Adozelesin Triggers DNA Damage Response Pathways and Arrests SV40 DNA Replication through Replication Protein A Inactivation*

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Jen-Sing Liu‡§, Shu-Ru Kuo‡§, Mary M. McHugh§, Terry A. Beerman¶ and Thomas Melendy‡¶

From the ‡Department of Microbiology and the Center for Microbial Pathogenesis, State University of New York School of Medicine and Biomedical Sciences, Buffalo, New York 14214 and the ¶Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263

The cyclopropylpyrroloindole anti-cancer drug, adozelesin, binds to and alkylates DNA. Treatment of human cells with low levels of adozelesin results in potent inhibition of both cellular and simian virus 40 (SV40) DNA replication. Extracts were prepared from adozelesin-treated cells and shown to be deficient in their ability to support SV40 DNA replication in vitro. This effect on in vitro DNA replication was dependent on both the concentration of adozelesin used and the time of treatment but was not due to the presence of adozelesin in the in vitro assay. A dose of adozelesin treatment of cells was shown to result in the following: induction of p53 protein levels, hyperphosphorylation of replication protein A (RPA), and disruption of the p53-RPA complex (but not disruption of the RPA-cdc2 complex), indicating that adozelesin treatment triggers cellular DNA damage response pathways. Interestingly, in vitro DNA replication could be rescued in extracts from adozelesin-treated cells by the addition of exogenous RPA. Therefore, whereas adozelesin and other anti-cancer therapeutics trigger common DNA damage response markers, adozelesin causes DNA replication arrest through a unique mechanism. The S phase checkpoint response triggered by adozelesin acts by inactivating RPA in some function essential for SV40 DNA replication.

The cyclopropylpyrroloindole (CPI) drugs are a group of DNA sequence-specific minor groove binders that alkylate the N-3 of adenine at the 3' end of the binding sites. CPI drugs are currently in clinical trials for several types of solid tumors (1, 2). The CPI drug, adozelesin, carries a single cyclopropyl group and alkylates a single adenine (3–6). CPI adduct formation on naked DNA is able to block progression of DNA polymerases and helicases (7–9). Previous studies have shown that two different CPI drugs, adozelesin and bizelesin, inhibit both the initiation and elongation stages of cellular and viral DNA replication in cultured cells (10–12). However, the concentrations of these drugs required to cause S phase arrest are 2–4 orders of magnitude lower than levels of drug required to cause detectable adducts and to block polymerase or helicase progression. These results suggest that inhibition of DNA replication and cell cycle progression in cultured cells upon treatment with CPI drugs occurs via a trans-acting mechanism rather than by directly blocking DNA replication fork progression. The most likely explanation for this trans-inhibition of DNA replication is through cellular DNA damage response pathways or checkpoints.

Since both viral, simian virus 40 (SV40), and cellular DNA replication are inhibited at similar CPI levels, it is possible to use the more easily studied viral system to elucidate how CPI treatment results in DNA replication arrest. SV40 DNA replication is the most well studied model for eukaryotic DNA replication. An in vitro system was developed that requires only one viral protein, SV40 large T antigen, an exogenous plasmid DNA template containing the SV40 origin sequence, and primate or human cell extracts, to support SV40 DNA replication (13, 14). All the cellular DNA replication factors required to support SV40 DNA synthesis have been identified and purified (for review, see Refs. 15–18), and SV40 DNA replication can be reconstituted in vitro with these purified factors (19–22). The in vitro SV40 DNA replication system has been used by others to investigate the effects of DNA-damaging agents on SV40 DNA replication. Extracts from UV-irradiated cells have a reduced ability to support in vitro SV40 DNA replication. This defect can be complemented by the addition of purified human replication protein A (RPA) (23), strongly suggesting that RPA is at least one target of the cellular DNA damage response pathways. However, following treatment of cells by the anti-cancer drug camptothecin or by gamma irradiation, a trans-dominant negative factor was found to be present in treated cell extracts (24, 25). Addition of purified RPA to these cell extracts could not rescue in vitro SV40 DNA replication activity (25, 26). The different effects on SV40 DNA replication and the differing nature of the DNA-damaging agents have made it difficult to draw general conclusions about the mechanisms of DNA replication arrest following DNA damage.

RPA is a major eukaryotic single-strand DNA-binding protein required for DNA replication, repair, and recombination (for a review, see Ref. 27). Human RPA is a heterotrimer with subunits of 70, 32, and 14 kDa. The 70-kDa subunit (RPA70) carries the primary single-strand DNA binding domain, whereas the 32-kDa subunit of RPA (RPA32) has been shown to be phosphorylated during the cell cycle and hyperphosphorylated in response to DNA damage or apoptosis (23, 28–32). Several kinases phosphorylate RPA including cyclin-dependent kinases, DNA-dependent protein kinase, and ataxia telangiectasia-mutated protein (29, 31, 33–39). DNA-dependent protein kinase and ataxia telangiectasia-mutated protein have been implicated in RPA phosphorylation upon DNA damage (29, 31, 36, 37, 40). Hyperphosphorylation of RPA32 induced by camp-

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§ Both contributed equally to this work.
¶ To whom correspondence should be addressed: Dept. of Microbiology, School of Medicine & Biomedical Sciences, 138 Farber Hall, State University of New York, Buffalo, NY 14214-3000. Tel.: 716-829-3381; Fax: 716-829-2158; E-mail: tmelendy@buffalo.edu.
† The abbreviations used are: CPI, cyclopropylpyrroloindole; RPA, replication protein A; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis;
Adozelesin Arrests DNA Replication by RPA Inactivation

tohecin or gamma radiation can be either completely or partially blocked by the addition of the DNA polymerase inhibitor, aphidicolin. This suggests that replication fork passage through sites of damaged DNA may be required to induce RPA32 hyperphosphorylation (31). The hyperphosphorylation of RPA, and its reduced ability to support SV40 DNA replication in extracts from UV-irradiated cells, suggested a relationship between phosphorylation of RPA and arrest of DNA synthesis upon UV treatment (23). However, after extensive in vitro studies, a relationship between the hyperphosphorylation of RPA32 and the inhibition of RPA function in SV40 DNA replication has not been shown (37, 41, for a review see Wold (27)).

RPA may also function in DNA damage responses through binding to and inactivation of the p53 protein. Interaction of RPA with p53 inhibits the ability of p53 to bind to its DNA-binding site (42). Upon UV irradiation, the RPA-p53 interaction is disrupted (43), and p53 is free to activate downstream transcriptional targets, resulting in cell cycle arrest. This suggests that RPA may be playing roles in both the S phase and the G1 checkpoints.

In this study, adozelesin was used as a model agent to investigate the link between DNA damage and DNA replication inhibition at the molecular level. Our previous studies have shown that a trans-acting inhibitor of SV40 DNA replication is induced in cell extracts upon treatment of HELa cells with bizelesin (11). These results are similar to effects reported for camptothecin treatment and gamma radiation (24, 25). In this study we show that adozelesin-induced DNA replication inhibition occurs through RPA inactivation, similar to results previously reported for UV radiation and hyperthermia (23, 44). This adozelesin response is clearly different from those induced by bizelesin treatment, camptothecin treatment, or gamma radiation.

EXPERIMENTAL PROCEDURES

Chemicals—[α-32P]dATP was obtained from Amersham Pharmacia Biotech. Adozelesin was generously supplied by The Upjohn Co. Adozelesin solutions in dimethyl sulfoxide were diluted in dimethyl sulfoxide prior to addition to 293 suspension cell cultures. Camptothecin and aphidicolin were obtained from Sigma.

Cell Cultures and Antibodies—Human 293 cells (transformed embryonic kidney cells) were grown as suspension cultures in Joklik-modified minimal essential medium (Life Technologies, Inc.) containing 5% fetal bovine serum (45). Monoclonal antibodies against human p53 protein (DO-1) and p34-cdc2 were purchased from PharMingen and Stressgen Biotech, respectively. Monoclonal antibodies specific to the human RPA (Marsh Biomedical Products).

RESULTS

Cell-free SV40 DNA Replication Is Reduced in Extracts from Adozelesin-treated Cells—Extracts from adozelesin-treated cells were tested for the ability to support SV40 DNA replication in vitro. Human 293 cells cultured in suspension were treated with 0, 5, 20, or 100 nM adozelesin for 2 h. The cell number was identical in all four cultures at the time of harvest. Cell extracts were prepared as described under “Experimental Procedures” and used in cell-free SV40 DNA replication assays with exogenous SV40 origin-containing plasmid template and SV40 large T antigen. As shown in Fig. 1, SV40 DNA replication activity in extracts from adozelesin-treated cells was suppressed as compared with activity in mock-treated cell extracts (Fig. 1, A and B). The maximal level of inhibition (~85%) was obtained with extracts from cells treated with the highest level of adozelesin used, 100 nM (Fig. 1A, lanes 12–15). Treatment up to 1 μM adozelesin showed little additional effect (data not shown).

Although the plasmid template was not present during adozelesin treatment of the cells, it was possible that the inhibition of SV40 DNA replication could have been due to the presence of residual drug in the prepared cell extracts. Binding of the drug to the DNA template during the in vitro reaction could result in inhibition of DNA replication. To test this possibility, adozelesin was added to control cell extracts that were then used in vitro SV40 DNA replication reactions. We calculated
the theoretical maximum level of adozelesin likely to be present in the in vitro reactions to be 17 nM. Therefore adozelesin was added to mock-treated 293 cell extracts at 25–100 nM. These mixtures were then used in in vitro SV40 DNA replication reactions. The addition of adozelesin to in vitro DNA replication reactions at up to 50 nM had virtually no effect on DNA replication (Fig. 1C). This is equivalent to treatment of cells with nearly 300 nM adozelesin. These results clearly indicate that extracts from cells treated with up to 100 nM adozelesin do not produce an appreciable cis-effect on SV40 DNA replication due to adozelesin binding to the plasmid template. Therefore, the inhibition of SV40 DNA replication in vitro appears to occur through a trans-effect, in agreement with results from studies of DNA replication in cell culture (10).

Adozelesin Treatment Triggers Known Cellular DNA Damage Markers—Several of the most commonly used molecular markers for DNA damage in human cells were evaluated following adozelesin treatment of cultured cells. The first marker tested was induction of p53 levels. The addition of adozelesin to 100 nM did not alter p53 protein levels in 293 cells (Fig. 2A, substantially less than that seen with the drug-treated cell extracts and would correspond to treatment of cells with well over 0.5 μM adozelesin.

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**Fig. 1.** Inhibition of in vitro SV40 DNA replication in extracts from human 293 cells treated with adozelesin. Human 293 cell cultures were treated with 0, 5, 20, or 100 nM adozelesin for 2 h prior to harvesting the cells. Extracts were prepared, protein levels normalized, and various amounts of each extract (as indicated) were used as the cellular extract source in in vitro SV40 DNA replication reactions. DNA products synthesized in these reactions were extracted and subjected to agarose gel electrophoresis. The gels were dried and analyzed using a Bio-Rad PhosphorImager. The electrophoretic migration patterns of the products are shown in A. B shows the quantitation of the relative amount of DNA synthesized (32P incorporation expressed as the total number of pixels per lane × 10^3) in each reaction (including both the θ form DNA replication intermediates (R.I.) and the completely replicated plasmids (from Form I through Form II)). C shows the electrophoretic migration pattern of DNA products synthesized in SV40 DNA replication reactions carried out with control cell extracts and exogenous adozelesin added directly to the reactions to the levels indicated.

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2 This calculation was based on an assumption of complete diffusion of adozelesin into the cells during the 2 h of drug treatment. The maximum level of adozelesin used to treat the cell cultures, 100 nM, was divided by the dilution factor of 2.4, which was due to the dilution of the total harvested cell pellet volume into the volume of lysis buffer used for hypotonic lysate extract preparation. Since a maximum volume of 4 μL of extract was used in each 10-μL DNA replication reaction, this result was further divided by a factor of 2.5, generating the predicted maximum level ofadozelesin in the DNA replication reactions at 17 nM.

3 Addition of higher levels of exogenous adozelesin (100 nM) did inhibit in vitro DNA replication slightly. However, this inhibition was

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lanes 3 and 4). However, human 293 cells are transformed by adenovirus E1A and E1B, which results in very high constitutive levels of transcriptionally inactive p53 protein (Fig. 2A, lane 3) (51, 52). Therefore primary cells were used to evaluate this marker. When primary human foreskin fibroblasts were treated with 100 nM adozelesin, p53 levels were substantially induced (Fig. 2A, lanes 1 and 2).

Following DNA damage, increased levels of p53 have been shown to lead to transcriptional activation of the p21Waf1/Cip1 promoter and increased levels of p21Waf1/Cip1 (an inhibitor of cyclin kinases that mediate G1 arrest) (for review see Ref. 53). p21Waf1/Cip1 levels are extremely low in 293 cells, likely due to the inactive nature of p53 in this transformed cell type (54). As anticipated from the inactive nature of p53 in 293 cells, no induction of p21Waf1/Cip1 levels was detected in 293 cells following adozelesin treatment (data not shown).

Another cellular marker for activation of the DNA damage response pathways, hyperphosphorylation of the 32-kDa subunit of RPA (RPA32), was also evaluated in adozelesin-treated cells. A monoclonal antibody specific to RPA32 was used for detection of RPA32 on immunoblots of adozelesin-treated cells. A population of slower-migrating hyperphosphorylated RPA32 (Pi-RPA32) was seen in both primary fibroblasts and 293 cells treated with 100 nM adozelesin (Fig. 2A, lanes 2 and 4) but was not seen in mock-treated cells (Fig. 2A, lanes 1 and 3). To confirm that the altered migration is due to phosphorylation, the slower migrating form was shown to be completely converted to the faster-migrating, hypophosphorylated form (RPA32) by treatment with calf intestine alkaline phosphatase (data not shown). Quantitative immunoblotting, using both RPA32 and RPA70 monoclonal antibodies, demonstrated that overall RPA protein levels varied by less than 10% between mock- and adozelesin-treated cells (Fig. 2A and data not shown).

The RPA-p53 association was also investigated following adozelesin treatment. It has been previously shown that p53 is co-immunoprecipitated with RPA from human cell lines and that pretreatment of cells with UV light dissociates this RPA-p53 complex (43). Monoclonal antibody against RPA32 was used to immunoprecipitate RPA from mock-treated 293 cell extracts as described under “Experimental Procedures.” The precipitates were then subjected to immunoblotting. As previously published for other cell types, p53 was co-immunoprecipitated with RPA from 293 cell extracts (Fig. 2B, lane 5). When RPA was immunoprecipitated from 100 nM adozelesin-treated 293 cell extracts, the levels of co-immunoprecipitated p53 were dramatically reduced as compared with the control cell extracts (Fig. 2B, lane 6). As a control, we analyzed whether p34Cdc2, which is known to phosphorylate RPA, was also co-immunoprecipitated with RPA from cell extracts. p34Cdc2 was found to co-immunoprecipitate with RPA at similar levels from both adozelesin- and mock-treated cell extracts (Fig. 2B, lanes 5 and 6). It should be noted that the levels of both p53 and RPA remain similar in mock- and adozelesin-treated 293 cell extracts (Fig. 2A).

Aphidicolin Effects on Adozelesin-induced RPA Hyperphosphorylation—Pretreatment of cells with aphidicolin, a DNA polymerase inhibitor, has been shown to prevent the hyperphosphorylation of RPA32 following treatment of cells with the anti-tumor drug, camptothecin (31). Therefore, the effects of aphidicolin on adozelesin-induced RPA32 hyperphosphorylation were examined and compared with those of camptothecin. As previously shown for other cell lines (31), treatment of 293 cells with 1 μM camptothecin for 2 h results in the hyperphosphorylation of RPA32 (Fig. 3, lane 3). Also as previously reported, this camptothecin-induced hyperphosphorylation of RPA32 can be blocked by pretreatment of the cells with 2.5 μM aphidicolin (Fig. 3, lane 4) (31). In a similar fashion, aphidicolin pretreatment blocks the hyperphosphorylation of RPA32 induced by 20 nM adozelesin (Fig. 3, lanes 5 and 6). However, the efficiency of aphidicolin blocking of RPA32 hyperphosphorylation is reduced when very high concentrations of adozelesin (100 nM or above) are used (Fig. 3, lanes 7–12).

Lack of a trans-Acting DNA Replication Inhibitor in Adozelesin-treated Cell Extracts—Adozelesin-treated cell extracts were evaluated for the presence of a trans-acting DNA replication inhibitor, which is characteristic of DNA replication inhibition in gamma radiation-, biselzin-, or camptothecin-treated cells (11, 24, 25). Extracts were prepared from 293 cells treated with 100 nM adozelesin for 2 h. The indicated amounts of adozelesin-treated cell extract were mixed with a fixed amount (40 μg) of control cell extract, and the extract mixtures were used to carry out in vitro SV40 DNA replication. Addition of 10–30 μg of mock-treated cell extract to the control cell extract resulted in increases in DNA replication (Fig. 4, open triangles). The addition of 10–30 μg of 100 nM adozelesin-treated cell extract, which can support little DNA synthesis on its own (Fig. 1), also increased DNA replication activity slightly, although to a much lesser degree than the addition of the same amounts of mock-treated cell extracts (Fig. 4, open circles). If the inhibition of DNA replication in adozelesin-treated cell extracts is primarily due to a trans-acting DNA replication inhibitor, the addition of drug-treated cell extract would have a negative effect on DNA synthesis by the control cell extract, resulting in DNA synthesis levels below that of the base-line reaction (100%). This has been shown to be the case for treatment of cells with other DNA-damaging agents (11, 24, 25) but is clearly not the case for adozelesin treatment (Fig. 4). Therefore, these results show that the 100 nM adozelesin-treated cell extracts do not contain a potent trans-inhibitor of DNA repli-
Adozelesin treatment induces a trans-acting inhibitor of DNA replication (11), addition of RPA cannot rescue SV40 DNA replication activity in bizelesin-treated cell extracts (data not shown). SV40 DNA replication assays were also carried out with extracts from cells treated with adozesin for extended periods. Following treatment of 293 cells with 0, 1, or 20 nM adozesin for up to 16 h, the cell number was found to vary by less than 10%. Extracts were prepared from mock- and adozesin-treated 293 cell cultures treated for the indicated times. Protein levels for all extracts were normalized as described under “Experimental Procedures,” and the extracts were used in in vitro DNA replication assays. For 20 nM adozesin treatment, longer drug treatment resulted in stronger inhibition of DNA synthesis (Fig. 6A). The level of inhibition of DNA replication varied from 15% at 1 h to more than 85% at 16 h. At all time points SV40 DNA replication activity could be fully restored by the addition of exogenous RPA (Fig. 6A). These results indicate that RPA is the primary target for adozesin-induced DNA replication arrest, even after prolonged drug treatment. No evidence of a trans-acting DNA replication inhibitor was seen in 20 nM adozesin-treated cells even after 16 h of treatment.

Immunoblot analysis of RPA32 in cells treated with either 1 or 20 nM adozesin for various lengths of time showed that hyperphosphorylated RPA32 was not detectable following a single hour of drug treatment. However, hyperphosphorylated RPA32 was detected following 2 h of treatment with 20 nM adozesin and 4 h of treatment with 1 nM adozesin (Fig. 6B). It should be noted that cells treated with 1 nM adozesin for 4 h showed a high level of hyperphosphorylated RPA32 (Fig. 6B); however, extracts from these cells were inhibited in their ability to support SV40 DNA replication by less than 30% (data not shown). These results suggest that hyperphosphorylation of RPA32 may not precisely correlate with inhibition of DNA replication.

**DISCUSSION**

The studies presented here demonstrate that at concentrations lower than 100 nM, adozesin inhibits DNA replication through inactivation of RPA. This effect appears similar to that previously described for UV irradiation and hyperthermia (23, 44). Addition of purified RPA protein is both necessary and sufficient to rescue SV40 DNA replication activity in extracts from heat-, UV-, or adozesin-treated cells (Figs. 5 and 6) (23, 44). This mechanism of DNA replication inhibition is different than that shown for other DNA-damaging chemotherapeutics. When bizelesin, camptothecin, or gamma radiation are used to treat cells, a trans-acting inhibitor is found in cell extracts. Addition of RPA into extracts from bizelesin- or camptothecin-treated cells cannot rescue SV40 DNA replication activity (data not shown and Refs. 11, 24, and 25). The presence of trans-inhibitors makes it difficult to evaluate whether RPA has also been inactivated in extracts from cells treated with bizelesin, camptothecin, or gamma radiation. One recent report indicates that treatment of cells with camptothecin both induces a trans-acting inhibitor (which appears to be the DNA-dependent protein kinase acting on SV40 T antigen) and results in a 50% decrease in levels of RPA (26). However, this report gave no
Adozelesin Arrests DNA Replication by RPA Inactivation

**FIG. 5.** RPA, but not RFC or PCNA, can rescue SV40 DNA replication in extracts from adozelesin-treated 293 cells. A, human 293 cells were treated with 0 or 20 nM adozelesin (as indicated) for 2 h prior to harvesting. Extracts were prepared and 40 μg of protein from each were used in *in vitro* SV40 DNA replication reactions (lanes 1 and 6). Highly purified DNA replication proteins were added to the mock-(lane 1) or 20 nM adozelesin-treated (lane 6) cell extracts, respectively, and used to carry out *in vitro* SV40 DNA replication reactions (RPA to 50 ng/μl final, lanes 2 and 7; PCNA to 16 ng/μl final, lanes 3 and 8; RFC to 0.4 ng/μl final, lanes 4 and 9; or a combination of all three at the indicated concentrations, lanes 5 and 10). Replication products were isolated and analyzed as in Fig. 1B. Quantitation of replication products shown in A are as described in Fig. 1.

**FIG. 6.** RPA can rescue SV40 DNA replication in extracts from cells treated with adozelesin for extended periods. A, human 293 cells were treated with 0 or 20 nM adozelesin as indicated for various times prior to harvesting. Extracts were prepared, and 40 μg of protein from each were used in *in vitro* SV40 DNA replication reactions as indicated. RPA was added to both the mock-treated (control) and 20 nM adozelesin-treated cell extracts to 50 ng/μl final, and the mixtures were used to carry out *in vitro* SV40 DNA replication. Levels of DNA synthesis were evaluated as described in Fig. 4B. Human 293 cells were treated with 0, 1, or 20 nM adozelesin (as indicated) for various times. Cells were lysed and lysates were subjected to SDS-PAGE and immunoblotted for RPA32 as in Fig. 2. The hypo-(RPA32) and hyperphosphorylated (P-RPA32) forms of RPA32 are indicated.

An indication that camptothecin treatment resulted in RPA inactivation. Decreased levels of RPA were not seen following treatment of cells with either adozelesin or bizelesin (Fig. 2 and data not shown).

Other potential mechanisms have been proposed for inhibition of SV40 DNA replication following DNA damage. These include the following: induction of p53 levels (which can inhibit the function of SV40 T antigen (55–57)) and induction of p21cip/Waf1 levels (which can inhibit PCNA function (58, 59)). However, since levels of neither p53 nor p21cip/Waf1 are induced in 293 cells following adozelesin treatment (Fig. 2), and since the addition of RPA alone rescues SV40 DNA replication activity in drug-treated cell extracts (Figs. 5 and 6), these postulated mechanisms appear to play no role in the inhibition of DNA replication upon treatment with adozelesin.

Consistent with the findings of others (37, 41), our results suggest that the hyperphosphorylation of RPA32 may not correlate with the inactivation of RPA. Less than 30% of the RPA32 in 20 nM adozelesin-treated cell extracts is hyperphosphorylated, whereas SV40 DNA replication activity is inhibited by more than 80% (see Fig. 6). Although it is possible that a small amount of modified RPA might act in a dominant manner to inhibit *in vitro* DNA replication, if this were the case then the extract-mixing experiments should show the presence of a trans-inhibitor, which they clearly do not (Fig. 4). Furthermore, treatment of cells with 1 nM adozelesin for 4 h induces levels of RPA32 hyperphosphorylation similar to those seen in cells treated with 20 nM adozelesin but results in far less inhibition of *in vitro* DNA replication (30% inhibition compared with 75% inhibition) (Fig. 6 and data not shown).

Currently, the mechanism of RPA inactivation remains unknown. Although hyperphosphorylation of RPA32 does not appear to correlate to RPA inactivation, it is possible that inactivation of RPA may be mediated by phosphorylation at a specific amino acid residue. Phosphorylation at this single site would not necessarily correlate with RPA32 hyperphosphorylation. Alternatively, inactivation may be regulated by some other type of modification, such as acetylation. Acetylation of p53 by p300 and PCAF is induced by UV or gamma radiation and enhances the DNA binding activity of p53 (60, 61). The association of RPA with p53 (43), the simultaneous phosphorylation of these two proteins upon DNA damage (29, 31, 36, 37, 40), the DNA damage-dependent acetylation of p53 (61), and the presence of an acetylation consensus site in RPA14 (XGKGXG) (62) suggest that RPA might also be acetylated following DNA damage. The mechanism of RPA inactivation is currently under investigation.

It was recently shown that RPA32 hyperphosphorylation induced by camptothecin or gamma radiation can be blocked by pretreatment of the cells with aphidicolin (31). We have found that this is also true for bizelesin.4 Since aphidicolin is a DNA polymerase (α, δ, and ε) inhibitor, it was suggested that replication fork passage through a camptothecin-DNA adduct may be required to generate recognizable DNA damage (31). Hyperphosphorylation of RPA32 induced by 20 nM adozelesin can also be blocked by treatment with aphidicolin, suggesting that replication fork passage through an adozelesin-DNA adduct may also be required to generate recognizable DNA damage (31).

**FIG. 7.** A, Percentage of SV40 DNA replication in cell-free extracts from human 293 cells treated with 0 nM (lane 1), 2 nM (lane 2), 10 nM (lane 3), or 20 nM (lane 4) adozelesin for 10 min, followed by 40 μg of protein from each were used in *in vitro* SV40 DNA replication reactions as described in Fig. 6. Quantitation of replication products shown in A are as described in Fig. 1B. B, Percentage of SV40 DNA replication activity in cell-free extracts from human 293 cells treated with 0 nM (lane 1), 2 nM (lane 2), 10 nM (lane 3), or 20 nM (lane 4) adozelesin for 10 min, followed by 40 μg of protein from each were used in *in vitro* SV40 DNA replication reactions as described in Fig. 6.

4 S.-R. Kuo, J.-S. Liu, M. M. McHugh, T. A. Beerman, and T. Melendy, manuscript in preparation.
adducts, such that DNA repair fork collisions with adozelesin-DNA adducts are more likely. This model predicts that RPA32 hyperphosphorylation would be triggered differently at different levels of drug. Low levels of drug would induce an aphidicolin-sensitive RPA hyperphosphorylation, whereas high levels of drug would trigger an aphidicolin-insensitive RPA hyperphosphorylation. This model is supported by the fact that Colin-sensitive RPA hyperphosphorylation, whereas high levels of DNA adducts are more likely. This model predicts that RPA32 adducts, such that DNA repair fork collisions with adozelesin.

Adozelesin Arrests DNA Replication by RPA Inactivation

Many anti-cancer chemotherapeutics act by inhibiting cell cycle progression. Although much has been learned in recent years about how G1 arrest is induced, relatively little is known about how the S phase checkpoint(s) act to block DNA replication. Elucidation of these S phase checkpoints, how they are triggered, and how they act to block DNA synthesis should prove helpful for both improved drug design and optimal selection of chemotherapeutic agents for specific types of cancer.

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REFERENCES

1. Cristofanilli, M., Bryan, W. J., Miller, L. L., Chang, A. Y., Gradishar, W. J., Kufe, D. W., and Hortobagyi, G. N. (1998) Anti-Cancer Drugs 9, 779–782
2. Von Hoff, D. D. (1998) Semin. Oncol. 25, (Suppl. 11) 47–52
3. Wymanowski, J. M., McHugh, M. M., Gawron, L. S., and Beerman, T. A. (1995) Biochem. Biophys. Acta 1294–1305
4. Wymanowski, J. M., Chapman, W. G., Napier, C., and Herzig, M. C. (1999) Biochem. Biophys. Acta 1444, 201–217
5. Mitchell, M. A., Kelly, R. C., Wicnienski, N. A., Hatzenbuhler, N. T., Williams, M. G., Petrilli, G. L., Slightom, J. L., and Siemienak, D. R. (1991) J. Am. Chem. Soc. 113, 8884–8886
6. Hurley, L. H., Warpehoski, M. A., Lee, C.-S., McGovern, J. P., Schall, T. A., Kelly, R. C., Mitchell, M. A., Wicnienski, N. A., Gebhard, I., Jihnson, P. D., and Bradford, V. S. (1991) J. Am. Chem. Soc. 113, 4633–4640
7. Sun, D., and Hurley, L. H. (1992) Biochemistry 31, 2822–2829
8. Sun, D., and Hurley, L. H. (1992) J. Med. Chem. 35, 1773–1782
9. Maine, I. P., Sun, D., Hurley, L. H., and Kodadek, T. (1992) Biochemistry 31, 3968–3975
10. Cobuzzi, R. J., Jr., Burhans, W. C., and Beerman, T. A. (1996) J. Biol. Chem. 271, 18952–18959
11. McHugh, M. M., Kuo, S.-R., Walsh-O’Beirne, M. H., Liu, J.-S., Melody, T., and Beerman, T. A. (1999) Biochemistry 38, 11508–11515
12. Wymanowski, J. M., and Beerman, T. A. (1997) Biochem. Biophys. Acta 1353, 50–60
13. Li, J. J., and Kelly, T. J. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6973–6977
14. Stillman, B. W., and Gluzman, Y. (1985) Mol. Cell. Biol. 5, 2051–2060
15. Brush, G. S., Kelly, T. J., and Stillman, B. (1985) Methods Enzymol. 262, 562–568
16. Hurwitz, J., Dean, F. B., Kwong, A. D., and Lee, S. H. (1990) J. Biol. Chem. 265, 18043–18046
17. Melody, T., and Stillman, B. (1992) Nucleic Acids Mol. Biol. 6, 129–158
18. Waga, S., and Stillman, B. (1998) Annu. Rev. Biochem. 67, 721–751
19. Waga, S., Bauer, G., and Stillman, B. (1994) J. Biol. Chem. 269, 10923–10934
20. Tourimoto, T., Melody, T., and Stillman, B. (1990) Nature 346, 534–539
21. Eki, T., Matsumoto, T., Murakami, Y., and Hurwitz, J. (1992) J. Biol. Chem. 267, 7284–7294
22. Weinberg, D. H., Collins, K. L., Simanek, P., Russo, A., Wold, M. S., Virshup, D. M., and Kelly, T. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8692–8696