

Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes

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Replication protein A (RPA) is a DNA single-strand binding protein essential for DNA replication, recombination and repair. In human cells treated with the topoisomerase inhibitors camptothecin or etoposide (VP-16), we find that RPA2, the middle-sized subunit of RPA, becomes rapidly phosphorylated. This response appears to be due to DNA-dependent protein kinase (DNA-PK) and to be independent of p53 or the ataxia telangiectasia mutated (ATM) protein. RPA2 phosphorylation in response to camptothecin required ongoing DNA replication. Camptothecin itself partially inhibited DNA synthesis, and this inhibition followed the same kinetics as DNA-PK activation and RPA2 phosphorylation. DNA-PK activation and RPA2 phosphorylation were prevented by the cell-cycle checkpoint abrogator 7-hydroxystaurosporine (UCN-01), which markedly potentiates camptothecin cytotoxicity. The DNA-PK catalytic subunit (DNA-PKcs) was found to bind RPA which was replaced by the Ku autoantigen upon camptothecin treatment. DNA-PKcs interacted directly with RPA1 *in vitro*. We propose that the encounter of a replication fork with a topoisomerase–DNA cleavage complex could lead to a juxtaposition of replication fork-associated RPA and DNA double-strand end-associated DNA-PK, leading to RPA2 phosphorylation which may signal the presence of DNA damage to an S-phase checkpoint mechanism.

Keywords: camptothecin/DNA damage/DNA-dependent protein kinase/RPA2 phosphorylation

Introduction

Replication protein A (RPA) is an ubiquitous eukaryotic single-stranded DNA (ssDNA)-binding protein complex, which was originally identified as an essential factor for simian virus 40 (SV40) DNA replication *in vitro* (Wobbe *et al.*, 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). Human RPA is composed of three subunits of ~70, 32/34 and 14 kDa (RPA1, RPA2 and RPA3, respectively) whose assembly as a heterotrimeric complex is required for biological activity in DNA replication, repair and recombination (for a recent review see Wold, 1997). The

p34 subunit (RPA2) consists of two functional domains: an essential C-terminus domain and a small N-terminal domain comprising the first 35 amino acids (Wold, 1997). This N-terminal domain is not required for ssDNA binding, RPA complex formation or DNA replication (Henricksen *et al.*, 1996; Lee and Kim, 1996; Lin *et al.*, 1996). However, phosphorylation of RPA2 takes place in the N-terminal domain.

RPA2 is found phosphorylated (primarily at Ser23 and Ser29) in HeLa cells, even in the absence of DNA damage (Zernik-Kobak *et al.*, 1997) and during DNA replication (Dutta and Stillman, 1992; Fotedar and Roberts, 1992; Brush *et al.*, 1994; Henricksen *et al.*, 1996; Lee and Kim, 1996). RPA2 phosphorylation appears during the G₁ to S transition and persists through the S phase (Din *et al.*, 1990; Dutta and Stillman, 1992; Fotedar and Roberts, 1992). As cells progress through the late M phase, RPA2 is dephosphorylated. RPA2 phosphorylation is probably important for initiation and maintenance of S phase (Dutta and Stillman, 1992; Fotedar and Roberts, 1992) and for signaling mechanisms that coordinate DNA replication and cell cycle (Brush *et al.*, 1994; Henricksen and Wold, 1994; Henricksen *et al.*, 1996). RPA2 is additionally phosphorylated in response to DNA damage (Liu and Weaver, 1993; Carty *et al.*, 1994; Fried *et al.*, 1996; Henricksen *et al.*, 1996; Shao *et al.*, 1997; Wang *et al.*, 1997; Zernik-Kobak *et al.*, 1997). After UV exposure, several additional sites within the N-terminal 33 amino acids become phosphorylated; two of these (Ser21 and Ser33) are consensus sites for DNA-PK (Niu *et al.*, 1997; Zernik-Kobak *et al.*, 1997). DNA damage-induced RPA2 phosphorylation is probably important for regulating the damage-response pathways and the S-phase checkpoint (Carty *et al.*, 1994; Santocanale *et al.*, 1995; Brush *et al.*, 1996; Cheng *et al.*, 1996).

Three protein kinases have been implicated in RPA2 phosphorylation: DNA-dependent protein kinase (DNA-PK) (Brush *et al.*, 1994; Boubnov and Weaver, 1995; Zernik-Kobak *et al.*, 1997), cyclin-dependent kinases (cdks) (Dutta and Stillman, 1992; Gibbs *et al.*, 1996; Niu *et al.*, 1997), and ataxia telangiectasia mutated protein (ATM) (Brush *et al.*, 1996; Gately *et al.*, 1998). DNA-PK has been primarily implicated in DNA damage-induced RPA2 phosphorylation because it can phosphorylate *in vitro* the sites of RPA2 that are detected *in vivo* (Niu *et al.*, 1997; Zernik-Kobak *et al.*, 1997). Active DNA-PK consists of a large catalytic subunit (DNA-PKcs, ~470 kDa) and the Ku autoantigen, which is a heterodimer of Ku70 and Ku80 (70 and 86 kDa, respectively). DNA-PKcs binds weakly to double-stranded ends of DNA, and its binding and activity are strongly enhanced by Ku (Anderson and Carter, 1996). The kinase domain of DNA-PKcs is located at its C-terminus and is related to the kinase domain of phosphatidylinositol

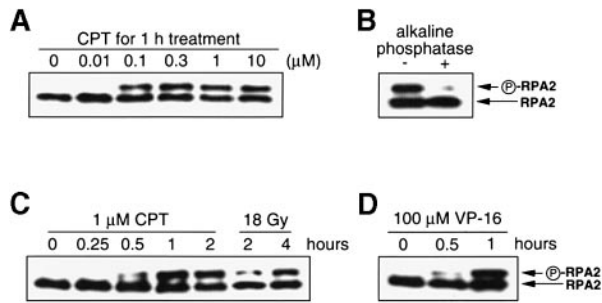


Fig. 1. DNA damage by camptothecin (CPT) induces rapid phosphorylation of RPA2 in human colon carcinoma HT29 cells. (A) Cells were treated with the indicated concentrations of CPT for 1 h. (B) Cell lysates from CPT-treated cells (1 μ M, 1 h) were immunoprecipitated using anti-RPA2 monoclonal antibody. Half of the immunoprecipitate was incubated with 20 U calf alkaline phosphatase (+) and the other half with phosphatase buffer lacking calf alkaline phosphatase (-). (C) Cells were treated with 1 μ M CPT or 18 Gy ionizing radiation for the indicated times. (D) Cells were treated with 100 μ M VP-16 for the indicated times. RPA2 phosphorylation was detected by Western blotting using anti-RPA2 monoclonal antibody.

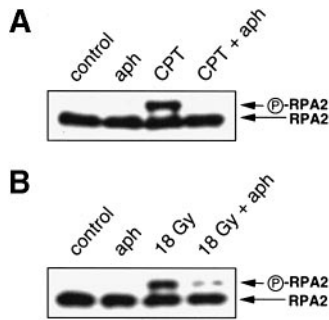


Fig. 2. Ongoing DNA replication is critical for RPA2 phosphorylation in response to DNA damage. HT29 cells were treated with 1 μ M camptothecin (CPT) (A) or 18 Gy ionizing radiation (B) in the absence or presence of aphidicolin (aph) (1 and 3 μ M in the case of CPT and ionizing radiation, respectively). Cell lysates were analyzed by SDS-PAGE and immunoblotting 1 h after addition of CPT or 4 h after irradiation.

3 kinases (PI3K). The genes of the PI3K-related family play key roles in the regulation of cell-cycle progression and cell-cycle checkpoints. In addition to DNA-PKcs, they include: *ATM*, the gene defective in ataxia telangiectasia patients; *ATR/FRP*, which is most closely related to *ATM*; *MEC1 (ESR1)* and *TEL1* in *Saccharomyces cerevisiae*; *rad3* in *Schizosaccharomyces pombe*; and *MEI-41* in *Drosophila* (Anderson and Carter, 1996). Other members of the family include *TOR1 (DDR1)* and *TOR2 (DDR2)* from *S.cerevisiae*, and their mammalian homologs *FRAP* and *RAF1* (also called *RAPT1*) (Anderson and Carter, 1996). Recently, a DNA-PK activity associated with ATM immunocomplexes has been found to phosphorylate RPA2 independently of DNA-PK (Gately *et al.*, 1998). Thus, both DNA-PK and ATM can be involved in DNA damage-induced RPA2 phosphorylation.

Camptothecin selectively poisons topoisomerase I by trapping topoisomerase I cleavage complexes, which correspond to enzyme-linked DNA breaks (for a review see Pommier, 1996). Camptothecin induces replication-dependent DNA lesions, and arrests cells in the S and G₂ phase of the cell cycle. DNA damage induced by camptothecin probably consists of replication-mediated DNA double-strand ends and formation of abnormal

replication intermediates, consequent to encounters of replication forks with camptothecin-stabilized topoisomerase I-DNA complexes (Tsao *et al.*, 1993; Pommier, 1996). When Chinese hamster lung fibroblast DC3F or human colon carcinoma HT29 cells are treated with the DNA polymerase inhibitor aphidicolin, the cytotoxicity of camptothecin is markedly suppressed, while the production of camptothecin-stabilized topoisomerase I-DNA complexes is not affected (Holm *et al.*, 1989; O'Connor *et al.*, 1991). Deficiency in S and G₂ phase arrest is correlated with enhanced cytotoxicity in human colon carcinoma cells (Goldwasser *et al.*, 1995, 1996; Shao *et al.*, 1997), and abrogation of the camptothecin-induced S-phase arrest/checkpoint by UCN-01 (7-hydroxystaurosporine) markedly enhances the cytotoxicity of camptothecin (Shao *et al.*, 1997).

We observed recently that pharmacological concentrations of camptothecin induce RPA2 phosphorylation in human colon carcinoma HT29 cells (Shao *et al.*, 1997). Wang *et al.* (1997) also reported that camptothecin induces RPA2 phosphorylation in HeLa cells. In the present study, we examined further RPA2 phosphorylation associated with camptothecin-induced DNA synthesis inhibition and a possible S-phase checkpoint. We investigated the kinetics of induction of RPA2 phosphorylation and the relationship between RPA2 phosphorylation and DNA-PK and ATM. Our results suggest that DNA replication itself is critical for activating DNA-PK, which can then lead to RPA2 phosphorylation. We also report for the first time that DNA-PKcs can form a multiprotein complex with RPA and that this complex is disrupted and switched to a DNA-PKcs-Ku complex in response to replication-dependent DNA lesions.

Results

RPA2 phosphorylation in response to DNA damage in human colon carcinoma and leukemia cells treated with topoisomerase inhibitors and ionizing radiations

RPA2 phosphorylation was determined by Western blotting of cell lysates using specific monoclonal antibody. Under these conditions, hyperphosphorylated forms of RPA2 exhibit reduced mobility corresponding to an increase of 2–4 kDa (Din *et al.*, 1990; Dutta and Stillman, 1992; Fotedar and Roberts, 1992; Carty *et al.*, 1994). We first tested the effect of camptothecin on RPA2 phosphorylation in human colon carcinoma HT29 cells. Figure 1 shows that RPA2 phosphorylation was induced at pharmacological camptothecin concentrations (Figure 1A) and within 30 min of drug treatment (Figure 1C). The total RPA2 protein levels did not change significantly in response to camptothecin treatment. To confirm that the RPA2 band with retarded electrophoretic migration corresponded to phosphorylated RPA2, cell lysates from camptothecin-treated cells were digested with alkaline phosphatase. Under these conditions, the upper band was depleted (Figure 1B), demonstrating that the upper band observed after camptothecin treatment corresponded to RPA2 phosphorylation. A positive control was included in these experiments by using ionizing radiation (Figure 1C), which is a well-established inducer of RPA2 phosphorylation (Liu and Weaver, 1993; Boubnov and Weaver,

1995; Brush *et al.*, 1996; Fried *et al.*, 1996; Morgan and Kastan, 1997). The topoisomerase II inhibitor etoposide (VP-16) (Pommier, 1997) was also an effective inducer of RPA2 phosphorylation in HT29 cells (Figure 1D), and RPA2 phosphorylation was observed in p53-null human leukemia HL60 cells treated with camptothecin (Figure 3C). These results demonstrate for the first time that inhibitors of topoisomerase I (camptothecin) and topoisomerase II (VP-16) induce rapid phosphorylation of RPA2 in human colon carcinoma and leukemia cells, independently of p53. In the rest of this study we used camptothecin to analyze further RPA2 phosphorylation.

Active DNA replication is required for RPA2 phosphorylation

Camptothecin-induced DNA damage is critically linked to DNA replication (Pommier, 1996). Arresting DNA replication with the DNA polymerase inhibitor, aphidicolin prevents the cytotoxicity of camptothecin without modifying the yield of cleavage complex (Holm *et al.*, 1989; Hsiang *et al.*, 1989). Figure 2A shows that aphidicolin inhibited camptothecin-induced RPA2 phosphorylation. This result indicates that replication-dependent DNA damage rather than top 1 cleavage complexes signals for RPA2 phosphorylation.

We also tested the effect of aphidicolin on ionizing radiation-induced RPA2 phosphorylation. Figure 2B demonstrates that aphidicolin blocked RPA2 phosphorylation in response to ionizing radiation. These observations suggest that ongoing replication is critical for inducing RPA2 phosphorylation in response to DNA damage.

RPA2 phosphorylation in ataxia telangiectasia and ATM-deficient cells

DNA damage-induced phosphorylation of RPA2 has been attributed to DNA-PK (Brush *et al.*, 1994; Boubnov and Weaver, 1995; Zernik-Kobak *et al.*, 1997) or ATM (Liu and Weaver, 1993; Morgan and Kastan, 1997; Gately *et al.*, 1998). To test the possible role of ATM, we compared camptothecin-induced RPA2 phosphorylation in normal (GM637) and AT (GM5849) fibroblasts (Figure 3A), and in RKO cells with a dominant-negative (DN) ATM after transfection of the ATM-DN fragments (RKO FB2F) (Figure 3B). RPA2 phosphorylation was comparable in ATM-deficient cells and their normal counterparts. Furthermore, HL60, which does not express detectable ATM (Gately *et al.*, 1998), also exhibited strong RPA2 phosphorylation after camptothecin treatment. Together, these data indicate that ATM is not required for camptothecin-induced RPA2 phosphorylation.

DNA replication-dependent activation of DNA-PK in camptothecin-treated HT29 cells

Since DNA-PK has been implicated in RPA2 phosphorylation (Brush *et al.*, 1994; Boubnov and Weaver, 1995; Zernik-Kobak *et al.*, 1997), we next investigated whether DNA-PK was activated in HT29 cells treated with camptothecin. DNA-PK activity was measured after immunoprecipitation of cell lysates with anti-DNA-PKs antibodies, and using dephosphorylated casein as a substrate. Figure 4 shows that DNA-PK activity was increased ~5-fold after 1 h of camptothecin treatment. The kinetics of DNA-PK activation coincided with RPA2 phosphorylation

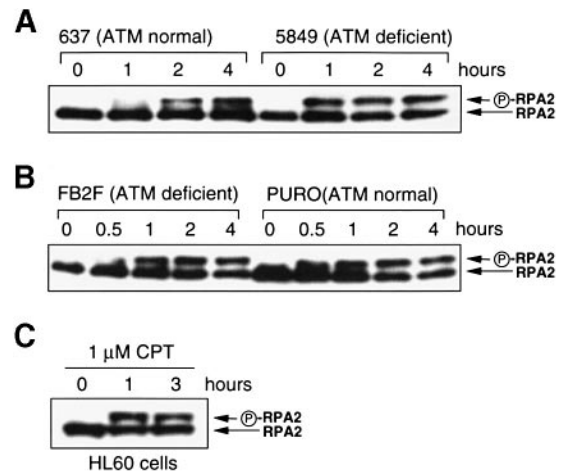


Fig. 3. ATM is not involved in RPA2 phosphorylation induced by camptothecin (CPT). (A) Normal (GM637) and AT fibroblasts (GM5849) were treated with 1 μM CPT for the indicated times. (B) Colon carcinoma RKO cells transfected with an ATM-dominant-negative vector (FB2F) and with control vector (PURO) were treated with 1 μM CPT for the indicated times. (C) ATM-deficient human leukemia HL60 cells (Gately *et al.*, 1998) were treated with 1 μM CPT for the indicated times. RPA2 phosphorylation was detected by Western blotting using anti-RPA2 monoclonal antibody.

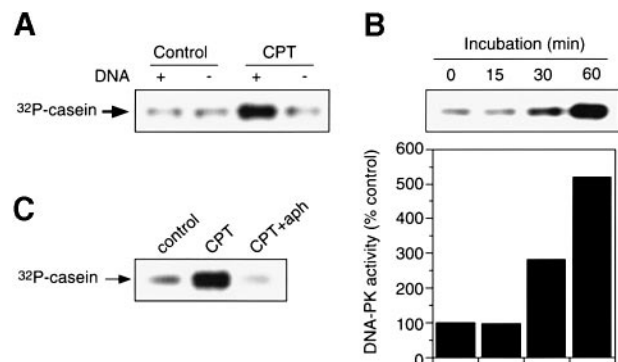


Fig. 4. DNA replication-dependent DNA damage activates DNA-PK in HT29 cells. (A) DNA-PK activity in cells treated with 1 μM camptothecin (CPT) for 1 h was measured in the absence or presence of DNA. (B) Kinetics of DNA-PK activation in cells treated with 1 μM CPT. (C) DNA-PK activity in cells treated with 1 μM CPT for 1 h in the absence or presence of 1 μM aphidicolin (aph).

(Figures 4B and 1C). These data are consistent with the possibility that DNA-PK is responsible for RPA2 phosphorylation in camptothecin-treated cells.

Since we observed (Figure 2) that RPA2 phosphorylation was blocked by the DNA polymerase inhibitor aphidicolin, we also investigated the effect of aphidicolin on DNA-PK activation by camptothecin. Figure 4C shows that aphidicolin blocked DNA-PK activation. These data are consistent with the possibility that ongoing DNA replication is required for DNA-PK activation, and RPA2 phosphorylation.

Inhibition of RPA2 phosphorylation and DNA-PK activation by the protein kinase inhibitors wortmannin and UCN-01

Next, we used wortmannin to obtain further evidence for a role of DNA-PK in RPA2 phosphorylation. As expected (Hartley *et al.*, 1995; Sarkaria *et al.*, 1998), 0.5 μM wortmannin completely inhibited the immuno-

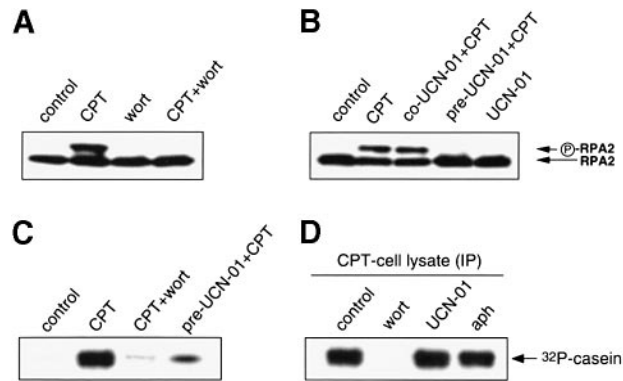


Fig. 5. Inhibition of RPA2 phosphorylation and DNA-PK activation by protein kinase inhibitors. (A) Wortmannin blocks RPA2 phosphorylation. Cells were treated with 1 μ M camptothecin (CPT) in the absence or presence of 5 μ M wortmannin (wort) for 1 h. (B) UCN-01 pre-treatment blocks RPA2 phosphorylation. Cells were co-treated with 1 μ M CPT and 10 μ M UCN-01 for 1 h either with UCN-01 pre-treatment for 15 min (pre-UCN-01+CPT) or without UCN-01 pre-treatment (co-UCN-01+CPT). (C) Wortmannin and UCN-01 pre-treatment inhibit DNA-PK activation in CPT-treated HT29 cells. (D) Wortmannin is a direct inhibitor of DNA-PK *in vitro* while aphidicolin (aph) and UCN-01 have no direct effect on DNA-PK activity. Cell lysates from CPT-treated cells were immunoprecipitated with anti-DNA-PK antibody. DNA-PK activity was measured in the absence or presence of 0.5 μ M wortmannin, 10 μ M UCN-01 or 1 μ M aphidicolin.

precipitated DNA-PK activity from camptothecin-treated cells (Figure 5D). We also found that wortmannin inhibited both RPA2 phosphorylation (Figure 5A) and DNA-PK activation (Figure 5C) induced by camptothecin in HT29 cells. These results are consistent with the role of DNA-PK in RPA2 phosphorylation.

We proposed previously that the protein kinase C inhibitor and cell-cycle checkpoint abrogator 7-hydroxystaurosporine (UCN-01) abrogates the S and G₂ checkpoints in camptothecin-treated cells (Shao *et al.*, 1997). Figure 5B shows that UCN-01 also prevented RPA2 phosphorylation when HT29 cells were preincubated with UCN-01 prior to the camptothecin treatment. However, under conditions of co-treatment, UCN-01 could not block camptothecin-induced RPA2 phosphorylation. Interestingly, UCN-01 also prevented DNA-PK activation in cells (Figure 5C), but had no effect on the immunoprecipitated DNA-PK from camptothecin-treated cells (Figure 5D). These results indicate that UCN-01 is not a direct inhibitor of DNA-PK, but that it affected RPA2 phosphorylation and DNA-PK activation indirectly, probably by acting upstream from DNA-PK. Thus, DNA-PK might be activated by other kinases in response to replication damage.

DNA-PKcs is associated with RPA in untreated cells and with Ku in camptothecin-treated cells

The above data indicated a relationship between DNA-PK activity and camptothecin-induced RPA2 phosphorylation; therefore we tested next whether DNA-PKcs could be detected in association with RPA2. Western blotting of cell lysates showed that DNA-PKcs, RPA or Ku protein levels did not change after camptothecin treatment (Figure 6A). Immunoprecipitation of cell lysates with

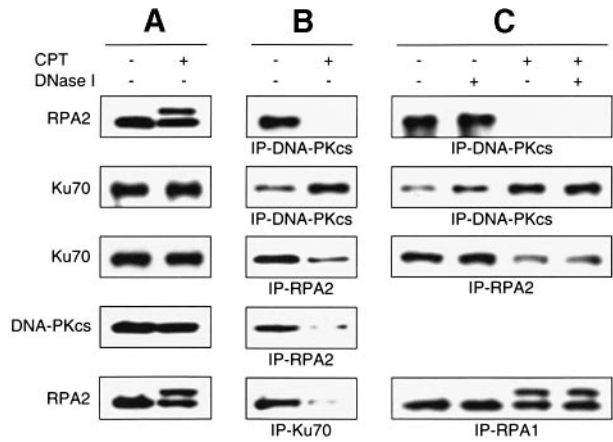


Fig. 6. DNA-PK forms complexes with RPA, and these complexes are disrupted by camptothecin (CPT) treatment. Cell lysates from untreated (control) or CPT-treated (1 μ M, 1 h) HT29 cells were directly tested for protein expression by Western blotting (A), or were immunoprecipitated (IP) and detected by Western blotting with the indicated antibodies (left of the figure) (B). (C) Cell lysates were treated with 100 U DNase I for 30 min before immunoprecipitation.

DNA-PKcs or RPA2 antibodies showed that RPA2 was associated with DNA-PKcs in untreated cells. This DNA-PKcs:RPA2 association was not affected by DNase I treatment of the cell lysates before immunoprecipitation (Figure 6C), suggesting that DNA was not required for the formation of the RPA2:DNA-PKcs complexes. The situation was different in the camptothecin-treated cells. The RPA2:DNA-PKcs association was markedly decreased and DNA-PKcs was prominently associated with Ku rather than with RPA2. This Ku:DNA-PKcs association in camptothecin-treated cells was not affected by DNase I treatment, suggesting that DNA was either absent from the Ku:DNA-PKcs complex or not accessible to DNase I. We also found that Ku-immunoprecipitation pulled down RPA2 more efficiently in untreated cells than in camptothecin-treated cells (Figure 6B), suggesting that both proteins can coexist in similar complexes. RPA2 was also found associated with RPA1 independently of RPA2 phosphorylation both in untreated and camptothecin-treated cells (Figure 6C, lower panel). To the best of our knowledge, these data indicate for the first time that DNA-PKcs can be found in association with RPA in untreated cells and that this association is disrupted as DNA-PKcs:Ku complexes form in the camptothecin-treated cells.

Direct interaction between RPA1 and DNA-PKcs *in vitro*

Immunoprecipitation experiments were performed to test whether DNA-PKcs could form a protein complex with RPA. Figure 7A and D shows that immunoprecipitation with DNA-PKcs pulled down the RPA trimeric complex (RPA1/2/3 = RPA1 + RPA2 + RPA3). This association was not enhanced by addition of double-stranded DNA and was insensitive to DNase I digestion (Figure 7A). This result indicates that DNA is not required for the RPA:DNA-PKcs interaction. To evaluate the possible contribution of Ku for the RPA:DNA-PKcs interactions, the DNA-PK preparation was first immunoprecipitated with anti-Ku antibodies to deplete Ku from the DNA-PK

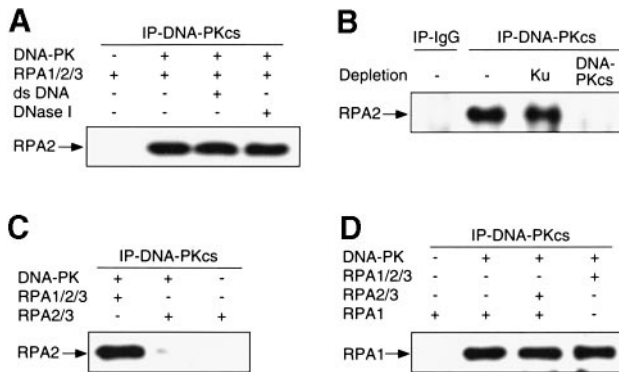


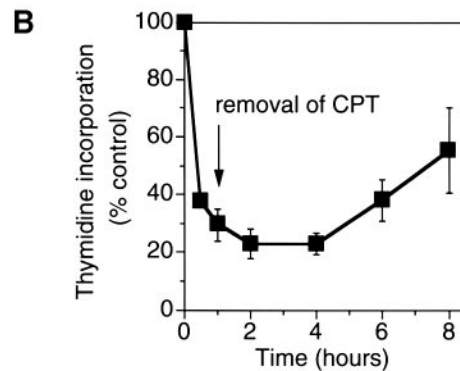
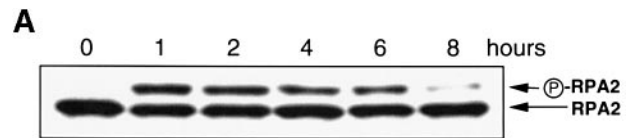
Fig. 7. Binding of RPA to DNA-PKcs *in vitro*. DNA-PK was mixed as indicated with RPA heterotrimer (RPA1/2/3) or with the heterodimer (RPA2/3), or with RPA1 alone in the absence or presence of DNase I and exogenous double-stranded DNA (ds DNA) for 30 min at 37°C. The mixture was incubated further with anti-DNA-PKcs or IgG for 2 h. After immunoprecipitation, RPA2 and RPA1 were detected by Western blotting (see text for details).

preparation (Figure 7B). Such an immunodepletion had no demonstrable effect on the formation of RPA:DNA-PKcs complexes. As a control, immunodepletion with anti-DNA-PKcs antibody completely inhibited the formation of the RPA:DNA-PKcs complex (Figure 7B). This result suggests that Ku is not required for the formation of the RPA:DNA-PKcs complexes. We tested next whether all three RPA subunits were required for the formation of the RPA:DNA-PKcs complex (Figure 7C and D). Figure 7C shows that complexes were not detectable in the absence of RPA1. To confirm the critical role of RPA1, RPA1 Western blotting was performed (Figure 7D). RPA1 alone was sufficient to form a complex with DNA-PKcs. Together these results indicate that RPA can form complexes with DNA-PK *in vitro*, and that the RPA1 and DNA-PKcs subunits are required to form the RPA:DNA-PK complex.

Kinetics of RPA2 phosphorylation, DNA synthesis inhibition and cell-cycle distribution after camptothecin treatment in HT29 cells

Cells treated with camptothecin (Goldwasser *et al.*, 1996) and DNA damaging agents generally respond by rapid inhibition of DNA synthesis. The kinetics of RPA2 phosphorylation and DNA synthesis inhibition were examined. Figure 8A shows that RPA2 phosphorylation was maximal within 1 h of drug treatment and that it persisted for several hours after camptothecin removal. Subsequently, RPA2 phosphorylation tended to reverse after 4 h. Consistent with previous observations (Goldwasser *et al.*, 1996), DNA synthesis was rapidly inhibited following camptothecin treatment and was restored partially after 4 h (Figure 8B). Thus, RPA2 phosphorylation is concomitant with DNA synthesis inhibition.

Under these same conditions, flow cytometry analyses (Figure 8C) indicated that camptothecin induced a progressive accumulation of cells in S phase, but that the RPA2 phosphorylation preceded detectable changes in cell-cycle distribution.



C

Time (hours)	0	1	2	4	6	8
% G1	67.6	66.9	65.3	65.0	61.7	53.8
% S	26.2	26.8	28.6	29.7	35.2	43.8
% G2/M	6.2	6.3	6.1	5.3	3.1	2.4

Fig. 8. RPA2 phosphorylation coincides with and follows DNA synthesis inhibition after camptothecin (CPT) treatment. HT29 cells were treated with 1 μ M CPT for 1 h. After camptothecin (CPT) removal, cells were incubated in fresh medium for the indicated times. (A) RPA2 phosphorylation was measured by Western blotting. (B) DNA synthesis was measured by pulse thymidine incorporation. (C) Cell-cycle distribution was measured by flow cytometry.

Camptothecin-induced RPA2 phosphorylation is reduced in DNA-PK-deficient cells; possible relationship with a DNA damage/replication checkpoint

To establish further the role of DNA-PK in RPA2 phosphorylation, the ability of camptothecin to induce RPA2 phosphorylation was tested in DNA-PK-deficient cells. We used the DNA-PK-wild-type control cell line M059K and its DNA-PK-deficient counterpart, M059J (Lees-Miller *et al.*, 1995). M059J cells do not express DNA-PKcs (Lees-Miller *et al.*, 1995). Figure 9A shows that RPA2 was phosphorylated in response to camptothecin in M059K cells in a time-dependent manner, while markedly less RPA2 phosphorylation was observed in DNA-PKcs deficient M059J cells under the same conditions. These results demonstrate that DNA-PK is important for RPA2 phosphorylation.

We next tested whether the DNA-PK-deficient cells exhibited differences in DNA synthesis and cell survival response. DNA synthesis inhibition appeared more pronounced in the DNA-PKcs wild-type M059K cells than in the DNA-PKcs-deficient M059J cells (Figure 9B). Clonogenic assays showed that the DNA-PKcs-deficient M059J cells were also more sensitive to camptothecin treatment compared with the M059K cells (Figure 9C). These results suggest that DNA-PK activity might be involved in a DNA replication checkpoint.

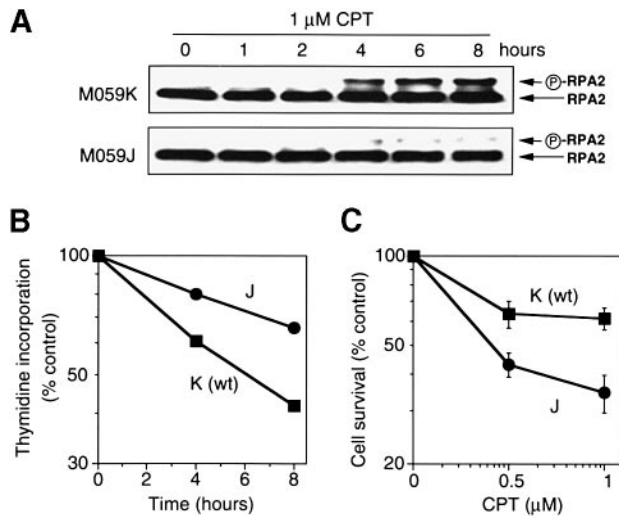


Fig. 9. Deficiencies of RPA2 phosphorylation, DNA synthesis inhibition and survival in DNA-PKcs-deficient M059J human glioblastoma cells treated with camptothecin (CPT). (A) Reduced RPA2 phosphorylation in M059K (normal for DNA-PK) and M059J (DNA-PKcs-deficient) cells treated with 1 μ M CPT for the indicated times. (B) Defective inhibition of DNA synthesis in DNA-PK-deficient M059J human glioblastoma cells. Cells were treated with 1 μ M CPT for the indicated times. DNA synthesis was measured by pulse thymidine incorporation. (C) Enhanced sensitivity to CPT of the DNA-PK-deficient M059J cells. Cells were treated with CPT for 8 h. Cell survival was determined by clonogenic assay. Squares and circles correspond to M059K and M059J, respectively.

Discussion

Each of the three subunits of RPA (RPA1, RPA2 and RPA3) is highly conserved among species and is essential for cell viability (Brill and Stillman, 1991; Wold, 1997). RPA2 forms a stable complex with RPA3 to which RPA1 binds (Henricksen and Wold, 1994; Stigger *et al.*, 1994). The heterotrimeric complex has high affinity for single-stranded and damaged DNA, and plays key roles in DNA replication, repair and recombination (for a review see Wold, 1997). Although RPA1 alone can bind to ssDNA, it cannot function in replication, suggesting that RPA2 and RPA3 are essential for RPA function.

In the present study, we demonstrate that the anticancer topoisomerases I and II inhibitors, camptothecin and etoposide (VP-16), respectively, induce marked RPA2 phosphorylation in human cells. Phosphorylation of RPA2 has previously been reported in cells exposed to ionizing radiations or UV (Liu and Weaver, 1993; Fried *et al.*, 1996; Henricksen *et al.*, 1996; Zernik-Kobak *et al.*, 1997). Generally, detection of radiation-induced RPA2 phosphorylation requires high-dose ionizing radiation [50 Gy in the studies of Morgan and Kastan (1997) and Fried *et al.* (1996), and at least 15 Gy in the study of Boubnov and Weaver (1995); doses that kill almost all the cells in the culture]. In the present study, the camptothecin concentrations used were within pharmacological range and compatible with survival of a large fraction of the cells (Goldwasser *et al.*, 1995) (Figure 9). Furthermore, camptothecin-induced RPA2 phosphorylation was extensive (between 30 and 50% of total RPA) and rapid (<30 min) (Figures 1, 3 and 9). During this short time period no change in cell-cycle distribution was detectable and cyclin/cdk2 kinase activity was unaltered (data not

shown). Thus, topoisomerase inhibitors, such as camptothecin, which selectively poison top1 and damage replicating DNA, are very effective pharmacological tools for studying RPA2 phosphorylation.

To the best of our knowledge, the observation that the DNA polymerase inhibitor, aphidicolin (Decker *et al.*, 1986) blocks RPA2 phosphorylation for both camptothecin and ionizing radiations is new and possibly important. It is consistent with the report that ionizing radiations do not induce RPA2 phosphorylation in non-cycling fibroblasts (Cheng *et al.*, 1996). We also found that RPA2 phosphorylation was slower in the relatively slowly growing M059K glioblastoma cells (Figure 9) than in the more rapidly proliferating colon carcinoma, SV40-transformed fibroblasts and human leukemia HL60 cells (Figures 1 and 3). Thus, DNA damage associated with DNA replication is a potent inducer of RPA2 phosphorylation.

Several lines of evidence suggested that DNA-PK is required for RPA2 phosphorylation following replication-associated DNA damage. First, RPA2 phosphorylation was markedly attenuated in the human glioblastoma cells, M059J (Figure 9), which lack the DNA-PK catalytic subunit (DNA-PKcs) (Lees-Miller *et al.*, 1995). Secondly, the DNA-PK inhibitor, wortmannin (Figure 5D) (Hartley *et al.*, 1995), blocked RPA2 phosphorylation in camptothecin-treated cells (Figure 5A). Thirdly, the kinetics of DNA-PK activation and RPA2 phosphorylation were similar (Figures 1C and 4B). Fourthly, both DNA-PK activation and RPA2 phosphorylation were abrogated by aphidicolin (Figures 2 and 4C) and UCN-01 (Figures 5B and C). And fifthly, ATM was not required for RPA2 phosphorylation, since camptothecin-induced RPA2 phosphorylation was normal in the ATM-deficient cell lines (Figure 3).

DNA-PK has also been implicated as the major RPA2 kinase in radiation response (Boubnov and Weaver, 1995; Niu *et al.*, 1997; Zernik-Kobak *et al.*, 1997). After UV irradiation, several sites within the N-terminal 33 amino acids of RPA2 become phosphorylated, and two of these (Ser21 and Ser33) are consensus sites for DNA-PK (Niu *et al.*, 1997; Zernik-Kobak *et al.*, 1997). Boubnov and Weaver (1995) reported that radiation-induced RPA2 hyperphosphorylation was incomplete in the DNA-PK-deficient *scid* cells compared with control or with human chromosome 8-complemented *scid* cells, containing the human DNA-PK catalytic subunit (Boubnov and Weaver, 1995). Both the results of Boubnov and Weaver (1995) and ours (present study) are in apparent contradiction with the conclusions of Fried *et al.* (1996) who reported that DNA-PK was not required for ionizing radiation-induced RPA2 phosphorylation in the DNA-PKcs-deficient, M059J cells. The difference might be that Fried *et al.* (1996) used a high radiation dose (50 Gy), considering that the IC_{99} has been reported to be 2.5 Gy for M059J and 8.5 Gy for M059K cells (Allalunis-Turner *et al.*, 1993). In contrast, the 1 μ M camptothecin dose that we used in the RPA2 phosphorylation experiments killed ~60% of the M059J and 35% of the M059K cells. The DNA damage produced by this camptothecin dose is approximately equivalent to the DNA single-strand breaks produced by a dose of 10 Gy ionizing radiation (Goldwasser *et al.*, 1995) (e.g.

approximately one single-strand break per 10^6 nucleotides).

It is possible that different pathways lead to RPA2 phosphorylation depending on the nature of the DNA damage. This would explain why we found camptothecin-induced RPA2 phosphorylation in the ATM-deficient cell lines (Figure 3), while RPA2 phosphorylation was markedly attenuated in these same cells following ionizing radiation (Morgan and Kastan, 1997). Recently, a DNA-PK activity associated with ATM immunocomplexes has been found to phosphorylate RPA2 independently of DNA-PK (Gately *et al.*, 1998). Thus, depending on the intensity and nature of the DNA damage, different pathways including either DNA-PK or ATM can lead to RPA2 phosphorylation. In the case of replication damage resulting from topoisomerase I trapping by camptothecin, we found that DNA-PK is involved rather than ATM.

Although the molecular target(s) of UCN-01 are not fully known and include protein kinase C (Seynaeve *et al.*, 1993) and cell cycle-related kinases (Akiyama *et al.*, 1997), our finding that short pre-treatment with UCN-01 prevented camptothecin-induced RPA2 phosphorylation *in vivo* (Figure 4B), while having no direct effect on DNA-PK *in vitro* (Figure 4D), is consistent with the possibility that other kinases probably upstream from DNA-PK regulate RPA2 phosphorylation in response to DNA damage.

The observation that RPA forms complexes with DNA-PKcs both in untreated cells and *in vitro* is novel. Such complexes probably involve direct protein-protein interactions because their formation is not enhanced by the addition of DNA and is not affected by DNase I (Figures 6 and 7). RPA binds to single-stranded DNA and stabilizes single-stranded regions that form transiently during replication, this action being essential for SV40 DNA replication (Wold, 1997). The evidence reported here for direct binding between RPA and DNA-PKcs, phosphorylation of RPA by DNA-PK, and dependence of this phosphorylation on both DNA damage and active replication suggests an intimate relationship among these components: RPA, DNA-PK, DNA damage and replication fork progression. DNA-PK has been shown to bind strongly to DNA double-stranded ends, including DNA double-strand breaks produced by radiation (Anderson and Carter, 1996; Jeggo, 1997; Jin *et al.*, 1997). We hypothesize, therefore, that RPA2 phosphorylation by DNA-PK occurs when a DNA single-stranded region and a double-stranded end are juxtaposed. DNA-PK could bind to the double-stranded end and then interact with RPA bound to the single-stranded region. Alternatively, DNA-PK may normally be bound to RPA at single-stranded regions and become activated when a double-stranded end is within reach (Figure 10).

The model depicted in Figure 10 proposes that RPA:DNA-PKcs complexes may exist bound to single-stranded regions near DNA replication forks. Our immunoprecipitation data suggest that Ku may also be associated with this complex (Figure 10A). The DNA-PKcs and Ku in the complex would interact with any double-stranded end that may appear in the vicinity as a result of an encounter between a DNA replication fork and a site of damaged DNA (Figure 10B). Ku enhances both the

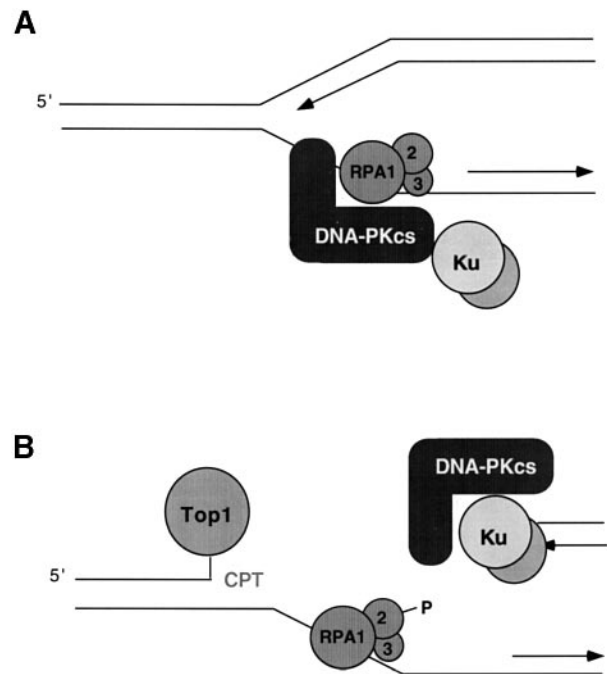


Fig. 10. Model for the roles of RPA and DNA-PK in replication-dependent DNA lesions induced by camptothecin (CPT). (A) Presence of RPA-DNA-PKcs complexes in the replicating DNA of untreated cells. (B) Phosphorylation of RPA2 by DNA-PK and formation of Ku:DNA-PKcs complexes in CPT-treated cells. The case of a replication fork encounter from the 3' side of a CPT-stabilized topoisomerase I-DNA complex is described. Leading-strand synthesis terminates at a CPT-stabilized topoisomerase I-DNA complex, forming a double-stranded DNA end to which the Ku:DNA-PKcs complex forms. Lagging-strand synthesis leaves a segment of unreplicated ssDNA to which RPA heterotrimer binds.

strength and stability of the association between double-strand ends and DNA-PKcs (Yaneva *et al.*, 1997; West *et al.*, 1998). As a consequence of the interaction with a double-stranded end, we propose that the DNA-PKcs becomes activated and phosphorylates RPA. The hyperphosphorylated RPA then dissociates from the Ku:DNA-PKcs complex (Figure 10B). Our understanding of mechanisms suggests that camptothecin would be much more effective than ionizing radiation in generating DNA double-stranded ends juxtaposed to DNA replication forks (Pommier, 1996). This is consistent with our finding that camptothecin induces RPA phosphorylation at much less toxic doses than does radiation.

RPA hyperphosphorylation may change how RPA complexes function in DNA repair and replication. RPA can interact with a large number of DNA replication, recombination and repair proteins (Wold, 1997), including SV40 large T-antigen, replication initiator proteins, DNA polymerase α , nucleotide excision repair proteins (XPA, XPG, XPF/ERCC1, XPE), uracil DNA glycosylase and the Rad52-recombinosome complex. Such interactions are species specific, which reflects their tight specificity. For instance, only human RPA can bind with high affinity to large T-antigen and stimulate SV40 replication (Melendy and Stillman, 1993), and human RPA cannot substitute for yeast RPA-mediated Rad52 DNA annealing (Sugiyama *et al.*, 1998). RPA can also bind to p53, and this binding is abrogated when RPA becomes phosphorylated in UV-irradiated cells (Abramova *et al.*, 1997). Since RPA is in

large excess in the cell, it may serve to sequester p53. As suggested by Abramova *et al.* (1997), RPA may participate in the regulation of p53. RPA2 phosphorylation would free p53 from its complexes with RPA and lead to p53 activation (Miller *et al.*, 1997). Thus, it is possible that the interaction of cell-cycle regulatory proteins with RPA is part of the mechanisms that coordinate DNA repair with checkpoint controls.

We found a temporal relationship between RPA2 phosphorylation and DNA synthesis inhibition (Figure 8). Camptothecin produces rapid and prolonged inhibition of DNA synthesis with S phase retardation, even after reversal of the top1 cleavage complexes (Goldwasser *et al.*, 1995; Pommier, 1996). It has been hypothesized that this camptothecin-induced DNA synthesis inhibition probably results from an active process (S-phase checkpoint) aimed at reducing further replication and DNA damage (Shao *et al.*, 1997; Wang *et al.*, 1997). Our data (Figure 8), showing that RPA2 phosphorylation coincides with camptothecin-induced DNA synthesis inhibition, are consistent with the possibility that RPA2 phosphorylation is related to DNA synthesis inhibition. Similarly, UV light-induced DNA synthesis arrest in HeLa cells is associated with increased phosphorylation of RPA2 (Carty *et al.*, 1994). These observations suggest that DNA synthesis arrest may be related to DNA-PK-induced phosphorylation of RPA2.

The existence of an S-phase checkpoint has been proposed from studies performed in MEC1-deficient yeast (Paulovich and Hartwell, 1995) and in human ataxia telangiectasia cells (ATM-deficient cells) (Painter and Young, 1980). In both cases, DNA damage-resistant DNA synthesis and enhanced cell killing characterize these cells. At the molecular level, both MEC1 and ataxia telangiectasia cells are defective in RPA2 phosphorylation following ionizing radiation-induced DNA damage. This suggests a relationship between RPA2 phosphorylation and S-phase checkpoint. Two results of the present study provide additional evidence for a relationship between RPA2 phosphorylation and S-phase checkpoint. First, the S-phase checkpoint abrogator, UCN-01 (Shao *et al.*, 1997) prevented RPA2 phosphorylation (Figure 5). Secondly, DNA-PK-deficient M059J cells, which exhibit marked reduction of RPA2 phosphorylation, also exhibited less DNA synthesis arrest and greater sensitivity in response to camptothecin than their normal counterpart (Figure 9). Together, these observations suggest that RPA2 phosphorylation is involved in a conserved eukaryotic S phase DNA damage-response pathway.

Materials and methods

Drugs, chemicals and antibodies

UCN-01 and camptothecin were provided by the Drug Synthesis Chemistry Branch, Division of Cancer Treatment, NCI. Aliquots were stored frozen at 10 mM in dimethylsulfoxide and diluted further in water immediately prior to each experiment. Purified RPA (RPA1/2/3), RPA2/3 and RPA1 were prepared as described previously (Henricksen and Wold, 1994). DNA-PK purified from HeLa cells extracts was purchased from Promega (Madison, WI) and used for *in vitro* binding assays. Unless otherwise mentioned, other reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Anti-RPA34 (anti-RPA2) monoclonal antibody (Ab-3) and anti-RPA70 (anti-RPA1) monoclonal antibody (Ab-1) were purchased from Oncogene Science Inc. (Cambridge, MA). Anti-DNA-PKcs monoclonal antibody MC-362 was from Kamiya Biomedical Co. (Seattle, WA) and anti-

DNA-PKcs monoclonal antibody Ab-2 was from NeoMarkers Inc. (Fremont, CA). Anti-Ku70 monoclonal antibody (C-19) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). For immunodepletion, anti-Ku (p70/p80) monoclonal antibody (clone 162) was purchased from NeoMarkers Inc.

[¹⁴C]Thymidine (53.6 mCi/mmol), [methyl-³H]thymidine (80.9 Ci/mmol) and [γ -³²P]ATP (4500 μ Ci/mmol) were purchased from New England Nuclear (Boston, MA).

Cell culture

Human colon carcinoma HT29 cells were obtained from the Developmental Therapeutics Program (National Cancer Institute) and grown at 37°C in the presence of 5% CO₂ in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Human leukemia HL60 cells were grown at 37°C in the presence of 5% CO₂ in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. The SV40-transformed fibroblast cell lines from normal individual (GM637) and from an AT homozygous patient (GM5849), and the RKO colorectal cell lines pBABEpuro and FB2F12 were obtained from Dr Michael Kastan, St Jude Children's Research Hospital, Memphis, TN. M059J and M059K human glioblastoma cells were a generous gift from Dr Joan Turner, University of Edmonton, Canada. They were grown in DMEM medium with 10% FBS at 37°C and 5% CO₂.

Preparation of cell lysates

Cells were grown to 50–80% confluence when treated with different agents. Whole-cell extracts were prepared as described (Shao *et al.*, 1997). Briefly, cells were harvested and washed twice with phosphate-buffered saline (PBS), then incubated on ice for 30 min in lysis buffer (1 \times PBS containing 1% NP-40, 1 μ g/ml leupeptin, 5 mM NaF, 1 mM Na₃VO₄, 2 mM AEBSF, 4 U/ml aprotinin and 1% bovine serum albumin). Cell debris was removed by centrifugation at 12 000 *g* for 15 min at 4°C. Supernatant was evaluated for protein concentration (Bio-Rad, Hercules, CA) and either used immediately for assays or stored at –70°C.

Western blotting

Cells lysates were electrophoresed in SDS-PAGE precast gels (Novex, San Diego, CA) and then electrophoretically transferred to Immobilon membranes (Millipore, Bedford, MA), for 2 h at 30 V. Membranes were saturated overnight in PBS–0.02% Tween-20 (PBS-T) containing 5% non-fat dried milk. They were probed for 1 h with primary antibody and then for an additional hour with secondary antibody (1/1000 dilution). Western blotting was visualized by enhanced chemiluminescence (ECL, DuPont NEN, Boston, MA), according to the manufacturer's instructions. All the presented data were confirmed in independent experiments.

DNA-PK activity assay

The kinase assay was performed using dephosphorylated casein as a substrate (Yaneva *et al.*, 1997). Protein A-Sepharose beads were mixed with anti-DNA-PKcs monoclonal antibody and incubated with rotating overnight at 4°C. The beads were washed three times with 10 mM Tris–HCl buffer, pH 7.5, 0.15 M NaCl, containing 0.02% Tween-20, and mixed with 500 μ l of cell lysates from control or treated HT-29 cells for 2 h at 4°C with gentle rotating. The beads with the immune complexes were washed three times with kinase buffer (20 mM Tris–HCl pH 8.0, 50 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 0.02% Tween-20, 10% glycerol) containing 0.5% NaCl and 0.1% NP-40, and twice with kinase buffer. Reactions were performed in kinase buffer containing 200 ng linearized SV40 DNA, 20 μ g/ml dephosphorylated casein and 5 μ Ci [γ -³²P]ATP for 10 min at 37°C. They were terminated by adding SDS-PAGE sample buffer (0.15 M Tris–HCl pH 6.8, 20% glycerol, 1% SDS, 0.005% Bromophenol Blue and 5% mercaptoethanol). Phosphorylated casein was separated by 12% SDS-PAGE gels. The gels were dried and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitations

Cell lysates (prepared as described above) were incubated with anti-DNA-PKcs, anti-RPA2, anti-RPA1 or anti-Ku70 monoclonal antibodies for 2 h at 4°C. Immune complexes were then linked to Protein A-Sepharose beads for an additional 2 h at 4°C. After five washes with lysis buffer, the immunoprecipitates were resolved by SDS-PAGE and immunoblotted with the either anti-RPA2, anti-DNA-PKcs or Ku70 monoclonal antibodies.

For *in vitro* binding analysis, 1 μg DNA-PK and 0.2 μg RPA heterotrimer (RPA1/2/3) or RPA heterodimer (RPA2/3) or RPA1 were mixed in 20 μl 1 \times PBS containing 1% bovine serum albumin, 1% glycerol in the absence or presence of double-stranded DNA (10 ng/ μl), and incubated at 4°C for 30 min. Anti-DNA-PKs monoclonal antibody and protein A–Sepharose beads were then added for 2 h. After centrifugation, beads were washed with lysis buffer. The immunoprecipitates were resolved by SDS–PAGE and immunoblotted with anti-RPA2 or anti-RPA1 antibodies.

For the immunodepletion experiments, anti-Ku or anti-DNA-PKs antibodies (5 μg) were incubated with protein A–Sepharose beads for 2 h at 4°C. The beads were collected by centrifugation, and after removal of the supernatant, the beads were washed with lysis buffer and incubated with DNA-PK for an additional 2 h at 4°C. The immunoprecipitates were resolved by SDS–PAGE and immunoblotted with anti-RPA2 antibody.

DNA synthesis assays

Thymidine incorporation assays were performed as described previously (Shao *et al.*, 1997). Briefly, cells were pre-labeled with 0.005 $\mu\text{Ci/ml}$ of [^{14}C]thymidine (53.6 mCi/mmol) for 48 h at 37°C. The rate of DNA synthesis was measured by 10 min pulses with 1 $\mu\text{Ci/ml}$ of [methyl- ^3H]thymidine (80.9 Ci/mmol). ^3H -incorporation was stopped by washing cell cultures twice in ice-cold Hanks balanced salt solution (HBSS), and then by scraping cells into 4 ml of ice-cold HBSS. Aliquots (1 ml) were then precipitated after addition of 100 μl of trichloroacetic acid in triplicate. Samples were vortexed, mixed, and centrifuged for 10 min at 12 000 g at 4°C. The precipitates were then dissolved overnight at 37°C in 0.5 ml of 0.4 M NaOH. Samples were counted by dual label liquid scintillation and [^3H]-values were normalized using [^{14}C] counts (Shao *et al.*, 1997). Inhibition of DNA synthesis was calculated as the ratio of [^3H]:[^{14}C] in the treated samples over the [^3H]:[^{14}C] ratio in the untreated control samples.

Flow cytometry

Assays were performed as described previously (Shao *et al.*, 1997). Briefly, cells were harvested and fixed in 70% ethanol. Before analysis, cells were washed with PBS, treated with 1 $\mu\text{g/ml}$ RNase and stained with 50 $\mu\text{g/ml}$ propidium iodide for at least 30 min. DNA content was determined by FACScan flow cytometry (Becton Dickinson Immunocytometry System, San Jose, CA).

Clonogenic assays

Briefly, exponentially growing cells were treated with 1 μM camptothecin for 8 h. Following camptothecin treatment, cells were washed in fresh medium and trypsinized. Two hundred cells were seeded in triplicate in T-25 tissue culture flasks and incubated for 10–14 days before colonies were counted.

Acknowledgements

We wish to thank Dr Michael Kastan, St Jude Children's Research Hospital, Memphis, TN, for providing us with the SV40-transformed fibroblast cell lines from normal individual (GM637) and from an ataxia telangiectasia homozygous patient (GM5849), and for the RKO colorectal cell lines pBABEpuro and FB2F12 cell lines. M059J and M059K human glioblastoma cells were a generous gift from Dr Joan Turner, University of Edmonton, Canada.

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Received June 16, 1998; revised December 8, 1998;
accepted January 7, 1999