

The Metallo- β -Lactamase/ β -CASP Domain of Artemis Constitutes the Catalytic Core for V(D)J Recombination

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Abstract

The V(D)J recombination/DNA repair factor Artemis belongs to the metallo- β -lactamase (β -Lact) superfamily of enzymes. Three regions can be defined within the Artemis protein sequence: (a) the β -Lact homology domain, to which is appended (b) the β -CASP region, specific of members of the β -Lact superfamily acting on nucleic acids, and (c) the COOH-terminal domain. Using in vitro mutagenesis, here we show that the association of the β -Lact and the β -CASP regions suffices for in vivo V(D)J recombination of chromosome-integrated substrates. Single amino acid mutants point to critical catalytic residues for V(D)J recombination activity. The results presented here define the β -Lact/ β -CASP domain of Artemis as the minimal core catalytic domain needed for V(D)J recombination and suggest that Artemis uses one or two Zn(II) ions to exert its catalytic activity, like bacterial class B β -Lact enzymes hydrolyzing β -lactam compounds.

Key words: Artemis • metallo- β -lactamases • β -CASP • V(D)J recombination • DNA repair

Introduction

V(D)J recombination, the mechanism by which immunoglobulin and T cell receptor variable domain encoding genes are assembled, is initiated through the introduction of a DNA double strand break (DNA-dsb) by the lymphoid-specific factors RAG1 and RAG2 (for a review of V(D)J recombination see references 1 and 2). RAG1 and RAG2 recognize recombination signal sequences (RSS) that flank all variable (V), diversity (D), and joining (J) gene units and introduce the DNA-dsb at the border of the RSS. The repair of the DNA gap is then achieved by at least six proteins that belong to the nonhomologous end joining apparatus: Ku70/Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4/LigaseIV, and Artemis (3). The DNA-dsb is first recognized by the DNA-PK complex formed by the Ku70/Ku80 heterodimer, which binds to DNA ends, and the DNA-PKcs, which belongs to the PI3 family of kinases and requires DNA association for enzymatic activity. Artemis then probably opens the hairpin structures at coding ends (CE) before ligation (see below). Finally, the XRCC4/DNA-LigaseIV complex catalyzes the ligation

step. Several animal models (2, 4) and human radiosensitive SCID (RS-SCID; references 3 and 5) have demonstrated the absolute prerequisite for these factors in terminating the V(D)J recombination process. Indeed, a defect in any non-homologous end joining factor results in a complete block of both B and T cell maturation, leading to SCID in all of these situations. However, in human Artemis-deficient patients (5), in Artemis KO mice (4), and to a lesser extent in the murine SCID condition (6), the signal joints are qualitatively and quantitatively unaffected, whereas the coding joint formation is severely impaired, suggesting a subtle difference in the resolution of the two RAG1/RAG2-generated DNA intermediates. Before DNA repair, CE are present on the chromosome as hairpin-sealed structures (7), whereas signal ends are excised from the chromosome as blunt and 5' phosphorylated termini (8, 9). Artemis, which is mutated in RS-SCID patients, was therefore postulated to represent the missing factor accountable for opening the hairpin at CE before religation (3). Ma et al. (10) dem-

Abbreviations used in this paper: β -Lact, metallo- β -lactamase; CE, coding ends; DNA-dsb, DNA double strand break; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; EGFP, enhanced green fluorescent protein; FL, full-length; GFP, green fluorescent protein; GST, glutathione-S-transferase; RS-SCID, radiosensitive SCID; RSS, recombination signal sequences; WB, Western blotting.

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the five conserved motifs (Fig. 1 B, I to IV), constituting the β -Lact-active center and mostly consisting of histidine and aspartic acid residues that participate in zinc coordination and hydrolysis reaction characteristic of enzymes of this superfamily (14). This region is followed by the β -CASP region (11), which is encoded by exons 7–13, up to S385. This region is always associated with β -Lact motifs I to IV within nucleic acid-processing enzymes of the β -CASP family such as murine SNM1, yeast PSO2, or the cleavage- and polyadenylation-specific factor. Two highly conserved residues (D165 and H319 in Artemis) constitute an outstanding signature of the β -CASP domain, whereas a third one (V341 in Artemis) appears to be specific of the kind of substrate that is highly conserved as a valine or a histidine in proteins acting on DNA or RNA, respectively (Fig. 1 B). Importantly, D165 or H319 in the β -CASP-specific region could represent the fifth motif of the β -Lact signature, which is not present in the β -Lact homology region per se. We propose that the β -CASP region participates with the β -Lact region in forming the catalytic site of Artemis. Lastly, the exon 14-encoded carboxy terminal half of the protein, from E386 to T692, designated hereafter as “C-Ter,” appears as a separate domain.

Definition of the Artemis Catalytic Core Required for V(D)J Recombination. We undertook deletional and single amino acid mutagenesis analyses to define the core catalytic region of Artemis in vivo. Given recent studies demonstrating a different RAG1/RAG2 requirement for V(D)J recombination on extrachromosomal versus chromosomal substrates (12, 15–17), we analyzed the function of Artemis in the context of in-chromosome V(D)J recombination by using the experimental strategy developed by Liang et al. (12). A chromosomal V(D)J recombination substrate was stably in-

tegrated in the Artemis-deficient GUETEL (GUETEL/RSS cells) and control OTEL (OTEL/RSS) cell lines by means of retroviral infection (Fig. 2). pMX-RSS-GFP/ires-huCD4 (Fig. 2 A) is a retroviral construct in which an RSS-flanked GFP cassette is inserted in reverse transcriptional orientation relative to the 5' LTR promoter. Cells carrying the V(D)J reporter cassette are detected through the cell surface expression of huCD4 (Fig. 2 B). V(D)J recombination of the construct is induced through the transient transfection of RAG1/RAG2 expression plasmids, which results in the inversion of the GFP cassette leading to green fluorescence expression. The control OTEL/RSS cells recombine the substrate (1.44% GFP⁺/CD4⁺) in the presence of both RAG1 and RAG2 but not in the sole presence of RAG1 (0.07%) as expected, and the addition of exogenous Artemis does not increase the recombination frequency (1.79%). In contrast, the Artemis-deficient GUETEL/RSS cells poorly rearrange the substrate in the presence of RAG1 and RAG2 (0.22%), a defect that is fully complemented by the addition of exogenous Artemis (1.36%). Interestingly, the recombination frequency of GUETEL/RSS cells in the absence of Artemis is significantly above background level (0.22 vs. 0.03%) in accord with the previously reported leakiness of Artemis-deficient cells in mice (4, 18). Sequencing the V(D)J coding joints from GUETEL/RSS cells transfected in the absence of Artemis revealed a high frequency of 1–8-bp-long P nucleotide addition (Fig. 3 B), a situation previously associated with Artemis deficiency in murine ES cells but not found in control, V(D)J-proficient, OTEL/RSS cells (Fig. 3 A). The addition of Artemis completely restores the quality of the V(D)J junctions in GUETEL/RSS cells (Fig. 3 C). The integrated results of six experiments show that the relative

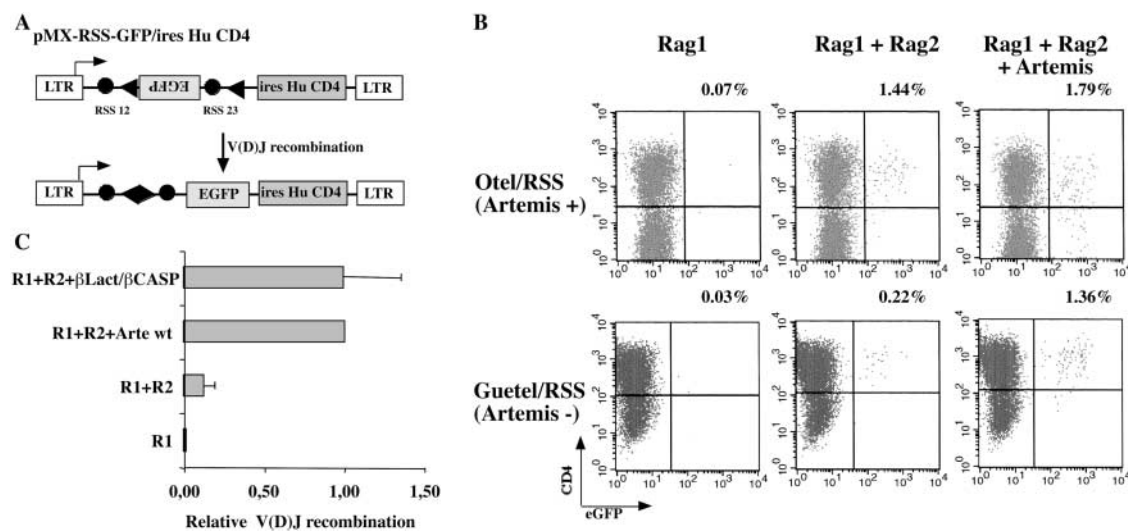


Figure 2. V(D)J recombination on chromosomal substrates. (A) structure of the MX-RSS-EGFP/ires-HuCD4 construct before and after inversional V(D)J recombination. In the germline configuration, an inverted EGFP cassette is flanked by two RSS. Upon V(D)J recombination, the EGFP gene is reoriented and productively transcribed from the LTR promoter. (B) Wild-type OTEL- and Artemis-deficient GUETEL cells were transfected with MX-RSS-EGFP/ires-HuCD4 resulting in 43 and 80% CD4⁺ cells, respectively. Recombination of the substrates is initiated by transient transfection of RAG1/RAG2 expression constructs with or without Artemis. (C) Mean results of 6 V(D)J recombination experiments in GUETEL/RSS cells. The V(D)J recombination activity is calculated relative to the recombination frequency obtained with RAG1 (R1), RAG2 (R2), and wild-type Artemis (Arte wt).

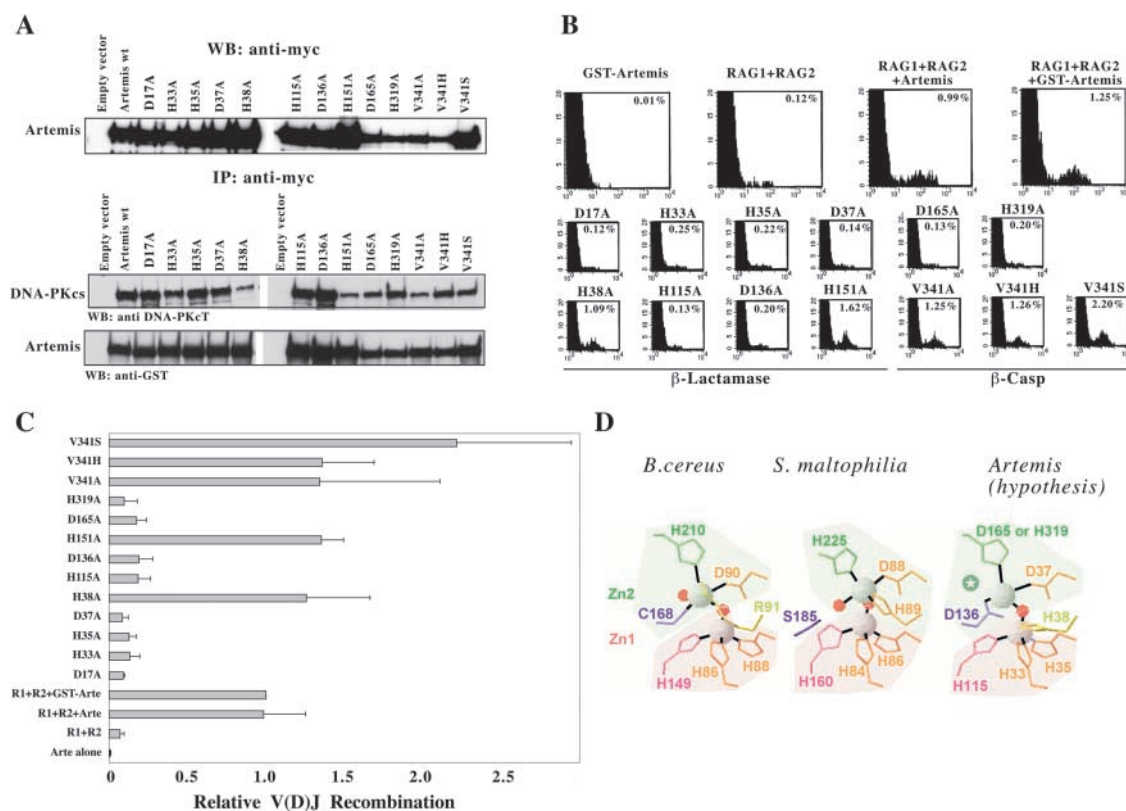


Figure 4. V(D)J recombinase activity of in vitro-generated Artemis mutants. (A) All mutants are expressed in 293T cells and retain their capacity to interact with DNA-PKcs. (B) FACS[®] analysis of GUETEL/RSS cells transiently transfected with RAG1, RAG2, and the various Artemis mutants. The percent of recombination refers to the frequency of EGFP⁺ cells among the CD4⁺ cells. (C) Integrated results of four experiments showing the relative V(D)J recombination activity of the mutants relative to the recombination frequency using GST-Arte. (D) Hypothetical model for the structure of the catalytic site of Artemis. This model is based on the structure of the *B. cereus* and *S. maltophilia* β-Lacts as adapted from Wang et al. (reference 14; PDB codes: 1BC2 and 1SML, respectively). Gray and red spheres indicate the position of zinc and water oxygen atoms, respectively. Shaded areas correspond to the two Zn(II)-binding domains. The green star indicates the position of the fifth Zn2 ligand, which may correspond to a water molecule, or to a conserved amino acid of the β-CASP region (possibly D165).

tions at these two positions. Although H38A mutant is fully active, D136A has lost most of the catalytic activity (Fig. 4 C). Therefore, this result suggests that the putative second Zn(II) center of the Artemis catalytic site would adopt a structure similar to that of the *B. cereus* enzyme with Asp136 substituting the conserved Cys (Figs. 1 B and 4 D).

The last residue of the second Zn(II) center (H210 in *B. cereus*) is not easily recognized in members of the β-CASP family, including Artemis as previously noticed (11). However, it could correspond to either one of the two highly conserved anchoring residues that constitute the signature of the β-CASP region, the Asp165 or His319 (Figs. 1 B and 4 D). Unfortunately, the loss of Artemis function in both D165A and H319A mutants does not allow discriminating between these two hypotheses. Altogether, these results demonstrate that Artemis has conserved not only the β-lactamase fold but also the functional catalytic residues and, therefore, suggest that the hairpin opening activity of Artemis described by Ma et al. (10) proceeds through an enzymatic mechanism similar to the one described for bacterial class B β-Lacts. We propose a model in which Artemis may possess a structure closest to that of the β-Lact enzyme of *B.*

cereus to account for the two putative Zn(II)-binding sites (Fig. 4 D). Another mutant, D17A, has also lost catalytic activity. Although this residue does not directly participate in the two Zn(II) pockets, its high degree of conservation among all β-Lacts suggested that it was important for enzymatic activity (14). Lastly, it was hypothesized that the Val/His341 could discriminate between DNA- and RNA-processing enzymes, respectively (11). However, the replacement of Val341 by alanine, histidine, or serine does not compromise the catalytic function of Artemis.

The COOH-terminal Region of Artemis Is Required for DNA Repair after Ionizing Radiation. Although the β-Lact/β-CASP domain of Artemis suffices for V(D)J recombination, the high degree of sequence conservation in the C-Ter domain (unpublished data) suggests that this region could have an important function, perhaps in relation with the repair of ionizing radiation-induced DNA damages. We analyzed the capacity of the FL and β-Lact/β-CASP forms of Artemis to complement the increased radiosensitivity of RS-SCID primary fibroblasts. FL and β-Lact/β-CASP versions of Artemis were introduced in RS-SCID primary fibroblasts by retroviral infection (Fig. 5). The resulting mixes

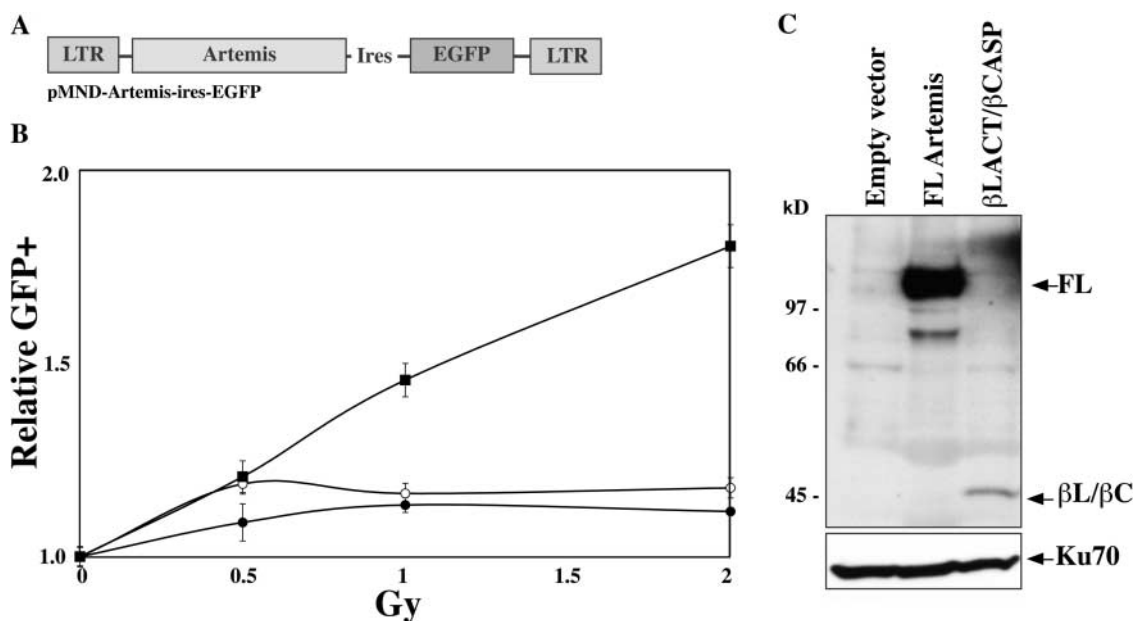


Figure 5. Complementation of the radiosensitivity of RS-SCID primary fibroblasts by Artemis. (A) Structure of the pMND-Arte-ires-GFP construct. (B) Index of GFP⁺ cells in transduced/untransduced mixed cell populations after irradiation. The index is calculated relative to the unirradiated cells. RS-SCID cells were transduced with empty virus (○), FL-Artemis-encoding virus (■), or β-Lact/β-CASP-expressing virus (●). (C) WB analysis of Artemis expression in transduced mixed populations using anti-myc antibody.

of transduced (enhanced GFP [EGFP]⁺) and untransduced (EGFP⁻) cells were subjected to increasing doses of γ irradiation (0–2 Gy) and the frequency of EGFP⁺ cells was determined 15 d later (Fig. 5 B). Although in sample using empty ires-EGFP retroviral vector the frequency of green fluorescent cells does not vary (index 1.18 ± 0.03 at 2 Gy), this value increases in a dose-dependent manner when using the Artemis (FL)-ires-EGFP virus, with an index of 1.81 ± 0.006 after irradiation at 2 Gy. This demonstrates that Artemis complements the increased radiosensitivity phenotype of RS-SCID cells, conferring a growth advantage of the transduced (GFP⁺) cells. In contrast, the same experiment using the Artemis β-Lact/β-CASP domain-only does not result in any growth advantage of the transduced cells (index 1.12 ± 0.01 at 2 Gy). Western blot analysis of the transduced cell populations (Fig. 5 C) revealed a much weaker expression of the truncated version of Artemis compared with FL. This decreased expression is apparently not caused by a difference in the transcription rate of the constructs as judged by the mean fluorescence intensity of GFP⁺ cells (unpublished data), but possibly by a decrease in protein stability. However, the barely detectable expression of endogenous Artemis protein in wild-type fibroblasts by WB (unpublished data) suggests that the low level of truncated Artemis expression in these experiments, which is already above physiological level, may not be directly and/or solely responsible for the absence of complementation of increased radiosensitivity of RS-SCID cells.

In conclusion, our study demonstrates that Artemis can be divided into two critical regions. The β-Lact homology domain associated with the β-CASP region defines the catalytic site *stricto sensu*, which probably exerts its enzymatic

activity through Zinc coordination like other β-Lact enzymes. This catalytic site suffices on its own to perform V(D)J recombination. However, this “core” of Artemis is not sufficient to guarantee the repair of DNA damages caused by ionizing radiation. The COOH-terminal region may play an important role through stabilization of the protein. Another explanation could be that the DNA damage during V(D)J recombination and after ionizing radiation are qualitatively different and therefore may not require exactly the same regions of Artemis for their repair. Although the precise function of Artemis in V(D)J recombination seems to be the opening of hairpin structures at CE (10), hairpin formation is not a common consequence of ionizing radiation.

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