DNA Damage by the Enediyne C-1027 Results in the Inhibition of DNA Replication by Loss of Replication Protein A Function and Activation of DNA-Dependent Protein Kinase†

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ABSTRACT: Treatment of cells with the enediyne C-1027 is highly efficient at inducing single- and double-strand DNA breaks. This agent is highly cytotoxic when used at picomolar levels over a period of days. For this study, C-1027 has been used at higher levels for a much shorter time period to look at early cellular responses to DNA strand breaks. Extracts from cells treated with C-1027 for as little as 2 h are deficient in SV40 DNA replication activity. Treatment with low levels of C-1027 (1−3 nM) does not result in the presence of a replication inhibitor in cell extracts, but they are deficient in replication protein A (RPA) function. Extracts from cells treated with high levels of C-1027 (10 nM) do show the presence of a trans-acting inhibitor of DNA replication. The deficiency in RPA in extracts from cells treated with low levels of C-1027 can be fully complemented by the addition of exogenous RPA, and may be due to a C-1027-induced decrease in the extractability of RPA. This decrease in the extractability of RPA correlates with the appearance of many extraction-resistant intranuclear RPA foci. The trans-acting inhibitor of DNA replication induced by treatment of cells with high levels of C-1027 (10 nM) is DNA-dependent protein kinase (DNA-PK). DNA-PK is activated by the presence of DNA fragments induced by C-1027 treatment, and can be abrogated by removal of the DNA fragments. Although it is activated by DNA damage and phosphorylates RPA, DNA-PK is not required for either RPA focalization or loss of RPA replication activity.

When DNA damage occurs during the S phase of the cell cycle, cellular checkpoint pathways are activated to arrest DNA replication and turn on DNA repair mechanisms, or to induce programmed cell death. Although many types of DNA damage are able to physically block replication fork movement, as little as a single damaged site is reported to be able to inhibit all cellular DNA replication (*1*). This indicates that trans-acting mechanisms must be induced to inhibit replication forks distal to the site of damage. Extensive genetic studies have identified multiple genes that are involved in S phase DNA damage checkpoint pathways (*2*, *3*). However, how these gene products participate in arresting DNA synthesis requires further study.

The most common agent used to study cellular responses to double-strand DNA breaks (DSBs)[1] is ionizing radiation. However, while gamma irradiation has been shown to induce DSBs, this type of damage makes up only ∼10% of the DNA lesions produced, which makes it difficult to separate cellular responses to DSBs from cellular responses to other types of DNA damage. The natural antibiotic enediyne C-1027 is a DNA scission agent that generates both single-strand DNA (ssDNA) breaks and DSBs (*4*, *5*). The absence of other types of adducts makes C-1027 a useful reagent for looking at cellular responses to DNA strand breaks.

The well-established in vitro simian virus 40 (SV40) DNA replication system has proven invaluable in investigating DNA damage-induced DNA replication arrest (*6*). Extracts from cells treated with a variety of genotoxic agents have been shown to be deficient in their ability to support SV40 DNA replication in vitro. We have recently shown this to also be true for C-1027 (*7*). However, the mechanisms of this DNA replication inhibition have been shown to be different for different genotoxic agents. Treatment of cells with gamma radiation, camptothecin, or a bifunctional DNA alkylator, bizelesin, induces the presence of a dominant trans-acting inhibitor of DNA replication (*8*−*11*). Wang et al. (*11*)

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[1] Abbreviations: RPA, replication protein A; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; ssDNA, single-strand DNA; SV40, simian virus 40.

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reported that the trans-acting inhibitor induced by camptothecin treatment is DNA-dependent protein kinase (DNA-PK). Their data suggest that inhibition of SV40 DNA replication by DNA-PK may be mediated through phosphorylation and inactivation of SV40 large T antigen. The identity of the trans-inhibitor induced by bizelesin treatment or gamma radiation remains unknown. However, treatment of cells with two other reagents, UV radiation or the DNA alkylator adozelesin, results in extracts that do not contain a trans inhibitor, but are deficient in replication protein A (RPA) function (*12*, *13*). This heterotrimeric (70, 32, and 14 kDa) ssDNA-binding complex is a relatively plentiful nuclear protein that plays important roles in DNA replication, repair, recombination, and transcription [for review, see (*14*, *15*)]. It has been shown that during the S phase of the cell cycle, or in response to gamma irradiation, RPA can be detected in focal points within cell nuclei (*16*−*20*). The 32 kDa subunit of RPA (RPA32) is also hyper-phosphorylated by DNA-PK in response to DNA damage. These modifications appear to have no effect on RPA's DNA replication functions; however, they may be involved in transcriptional activation of DNA repair genes (*14*, *21*−*29*).

We have recently demonstrated that treatment of cells with the DSB agent C-1027 results in a decrease in the ability of extracts to support SV40 DNA replication in vitro. Further, we have shown that such treatment also results in a substantial decrease in the levels of RPA in extracts from treated cells, and a concomitant increase in the levels of RPA in the pellet following extract preparation (*7*). Here we investigate this C-1027-induced redistribution of RPA, and the mechanisms of how DNA replication activity is inhibited in extracts from treated cells.

MATERIALS AND METHODS

Chemicals and Kinase Assays. [α-^{32}P]dATP and [γ-^{32}P]-ATP were obtained from Amersham Pharmacia Biotech. C-1027, a gift from Taiho Pharmaceuticals Co., Ltd., Tokushima, Japan, was diluted in water and stored at −20 °C. DNA-PK activity in cell extracts was evaluated using the SignaTECT DNA-Dependent Protein Kinase System (Promega).

Plasmids and Proteins. The SV40 origin-containing plasmid pSV011 has been described previously (*30*). SV40 large T antigen (Tag) was purified from recombinant baculovirus-infected High-Five insect cells (Invitrogen) using immunoaffinity chromatography (*31*).

Cell Cultures and Antibodies. Human 293 cells were grown as suspension cultures in S-MEM (Life Technologies) containing 5% (v/v) calf serum. MO59J cells (ATCC) were maintained in DMEM/F12 supplemented with 0.05 mM nonessential amino acids and 10% fetal bovine serum. HeLa cells (ATCC) were maintained in DMEM with 10% fetal bovine serum. Monoclonal antibodies specific to the human RPA 70 and 32 kDa subunits have been described previously (*32*).

Indirect Immunofluorescent Staining. MO59J and HeLa cells grown in two-well chamber slides were treated with 0, 0.1, or 1 nM C-1027 at 37 °C for 2 h. RPA was immunostained following the procedure of Swindle et al. (*33*) with minor modifications. Briefly, the cells were washed with PBS with 0.5% Triton X-100, fixed with 3% paraformaldehyde in PBS, and blocked with 50% normal goat serum in PBS. Monoclonal antibody against RPA32 was used as the primary antibody and incubated overnight at 4 °C. After extensive washing, fluorescein-conjugated goat anti-mouse antibody (Vector Laboratory Inc.) was added as the secondary antibody and incubated at room temperature for 1 h. The cells were washed with 0.5% Triton X-100 in PBS and examined under a Nikon Microphot microscope using a Bio-Rad MRC-1024 confocal imaging system. Adobe Photoshop was used for image processing and printing.

Subcellular Fractionation. The cellular fractionation was a slight modification of the protocol of Zou et al. (*34*). Briefly, 5×10^7 suspension-cultured 293 cells were treated with either 0 or 3 nM C-1027 for 2 h. The cells were harvested and washed with PBS. The cyto/nucleosolic fraction was prepared by extracting protein from the harvested cells for 10 min on ice using 1 cell pellet volume of 0.5% Triton X-100 in CSK buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA). Following centrifugation (10 min at 13000*g*), the residual nuclear structures were washed 3 times with PBS, and then incubated with 100 μg/mL DNase I in CSK buffer at 37 °C for 15 min. Following digestion, ammonium sulfate was added to 0.25 M, and the suspension was further incubated at RT for another 10 min. The suspension was subjected to centrifugation (10 min at 13000*g*), and the supernatant was collected and designated the chromatin fraction. The remaining insoluble material was washed with PBS and designated the nuclear matrix. The insoluble nuclear matrix was resuspended in SDS sample buffer with 20 mM Tris-HCl, pH 7.5, 1 M 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, and 10% glycerol.

Cell Extract Preparation and Cell-Free SV40 DNA Replication Assays. Cell extracts were prepared from 293 suspension cells treated with DNA damaging agents at the indicated concentrations for 2 h. In vitro SV40 DNA replication conditions and analyses were described previously (*13*). Quantitation of DNA replication activity was achieved using a Bio-Rad Phosphorimager system to quantitate the [^{32}P]dAMP incorporated in DNA products that ranged from the replication intermediates (RI) to Form I positions on agarose gel analyses. Levels of synthesis are displayed as relative activity, which represents the percent of DNA replication compared to the control reaction for each experiment. Control reactions contained 40 μg of control cell extract used in the standard SV40 in vitro replication reaction conditions. Each experiment was carried out between 4 and 8 times; representative results are presented.

Immunoblotting. Each subcellular fraction from mock- or C-1027-treated 293 cells (5×10^6) was mixed with an equal volume of 2× SDS sample buffer (40 mM Tris-HCl, pH 7.5, 4% SDS, 2 M 2-mercaptoethanol). Proteins were then resolved by electrophoresis on 12.5% (w/v) SDS−polyacrylamide gels and transferred to Hybond-P membrane (Amersham Pharmacia Biotech) using NovaBlot (Amersham Pharmacia Biotech) as per the manufacturers' instructions. Membranes were probed with monoclonal antibodies against the two largest subunits of RPA, RPA70 and RPA32. Peroxidase-conjugated goat anti-mouse IgG (Pierce) was used as the secondary antibody and was detected using the Supersignal enhanced chemiluminescent reagent (Pierce) and exposure to X-ray film (Marsh).

Preparation of Protein-Free and DNA-Free Fractions of Cell Extracts. Whole cell extracts from mock- or 10 nM C-1027-treated cells were incubated with 200 ng/μL proteinase K at 37 °C for 30 min, followed by deproteination using phenol/chloroform extraction. The fractions were precipitated with 70% ethanol and 0.3 M sodium acetate, pH 5.0. This nucleic acid fraction was resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA to half the original cell extract volume. In the DNA-PK activation assays, the amount of the nucleic acid fraction added is equivalent to that from the control cell extract used to test for DNA-PK activity.

DNase I (Life Technologies) was immobilized on Affigel 10 (Bio-Rad) at 30 units/μL of matrix according to the manufacturers' instructions. After several washes with ethanolamine and PBS, the immobilized DNase I was resuspended in 50 μL of DNase I reaction buffer (20 mM Tris-HCl, pH 8.0, 1 mM $ZnCl_2$, and 1 mM DTT). One microliter of the immobilized enzyme matrix was capable of complete digestion of 500 ng of pUC-19 plasmid at room temperature in less than 20 min. Two hundred microliters of extract from mock- or 10 nM C-1027-treated 293 cells was incubated with 10 μL of immobilized DNase I at room temperature for 20 min. The DNase I matrix was pelleted by centrifugation and removed, and the digested DNA was removed from the extracts by dialysis against Buffer A (Tris-HCl, pH 8.0, and 10 mM 2-mercaptoethanol) with 10% glycerol at 4 °C for 2 h. Control cell extracts without DNase I treatment were dialyzed against the same buffer.

RESULTS

Extracts from C-1027-Treated 293 Cells Show Reduced Levels of in Vitro SV40 DNA Replication. We have previously demonstrated that treatment of human cells with C-1027 results in lower levels of in vitro SV40 DNA replication activity (*7*). To elucidate the mechanism of this replication arrest, suspension cultured human 293 cells were treated with a wider range of C-1027, and extracts were prepared and tested for their ability to support SV40 DNA replication. As previously shown, compared to mock-treated cell extracts, extracts from C-1027-treated cells are clearly deficient in their ability to support SV40 DNA replication (Figure 1A). This was not due to the presence of drug in the extracts as control experiments showed that addition of up to 20 nM C-1027 to extracts from control cells had little effect on in vitro DNA replication (data not shown) (*7*).

Reduced DNA replication activity can be due either to the loss of essential DNA replication activities or to the induction of trans-acting inhibitors of DNA replication. These two mechanisms can be differentiated by using cell extract mixing experiments to see if extracts from treated cells are capable of inhibiting the replication activity of control cell extracts (*8*−*10*, *13*). Similar to previously published results, extracts from cells treated with low levels (1 or 3 nM) of C-1027 do not contain a trans-acting inhibitor (Figure 1B), consistent with the loss of one or more activities essential for DNA replication (*7*). However, when cells were treated with higher levels of C-1027 (10 nM), a potent trans-acting DNA replication inhibitor was induced (Figure 1B, filled squares). These results indicate that the cellular concentration response curve to treatment with C-1027 is biphasic, an observation not previously reported. Upon treatment of cells

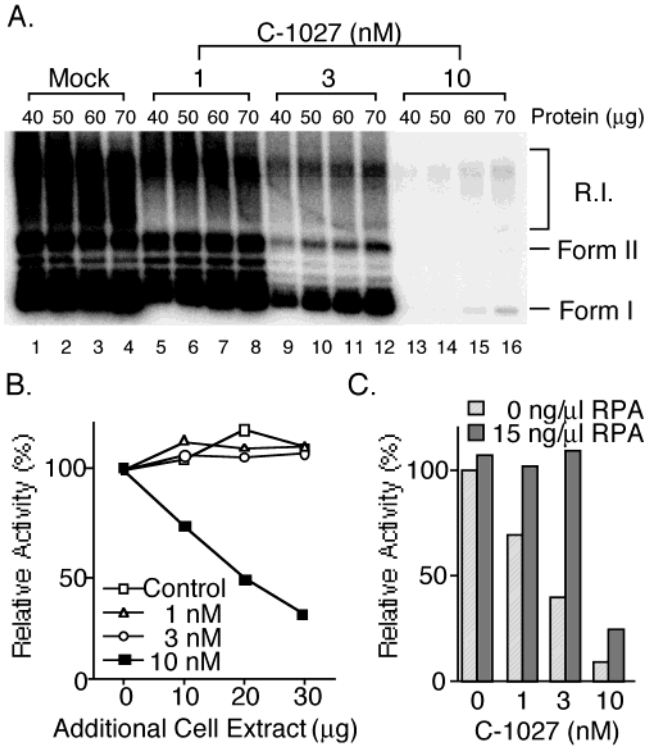

FIGURE 1: Treatment of cells with C-1027 results in inhibition of in vitro SV40 DNA replication through loss of RPA activity and, at high levels of C-1027, induction of a trans-acting inhibitor. (A) 40−70 μg of extract from cells treated with 0, 1, 3, or 10 nM C-1027 for 2 h was used in in vitro SV40 DNA replication assays. The migration of supercoiled (Form I) DNA, open circular (Form II) DNA, and θ form replication intermediates (R.I.) is indicated to the right of the panel. (B) 0−30 μg of extract from cells treated with 0 (open squares), 1 (triangles), 3 (circles), or 10 nM (filled squares) C-1027 was mixed with 40 μg of mock-treated cell extract and tested in SV40 DNA replication assays. In 6 independent experiments, no trans inhibition was detected in extracts from 1 or 3 nM treated cells. Levels of trans inhibition by extracts from cells treated with 10 nM C-1027 were similar in all experiments. (C) 0 or 15 ng/μL RPA was added to SV40 DNA replication reactions with extracts from cells treated with 0−10 nM C-1027. Four independent experiments showed the addition of RPA to extracts from 1 or 3 nM C-1027-treated cells rescued DNA replication levels to 100 ± 5% of the control. Rescue of DNA replication in extracts from cells treated with 10 nM C-1027 by addition of RPA was always <25% of control reactions. Relative activity (%) indicates the percent of DNA synthesis seen in comparison to levels of synthesis seen with the control reaction, 40 μg of extract from mock-treated cells with no additional RPA.

with low levels of drug, one or more essential DNA replication activities are lost, while at high levels of drug a trans-acting DNA replication inhibitor is induced.

Reduced SV40 DNA Replication in Extracts from Cells Treated with Low Doses of C-1027 Is Mediated through Loss of RPA Function. It has been shown that for some types of DNA damage, addition of exogenous replication protein A (RPA) is capable of rescuing the DNA replication activity of extracts from treated cells (*12*, *13*), while for other types of DNA damage it cannot (*8*−*11*). We therefore tested whether the inhibition of DNA replication upon treatment of cells with low doses of C-1027 is due to a loss of RPA function. Addition of purified RPA (to 15 ng/μL) was sufficient to fully rescue SV40 DNA replication activity in extracts from cells treated with either 1 nM or 3 nM C-1027 (Figure 1C). Addition of RPA to mock-treated extracts

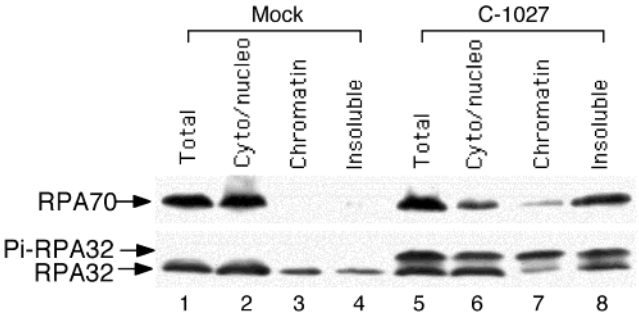

FIGURE 2: A large fraction of the cellular RPA becomes stably associated with the nuclear matrix following treatment with C-1027. Subcellular fractions from mock- and C-1027-treated 293 cells (5×10^5) were analyzed via immunoblotting with RPA70 and RPA32 monoclonal antibodies. The amount of each fraction loaded onto the gel represents the following percentage of the total volume of that fraction: Total (lanes 1 and 5), 0.8%; Cyto/nucleo (lanes 2 and 6), 1%; Chromatin (lanes 3 and 7), 3%; Insoluble (nuclear matrix) (lanes 4 and 8), 3%.

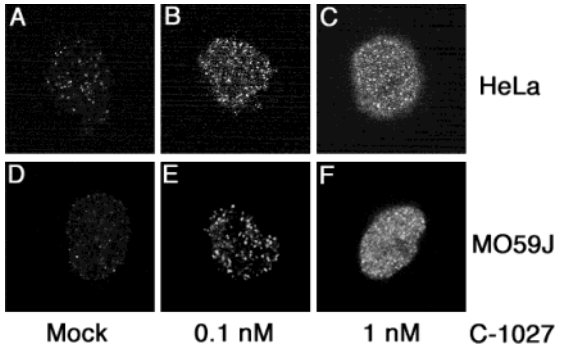

FIGURE 3: C-1027 treatment induces rapid, DNA-PK-independent, focalization of RPA. Monolayer cultured HeLa cells (A−C) or DNA-PK$^{(-/-)}$ (MO59J) cells (D−F) were grown on coverslips and treated with 0 (A and D), 0.1 (B and E), or 1 nM (C and F) C-1027 for 2 h. The cells were then washed with 0.5% Triton X-100 to permeabilize the cell and nuclear membranes and remove the free nucleosolic RPA. The slides were then treated with paraformaldehyde to fix the remaining proteins, and then stained for RPA with monoclonal antibody against RPA32 and fluorescein-labeled goat anti-mouse antibody. The nuclei were visualized using a Bio-Rad MRC-1024 confocal microscopic imaging system.

showed little effect on DNA replication activity (tested up to 30 ng/μL; Figure 1C and data not shown). Since the role of RPA in DNA replication is unique, this indicates that the primary mechanism of inhibition of DNA replication following treatment of cells with low doses of C-1027 is through loss of RPA function. Since RPA has never been shown to be able to compensate for deficiency in any other DNA replication protein (*14*, *15*), this also demonstrates that all the other essential factors in these extracts remain fully functional for in vitro DNA replication. Conversely, addition of RPA was not sufficient to rescue SV40 DNA replication activity in extracts from 10 nM C-1027-treated cells (Figure 1C). This is consistent with the cell extract mixing experiments showing that a trans inhibitor is induced upon treatment with 10 nM C-1027 (Figure 1B).

Intranuclear Redistribution of RPA. We have previously shown that RPA becomes resistant to extraction by hypotonic lysis in cells treated with C-1027 (*7*). The fact that more than 60% of the total RPA becomes extraction-resistant (Figure 2) (*7*) suggested that this could be the cause of the loss of RPA function in in vitro SV40 DNA replication assays (Figure 1C). To investigate whether RPA is being targeted to the chromatin or to the nuclear matrix, 293 cells were mock-treated or treated with low levels of C-1027 and then separated into three fractions: a combined cytosolic and nucleoplasmic fraction (prepared by treatment of cells with nonionic detergent under low ionic strength to solubilize the cell and nuclear membranes), a chromatin-bound fraction (prepared by extensive DNase digestion of the remaining pellet followed by high-salt extraction), and an insoluble nuclear matrix fraction (see Materials and Methods for details). These fractions were monitored for RPA levels using immunoblotting (Figure 2). For mock-treated cells, the majority of RPA (70−80%) was found in the cyto/nucleosolic extract (lanes 1 and 2), while very little RPA was found in the chromatin-bound or insoluble nuclear matrix fractions (lanes 3 and 4), consistent with previously published results (*35*). However, cyto/nucleosolic extracts from cells treated with low levels of C-1027 showed a substantial reduction in levels of the RPA complex (as evaluated by levels of RPA70, lanes 5 and 6). Following drug treatment, RPA levels were increased in both the chromatin-bound fraction (lane 7) and to an even greater degree in the insoluble nuclear matrix fraction (lane 8). In C-1027-treated cells, the chromatin-bound RPA population showed a much higher percentage of hyper-phosphorylated RPA32 than the other fractions (lane 7).

To further understand this RPA redistribution, indirect immunostaining was used to monitor the extraction-resistant RPA in drug-treated cells. HeLa cells were treated with low levels of C-1027 for 2 h. The cells were then washed with nonionic detergent (using the same buffer conditions as in Figure 2) before fixation (see Materials and Methods). This nonionic detergent wash results in the solubilization of both the cell and nuclear membranes, thereby washing away both cytoplasmic and nucleoplasmic proteins. This treatment has been shown to remove the vast majority of the RPA from untreated cells, which is present in the loosely bound nucleosolic fraction (*20*). The extraction-resistant RPA was then visualized using a monoclonal antibody against RPA32 and fluorescein-conjugated secondary antibody. Less than half (~40%) of mock-treated cells showed very low levels of RPA present as small faint nuclear foci (Figure 3A), consistent with previous studies showing RPA's presence at replication foci (*16*−*20*). Nuclei from the remaining mock-treated cells showed no detectable staining of RPA higher than background. However, cells treated with C-1027 showed strong staining of intranuclear RPA foci in more than 90% of treated cells (Figure 3B). Furthermore, the intensity of the RPA signal and the number of foci increased with increasing levels of drug (Figure 3B,C).

DNA-dependent protein kinase (DNA-PK) is the primary kinase responsible for DNA damage-dependent hyperphosphorylation of RPA32 (*14*). Therefore, DNA-PK$^{(-/-)}$ MO59J cells were used to test whether RPA focus formation is dependent on DNA-PK. As shown in Figure 3, RPA focalization appears to be unaffected by the absence of DNA-PK in these cells (compare panels A−C to panels D−F). Therefore, we conclude that RPA focalization is not dependent on RPA32 hyper-phosphorylation by DNA-PK.

Extracts from Cells Treated with High Levels of C-1027 Show Induction of DNA-PK Activity. Since treatment of 293 cells with high levels of C-1027 induces the presence of a

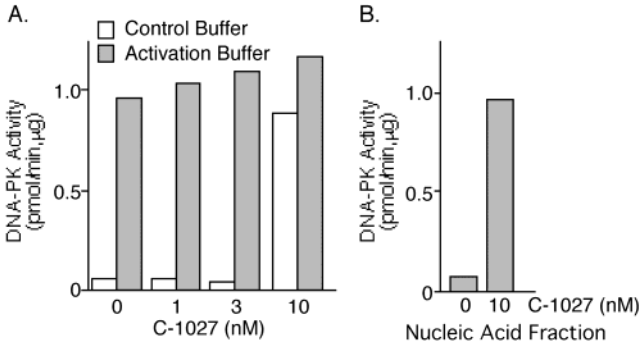

FIGURE 4: Treatment of cells with high levels of C-1027 generates DNA fragments sufficient to induce DNA-PK activity. (A) 40 μg of total protein from hypotonic extracts of 293 cells treated with the indicated concentrations of C-1027 for 2 h (prepared as in Figure 1) was assayed for DNA-PK activity. Control buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) assays show the level of preactivated DNA-PK kinase activity. Activation buffer is the same as control buffer but also contains 100 ng/μL sheared calf thymus DNA to activate any additional DNA-PK in the extracts. In three independent experiments, DNA-PK activity levels by extracts from cells treated with 0, 1, or 3 nM C-1027 ranged from 0.05 to 0.1 pmol min^{-1} μg^{-1}, while activity in extracts from cells treated with 10 nM C-1027 ranged from 0.8 to 1.0 pmol min^{-1} μg^{-1}. Addition of activation buffer to any cell extract resulted in DNA-PK activity levels ranging from 0.9 to 1.2 pmol min^{-1} μg^{-1}. Assays are capable of measuring from 0.02 to 2.0 pmol min^{-1} μg^{-1}. (B) Nucleic acid fractions were prepared from 40 μg of extract from the cells treated with either 0 or 10 nM C-1027 (as in panel A, 0 and 10 nM, open bars) by protein digestion, extraction, and ethanol precipitation of the nucleic acids. These nucleic acid fractions were each added to 40 μg of control extract (as in panel A, 0 nM, open bar), which was then assayed for DNA-PK activity. In three independent experiments, addition of the nucleic acid fraction from control cells to control cell extract resulted in <0.15 pmol min^{-1} μg^{-1} of DNA-PK activity. Addition of nucleic acid fraction from cells treated with 10 nM C-1027 to control cell extracts resulted in DNA-PK activity ranging from 0.9 to 1.1 pmol min^{-1} μg^{-1}.

trans-acting inhibitor of SV40 DNA replication (Figure 1B), and elevated DNA-PK activity has been reported to act as an inhibitor of in vitro SV40 DNA replication (*11, 24*), we examined DNA-PK activity in extracts from cells treated with C-1027. DNA-PK activity levels in extracts from cells treated with 1 and 3 nM C-1027 were indistinguishable from DNA-PK activity levels in control cell extracts (Figure 4A, open bars), indicating that DNA-PK activity is not induced in these extracts. However, extracts from cells treated with 10 nM C-1027 showed a dramatic increase in DNA-PK activity, almost to levels seen with addition of DNA-PK activation buffer to any of the extracts (Figure 4A, compare open bars at 10 nM to striped bars). DNA-PK activation buffer contains fragmented calf thymus DNA sufficient to activate endogenous DNA-PK present in tested extracts (Figure 4A, compare striped bar with open bar for DNA-PK assay results with extracts from '0 nM' C-1027-treated cells). The fact that DNA-PK activity is induced only upon treatment with 10 nM C-1027 is consistent with the possibility of DNA-PK activity being the trans-acting replication inhibitor induced in extracts from cells treated with high levels of C-1027 (Figure 4A and Figure 1B).

The similarity in the DNA-PK activity levels in extracts from cells treated with high levels of C-1027 and in control extracts with the addition of exogenous DNA fragments (present in the DNA-PK activation buffer) suggested that the activation of DNA-PK in extracts from drug-treated cells might be due to the presence of DNA fragments in these extracts. Nucleic acid fractions were prepared from mock- and C-1027-treated cell extracts and compared for their ability to activate the DNA-PK present in control cell extracts. The addition of the nucleic acid fraction from mock-treated cell extracts to extracts from mock-treated cells did not result in an appreciable increase in DNA-PK activity (Figure 4, compare the striped bar labeled '0 nM C-1027' in panel B to the open bar of '0 nM C-1027' in panel A). However, the addition of the nucleic acid fraction from extracts from cells treated with high levels of C-1027 (10 nM) to extracts from mock-treated cells resulted in a dramatic increase in DNA-PK activity (Figure 4, compare '10 nM C-1027' bar in panel B to the striped bars in panel A). These results clearly indicate that treatment of cells with 10 nM C-1027 causes enough DSBs to release sufficient DNA fragments to fully activate the DNA-PK present in hypotonically prepared cell extracts.

DNA Fragment-Induced DNA-PK Activity Is the Trans-Acting Inhibitor in Extracts from Cells Treated with High Levels of C-1027. To determine whether the C-1027-induced DNA fragments were responsible for the trans-acting DNA replication inhibitory activity in extracts from cells treated with high levels of C-1027, the DNA fragments were removed from these extracts. Immobilized DNase I was used to exhaustively digest any DNA present in extracts from 10 nM C-1027-treated cells, and the remaining small fragments were removed by dialysis (see Materials and Methods). DNA-PK assays were used to demonstrate that after this treatment, DNA-PK activity was reduced to levels similar to those seen in mock-treated cell extracts (data not shown). Cell extract mixing experiments were then used to test for the presence of trans-inhibitor activity as in Figure 1B. Before DNase I treatment, addition of extract from cells treated with high levels of C-1027 showed a strong inhibition of DNA replication by control cell extracts (Figure 5A, filled triangles; and Figure 1B, filled squares). After DNase I treatment of the extract, trans inhibition was no longer seen (Figure 5A, open triangles). DNase I treatment had little effect on mock-treated cell extracts (Figure 5A, open circles and squares). These results indicate that the trans inhibitor induced in extracts from cells treated with 10 nM C-1027 is dependent upon DNA fragments in these extracts.

DNase I-treated extracts from cells treated with high levels of C-1027 also did not show the ability to appreciably stimulate the basal level of DNA replication (Figure 5A, compare open triangles to open squares and circles). This result could be explained if these extracts were also deficient in limiting DNA replication activity. Indeed, the DNase I-treated extracts from cells treated with high levels of C-1027 were not capable of supporting appreciable levels of SV40 DNA replication on their own (Figure 5B, striped bars). Since RPA activity is deficient in extracts from cells treated with low levels of C-1027 (Figure 1C), we anticipated that this would also be true for extracts from cells treated with high levels of C-1027. RPA was added back to the DNase I-treated extracts from cells treated with high levels of C-1027 to see if RPA could complement these extracts for in vitro SV40 DNA replication activity. The addition of 15 ng/μL RPA to DNase I-treated extracts from cells treated with high levels of C-1027 was able to rescue SV40 DNA replication to levels consistent with control cell extracts

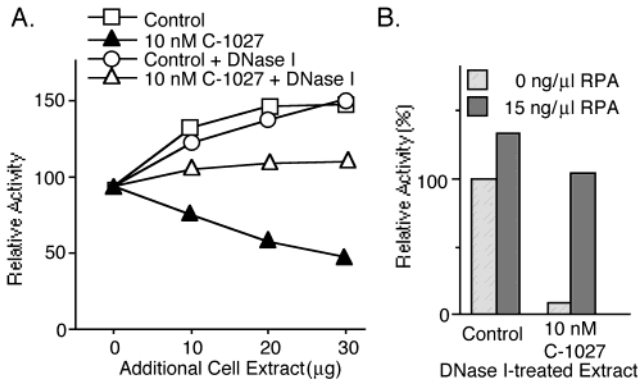

FIGURE 5: DNase I treatment eliminates the trans inhibitor in extracts from cells treated with high levels of C-1027. (A) 40 μg of extract from mock-treated cells was mixed with 0−30 μg of DNase I-treated extract from mock- or 10 nM C-1027-treated cells. These mixtures were then tested for the ability to support in vitro DNA replication. In three independent experiments, the addition of 30 μg of extract from cells treated with 10 nM C-1027 resulted in >40% inhibition of DNA replication by 40 μg of control cell extract. The addition of 30 μg of the same extract first treated with DNase I to 40 μg of control cell extract always showed DNA synthesis levels greater than that seen with 40 μg of control cell extract alone. (B) 0 or 15 ng/μL purified RPA was added to SV40 DNA replication assays carried out with 40 μg of DNase I-treated extracts from either mock- or 10 nM C-1027-treated cells. In three independent experiments, the level of DNA replication by 40 μg of extract from cells treated with 10 nM C-1027 was always < 10% of levels seen with 40 μg of control cell extract. Addition of 15 ng/μL RPA to 40 μg of extract from cells treated with 10 nM C-1027 resulted in DNA replication levels of 90−110% of that seen with 40 μg of control cell extract. Relative activity (%) indicates the percent of DNA synthesis seen in comparison to levels of synthesis seen with the control reaction, 40 μg of untreated extract from mock-treated cells with no additional RPA.

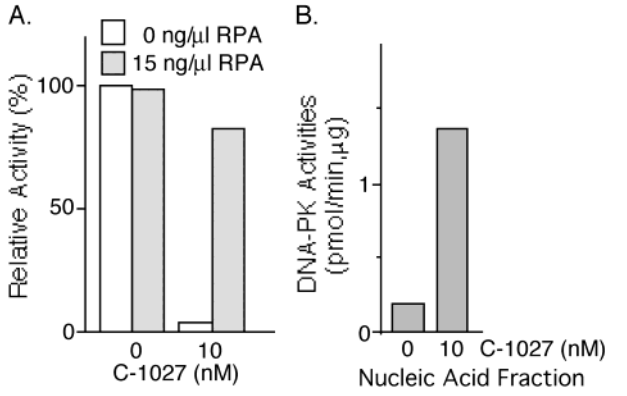

FIGURE 6: SV40 DNA replication in extracts of C-1027-treated DNA-PK$^{(-/-)}$ cells. (A) SV40 DNA replication was carried out with extracts from 0 or 10 nM C-1027-treated MO59J (DNA-PK$^{(-/-)}$) cells in either the presence (open bars) or the absence (striped bars) of 15 ng/μL exogenously added RPA. In three independent experiments, the levels of DNA replication by 40 μg of extract from MO59J cells treated with 10 nM C-1027 resulted in <5% of the levels of DNA synthesis seen with 40 μg of control extract from MO59J cells. Addition of 15 ng/μL RPA to the extracts from the C-1027-treated cells resulted in levels of DNA synthesis ranging from 77 to 83% of that seen with the control cell extract alone. Relative activity (%) indicates the percent of DNA synthesis seen in comparison to levels of synthesis seen with the control reaction, 40 μg of extract from untreated MO59J cells with no additional RPA. (B) Nucleic acid fractions from 40 μg of extract from 0 or 10 nM C-1027-treated MO59J cells were added to 40 μg of extract from mock-treated 293 cells and assayed for DNA-PK activity. In three independent experiments, addition of the nucleic acid fraction from 40 μg of extract from untreated MO59J cells to 40 μg of untreated 293 cell extract resulted in 0.1−0.2 pmol min^{-1} μg^{-1} of DNA-PK activity. Addition of the nucleic acid fraction from 40 μg of extract from MO59J cells treated with 10 nM C-1027 resulted in DNA-PK activity ranging from 1.2 to 1.4 pmol min^{-1} μg^{-1}.

(Figure 5B, compare 10 nM solid bar to control bars). These results are consistent with those in Figure 1C, and show that once the trans-acting inhibitory activity is removed from extracts of cells treated with high levels of C-1027, the only other mechanism of SV40 DNA replication inhibition is through loss of RPA function.

It has been reported that DNA fragments are capable of competing for essential DNA replication factors and can thus inhibit in vitro SV40 DNA replication (*36*). To verify that the trans inhibitor of DNA replication is activated DNA-PK, and not the DNA fragments themselves, experiments similar to those described above were performed using DNA-PK$^{(-/-)}$ MO59J cells. MO59J cells were treated with 0 or 10 nM C-1027 as above and tested for the presence of a trans-acting DNA replication inhibitor in the cell extracts. Using cell extract mixing experiments, no inhibitor was detected in extracts from MO59J cells treated with 10 nM C-1027 (data not shown). Furthermore, the addition of RPA alone to these extracts was capable of rescuing SV40 DNA replication activity (Figure 6A). As a control to demonstrate that C-1027 is capable of inducing comparable fragmentation of DNA in MO59J cells, nucleic acid fractions were prepared from extracts of MO59J cells treated with 10 nM C-1027 and used to stimulate DNA-PK activity in control 293 cell extracts (as was done in Figure 4B). Figure 6B clearly demonstrates that treatment of MO59J cells with 10 nM C-1027 does induce the presence of DNA fragments in cell extracts, sufficient to activate DNA-PK in control cell extracts. Taken together, these results indicate that the activation of DNA-PK, not the DNA fragments themselves, is responsible for the trans-acting inhibition of SV40 DNA replication seen in extracts from 293 cells treated with high levels (10 nM) of C-1027.

DISCUSSION

We have shown that treatment of cells with the DNA strand break agent, C-1027, causes both loss of RPA replication activity and induction of DNA-PK activity. Both of these effects have been demonstrated previously in cells treated with different DNA damaging agents. However, this is the first report showing that both mechanisms can be induced by a single DNA damaging agent in a dose-dependent manner. This is further evidence that there appear to be multiple S phase DNA damage checkpoint pathways to turn off DNA synthesis; and that these pathways can be activated differentially, in response both to different types of DNA lesions as well as to the amount of DNA damage.

We have demonstrated that RPA becomes extraction-resistant (associating with the nuclear matrix fraction) and forms foci in cells treated with C-1027 (Figures 2 and 3). These foci arise rapidly following DNA damage and are independent of DNA-PK (Figure 3). Other proteins have also been shown to form intranuclear foci in response to treatment of cells with genotoxic agents. Mre11, 53BP1, and histone H2AX are reported to form nuclear foci at DNA DSBs within a short time after induction of damage (*37*−*39*). RPA has been shown to form foci in response to ionizing radiation,

and is believed to bind to these DSBs through subsequent nuclease action on the DSBs, which creates areas of ssDNA. RPA is also capable of binding damaged dsDNA in the absence of strand breaks (*40, 41*). Since RPA binds to both ssDNA and damaged dsDNA and is involved in several DNA repair pathways, one would expect that RPA would form a wide variety of DNA damage-induced foci, only a subset of which would co-localize with these other markers. This suggests that RPA could be a more universal DNA damage marker than other proteins that focalize in response to DNA damage. These questions are currently under investigation.

Another question remaining is whether the transfer of RPA to DNA damage-induced intranuclear foci is responsible for the lack of sufficient RPA to support DNA replication. When cells are treated with 3 nM C-1027, it is estimated that ~60% of the total RPA is transferred to the extraction-resistant fraction, resulting in a concomitant decrease in the amount of RPA in the hypotonic cell lysates (Figure 2). A decrease of 60% of the RPA in these extracts would result in a substantial decrease in DNA replication activity. It has also been suggested that RPA might be inactivated in UV- or adozelesin-treated cells (*12, 13, 36*). If this is the case, loss of RPA function upon DNA damage could be due to more than one mechanism. The biochemical function of RPA purified from cells subjected to DNA damage is currently under investigation.

The available biochemical and cell staining data suggest a unique role for RPA in cellular responses to DNA damage. Extrapolating to the cellular level, we hypothesize that there is a large pool of RPA in normal cell nuclei that is available for DNA replication, recombination, or repair. Once the appropriate signals are triggered, this pool of available RPA is rapidly targeted to sites of DNA replication (at replication foci once S phase begins) or to DNA repair complexes (at sites of damaged DNA, which initiate foci when cells are subjected to DNA damage). The amount of RPA recruited to DNA replication foci is a small proportion of the intranuclear RPA population (*20*), suggesting that during S phase the majority of RPA is still available to form DNA damage-induced foci. However, DNA damage results in a much more substantial depletion of the pool of available RPA, which likely helps inhibit DNA replication, thereby assisting in S phase DNA damage checkpoint responses.

Whether DNA-PK is directly involved in cellular DNA damage-induced replication arrest remains unclear. We have demonstrated in this study that the induction of DNA-PK activity, and the resultant inhibition of SV40 DNA replication in extracts from cells treated with high levels of C-1027, can be directly attributed to the presence of DNA fragments in extracts from treated cells (Figures 4 and 5). And it has been shown that activated DNA-PK is capable of inhibiting SV40 DNA replication in cell-free assays (*11, 24*) (Figures 4 and 5). However, it has been suggested by Wang et al. (*11*) that the inhibition of SV40 DNA replication by DNA-PK may act through phosphorylation and inactivation of the viral replication protein, SV40 large T antigen. This preliminary result needs to be verified; further, it is unknown whether there may be a cellular replication protein whose function is also targeted by DNA-PK. It is currently believed that DNA-PK does not play a primary role in DNA damage checkpoint control (*42, 43*). However, it is possible that DNA-PK activation may play a secondary checkpoint role at higher levels of DNA damage. In addition, ATM and ATR kinases, both close relatives of DNA-PK, are directly involved in primary DNA damage checkpoint responses (*43*), and ATM, like DNA-PK, has been shown to be activated in response to DNA strand breaks (*44*). Therefore, whether or not DNA-PK is directly involved in the initial inhibition of cellular DNA replication in response to DNA damage, it may be involved in a secondary response at higher levels of DNA damage. Further, DNA-PK's effect on SV40 DNA replication may also prove to be an important model for cellular responses mediated through the DNA-PK-related kinases, ATM and ATR.

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