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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.

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 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].

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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 [gamma-^{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 [alpha-^{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 [alpha-^{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 AAA-3′
JG168: 5′-CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC-3′
JG169: 5′-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT-3′
JG282: 5′-[P]TTT 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 (×8) 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 NaH_2PO_4 (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 μg 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 $MgCl_2$, 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 347–692 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 μl with a buffer composition of 25 mM Tris–HCl (pH 8.0), 10 mM KCl, 10 mM $MgCl_2$, 1 mM DTT and 100 ng/μl 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 μM of 35 bp 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 9–11 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 ∼100 kDa) 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 μg of fraction 9 and 4 μg 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