Processing of 3’-Phosphoglycolate-terminated DNA Double Strand Breaks by Artemis Nuclease

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The Artemis nuclease is required for V(D)J recombination and for repair of an as yet undefined subset of radiation-induced DNA double strand breaks. To assess the possibility that Artemis acts on oxidatively modified double strand break termini, its activity toward model DNA substrates, bearing either 3’-hydroxy or 3’-phosphoglycolate moieties, was examined. A 3’-phosphoglycolate had little effect on Artemis-mediated trimming of long 3’ overhangs (>9 nucleotides), which were efficiently trimmed to 4–5 nucleotides. However, 3’-phosphoglycolates on overhangs of 4–5 bases promoted Artemis-mediated removal of a single 3’-terminal nucleotide, while at least 2 nucleotides were trimmed from identical hydroxyl-terminated substrates. Artemis also efficiently removed a single nucleotide from a phosphoglycolate-terminated 3-base 3’ overhang, while leaving an analogous hydroxyl-terminated overhang largely intact. Such removal was completely dependent on DNA-dependent protein kinase and ATP and was largely dependent on Ku, which markedly stimulated Artemis activity toward all 3’ overhangs. Together, these data suggest that efficient Artemis-mediated cleavage of 3’ overhangs requires a minimum of 2 nucleotides, or a nucleotide plus a phosphoglycolate, 3’ to the cleavage site, while at least 2 unpaired nucleotides 5’ to the cleavage site. Shorter 3’-phosphoglycolate-terminated overhangs and blunt ends were also processed by Artemis but much more slowly. Consistent with a role for Artemis in repair of terminally blocked double strand breaks in vivo, human cells lacking Artemis exhibited hypersensitivity to x-rays, bleomycin, and neocarzinostatin, which all induce 3’-phosphoglycolate-terminated double strand breaks.

The Artemis genetic locus was identified by virtue of its association with a form of B- T- NK+ severe combined immune deficiency (SCID) in humans, designated RS-SCID (radiation-sensitive SCID) (1) or SCIDA (Athabascan SCID) (2). The Artemis protein is a nuclease that is activated by DNA-dependent protein kinase (DNA-PK) and is required for the opening of hairpin ends formed during V(D)J recombination (3), thus accounting for the SCID phenotype associated with Artemis deficiency. SCIDA and RS-SCID fibroblasts are radiation-sensitive but fail to repair only a small fraction of radiation-induced DNA double strand breaks (DSBs) (4–6). In vitro, activated Artemis removes 5’ overhangs from DNA ends and shortens 3’ overhangs (7), raising the possibility that during DSB repair in vivo, Artemis may trim overhangs that otherwise cannot be processed to give ligatable ends.

About half of DNA breaks induced by ionizing radiation bear 3’-phosphoglycolate (3’-PG) termini in various contexts (7) that must be removed in order to allow gap filling by DNA polymerases μ and λ and ligation by DNA ligase IV (8). Although tyrosyl-DNA phosphodiesterase (TDP1) is the only identified enzyme capable of processing 3’-PGs on 3’ overhangs (9), TDP1 mutant cells show only marginal radiosensitivity (10), suggesting the existence of an alternative pathway for processing of these lesions in vivo.

Because Artemis-deficient cells exhibit significant sensitivity to ionizing radiation, yet have only a small DSB repair defect, we assessed the possibility that Artemis functions in processing overhanging 3’-PG termini. We purified recombinant Artemis and examined the activity of this protein on a variety of oligomeric and plasmid substrates bearing 3’-overhangs. We further investigated the relevance of Artemis to the repair of 3’-PG terminated DSBs by measuring the toxicity of drugs known to induce such breaks in normal and Artemis-deficient cells.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Artemis expression constructs were derived from full-length Artemis cDNA as described previously (2). Full-length Artemis protein was purified from SF9 insect cells infected with recombinant baculovirus generated by subcloning Artemis cDNA into pFASTBAC-HT (Invitrogen). Briefly, the amino-polyhistidine-tagged Artemis was extracted and purified using immobilized metal affinity chromatography with standard protocols (nickel-
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nitrilotriacetic acid; Amersham Biosciences). Fractions containing Artemis were dialyzed into 50 mM HEPES, pH 7.5, 10% glycerol, 2 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P-40, 20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, pepstatin A, and leupeptin (HCB), containing 0.1 mM NaCl. The affinity-purified protein was loaded onto a Mono Q column (Amersham Biosciences) and eluted with a linear gradient of 100–500 mM NaCl in HCB. Aliquots of Artemis-containing fractions were snap-frozen and stored at −70°C. Protein from these aliquots was subjected to tandem mass spectrometry and identified as Artemis. Endonuclease and exonuclease activities were verified by standard biochemical assays, and aliquots were discarded after four or fewer freeze/thaw cycles. Ku70/80 was purified from insect cells coinfected with a mixture of recombinant baculovirus harboring the human KU70 and KU80 genes, and DNA-PKcs was purified from HeLa cells, both as described previously (11). All protein concentrations were determined by Bradford assays using bovine serum albumin as a protein standard (Bio-Rad), and protein purity was evaluated by Comassie Blue-stained SDS-polyacrylamide gels. When necessary, proteins were diluted immediately before use in reaction buffer lacking ATP either on ice (Artemis, DNA-PKcs) or at 22°C (Ku).

DNA Substrates—Oligonucleotides were purchased from Qiagen or Integrated DNA Technologies. All labeled oligomers were purified by gel electrophoresis followed by reverse-phase HPLC. The yield of each oligomer was determined by integrating the absorbance at 260 nm recorded on an in-line UV monitor. To generate 3’-PG oligomers with the sequence CGAGAACGGC(Aₙ)CG (0 ≤ n ≤ 4), 5’-32P-3’-end-labeled oligomers CGAGGAACGGC(Aₙ)CGCCC were treated with bleomycin plus H₂O₂, and the desired 14–17-base 3’-PG products were isolated from a sequencing gel and purified by HPLC (12). The terminal structure and purity of the 14-base (n = 1) 3’-PG oligomer was verified by electrospray mass spectrometry and fragmentation-based chemical sequencing. Treatment of the other (n = 0 and 2–4) oligomers with bleomycin likewise resulted in efficient site-specific cleavage at the -CGCCCC site near the 3’ end, as judged by the appearance of a prominent band with the expected mobility (supplemental Fig. 1). Upon extraction from the gel, each such oligomer eluted as a single well defined peak from reverse-phase HPLC. The terminal structure and purity of each putative 3’-PG oligomer was confirmed by quantitative conversion to the corresponding 3’-phosphate and 3’-hydroxyl oligomers by treatment with tyrosyl-DNA phosphodiesterase and polynucleotide kinase/phosphatase, which in every case induced the expected shifts in electrophoretic mobility (supplemental Fig. 1). An analogous oligomer (n = 1) bearing a 3’-phosphotyrosyl terminus was purchased from Midland Certified Reagents. The 9- and 11-base 3’-PG oligomers CGAGGAAACGGC and CGAGGAACGGC were similarly prepared as described previously and their structures verified by mass spectrometry (12). The 36-base 3’-PG oligomers were prepared by ligating the corresponding labeled 14-mers to the 22-mer GCCATGTACTTTGATGATCATTTC and were again gel/HPLC-purified. Partial duplexes were annealed in 10 mM Tris-HCl, pH 8, 0.1 mM NaCl, 1 mM EDTA by heating a mixture of labeled oligomer and a 1.5-fold excess of unlabeled complement to 80°C followed by slow cooling to 10°C over a period of 3 h. Internally labeled plasmid substrates with various overhangs were constructed by ligating 3’-PG, 3’-phosphotyrosyl, or 3’-hydroxyl oligomers (9–24 bases in length) into plasmids with an 11-base 5’ overhang, as described (13). Each plasmid was gel-purified and eluted, and the concentration was determined from the 260 nm peak of the absorbance spectrum.

Nuclease Assays—Reaction mixtures (10 μl) containing 25 mM Tris-HCl, pH 8, 25 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.25 mM ATP, 50 μg/ml bovine serum albumin, and either 5 nm oligomeric substrate or 1 nm plasmid substrate were prepared at 22°C. In some cases, a blunt-ended double-stranded 35-mer (4 nt to 1 μm) was also included (3). Ku was added, and the mixture was immediately vortexed and briefly centrifuged. DNA-PKcs was then added followed immediately by Artemis, and the reaction was mixed by pipeting and placed in a 37°C bath. Reactions with oligomeric substrates were stopped by addition of 10 μl of formamide containing 20 mM EDTA, and the DNA was heat-denatured and analyzed on sequencing gels. Reactions containing plasmid substrates were stopped by addition of 20 μl of 10 mM EDTA, 0.45 mM sodium acetate, 100 μg/ml tRNA, followed immediately by phenol extraction and ethanol precipitation. DNA was cut with Aval (20 units, 6 h, 37°C) or TaqI (hereafter referred to as TaqI; 20 units, 4 h, 65°C) in 50 μl of the buffer supplied by the vendor (New England Biolabs). In some cases 0.25 mM CoCl₂ and 0.1 mM ddGTP were subsequently added, and the samples were treated with 20 units of terminal deoxynucleotidyltransferase (New England Biolabs) for 1 h at 37°C. DNA was precipitated, dissolved in formamide, heat-denatured, and analyzed on sequencing gels. The Aval and TaqI cleavage sites differ by one nucleotide; however, as expected these two enzymes always gave essentially identical results in parallel experiments.

Cell Lines and Proliferation Assays—Fibroblast lines were established from skin biopsies from SCIDA patients 04 and 05 and an unrelated immunologically normal individual (AK). Early passage cultures were immortalized by introduction of human telomerase reverse transcriptase cDNA using methods previously described (6, 11). Cells were either irradiated using a Pantak® x-ray generator operating at 320 kV/10 mA with 0.5 mm copper filtration or exposed to genotoxic drugs for 1 h, washed three times with PBS, and then labeled by the addition of fresh medium containing 10 μg/ml bromodeoxyuridine (BrdUrd) (Sigma) for 24–35 h. Cells were harvested, fixed, and stained using standard procedures, and cell cycle distribution and BrdUrd incorporation were analyzed with a Beckman-Coulter EPICS XL-MCL flow cytometer using XL Data Acquisition software and WinMDI 2.8 or FlowJo 8.1 software packages. The fraction of proliferating cells (F) was calculated by scoring the percent of intact cells staining positive for BrdUrd and normalizing to the untreated control of the same cell line (≥10,000 events were scored for each point). To quantitatively compare the toxicity of each agent toward SCIDA and normal cells, relative toxicity at each radiation dose or drug concentration was calculated as ln(F1)/ln(F2), where F1 and F2 are the proliferating fractions, normalized to untreated controls, for...
SCIDA and normal cells, respectively. This parameter was relatively constant over a range of concentrations even in the case of bleomycin, which typically shows a distinct upward concavity in the response.

RESULTS

Trimming of Long 3'-PG-terminated Overhangs by Artemis—DSBs induced by radiation and other free radical-based toxins commonly bear 3'-PG termini (7, 14–16), which block polymerase and ligase activities as well as most human exonucleases. The Artemis nuclease, which reportedly shortens long 3’ overhangs, could thereby resolve PG and other blocked termini on 3’ overhangs of DSBs. To determine whether the endonucleolytic activity of Artemis was affected by the presence of PG termini, an internally labeled 3'-PG or 3'-hydroxyl-terminated 36-mer was annealed to 21-, 23-, and 27-base complementary strands to yield substrates with 15-, 13-, and 9-base 3’ overhangs.

As expected, Artemis treatment of the 3’-hydroxyl substrates resulted in efficient trimming of overhangs (Fig. 1A) (17). The 15-base (36/21 substrate) and 13-base (36/23 substrate) overhangs were each predominantly shortened to a 5-base overhang, yielding 26- and 28-base products, respectively. The 9-base overhang (36/27p substrate) was trimmed predominantly to a 4-base overhang (31-mer) rather than 5-base overhang. The PG-terminated 15- and 13-base overhangs were likewise trimmed predominantly to 5-base overhangs, whereas the 9-base overhang was trimmed to a 4-base overhang. These cleavage patterns and levels of activity are essentially identical to those obtained with the analogous 3’-hydroxyl overhangs (compare Fig. 1, A and B), indicating that PG moieties several bases from the cleavage site had little or no effect on the efficiency or specificity of Artemis-mediated DNA cleavage. In all cases, 38–60% of the substrate was cleaved, with the major product accounting for 50–66% of the total.

A substrate composed of a 19-mer annealed to the labeled 36-mer, with 19 bp of duplex DNA and a 17-base overhang, sustained at least 3-fold less Artemis-mediated cleavage than the other substrates and showed altered specificity (Fig. 1A). To determine whether this inefficiency was because of the 19-bp duplex being too short to accommodate the Ku-DNA-Artemis complex, or the 17-base overhang being too long to allow efficient cleavage, we prepared substrates having the same 23-bp duplex DNA and differing only in the length of the 3’ single-stranded overhangs, 13, 19, and 25 bases. Artemis nuclease cleaved all three substrates, with comparable efficiency and essentially identical specificity, with the dominant 5-base overhang (28-mer) product accounting for 44–52% of total cleavage in each case (Fig. 1C). A substrate having only 19 bp of duplex DNA (48/19) sustained 50% less cleavage, and the usual 5-base overhang specificity was lost, with an 11-base overhang (30-mer) being the most frequent product (Fig. 1C).

Taken together, these data indicate that Artemis is capable of trimming 3’ overhangs at least as long as 25 bases, with the incision site being predominantly 5 nucleotides from the double strand/single strand transition. As footprinting studies indicate that Ku plus DNA-PKcs protects ~28 bp at a DNA end (18), the apparent requirement for at least ~20 bp of duplex DNA for proper binding and alignment of the nuclease complex is not surprising.

Control reactions here and throughout reveal that Artemis, Ku, and DNA-PKcs protein preparations are free of significant contaminating nucleases. A Coomassie Blue-stained polyacrylamide gel showing the purity of preparations of DNA-PKcs, Ku70/80, and Artemis is shown (Fig. 1D).

DNA-PKcs and Ku Dependence of Artemis Activity on Long 3’ Overhangs—Previous work with immunocomplexed Artemis immobilized on agarose beads suggested that Artemis nuclease cleaved long 3’ overhangs to a length of ~5 bases, in a reaction that required DNA-PKcs but was unexpectedly independent of the presence or absence of Ku (3). To assess the DNA-PKcs and Ku dependence of DNA cleavage by purified soluble Artemis, a substrate having 23 bp of duplex DNA and a 13-base 3’-hydroxyl-terminated 3’ overhang (36/23-mer, as above but labeled at

FIGURE 1. Effect of substrate length and PG termini on cleavage of 3’ overhangs by Artemis nuclease. All samples were treated with 65 nM Ku, 55 nM DNA-PKcs, and/or 240 nM Artemis, as indicated. A, internally labeled oligomeric substrates with a 17-base (36/19), 15-base (36/21), 13-base (36/23), or 9-base (36/27p) 3’ overhang and bearing a 3’-hydroxyl terminus were treated with Artemis plus DNA-PK.B, analogous internally labeled substrates with the indicated PG-terminated (○) 3’-overhangs were treated with Artemis plus DNA-PK. C, end-labeled substrates with 13-base (36/23), 19-base (42/23), 25-base (48/23), and 29-base (48/19) 3’ overhangs were treated with Artemis and/or DNA-PK. Replicate experiments yielded essentially identical results. The 27p oligomer was 5’-phosphorylated, but the other complementary strands were not. Note that the substrates in A and B are internally labeled 14 bases from the 3’ end. Thus, the Artemis cleavage products, which bear both 5’- and 3’-hydroxyls, run a full nucleotide position more slowly than Maxam-Gilbert markers of the same length, which bear 5’- and 3’-phosphates; for example, the 31-base cleavage product from the 36/27p duplex comigrates with the 32-base marker generated by chemical cleavage of the G at position 33 in the sequence (…CGCGACG)D, purity of proteins used in Artemis cleavage reactions. Recombinant Artemis (10 μg), recombinant Ku (10 μg), and DNA-PKcs (5 μg) purified from HeLa cells were subjected to denaturing PAGE and stained with Coomassie Blue. Leftmost lane contains molecular size markers (kDa).
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FIGURE 2. Stimulation of Artemis-mediated DNA cleavage by Ku and inhibition by excess DNA ends. A, reaction requirements. The end-labeled 36/23 substrate bearing a 13-base hydroxyl-terminated 3′ overhang (5 nM) was incubated for 30 min in the presence of 13 nM Ku, 35 nM DNA-PKcs, 90 nM Artemis, and/or 0.5 μM unlabeled 35-mer duplex. B, reaction time course. The same 36/23 substrate was treated with 35 nM DNA-PKcs, 13 nM Ku, and 90 nM Artemis for the times indicated. Inset at right shows a 20-fold overexposure of lane 3, C, concentration-dependent inhibition by excess DNA ends. The 36/23 substrate was treated for 30 min with 65 nM Ku, 55 nM DNA-PKcs, and 240 nM Artemis in the presence of 4, 16, 60, 250, or 1000 nM 35-mer duplex. D, effect of Ku and excess DNA ends on overhang cleavage. The 36/23 substrate was treated with 240 nM Artemis, 55 nM DNA-PKcs, and 13 nM (+) or 65 nM (+ +) Ku. Error bars show S.E. of 3–5 determinations from at least three independent experiments. There was no detectable cleavage by either Artemis or DNA-PK alone. E, titration of overhang cleavage with limiting Artemis. The presence of 35 nM DNA-PKcs and the presence (●) or absence (□) of 65 nM Ku. F, titration of cleavage of a plasmid substrate with limiting Artemis. An internally labeled plasmid substrate bearing a hydroxyl-terminated 13-base overhang (1 nM) was treated with the indicated concentrations of Artemis in the presence of 35 nM DNA-PKcs and the presence (●) or absence (□) of 13 nM Ku and cut with TaqI. G, titration of cleavage of the same plasmid substrate with limiting DNA-PKcs by 90 nM Artemis in the presence (●) or absence (□) of 13 nM Ku. Error bars in E–G show S.E. of three independent experiments when larger than the symbols.

Although Ku was not strictly required for cleavage, the addition of purified Ku70/80 stimulated Artemis nuclease activity 4-fold, in a DNA-PKcs- and ATP-dependent manner (Fig. 2A, lanes 5–8). Previous studies, showing Ku-independent Artemis activity, included excess 35-bp blunt-ended DNA in Artemis nuclease reactions to ensure robust activation of DNA-PKcs and phosphorylation of Artemis (3). However, in reactions containing soluble recombinant Artemis, addition of the same 35-bp duplex did not stimulate cleavage, even in the absence of Ku (Fig. 2A, compare lanes 7 and 9). Moreover, in reactions containing Ku, the addition of excess 35-bp duplex reduced Artemis-mediated cleavage of the radiolabeled substrate 3-fold (Fig. 2A, compare lanes 8 and 10; see supplemental Fig. 2). A titration of 35-bp duplex into Artemis nuclease reactions containing Ku clearly showed that there was no stimulatory effect at any concentration (Fig. 2C). Thus, the labeled substrate, at a concentration of ~5 nM, appears to be sufficient to promote activation of DNA-PK and Artemis. Quantification of triplicate experiments with 240 nM Artemis revealed that Ku stimulated overall cleavage by ~2-fold at 13 nM and 4-fold 65 nM (Fig. 2D). Most notably, this stimulatory effect was almost completely abolished by the presence of excess DNA ends (Fig. 2D), thus explaining the lack of stimulation by Ku in previous work where excess the 5′ end rather than internally) was reacted with Artemis plus DNA-PK components proteins (Fig. 2A). As expected, cleavage of this substrate was completely dependent on Artemis, DNA-PKcs, and ATP and yielded a 5′-base overhang as the predominant product (49% of total cleavage in Fig. 2A, lanes 3 and 8). A time course showed that the cleavage pattern was established within the first few minutes of the reaction (Fig. 2B), consistent with endo- rather than exonucleolytic cleavage. In addition, the similarity of the cleavage patterns between the 35-bp duplex was routinely added to Artemis reactions (3).

Titrations with limiting Artemis revealed that Ku stimulated Artemis activity over a wide range of Artemis concentrations (Fig. 2E). In the presence of Ku, the concentration of Artemis required for a given level of cleavage was 10-fold lower than in its absence.

To generate substrates more similar to DSBs produced in vivo (i.e. having very long duplex regions and short overhangs), a 3′-resected plasmid with an 11-base 5′ overhang was pre-
Ku-dependent Serial Cleavage of 3’ Overhangs—To examine in more detail the effect of Ku on the kinetics and specificity of cleavage, the same plasmid substrate used above was treated with Artemis in the presence or absence of Ku for various times, and the percentage of uncleaved substrate as well as of individual cleavage products was quantified (Fig. 3). Quantification of uncleaved substrate as a function of time reveals that the rate of Artemis/DNA-PKcs-mediated cleavage was three times faster in the presence of Ku than in its absence (Fig. 3A). As expected, bands corresponding to the trimmed overhangs were only detected when the internally labeled DNA end was released as an oligomer by treatment with TaqI, and nuclease activity was ATP-, DNA-PKcs-, and Artemis-dependent (Fig. 3B). Notably, the addition of Ku also markedly altered the digestion pattern for the 3’ overhang (Fig. 3B). In the absence of Ku, the predominant cleavage product was a 5-base overhang (40% of total cleavage), with significant amounts of 2–4- and 6-base overhang products also being apparent. In contrast, a 3-base overhang product consistently dominated the products in reactions containing Ku.

To investigate further this Ku-induced change in product distribution, reaction products were examined at various reaction times (Fig. 3C). In the presence of Ku, the proportion of 5-base overhang decreased from 56% of total cleavage at 2 min to only 6% at 60 min, whereas the proportion of 3-base overhang increased from 10 to 54%, becoming the dominant product by 60 min. Moreover, the appearance of the 3-base overhang product was concomitant with the decrease in the 5-base product (Fig. 3D). These data are consistent with a two-step reaction, wherein the 5-base product is the substrate for a secondary nuclease cleavage yielding the 3-base product. Notably, in the absence of Ku this crossover pattern was not apparent (Fig. 3E), and the distribution of products was essentially invariant over the course of the reactions. In addition, this same effect was observed in titrations with limiting Ku, namely the appearance of the 3-base product accompanied by the loss of the 5-base product (as well as the 6-base product) as Ku concentrations increased (Fig. 3F). A more detailed curve-fitting analysis indicated that these kinetics could be accurately described by a two-step model, and that although Ku accelerated the initial generation of the 5-base
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5-base overhang product was evident only at early time points. At later times, the abundance of both the 5-base and 4-base overhangs diminished, with concomitant increases in the 3- and 2-base overhangs (Fig. 4D). These results are consistent with a secondary cleavage that removes two additional bases, as seen above with the 13-base overhang substrate. The analogous hydroxyl-terminated substrate (Fig. 4B) also showed a time-dependent increase in the relative abundance of shorter reaction products, albeit less apparent than with the 3'-PG substrate. This is likely because the initial cleavage reaction proceeded more slowly (see supplemental Fig. 4).

The influence of 3'-PG on Artemis nuclease specificity was much more evident on substrate sets with 5- and 4-base overhangs (Fig. 5). For a 5-base 3'-hydroxyl overhang, two-nucleotide trimming was dominant (78% of cleavage products at 5 min), and single-nucleotide trimming was <5%, whereas for the analogous PG-terminated substrate single-nucleotide removal accounted for nearly half (48%) of the total initial cleavage (Fig. 5A). This single-nucleotide removal was 10-fold greater in the presence of Ku than in its absence, reflecting both an overall increase in cleavage and a change in cleavage specificity. The resulting 4-base overhang reached a maximum by 10 min and appeared to decrease slightly between 10 and 30 min, whereas shorter reaction products continued to accumulate. Just as with the 13-base overhang, this shift from longer to shorter overhangs at 10–30 min was seen only in those reactions containing Ku, consistent with Ku-dependent secondary cleavage of a portion of the longest initial product; without Ku, the three cleavage products accumulated in parallel (Fig. 5A, graphs). At longer times, there was little change in the overall cleavage pattern, although traces of shorter fragments (*), indicating trimming into duplex DNA, began to accumulate (Fig. 5A, top right). These data do not distinguish whether this late trimming was endo- or exonucleolytic, but subsequent experiments wherein a blunt-ended substrate was cleaved at the same sites (see Fig. 7) indicated the same dependence on activated DNA-PK.

A similar effect of PG termini on nuclease specificity was apparent with substrates having 4-base overhangs (Fig. 5B), i.e. almost exclusively (88%) 2-base trimming for the hydroxyl-terminated substrate, but approximately equal 1-base and 2-base trimming for the PG-terminated substrate. Unlike the longer overhangs, the hydroxyl- and PG-terminated 4-base overhang substrates did not show loss of longer reaction products or a significant change in product distribution over time, suggesting that the 2- and 3-base overhangs are poor substrates for secondary Artemis-mediated cleavage even in the presence of Ku. Inasmuch as there was almost no 3-base overhang produced from the hydroxyl-terminated 4-base substrate, the 3-base overhang generated from the 5-base PG-terminated substrate must have arisen by 2-nucleotide trimming of the initial substrate.

A titration with limiting Artemis (Fig. 5C) revealed that cleavage of the hydroxyl-terminated 5-base substrate by Artemis/DNA-PK shows the same 2-base removal specificity, as well as nearly complete Ku dependence, over a range of Artemis concentrations. When Artemis was limiting (5.6 nM), this cleavage was 17 times greater in the presence of Ku (26 ± 2%, n = 4)
than in its absence (1.5 ± 0.1%, n = 3), and increasing the Artemis concentration more than 20-fold did not fully compensate for the lack of Ku (Fig. 5C).

Finally, when substrates with 3-base overhangs were treated with Artemis, there was almost no processing of the hydroxyl-terminated substrate, whereas the PG-terminated substrate was processed almost exclusively (85–90%) by single-nucleotide removal, 90% of which was Ku-dependent (Fig. 6A and B). An analogous phosphotyrosyl-terminated substrate, typical of strand breaks induced by topoisomerase I inhibitors (20), was likewise processed primarily by single-nucleotide removal, albeit 3-fold less efficiently (Fig. 6A and B). To verify the terminal structure of the putative 2-base overhang product generated by removal of the terminal 3'-PG nucleotide, this product was subsequently treated with terminal transferase plus ddGTP, which produced the expected +1 nucleotide shift, demonstrating that the Artemis reaction product has a 3'-hydroxyl rather than a 3'-PG terminus (Fig. 6C, lane 6). Moreover, both the putative 14-mer and the resulting 15-mer precisely comigrated with authentic markers of the predicted sequence. As expected, the initial 3'-PG substrate was unaffected by treatment with terminal transferase, confirming that it had a blocked 3'-terminus (Fig. 6C, lane 5). As expected for Artemis-mediated cleavage, the band corresponding to the 2-base overhang product was only seen when Artemis, DNA-PKcs, and ATP were present in the reaction and when the substrate was subsequently cleaved with AvaI or TaqI (Fig. 6D).

Thus, Artemis nuclease trims short hydroxyl-terminated 3' overhangs predominantly by removal of two terminal nucleotides, whereas 3'-PG termini (and to a lesser extent 3'-phosphotyrosyl) alter this specificity, promoting trimming of a single nucleotide. For unmodified substrates, the minimum length...
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**FIGURE 6.** Single-nucleotide trimming of PG- and phosphotyrosyl-terminated 3-base 3’ overhangs by Artemis. A, time course and cleavage specificity. Plasmid substrates with the structures indicated (pTy r = phosphotyrosyl) were treated with Artemis plus DNA-PK as in Fig. 3 and then cut with AvaI. Numbers to the right indicate overhang length. B, quantitation of single-nucleotide trimming of a 3’-PG- (Col.), 3’-phosphotyrosyl- (C), or 3’-hydroxy terminated (A) overhang in the presence (closed symbols) or absence (open symbols) of Ku. Error bars show S.E. of three independent experiments with each substrate, when larger than the symbols. C, terminal transferase-mediated extension of the 3’-hydroxyl terminus generated by Artemis-terminated trimming of a 3’-PG-terminated 3-base overhang. Following treatment with Artemis and Aval as in A, samples were treated with terminal transferase (TdT) plus ddGTP. Lanes marked M contain 5’-end-labeled 14- or 15-base fragments of the expected sequence (TCGAGGAACGC- GAC and TCGAGGACCCGACGAC, respectively). For the 3’-PG substrate, essentially identical results were obtained when TaqI was used instead of Aval (data not shown). D, requirements for single-nucleotide trimming. The PG-terminated 3-base overhang was treated with Artemis plus DNA-PK for 30 min as in A, but various reaction components were omitted as indicated.

of overhang that will support efficient Artemis-mediated cleavage is 4 bases.

Slow Trimming of 3’-PG-terminated Blunt Ends—To further investigate the specificity of Artemis/DNA-PK, a series of plasmid substrates bearing a 3’-PG terminus on a 2-base overhang, a blunt end, or a 2-base recessed 3’ end were generated and assayed for susceptibility to Artemis nuclease (Fig. 7A). Although all substrates were subject to some degree of digestion, there was a pronounced decrease in nuclease efficiency and specificity for PG-terminated substrates having overhangs of less than 3 bases. For example, a 30-min Artemis treatment of a substrate with a PG-terminated blunt end or 2-base 3’ overhang yielded small amounts of multiple cleavage products, each of which accounted for <10% of the initial substrate (Fig. 7A and B). To assess the requirements, specificity, and extent of this processing, nuclease assays with the 3’-PG blunt-ended plasmid substrate were carried out for up to 2 h (Fig. 7B). Although control reactions with Ku and/or DNA-PKcs alone showed no significant trimming, those containing Artemis indicated time-dependent trimming corresponding to removal of 2–4 bases from the 3’-PG-terminated blunt ends (Fig. 7B). Although this trimming proceeded about seven times more slowly than trimming of a 5-base 3’ overhang, by 2 h nearly half (43 ± 3%, n = 3) of the blunt ends were trimmed (Fig. 7, C and D) in an Artemis-, DNA-PKcs-, and ATP-dependent manner. The addition of Ku stimulated this activity 5-fold but did not markedly alter the product profile. Thus, this processing, although slower and having different cleavage specificity than that of 3’ overhangs, exhibited the same protein and cofactor requirements. Similar results (not shown) were obtained with a PG-terminated 1-base 3’ overhang.

To assess whether the unlabeled 5’-terminal strand was still intact following trimming of the 3’ terminus, similar experiments were performed wherein the labeled oligomer was released by treatment with Aval, which requires a double strand substrate. Aval yielded essentially the same product profile as TaqI, which can cleave both single- and double-stranded DNA (Fig. 7E). This result implies that, at least for the majority of trimmed molecules, both strands were still intact at the Aval site and that trimming of the 3’ end was accompanied by neither 5’ → 3’ exonucleolytic resection or trimming of at most a few (<10) nucleotides from the 5’ terminus. Thus, Artemis plus DNA-PK is clearly able to process blunt 3’-PG ends, albeit more slowly than short overhangs, with removal of a few adjacent nucleotides.

Toxicity of 3’-Phosphoglycolate-terminated DSBs in Artemis-deficient Cells—The above data clearly show that Artemis can process 3’-PG-terminated DSBs. However, although hypersensitivity of Artemis-deficient mouse fibroblasts to bleomycin has been reported (21), a role for Artemis in processing of PG-terminated DSBs in intact human cells has not been established. To address this question, we assayed the toxicity of two drugs that induce different types of 3’-PG-terminated DSBs in vivo. Bleomycin treatment gives rise to DSBs, nearly all of which have either blunt ends or single-base 5’ overhangs, with 5’-phosphate and 3’-PG termini at both ends of the break. Neocarzinostatin-induced DSBs have at one end a 5’-phosphate and a 3’-phosphate on a 2-base 3’ overhang; the opposite end has a 5’-aldehyde and either a 3’-PG (~75%) or a 3’-phosphate (~25%) on a 1-base 3’ overhang (14, 15). Following treatment of exponentially growing cells with each of these agents, bromodeoxyuridine incorporation into ~10,000 cells was measured ~24 h after treatment, and from these data the proliferating fraction of cells was calculated (Fig. 8).

We previously showed that fibroblasts from Artemis-deficient SCIDA patients exhibit significantly elevated sensitivity to x-irradiation but a much lesser sensitivity to DSBs induced by
etoposide (6). Here we find that human telomerase reverse transcriptase-immortalized SCIDA fibroblasts show hypersensitivity to the DSB-inducing agent bleomycin, albeit not as great as their sensitivity to x-rays (Fig. 8A). Interestingly, DSBs produced by neocarzinostatin (~75% of which bear an overhanging 3'-PG terminus at one end of the break) appear to be nearly as toxic to SCIDA cells as x-rays (Fig. 8A and Table 1). Similarly, treatment with x-rays, neocarzinostatin, and bleomycin, all of which induce 3’-PG-terminated DSBs, results in at least 2-fold greater accumulation in G2/M in cycling SCIDA cells than in normal cells (Fig. 8B). Comparison of the relative toxicities of these three agents in multiple experiments reveals that the hypersensitivity of SCIDA cells is about the same for x-rays and neocarzinostatin but less for bleomycin (Table 1). Together, these data suggest that x-rays and radiomimetic drugs induce DSBs or other lesions that require Artemis for resolution prior to continued cellular proliferation.

DISCUSSION

Data presented here show that in the context of a variety of model DSB substrates, purified histidine-tagged Artemis efficiently cleaves long 3’ overhangs to a length of 4–5 nucleotides, in a reaction dependent on ATP and catalytically active DNA-PKcs. Our reactions with soluble purified proteins also reveal significant stimulation of Artemis activity by Ku for all substrates tested (Figs. 2, 3, and 5–7). Inasmuch as Ku is normally required for efficient DNA-PK assembly and self-activation on DNA ends, except at very low ionic strength (22, 23), and catalytically active DNA-PK is required to activate Artemis nuclease, it is expected that Ku would have a stimulatory effect on Artemis-mediated DNA cleavage. The reported Ku independence for immobilized Artemis activity (3) is likely because of the presence of excess DNA ends in those reactions, which in our hands almost completely suppresses the stimulatory effect of Ku (Fig. 2).

At low DNA terminus concentrations (1–5 nM), a Ku-mediated recruitment of Artemis/DNA-PKcs to DNA ends should markedly improve the reaction rate, as is observed (Fig. 2D; Fig. 3, A and C). At high DNA terminus concentrations (1 μM), the effect of any increase in affinity will be at least partially abrogated by increased binding to unlabeled competitor DNA, and the increase in cleavage will be less pronounced, as seen in Fig. 2. The finding that unlabeled 35-bp duplex does not compete with labeled substrate in the absence
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**A**

![Graph A](image1)

**B**

![Graph B](image2)

**FIGURE 8.** Cellular proliferation assays showing hypersensitivity of SCIDα cells to x-rays, neocarzinostatin, and bleomycin. A, percent of proliferating (BrdUrd-positive) cells 29 h after treatment with each agent was determined and normalized to the untreated control. Shown above the toxicity data are the structures of typical bleomycin and neocarzinostatin-induced DSBs, in some cases bearing 3′-PG (•) or 3′-aldehyde (■) termini. An estimated 75% of neocarzinostatin-induced DSBs have the structure shown, whereas the remainder have a 3′-phosphate rather than an 3′-phosphoglycolate. Radiation-induced DSBs will have short 5′ or 3′ overhangs of varied length with predominantly 5′-phosphate termini and ~50% 3′-PG termini. B, cell cycle distribution of BrdUrd-labeled cells 29 h after treatment with 5 grayscale (Gy) x-rays, 10 nM neocarzinostatin, or 8 µg/ml bleomycin.

Artemis under the same conditions resulted almost exclusively in 2-base trimming to a 3-base overhang, in a reaction that was almost completely dependent on Ku (Fig. 5C). Other substrates (Figs. 4C and 5A) show, to varying degrees, similar progression from longer to shorter cleavage products with time, and although time-dependent changes in Artemis specificity cannot be formally ruled out, all of the data can be explained by the same two-step mechanism wherein a portion of the initial cleavage products are subsequently trimmed, 2 bases at a time, until the overhang length is reduced to 3 bases or less.

Although 3′-blocking PG moieties on long 3′ overhangs have no discernible influence on Artemis endonuclease activity, the presence of 3′ PG closer to the site of cleavage significantly alters Artemis nuclease specificity, promoting single-nucleotide removal from short overhangs. Most strikingly, Artemis trims a 3-base 3′-PG overhang almost exclusively by single-nucleotide removal, while leaving an analogous 3′-hydroxyl substrate largely intact (Fig. 6).

Based on these and other results (Figs. 1–7), the site of Artemis-mediated cleavage of 3′ overhangs appears to be dependent on the following three criteria: 1) a requirement for either 2 nucleotides or a nucleotide plus a 3′-PG 3′ to the cleavage site; 2) a requirement for at least 2 unpaired nucleotides 5′ to the cleavage site; and 3) a preference for cleavage 4–5 bases from the single strand.double strand transition. It is likely that for long overhangs, DNA-PK at the double strand/single strand transition positions the Artemis active site for cleavage 4–5 bases from the transition. However, on shorter overhangs this positioning is preempted by the more stringent requirement for 2 nucleotides 3′ to the cleavage site, thus shifting the cleavage site closer to the double strand/single strand transition. A 3′-PG can apparently partially substitute for the terminal nucleotide, resulting in single-nucleotide removal from short PG-terminated overhangs. Unmodified overhangs shorter than 4 bases, and PG-terminated overhangs shorter than 3 bases, are much less efficiently processed, likely due to the fact that the requirements for 2 bases 3′ and 2 unpaired bases 5′ to the lesion cannot be simultaneously satisfied. Nevertheless, very short overhangs, blunt ends, and even recessed ends bearing 3′-PG termini show significant Artemis-mediated processing (Fig. 7), albeit at a much slower rate than longer overhangs, and after 2 h the 5-base overhang also shows traces of trimming into duplex regions. Overall, the observed patterns of cleavage are consist-

of Ku, but is an effective competitor in the presence of Ku (Fig. 2A), also suggests that the Artemis-DNA-PK complex has higher affinity for blunt ends in the presence of Ku than in its absence. Conversely, the finding that cleavage of very short overhangs is more strongly dependent on Ku than is cleavage of longer overhangs (compare Figs. 2F and 5C) is consistent with a model wherein contacts between Artemis and the long overhang can enhance affinity and thereby partially compensate for the lack of Ku. It is also notable that excess DNA ends, which should stimulate global DNA-PK kinase activity, do not stimulate Artemis nuclease activity (Fig. 2, A, C, and D). Rather, optimal nuclease activity is seen with the lowest concentrations of DNA ends, consistent with a model in which Ku and autophosphorylated DNA-PKcs form a stable complex with long 3′ overhangs (24), so that only local activation of DNA-PK on a given DNA end is important for Artemis nuclease activation.

In the case of a long (13-base) overhang on a plasmid substrate, the presence of Ku not only stimulated the cleavage reaction but also markedly altered the product distribution (Fig. 3). The data are most consistent with a two-step process, wherein the initial cleavage removes the extended 3′ single strand tail, leaving a 5-base overhang which, only in the presence of Ku, is subsequently trimmed by an additional 2 bases. Consistent with this idea, direct treatment of a 5-base overhang substrate with
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DSBs induced by neocarzinostatin bear PG termini, and then at only one end of the break (14, 15, 25), these PGs on 1-base 3’ overhangs will be refractory to Ape1 (26), as will PG termini of any x-ray-induced DSBs that have 3’ overhangs. Although radiation-induced DSBs are much more heterogeneous in both chemistry and geometry than those induced by neocarzinostatin, the fraction of DSB ends with protruding 3’-PG termini is predicted to be comparable, i.e. ~25% for radiation (the proportion of breaks with PG termini (~50%) times the proportion with 3’ overhangs (~50%)) versus ~37% for neocarzinostatin (one end of ~75% of the breaks). Within experimental error, this prediction correlates with the similar sensitivity of SCIDA cells to these two agents. Alternative PG removal from the blunt and 3’-recessed bleomycin-induced DSBs by Ape1 could account for the milder sensitivity of SCIDA cells to bleomycin (Table 1).

The only other enzyme known to be capable of processing 3’-PG termini is TDP1 (9), which has substrate preferences opposite those of Ape1, acting more efficiently on 3’ overhangs. However, processing of PG termini by TDP1 is also relatively inefficient, at least compared with its canonical 3’-phosphotyrosyl substrate (9), and TDP1 mutant cells are at best only marginally sensitive to x-rays (10). Thus, PG termini on recessed 3’ ends, blunt ends, and very short 3’ overhangs are not particularly favorable substrates for any of the three candidate enzymes (Ape1, TDP1, and Artemis), and of the three, only Artemis has been shown to interact with and be stimulated by other components of the nonhomologous -joining pathway. Although in vitro studies (Fig. 7) indicate that Artemis trims PG-terminated blunt ends very slowly (1–2 h), these kinetics are consistent with the slow time course of Artemis-dependent DSB repair in intact cells (5). Overall, the moderate hypersensitivity of SCIDA cells to radiation and radiomimetic drugs is consistent with Artemis providing an important pathway, but not the sole pathway, for repair of PG-terminated DSBs.

Alternatively, rather than being strictly required for processing 3’-PG DSBs, a more critical function for Artemis may be the resolution of derivative structures that can arise from them. In principle, a 3’-PG terminus will block gap filling and ligation but allow 5’ → 3’ resection by the Mre11-Rad50-NBS1 complex or other nucleases. Artemis, in the presence of DNA-PK, is capable of trimming any resulting blocked 3’ overhangs while they are still relatively short, at which point gap filling and ligation can proceed. However, blocked 3’ termini not resolved at this point may result in more extensive 5’ resection. Such resection in the context of an inverted repeat may lead to the formation of hairpin-like termini via annealing of the 3’ overhangs. This sequence of events would convert a fraction of 3’-blocked DSBs that were initially repairable by DNA-PK, TDP1, polynucleotide kinase/phosphatase, polymerase λ, and ligase IV (8) to hairpin structures, akin to V(D)J recombination intermediates, that strictly require Artemis for resolution. Thus, a failure to resolve 3’-PG structures at an early stage in the repair pathway could lead to an increased incidence of repair-resistant derivative structures, either the long 3’ overhangs themselves (25) or resulting hairpins. The observed accumulation of cycling Artemis-deficient cells in G2/M is consistent with the evolution of unresolved derivative DNA

### Table 1: Relative toxicity of DSB-inducing agents toward Artemis-deficient SCIDA cells (04 and 05) compared to normal cells (AK)

| Agent          | 04/AK       | 05/AK       |
|----------------|-------------|-------------|
| X-rays         |             |             |
| Exp. 1         | 3.88 ± 0.59 | 3.77 ± 0.60 |
| Exp. 2         | 2.08 ± 0.61 | 2.54 ± 0.57 |
| Exp. 3         | 2.54 ± 0.34 | 2.45 ± 0.47 |
| Average        | 2.83 ± 0.55 | 2.92 ± 0.43 |

Neocarzinostatin

| Exp. 1         | 2.0 ± 0.37  | 2.30 ± 0.34 |
| Exp. 2         | 2.55 ± 0.20 | 3.15 ± 0.20 |
| Exp. 3         | 3.66 ± 0.57 | 3.35 ± 0.34 |
| Average        | 2.74 ± 0.50 | 2.93 ± 0.33 |

Bleomycin

| Exp. 1         | 1.29 ± 0.29 | 1.42 ± 0.11 |
| Exp. 2         | 1.38 ± 0.05 | 1.61 ± 0.05 |
| Exp. 3         | 1.78 ± 0.10 | 1.92 ± 0.09 |
| Average        | 1.48 ± 0.15 | 1.65 ± 0.15 |

*Ratio of the toxicity of a given x-ray dose or drug concentration toward SCIDA cells to the toxicity of the same treatment in normal cells. Toxicity is defined as the change in ln(F), where F is the proliferating fraction of cells.

*Mean ± S.E. or range when n = 2, n = number of x-ray doses or drug concentrations analyzed. Treatments that reduced the proliferating fraction by less than 10% in normal cells were excluded.

*Mean ± S.E. of the average values obtained in the three independent experiments shown.

*Difference of significance between SCIDA and normal cells by Student’s t test.

*The hypersensitivity of SCIDA 05 cells to bleomycin was less than its hypersensitivity to x-rays (p < 0.05) or neocarzinostatin (p < 0.03). For SCIDA 04 cells these differences were not significant (p > 0.08).
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structures in these cells. This mechanism would also account for the relative lack of hypersensitivity of SCIDA cells to etoposide (5, 6), as etoposide-induced DSBs have 5' termini blocked with covalently bound topoisomerase II and would therefore not be subject to 5' resection.

Although there is evidence that Artemis is phosphorylated by ataxia telangiectasia-mutated, it is unlikely that hypersensitivity is because of a defect in cell cycle checkpoints, as Artemis deficiency does not confer a significant checkpoint defect (6, 27). Given that Artemis has no known activities other than DNA end-processing, and given that the radiomimetic compounds used here specifically induce DNA breaks and abasic sites almost exclusively, it seems unlikely that hypersensitivity is because of a defect wholly unrelated to DSB processing. Nevertheless, the possibility that efficient repair of some radiomimetic induced DSBs requires Artemis for reasons other than removal of chemically modified termini cannot be entirely excluded.

In summary, our results demonstrate that Artemis has the capability to carry out an essential step in repair of radiation-induced DNA damage, elimination of the 3'-PG blocking lesions commonly found at DSB termini. Although Artemis lacks the specificity to remove only the 3'-PG, it can nevertheless resolve this type of lesion by excising one or a few terminal nucleotides along with the contiguous 3'-PG moiety. This Artemis endonuclease activity is dependent on DNA-PKcs and stimulated by Ku, two core nonhomologous end-joining proteins. Moreover, like x-rays, agents that induce well characterized PG-terminated DSBs induce elevated toxicity in Artemis-deficient human cells. All of these findings are consistent with the proposal that the processing of PG-terminated DSBs, and/or derivative structures arising from them, is a biologically relevant activity of Artemis.

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