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DNA-PKcs Regulates a Single-stranded DNA Endonuclease Activity of Artemis

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Abstract

Human nuclease Artemis belongs to the metallo-beta-lactamase protein family. It acquires double-stranded DNA endonuclease activity in the presence of DNA-PKcs. This double-stranded DNA endonuclease activity is critical for opening DNA hairpins in V(D)J recombination and is thought to be important for processing overhangs during the nonhomologous DNA end joining (NHEJ) process. Here we show that purified human Artemis exhibits single-stranded DNA endonuclease activity. This activity is proportional to the amount of highly purified Artemis from a gel filtration column. The activity is stimulated by DNA-PKcs and modulated by purified antibodies raised against Artemis. Moreover, the divalent cation-dependence and sequence-dependence of this single-stranded endonuclease activity is the same as the double-stranded DNA endonuclease activity of Artemis:DNA-PKcs. These findings further expand the range of DNA substrates upon which Artemis and Artemis:DNA-PKcs can act. The findings are discussed in the context of NHEJ.

Keywords

nonhomologous DNA end joining; NHEJ; double-strand DNA break repair; V(D)J recombination; single-stranded DNA endonuclease

1. INTRODUCTION

Nonhomologous DNA end joining (NHEJ) is the primary DNA repair pathway for double-strand breaks in multicellular eukaryotes. Like most DNA repair pathways, NHEJ includes a nuclease to resect damaged DNA, polymerases to fill-in DNA, and a ligase to restore integrity of the DNA strands [1]. Among vertebrates, it appears that a complex of Artemis:DNA-PKcs provides endonuclease activity [2,3]. DNA-PKcs is a protein kinase which is only active when

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it is bound to a DNA end [4]. DNA-PKcs phosphorylates itself as well as other proteins [5]. DNA-PKcs autophosphorylation appears to change its conformation [6]. Artemis is a nuclease which only acquires endonuclease activity on double-stranded (ds) DNA when it is bound to an autophosphorylated DNA-PKcs [2,7–9]. DNA-PKcs also phosphorylates Artemis at 11 sites in the C-terminal portion of Artemis [7], and this region of Artemis is important for Artemis function in vivo [10]. In vitro, removal of this C-terminal region of Artemis permits it to function as an endonuclease independent of DNA-PKcs on short hairpins, indicating that the autophosphorylation-induced conformational change within DNA-PKcs appears to affect the position of the C-terminal region of Artemis.

The Artemis:DNA-PKcs complex endonuclease activities are interesting. The complex appears to localize to the junction of dsDNA and ssDNA and nick the DNA. For 5' overhangs, the complex cuts directly at the junction, generating a blunt duplex product [2]. For 3' overhangs, the complex prefers to cut on the single-stranded DNA (ssDNA) overhang 4 nt out from the ssDNA/dsDNA junction. Interestingly, the Artemis:DNA-PKcs complex also cuts perfect hairpins 2 nt past the hairpin tip and on the 3' side. All of these endonuclease activities are most consistent with a model in which DNA-PKcs helps Artemis localize to the junction of dsDNA and ssDNA; but the Artemis:DNA-PKcs complex appears to require 4 nt of ssDNA to bind, and then it cuts on the 3' side of that 4 nt stretch [2]. This would explain cutting directly at the ssDNA/dsDNA junction for 5' overhangs, but 4 nt away from the ssDNA/dsDNA junction on 3' overhangs [2,11]. Moreover, the last 2 bp of a perfect hairpin are known to be largely unpaired, thus providing 4 nt of ssDNA at the hairpin tip where Artemis:DNA-PKcs can bind [12–14]. Therefore, the model explains nicking of hairpins not at the very tip, but 2 nt past the tip on the 3' side [2].

Here we describe a new endonucleolytic property of Artemis. We find that Artemis alone has weak endonucleolytic activity on ssDNA (homopolymers). When DNA-PKcs is added, the Artemis:DNA-PKcs complex is markedly stimulated in this ssDNA endonuclease activity, and this is ATP-dependent and requires duplex DNA to stimulate DNA-PKcs kinase activity. Immunoinhibition studies using antibodies against Artemis confirm that the ssDNA endonuclease activity is intrinsic to Artemis. These findings further expand the range of substrates that Artemis and Artemis:DNA-PKcs can act upon. The relevance for NHEJ is discussed.

2. MATERIALS AND METHODS

2.1 Oligonucleotides

Oligonucleotides used in this study were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL, USA) and Integrated DNA Technologies, Inc. (San Diego, CA, USA). We purified the oligonucleotides using 12% or 15% denaturing polyacrylamide gel electrophoresis (PAGE) and determined the concentration spectrophotometrically.

DNA substrate 5' end labeling was done with [γ - 32 P]ATP (3000 Ci/mmol) (PerkinElmer Life Sciences, Boston, MA, USA) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) according to the manufacturer's instructions. Unincorporated radioisotope was removed by using G-25 Sephadex (Amersham Biosciences, Inc., Piscataway, NJ, USA) spin-column chromatography. For the hairpin substrate, YM164, labeled oligonucleotide was diluted in a buffer containing 10 mM Tris-hydrochloride, pH 8.0, 1 mM EDTA, pH 8.0 and 100 mM NaCl and then heated at 100°C for 5 min, allowed to cool to room temperature for 3 h, and then incubated at 4°C overnight.

DNA substrate 3' end labeling was done with [α - 32 P]TTP (3000 Ci/mmol) (PerkinElmer Life Sciences, Boston, MA, USA), ddTTP and terminal deoxynucleotidyl transferase

(Promega, Madison, WI, USA) according to the manufacturer's instructions. The molar ratio of [α - 32 P]TTP to ddTTP used was 1:5. Unincorporated radioisotope was removed by using G-25 Sephadex (Amersham Biosciences, Inc., Piscataway, NJ, USA) spin-column chromatography. Though this method generates a predominant substrate species, addition of more than one T at the 3' end results in a small amount of substrate that is 1 nt longer (and an even smaller amount that is 2 nt longer).

The sequences of the oligonucleotides used in this study are as follows:

JG167: 5'-AAA AAA AAA AAA AAA AAA AAA AAA AAA-3'

JG168: 5'-CCC CCC CCC CCC CCC CCC CCC CCC CCC-3'

JG169: 5'-TTT TTT TTT TTT TTT TTT TTT TTT TTT-3'

JG282: 5'-[P]TTT TTT TTT TTT TTT TTT TTT TTT TTT-3'

YM8: 5' AGG CTG TGT TAA GTA TCT GCG CTC GCC CTC AGA GG 3'

YM9: 5' CCT CTG AGG GCG AGC GCA GAT ACT TAA CAC AGC CT 3'

YM164: 5'-TTT TTG ATT ACT ACG GTA GTA GCT ACG TAG CTA CTA CCG TAG
TAA T-3'

2.2 Protein expression and purification

Artemis recombinant baculovirus with a C-terminal His (x8) tag (and an intervening TEV site) was a gift from Dr. John Harrington (Athersys, Ohio). Soluble Artemis-his is expressed and purified from Sf9 insect cells. Briefly, harvested Sf9 cells were resuspended in Ni-NTA binding buffer (50 mM NaH₂PO₄ (pH 7.8), 0.5 M KCl, 2 mM beta-mercaptoethanol, 10% glycerol, 0.1% Triton X-100, and 20 mM imidazole (pH 7.8)) supplemented with protease inhibitors and lysed by sonication. The cell lysate was applied to a Ni-NTA agarose resin (Qiagen). Artemis-his was eluted off with binding buffer plus 250 mM imidazole. Eluted fractions were dialyzed against DEAE binding buffer (50 mM Tris-HCl (pH 7.5), 10% glycerol, 2 mM EDTA, 1 mM DTT, 100 mM NaCl, and 0.02% NP-40), loaded onto preequilibrated DEAE-Sepharose column, and eluted with a linear gradient to 1 M NaCl over 20 mL. The yield at this point was typically 300 ug per 200 ml culture of Sf9. Artemis-his containing fractions were concentrated with Vivaspin 500 (Sartorius Stedim Biotech) and further purified by gel filtration on a Superose 12 column using a SMART system (Amersham Pharmacia) in gel filtration buffer (25 mM HEPES (pH 7.5), 0.5 M KCl, 10 mM MgCl₂, and 1 mM DTT). Eluted Artemis-his from Superose 12 was aliquoted and stored at -80°C.

The expression and purification of DNA-PKcs from Hela cells has been previously described [2].

2.3 Antibody production

Anti-Artemis antibody was produced as described [15]. In brief, a fragment encoding amino acid residues 347 to 692 of Artemis was fused to a hexahistidine tag by insertion into pET28 (Novagen). Purified recombinant protein from *E. coli* was used to raise antiserum in rabbits using standard protocols. Antisera were affinity purified using antigen that had been blotted and immobilized on nitrocellulose paper or by affinity chromatography.

2.4 In vitro nuclease assay

In vitro DNA nuclease assays were performed in a total volume of 10 μ l with a buffer composition of 25 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM MgCl₂, 1 mM DTT and 100 ng/ μ L BSA. In the reaction, 50 nM single-stranded DNA substrate or 20 nM hairpin substrate

were incubated with 50 nM Artemis and 50 nM DNA-PKcs unless otherwise specified. When DNA-PKcs was present, 0.25 mM of ATP and 0.25 μ M of 35bp blunt end DNA (YM 8/9) were also included in specified reactions. Reactions were then incubated at 37°C for 30 min. After incubation, reactions were stopped and analyzed on 12% denaturing PAGE gels. Gels were dried, exposed in a phosphorimager cassette and scanned.

3. RESULTS

3.1 Size exclusion chromatography of purified Artemis

Human Artemis-His was overexpressed with a baculovirus-insect cell system as described in the Methods. Purified Artemis from Ni-NTA columns and DEAE-Sepharose columns was further fractionated and purified on Superose 12 gel filtration columns (Fig. 1A). The predominant absorbance material elutes off the column as a single peak, which corresponds to a MW range of 239–292 kDa, based on the calibration curve generated with standard molecular weight markers (Fig. 1B). SDS-PAGE protein gels were run on all fractions and demonstrated a strong band visible in fractions 9 to 11 of the Superose 12 elution at 100 kDa, which is precisely the PAGE mobility of denatured Artemis (Fig. 1A, upper). Western blot analysis demonstrated that all bands in the lane are either full-length Artemis (mobility ~100kDa) or N-terminal proteolytic products of it (Suppl. Fig. 1).

The identity of the band was further confirmed on a linear ion trap LTQ (Thermo-Fisher) mass spectrometer. The Superose 12 fractions (6 μ g of fraction 9 and 4 μ g of fraction 11) containing active Artemis were concentrated and in-solution digested with trypsin. The digested peptide mixtures were analyzed by LC/MS/MS on a linear ion trap LTQ (Thermo-Fisher). For fractions 9 and 11, 31 and 50 peptides were identified, respectively. Only one protein was identified, and this was the recombinant human Artemis.

We searched two insect protein databases for any proteins that might co-purify with the Artemis, given that it was purified from baculovirus-infected insect cells. No insect proteins (or any other proteins) were identified.

3.2 Artemis has endonuclease activity on single-stranded DNA

After we obtained pure and homogeneous Artemis protein from the gel filtration Superose 12 column, we tested its activity on a variety of DNA substrates. Among these, we tested for activity on ssDNA. We designed four different homopolymer substrates to investigate whether there is any sequence preference for Artemis activity on ssDNA (Fig. 2). As previously reported, Artemis has 5' exonuclease activity on all of the substrates (see Section 4.1). Interestingly, Artemis also shows a pattern of nuclease activity on poly (dC) and poly (dT) substrates that cannot be explained by 5' exonuclease activity. This activity exhibits a substantial pyrimidine preference, because it does not act on poly (dA) or poly (dG) substrates (Fig. 2A, lanes 1–4 versus 5–12 and Fig. 2B), and a substrate of random sequence is cleaved (Fig. 2C). The nucleolytic products can be explained by either a sequence-dependent 3' exonuclease activity on ssDNA or a sequence-dependent endonuclease activity on ssDNA.

In order to differentiate these two possibilities, we performed time course studies. We used two poly (dT) substrates: one was labeled on the 5' end, and the other on the 3' end. To be comparable, the 3' end labeled substrate had a cold phosphate group on the 5' end. Then, Artemis and DNA substrates along with DNA-PKcs were incubated for various times (Fig. 3). As we can see from both panels, the products in 30 min reactions have the same pattern as in the 6, 10, and 20 min reactions, but with increased intensity with increasing time, which indicates that Artemis has endonuclease activity on ssDNA rather than 3' exonuclease activity. The ssDNA endonuclease activity of Artemis:DNA-PKcs seems to have a slight preference

for sites closer to the 5' portion of this 30 nt substrate, perhaps reflecting how the Artemis:DNA-PKcs loads onto ssDNA substrates (e.g., Artemis may bind more tightly when there is more DNA length 3' of the cleavage site). The amount of the endonuclease activity correlates with the amount of the Artemis protein (Fig. 1C).

3.3 Autophosphorylation of DNA-PKcs regulates the single-stranded DNA endonuclease activity of Artemis

Artemis alone has a basal level of ssDNA endonuclease activity (Fig. 2A, lane 6 and 10). However, with activated DNA-PKcs present in the reaction, this ssDNA endonuclease activity of Artemis is dramatically increased (Fig. 2A, lane 8 and 12). To further understand the regulation of the ssDNA endonuclease activity of Artemis by DNA-PKcs, we carried out additional experiments. It is interesting to see this regulation is not only dependent on DNA-PKcs, but also on ATP and the required duplex DNA to stimulate DNA-PKcs (Fig. 4A, lanes 6, 7, 9 versus lane 8), which suggests that protein phosphorylation plays an important role in this regulation.

It is clear that the dsDNA endonuclease activity of Artemis requires DNA-PKcs and ATP [2]. However, the underlying mechanism for activating the endonuclease activity of Artemis by DNA-PKcs is still a subject of investigation. Jeggo and colleagues showed that autophosphorylation of DNA-PKcs is necessary and sufficient to activate the dsDNA endonuclease activity of Artemis [16]. In order to elucidate the mechanism of regulation of the ssDNA endonuclease activity of Artemis by DNA-PKcs, we performed a corresponding experiment (Fig. 4B). We pre-autophosphorylated DNA-PKcs in the presence of ATP and unlabeled dsDNA. We then added this preparation to Artemis (Fig. 4B, lane 9). For comparison, in order to prevent DNA-PKcs from phosphorylating Artemis, we added wortmannin to the Artemis before adding the pre-autophosphorylated DNA-PKcs (Fig. 4B, lane 8). [Wortmannin blocks the ATP binding pocket for many PI3 kinases (see [16]).] The Artemis ssDNA endonuclease activity was similar for both reactions. This shows that pre-autophosphorylation of DNA-PKcs is sufficient to stimulate the ssDNA endonuclease activity of Artemis, just like the dsDNA endonuclease of Artemis:DNA-PKcs (Fig. 4B lane 4 versus lane 8). We have previously shown that Artemis and DNA-PKcs form a complex [2]. The conformational change within DNA-PKcs upon autophosphorylation may cause a conformational change within the bound Artemis, which stimulates Artemis activity, similar to what we have previously described for duplex DNA with overhangs and for hairpin DNA [2]. Based on this, we speculate that the ssDNA endonuclease activity and dsDNA endonuclease activity of Artemis are properties of the same active site within Artemis. (Though we do not favor it, another formal possibility is that DNA-PKcs autophosphorylation changes how DNA-PKcs binds to the DNA, thereby affecting Artemis recognition of the DNA [16].)

3.4 Single-stranded DNA endonuclease activity is intrinsic to Artemis

To further verify that this ssDNA endonuclease activity is intrinsic to Artemis, we performed an immunoinhibition assay with purified anti-Artemis antibody (Figure 5). Purified anti-Artemis antibody successfully abolished the 5' overhang and hairpin opening endonuclease activity of Artemis:DNA-PKcs, while heat-inactivated purified anti-Artemis antibody did not have any effect (Fig. 5, lanes 11–14) nor did an anti-RAG2 control antibody (not shown). When DNA-PKcs is present, anti-Artemis antibody reduced the ssDNA endonuclease activity of Artemis nearly down to the basal level (Fig. 5, lane 7 versus lane 2). This indicates that the anti-Artemis antibody likely interferes with a productive conformational interaction between Artemis, DNA-PKcs and the DNA substrate. This may explain why there is no effect of the antibody on the 5' exonuclease activity (see Section 4.1). It is interesting to note that anti-Artemis antibody up-regulates the ssDNA endonuclease activity of Artemis in the absence of DNA-PKcs (Fig. 5, lane 5 versus lane 2). Since the anti-Artemis antibody itself does not carry

any nuclease activity (Fig. 5, lane 10), this suggests that Artemis adopts a conformation with higher activity when the anti-Artemis antibody is bound, which illustrates that the basal level of the ssDNA endonuclease activity is also intrinsic to the Artemis protein.

We and others have observed an interesting phenomenon that Artemis can acquire dsDNA endonuclease activity in the absence of DNA-PKcs under Mn^{2+} conditions [10](Lu, H & MRL, unpublished). The single-stranded DNA endonuclease activity of Artemis was also tested under Mn^{2+} condition (Fig. 6). Interestingly, Mn^{2+} up-regulates the ssDNA endonuclease activity of Artemis relative to the level with Mg^{2+} , similar to the effect by DNA-PKcs (Fig. 6, lane 3 versus lane 2). This is consistent with the conclusion above that the ssDNA endonuclease activity is intrinsic to Artemis, and it is closely related to its dsDNA endonuclease activity.

4. DISCUSSION

4.1 Single-stranded endonuclease activity of Artemis and Artemis:DNA-PKcs

Using Artemis purified on myc-antibody immunobeads, we previously showed that Artemis:DNA-PKcs can cut 5' overhangs, 3' overhangs, and hairpins endonucleolytically [2]. Artemis alone also appears to have 5' exonuclease activity (see below) [2,3,9,17]. We have now further purified Artemis using an additional high-resolution size exclusion chromatography step beyond the affinity tag and ionic exchange chromatography steps. This more highly purified preparation has all of the nucleolytic properties that we described previously. But, in addition, we have been able to elucidate a ssDNA endonuclease activity of Artemis alone, which profiles the amount of Artemis protein quite tightly (Fig. 1), and is not present in an identical purification of mock-infected insect cells (not shown). Human Artemis is the only protein detectable in our protein preparations by mass spectrometry. The ssDNA endonuclease activity is stimulated by DNA-PKcs and modulated by Mn^{2+} in a manner that is quite similar to modulation of Artemis:DNA-PKcs activity on dsDNA overhang and hairpin substrates (Fig. 2, Fig. 4, Fig. 5 and Fig. 6). In addition, purified antibodies raised against Artemis modulate this ssDNA endonuclease activity at the same concentrations as those that modulate the previously described activities of Artemis:DNA-PKcs (Fig. 5). Finally, the ssDNA endonuclease and the dsDNA endonuclease of Artemis:DNA-PKcs have the same polypyrimidine >> polypurine preference (Fig. 2). We conclude that the ssDNA endonuclease activity is intrinsic to Artemis.

Upon stimulation with DNA-PKcs, we note that mutants of Artemis that have lost the dsDNA endonuclease activity also clearly have lost the ssDNA endonuclease (not shown). We have not included this data because these Artemis mutants, unlike the wild type Artemis described in this paper, are purified from mammalian cells rather than insect cells, and the purity is slightly lower, resulting in the presence of a contaminating exonuclease. The ssDNA endonuclease of Artemis or Artemis:DNA-PKcs is weaker than the dsDNA endonuclease of Artemis:DNA-PKcs, and thus a small amount of contaminating nuclease is easily apparent. Though the mammalian mutants reassure us that the ssDNA endonuclease is intrinsic to Artemis, we have only presented studies here with the much higher purity Artemis derived from an insect cell expression system.

Could the ssDNA endonuclease activity account for the 5' exonuclease activity of Artemis alone? This is clearly not the case because these have different nucleolytic properties. Moreover, the 5' exonuclease activity of Artemis is not stimulated by DNA-PKcs. In fact, the 5' exonuclease activity of Artemis is the only activity of Artemis that is not responsive to DNA-PKcs. This raises the question of whether the 5' exonuclease could be a contaminant. In this regard, point mutants of Artemis that destroy the overhang and hairpin opening activities of Artemis:DNA-PKcs do not affect the 5' exonuclease activity of Artemis [18]. However, the point mutants thus far may affect the binding of the DNA substrate rather than the catalytic

active site, which is still not fully defined [18]. The binding of substrates for the 5' exonuclease activity may be different than the binding for endonucleolytic cleavage. In addition, the 5' exonucleolytic activity is present not only after the Ni-NTA affinity tag step and DEAE-Sephacrose chromatography step, but also after the additional high resolution gel filtration step. Finally, mass spectrometry on the Artemis peak from the gel filtration column identified no other proteins than human Artemis (including no insect cell proteins). Despite this, definitive determination of whether the 5' exonuclease is intrinsic to Artemis must await further mutagenesis. (The reader should not confuse this discussion about the 5' exonuclease in this paragraph with the ssDNA endonuclease discussed in the rest of this study. Admittedly, one cannot rule out contamination of any activity without more extensive analysis of mutant forms of the enzyme. But we are convinced that the ssDNA endonuclease activity of Artemis described here is intrinsic to Artemis for the reasons enumerated in the first paragraph of this section.)

4.2 Model unifying all of the endonucleolytic activities of Artemis

All of the endonucleolytic activities of Artemis appear to function similarly. These lead to a model in which Artemis prefers ~4 nt of ssDNA to bind any type of DNA substrate (Fig. 7). After binding, Artemis nicks on the 3' side of that 4 nt binding site. When DNA-PKcs is present, Artemis exists in an Artemis:DNA-PKcs complex. Upon autophosphorylation of DNA-PKcs, a conformational change occurs which stimulates Artemis endonucleolytic activity on single-stranded substrates. DNA-PKcs causes the Artemis:DNA-PKcs complex to favor junctions of ssDNA and dsDNA. All of the endonucleolytic properties of Artemis and Artemis:DNA-PKcs are consistent with this model. We acknowledge (but do not favor) the possibility that autophosphorylated DNA-PKcs may alter the conformation of the DNA substrate [16] [8].

4.3 Artemis pyrimidine preference and relationship to such preferences by polymerase mu and the DNA ligase IV complex

The endonucleolytic action of Artemis on ssDNA is much greater for poly (dC) and poly (dT) substrates than for poly(dA) and poly (dG) substrate. This preference also exists for Artemis:DNA-PKcs action on overhangs. For example, a 3' overhang of four Gs followed by six Ts is not cleaved nearly as efficiently as one with entirely consecutive Ts (data not shown). Proteins that bind ssDNA often show biases for either purines or pyrimidines [19]. For a random ssDNA sequence, the strongest cleavage positions appear to be on the 5' side of each pyrimidine nucleotide (Fig. 2C), though cleavage at purine nucleotides does occur; this suggests that the sequence effect on cleavage activity may be at least partly on the binding of Artemis to the ssDNA.

It is interesting that the XRCC4:DNA ligase IV complex can ligate ssDNA, but this is only efficient for poly (dT) substrates [20]. Moreover, polymerase mu, one of the primary polymerases for NHEJ, can add dC or dT in a template-independent manner much more efficiently than dA or dG [21]. Hence, the nuclease, major polymerase, and the ligase of NHEJ all have pyrimidine preferences. It will be interesting to see if there is some unifying physiologic reason for such consistent pyrimidine preferences by the various NHEJ enzymatic activities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Size exclusion chromatography of purified Artemis

(A) Superose 12 gel filtration chromatogram of purified Artemis and corresponding SDS-PAGE gel stained with Coomassie Blue on which Artemis has a gel mobility position at ~100 kDa. The protein markers are in the left-most lane, and the fraction numbers are below each lane.

(B) Superose 12 elution volume plot of Artemis and molecular weight markers. The Artemis identity was further confirmed by mass spectrometry, and no other proteins were identified by mass spectrometry (see text).

(C) Fractions across the Superose 12 elution peak were assayed for nuclease activity using the poly (dT) substrate (JG169). In each reaction, 50 nM single-stranded DNA substrate (JG169) was incubated with the protein(s) indicated above the lane in a 10 μ l reaction for 30 min at 37 $^{\circ}$ C. After incubation, reactions were stopped and analyzed by 12% denaturing PAGE.

Concentrations are as follows: Artemis, 50 nM; DNA-PKcs, 50 nM, and 0.25 mM ATP.

Figure 2. Endonuclease activity of Artemis on single-stranded DNA
In each reaction, 50 nM single-stranded DNA substrates (JG167, JG168, JG169, JG175 or YM8) or 20 nM hairpin opening substrate (YM164) were incubated with the protein(s) indicated above the lane in a 10 μ l reaction for 30 min at 37°C. After incubation, reactions were stopped and analyzed by 12% denaturing PAGE. Protein concentrations are as follows: Artemis, 50 nM; DNA-PKcs, 50 nM. As specified, 0.25 mM of ATP and 0.25 μ M of YM8/9 were also included in some of the reactions. YM8/9 is a 35 bp blunt end double-stranded DNA, which is used as DNA-PKcs cofactor. The hairpin DNA substrate (YM164) is sufficient to stimulate DNA-PKcs directly [22], but YM8/9 further stimulated the autophosphorylation level of DNA-PKcs and the endonuclease activity of Artemis (lane 15 versus 16).

Figure 3. Time course of endonuclease activity of Artemis and DNA-PKcs on single-stranded DNA (A) In each reaction, 50 nM 5' labeled single-stranded DNA substrate, JG169, was incubated with 45 nM Artemis, 50 nM DNA-PKcs, 0.25 mM ATP and 0.25 uM YM8/9 in a 10 μ l reaction for indicated times at 37°C. After incubation, reactions were stopped and analyzed by 12% denaturing PAGE.

(B) In each reaction, 50 nM 3' labeled single-stranded DNA substrate, JG282, was incubated with 45 nM Artemis, 50 nM DNA-PKcs, 0.25 mM ATP and 0.25 uM YM8/9 in a 10 μ l reaction for indicated times at 37°C. After incubation, reactions were stopped and analyzed by 12% denaturing PAGE.

Figure 4. Autophosphorylation of DNA-PKcs regulates the single-stranded DNA endonuclease activity of Artemis

(A) In each reaction, 50 nM single-stranded DNA substrate, JG169, was incubated with the reagents indicated above the lane in a 10 μ l reaction for 30 min at 37°C. After incubation, reactions were stopped and analyzed using 12% denaturing PAGE. Reagent concentrations were as follows: Artemis, 50 nM; DNA-PKcs, 50 nM; ATP, 0.25 mM; YM8/9, 0.25 μ M.

(B) In each reaction, 50 nM single-stranded DNA substrate JG169 was incubated with the reagents indicated above the lane in a 10 μ l reaction for 30 min at 37°C. After incubation, reactions were stopped and analyzed by 12% denaturing PAGE. Reagents demarcated by dotted rectangles were preincubated with nuclease buffer on ice for 30 min. Reagent

concentrations were as follows: Artemis, 45 nM; DNA-PKcs, 50 nM; ATP, 0.25 mM; YM8/9, 0.25 uM; wortmannin, 5 uM. “W” indicates wortmannin. “D” indicates DMSO. Wortmannin was dissolved in DMSO. An equal amount of DMSO was used as a control in lanes 5 and 9.

Substrate	JG*169 (Poly dT)										YM*164 (Hairpin)			
Artemis	-	+	+	+	+	+	+	+	+	-	-	+	+	+
Antibody (uL)	-	-	-	0.2	0.5	0.2	0.5	<u>0.5</u>	<u>0.5</u>	0.5	-	-	0.5	<u>0.5</u>
DNA-PKcs	-	-	+	-	-	+	+	-	+	-	-	+	+	+
ATP	-	-	+	-	-	+	+	-	+	-	-	+	+	+
YM8/9	-	-	+	-	-	+	+	-	+	-	-	+	+	+
	1	2	3	4	5	6	7	8	9	10	11	12	13	14

Figure 5. Anti-Artemis antibody down-regulates the single-stranded DNA endonuclease activity of Artemis in the presence of DNA-PKcs

Artemis and purified anti-Artemis antibody were preincubated with nuclease buffer on ice for 20 min. Double-underlined purified anti-Artemis antibody amounts designate that this is heat-inactivated ($100^{\circ}\text{C} \times 5 \text{ min}$) and used as negative control. After preincubation, 50 nM single-stranded DNA substrate, JG169, or 20 nM hairpin opening substrate, YM164, was added to each 10 uL reaction along with other reagents indicated above the lane. Reactions were further incubated for 30 min at 37°C . After incubation, reactions were stopped and analyzed using 12% denaturing PAGE. Reagent concentrations were as follows: Artemis, 50 nM; DNA-PKcs,

50 nM; ATP, 0.25 mM; YM8/9, 0.25 uM. Amounts of purified anti-Artemis antibody are specified.

Substrate	JG*169 (Poly dT)						
Artemis	-	+	+	+	+	+	+
DNA-PKcs	-	-	-	-	+	+	+
ATP	-	-	-	-	+	+	+
YM8/9	-	-	-	-	+	+	+
Cation	Mg	Mg	Mn	Zn	Mg	Mn	Zn
	1	2	3	4	5	6	7

Figure 6. Artemis single-stranded DNA endonuclease activity with different divalent cations

In each reaction, 50 nM single-stranded DNA substrate, JG169, was incubated with the reagents indicated above the lane with different divalent cations (MgCl_2 , MnCl_2 and ZnCl_2) in a 10 μl reaction for 30 min at 37°C. After incubation, reactions were stopped and analyzed by 12% denaturing PAGE. Reagent concentrations were as follows: Artemis, 17.5 nM; DNA-PKcs, 50 nM; ATP, 0.25 mM; YM8/9, 0.25 μM ; MgCl_2 , 10 mM; MnCl_2 , 5 mM; ZnCl_2 , 1 mM. Artemis fractions used in this experiment were purified using Ni-NTA followed by a DEAE-Sepharose column, without the gel filtration step.

Action of Artemis on ssDNA

Action of Artemis:DNA-PKcs on overhangs of dsDNA

Figure 7. Artemis endonuclease activity on single-stranded DNA and double-stranded DNA

The model shows Artemis as a grey-filled oval and DNA-PKcs as a rectangle. Artemis appears to prefer to bind to 4 nt of ssDNA and cut immediately 3'. This behavior is most noticeable for duplex substrates with overhangs. But for ssDNA substrates, the preferred 4 nt zone can be anywhere. We have previously raised the possibility that DNA-PKcs might bend the single-stranded overhang into a near-hairpin conformation, in order for the associated Artemis to cut [7,8]; but for simplicity, we have drawn the overhangs and ssDNA as artificially straight (unbent).