The DNA-dependent Protein Kinase Catalytic Subunit Phosphorylation Sites in Human Artemis

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Artemis protein has irreplaceable functions in V(D)J recombination and nonhomologous end joining (NHEJ) as a hairpin and 5' and 3' overhang endonuclease. The kinase activity of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is necessary in activating Artemis as an endonuclease. Here we report that three basal phosphorylation sites and 11 DNA-PKcs phosphorylation sites within the mammalian Artemis are all located in the C-terminal domain. All but one of these phosphorylation sites deviate from the SQ or TQ motif of DNA-PKcs that was predicted previously from in vitro phosphorylation studies. Phosphatase-treated mammalian Artemis protein that is mutated at the three basal phosphorylation sites still retain DNA-PKcs-dependent endonucleolytic activities, indicating that basal phosphorylation is not required for the activation. In vivo studies of Artemis lacking the C-terminal domain have been reported to be sufficient to complement V(DJ) recombination in Artemis null cells. Therefore, the C-terminal domain may have a negative regulatory effect on the Artemis endonucleolytic activities, and phosphorylation by DNA-PKcs in the C-terminal domain may relieve this inhibition.

V(D)J recombination is the somatic DNA recombination that occurs in precursors of lymphocytes. During this process, the variable (V), diversity (D), and joining (J) exons are recombined to generate a functional variable domain exon coding for immunoglobulins and T cell receptors (1–3). V(D)J recombination begins with the binding of the RAG complex (RAG1, RAG2, and HMG1) to the recombination signal sequences. The RAG complex nicks between the coding end and the recombination signal sequences and converts the nicks to double-strand DNA breaks, thereby creating hairpin sealed coding ends and blunt signal ends (2, 4, 5). Subsequently, the joining of the two coding ends is carried out by the general NHEJ machinery (6).

NHEJ factors include Ku (composed of the Ku70 and Ku86 subunits), DNA-PKcs, Artemis, and DNA ligase IV-XRCC4 complex (6, 7). The absence of any of these components in vivo typically results in an ionizing radiation sensitivity and severe combined immune deficiency (SCID) phenotype (8). Artemis is the most recent NHEJ factor discovered. It was cloned from human patients that have radiosensitivity and a SCID phenotype (RS-SCID) (9). The hairpin opening (critical for V(DJ) recombination) and 5' and 3' overhang processing (necessary for NHEJ) activities of Artemis explain the dual phenotype of the human patients (10). Animals missing Artemis also have the RS-SCID phenotype. The presence of unresolved coding end hairpins in these animals confirmed the pivotal role of Artemis in the hairpin opening step of V(DJ) recombination (11, 12). The fact that human patients and animals deficient for the Artemis protein both have the RS-SCID phenotype suggests that other nucleases do not functionally fulfill the roles of Artemis.

Artemis seems to recognize structural discontinuity of single- to double-strand DNA transitions. The activities of Artemis are regulated by association with and phosphorylation by DNA-PKcs. Artemis interacts with DNA-PKcs in vitro and in vivo in the absence of DNA and ATP, and it can only be activated as a hairpin and overhang endonuclease once it is phosphorylated by DNA-PKcs (10). It is still an open question how DNA-PKcs activates Artemis by phosphorylation.

The N-terminal domain (amino acid 1–155, exons 1–6) of Artemis has significant homology to the β-lactamase fold of several members of the metallo-β-lactamase superfamily (9) and contains multiple catalytically important residues (13, 14). Amino acids 156–385 (exons 7–13) of Artemis comprise the β-CASP region, found only in members of the metallo-β-lactamase superfamily that process nucleic acid (15). Most interestingly, Artemis truncated for the C-terminal domain can functionally complement cells derived from an RS-SCID patient for V(DJ) recombination (14). Therefore, it seems that the metallo-β-lactamase domain and the β-CASP region together constitute the "catalytic core" of Artemis. However, the function of the remaining C-terminal domain (amino acids 386–692), composed of a single exon (exon 14), has yet to be determined.

In this study, we have discovered 11 DNA-PKcs phosphorylation sites and 3 basal phosphorylation sites of Artemis, all of which are located within the C-terminal domain. Our data suggest that the uncharacterized C-terminal domain of Artemis has important regulatory roles. These results lead to a model for how DNA-PKcs activates Artemis by phosphorylation.

**EXPERIMENTAL PROCEDURES**

Oligonucleotides—The 46-nt hairpin substrate (YM-164) has been described previously (Fig. 4A of Ref. 13). The sequences of the 35-bp DNA used as a DNA-PKcs cofactor are the same as described (10).

Protein Expression Constructs—Wild-type Artemis expression plasmids were constructed as described (13). The triple point mutant ARM49 (S385A, S516A, and S518A) as well as the other substitution...
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In Vitro Nuclease Assay—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 DTT, 0.25 mM ATP, and 50 μg/ml bovine serum albumin in a total volume of 10 μl. Where indicated, 50 nM DNA-PKcs and 0.25 μM of 35-35 BPNA (nonspecific DNA-PKcs cofactor) 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 bromophenol blue, and 0.025% of xylene cyanol FF). Reaction mixtures were resolved by 12% denaturing PAGE, and the gel image was obtained with PhosphoImager SI445 (Amersham Biosciences).

DNA-PKcs Phosphorylation and Phosphatase Dephosphorylation Assays—For the DNA-PKcs phosphorylation reactions, Artemis was incubated with DNA-PKcs in 10 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.25 mM ATP, and 1 μl 35-bp DNA at 37 °C for 30 min. If the kinase reactions are followed by dephosphorylation, the Artemis immunobeads were washed in 25 mM HEPES, pH 7.9, 650 mM KCl, 10 mM MgCl₂, and 0.1% Nonidet P-40 three times, and in 25 mM HEPES, pH 7.9, 10 mM MgCl₂, and 2 mM DTT twice, and the immunobeads were then aliquoted and treated with either A protein phosphatase (A Pase) (New England Biolabs, Beverly, MA) or calf intestine phosphatase (CIP) (Roche Applied Science) under conditions suggested by the vendor. DNA-PKcs, or protein phosphatase-treated samples were resolved by 7 or 8% SDS-PAGE and stained with Coomassie Blue R-250.

Immunoprecipitation Assay—The immunoprecipitation assay was done as described (10). DNA-PKcs was detected with anti-DNA-PKcs antibody 25-4 (Neomarkers, Fremont, CA). Myc-tagged Artemis was detected with anti-Myc antibody (Invitrogen). Equal transfection efficiencies were confirmed by co-transfection of an enhanced green fluorescent protein expression plasmid and FACS analysis. SV40 T-antigen was used as a sample loading control and was detected with an anti-SV40 T-Ag antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Artemis Can Be Phosphorylated at Multiple Positions in the C-terminal Domain by DNA-PKcs—DNA-PKcs is homologous to phosphatidylinositol 3-kinases, yet in vitro it phosphorylates many protein targets on serine and threonine residues (17, 18). It has been shown that the DNA-PKcs kinase activity is necessary to activate Artemis as a hairpin and 5’ and 3’ overhang endonuclease (10), and these activities are very likely to be critical to V(D)J recombination and NHEJ in vivo. We were interested in mapping the phosphorylation positions of Artemis by DNA-PKcs in vitro by mass spectrometry (MS). Artemis-myc-His immunobeads were incubated with DNA-PKcs under kinase permissive conditions and then analyzed by SDS-PAGE and Coomassie staining (see "Experimental Procedures"). In order to confirm that this in vitro phosphorylation system mimics other DNA-PKcs kinase reactions, we first analyzed the protein band corresponding to autophosphorylated DNA-PKcs. Nine phosphoserines and phosphothreonines were detected by MS (Fig. 1A). Six of these sites (Thr-2609, Ser-2612, Thr-2624, Thr-2638, Thr-2647, and Ser-3205) have been reported by Douglas et al. (19), and three (Ser-3821, Ser-4026, and Thr-4102) are novel.

Next, the untreated and DNA-PKcs-phosphorylated mammalian Artemis was analyzed by MS. Whereas the untreated Artemis (purified from mammalian cells) has only 3 phosphorylated serines, the phosphorylated Artemis has 14 phosphorylated serines and threonines (including the three basal phosphorylated residues) (Fig. 1B). Because of the limitations of MS, phosphorylation positions on some of the adjacent serines or threonines cannot be definitively determined (see Fig. 1B). It was suggested previously that the DNA-PKcs consensus phosphorylation sequence is 5’-Thr-Glu-Xaa-Thr (where Xaa is any amino acid) (17). The presence of this consensus sequence at several sites in Artemis supports the general mechanism postulated for DNA-PKcs.

The ArtemisFLAG baculovirus, full-length Artemis containing a SapI site in front of its stop codon was generated and cloned into pENTR1a-TEV (a modified version of pDEST8 (Invitrogen)). The resulting plasmid (pENTR1a-TEV-Artemis-SapI) was recombined with pDEST8-FLAG (a modified version of pDEST8 (Invitrogen), which contains a SapI site in front of a TEV protease recognition site followed by a FLAG epitope) vector using the Gateway system (Invitrogen). The resulting plasmid was digested with SapI and ligated back together to generate an in-frame fusion to the TEV cleavage site and the FLAG tag at the C terminus. This construct was then used in the Bac-to-Bac system (Invitrogen) to generate the baculovirus following the manufacturer's recommendation.

In order to generate the mammalian expression plasmid for ArtemisFLAG, full-length Artemis was amplified by PCR using Pfu Turbo Hot Start polymerase (Stratagene, La Jolla, CA) and primers Artemis-BamHI R (5’-CGCCGATTCCTACTGATCTCGATCTCGATCTGTAATTCTCGGGGTATCTAAGAGTGAGCATTTTCTTTT-3’) and Artemis-Sbl R (5’-CATGCGTGCAGATGGAGTTTCGAGGGGCAGATGGGG-3’). The PCR product was then cloned into the BamHI and SbfI sites of pEXA11.1. The ArtemisFLAG insert was sequenced to confirm that no mutations were introduced during the cloning process. The DNA-PKcs expression plasmid pPK1 was constructed as described (16).

In Vivo V(D)J Recombination Assay—The cellular V(D)J recombination assay and FACS analysis of transfected cells were carried out as described (13).

Protein Purification—DNA-PKcs and Artemis-myc-His were purified as described (10). pEXA11.1-ArtemisFLAG was transfected into 293T cells by calcium phosphate precipitation, and the cells were harvested 48 h after transfection. Artemis-FLAG was immunoprecipitated as described (10) with an anti-FLAG M2 monoclonal antibody (Sigma). The FLAG-tagged Artemis was left on the resin or, in the case that the protein was eluted, the immunobeads were washed three times in 25 mM Tris, pH 8.0, 50 mM NaCl, 10 mM MgCl₂, 1% glycerol, and 1 mM EDTA, 1 mM DTT, 0.25 mM ATP, and 50 μM of 35-bp DNA (nonspecific DNA-PKcs cofactor) 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 bromophenol blue, and 0.025% of xylene cyanol FF). Reaction mixtures were resolved by 12% denaturing PAGE, and the gel image was obtained with PhosphoImager SI445 (Amersham Biosciences).
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FIGURE 1. Positions of phosphorylated serines and threonines in DNA-PKcs and Artemis identified by mass spectroscopy. A, DNA-PKcs autophosphorylation sites identified by MS. The positions of phosphorylated serines and threonines are shown on the top of the schematic DNA-PKcs protein. The autophosphorylation sites identified by Douglas et al. (19) are shown on the bottom for comparison. The boldface and italic text (T2620) indicates the only unconfirmed phosphorylation site. B, phosphorylation sites of Artemis identified by MS. The schematic structure of Artemis is shown to emphasize the metallo-β-lactamase domain (dark gray), the β-CASP domain (light gray), and the C-terminal domain (white). Positions of phosphorylated serines from untreated Artemis are shown in the top panel; and positions of phosphorylated serines and threonines of DNA-PKcs phosphorylated Artemis are shown in the bottom panel with the three basal phosphorylation sites highlighted by boldface and italic letters. The positions of the SQ and TQ sites are labeled on the bottom. The source of the cells from which Artemis was purified and the treatment of Artemis are indicated on the right. Adjacent serines and threonines that cannot be distinguished by MS are noted.

TABLE ONE

V(D)J recombination assay in Artemis-deficient human primary skin fibroblasts complemented with Artemis serine or threonine point mutants

| T. E. Controls | T91A | T251A | S362A | S516A | S534A | S538A | S548A | S553A | S560A | S645A |
|---------------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Artemis-positive fibroblasts |
| Exp. 1        | 57.25 | 0.02  | 3.54  | 3.19  |       |       |       |       |       |       |
| Exp. 2        | 57.63 | 0.05  | 4.06  | 4.24  |       |       |       |       |       |       |
| Artemis-deficient fibroblasts |
| Exp. 1        | 56.47 | 0.04  | 0.10  | 5.30  | 3.22  | 3.87  | 3.70  | 3.05  | 3.50  | 1.73  |
| Exp. 2        | 62.18 | 0.11  | 0.11  | 4.67  | 3.58  | 3.39  | 4.67  | 4.26  | 3.73  | 4.17/3.18 |

* Two independent values were ascertained.

Artemis phosphorylation sites are serines or threonines followed by a glutamine (SQ or TQ) (20). However, none of the Artemis phosphorylation sites by DNA-PKcs is at an SQ or TQ, despite the fact that there is a cluster of SQ and TQ sites in the C-terminal domain of Artemis (Fig. 1B). Only one of the basal phosphorylation sites within Artemis is at an SQ site (Ser-516).

It is interesting that even untreated Artemis purified from human 293T cells has three basal phosphorylation sites. In order to determine whether this modification is conserved, human Artemis protein expressed from a baculovirus in insect cells was subjected to MS analysis. FLAG-tagged Artemis purified from insect cells is also phosphorylated at the three basal phosphorylation sites in addition to a fourth one (Fig. 1B). Therefore, the kinase activity that is responsible for the basal phosphorylation of Artemis exists in invertebrates.

As an initial effort to screen for critical DNA-PKcs phosphorylation site(s) of Artemis, we mutated each serine or threonine residue located at SQ or TQ into an alanine residue (Ser to Ala or Thr to Ala mutations). Next, the 10 plasmids expressing different point mutants of Artemis were tested for their ability to functionally complement human primary skin fibroblasts deficient for Artemis in a V(D)J recombination assay (TABLE ONE) as described (13). All the mutants showed wild-type levels of complementation, indicating that mutations at these SQ and TQ sites individually do not have an effect on the functions of Artemis. This is in agreement with our finding that DNA-PKcs phosphorylation sites of Artemis are not at SQ or TQ sites (Fig. 1B).

Full-length Artemis Purified from Mammalian and Insect Cells Has DNA-PKcs-dependent Hairpin Opening and 5′ Overhang Processing Activities—It is interesting that insect cell-purified Artemis has similar basal phosphorylation as the mammalian cell-purified Artemis (Fig. 1B). We were interested in testing if the insect-cell derived Artemis is functional.
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Human Artemis expression constructs were introduced into different protein expression systems. FLAG-tagged Artemis from insect and mammalian cells were generated. Subsequently, the protein preparations from these sources were subjected to an in vitro nuclease assay. Artemis-FLAG purified from baculovirus-infected insect cells did demonstrate DNA-PKcs-dependent hairpin opening and 5’ overhang processing activities similar to the wild-type protein (Fig. 2, A and B, compare lanes 4 and 6).

In the absence of DNA-PKcs, Artemis has exonucleolytic activity (Fig. 2, lanes 3 and 5). In order to rule out the possibility that the exonucleolytic activity was from a protein co-purifying with Artemis, C-terminally FLAG-tagged Artemis expression plasmid and the corresponding empty vector were transfected into mammalian cells. Proteins were purified by anti-FLAG antibody from both the Artemis-transfected and the mock-transfected cells. In this case, Artemis was eluted from the immunobeads (see “Experimental Procedures”) to minimize contamination from the agarose resin. Both protein preparations were assayed for hairpin opening activity (Fig. 2B), and only the protein preparation from Artemis-transfected cells showed significant exonucleolytic activity (Fig. 2B, compare lanes 5 and 7), suggesting that Artemis has intrinsic exonucleolytic activity.

Phosphatase-treated Artemis Has Increased Gel Mobility—Although phosphorylation by DNA-PKcs is necessary to activate Artemis as an endonuclease, we were interested in whether basal phosphorylation at the three sites identified by MS plays a role in activating Artemis. To address this question, we first tested protein phosphatase-treated Artemis. Immunobead-immobilized Artemis-RC-His was first mock-treated or phosphorylated by DNA-PKcs. Then after extensive washes with a high salt buffer, each sample was divided into 5 aliquots and was mock-treated, treated with λ PPase, or treated with CIP (Fig. 3A). Finally, the phosphatase-treated immunobeads were analyzed by SDS-PAGE followed by Coomassie staining.

As observed previously, untreated Artemis appears as a doublet after resolution by SDS-PAGE (Fig. 3B, lane 1). However, after phosphatase treatment, the Artemis protein appeared less diffuse on the gel and migrated faster than the untreated protein (Fig. 3B, lanes 2–5). Compared with the untreated Artemis, DNA-PKcs-phosphorylated Artemis migrated even slower, and the resulting band (potentially consisting of multiple bands) appeared even more diffuse (Fig. 3B, lane 6). Phosphatase treatment of DNA-PKcs-phosphorylated Artemis restored the sharpness of the protein band and increased the mobility of the protein in the gel (Fig. 3B, lanes 7–10).

**FIGURE 2.** Insect cell purified Artemis has wild-type DNA-PKcs-dependent endonucleolytic activities. C-terminally tagged Artemis was incubated with the substrate in the presence and absence of DNA-PKcs, and then the reaction mixtures were resolved by 12% denaturing PAGE. A, Artemis-FLAG immunobeads prepared from baculovirus-infected insect cells has exonucleolytic activity and DNA-PKcs-dependent hairpin and 5’ overhang processing activities. B, soluble Artemis-FLAG purified from mammalian cells has intrinsic exonucleolytic activity compared with the mock-purified protein. Lane M contains an oligonucleotide identical to the fragment 5’ to the hairpin tip (26 nt) of the substrate. The sizes (in nt) and the schematic representations of the products are indicated on the right.

**FIGURE 3.** Artemis can be phosphorylated by DNA-PKcs and dephosphorylated by protein phosphatases in vitro. A, flow chart of phosphorylation/dephosphorylation of Artemis-RC-His immunobeads. B, SDS-PAGE analysis and Coomassie staining of DNA-PKcs and then protein phosphatase-treated Artemis. Mock-treated or DNA-PKcs-phosphorylated (3 pmol of DNA-PKcs) Artemis-RC-His (15 pmol) immunobeads were washed and divided into 5 aliquots. Each aliquot was then dephosphorylated by different amounts of λ Pase or CIP as indicated. Positions of auto-phosphorylated DNA-PKcs, CIP, λ Pase, immunoglobulin heavy chain (lg H chain), and light chain (lg L chain) are indicated by arrowheads. The position of Artemis changes with its phosphorylation status and is indicated by a bracket. The sizes of protein molecular weight standards (lane M, in kDa) are shown on the left.
We have reported that after treating the Artemis immunobeads with DNA-PKcs and extensive washing, the phosphorylated Artemis is not able to demonstrate any endonucleolytic activities (10). We had suggested that this was because DNA-PKcs, which possibly recruits Artemis to the substrate, was washed away; the same washing buffer used in purifying Artemis by immunoprecipitation was sufficient to remove DNA-PKcs. We have now found that DNA-PKcs remains associated with the Artemis immunobeads after the phosphorylation and stringent washes (Fig. 3B, lanes 6–10). It is possible that DNA-PKcs or autophosphorylated DNA-PKcs has a higher affinity for phosphorylated Artemis.

**Phosphatase-treated Artemis Retains DNA-PKcs-dependent Endonucleolytic Activities**—In order to study the role of basal phosphorylation on the activation of Artemis as an endonuclease, untreated, λ-PPase-treated, and CIP-treated Artemis immunobeads were prepared as described in Fig. 3A, and an aliquot of these beads was subjected to an *in vitro* nuclease assay in the presence or absence of DNA-PKcs. Mock-treated Artemis demonstrated DNA-PKcs-dependent hairpin opening, 5′ overhang processing activities (Fig. 4A, lanes 1 and 2), as well as 3′ overhang processing activity (Fig. 4B, lanes 7 and 8). Most interestingly, both λ-PPase-treated and the CIP-treated Artemis were able to demonstrate DNA-PKcs-dependent endonucleolytic activities at levels comparable with the untreated protein (Fig. 4, A and B, lanes 3–6 and 9–12).

**The Triple Point Mutant of Artemis at the Three Basal Phosphorylation Sites Has DNA-PKcs-dependent Endonucleolytic Activities**—In order to confirm our phosphatase treatment studies of Artemis, a triple point mutant of Artemis was generated where all three basal phosphorylation sites (Ser-385, Ser-516, and Ser-518) were substituted by ala-nines (mutant ARM49). First, the ability of ARM49 to interact with DNA-PKcs was examined by an immunoprecipitation assay. Similar to the wild-type protein, ARM49 can interact with DNA-PKcs (Fig. 5A), and it can also be phosphorylated by DNA-PKcs to a level comparable with the wild-type protein (data not shown). The mutations did not...
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FIGURE 6. Triple basal site mutant of Artemis (ARM49) has DNA-PKcs-dependent endonucleolytic activities. Wild-type Artemis or ARM49 was incubated with the hairpin and 5′ overhang substrate (A) or the 3′ overhang substrate (B) in the presence or absence of DNA-PKcs. Labels are the same as described in the legend to Fig. 4.

Artemis Phosphorylated by DNA-PK Only in C-terminal Domain—In light of the finding that the kinase activity of DNA-PKcs is necessary to activate Artemis as a hairpin and 5′ overhang endonuclease, we sought to map the DNA-PKcs phosphorylation sites in Artemis as part of an effort to study the regulation of Artemis activities. We also mapped the phosphorylation sites within DNA-PKcs itself. A total of nine phosphorylated serines and threonines are detected in autophosphorylated DNA-PKcs. Six of these sites were reported before (19), and three of them were previously unknown, indicating that our DNA-PKcs in vitro phosphorylation system is similar to what has been used by others. In agreement with our finding of the three novel sites, Lees-Miller and co-workers (in collaboration with others) (21, 22) have recently suggested that additional autophosphorylation sites of DNA-PKcs exist besides the cluster of sites (Thr-2609 to Thr-2647) that they identified.

Regarding Artemis itself, none of the in vitro DNA-PKcs-phosphorylated sites on Artemis is located at an SQ or TQ site. Although some of the well characterized in vitro DNA-PKcs phosphorylation sites are at SQ or TQ sites (such as p53 (23)), phosphorylation sites deviating from this “consensus” have been reported. In fact, three (Thr-2624, Ser-3205, and Ser-4026) out of the nine autophosphorylation sites of DNA-PKcs are not at SQ or TQ sites. The DNA-PKcs phosphorylation sites on Ku70, Ku86, and XRCC4 are also at sites other than this consensus (24–26). Therefore, DNA-PKcs does not have a strict preference for SQ or TQ. It has been suggested that DNA-PKcs prefers serines and threonines followed by a hydrophobic amino acid in vitro (18). Despite studies of other proteins, Artemis is among the first to illustrate that DNA-PKcs phosphorylation can have a physiological effect on the function of a protein.

Furthermore, the DNA-PKcs phosphorylation sites and the three basal phosphorylation sites of Artemis are all located in the C-terminal domain (Fig. 1B). Considering the crucial role of DNA-PKcs kinase activity in activating Artemis endonuclease activities, the C-terminal domain may be an important regulatory domain of Artemis. Most likely, the C-terminal domain has an inhibitory role in the activation of Artemis. In line with this thinking, it has been shown recently that the “core” Artemis (metallo-β-lactamase domain/β-CASP region) can complement Artemis-deficient cells for V(D)J recombination (14).4

The Role of the C-terminal Domain of Artemis—Artemis is a structure-specific endonuclease and recognizes single- to double-strand

4 U. Pannicke and K. Schwarz, unpublished data.
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**TABLE TWO**

| V(D)J recombination assays in Artemis-deficient human primary skin fibroblasts complemented with ARM49 |
|---------------------------------------------------------------|
| The experiments were carried out as described for TABLE ONE. T. E. indicates transfection efficiency. |
| | T. E. | −RAG2 | −wt Art | +wt Art | +ARM49 |
| --- | --- | --- | --- | --- | --- |
| Artemis-positive fibroblasts | | | | | |
| Exp. 1 | 61.54 | 0.09 | 2.17 | 4.54 |
| Exp. 2 | 58.37 | 0.05 | 6.08 | 6.69 |
| Artemis-deficient fibroblasts | | | | | |
| Exp. 1 | 66.43 | 0.02 | 0.03 | 2.75 | 0.99 |
| Exp. 2 | 67.96 | 0.01 | 0.05 | 6.66 | 4.49/4.04 |

* Two independent values were ascertained.

Transitions. If the activity of Artemis is not properly controlled, it could potentially cause genetic instability because some DNA metabolic intermediates mimic Artemis substrates. For example, DNA replication, DNA transcription, and DNA recombination all involve separation of the two DNA strands and generation of DNA single- to double-strand transitions. The role of the C-terminal domain may be to inhibit Artemis in the absence of DNA double-strand breaks. Once a double-strand DNA break is generated, DNA-PKcs is activated upon association with DNA ends, and then Artemis can be turned on by association with and phosphorylation by DNA-PKcs and function in V(D)J recombination and NHEJ.

The Role of Basal Phosphorylation—Phosphatase-treated Artemis and Artemis that has mutations at the three MS-identified basal phosphorylation sites (ARM49) still retain DNA-PKcs-dependent endonucleolytic activities (Figs. 4 and 6), and ARM49 can support V(D)J recombination in a DNA-PKcs-dependent manner (TABLE TWO and data not shown). This illustrates that the DNA-PKcs-independent basal phosphorylation does not affect the ability of Artemis to be activated by DNA-PKcs. Recently, there have been reports that, in addition to DNA-PKcs, activation of ataxia telangiectasia mutated (ATM) kinase and possibly the ATM- and Rad3-related kinase (ATR) can lead to the phosphorylation of Artemis, although it was not clear whether this is via direct phosphorylation or via an indirect pathway (27–30). We considered the possibility that the basal phosphorylation identified in our study corresponds to ATM- and/or ATR-dependent phosphorylation of Artemis. Hence, in addition to V(D)J recombination, we also examined the ionizing radiation (IR) sensitivity of cells transfected with wt Artemis or the ARM49 expressing plasmid (supplemental Fig. S1). The ARM49 mutant was indistinguishable from wt Artemis regarding IR resistance. This is consistent with the fact that ARM49 demonstrated a mobility shift in a manner that is indistinguishable from wt Artemis in response to IR (supplemental Fig. S2). Because IR-induced phosphorylation of Artemis is lost in A-T cells (29), these three basal sites are unlikely to be the phosphorylation sites of ATM (or of ATR) upon exposure to ionizing radiation. Perhaps a common kinase present in vertebrates and invertebrates (Fig. 1B) phosphorylates these three sites in vivo.

One group has shown that Ser-645 of Artemis is phosphorylated in cells treated with IR in an ATM-dependent manner (31). We created a S645A mutant and found that cells expressing this Artemis mutant were indistinguishable from wt in their survival in response to IR (supplemental Fig. S1). Therefore, although this site may be phosphorylated in response to IR in an ATM-dependent manner, it does not appear to be functionally critical.

Is DNA-PKcs Required to Recruit Artemis to the DNA Substrate?—We have shown previously that after pre-phosphorylation of Artemis immunobeads by DNA-PKcs and extensive washes, the phosphorylated Artemis does not have endonucleolytic activities. We suggested that this was likely because DNA-PKcs was washed away, and therefore, the physical presence of DNA-PKcs is required to recruit Artemis to the DNA substrate (10). However, data from this study suggest that autophosphorylated DNA-PKcs has very high affinity for Artemis and remains associated with the Artemis immunobeads after stringent washes.

Work from others may shed light on the physical roles of DNA-PKcs in complex with Artemis. Some have suggested that autophosphory-
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lated DNA-PKcs dissociates from DNA ends (32, 33), whereas others have raised the possibility that autophosphorylated DNA-PKcs simply changes its conformation (but remains associated with DNA ends) to allow the downstream NHEJ factors to access DNA ends (21, 22). Thus, upon autophosphorylation of DNA-PKcs, the inhibitory role in the binding of Artemis to its substrate, and the protein structure of Artemis is regulated by interaction with and phosphorylation by DNA-PKcs. Based on the phosphorylation status, there are at least two conformations that Artemis may adopt (Fig. 7). In the absence of DNA-PKcs, the active center of Artemis is masked by the C-terminal domain. This inactive conformation does not allow Artemis to process DNA. Upon association with and phosphorylation by DNA-PKcs, at multiple sites within the C-terminal domain, the inhibition of the C-terminal domain is alleviated probably by conformational changes, and the catalytic center of Artemis is thus completely exposed. This activated form of Artemis can then process DNA hairpins and 5’ and 3’ overhangs.

Model of Activation of Artemis by DNA-PKcs—Based on the above observations, we propose that the C-terminal domain of Artemis may have an inhibitory role in the binding of Artemis to its substrate, and the protein structure of Artemis is regulated by interaction with and phosphorylation by DNA-PKcs. Based on the phosphorylation status, there are at least two conformations that Artemis may adopt (Fig. 7). In the absence of DNA-PKcs, the catalytic center of Artemis is masked by the C-terminal domain. This inactive conformation does not allow Artemis to process DNA. Upon association with and phosphorylation by DNA-PKcs, at multiple sites within the C-terminal domain, the inhibition of the C-terminal domain is alleviated probably by conformational changes, and the catalytic center of Artemis is thus completely exposed. This activated form of Artemis can then process DNA hairpins and 5’ and 3’ overhangs.

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