Roles of Replication Protein A and DNA-dependent Protein Kinase in the Regulation of DNA Replication following DNA Damage*

Ya Wang‡, Xiang-Yang Zhou, Hongyan Wang, M. Saiful Huq, and George Iliakis

From the Department of Radiation Oncology, Kimmel Cancer Center of Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Inhibition of DNA replication in eukaryotic cells is one of the earliest effects of radiation to be reported. Camptothecin (CPT)1 and other DNA damage-inducing agents (DDIA) exert similar inhibitory effects on DNA replication in actively growing cells. It has been documented that a major component of this inhibition derives from a delay in the initiation of unreplicated origins (1, 2). Although this response was first observed several decades ago, the underlying molecular mechanisms remain largely unknown but ATM is thought to play an important role. More recently, evidence has been presented suggesting that the inhibition observed in DNA replication in response to DDIA is equivalent to the activation of a checkpoint in the S-phase and that it requires in yeast the products of MEC1 and RAD53 genes, homologues of ATM and Chk2, respectively (3–6). It is important to establish the mechanisms of regulation of DNA replication after DNA damage and suggest that analogous processes regulate cellular DNA replication with the DNA-PK targeting the functional homologues of Tag.

Exposure of mammalian cells to DNA damage-inducing agents (DDIA) inhibits ongoing DNA replication. The molecular mechanism of this inhibition remains to be elucidated. We employed a simian virus 40 (SV40) based in vitro DNA replication assay to study biochemical aspects of this inhibition. We report here that the reduced DNA replication activity in extracts of DDIA-treated cells is partly caused by a reduction in the amount of replication protein A (RPA). We also report that the dominant inhibitory effect is caused by the DNA-dependent protein kinase (DNA-PK) which inactivates SV40 T antigen (Tag) by phosphorylation. The results demonstrate that RPA and DNA-PK are involved in the regulation of viral DNA replication after DNA damage and suggest that analogous processes regulate cellular DNA replication with the DNA-PK targeting the functional homologues of Tag.

In the SV40-based DNA replication assay, replication of plasmids containing the SV40 origin of DNA (ori1 DNA) replication is accomplished in vitro with either crude cytoplasmic extracts or proteins purified from such extracts with SV40 T antigen (Tag) as the only noncellular protein (9–13). It is believed that all cellular proteins required in this assay function in a manner similar to that in vivo (10–14). We have reported that the degree of inhibition of DNA replication in cells exposed either to x-rays or CPT is comparable with that measured in vitro using extracts prepared from cells exposed to these agents (7, 8). We subsequently focused on the molecular characterization of this inhibition. In the present study, we provide evidence that following DNA damage two processes contribute to the inhibition of in vitro DNA replication: reduced availability of replication protein A (RPA) and activation of DNA-dependent protein kinase (DNA-PK).

EXPERIMENTAL PROCEDURES

Cell Culture, X-ray Radiation, and CPT Treatment—HeLa cell culture, x-ray radiation, and CPT treatment are as described (7, 8). MO59J cells were grown in Dulbeco's modified Eagle's medium-F-12 supplemented with 10% iron-supplemented calf serum (Hyclone). Cytoplasmic Extract Preparation and Extract Modification—Procedures used for cytoplasmic extract preparation either in large scale (109 cells) or in small scale (106 cells) have been described previously (7, 8, 15). In some experiments, double-stranded DNA (dsDNA) fragments (Stratagene, sonicated salmon sperm DNA, length range from 0.5 to 2 kilobase pairs) in TE buffer (10 mmt Tris-Cl, pH 7.5, 1 mmt EDTA) were added in the cytoplasmic extracts at 30 °C for 10 min. Wortmannin (Sigma) was added to the extracts (20 μm) at 30 °C for 10 min before assembling replication reactions. In some experiments, purified recombinant RPA (rRPA) or Tag was added to the extracts at 30 °C for 10 min before assembling the reactions. RPA, wild type or mutant forms, were prepared as described (8). Detection of RPA was by Western blotting using standard procedures. In Vitro Assay for DNA Replication—The plasmid, pSV01ΔEP, carrying the minimal origin of SV40 DNA replication (ori1) was used as a template in the replication reactions. The reaction mixture was incubated at 37 °C for 1 h. Activity present in acid-insoluble material was determined (6, 7). Replication products were analyzed by agarose gel electrophoresis as described (7, 8).

Single-stranded DNA (ssDNA) Binding—A 25-mer DNA oligonucleotide (5′-GATCTCCTCTCCTCTCCTCTCTCCTCCTCT-3′) was end-labeled by T4 polynucleotide kinase using 5 pmol of [γ-32P]ATP (Life Technologies, Inc.). Extracts were prepared as described above. Binding reactions were performed at 37 °C for 15 min with 30 fmol of radiolabeled probe (2000 cpm/fmol) and 10 μg of total cell protein as described (16). Bound and free probes were separated on 10% native polyacrylamide gels in 0.25 × TBE buffer (45 mtt Tris borate, 1 mtt EDTA). Gels were dried and exposed to autoradiography film. A monoclonal antibody against RPA 70 subunit was used during DNA binding reactions when indicated.

DNA-PK Activity—DNA-PK activity was assayed by using a commercially available kit following the manufacturer's protocols (SignaTECT DNA-PK assay system, Promega). Tag Phosphorylation by DNA-PK—The reaction mixture (25 μl) con-
Roles of RPA and DNA-PK in Regulation of DNA Replication

RPA, a ssDNA-binding protein, is a heterotrimeric complex with subunits of 70, 34, and 14 kDa required in the initiation stages of in vitro DNA replication and for multiple other processes of DNA metabolism (17–20). To test whether RPA plays a role in the inhibition of DNA replication in the in vitro DNA replication assay, we measured, by immunoblotting, its abundance in the extracts of cells treated with radiation or CPT. The amount of RPA p70, the subunit responsible for ssDNA binding in the extracts of cells treated with radiation or CPT, was reduced by approximately 50% (Fig. 1a, top). In line with a reduction in RPA p70, the binding activity of RPA to ssDNA was also reduced by about 50% in the same extracts (Fig. 1a, bottom, lanes 4 and 5). Post-translational modifications of RPA p34 have been implicated in cell cycle regulation and S-phase checkpoint activation (21, 22). We observed phosphorylation of RPA p34 (8), but could not establish alterations in the level of this subunit after DNA damage, probably due to the presence of a form not associated with the RPA holoenzyme (23). A monoclonal anti-RPA p70 antibody slowed the mobility of the DNA-protein complex in reactions assembled using cell extracts, as well as in reactions assembled with rRPA (Fig. 1b, lanes 2 and 4), confirming that the ssDNA binding protein is RPA. The amount of RPA p70 in the whole cell lysates of treated cells was similar to that of nontreated cells (data not shown). Therefore, we hypothesized that RPA may bind to DNA damage sites after exposure of cells to DDIA and be retained in the nucleus during extract preparation, reducing thus its level in the cytoplasmic component. In support of this hypothesis, addition of dsDNA fragments reduced RPA binding to ssDNA (Fig. 1c). It is possible that competition for RPA between damage sites and replication sites contributes to the inhibition of DNA replication in DDIA-treated cells.

Although the amount of RPA was reduced in extracts from DDIA-treated HeLa cells, addition of rRPA could not recover DNA replication activity (Fig. 1d, compare lanes 7 and 8). Also, a small amount of such extracts mixed with control extracts strongly inhibited DNA replication (Fig. 1d, compare lanes 1 and 6). We discovered that the inhibition of DNA replication could be reversed when extracts of DDIA-treated HeLa cells were preincubated with wortmannin, a nonspecific inhibitor of protein kinases, and were supplemented with rRPA (Fig. 1d, lane 10). A similar reversion of DNA replication inhibition was also observed when extracts of DDIA-treated HeLa cells were preincubated with wortmannin and mixed with control extracts (Fig. 1d, lane 11). Thus, the reduction in DNA replication activity observed in cytoplasmic extracts of cells exposed to DNA damage was due to the denuding activity and an activation of a wortmannin-sensitive kinase. Other factors essential for in vitro SV40 DNA replication remain practically unaffected in such extracts.

As mentioned above, dsDNA fragments could reduce RPA binding to ssDNA. We examined whether addition of dsDNA fragments to extracts of nontreated cells could simulate some of the characteristics of inhibition observed in extracts of treated cells. The presence of dsDNA fragments in the extracts of nontreated cells inhibited DNA replication in a dose-dependent manner (Fig. 2a, HeLa), and the inhibition reached a plateau at 50 ng (Fig. 2a, HeLa). Addition of rRPA relieved somewhat the observed inhibition, but the effect was relatively small (Fig. 2a, HeLa, and Fig. 2b, lane 7). Pretreatment of extracts with wortmannin did not affect the inhibition caused by 50 ng of dsDNA (Fig. 2b, lane 6). However, addition of rRPA to wortmannin-treated extracts completely reversed DNA replication inhibition (Fig. 2b, lane 8). Thus, dsDNA fragments inhibit in vitro DNA replication via a mechanism similar to that identified for extracts of cells exposed to radiation or CPT, i.e. by sequestering RPA and by activating a wortmannin-sensitive kinase. In subsequent experiments, dsDNA fragments were added to the extracts of untreated cells to mimic the DNA damage effect.

The inhibition of DNA replication after DNA damage is thought of as an active process that favors DNA repair. To identify the target of wortmannin in the extracts from DDIA-treated cells, we tested DNA replication activity in extracts of repair-deficient cell lines. MO59J cells are deficient in dsDNA DNA-PK.

RESULTS AND DISCUSSION

Fig. 1. RPA DNA binding activities and in vitro DNA replication in cytoplasmic extracts of DDIA treated cells. a, top, Western blot for RPA p70 level in cytoplasmic extracts of HeLa cells (C, control; R, irradiated, CPT, CPT-treated). Extracts prepared as described under “Experimental Procedures.” a, bottom, RPA binding to ssDNA measured by a gel-shift assay with the same extracts. rRPA was used as a positive control. b, a monoclonal anti-RPA antibody against p70 suppressed the DNA-protein complex in HeLa cell extracts and in a reaction assembled with rRPA, c, as in a, but for reactions incubated in the presence of different amounts of dsDNA fragments (0, 1, 5, 10, 20, 50, and 100 ng in lanes 1, 2, 3, 4, 5, 6, and 7, respectively). d, SV40 DNA replication reactions (25 μl) were assembled using the indicated amount of extracts (S-100) from untreated, irradiated (upper gel) or CPT-treated (lower gel) cells. Shown are the effects of added rRPA (2 μg) and wortmannin (20 μM) on DNA replication activity. Results of reactions assembled by mixing extracts of treated (R or CPT) and nontreated cells are also shown. Reactions assembled without TAg were included in all experiments and consistently showed only background levels of DNA synthesis (about 1 pmol incorporation; results not shown).
break rejoining and sensitive to ionizing radiation-induced cell killing (24). We found that when MO59J cell extracts were supplemented with DNA fragments, both RPA binding to ssDNA (Fig. 2a, MO59J) and in vitro DNA replication (Fig. 2a, MO59J) were reduced to a degree similar to that observed in HeLa cell extracts. However, contrary to the results with the extracts of HeLa cells, RPA alone completely reversed this inhibition (Fig. 2a, MO59J, and Fig. 3c). These results indicated that the wortmannin-sensitive protein kinase was absent from MO59J cell extracts.

It has been reported that MO59J cells lack any detectable DNA-PK activity (25) and have a low expression of ATM (26). It is well known that AT cells show reduced inhibition of DNA replication after DNA damage (27). At the concentration used (20 μM), wortmannin inhibits both DNA-PK and ATM kinase. To examine whether wortmannin acts in MO59J cell extracts by inhibiting ATM or by inhibiting DNA-PK, we prepared extracts from AT cells and performed experiments similar to those outlined with MO59J cell extracts. Unexpectedly, restoration of DNA replication in AT cell extracts after addition of DNA fragments required both RPA and wortmannin, indicating that the inhibitory mechanism of in vitro DNA replication does not utilize ATM (data not shown). We measured, therefore, the DNA-PK activity in the extracts of DDIA-treated HeLa cells. The DNA-PK activity was higher in the extracts of treated HeLa cells than in control extracts when no activating dsDNA was added to the reactions (Fig. 3b, control buffer). Importantly, addition of purified DNA-PK to MO59J cell extracts supplemented with DNA fragments re-established the wortmannin requirement for the restoration of DNA replication activity (Fig. 3c). These results identify the wortmannin-sensitive kinase that inhibits DNA in vitro replication as DNA-PK.

DNA-PK (28, 29) is a serine/threonine kinase consisting of a 465-kDa catalytic subunit (DNA-PKcs) and a heterodimeric regulatory complex termed Ku. As targets of DNA-PK we considered two essential factors for in vitro SV40 DNA replication: RPA and TAg. Both proteins are known to be phosphorylated by DNA-PK and have been implicated in regulatory steps of DNA replication (29, 30). To investigate whether RPA or TAg contribute to the inhibition of DNA replication observed in extracts of DDIA-treated HeLa cells, we separately preincubated these proteins with a small amount of extract from treated HeLa cells to allow for possible post-translational modifications. The mixture was then used to assemble reactions with extracts of nontreated cells. Wortmannin was added to these reactions after the end of the preincubation period to neutralize the inhibitory effect of the treated cell extract that accompanied RPA or TAg. The results indicated that this preincubation did not alter RPA activity (data not shown), but dramatically reduced TAg activity for DNA replication (Fig. 4a). To further confirm that the modulation of RPA by DNA-PK has no effect on the DNA replication activity, we used a mutant RPA with a deletion in the N-terminal domain of RPA p34. The deletion removes DNA-PK phosphorylation sites without affecting the activity of the enzyme for in vitro DNA replication (31, 32). The effect of this mutant protein (data not shown) on DNA replication was practically identical to the effect of the wild type protein. This suggests that phosphorylation of RPA by DNA-PK does not modify its activity in supporting DNA
replication in extracts of either treated or nontreated cells. Despite the fact that in vitro observations suggest no role for phosphorylated RPA in the regulation of DNA replication after DNA damage, the possibility that this form of RPA plays a role in vivo should be left open.

To confirm that the modification of TAg in extracts of treated HeLa cells was mediated by DNA-PK, we preincubated TAg with purified DNA-PK. The in vitro DNA replication activity decreased dramatically in reactions assembled using TAg preincubated with DNA-PK, and this inhibition could be completely reversed by nontreated TAg (Fig. 4b). Addition of [γ-^32P]ATP during the preincubation period confirmed phosphorylation of TAg by DNA-PK (Fig. 4c). Phosphorylation and inactivation of TAg for DNA replication was inhibited by the presence of ori^− DNA in the preincubation reaction (Fig. 4c), suggesting that binding of TAg to the replication origin reduced the phosphorylation of TAg and thus its inactivation.

The results presented here provide evidence that following DNA damage two processes contribute to the inhibition of in vitro DNA replication: reduced availability of RPA and activation of DNA-PK. Similar processes may also be involved and partly regulate cellular or viral DNA replication in vivo after exposure of cells to DNA damage. DNA-PK may inhibit DNA replication by phosphorylating functional homologues of TAg. Recruitment of RPA to DNA damage sites may contribute to this down-regulation. The latter pathway may explain the inhibition of DNA replication in irradiated MO59J cells, which is found to be similar to that observed in cell lines with normal DNA-PK activity (24). The results shown in Fig. 2a support this hypothesis.

There is strong evidence that severe combined immunodeficiency mice have greatly reduced DNA-PK activity (33–35) and are as a result hypersensitive to radiation and deficient in DNA double strand breaks rejoicing. Recently it has been reported that DNA-PK may play a role in tumor suppression (36). Here, we report yet another function of DNA-PK: the regulation DNA replication after DNA damage. Although our results did not implicate ATM in the inhibition of in vitro DNA replication following DNA damage, the evidence for an important role of this kinase in the regulation of DNA replication in vivo is overwhelming and suggests that multiple processes regulate DNA replication after DNA damage. The in vitro assay allowed us to study one specific aspect of this regulation.

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