The Cellular Response to DNA Damage Induced by the Enediynes C-1027 and Neocarzinostatin Includes Hyperphosphorylation and Increased Nuclear Retention of Replication Protein A (RPA) and Trans Inhibition of DNA Replication†

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ABSTRACT: This study examined the cellular response to DNA damage induced by antitumor enediynes C-1027 and neocarzinostatin. Treatment of cells with either agent induced hyperphosphorylation of RPA32, the middle subunit of replication protein A, and increased nuclear retention of RPA. Nearly all of the RPA32 that was not readily extractable from the nucleus was hyperphosphorylated, compared to ≤50% of the soluble RPA. Enediyne concentrations that induced RPA32 hyperphosphorylation also decreased cell-free SV40 DNA replication competence in extracts of treated cells. This decrease did not result from damage to the DNA template, indicating trans-acting inhibition of DNA replication. Enediyne-induced RPA hyperphosphorylation was unaffected by the replication elongation inhibitor aphidicolin, suggesting that the cellular response to enediyne DNA damage was not dependent on elongation of replicating DNA. Neither recovery of replication competence nor reversal of RPA effects occurred when treated cells were further incubated in the absence of drug. C-1027 and neocarzinostatin doses that caused similar levels of DNA damage resulted in equivalent increases in RPA32 hyperphosphorylation and RPA nuclear retention and decreases in replication activity, suggesting a common response to enediyne-induced DNA damage. By contrast, DNA damage induced by C-1027 was at least 5-fold more cytotoxic than that induced by neocarzinostatin.

Enediyne drugs are highly cytotoxic protein antitumor antibiotics containing a reactive chromophore which binds in the minor groove of DNA (*1*, *2*). The cytotoxicity of enediyne drugs is directly correlated with chromophore-induced single- or double-strand DNA damage (*3*) which occurs via formation of a benzenoid diradical.

Despite their common dependence on a chromophore for DNA damage, enediynes exhibit striking differences. For example, the enediyne C-1027, which induces primarily double-stranded DNA damage (*4*, *5*), has significant cytotoxicity in the picomolar range (*6*, *7*). By contrast, primarily single-strand DNA damage is induced by the enediyne neocarzinostatin (*8*) which is 3 orders of magnitude less cytotoxic than C-1027 (*9*). C-1027 and neocarzinostatin also differ in the chemical structure of the activated chromophore (*10*) and in its nucleotide base preference (*11*). Despite these differences, within minutes of treatment with cytotoxic doses of either C-1027 or neocarzinostatin, both DNA synthesis and progression through the S phase are decreased.

Recently, we showed that C-1027 inhibited intracellular replication of SV40 DNA at drug levels that induced less than one lesion per viral DNA molecule (*12*). That the presence of a DNA lesion was not crucial for reduced replicative synthesis suggested a trans mechanism of C-1027 inhibition. Trans inhibition of replication can occur by induction of an inhibitor or by reduction in the amount or activity of essential replication factors. DNA damaging agents which induce an inhibitor of cell-free SV40 DNA replication include X irradiation (*13*), the topoisomerase I inhibitor camptothecin (*13*), and the DNA alkylator bizelesin (*14*). In contrast, reduction in the amount of an essential replication factor rather than induction of an inhibitor is observed after treatment with adozelesin, a DNA alkylator similar to bizelesin (*15*). To date, trans inhibitory effects on replication have not been reported for neocarzinostatin.

One essential replication factor that has been associated with the cellular trans response to DNA damage is the heterotrimeric replication protein A (RPA) (*16*). Damage to cellular DNA by X irradiation, camptothecin (*13*), and the DNA alkylator bizelesin (*15*) result in hyperphosphorylation of the middle subunit of RPA (RPA32), possibly via kinases such as DNA-PK and ATM (*17*). Addition of exogenous RPA failed to restore replication activity in cell-free studies of these agents, indicating that additional factors were involved in inhibiting replication activity. By contrast, decreased functional levels of RPA alone may account for

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the reduced replication activity observed when RPA hyperphosphorylation results from UV irradiation (*18*, *19*) or from treatment with the DNA alkylator adozelesin (*15*), since replication activity can be restored with exogenous RPA.

This study examined the relationship between DNA damage and inhibition of DNA replication induced by the enediynes C-1027 and neocarzinostatin. A cell-free SV40 DNA replication assay was used to examine whether enediynes inhibited replication competence in extracts of treated cells in a trans-acting manner. The role of phosphorylation and intracellular localization of RPA32 in enediyne-induced decreased replication competence was examined by Western blotting. The extent of enediyne-induced genomic DNA damage was quantitated to identify threshold levels of strand breaks necessary to reduce replication competence.

EXPERIMENTAL PROCEDURES

Chemicals. C-1027, a generous gift from Taiho Pharmaceutical Co., Ltd. (Saitama, Japan), was adjusted to 2 mg/mL in H_2O. Neocarzinostatin was obtained from Bristol-Myers Co. (Syracuse, NY) as a 2 mg/mL solution in 15 mM sodium acetate. Both stock solutions were stored at −20 °C. [2-^{14}C]Thymidine (55 mCi/mmol) was from Moravek Biochemicals, Inc. (Brea, CA). [α-^{32}P]dATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Aphidicolin from Sigma-Aldrich (St. Louis, MO) was diluted in ethanol and stored at −20 °C. All other chemicals were of reagent grade.

Cell Culture and Antibodies. Human 293 cells (adenovirus 5 DNA-transformed embryonic kidney cells) were maintained in monolayer culture in DMEM with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2. Monoclonal antibodies against human RPA-70 and RPA-32 have been described previously (*20*).

Plasmids and Proteins. SV40 large T-antigen was purified from recombinant baculovirus-infected High-Five insect cells (Invitrogen) using immunoaffinity chromatography (*21*). SV40 origin-containing plasmid pSV011 has been described elsewhere (*22*).

Cytotoxicity Assays. Cells (3×10^5) were seeded in 60 mm plates. After 2 days, growth medium was replaced with 1.0 mL of medium containing C-1027 (0.1−100 pM) or neocarzinostatin (0.1−20 nM). After 2 h at 37 °C, cells were rinsed with warm phosphate-buffered saline (PBS), trypsinized, and resuspended in fresh medium. Cells were seeded at 500, 1000, or 3000 per 60 mm plate and incubated at 37 °C for 12 days. Plates were stained with 1% methylene blue and colonies counted. The number of colonies in plates containing drug-treated cells was compared to that in control plates containing nondrug-treated cells, and results were expressed as percent inhibition of colony formation.

Detection of Genomic DNA Damage. The procedure for assaying genomic DNA damage was described previously (*23*). Briefly, 293 cells (1×10^6/100 mm plate) were radiolabeled for 48 h with [2-^{14}C]thymidine (0.0125 μCi/mL) and then treated with C-1027 or neocarzinostatin for an additional 2 h. X-ray treatment of nondrug-treated cells was used as a positive control for DNA double-strand breaks. Drug-treated or irradiated cells were harvested and washed once in PBS. Cells were resuspended in PBS, and low gelling temperature agarose was added to a final concentration of 0.66%. Seven microliters of lysis buffer (1% Sarcosyl, 0.5 M EDTA, pH 8.0, 10 mg/mL proteinase K) was added to 21 μL of the cell−agarose mixture, and samples were incubated at 55 °C for 2 h. After incubation, samples were spun briefly and placed at 4 °C to allow a hardened agarose plug to form. Plugs were stored overnight in 0.5 mL of TE before loading on a 0.8% agarose gel. After pulsed-field gel electrophoresis for 90 h at 64 V with a 35 min pulse between field changes, gels were dried and exposed to a phosphorimager screen for detection of ^{14}C-radiolabeled DNA. The image was scanned, and lanes were quantitated using a Molecular Dynamics phosphorimager and ImageQuant software.

Cell-Free DNA Cleavage Assay. Drug-induced DNA breaks in the superhelical pSV011 plasmid DNA were measured using a topological form conversion assay. One hundred nanograms of DNA was incubated with C-1027 or neocarzinostatin in 10 mM Tris, pH 7.5, for 15 min at 37 °C. Dithiothreitol (0.5 mM) was included in the neocarzinostatin reaction buffer as an activating agent. After addition of 1% sodium dodecyl sulfate (SDS) to terminate the reaction, samples were electrophoresed on a 0.8% agarose gel. Gels were stained with ethidium bromide and analyzed using an Alpha Innotech Corp. (San Leandro, CA) Chemi-Imager.

Preparation of Cell Extracts for Cell-Free Replication Assays. Mid-log phase growing cells were seeded at 3.8×10^6/100 mm plate and grown overnight before treatment with C-1027 or neocarzinostatin. After 2 h at 37 °C, cells were harvested and extracts prepared as described previously (*14*). All steps were performed at 4 °C. Briefly, after one wash each in PBS and hypotonic buffer, cells were incubated in hypotonic buffer for 10 min and then lysed by seven passages through a 25-gauge needle. Samples were placed on ice for 30 min and centrifuged at 9000*g* for 10 min. The supernatants (soluble extracts) were removed, quick frozen in dry ice−propanol, and stored at −80° C until use. The pellets were washed twice in cold PBS, and 2 × SDS loading buffer (*24*) was added to each pellet to obtain the extraction-resistant nuclear fraction. Protein concentrations of the extracts were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA).

Cell-Free SV40 DNA Replication Assays. Cell-free replication assays were carried out as described previously (*14*, *15*) in a total volume of 10 μL. Thirty nanograms of SV40 origin containing plasmid pSV011, 500 ng of T-antigen, and 40 μg of soluble extract protein from control (no drug treatment) or enediyne-treated cells (or as indicated in the figure legends) were combined with replication assay buffer (4 mM ATP, 0.2 mM CTP, GTP, and UTP, 0.1 mM dCTP, dGTP, and dTTP, 0.025 mM dATP, 7.0 mM $MgCl_2$, 0.024 unit of creatine phosphokinase, 40 mM phosphocreatine, and 2 μCi of [α-^{32}P]dATP) and incubated at 37 °C for 60 min. Total incorporation of ^{32}P was determined by spotting samples on Whatman DE-81 paper filters and washing the filters. For analysis of DNA products, samples were separated by electrophoresis on a 0.8% (w/v) agarose gel in 1 × TAE.

Western Blots. Extracts prepared from control and treated cells as described above were electrophoresed on 10% SDS−PAGE and transferred to Hybond-P membrane [Amersham Pharmacia Biotech, Inc. (Piscataway, NJ)]. Membranes were

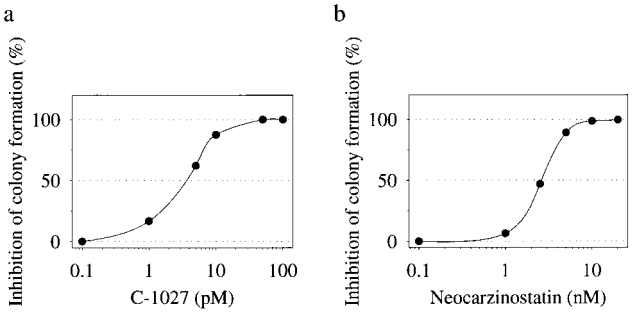


FIGURE 1: Cytotoxicity of C-1027 and neocarzinostatin. 293 cells were treated with the indicated concentrations of C-1027 (a) or neocarzinostatin (b). After 2 h, cells were washed in PBS, trypsinized, reseeded, and incubated at 37 °C for 12 days when colonies were counted. Data are the average of two independent experiments.

first probed with monoclonal antibodies against human RPA-70 and RPA-32 and then with peroxidase-conjugated anti-mouse IgG antibody. Blots were developed using the chemiluminescent ECL kit (Amersham Pharmacia Biotech).

RESULTS

Cytotoxicity of C-1027 and Neocarzinostatin in 293 Cells. This study investigates the cellular response to DNA damage induced by enediynes. Since response to DNA damage would likely be dependent on cytotoxic activity, it was necessary to quantitate C-1027- and neocarzinostatin-induced cytotoxicity in 293 cells (*7*, *25*, *26*). The cytotoxic effects of C-1027 and neocarzinostatin were assayed by colony formation. A 50% decrease in colony formation was observed 12 days after treatment with 3.6 pM C-1027 or 2.6 nM neocarzinostatin (see Figure 1). Thus, C-1027 was more than 700-fold more cytotoxic to 293 cells than was neocarzinostatin.

Genomic DNA Damage by C-1027 and Neocarzinostatin. To study the relationship between DNA damage and cellular responses that affect DNA replication, it was necessary to quantitate enediyne-induced intracellular DNA damage. Induction of genomic DNA double-strand damage was measured by pulsed-field gel electrophoresis.

Figure 2a is a representative phosphorimage of ^{14}C-radiolabeled DNA isolated from cells treated with increasing doses of X-ray, C-1027, or neocarzinostatin. The amount of DNA remaining in the well after pulsed-field gel electrophoresis was used to quantitate the extent of DNA double-strand damage (*23*). In the controls (lanes 1 and 7), the majority of radiolabel remained within the well. However, increased migration of ^{14}C-radiolabeled DNA into the gel was apparent with 0.5−5 nM C-1027 (lanes 8−10) and 0.5−2 μM neocarzinostatin (lanes 11−13). DNA from cells X-irradiated with 2−120 Gy (lanes 1−6) was used to compare enediynes with an agent that produces a predictable amount of DNA damage

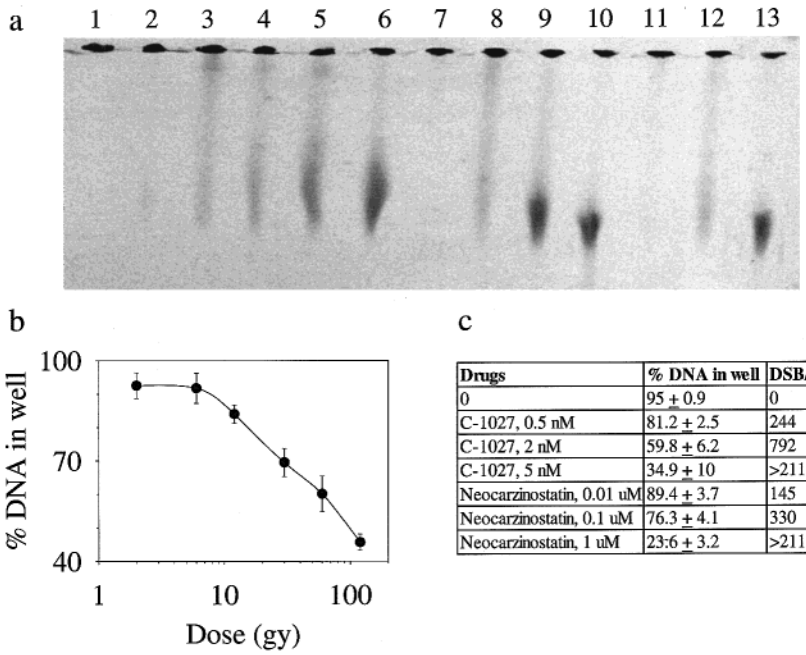
Figure 2b is a graphic representation of data from multiple pulse-field gels showing that the fraction of ^{14}C-radiolabeled DNA remaining in the well decreased as the X-ray dose increased. In control samples (no drug or X-ray treatment), the amount of DNA remaining in the well was typically 90−95% of the total ^{14}C signal. In samples subjected to X irradiation, a slight decrease in ^{14}C-radiolabeled DNA in the well was observed with 7 Gy and a 50% loss with 90 Gy.

The percent of DNA remaining in the well after C-1027 or neocarzinostatin treatment also decreased in a dose-dependent manner (Figure 2c). Double-strand DNA breaks (DSB) per cell were estimated by comparing the loss of DNA from the well in C-1027- or neocarzinostatin-treated samples with that observed after X irradiation. Similar levels of DNA damage were induced by 0.5 nM C-1027 and 0.1 μM neocarzinostatin (244 and 330 double-strand breaks per cell, respectively).

If double-strand DNA damage is assumed to be linear, the formation of double-strand breaks at cytotoxic doses of C-1027 and neocarzinostatin can be calculated from the data in Figures 1 and 2. At doses necessary to reduce colony formation by 50% (i.e., 3.6 pM C-1027 or 2.6 nM neocarzinostatin), the number of double-strand breaks induced per cell was 1.4−1.8 for C-1027 (calculated from DSB induced by 0.5 and 2 nM C-1027) and 8.6−37.7 for neocarzinostatin (calculated from DSB induced by 0.1 and 1.0 μM neocarzinostatin). Thus, at least five times more neocarzinostatin- than C-1027- induced double-strand breaks were necessary to cause cytotoxicity.

Reduced Cell-Free DNA Replication Competence in Soluble Extracts of Drug-Treated Cells. To examine whether damage to cellular DNA by enediynes leads to a loss of replication competence, the replication activity of soluble extracts of enediyne-treated 293 cells was determined using a cell-free SV40 DNA replication assay that has been described elsewhere (*27*, *28*). Superhelical pSV011 plasmid DNA which contains an SV40 origin fragment (*22*) was used as a template. Cells were treated for 2 h at 37 °C with C-1027 or neocarzinostatin, and soluble extracts were prepared. Figure 3a is a phosphorimage of a representative agarose gel after electrophoresis of cell-free DNA replication reactions. Twenty, thirty, or forty micrograms of total protein from each untreated control or C-1027-treated cell extract was used in replication assays. A slight decrease in total ^{32}P incorporation into newly replicated DNA was observed with as little as 0.5 nM C-1027 at all three protein levels. At 2.0 and 5.0 nM C-1027, the radioactive signal was reduced dramatically compared to the control (0) samples. Thus, C-1027 treatment of intact cells reduced in a dose-dependent manner the ability of cell extracts to replicate SV40 DNA.

A graphic representation of reduced replication activity in extracts from cells treated with C-1027 is shown in Figure 3b. When the amount of protein extract was increased from 20 to 40 μg, the replication activity increased in extracts from untreated (control) and 0.5 and 2.0 nM C-1027-treated cells, although drug-treated samples remained less active than control samples. A similar pattern of replication inhibition was observed after cells were treated with 0.01−1.0 μM neocarzinostatin (see Figure 3c). Treatment with 0.5 nM C-1027 reduced replication 20−30%, while treatment with 0.1 μM neocarzinostatin reduced activity by 31−39%. Thus, drug doses that induced similar levels of DNA damage [i.e., C-1027 (0.5−5.0 nM) or neocarzinostatin (0.1−1.0 μM) (see Figure 2)] caused equivalent decreases in cell-free replication competence. Replication activity observed in extracts of cells treated with the highest dose of C-1027 (5.0 nM) or neocarzinostatin (1.0 μM) increased only slightly when the amount of extract was doubled from 20 to 40 μg, indicating that inhibition at higher doses may not be reversed by



Drugs	% DNA in well	DSB/cell
0	95 ± 0.9	0
C-1027, 0.5 nM	81.2 ± 2.5	244
C-1027, 2 nM	59.8 ± 6.2	792
C-1027, 5 nM	34.9 ± 10	>2112
Neocarzinostatin, 0.01 uM	89.4 ± 3.7	145
Neocarzinostatin, 0.1 uM	76.3 ± 4.1	330
Neocarzinostatin, 1 uM	23:6 ± 3.2	>2112

FIGURE 2: DNA damage in 293 cells induced by C-1027 and neocarzinostatin detected by pulsed-field gel electrophoresis (PFGE). (a) Representative phosphorimage showing increased migration of DNA into the gel when cells were treated with DNA damaging agents. Lanes 1 and 7: DNA from untreated cells. Lanes 2−6 contain DNA from cells irradiated prior to proteinase K digestion with 2 (1), 6 (2), 12 (3), 30 (4), 60 (5), or 120 (6) gray (Gy). Lanes 8−10: DNA from cells treated with 0.5 (8), 2 (9), or 5 (10) nM C-1027. Lanes 11−13: DNA from cells treated with 0.01 (11), 0.1 (12), or 1.0 (13) μM neocarzinostatin. (b) Graphic representation of the amount of DNA remaining in the well after PAGE of X-irradiated cells. (c) Summary of DNA damage induced by C-1027 and neocarzinostatin. Rad equivalent damage, estimated by comparing the loss of DNA from the well in C-1027-treated samples with that observed after X irradiation, was converted to double-strand breaks (DSB). Using a molecular mass of 320 Da per nucleotide, DSB per cell (6.6×10^9 nucleotides) were calculated by multiplying the rad equivalent damage by 8.3×10^{-14} DSB Da^{-1} rad^{-1} $\times$ 320 Da $\times$ 6.6×10^9 nucleotides.

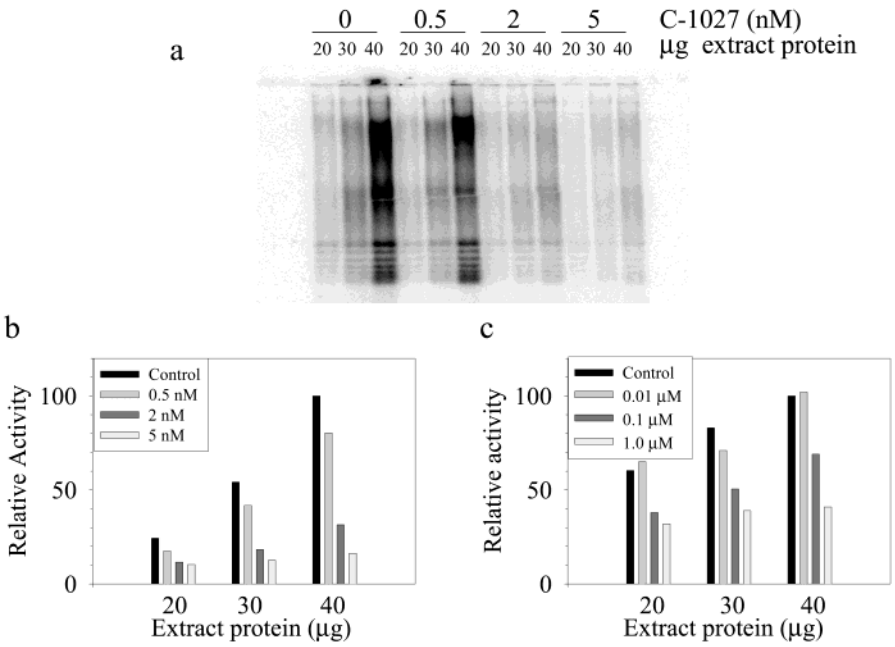


FIGURE 3: Cell-free SV40 DNA replication using soluble extracts from drug-treated cells. 293 cells were treated with the indicated concentrations of drugs for 2 h, and soluble extracts were prepared. A total of 20−40 μg of extract protein was used in reactions. (a) Phosphorimage of an agarose gel after electrophoresis of DNA replication reactions containing soluble extracts from cells treated with the indicated concentrations of C-1027. (b, c) Graphic representation of SV40 replication activity in soluble extracts from C-1027- or neocarzinostatin-treated cells, respectively.

supplementation with additional cellular replication proteins (i.e., additional extract).

Reduced Cell-Free Replication Competence Was Not Due to Damage to the DNA Template. To exclude the possibility that reduced cell-free replication competence was due to direct drug-induced damage to the DNA template, a cell-free DNA cleavage assay was carried out. Cleavage was identified as conversion of pSV011 supercoiled (form I) to nicked circular (form II) or linear (form III) DNA. Figure 4a shows the electrophoretic migration of pSV011 topological forms after incubation in the absence (control) or presence of 1−500 nM C-1027. Only high concentrations of C-1027

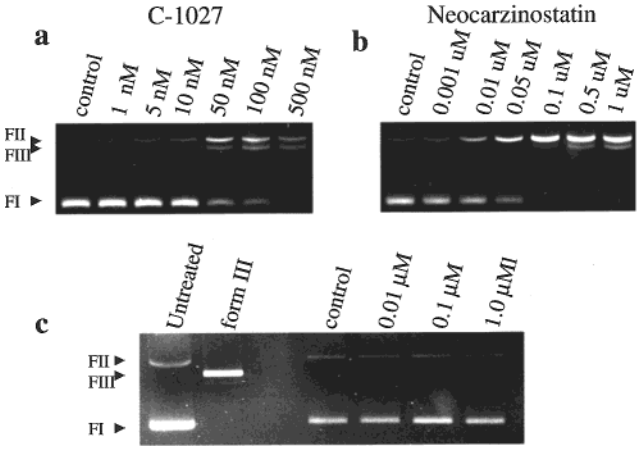


FIGURE 4: Cleavage of purified SV40 DNA by C-1027 or neocarzinostatin. pSV011 DNA was treated with the indicated concentrations of C-1027 (a) or neocarzinostatin (b) for 15 min at 37 °C and electrophoresed on a 1% agarose gel in 1 μg/mL ethidium bromide. A decrease in supercoiled form I (FI) and an increase in nicked circular form II (FII) or linear form III (FIII) were indicative of DNA damage. (c) A total of 40 μg of soluble extract protein from neocarzinostatin-treated cells was incubated with pSV011 DNA for 1 h at 37 °C. After proteinase K digestion and phenol/chloroform extraction, samples were electrophoresed as above. Lane 1 contained FI and FII and lane 2 contained FIII pSV011. Lanes 3−6 contain DNA incubated with soluble extracts from cells treated with the indicated concentrations of neocarzinostatin.

(10−500 nM) induced a progressive increase in form III and decrease in form I, indicative of DNA double-strand damage. However, after treatment with 1 and 5 nM C-1027, 85−90% of the DNA migrated as intact superhelical form I, similar to control samples. Since this was the dose range used for treating intact cells, damage to the template probably does not contribute to C-1027 inhibition of cell-free SV40 DNA replication.

However, with neocarzinostatin, damage was observed with as little as 0.01 μM and complete conversion of form I to form II or form III was noted with 1 μM neocarzinostatin treatment (see Figure 4b). While this is the neocarzinostatin range used to treat intact cells, it is likely that the actual drug levels present in the extract are lower, since cells were washed with PBS after neocarzinostatin treatment and the drug levels diluted during extract preparation. To determine whether any neocarzinostatin remained in the extract that could damage the DNA template, pSV011 was incubated with cell extract for 1 h at 37 °C. Figure 4c shows pSV011 DNA after incubation with soluble extracts from cells treated with 0, 0.01, 0.1, or 1.0 μM neocarzinostatin. No damage to DNA (i.e., no loss of form I or increase in forms II or III) was observed. Thus, direct damage to the DNA template due to residual amounts of neocarzinostatin in the cell extract probably does not contribute to the inhibition of cell-free replication.

Lack of a Trans-Acting Replication Inhibitor in C-1027- or Neocarzinostatin-Treated Extracts. C-1027 was reported earlier to inhibit DNA replication in trans (*12*, *29*), and a trans-acting inhibitor has been described in extracts from X-irradiated cells and in camptothecin- and bizelesin-treated cells (*13*, *14*). Mixing experiments were designed to determine whether enediynes also induce a trans-acting inhibitor. To 40 μg of control extract was added additional extract (10, 20, or 30 μg) from control or drug-treated cells, and cell-free SV40 DNA replication activity was measured. Figure 5a shows the effect of extracts from 2 or 5 nM C-1027-treated cells on the replication activity of control cell extracts. In the absence of additional extract the relative activity of 40 μg of control extract was 100%. Addition of 10−30 μg of control extract to the original 40 μg caused a progressive increase in replication activity. An increase in activity was also noted when extracts from cells treated with 2 or 5 nM C-1027 were added, although the increase was less pronounced. Since the effect of extracts from C-1027-treated cells on replication by control cell extract was additive, reduced replication activity in the drug-treated extracts was not due to the presence of an inhibitor.

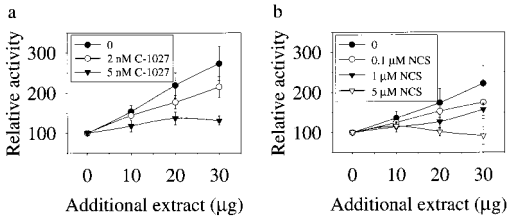


FIGURE 5: Mixing of enediyne-treated soluble extracts with control cell extracts does not indicate the presence of a trans-acting DNA replication inhibitor. Ten, twenty, or thirty micrograms of additional soluble extracts from mock- or enediyne-treated cells was added to 40 μg of mock-treated cell extract in cell-free SV40 DNA replication reactions. DNA products were isolated and analyzed as in Figure 3. DNA synthesis levels for each reaction are plotted as a percent of the activity present in the control (40 μg) reaction. Control soluble extract was mixed with the indicated amount of soluble extract from (a) C-1027-treated or (b) neocarzinostatin-(NCS-) treated cells. Data are the average of three experiments ± SEM.

Similar effects were observed with extracts from cells treated with 0.1 or 1.0 μM neocarzinostatin (Figure 5b). However, adding 20−30 μg of extract from cells treated with 5 μM neocarzinostatin to replication reactions did not increase activity over that in the presence of 40 μg of control extract alone. Although no additive effect was observed, the presence of an inhibitor of replication also was not indicated, since a reduction in activity below control levels did not occur.

Increased Retention in the Nucleus of RPA and Altered Electrophoretic Migration of RPA32 in Response to DNA Damage by C-1027 and Neocarzinostatin. Recent reports have described changes in RPA in response to DNA damage induced by various agents and suggest that RPA modification may be associated with a loss of replication competence similar to that seen in Figure 3 (for review, see ref *17*). To examine whether RPA was altered by enediyne treatment, the levels of RPA70 and RPA32 in the soluble and in the extraction-resistant nuclear fraction were determined by Western blotting. Figure 6a shows the distribution of RPA32 and RPA70 in cells treated with 0.5−5 nM C-1027 or 0.01−1 μM neocarzinostatin. With increasing C-1027 or neocarzinostatin treatment, the amount of RPA in the soluble extract decreased in a dose-dependent manner. The decrease in soluble RPA was accompanied by an increase in both RPA70 and RPA32 in the extraction-resistant nuclear fraction (see lower panel, Figure 6a). In addition, at higher drug doses (i.e., 2 and 5 nM C-1027 and 0.1 and 1 μM neocarzinostatin) a slower migrating band recognized by the RPA32 antibody was observed. At the higher drug doses, the slower migrating band accounted for about 50% of soluble and nearly 100%

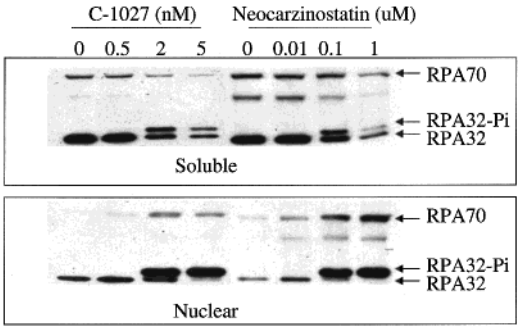


FIGURE 6: Changes in RPA induced by C-1027 or neocarzinostatin. Soluble extracts and extraction-resistant nuclear fractions prepared from cells treated with the indicated concentrations of C-1027 or neocarzinostatin were electrophoresed on 10% PAGE, subjected to Western blotting, and probed with monoclonal anti-RPA32 and RPA70 antibodies. Shown are phosphorimages of representative Western blots. A single RPA70 band and two RPA32 bands (RPA32-P_i and RPA32) are indicated by arrows.

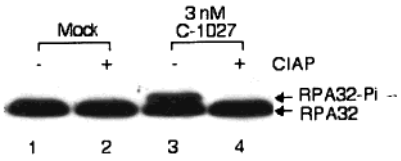


FIGURE 7: The enediyne-induced RPA32-P_i band is hyperphosphorylated RPA32. To prove that the slower migrating RPA32 band (RPA32-P_i) in Figure 6 was hyperphosphorylated RPA32, samples were treated with alkaline phosphatase as described in Experimental Procedures. A total of 10 μg of proteins from extracts of control (lanes 1 and 2) or 3 nM C-1027- (lanes 3 and 4) treated cells was incubated with (lanes 2 and 4) or without (lanes 1 and 3) 7 units of calf intestine alkaline phosphatase (CIAP).

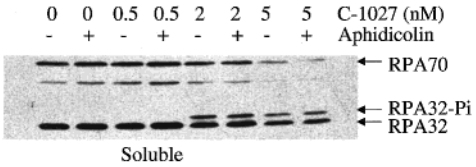


FIGURE 8: Aphidicolin does not prevent enediyne-induced RPA alterations. Cells were incubated for 15 min with 5 μM aphidicolin prior to addition of C-1027 at the indicated concentrations. Preparation of extracts and Western blot analysis were performed as described in Figure 6.

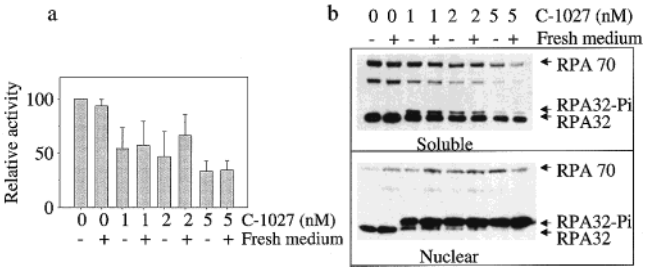


FIGURE 9: Replication inhibition and RPA hyperphosphorylation are not reversible by incubation in enediyne-free medium. Cells were treated with 1, 2, or 5 nM C-1027. After 2 h, half of the cells were harvested. The remaining cells were washed with fresh medium and further incubated in fresh medium for 4 h before harvesting, and extracts were prepared. (a) Cell-free SV40 DNA replication activity of 30 μg of soluble extract protein. Data are the average of four to six independent experiments and are expressed as the percent activity in control cell extracts. (b) Equal amounts of soluble and extraction-resistant nuclear fractions from a representative experiment were electrophoresed on 10% SDS−PAGE. RPA was detected by Western blotting using anti-RPA70 and anti-RPA32 monoclonal antibodies.

of extraction-resistant nuclear RPA32 signal. Thus, treatment with either enediyne caused increased RPA retention in the nucleus and altered RPA32 electrophoretic migration.

The Enediyne-Induced RPA32 Band with Decreased Electrophoretic Migration Is Hyperphosphorylated RPA32. DNA damage induces hyperphosphorylation of RPA32 which exhibits reduced electrophoretic migration compared to underphosphorylated RPA32 (*15*). To confirm that the slower migrating band seen after treatment with C-1027 or neocarzinostatin was hyperphosphorylated RPA, soluble extracts were treated with alkaline phosphatase as described in Experimental Procedures. Figure 7 is a Western blot showing the effect of alkaline phosphatase treatment on extracts from untreated (mock) and 3 nM C-1027-treated cells. Alkaline phosphatase had no effect on the faster migrating RPA32 band from mock- or C-1027-treated cells (lanes 1−4). However, phosphatase treatment caused the disappearance of the slower migrating RPA32 band (lane 3 compared to lane 4), indicating that hyperphosphorylation of RPA32 was responsible for the reduced band migration. Thus, the slower migrating RPA32 band in Figure 6 was defined as RPA32-P_i.

Enediyne-Induced RPA32 Hyperphosphorylation Is Not Dependent upon Continuing DNA Replication. Hyperphosphorylation of RPA induced by DNA damaging agents such as adozelesin, bizelesin, X irradiation, camptothecin, and UV is dependent on continuing DNA replication (*15*, *30*, *31*). Whether enediyne-induced hyperphosphorylation was similarly dependent was assayed by incubating cells with the well-known DNA fork elongation inhibitor aphidicolin (*32*−*34*). Pretreatment of cells with 5 μM aphidicolin prevented the appearance of a hyperphosphorylated RPA32 band in Western blots of extracts from 293 cells treated with 1 μM camptothecin (data not shown). However, aphidicolin had no effect on RPA32 hyperphosphorylation induced by C-1027 (see Figure 8) or neocarzinostatin (data not shown). Thus, control of RPA hyperphosphorylation induced by either enediyne differs from that of other DNA damaging agents.

Reversal of Enediyne-Induced Replication Inhibition and RPA Alterations Was Not Observed. The ability of C-1027-treated cells to recover their replication competence and reverse drug-induced changes in RPA was determined as described in Experimental Procedures. Cells were treated for 2 h with C-1027 and either harvested immediately or further incubated in fresh medium for 4 h. Figure 9a shows the relative replication activity in extracts from control cells or cells treated with 1−5 nM C-1027. Replication competence was unchanged whether cells were harvested immediately or further incubated to allow for reversal of drug-induced effects. The amount of enediyne-induced hyperphosphorylated RPA associated with the extraction-resistant nuclear fraction was the same with or without 4 h postdrug incubation in fresh medium (see Figure 9b). A similar lack of reversibility was observed with neocarzinostatin, even when the incubation time in fresh medium was increased to 16 h (data not shown). Thus, enediyne-induced effects on replication and RPA hyperphosphorylation are essentially irreversible.

DISCUSSION

A decrease in cell-free replication competence was observed in soluble extracts of cells treated with C-1027 or

neocarzinostatin doses that induced threshold levels of intracellular DNA damage. This decrease was not dependent on damage to the DNA template in the cell-free replication reaction, indicating for the first time that neocarzinostatin, like C-1027 (*12*), can inhibit DNA replication in trans.

Enediyne concentrations that decreased replication activity also caused hyperphosphorylation of RPA32, the middle subunit of RPA. Changes in RPA phosphorylation after DNA damage induced by a wide variety of agents [e.g., X irradiation (*35*, *36*), UV irradiation (*19*, *31*), and the topoisomerase inhibitor camptothecin (*30*, *37*), as well as the DNA alkylating agents bizelesin and adozelesin (*14*, *15*)] have been reported elsewhere. As with X irradiation and camptothecin, nearly 50% of soluble RPA32 becomes hyperphosphorylated after treatment with enediynes. The present study also showed that enediynes effected a large change in the intracellular distribution of RPA leading to association of the majority of cellular RPA with the extraction-resistant nuclear fraction. Nearly 100% of the RPA32 tightly associated with the nuclear fraction was hyperphosphorylated. This is the first report of a DNA damaging agent causing increased tight binding to the nuclear fraction of RPA70 and underphosphorylated and hyperphosphorylated RPA32. Thus, the RPA phosphorylation response to enediyne treatment may differ from that reported for other DNA damaging agents.

RPA32 is phosphorylated during the S phase of a normal cell cycle (*20*, *38*) as well as in response to DNA damage. Hyperphosphorylated RPA reportedly localizes to DNA single-strand regions (*39*), such as sites for DNA replication and repair. Enediyne-induced nuclear extraction-resistant hyperphosphorylated RPA32 may be localized at repair sites, limiting the ability of RPA to assemble at replication foci and to function in replication. Thus, in intact cells, enediyne-induced DNA damage may inhibit replication by altering RPA subunits to prevent their functional association with replicating foci.

In addition to increased RPA32 hyperphosphorylation, reduced levels of RPA70 and RPA32 were observed in the soluble extract from cells treated with either C-1027 or neocarzinostatin (see Figure 6). This loss, accompanied by decreased cell-free SV40 DNA replication activity, was observed after treatment with 0.5−5.0 nM C-1027 or 0.1−1.0 μM neocarzinostatin. Replication activity can be restored to soluble extracts from cells treated with 1 or 3 nM C-1027 by adding back RPA (manuscript in preparation). Thus, at the C-1027 concentrations used in the present study, reduced amounts of functional RPA, rather than induction of a replication inhibitor, likely account for the decreased replication competence of the soluble cell extract. This response to DNA strand damage is similar to that observed with the DNA alkylator adozelesin,which also decreased cell-free SV40 DNA replication competence in an RPA-associated manner (*15*). While no decrease in RPA levels in the soluble extract was noted after adozelesin treatment, RPA hyperphosphorylation was observed, and replication activity could be restored to the soluble extract by addition of RPA. Thus, a similar mechanism for inhibition of DNA replication resulting from decreased functional levels of RPA was observed with agents that differed dramatically in the type of DNA damage induced.

Decreased replication competence and reduced levels of RPA also have been described in soluble extracts from X-ray- and camptothecin-treated cells (*13*). However, replication activity inhibited by X-ray, by camptothecin, or by bizelesin, the bifunctional analogue of adozelesin (*14*), could not be restored by addition of exogenous RPA. Rather, the dominant replication effect of these agents was induction of an inhibitor, possibly DNA-PK. Thus, while the cellular response to treatment with a variety of DNA damaging agents may include a reduction in RPA levels, not all decreases in replication activity can be restored by adding back RPA, and the precise mechanism for replication inhibition may differ with specific DNA damaging agents.

Another example of the differing effects on replication caused by DNA reactive agents is the response to DNA damage after treatment with the DNA elongation inhibitor, aphidicolin. In contrast to RPA hyperphosphorylation induced by camptothecin, or the DNA alkylators adozelesin or bizelesin, that induced by C-1027 or neocarzinostatin is unaffected by aphidicolin pretreatment (Figure 8 and data not shown). Thus, cells can detect enediyne-induced DNA damage even in the absence of replication fork movement.

Recent studies on cellular recognition of DNA damage may partially explain this finding. An early step in DNA damage recognition reportedly involves thermodynamic probing of the duplex to identify agents which either stabilize (e.g., the alkylator CC-1065) or destabilize the DNA helix (*40*). Adozelesin and bizelesin, both analogues of CC-1065 (*60*,*61*) form bulky single- or double-strand adducts, but not DNA breaks (*41*), at the DNA−drug binding site, presumably stabilizing the DNA helix. Readily reversible protein-associated single-strand breaks are induced by camptothecin treatment (*42*), and these breaks also should have limited effects on helix destabilization. Thus, collision of the moving replication fork with these lesions might be required to enable DNA damage recognition and subsequent phosphorylation of RPA. By contrast, C-1027 and neocarzinostatin induce strand breaks which result from site-specific free radical attack on sugar moieties in both strands of DNA (*26*). These lesions directly reduce DNA superhelicity (*5*, *43*), effectively altering the tertiary structure of the DNA helix. Recently, RPA, which participates in multiple steps of nucleotide excision repair (NER) including the damage recognition step, was found to have increased affinity for sites where the double helix had been disrupted (*44*). RPA recognition of enediyne-induced effects on helical structure provides a possible pathway for recognition of enediyne-induced DNA damage which is independent of continuing DNA replication.

The minimum number of DNA double-strand breaks per cell necessary to decrease replication competence and induce RPA32 hyperphosphorylation and increased levels of nuclear extraction-resistant RPA was similar for C-1027 and neocarzinostatin. This suggested that a threshold level of damage was necessary to inhibit replication regardless of the enediyne examined. By contrast, when the cytotoxicity of the DNA lesions was assayed, differences between C-1027 and neocarzinostatin were observed. Extrapolation of the double-strand break damage shown in Figure 2 to drug levels, which caused a 50% reduction in colony formation, revealed a double-strand break frequency per cell of 1.3−1.6 for C-1027 compared to 7.8−34 for neocarzinostatin. Thus, damage

induced by C-1027 was at least 5-fold more lethal than that induced by neocarzinostatin.

One explanation for this difference may be the repairability of C-1027- and neocarzinostain-induced DNA lesions. C-1027 affects primarily double-strand damage, while double-strand breaks induced by neocarzinostatin probably result from single-strand breaks closely spaced on opposite strands (*45*). Single-strand DNA breaks induced by neocarzinostatin are readily repaired, and such repair should lead over time to reduced numbers of double- as well as single-strand breaks. Studies with repair enzymes also indicate a difference in repairability of lesions induced by differing enediynes. Other workers have shown that human apurinic/apyrimidinic endonuclease 1 (Ape 1) and *Escherichia coli* exonuclease III can partially repair DNA strand breaks produced by neocarzinostatin but not those induced by the enediyne calicheamicin (*46*). C-1027 contains an enediyne chromophore of the esperamicin/calicheamicin type, and it is possible that C-1027-induced lesions are not repaired as readily as those induced by neocarzinostatin. However, it is unlikely that differences in lesion repairability influence replication effects since RPA hyperphosphorylation induced by either enediyne was irreversible (see Figure 8), and the replication response to both drugs was very similar at concentrations affecting equivalent amounts of DNA damage.

In summary, both C-1027 and neocarzinostatin produced similar decreases in cell-free SV40 DNA replication competence with equivalent amounts of DNA double-strand damage. Future studies will examine enediyne-induced alterations in other replication and proliferation factors (e.g., the cyclin/cdk and Rb families) that may contribute to the DNA damage response pathways leading to replication inhibition. Whether a threshold of genomic damage similar to that induced by C-1027 and neocarzinostatin is crucial for triggering cellular response by other types of DNA damaging agents will be investigated.

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