\(^{P}\)\(^{29}\) and Ser\(^{P}\)\(^{4}\)/Ser\(^{P}\)\(^{8}\), we also tested T21A and S33A single mutants. Comparing the two mutations, both similarly reduced formation of Ser\(^{P}\)\(^{4}\)/Ser\(^{P}\)\(^{8}\) and Ser\(^{P}\)\(^{29}\) to levels intermediate between that of wt-RPA2 and T21A/S33A-RPA2. Somewhat surprisingly, although Thr\(^{P}\)\(^{21}\) is only found in the hyperphosphorylated position, the T21A mutation also had severe inhibitory effects on the level of Ser\(^{P}\)\(^{33}\) in the hyperphosphorylated position (H), although Ser\(^{P}\)\(^{33}\) in RPA2 of a low phosphorylation state (L) was unaffected. Similarly, mutation of Thr\(^{21}\) did not alter the level of Ser\(^{P}\)\(^{29}\) migrating at the intermediate position. Therefore, these data indicate that phosphorylation of Thr\(^{21}\) most strongly influences events occurring late in the RPA2 phosphorylation pathway.

It is unlikely that the T21A mutation creates a defect in the general conformation of the RPA2 N terminus, because this region is thought to be unstructured (16, 17). It is also highly unlikely that antibody recognition of any of the RPA2 phosphoresidues is directly affected by mutation of other sites for the following reasons. For Ser\(^{P}\)\(^{4}\)/Ser\(^{P}\)\(^{8}\), the immunizing peptide used to generate the antibody did not contain Thr\(^{21}\) or other residues to the C terminus. For Ser\(^{P}\)\(^{29}\), the T21A/S33A mutation did not affect recognition of the phosphoepitope by this antibody, because this mutation affected neither the total level of mitotic RPA2 nor Ser\(^{P}\)\(^{29}\).RPA2 (supplemental Fig. 3, lane 2). Similarly, although the S23A mutation changed the position of mitotic RPA2 and the migration of the primary Ser\(^{P}\)\(^{29}\) band, it was nevertheless efficiently detected by the Ser\(^{P}\)\(^{29}\) antibody (lane 3). For Ser\(^{P}\)\(^{33}\), we demonstrated above that mutation of the adjacent Ser residue (S29A) did not alter the level of Ser\(^{P}\)\(^{33}\) (Fig. 4C). The next closest residue is 10 amino acids away (Ser\(^{35}\)), making it highly unlikely that Ser\(^{23}\) or Thr\(^{21}\) would be a determinant in the Ser\(^{P}\)\(^{33}\) recognition motif. This conclusion is strengthened by the finding that, for both the S23A and T21A single mutants, the L form of Ser\(^{P}\)\(^{33}\) was recognized at least as well as that of the wild type. It must be noted that although the S23A mutation reduced the level of Ser\(^{P}\)\(^{33}\), this mutation also abolished RPA2 hyperphosphorylation as assayed with a monoclonal antibody that does not target phosphorylated residues. Thus, the reduction of the Ser\(^{P}\)\(^{33}\) signal is a consequence of the general loss of RPA phosphorylation. For Thr\(^{P}\)\(^{21}\), a peptide containing RPA2 residues 16–26 was used to generate the anti-Thr\(^{P}\)\(^{21}\) antibody. This is consistent with the fact that the S29A mutation did not affect formation of Thr\(^{P}\)\(^{21}\), indicating that this mutation does not affect recognition of the epitope (Figs. 4C). Although the S23A mutation could affect formation of Thr\(^{P}\)\(^{21}\), the strong effect on RPA2 hyperphosphorylation (using the general antibody) and on Ser\(^{P}\)\(^{4}\)/Ser\(^{P}\)\(^{8}\) formation (Fig. 4C) indicate that the loss of Thr\(^{P}\)\(^{21}\) is caused by the S23A mutation causing a great reduction in the overall phosphorylation of RPA2. It must be emphasized that, in all cases where a particular mutation had an effect on the level of hyperphosphorylated RPA2, this effect was also seen when using a general RPA2 antibody. These data demonstrate that the information obtained using the phospho-specific antibodies in combination with the various RPA2 mutations yields valid data.

Cell Cycle Dependence of RPA2 Phosphorylation in Response to Genotoxic Stress—We have shown that phosphorylation of cyclin-Cdk sites influences the modification of non-Cdk sites on RPA2. Because Cdk2 activity is strongly up-regulated beginning at the G1/S transition, it can be predicted that the RPA phosphorylation pattern in response to genotoxic stress will be cell-cycle dependent. We tested this hypothesis using either

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4 E. McIntosh, personal communication.

5 Monica Giddings, personal communication.
CPT or Ble, agents which have different mechanisms of action. CPT indirectly causes damage through the collision of DNA replication forks with trapped topoisomerase I-DNA complexes (33). In contrast, Ble directly causes single and double strand DNA breaks (44). For cell synchronization, mitotic U2-OS cells were first prepared by treatment with nocodazole, and the cells were then released and allowed to proceed through the cell cycle for 4 h (early G1), 8 h (late G1), 16 h (S phase), and 20 h (a mixture of cells in late S and G2). Cell cycle positions were determined by FACS (Fig. 5A). For 1 h prior to harvest, cells were treated with either CPT or Ble, and the resulting lysates were analyzed by Western for total RPA2, for specific phosphorylated RPA2 residues, or for γH2AX (a DNA damage marker).

Although the level of γH2AX is not a direct quantitative measurement of the amount of DNA damage, at least at moderate to high levels of DSBs (45), it is nevertheless a useful indicator of the overall extent of damage and the relative amount of DNA repair. Although it is conceivable that defects in DNA damage signaling could affect γH2AX formation and thereby complicate our analysis, current evidence indicates that RPA phosphorylation does not have obvious effects on checkpoint activation (e.g. see Refs. 24 and 46).

Testing CPT, we found no significant RPA phosphorylation in G1 cells at 4 or 8 h (Fig. 5B, lanes 2 and 4), apart from a slight Ser(P)4/Ser(P)8 signal. In contrast, the 16-h S phase sample showed a very strong signal for Ser(P)29, Ser(P)33, Thr(P)21, and Ser(P)4/Ser(P)8 (lane 6). These signals decreased slightly at the 20 h time point as an increased portion of cells entered G2 (lane 8). The increase in RPA phosphorylation was CPT-dependent, since no significant phosphorylation of RPA2 (or H2AX) was observed in 16 and 20 h samples prepared from mock-treated cells (lanes 9 and 10).

Adding Ble to G1 cells did not cause significant RPA2 modification except for a weak Ser(P)4/Ser(P)8 signal (Fig. 5B, lanes 1 and 3). Even so, robust formation of γH2AX was detected, demonstrating the presence of a high level of DSBs. S-phase cells treated with Ble had only a minor increase in RPA phosphorylation, specifically of the Ser(P)29 and Ser(P)33 signals (lane 5). The level of RPA phosphorylation in these cells was dramatically less than RPA from S-phase cells treated with CPT (lane 6), although the level of DSBs caused by Ble was significantly greater, as indicated by the markedly higher γH2AX levels. When cells in late S and G2 were treated with Ble (20 h), Thr21 modification was also detected, along with an increase in the level of Ser(P)4/Ser(P)8 (lane 7). These data indicate that although Ble treatment of S-phase cells causes a higher level of DNA breaks compared with CPT treatment, it generates weaker RPA phosphorylation. These data also demonstrate that RPA is not subject to significant phosphorylation in response to all DNA breakage events. We take the results of these experiments to indicate that the collision of DNA replication forks with trapped topoisomerase I complexes generates significant amounts of ssDNA that facilitate RPA phosphorylation.

Expression of the S23A/S29A-RPA2 Mutant Alters the Cell Cycle Profile—Because Ser29 and presumably Ser23 are phosphorylated in response to genotoxic stress, we tested if expression of S23A/S29A-RPA2 had any gross biological effects. The cell cycle profiles of U2-OS clones replaced for wt- or S23A/S29A-RPA2 were examined by FACS (Fig. 6A). Cells were tested both under normal conditions and 12 h following a 3-h exposure to 4 μM CPT. In the absence of stress (Normal), a significantly greater fraction of cells expressing the mutant RPA2 were in the G2 phase as compared with cells expressing wt-RPA2. Following stress, both wild type and mutant cells had a higher fraction in S phase (1.5–2.0-fold over nonstressed

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**FIGURE 5.** Cell cycle dependence of RPA2 phosphorylation in response to genotoxic stress. U2-OS cells were released from a nocodazole arrest and harvested at different time points postrelease. One h prior to harvest, cells were treated with CPT or Ble. A, FACS analysis of cell cycle positions. Aliquots of the nontreated cells were subject to FACS to determine the relative fraction of cells in G1 (black bars), S (stippled bars), or G2/M phases (white bars). B, lysates of harvested cells were analyzed by Western using the indicated antibodies. White lines have been added to more clearly indicate samples with identical treatment times and are not meant to indicate splicing of the figure. Protein loading is shown using a portion of the Ponceau S-stained transfer membrane. The relative migration of the various RPA2 species is indicated using the same scheme as described in the legend to Fig. 3. pS33, Ser(P)29-RPA2; pS29, Ser(P)33-RPA2; pT21, Thr(P)21-RPA2.

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6 V. M. Vassin and J. A. Borowiec, unpublished observations.
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cells. This result is expected, since U2-OS cells have an impaired G1/S arrest, allowing cells with damaged DNA to enter S (47). CPT-induced damage would lead to an extended S phase as replication forks encounter unrepaired DNA. Even so, cells expressing the S23A/S29A-RPA2 mutant had a significantly higher level in S phase as compared with cells expressing wt-RPA2 and a reduced fraction in G2. These data suggest that, compared with cells expressing wt-RPA2, S23A/S29A-RPA2 cells had a greater level of DNA damage at later times following CPT treatment.

We examined the effect of RPA2 mutation on the formation of foci in the presence or absence of stress caused by CPT treatment. In untreated cells, those expressing wt- or S23A/S29A-RPA2 had apparently similar levels of RPA2 staining (Fig. 6B). Following a 1-h exposure to CPT, RPA2 in apparent DNA damage foci showed a significantly greater immunofluorescence signal compared with untreated cells. Although a spectrum of RPA2 staining intensities was noted, it appeared that cells replaced for S23A/S29A-RPA2 had an average RPA2 staining intensity that was greater than that seen in cells expressing wt-RPA2. Cells representative of average staining patterns are shown (Fig. 6B).

We quantitated the differences in RPA2 staining in cells that were fixed either 0 or 8 h following CPT treatment. In cells fixed immediately after treatment (0 h), a slightly greater fraction of S23A/S29A-RPA2 cells had robust RPA2 staining (39%; n = 224) compared with wt-RPA2 cells (34%; n = 322). Of these, cells expressing S23A/S29A-RPA2 had a significantly higher staining intensity (average = 54.8, mean = 53.6) compared with wt-RPA2 cells (average = 40.2, mean = 38.7) (Fig. 6C). Eight h post-treatment, the fraction of cells with damage-dependent RPA2 staining increased slightly for both cell types (44% of wt-RPA2 cells (n = 294); 45% of S23A/S29A-RPA2 cells (n = 265)), and the average RPA2 staining level in both cell types increased. However, cells expressing the mutant were seen to have more intense RPA2 staining intensity (average = 90.5, mean = 82.4) relative to wt-RPA2 cells (average = 59.1, mean = 53.1) (Fig. 6C). These data suggest that, in response to CPT treatment, the phosphorylation site mutation impairs the ability of cells to repair the induced DNA damage, leading to higher levels of RPA binding.

Mutation of Ser23 and Ser29 Cdk Sites Inhibits Chromosomal DNA Repair—We tested the hypothesis that RPA2 phosphorylation stimulates DNA repair. Cells replaced for either wt- or S23A/S29A-RPA2 were treated with CPT or Ble and then fixed immediately (0 h) or at 8 h post-treatment for CPT or at 15 h in the case of Ble. To assay for DNA damage, we quantitated the levels of γH2AX by immunofluorescence microscopy. Both the fraction of γH2AX-positive cells and the level of γH2AX staining were assayed.

Testing CPT, it was found that the RPA2 variant expressed had no significant effects on the fraction of cells showing significant γH2AX staining (Fig. 7A). In contrast, the phosphorylation site mutation had a significant effect on the average γH2AX signal in the γH2AX-positive cells. For example, at 8 h following treatment, the relative γH2AX signal in cells expressing wt-RPA2 was 2.6 ± 1.7 units compared with 8.6 ± 4.0 units for S23A/S29A-RPA2 cells. Representative cells reflecting this

FIGURE 6. Expression of S23A/S29A-RPA2 alters the cell cycle profile and the response to DNA damage. A, FACS analysis of U2-OS cells “replaced” for wt- or S23A/S29A-RPA2. Cells were examined in the absence of exogenous stress (Normal), and 12 h following a 3-h exposure to 4 μM CPT. To determine statistical significance, data from cells expressing wt- and S23A/S29A-RPA2 were analyzed using Student’s t test (*, p < 0.05; **, p < 0.01), comparing either mock or CPT treatment conditions. B, representative RPA2 staining patterns of cells replaced for wt-RPA2 (left two panels) or S23A/S29A-RPA2 (DM; right two panels) under control conditions (top two panels) or following DNA damage induced by CPT treatment (2 μM for 1 h; bottom two panels). Images were analyzed by confocal microscopy, using identical image capture conditions. C, analysis of the distribution of RPA2 signals in cells replaced for wt- or S23A/S29A-RPA2. Each cell type was treated with 2 μM CPT for 1 h and then either immediately fixed (0 h post-CPT) or fixed 8 h after CPT treatment (8 h post-CPT). Following image capture under identical conditions, the level of RPA2 in each cell type and damage condition was quantitated. In those cells with RPA2 signals consistent with the presence of genotoxic stress (~100 cells for each condition), the number of cells is plotted against the level of RPA2 in each cell, using a bin range of 10 fluorescence units (e.g. 20 indicates those cells with staining intensities between 20.0 and 29.9 units).
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FIGURE 7. Mutation of RPA2 Cdk sites lengthens retention of γH2AX foci following genotoxic stress. Cells were silenced for endogenous RPA2 and ectopic RPA2 induced. Cells were then treated with 5 μM CPT (70 h postsilencing) or 30 μg/ml Ble (56 h postsilencing). Following a 2.5-h treatment, cells were washed twice with PBS and then fixed either immediately (0 h) or at 8 h (CPT) or 15 h (Ble) postwash. Cells were stained for γH2AX and DAPI, and images were captured by epifluorescence microscopy. The fraction of comparison with DAPI images (200–300 cells total). The average expressing wt- or S23A/S29A-RPA2, following treatment with CPT (That is, cells expressing RPA2 mutated at two Cdk sites had increased γH2AX levels in response to either CPT or Ble). Treatment of such cells also caused a greater fraction to have an elongated S phase, compared with cells expressing wt-RPA2, and led to more intense RPA2 foci. We postulate that the inability of cyclin-Cdk to phosphorylate RPA2 impairs recruitment of specific DNA repair factors to DNA lesions. The defective ability of RPA to recruit these factors leads to longer retention of γH2AX foci, increased binding of RPA to damaged DNA, and altered cell cycle progression.

A second significant conclusion of this study is that RPA phosphorylation in response to CPT treatment follows a preferred pathway involving both the cyclin-Cdk and PIKK families of kinases. The two kinase classes act synergistically to yield hyperphosphorylated RPA that is modified on the extreme N-terminal Ser4 and Ser8 residues. The major observations are as follows: 1) Ser33 is phosphorylated by ATR (24); 2) mutation of both of the cyclin-Cdk sites (S23A/S29A) causes a reduction of the Ser(P)33 signal in hyperphosphorylated RPA2 yet retains a Ser(P)33 signal migrating slightly above nonphosphorylated RPA2 (e.g., Fig. 4B); 3) RPA2 containing a S23D/S29D phosphomimetic mutation restores hyperphosphorylation; 4) mutation of Thr21 causes a loss of the Ser(P)29 signal from the hyperphosphorylated RPA2 foci. We postulate that the inability of cyclin-Cdk to phosphorylate RPA2 impairs recruitment of specific DNA repair factors to DNA lesions. The defective ability of RPA to recruit these factors leads to longer retention of γH2AX foci, increased binding of RPA to damaged DNA, and altered cell cycle progression.

>3-fold difference are shown (Fig. 7, B (wt-RPA2) and C (S23A/S29A-RPA2)). These data indicate that cells expressing the mutant RPA2 were defective in repair of CPT-induced lesions.

The RPA2 mutation also had effects following Ble treatment, with cells expressing S23A/S29A-RPA2 showing a significant increase in the fraction of cells that were γH2AX-positive (Fig. 7D). Immediately following treatment (0 h), the percentage of S23A/S29A-RPA2 cells with significant γH2AX staining was 2-fold higher than in wt-RPA2 cells (68 ± 0.1 versus 32.2 ± 0.1, respectively). At 15 h post-treatment, the fraction of γH2AX-positive cells expressing S23A/S29A-RPA2 was >4-fold higher (37.0 ± 5.7% versus 8.8 ± 0.1%, respectively). Cells replaced for S23A/S29A-RPA2 also had a slightly higher level of γH2AX staining compared with cells expressing wt-RPA2. Interestingly, although the fraction of γH2AX-positive cells decreased at 15 h post-Ble treatment compared with immediately following treatment, the level of γH2AX staining in these positive cells was seen to increase, perhaps reflecting an increased fraction of cells with intense γH2AX staining. Representative images of the different γH2AX staining patterns seen at 15 h after Ble treatment are shown (Fig. 7, E (wt-RPA2) and F (S23A/S29A-RPA2)). Images of untreated wt- and S23A/S29A-RPA2 cells are also provided, which demonstrate that both cell types show a low basal level of γH2AX staining in the absence of exogenous stress (Fig. 7G). In summary, these data indicate that cells expressing RPA unable to undergo cyclin-Cdk phosphorylation are defective in repair of lesions induced either by Ble or CPT.

DISCUSSION

The significance of RPA phosphorylation has been unclear since its discovery nearly 2 decades ago. Our data demonstrate that the role of RPA phosphorylation is to stimulate chromosomal DNA repair. (That is, cells expressing RPA2 mutated at two Cdk sites had increased γH2AX levels in response to either CPT or Ble). Treatment of such cells also caused a greater fraction to have an elongated S phase, compared with cells expressing wt-RPA2, and led to more intense RPA2 foci. We postulate that the inability of cyclin-Cdk to phosphorylate RPA2 impairs recruitment of specific DNA repair factors to DNA lesions. The defective ability of RPA to recruit these factors leads to longer retention of γH2AX foci, increased binding of RPA to damaged DNA, and altered cell cycle progression.

A second significant conclusion of this study is that RPA phosphorylation in response to CPT treatment follows a preferred pathway involving both the cyclin-Cdk and PIKK families of kinases. The two kinase classes act synergistically to yield hyperphosphorylated RPA that is modified on the extreme N-terminal Ser4 and Ser8 residues. The major observations are as follows: 1) Ser33 is phosphorylated by ATR (24); 2) mutation of both of the cyclin-Cdk sites (S23A/S29A) causes a reduction of the Ser(P)33 signal in hyperphosphorylated RPA2 yet retains a Ser(P)33 signal migrating slightly above nonphosphorylated RPA2 (e.g., Fig. 4B); 3) RPA2 containing a S23D/S29D phosphomimetic mutation restores hyperphosphorylation; 4) mutation of Thr21 causes a loss of the Ser(P)29 signal from the hyperphosphorylated RPA2 foci. We postulate that the inability of cyclin-Cdk to phosphorylate RPA2 impairs recruitment of specific DNA repair factors to DNA lesions. The defective ability of RPA to recruit these factors leads to longer retention of γH2AX foci, increased binding of RPA to damaged DNA, and altered cell cycle progression.

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only migrates with hyperphosphorylated RPA2 (e.g. see Fig. 4C); 6) individual mutation of Ser\(^{33}\), Ser\(^{29}\), Ser\(^{23}\), and Thr\(^{21}\) all inhibit formation of Ser(P)\(^{33}\)/Ser(P)\(^{29}\), to varying degrees; and 7) test of the role of DNA-PK in this (see Fig. 3D) and previous studies (22, 23) indicates that DNA-PK phosphorylates Thr\(^{21}\) and Ser\(^{4}/\)Ser\(^{8}\).

We therefore propose the following scheme of RPA phosphorylation (Fig. 8A). In response to CPT, ATR becomes activated and modifies Ser\(^{33}\). Formation of Ser(P)\(^{33}\) stimulates formation of Ser(P)\(^{29}\) and Ser(P)\(^{23}\) through the action of a cyclin-Cdk complex, yielding a RPA2 species with intermediate migration by SDS-PAGE. Modification of Ser\(^{33}\), Ser\(^{29}\), and Ser\(^{23}\) stimulate the subsequent phosphorylation of Thr\(^{21}\) primarily by DNA-PK. Phosphorylation of these four residues leads to final modification of Ser\(^{4}\) and Ser\(^{8}\) and perhaps residues within the Ser\(^{11}\), Ser\(^{12}\), and Ser\(^{13}\) cluster, by the action of DNA-PK. Lack of Cdk phosphorylation inhibits further RPA2 phosphorylation events, leading to the aforementioned effects on DNA repair.

Although the large bulk of our data leads us to propose this model of a preferred pathway of RPA phosphorylation, this may not be the only pathway followed, and other explanations are conceivable. For example, one could argue that phosphorylation of Ser\(^{23}\) and Ser\(^{29}\) is constitutive, but the modifications are normally removed by a phosphatase that becomes inhibited by Ser\(^{33}\) phosphorylation. However, such a model appears less likely because we do not detect Ser(P)\(^{29}\) outside of mitosis in nonstressed cells (see Fig. 2C). We also emphasize that the pattern of phosphorylation and the kinases we propose in our model (Fig. 8A), were deduced from treatment of cells with CPT and are not meant to represent the pathway of events occurring in response to other genotoxic stresses or under all circumstances.

Our data also indicate that cyclin-Cdk and PIKK act synergistically in catalyzing RPA phosphorylation. In other words, mutation of the two cyclin-Cdk sites inhibits phosphorylation of the two PIKK sites, and loss of the two PIKK sites reduces Ser\(^{29}\), and presumably Ser\(^{23}\), modification (Fig. 4B). We hypothesize that this synergy exists in two forms, cis and trans. First, modification of particular residues on RPA2 stimulates phosphorylation of residues on the same RPA2 molecule (i.e. in cis; Fig. 8A). This could conceivably be caused by the modified residue(s) stabilizing the binding of a kinase, thereby increasing the efficiency of another phosphorylation event (e.g. formation
of Ser(P)\textsuperscript{33} facilitates Ser\textsuperscript{29} phosphorylation). In addition, our data also suggest that modification of one RPA molecule bound to ssDNA stimulates the phosphorylation of a different RPA molecule bound to the same ssDNA (Fig. 8B). In other words, we find that mutation of Thr\textsuperscript{21} affects modification of Ser\textsuperscript{29} and Ser\textsuperscript{33}, although formation of Thr\textsuperscript{21} appears to occur after Ser\textsuperscript{29} and Ser\textsuperscript{33} phosphorylation (i.e. Thr\textsuperscript{21} is only found in the hyperphosphorylated form). In addition, we also observe that loss of DNA-PK\textsubscript{cs} not only prevents Thr\textsuperscript{21} and Ser\textsuperscript{4}/Ser\textsuperscript{8} modification but also generation of Ser(P)\textsuperscript{29} by cyclin-Cdk. From these data, we propose that phosphorylation of Thr\textsuperscript{21}-RPA\textsubscript{2} on one RPA molecule stimulates the phosphorylation of Ser\textsuperscript{33}-RPA\textsubscript{2} by ATR on a different RPA molecule in trans. Such a regulatory device would allow all of the RPA bound to a particular lesion to achieve a similar phosphorylation state. This notion is conceptually similar to the propagation of γH2AX outward from a DSB by ATM (e.g. see Ref. 48).

The dual control of RPA phosphorylation by Cdk and PIKK members is reminiscent of a similar requirement for the cell cycle-dependent activation of ATR at DSBs. In this case, DSBs are resected by the Mre11 exonuclease in an ATM- and Cdk-dependent process to generate ssDNA tails, which are then bound by RPA (31). The ATR-ATRIP complex loads onto the RPA-bound ssDNA structure, leading to activation of the ATR kinase (28, 29). A similar reaction occurs in budding yeast (49). The co-regulation of ATR and RPA (i.e. both require upstream Cdk and PIKK activities) would be expected to facilitate coordination of these two factors in the signaling and repair of DNA damage. In other words, ATR activation or RPA phosphorylation alone would be unable to facilitate further downstream repair events. The need to simultaneously activate both factors for repair may serve as a check against spurious recruitment of DNA repair factors.

Our results extend those of Pan et al. (32) examining RPA phosphorylation in extracts from HeLa cells prepared at different cell cycle phases. These in vitro experiments led to the conclusion that phosphorylation of RPA\textsubscript{2} by cyclin A-Cdk\textsubscript{2} was critical for subsequent phosphorylation by DNA-PK. However, our in vivo study indicates that cyclin-Cdk is both facilitated by prior ATR action (at Ser\textsuperscript{33}) and stimulates subsequent phosphorylation of other residues by DNA-PK (Thr\textsuperscript{21} and probably Ser\textsuperscript{4} and Ser\textsuperscript{8}).

Use of various strategies (i.e. test of the Cdk inhibitor roscovitine and the MEK inhibitor U0126) lead us to conclude that the primary kinase that phosphorylates Ser\textsuperscript{29}-RPA\textsubscript{2} following CPT treatment is Cdk2. Because the phosphorylation occurs selectively in the S and G\textsubscript{2} phases and cyclin E-Cdk\textsubscript{2} is unable to modify RPA in vitro (32, 39), we postulate that the responsible kinase is cyclin A-Cdk\textsubscript{2}. Although increased phosphorylation of Cdk sites in response to genotoxic stress is rare, it is not unprecedented. Human p53, for example, also contains a site (Ser\textsuperscript{151}) that is apparently modified by cyclin A-Cdk\textsubscript{2} in response to UV or ionizing irradiation (48, 50). Although the general activity of cyclin A-Cdk\textsubscript{2} decreases in response to stress, we detect Ser\textsuperscript{29}-RPA\textsubscript{2} phosphorylation at early times after DNA damage when cyclin A-Cdk\textsubscript{2} would be expected to retain significant activity. Even so, it remains unclear why only a few cyclin A-Cdk\textsubscript{2} substrates show increased modification in response to genotoxic stress. In the case of RPA, it is possible that phosphorylation of Ser\textsuperscript{33} creates a more favorable binding site for cyclin A-Cdk\textsubscript{2}, stimulating Ser\textsuperscript{29} and Ser\textsuperscript{29} modification.

It is notable that the level of RPA phosphorylation in response to Ble is relatively low in G\textsubscript{1}, increases in S, and reaches a maximal level in G\textsubscript{2} phase. DSBs in vertebrate cells are repaired by nonhomologous end joining in G\textsubscript{1} and early S phases and by HR during late S and G\textsubscript{2} phases (51). Because we find maximum RPA\textsubscript{2} phosphorylation in cells treated with Ble in the late S and G\textsubscript{2} phases, we speculate that these RPA species are selectively generated at DSBs repaired by HR, presumably when bound to the ssDNA formed after 5’ to 3’ resection of DSB ends. The weak phosphorylation of RPA in G\textsubscript{1} cells treated with Ble may reflect the apparent lack of RPA in repair of DSBs by nonhomologous end joining. Hence, RPA phosphorylation may facilitate repair of DSBs by HR. We are currently exploring this possibility.

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