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Advances in Brief

Dissociation of Radiation-induced Phosphorylation of Replication Protein A from the S-Phase Checkpoint[1]

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Abstract

Replication protein A (RPA) is a trimeric single-stranded DNA-binding protein complex involved in DNA replication, repair, and recombination. DNA damage induces phosphorylation of the RPA p34 subunit, and it has been speculated that this phosphorylation could contribute to the regulation of the DNA damage-induced S-phase checkpoint. To further examine this potential relationship, human cell lines expressing ataxia telangiectasia (AT)-mutated dominant-negative fragments, which fail to arrest in S phase in response to ionizing radiation (IR), and AT cells expressing AT-mutated-complementing fragments, which regain the ability to arrest replicative DNA synthesis in response to IR, were analyzed for radiation-induced RPA phosphorylation. Results from these studies demonstrate that IR-induced RPA phosphorylation can be uncoupled from the S-phase checkpoint, suggesting that RPA phosphorylation in response to IR is neither necessary nor sufficient for an S-phase arrest.

Introduction

IR[3] and other DNA-damaging agents can induce arrest of the cell cycle in G_1, S, and G_2 (for review, see Ref. 1). Although p53, p21, and pRb have been implicated in the irradiation-induced G_1 arrest, little is known about the molecular mediators of the arrests in S or G_2 in mammalian cells. Some studies have suggested that inhibition of DNA replication activity causing the radiation-induced S-phase arrest may be modulated in part by the phosphorylation status of a eukaryotic heterotrimeric single-stranded DNA-binding protein, RPA (2–4). RPA was initially characterized as a required component for the initiation and elongation stages of SV40 DNA replication *in vitro* (5–7). Human RPA consists of three subunits (M_r 14,000, M_r 32,000–34,000, and M_r 70,000) and is a member of a ubiquitous family of eukaryotic single-stranded DNA-binding proteins involved in DNA replication, DNA repair, and recombination (8–10). The RPA p34 subunit, in particular, is phosphorylated at multiple serine residues during the S and G_2 phases of the cell cycle and in response to ionizing or UV radiation (2, 3, 11–14). Several cellular kinases found to phosphorylate RPA *in vitro* include cyclin-dependent kinases (3, 15) and DNA-PK (16–18). DNA-PK is activated by DNA double-strand breaks and is required for V(D)J recombination and DNA repair (16, 19–21).

Although the physiological role of phosphorylation of RPA is unclear, the observation that UV or IR exposure causes both phosphorylation of RPA p34 and an S-phase arrest led to the suggestion that this phosphorylation event may contribute to the inhibition of DNA replication activity (12, 13). These studies, in conjunction with the observation that cell lines derived from AT patients lack the irradiation-induced S-phase arrest (*i.e.*, exhibit RDS; Refs. 22 and 23) and are deficient in IR-induced RPA phosphorylation (13, 14), suggested that RPA phosphorylation may play a functional role in the regulation of the S-phase checkpoint.

There are, however, several observations that would argue against such a functional relationship between irradiation-induced RPA phosphorylation and the S-phase checkpoint. First, inhibition of DNA synthesis in response to irradiation occurs within 30 min after treatment (22, 23), whereas optimal RPA p34 phosphorylation is not detected until 1–2 h postirradiation (24). Second, such phosphorylation of RPA is difficult to detect until radiation doses as high as 50 Gy are used (14), whereas the S-phase checkpoint is measurable at much lower (*i.e.*, 2 Gy) doses of IR.

To further explore the relationship between IR-induced RPA p34 phosphorylation and the S-phase checkpoint, we used recently developed human cell lines that express either DN or complementing fragments of the ATM gene product (25–27). Cells expressing the DN fragment lose the IR-induced S-phase checkpoint, whereas AT cells expressing the complementing fragment regain the ability to arrest replicative DNA synthesis in response to IR (27). We analyzed these cells with manipulated ATM function to ask whether IR-induced RPA phosphorylation could be uncoupled from the IR-induced S-phase checkpoint. Results from our studies demonstrate that IR-induced RPA p34 phosphorylation is neither necessary nor sufficient for the S-phase checkpoint.

Materials and Methods

Cell Lines and Irradiation Treatment. SV40-transformed fibroblast cell lines from normal individuals (GM637) and AT homozygotes (GM5849), and the RKO colorectal carcinoma cell line were grown as described previously (27). HeLa cells were grown in DMEM with 10% FCS at 37°C and 5% CO_2. Cells were irradiated with a ^{137}Cs source at a dose rate of approximately 90 rad/min.

Immunoblot and RDS Analysis. Cells were grown in 100-mm^2 dishes to 50–80% confluence and treated with 0, 10, 20, or 50 Gy of IR. For immunoblots, cells were harvested and solubilized in a buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium molybdate, 1 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 10 μg/ml leupeptin, 2.5 μg/ml antipain, and 1 μg/ml chymostatin (Sigma Chemical Co.). All steps were performed at 4°C. Whole-cell extracts (100 μg) were fractionated on a 12% denaturing SDS-polyacrylamide gel and transferred to nitrocellulose in 10 mM 3-(cyclohexylamino)propanesulfonic acid (pH 11.0)-10% methanol at 500 mA for 5 h at 4°C. After transfer, equal loading was confirmed by fast green staining of the membrane. Membranes were blocked with 5% milk in TBS-T (20 mM Tris-HCl, 137 mM NaCl, and 0.2% Tween 20) and were incubated with a monoclonal antibody (71-9A; Ref. 28) that was specific for the p34 subunit of RPA. Immunoreactive bands were visualized using the ECL detection system (Amersham). To verify the identity of the phosphorylated

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[3] The abbreviations used are: IR, ionizing radiation; RPA, replication protein A; DNA-PK, DNA-activated protein kinase; AT, ataxia telangiectasia; RDS, radioresistant DNA synthesis; DN, dominant-negative; ATM, AT-mutated; ssDNA, single-stranded DNA.

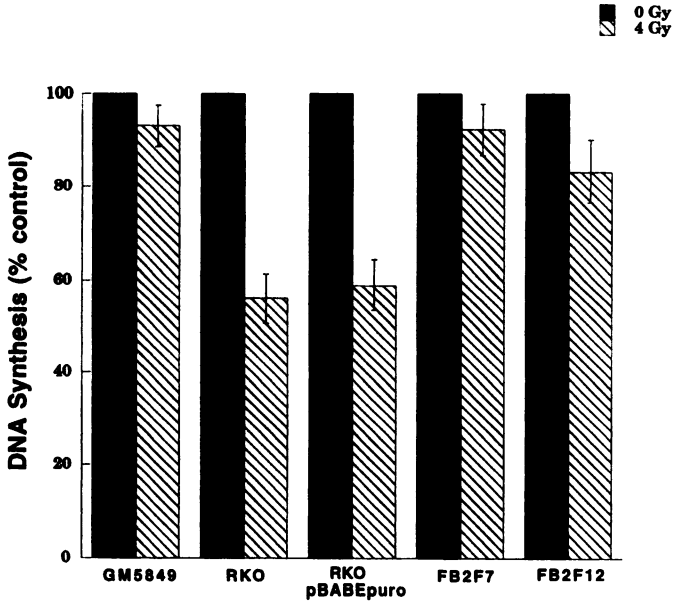

Fig. 1. Inhibition of DNA synthesis following IR. AT cells (GM5849), RKO parental cells, or RKO clones expressing either vector alone or DN-FB2F fragments were assessed for DNA synthesis 30 min following treatment with 0 (■) or 4 Gy (▨) of IR. S-phase arrest is manifest as a decrease in thymidine incorporation in irradiated cells relative to unirradiated cells. *Error bars*, SE.

RPA form, protein extracts were treated with λ phosphatase (400 units; New England Biolabs) for 30 min at 30°C before electrophoresis.

Inhibition of DNA synthesis following IR (RDS analysis) was assessed as described previously (27).

***In Vitro* RPA Kinase Assays.** HeLa cell extract (75 μg) was incubated in a reaction mixture (80 μl) containing 40 mM Hepes (pH 7.8), 7 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 200 ng of M13 ssDNA as described previously (18). The reactions were incubated for 15 min at 37°C, terminated with 2× Laemmli sample buffer, and analyzed by Western blotting.

Results and Discussion

ATM-DN fragments have been previously generated (27), which, when expressed in a human tumor cell line, abrogate the S-phase checkpoint following IR treatment (Fig. 1). Inhibition of DNA synthesis after IR is manifested as a decrease in thymidine incorporation in irradiated cells relative to unirradiated cells. Following exposure to 4 Gy of IR, the parental RKO and control RKO-pBABEpuro (vector alone) cells exhibited an approximately 50% decrease in DNA syn-

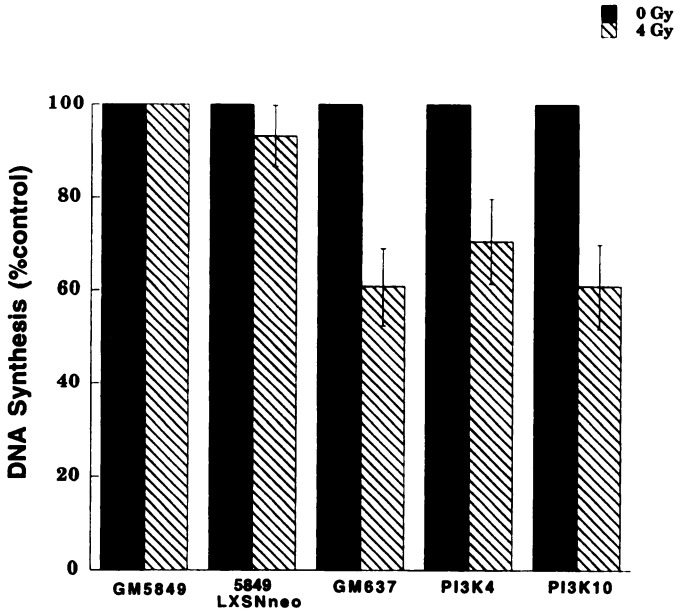

Fig. 3. Inhibition of DNA synthesis following IR. Normal control (GM637) and AT (GM5849) SV40-transformed fibroblasts expressing the PI3K domain were measured for RDS following treatment with 0 (■) or 4 Gy (▨) of IR as described in the legend for Fig. 1. *Error bars*, SE.

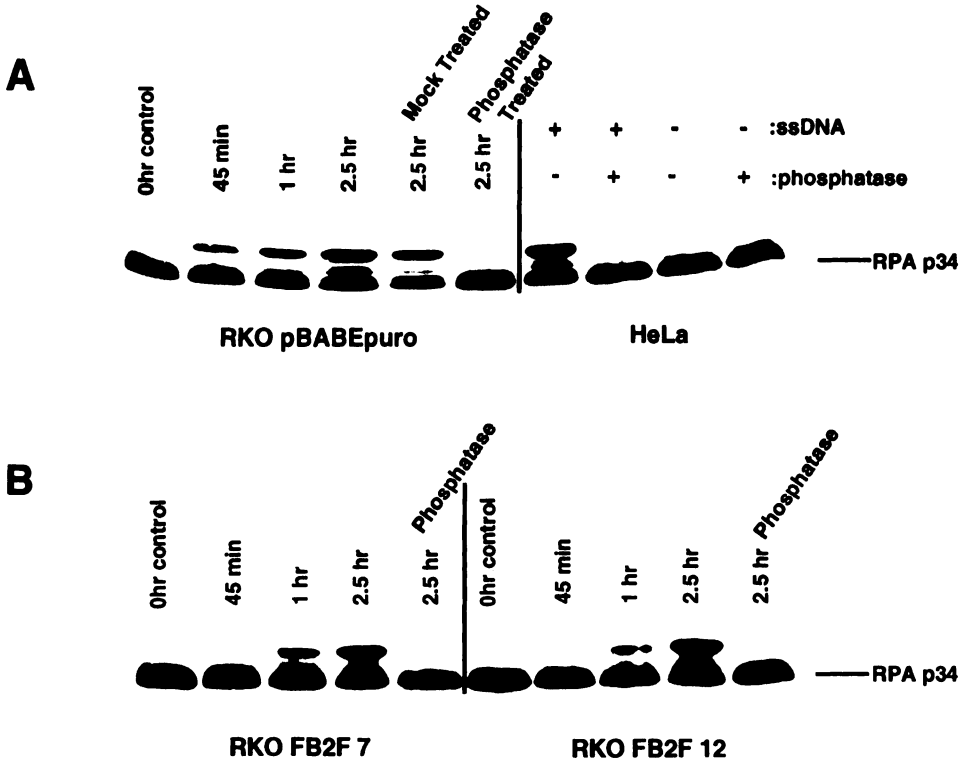

Fig. 2. Phosphorylation of RPA p34. *A*, control RKO cells were irradiated at 50 Gy and harvested 45 min, 1 h, and 2.5 h after treatment and analyzed for RPA p34 by immunoblotting. At 2.5 h post-IR, cell extracts were also mock-treated or treated with λ phosphatase. HeLa cell extracts were incubated with (+) or without (−) M13 ssDNA in the absence (−) or presence (+) of λ phosphatase. *B*, RKO DN clones were irradiated at 50 Gy, harvested at the times indicated, treated or untreated with λ phosphatase, and analyzed for RPA p34 phosphorylation by immunoblotting.

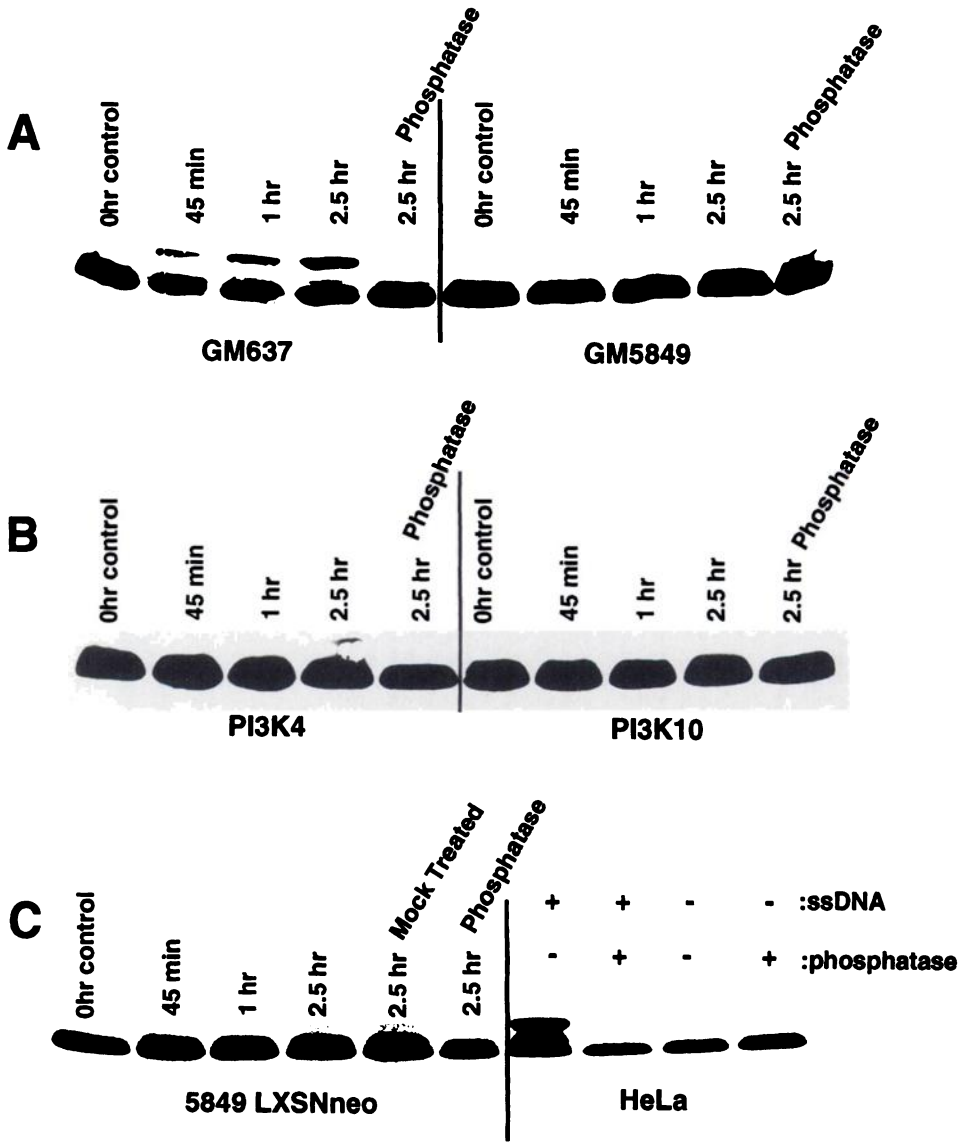

Fig. 4. Phosphorylation of RPA p34. *A* and *B*, control normal (GM637) and AT (GM5849) cells and AT 5849 clones PI3K4 and PI3K10 were irradiated at 50 Gy, harvested at the times indicated, treated or untreated with λ phosphatase, and analyzed for RPA p34 by immunoblotting. *C*, control 5849-LXSNneo and HeLa cells were treated as described in the legend for Fig. 2*A*.

thesis. In contrast, an AT fibroblast cell line, GM5849, and RKO clones expressing the DN fragments, FB2F7 and FB2F12, exhibited a lack of inhibition of DNA synthesis after irradiation (Fig. 1). Cells used in the above cell cycle experiments were the same cells that were used for Western blot analysis described below.

To determine whether manipulation of this S-phase checkpoint by ATM-DN fragment expression in RKO cells also affects RPA phosphorylation status in response to IR, RPA phosphorylation levels were assessed in the same experiment by immunoblotting. The RPA p36 form was undetectable using doses of 4, 10, or 20 Gy (data not shown); however, phosphorylation of RPA was detectable by 45 min following treatment with 50 Gy of IR with an approximate increase in size of M_r 2000 compared to the unirradiated control (Fig. 2*A*). The amount of RPA phosphorylation detected was both dose and time dependent in all cell lines tested. RPA p34 phosphorylation in control RKO-pBABEpuro cells was detected by 45 min, and the amount of p34 phosphorylation increased to a maximum at 2.5 h following IR treatment (Fig. 2*A*). Similar to the control transfectant, RPA phosphorylation was also detected in extracts derived from RKO DN clones FB2F7 and FB2F12 following IR treatment (Fig. 2*B*). The fact that these RKO clones failed to arrest in S-phase in response to IR while they maintained RPA phosphorylation patterns similar to those of the RKO control transfectant demonstrates that IR-induced RPA phosphorylation is not sufficient for an S-phase arrest.

The identity of the phosphorylated form of RPA p34 was confirmed using two independent methods. First, RKO cell extracts were treated with λ phosphatase, which eliminated the RPA p36 protein band (Fig. 2). Second, IR-induced RPA p34 phosphorylation was found to be the same phosphorylated species as that seen in crude HeLa cell extracts using an *in vitro* kinase assay (Fig. 2*A*). As previously reported, the enzyme responsible for ssDNA-activated phosphorylation of RPA p34 in crude HeLa cell extracts was identified as DNA-PK (18). Phosphorylation of RPA p34 by DNA-PK is dependent upon ssDNA template and the reaction can be abolished by addition of λ phosphatase (Fig. 2*A*).

We have also recently demonstrated that AT SV40-transformed fibroblast cells expressing the ATM PI-3 kinase domain exhibit restoration of an IR-induced S-phase arrest (27). Fig. 3 represents an RDS assay in which the AT fibroblast cell lines GM5849 and control 5849-LXSNneo exhibited a lack of inhibition of DNA synthesis in response to IR. In contrast, the normal GM637 cell line, along with the AT 5849 clones PI3K4 and PI3K10, exhibited an approximately 40% decrease in DNA synthesis following treatment with 4 Gy of IR.

To determine whether restoration of this S-phase checkpoint by

expression of the ATM kinase domain in AT cells affects IR-induced RPA phosphorylation status, RPA phosphorylation levels in response to IR were assessed by immunoblotting. RPA p34 phosphorylation in normal GM637 fibroblast cells was apparent at 45 min in response to IR, whereas in AT GM5849 fibroblast cells, the fraction of IR-induced RPA p34 phosphorylation was absent or barely detectable even at 2.5 h (Fig. 4*A*). The observation that AT cells are deficient in IR-induced RPA phosphorylation is in agreement with previously published reports (13, 14). The 5849 PI3K clones, which exhibit restoration in IR-induced S-phase arrest, revealed a similar deficiency in IR-induced RPA phosphorylation as the parental AT GM5849 cell line and the control transfectant (Fig. 4, *B* and *C*). These data therefore demonstrate that RPA phosphorylation is not necessary for an IR-induced S-phase arrest.

In summary, manipulation of the S-phase checkpoint, by expression of either ATM-DN fragments in a human tumor cell line or complementing ATM-PI3K fragments in an AT cell line, did not appear to affect IR-induced RPA phosphorylation status. Taken together, results from these data suggest that RPA phosphorylation in response to IR is neither necessary nor sufficient for the irradiation-induced S-phase cell cycle checkpoint. The following two observations further support such a conclusion: the S-phase checkpoint occurs within 30 min following IR treatment, whereas RPA phosphorylation is not optimally detected until 1–2 h post-IR; and the S-phase arrest can be induced in response to doses as low as 2–4 Gy of IR, whereas RPA phosphorylation is difficult to detect until radiation doses as high as 50 Gy are delivered. Recent studies have also suggested that activation of an S-phase checkpoint, in response to a topoisomerase I inhibitor, camptothecin, is not directly caused by the phosphorylated form of RPA because addition of nonphosphorylated RPA p34 to extracts of camptothecin-treated cells did not appear to restore DNA replication activity (29).

The observation that expression of the PI-3 kinase domain in AT cells failed to restore IR-induced RPA phosphorylation suggests that complementation of this phosphorylation event, which only occurs at very high doses of IR, may require the remainder of the ATM protein. However, a functional link between ATM and RPA has yet to be determined.

What can be concluded from these studies is that there is not a functionally dependent relationship between IR-induced RPA phosphorylation and the IR-induced S-phase checkpoint; however, these studies do not exclude the possibility that RPA phosphorylation is a participant in the S-phase checkpoint. The fact that IR-induced RPA phosphorylation can be uncoupled from the DNA-damaged induced S-phase checkpoint suggests that the phosphorylation change induced in RPA by IR may not directly be involved in the regulation of DNA synthesis but could instead be involved in a signal transduction pathway that coordinates cell cycle events with DNA damage and/or repair.

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