DNA-PKcs Dependence of Artemis Endonucleolytic Activity, Differences between Hairpins and 5’ or 3’ Overhangs

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Doris Niewolik§1, Ulrich Pannicke§1, Haihui Lu§1, Yunmei Ma§1, Ling-Chi Vicky Wang§, Peter Kulesza§, Ebrahim Zandi§, Michael R. Lieber§2, and Klaus Schwarz§3

From the §1Institute for Clinical Transfusion Medicine and Immunogenetics and the Department of Transfusion Medicine, the University Hospital Ulm, D-89081 Ulm, Germany and the §Departments of Pathology, Biochemistry and Molecular Biology, Biological Sciences, and Molecular Microbiology and Immunology and the §Department of Molecular Microbiology and Immunology, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California 90089-9176

During V(D)J recombination, the RAG proteins create DNA hairpins at the V, D, or J coding ends, and the structure-specific nuclease Artemis is essential to open these hairpins prior to joining. Artemis also is an endonuclease for 5’ and 3’ overhangs at many DNA double strand break events by ionizing radiation, and Artemis functions as part of the nonhomologous DNA end joining pathway in repairing these. All of these activities require activation of the Artemis protein by interaction with and phosphorylation by the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). In this study, we have identified a region of the Artemis protein involved in the interaction with DNA-PKcs. Furthermore, the biochemical and functional analyses of C-terminally truncated Artemis variants indicate that the hairpin opening and DNA overhang endonucleolytic features of Artemis are triggered by DNA-PKcs in two modes. First, autoinhibition mediated by the C-terminal tail of Artemis is relieved by phosphorylation of this tail by DNA-PKcs. Thus, C-terminally truncated Artemis derivatives imitate DNA-PKcs-activated wild type Artemis protein and exhibit intrinsic hairpin opening activity. Second, DNA-PKcs may optimally configure 5’ and 3’ overhang substrates for the endonucleolytic function of Artemis.

V(D)J recombination is the somatic recombination in precursor lymphocytes by which functional and highly diverse immunoglobulin and T cell receptor genes are generated (1–3). This process is initiated by the recombination activating complex (composed of the RAG1, RAG2, and HMG1 proteins). The RAG proteins cleave the DNA at the border between signal sequences and their adjacent coding segments and thereby generate hairpin structures at the DNA termini of these double strand breaks. Subsequently, the factors of the nonhomologous end joining (NHEJ) pathway reopen the hairpins and join the coding ends (2, 4–6). Components involved are the Ku proteins (Ku70 and Ku86), the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), the nuclease Artemis, and the ligase complex XRCC4-DNA ligase IV (6, 7). The role of the recently identified NHEJ factor XLF/Cernunnos in this process remains to be clarified (8, 9).

In humans, a defect in either RAG-1/-2, Artemis, DNA ligase IV, or XLF/Cernunnos leads to the phenotypes of severe combined immunodeficiency, Omenn syndrome, or other immunodeficiencies with perturbed lymphocyte development, because of the impaired generation of immunoglobulin and T cell receptor genes. Except for RAG deficiencies, cells of these patients also display an increased radiosensitivity, because of a general deficiency in NHEJ (8–18).

The nuclease Artemis is a recently discovered factor involved in V(D)J recombination and NHEJ (14). When associated with and phosphorylated by DNA-PKcs, Artemis possesses hairpin opening and DNA overhang endonucleolytic activities in vitro. Artemis recognizes structural discontinuities of single to double strand DNA transitions as targets for these enzymatic activities (19, 20). Without DNA-PKcs activation, Artemis exhibits 5’- to 3’-exonuclease activity in vitro on single-stranded DNA and on 5’ overhangs of double-stranded DNA (19).

In the process of DNA double strand break (DSB) repair, Artemis and the kinase ataxia telangiectasia mutated protein (ATM) function in a common pathway, which also requires H2AX, 53BP1, Nbs1, Mre11, and DNA-PKcs. Following ionizing radiation treatment, about 10% of the DSBs are rejoined by an ATM-dependent pathway (21). Importantly, ATM-negative cells are proficient in V(D)J recombination, demonstrating that ATM is dispensable for hairpin opening activities of Artemis.

Artemis is a member of the large metallo-β-lactamase superfamily, since a metallo-β-lactamase domain was identified in the N-terminal part of the protein (amino acids 1–155) (22). Additionally, amino acids (aa) 156–385 of Artemis share several conserved features with other metallo-β-lactamases specifically acting on nucleic acids. This domain was named β-CASP motif (metallo-β-lactamase-associated CPSF Artemis SNM1...
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It was shown that the catalytic core of Artemis is located in these two domains (23, 24) and that an Artemis variant (aa 1–385) with the C-terminal domain (missing aa 386–692) is able to restore V(D)J recombination capability of Artemis-deficient cells (24). The function of the C-terminal domain (aa 386–692), which is encoded by a single exon (exon 14), has yet to be determined. Recently, 11 in vitro DNA-PKcs phosphorylation sites on Artemis were identified in addition to three basal phosphorylation sites, the latter three being dispensable for Artemis activation (25). All of these phosphorylation sites are located within the C-terminal domain of the protein.

In this study, we demonstrate that amino acids 398–403 are important for the interaction of Artemis with DNA-PKcs. In vivo V(D)J recombination assays performed with C-terminally truncated variants of Artemis show that deletion of amino acids 383–692 still allows for V(D)J recombination, albeit at a significantly reduced level, and this correlates with loss of DNA-PKcs binding activity. Furthermore, we found that Artemis variants missing the C-terminal domain in vitro exhibit, in contrast to wild type Artemis, hairpin opening activities that are independent of the presence of the DNA-PKcs and ATP. These results confirm our previously suggested model of a regulatory function of the C-terminal domain of the wild type Artemis protein.

In the absence of ATP, DNA-PKcs blocks the hairpin opening activities of the C-terminally truncated Artemis variants suggesting that DNA-PKcs must be autophosphorylated to give Artemis access to the hairpin structures. The 5’ and 3’ overhang endonucleolytic activities of the C-terminally truncated Artemis derivatives remain, at least partially, dependent on the presence of DNA-PKcs and ATP. However, this DNA-PKcs dependence is neither associated with a physical interaction between the C-terminally truncated Artemis protein and DNA-PKcs nor with specific in vitro phosphorylation of this Artemis mutant by DNA-PKcs. We propose that in the in vitro nuclease assay, DNA-PKcs and ATP are needed to force the 5’ and 3’ overhangs into the appropriate substrate conformation for Artemis endonucleolytic activity.

**Experimental Procedures**

Protein Expression Constructs—Human wild type Artemis expression plasmids were constructed as described (23). For construction of expression plasmids coding for ARM32 (Artemis amino acids 1–382), ARM37 (aa 1–413), ARM57 (aa 1–408), ARM58 (aa 1–403), ARM59 (aa 1–398), ARM60 (aa 1–392), and ARM61 (aa 1–403), Artemis cDNA was amplified using primers Kpn1ARTcDNA5′-ATAAGAATGCGGCCGCATGGCAGAGGATCATCAAA-3′, Not1ARM37reverse (5′-GGGGTACCGCTATG-GAG-3′), Not1ARM59reverse (5′-ATAAGAATGCGGCCGCCAGTGAACTGTTCTAGCTCT-3′), Not1ARM57reverse (5′-ATAAGAATGCGGCCGCACGGGTATGGAACTTTGT-3′), Not1ARM58reverse (5′-ATAAGAATGCGGCCGCCTTTCCTTCGAGGG-3′), and Not1ARM61reverse (5′-ATAAGAATGCGGCCGCCTTTCCTTCGAGGG-3′), respectively. Subsequently, the cDNA fragments were cloned into the vectors pcDNA6/myc-His version A and pcDNA6/V5-His version A (Invitrogen).

The expression plasmid pPK1 encoding full-length human DNA-PKcs was constructed as described (26). The correctness of the cDNAs encoding ART-WT, its variants, and DNA-PKcs were confirmed by sequencing.

**Cells and Transfections**—HEK293T cells were transfected using the calcium phosphate precipitation method (27). Human primary Artemis-negative fibroblasts were transfected using AMAXA NHDF-Neo nucleofector kit (AMAXA Biosystems, Cologne, Germany) as described (23). Cell lines (AA8, V-3, and V-3R5) derived from Chinese hamster ovary (CHO) cells were transfected using AMAXA nucleofector kit T (AMAXA Biosystems). Equal transfection efficiencies were confirmed by co-transfection of an enhanced green fluorescent protein expression plasmid followed by FACS analysis.

**Immunoprecipitation Assay and Immunoblotting**—The immunoprecipitation assay was performed as described (19). Endogenous DNA-PKcs was detected using anti-DNA-PKcs antibodies 254 and 42psc (Neomarkers, Fremont, CA). Myc-tagged DNA-PKcs and Myc-tagged Artemis were immunoprecipitated and detected with anti-Myc antibody (Invitrogen). V5-tagged Artemis was detected with anti-V5 antibody (Invitrogen). SV40 T-antigen was used as sample loading control and was detected with anti-SV40 T-Ag antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**In Vitro V(D)J Recombination Assay**—The cellular V(D)J recombination assay and FACS analysis of transfected cells were carried out as described (23). Cells used were primary human dermal fibroblasts and the DNA-PKcs-negative CHO-derivatives V-3.

**In Vitro Nuclease Assay**—Details of the DNA-PKcs and Artemis purification are described in Refs. 19 and 28. Substrates used in the in vitro nuclease assays were described elsewhere (19, 23). 25 nm of the 5′-labeled substrate was incubated with 100 nm Artemis in 25 mm Tris, pH 8.0, 10 mm KCl, 10 mm MgCl₂, 1 mm dithiothreitol, 0.25 μM of 40-bp DNA (nonspecific DNA-PKcs cofactor), and 50 μg/ml bovine serum albumin in a total volume of 10 μl. Where indicated, 50 nm DNA-PKcs and 0.25 mm ATP were included in the reactions. Reactions were incubated for 1 h at 37 °C and then denatured for 5 min at 100 °C in an equal volume of denaturing gel loading dye (98% formamide, 10 mm EDTA, 0.025% of bromphenol blue, and 0.025% of xylene cyanol FF). Reaction mixtures were resolved by 12% denaturing PAGE, and the gel image was obtained with PhosphorImager SI445 (Amersham Biosciences).

**DNA-PKcs Kinase Assay**—The DNA-PKcs kinase assay was carried out as described (19).

**Immunostaining of AA8 and V-3 Cells**—The Chinese hamster ovary cell lines AA8 (DNA-PKcs-positive) and V-3 (DNA-PKcs-negative) were transfected by nucleofection using the AMAXA nucleofector kit T (AMAXA Biosystems) with empty pcDNA6/myc-His version A or plasmid coding for wild type or mutant forms of Artemis. After 48 h, cells were washed twice in 1× PBS (without Ca²⁺ and Mg²⁺). The cells were fixed in 1.5% paraformaldehyde, washed twice in 1× PBS (without Ca²⁺ and Mg²⁺), and treated with 50 mm NH₄Cl. Afterward, the cells were permeabilized in buffer A (0.25% gelatin, 0.01% saponin in...
PBS (without Ca\(^{2+}\) and Mg\(^{2+}\)) supplemented with 0.2% Nonidet P-40. The cells were washed three times in buffer A and incubated with mouse anti-Myc antibody (diluted 1:2000 in buffer A; Invitrogen) overnight at room temperature. After three washes in buffer A, the cells were incubated with goat anti-mouse IgG-fluorescein isothiocyanate (diluted 1:400 in buffer A; Santa Cruz Biotechnology) for 1 h at room temperature followed by two washes in buffer A and two washes in 1× PBS (without Ca\(^{2+}\) and Mg\(^{2+}\)). Subsequently, cells were analyzed by confocal fluorescence microscopy.

Ionizing Radiation Assay—We generated vectors that express EGFP only or simultaneously express EGFP and either wild type Artemis (ART-WT) or mutant Artemis (ARM23) (25). Artemis-deficient primary human dermal fibroblasts were transfected with these constructs. 48 h after transfection, the amount of green fluorescent cells was adjusted to about 10% with mock-transfected cells. Half of the cells were exposed to 3 gray of \(\gamma\)-irradiation, and the other half remained untreated. After 4, 7, and 10 days, the percentage of fluorescent cells was scored by FACS analysis. Similar experiments were conducted with Artemis-positive cells. The relative survival advantage was calculated as percentage of green cells in irradiated samples divided by the percentage of green cells in unirradiated samples. This ratio was normalized to the corresponding value obtained with the control (only EGFP expressing) vector.

Mass Spectrometry—Mass spectrometry was performed as described (25). Proteins were digested by trypsin directly in gel. Peptides were analyzed by capillary electrospray ionization-liquid chromatography /MS/MS on a linear ion trap LTQ (Thermo Finnigan) mass spectrometer. Each sample was analyzed by two experiments using different scan modes, a general MS/MS scan mode, and an MS\(^3\) data-dependent neutral loss mode for specific detection of phosphopeptides.

RESULTS

Artemis Amino Acids 398–403 Are Important for Interaction with DNA-PKcs—Artemis interacts with DNA-PKcs in vivo (19) and is phosphorylated in vitro by DNA-PKcs at 11 threonine and serine residues in its C-terminal domain (25). We constructed various expression plasmids, in which the C-terminal domain of Artemis was deleted stepwise and which contained the Myc epitope as an immunological tag. These expression plasmids were transfected into HEK293T cells, and the ability of the Artemis mutant proteins to interact with endogenous DNA-PKcs was analyzed in co-immunoprecipitation assays. We found that C-terminal deletion of amino acids up to position 403 still allowed interaction of the truncated Artemis protein with DNA-PKcs (Fig. 1). In fact, truncation of the C-terminal domain up to position 413 even resulted in increased binding capability of the mutant as compared with the wild type protein (compare ART-WT to ARM37, short exposure in Fig. 1A). Taking into account that the expression of ART-WT was significantly higher than the expression of the mutant proteins (Fig. 1B), the increase in co-immunoprecipitated DNA-PKcs by ARM37 as compared with ART-WT was even more pronounced. Deletion of the five amino acids between positions 403 and 398 led to a dramatic reduction in the amount of co-immunoprecipitated DNA-PKcs. The remaining binding activity was lost upon further

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V(D)J Recombination Activities Are Reduced in Artemis Mutants That Have Lost DNA-PKcs Binding Capacities—To examine whether the DNA-PKcs binding capacities of wild type Artemis and the different C-terminally truncated Artemis mutants (ARM23, ARM37, and ARM57–61) influence in vivo V(D)J recombination capabilities, we performed a test for inversional V(D)J recombination on an extrachromosomal substrate in primary human dermal Artemis-negative fibroblasts (Table 1) (23). All tested mutants were capable of carrying out inversional V(D)J recombination, which includes both coding and signal joint formation. While ARM37 exhibited recombination rates in the range of wild type Artemis, the V(D)J recombination activities decreased as the C-terminal truncations become more severe. Approximately 50% of the V(D)J recombination activity was lost upon deletion of amino acids between 403 and 398 (compare ARM58 and ARM59). ARM23, the shortest Artemis fragment tested, showed activities of about 10% as compared with wild type Artemis.

Because V(D)J recombination is a nuclear process, we wondered if all tested Artemis mutants had been successfully localized to the nucleus. To address this question we did an immunostaining assay using CHO-derived AA8 and V-3 cells transfected with expression plasmids coding for either wild type Artemis, ARM23, or ARM37. Because wild type Artemis and both Artemis derivatives localized to cell nuclei, we ruled out the possibility that the decrease in V(D)J recombination activity of ARM23 is based on diminished nuclear localization (supplemental Fig. 2). Therefore, the results of the V(D)J recombination assay combined with the results of the immunoprecipitations indicate that the ability of the truncated Artemis mutants to support V(D)J recombination correlates with their ability to interact with DNA-PKcs.

C-terminally Truncated Artemis Can Restore Radiation Resistance When Transfected into Artemis-deficient Human Primary Skin Fibroblasts—Knowing that the C-terminally truncated Artemis mutant ARM23 is capable of inversional V(D)J recombination on an extrachromosomal substrate, we were interested to see whether the C-terminal domain is also dispensable for Artemis function in the repair of γ-irradiation-induced DNA damage. To this end, we performed transient transfections of either wild type or truncated Artemis (ARM23) into Artemis-deficient human primary skin fibroblasts and monitored the relative survival advantage. Like wild type Artemis, ARM23 protein could restore cell survival after γ-irradiation (Fig. 2).

Analysis of the Dependence of Wild Type Artemis and C-terminally Truncated Artemis Mutants on Activation by DNA-PKcs in Vitro—Two different substrates were used to investigate whether the Artemis mutants, like wild type protein, still exhibit DNA-PKcs-independent exonuclease activity and DNA-PKcs-dependent endonuclease activities in an in vitro cleavage assay. The first substrate was a DNA hairpin with a 5’ overhang, 6 nucleotides in length (Fig. 3A). As described previously, wild type Artemis showed hairpin opening activity (preferential cut at position 9 +2) and endonuclease activity on the 5’ overhang (preferential cut at blunt end position) (19). These activities were dependent on the presence of DNA-PKcs and ATP. Furthermore, in the absence of DNA-PKcs, wild type Artemis exhibited exonuclease activity. When a 21-bp double strand DNA substrate with a 15-nucleotide 3’ overhang was used (Fig. 3B), wild type Artemis also showed strictly DNA-PKcs-dependent endonuclease activity on the 3’ overhang (preferential cuts at positions centered around the 4th nucleotide into the overhang).

When the nuclease assay was performed with ARM37 instead of wild type Artemis, the hairpin opening activity was independent of the presence of DNA-PKcs and ATP but somewhat weaker as compared with wild type Artemis in the presence of ATP-activated DNA-PKcs (Fig. 3A, lanes 4 and 9). The level of hairpin opening activity of ARM37 was not enhanced by the addition of DNA-PKcs and ATP (Fig. 3A, lanes 9 and 12). In contrast to ART-WT, ARM37 exhibited some endonuclease activity on 5’ overhangs in the absence of DNA-PKcs and ATP with an equal distribution of cleavage products 4–6 nucleotides long (Fig. 3A, lanes 1 and 9). But upon addition of DNA-PKcs and ATP, the activity of ARM37 on 5’ overhangs increased to DNA-PKcs-induced wild type levels and resulted in one major cleavage product 6 nucleotides in length (Fig. 3A, lanes 4 and 12). Endonuclease activities on both hairpins and

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**TABLE 1**

<table>
<thead>
<tr>
<th>Controls</th>
<th>+ ARM37 (aa 1–413)</th>
<th>+ ARM57 (aa 1–408)</th>
<th>+ ARM58 (aa 1–403)</th>
<th>+ ARM59 (aa 1–398)</th>
<th>+ ARM60 (aa 1–392)</th>
<th>+ ARM61 (aa 1–387)</th>
<th>+ ARM23 (aa 1–382)</th>
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<tr>
<td>Pos</td>
<td>− RAG2</td>
<td>− WT Art</td>
<td>+ WT Art</td>
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<tr>
<td>Exp. 1</td>
<td>81.80</td>
<td>0.06</td>
<td>3.18</td>
<td>3.02</td>
<td>2.89</td>
<td>3.29</td>
<td>1.74</td>
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<tr>
<td>Exp. 2</td>
<td>63.08</td>
<td>0.10</td>
<td>4.40</td>
<td>2.04</td>
<td>3.58</td>
<td>3.68</td>
<td>1.83</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>67.38</td>
<td>0.04</td>
<td>3.02</td>
<td>3.00</td>
<td>2.97</td>
<td>2.95</td>
<td>1.58</td>
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Human primary skin fibroblasts were co-transfected with V(D)J recombination substrate plasmid and plasmids expressing RAG1, RAG2, and one of the Artemis proteins (wild type or mutant). As negative controls, the same co-transfections without the plasmids expressing RAG2 (− RAG2) or wild-type Artemis (− WT Art) were performed. As positive controls, a pre-recombined substrate (Pos) plasmid was transfected or wild type Artemis (+ WT Art) was used in the recombination assay. In each assay 5 × 10⁴ fibroblasts were analyzed by FACS analysis. The percentages of recombination-positive cells out of the subpopulation of transfected fibroblasts are shown.

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Two Modes of DNA-PKcs Dependence of Artemis Activity
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5’ overhangs were blocked when DNA-PKcs was added to the assay without ATP (Fig. 3A, lanes 9–12). Similarly, the majority of endonucleolytic activity on 3’ overhangs remained dependent on the combined presence of DNA-PKcs and ATP, and the activity appeared weaker than that of wild type Artemis (Fig. 3B, lanes 13–16 and 21–24). However, as in the case of 5’ overhangs, differences in the banding pattern observed with ARM37 as compared with wild type Artemis suggested that some endonucleolytic activity on 3’ overhangs was apparent in the absence of DNA-PKcs and ATP (Fig. 3B, compare lanes 13–14 and 21–22). Again the cleavage products were equally distributed over a range of several nucleotides (Fig. 3B, lane 21–22), whereas in the presence of DNA-PKcs and ATP, the majority of nucleolytic cleavage occurred at two positions, resulting in fragments 25 and 26 nucleotides in length (Fig. 3B, lane 24). The exonuclease activity of ARM37 showed features comparable with wild type Artemis.

Using the shortest Artemis variant, ARM23, in the nuclease assay, we found the same enzymatic properties regarding activation and blocking by DNA-PKcs/ATP as observed for ARM37 (Fig. 3, A and B). In the absence of DNA-PKcs, again weak endonucleolytic activities on the 5’ and 3’ overhangs were observed, resulting in evenly distributed cleavage products. Upon addition of DNA-PKcs and ATP, again we observed an enhanced cleavage at the same specific sites, as were seen with ARM37. Except for the exonuclease function, all activities of ARM23 were reduced compared with ARM37 or wild type Artemis.

C-terminally Truncated Artemis Mutants Have Lost Nearly All of DNA-PKcs Phosphorylation in Vitro—Having observed that both C-terminally truncated Artemis variants showed significant enhancement of their endonucleolytic activities on 5’ and 3’ overhangs by DNA-PKcs and ATP in the in vitro nuclease assay (Fig. 3), we wondered whether these mutants are still targets for phosphorylation by DNA-PKcs. To address this question we performed an in vitro phosphorylation assay for wild type Artemis, the longest (ARM37) and the shortest (ARM23) deletion variants of Artemis (Fig. 4). Full-length Artemis was well phosphorylated, as described previously (21). As expected, ARM37 was phosphorylated at a lower level because it retains only residues Ser-385 and Thr-410 of the previously described phosphorylation sites. ARM23 was the least phosphorylated, and the very weak phosphorylation signal observed could not be assigned to any specific residues as analyzed by mass spectrometry on three independent preparations in our work,5 and also by the University of Virginia Mass Spectrometry Core Facility (data not shown).

In Vivo V(D)J Recombination Activities Mediated by C-terminally Truncated Artemis Mutants Are Still Dependent on DNA-PKcs—Because the C-terminally truncated Artemis variants ARM23 and ARM37 showed in vitro DNA-PKcs-independent hairpin opening activities, we wondered whether these proteins were able to function in the process of V(D)J recombination in vivo in the absence of DNA-PKcs. Therefore, both mutants as well as wild type Artemis were tested in V(D)J recombination assays performed on an extrachromosomal substrate in DNA-PKcs-negative CHO-V-3 cells (Table 2). In these cells, the endogenous, DNA-PKcs-dependent Artemis cannot mediate V(D)J recombination, so upon transfection of the C-terminally truncated Artemis proteins, we could test whether the DNA-PKcs-independent hairpin opening activity of the truncated proteins was sufficient to allow for in vivo V(D)J recombination on the extrachromosomal substrate. Our experiments clearly demonstrated that in the absence of DNA-PKcs, neither cells transfected with wild type Artemis, ARM23, nor ARM37 exhibited V(D)J recombination activities above background levels. When DNA-PKcs was successfully provided by co-transfection of the expression plasmid pPK1, which encodes full-length human DNA-PKcs (26), cells transfected with ARM23 or ARM37 support V(D)J recombination activities in ranges comparable with cells transfected with wild type Artemis. (Note: In all cases, the endogenous Artemis is also activated by the exogenously provided DNA-PKcs.) This result indicates that without DNA-PKcs, in vivo V(D)J recombination appears not to be possible, even if a hairpin opening and exonuclease-proficient Artemis mutant is provided.

DISCUSSION

We have experimentally confirmed the previously suggested model of autoinhibition of Artemis endonucleolytic functions via

5 V. L. C. Wang and E. Zandi, personal communication.
its C-terminal domain. Deletion of the C terminus of Artemis results in DNA-PKcs-independent hairpin opening activity. However, there is an additional important mode of DNA-PKcs dependence of the endonucleolytic activities of Artemis on 5' and 3' overhangs. Fig. 5 summarizes the different endonucleolytic activities of Artemis and the C-terminally truncated Artemis proteins, as well as the different modes of dependence on DNA-PKcs.

**Artemis Hairpin Opening Activity Is Regulated by Autoinhibition via Its C Terminus and Is Relieved by DNA-PKcs-mediated Phosphorylation**—Previously, we have proposed the C-terminal domain of Artemis to have a negative regulatory role on the Artemis endonucleolytic activities and that phosphorylation by DNA-PKcs can relieve this inhibition (25). Now we have shown that indeed the C-terminal domain is dispensable for all catalytic activities of Artemis, namely its 5'-to-3' exonuclease as well as the endonucleolytic activities on hairpins and 5' and 3' overhangs. A detailed mutational analysis had previously identified 8 residues within the C-terminal domain of Artemis to be critical for the endonucleolytic activities, whereas exonuclease

**FIGURE 3.** ARM23 and ARM37 have DNA-PKcs-independent hairpin opening activities, but the endonucleolytic activities on 5' and 3' overhangs are still largely DNA-PKcs-dependent. In vitro hairpin opening and 5' overhang processing activity assay (A) and 3' overhang processing activity assay (B) of wild type Artemis (ART-WT), ARM23, and ARM37 are shown. 25 nM of the 5'-labeled substrates were incubated with 100 nM Artemis as well as 50 nM DNA-PKcs and 50 nM ATP where indicated. Positions in size of the major products are indicated. The exonucleolytic product (1 nucleotide) is labeled by exonuclease (exo). Diagrams adjacent to the arrows are depicted to emphasize the cleavage positions in the substrate that result in corresponding products. The hairpin is opened at position tip + 2 nucleotides in 3' direction resulting in a product 28 nucleotides in length as indicated. The structures of the substrates are illustrated on the top, with an asterisk indicating the position of the radioactive labeling. Notice the left-most panel in A has three times shorter exposure time than the right one (20 h).

**FIGURE 4.** Markedly reduced phosphorylation of truncated Artemis by DNA-PKcs in vitro. An in vitro DNA-PKcs phosphorylation assay for ART-WT, ARM23, and ARM37 was performed without or with addition of activating 40-bp DNA fragments. The sizes of phosphorylated forms of DNA-PKcs, ART-WT, ARM37, and ARM23 are shown on the left.
Two Modes of DNA-PKcs Dependence of Artemis Activity

**TABLE 2**

<table>
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<tr>
<th>V(D)J recombination assay in DNA-PKcs-negative CHO-V3 cells</th>
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<tr>
<td>Chinese hamster ovary cells were co-transfected with V(D)J recombination substrate plasmid and plasmids expressing RAG1, RAG2, and one of the Artemis proteins (wild-type or C-terminally truncated mutants). DNA-PKcs expression plasmid pPK1 was added (+pPK1) or not (−pPK1). As negative control, a co-transfection without the plasmid expressing RAG2 (−RAG2 + pPK1) was performed. As positive control a pre-recombined substrate (Pos) was transfected. In each assay, 5 × 10⁴ fibroblasts were analyzed by FACS analysis. The percentages of recombination-positive cells out of the subpopulation of transfected fibroblasts are shown.</td>
</tr>
<tr>
<td>Controls</td>
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<tr>
<td>Exp. 1</td>
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Activity was not affected by any of the mutations tested (23). However, analyses of the C-terminally truncated Artemis proteins clearly show that the exonuclease activity also resides within the N-terminal half of the protein (Fig. 3). The in vivo role of the exonuclease activity of Artemis is still unclear.

Deletion of the C-terminal domain from amino acids 383 to 692 renders the truncated Artemis mutant DNA-PKcs independent with respect to its hairpin opening activity (Fig. 3), thereby confirming the autoinhibitory role of the Artemis C terminus. DNA-PKcs is activated by binding to a DNA DSB. It phosphorylates 11 sites in the C-terminal portion of Artemis, and this causes a conformational change in Artemis that results in the C-terminal tail no longer inhibiting the hairpin opening activity of Artemis. This relief of autoinhibition could be a movement of the inhibitory tail away from the binding pocket for DNA hairpins. The ARM23 (aa 1–382) and ARM37 (aa 1–413) truncation mutants support the point that Artemis alone can function in hairpin opening, even without DNA-PKcs, as long as the inhibitory tail is removed (Fig. 3).

Autoinhibition is a regulatory strategy frequently found in the control of enzymatic activities, DNA binding of transcription factors, and subcellular localization (29). In the case of Artemis, inhibition is counteracted by phosphorylation, a mechanism also found in other systems, for example in the relief of autoinhibition of NF-κB p65 (30). Co-immunoprecipitation experiments using N- and C-terminal Artemis fragments have not provided any evidence for intramolecular contacts (data not shown), as have been found for some autoinhibitory systems (reviewed in Ref. 29). In the case of Artemis, it can be envisaged that inhibition of the endonuclease activity is necessary to prevent its inappropriate activity on DNA at times when single to double strand transitions occur (e.g. replication). Artemis is specifically activated at sites of DNA damage and naturally occurring DNA DSBs (e.g. during V(D)J recombination), where active DNA-PKcs is found associated with Ku70 and Ku86 (31).

The DNA-PKcs Interaction Domain Maps to the Center of Artemis—Systematic analyses of C-terminal deletion mutants in co-immunoprecipitation experiments and in vivo V(D)J recombination assays in Artemis-deficient primary human dermal fibroblasts led to the identification of amino acids 398–403 as critical residues for DNA-PKcs interaction (Fig. 1 and supplemental Fig. 1) and in vivo V(D)J recombination (Table 1). Although the majority of DNA-PKcs binding activity was lost upon deletion of amino acids between residues 398 and 403, this correlates with loss of 50% of the V(D)J recombination activity. ARM23 (aa 1–382) shows no detectable DNA-PKcs binding activity as analyzed in reciprocal immunoprecipitation assays, and this corresponds to a 10-fold reduction of V(D)J recombination as compared with ART-WT or ARM58 (aa 1–403), both of which interact with DNA-PKcs. Therefore, amino acids 398–403 in Artemis most likely are important for DNA-PKcs to bind and subsequently phosphorylate the Artemis C-terminal tail, a prerequisite for wild type Artemis function in V(D)J recombination. However, because deletion of the DNA-PKcs interaction domain results in reduced in vivo V(D)J recombination as well as reduced DNA-PKcs-independent hairpin opening activity in vitro (compare ARM23 and ARM37 in Table 1 and Fig. 3), amino acids 403 to 382 clearly also have an effect on the efficiency of Artemis endonuclease activities. Conformational changes that effect DNA substrate utilization can be envisaged.

While this manuscript was in preparation, Soubeyrand et al. (32) described amino acids 360–486 to be necessary and sufficient for the physical interaction between Artemis and DNA-PKcs with residues 401 and 402 being critical for binding. These data are in agreement with our own findings. However, Soubeyrand et al. (32) also report that an Artemis protein in which residues 401 and 402 are mutated, and which cannot bind DNA-PKcs, still confers V(D)J recombination activity indistinguishable from wild type Artemis. Similarly the authors previously had not observed any difference between wild type and C-terminally truncated Artemis in their in vivo V(D)J recombination assay (24). The discrepancy between these results and ours, with respect to a correlation between DNA-PKcs binding capacity and the ability to mediate in vivo V(D)J recombination, could be related to the differences in V(D)J recombination assays employed, using either extrachromosomal or stably integrated DNA substrates. In addition, GUETEL/RSS cells are a transformed cell line, whereas our V(D)J recombination experiment was performed in human primary Artemis-deficient fibroblasts. Because in our system Artemis is the limiting component for the inversional recombination of the extrachromosomal substrate, most likely we can detect differences in V(D)J recombination activity of the truncated Artemis mutants, which were not apparent in the GUETEL/RSS cell line-based V(D)J recombination assay employed by Soubeyrand et al. (32) and Poinsignon et al. (24).

Other NHEJ proteins have been described as in vitro substrates of DNA-PKcs, but so far the functional significance is largely unclear. XRCC4 was shown to interact with and to be phosphorylated by DNA-PKcs, but mutation of the phospho-
rylation sites does not inhibit function (33, 34). Ku86 also is a substrate of DNA-PKcs, and its interaction domain was localized to the 20 C-terminal amino acids (35, 36). Recently a conserved motif has been identified in the C-terminal domains of Nbs1, ATRIP, and Ku86 that interact with different members of the phosphoinositide 3-kinase-related protein kinase family as follows: ATM, ATM and Rad3-related protein, and DNA-PKcs, respectively (37). The interaction between Ku86 and DNA-PKcs is important for recruiting DNA-PKcs to the sites of DNA DSBs. The region in Artemis, which we have shown here to be most important for DNA-PKcs interaction (398–403), shows no obvious sequence similarity to the conserved motif found in Ku86.

The Endonucleolytic Activities on 5'H11032 and 3'H11032 Overhangs of the C-terminally Truncated Artemis Protein Show Dependence upon DNA-PKcs and ATP—In contrast to the DNA-PKcs-independent hairpin opening activity of the truncated Artemis proteins, both DNA overhang activities are still largely dependent on DNA-PKcs (Fig. 3). Because there is weak and barely detectable phosphorylation of mutants ARM37 and ARM23, respectively (Fig. 4), it cannot be ruled out completely that DNA-PKcs does act on these mutant Artemis proteins, resulting in the gain of endonucleolytic activities on 5’ and 3’ overhangs. However, for ARM23 we could not identify phosphorylation on any specific residues in mass spectrometry and also have no evidence for any physical association between ARM23 and DNA-PKcs as analyzed by co-immunoprecipitation experiments (Fig. 1 and supplemental Fig. 1). We therefore favor the interpretation that DNA-PKcs is needed for positioning the DNA overhangs in such a way that Artemis can endonucleolytically act on them. Perhaps DNA-PKcs makes the 5’ and 3’ overhangs more similar in orientation to that of the hairpin conformation. This is a more specific statement of a point that we made previously (19) when we hypothesized that hairpins, 5’ overhangs, and 3’ overhangs may all have structural features in common. It would not be surprising if the endonucleolytic substrate binding pocket of Artemis was optimal for hairpins but not for overhangs. The second role of DNA-PKcs (the first being phosphorylation of the C-terminal domain of Artemis) would be to “mold” overhangs into a hairpin-like configuration (Fig. 5) to facilitate efficient Artemis endonucleolytic activity. This feature of DNA-PKcs

**FIGURE 5. Model of the activation of endonucleolytic activities of Artemis proteins. A, the autoinhibition of wild type Artemis (dark blocks) by its C-terminal domain is depicted on the left. On the right, binding of and phosphorylation by DNA-PKcs (light blocks) results in relief of autoinhibition via a conformational change of wild type Artemis protein, which activates hairpin opening activity. P indicates protein phosphorylation of either Artemis or of DNA-PKcs itself. The exonuclease activity, which is DNA-PKcs-independent, is omitted in this model. B, endonucleolytic activities on DNA overhangs by C-terminally truncated Artemis mutants (ARM). In the absence of DNA-PKcs (top), at a low frequency, DNA overhangs spontaneously bend into hairpin-like structures and can be cleaved by C-terminally truncated Artemis. In the presence of DNA-PKcs and ATP (bottom), overhangs are molded into specific hairpin-like configurations by DNA-PKcs and can be efficiently cut by the Artemis proteins. The activities on 5’ and 3’ overhang substrates are depicted on the left and right side, respectively. The major cleavage sites are symbolized by arrows. The size of the oligonucleotides and the proteins are not drawn to scale.
Two Modes of DNA-PKcs Dependence of Artemis Activity

would be in accordance with the findings of Hammarsten et al. (38) who have provided evidence that DNA-PKcs has adjacent to its DNA double strand binding channel a separate binding cavity for single-stranded DNA.

The weak DNA-PKcs-independent endonucleolytic activities of C-terminally truncated mutants on 5’ and 3’ overhangs most likely reflect the spontaneous bending of free overhangs into a hairpin-like conformation that is suitable for Artemis endonucleolytic action. Our observation, on the one hand, of several evenly distributed cleavage products in the absence of DNA-PKcs and, on the other hand, enhanced cutting at specific positions in the presence of DNA-PKcs and ATP is in agreement with the above proposed mode of DNA-PKcs dependence, regarding the configuration of DNA overhangs for Artemis endonucleolytic activity. This also makes quite clear that there is only one endonucleolytic function elicited by the catalytic domain in the Artemis protein, and this is opening of hairpins and hairpin-like structures. DNA-PKcs therefore has a dual role. First, it activates Artemis for endonucleolytic activity by phosphorylation of the C-terminal tail, thereby relieving autoinhibition; second, it configures the DNA substrate to Artemis for hairpin opening activity.

The experiments shown in Fig. 3 also indicate that autophosphorylation of DNA-PKcs plays a critical role in the activation of Artemis endonucleolytic activities. The DNA-PKcs-independent hairpin opening activities of ARM37 and ARM23 are blocked if DNA-PKcs is added in the absence of ATP, whereas they reappear in the presence of both DNA-PKcs and ATP (compare lanes 7 and 8, as well as 11 and 12 in Fig. 3). This is in accordance with models in which DNA-PKcs blocks DNA ends (e.g. hairpins) until autophosphorylation causes a conformational change, resulting in the release of DNA-PKcs from the DNA ends, which is a prerequisite of DNA end processing (39–42). In the case of the endonucleolytic activities on 5’ and 3’ overhangs, our experiments show that the C-terminally truncated Artemis proteins are still largely dependent on the presence of ATP and DNA-PKcs. Despite relief of autoinhibition, as imitated by C-terminal truncation, these proteins cannot process overhangs by themselves but need the presence of both DNA-PKcs and ATP. We consider it unlikely that this reflects solely a necessity for autophosphorylation-induced conformational changes of DNA-PKcs as a critical step for DNA end processing or else we would expect to see DNA-PKcs-independent activity as observed at hairpins. Rather we prefer to believe that in addition a “molding” of the DNA overhangs into hairpin-like configurations is necessary for Artemis to cleave these endonucleolytically. Thus, the phosphorylation at the various known sites in DNA-PKcs permits consideration of iteration of conformational changes that may result in binding, release, molding, and degrading of DNA ends (39–42).

The DNA-PKcs Dependence of Artemis Function in Vivo V(D)J Recombination—The C-terminally truncated mutants ARM37 and ARM23, which have DNA-PKcs-independent hairpin opening activities, could not mediate V(D)J recombination on an extrachromosomal substrate in DNA-PKcs negative CHO-V-3 cells (Table 2). Although this result could be explained by the DNA-PKcs dependence of an essential NHEJ factor, to date no definitive role of DNA-PKcs-mediated phosphorylation of either Ku86, XRCC4, or DNA ligase IV has been described (33, 34, 43). DNA-PKcs also seems not to be important for recruiting Artemis to the site of DNA DSBs, because ARM23, which does not bind DNA-PKcs, could restore cell survival to Artemis-deficient human primary skin fibroblasts after γ-irradiation (Fig. 2). In DNA-PKcs-negative CHO-V-3 cells, all Artemis proteins were found in the cell nucleus (supplemental Fig. 2), indicating that DNA-PKcs was not necessary for recruiting Artemis to the nucleus. The failure of ARM23 and ARM37 to mediate V(D)J recombination in CHO-V-3 cells can also be interpreted to mean that the endonucleolytic activities of Artemis on 5’ and 3’ overhangs, which are still largely DNA-PKcs-dependent because the overhangs need to be molded into a hairpin-like conformation by DNA-PKcs (see above), are critical for an efficient in vivo V(D)J recombination. The experiments in CHO-V-3 cells also show that no other related protein kinase (e.g. ATM/ATM and Rad3-related proteins) could replace DNA-PKcs and activate Artemis function in V(D)J recombination.

In summary, we have provided experimental evidence for the autoinhibitory role of the C-terminal tail of Artemis, which can be relieved by DNA-PKcs-mediated phosphorylation. In addition DNA-PKcs may play an important role in configuring 5’ and 3’ overhangs in a hairpin-like manner so that Artemis can process these ends (Fig. 5).

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Doris Niewolik, Ulrich Pannicke, Haihui Lu, Yunmei Ma, Ling-Chi Vicky Wang, Peter Kulesza, Ebrahim Zandi, Michael R. Lieber and Klaus Schwarz

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