doi:10.1016/j.jmb.2006.02.061 *J. Mol. Biol.* (2006) **358**, 1200–1211

JMB

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Artemis Phosphorylated by DNA-dependent Protein Kinase Associates Preferentially with Discrete Regions of Chromatin

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Artemis is a nuclear phosphoprotein required for genomic integrity whose phosphorylation is increased subsequent to DNA damage. Artemis phosphorylation by the DNA-dependent protein kinase (DNA-PK) and the association of Artemis with DNA-PK catalytic subunit (DNA-PKcs) have been proposed to be crucial for the variable, diversity, joining (V(D)J) reaction, genomic stability and cell survival in response to double-stranded DNA breaks. The exact nature of the effectors of Artemis phosphorylation is presently being debated. Here, we have delimited the interface on Artemis required for its association with DNA-PKcs and present the characterization of six DNA-PK phosphorylation sites on Artemis whose phosphorylation shows dependence on its association with DNA-PKcs and is induced by double-stranded DNA damage. Surprisingly, DNA-PKcs Artemis association appeared to be dispensable in a V(D)J recombination assay with stably integrated DNA substrates. Phosphorylation at two of the sites on Artemis, S516 and S645, was verified *in vivo* using phosphospecific antibodies. Basal Artemis S516 and S645 phosphorylation *in vivo* showed a significant dependence on DNA-PKcs association. However, regardless of its association with DNA-PKcs, phosphorylation of Artemis at both S516 and S645 was stimulated in response to the double-stranded DNA-damaging agent bleomycin, albeit to a lesser extent. This suggests that additional factors contribute to promote DNA damage-induced Artemis phosphorylation. Intriguingly, pS516/pS645 Artemis was concentrated in chromatin-associated nuclear foci in naïve cells. These foci were maintained upon DNA damage but failed to overlap with the damage-induced γH2AX. These results provide the expectation of a specific role for DNA-PK-phosphorylated Artemis in both naïve and damaged cells.

Keywords: Artemis; DNA damage; DNA-PK; phosphorylation; variable, diversity, joining

Introduction

Artemis/SNM1C is a member of the SNM1 nuclease family recently shown to be a key component of the variable, diversity, joining (V(D)J) recombination machinery and to be required for cell survival in response to double-stranded DNA-breaks (DSBs).[1–4] Artemis deficiency leads to genomic instability and chromosomal aberrations, consistent with a role as a tumor suppressor.[5–10]

Artemis is encoded by a gene whose transcript is subject to a number of differential splicing

Abbreviations used: V(D)J, variable, diversity, joining; NHEJ, non-homologous DNA end-joining; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; CIP, calf intestinal phosphatase; pArtemis, phosphoArtemis; PIKK, phosphoinositide 3-kinase-related kinase; DSB, double-stranded DNA breaks; DSBR, double-stranded DNA break repair; Wt, wild-type; GST, glutathione-*S*-transferase; ATM, ataxia telangiectasia-mutated; ATR, ATM and Rad3-related.

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variations within the RNA region coding for the N-terminal region of the protein, which contains the catalytically important β-lactamase and β-CASP domains that mediate its endonucleolytic activity.[11–14] By contrast the C-terminal region of Artemis is encoded by a single exon, which, although not directly involved in the catalysis, is projected to have important regulatory functions. For example, a patient expressing Artemis with a C-terminal mutation exhibits a variant of radiosensitive severe combined immunodeficiency (RS-SCID) featuring a poorly developed immune system and a defect in non-homologous DNA end-joining (NHEJ), the major DSB repair pathway in mammals.[6]

Recent studies have shown that Artemis is constitutively phosphorylated in proliferating cells and that its phosphorylation increases following exposure to several types of DNA-damaging agents.[15–17] This has led to the proposal that Artemis may play a role in cell cycle regulation following DNA damage. These studies also have suggested that the majority of Artemis phosphorylation in response to DNA damage occurs in a region of the extended C-terminal domain containing a cluster of SQ motifs that reflect the consensus phosphorylation sites of the large phosphoinositide 3-kinase-related Ser/Thr kinase family (PIKK), ataxia telangiectasia-mutated (ATM), ATM and Rad3-related (ATR) and DNA-dependent protein kinase catalytical subunit (DNA-PKcs).[18–20] However, the proposed SQ phosphorylation has not been directly demonstrated and another recent study suggests extensive PIKK phosphorylation of Artemis at non-SQ/TQ motifs.[21] Indeed the importance of Artemis phosphorylation for regulation of its endonucleolytic activity and its involvement in V(D)J recombination remains the subject of debate.[16,21]

Artemis has been demonstrated to form a complex with DNA-PKcs, which has been proposed to promote cleavage of coding end-like hairpin DNA structures by Artemis *in vivo*.[21,22] Since processing of the coding end is a critical intermediate in V(D)J recombination,[23] the DNA-PKcs/Artemis interaction has important implications. Further, in view of the significant overlap in substrate recognition amongst PIKKs *in vitro*, colocalization and physical association of DNA-PK with Artemis is hypothesized to be important for the specific and preferential phosphorylation of Artemis by DNA-PK *in vivo*.[20,24–28]

Here, we show that DNA-PK-mediated phosphorylation of Artemis *in vivo* occurs on six SQ motifs clustered in the Artemis C-terminal region. Specific antiphosphoserine antibodies confirmed that Artemis is phosphorylated on S516/S645 in a manner that depends on its association with DNA-PKcs, but also revealed that a further DNA damage-dependent induction in phosphorylation at these sites proceeds in the absence of the stable association of Artemis with DNA-PK. By contrast S516/S645-phosphorylated Artemis localized to discrete chromatin-associated nuclear foci that failed to overlap with sites of DNA damage marked by the γH2AX and the recruitment of 53BP1. These results suggest a role for phosphorylation of S516/S645 in normally growing cells beyond its role in NHEJ.

Results

Artemis is phosphorylated at multiple sites by DNA-PK *in vitro*

While Artemis hyperphosphorylation has been described in response to several DNA-damaging agents,[15–17] the precise nature of sites of phosphorylation and kinases involved remain to be identified. Artemis phosphorylation by DNA-PK has been proposed to play an important role in V(D)J recombination[22] and DNA-PK is also required for NHEJ, making DNA-PK an obvious candidate kinase for Artemis phosphorylation in response to DSBs.

To initiate direct analysis of the Artemis residues that could be phosphorylated by DNA-PK, we examined the phosphorylation of recombinant Artemis (Figure 1(a)–(c)). Full-length Artemis was efficiently phosphorylated by purified DNA-PK in a DNA-dependent manner (Figure 1(a)). Truncation of Artemis from amino acid residues 445–647,

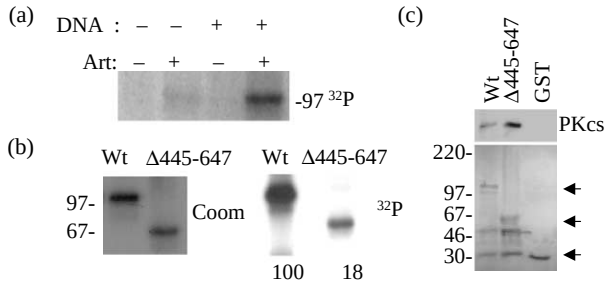

Figure 1. Phosphorylation of Artemis by DNA-PK. (a) SDS-PAGE analysis of Artemis phosphorylation by DNA-PK. Artemis expressed as a GST fusion protein and purified from the GST tag was incubated with purified DNA-PK in the presence (+) or absence (−) of 10 ng of linearized pBluescript DNA and 50 μM [γ-^{32}P]ATP as indicated, then resolved through 8% SDS-PAGE. (b) Deletion of amino acid residues 445–647 of Artemis reduces phosphorylation by DNA-PK. Input of Artemis and Artemis$_{\Delta 445\text{-}647}$ visualized by Coomassie staining (left) and DNA-PK-mediated ^{32}P incorporation (right) analyzed by SDS-PAGE. Quantification below the right panel indicates relative ^{32}P incorporation into the constructs corrected for inputs. Data are representative of two independent determinations. (c) Pulldown of HEK293T whole cell extracts from cells with full-length GST-Artemis (Wt), GST-Artemis$_{\Delta 445\text{-}647}$ or GST alone as indicated. Interactions were detected by Western blot with an antibody to DNA-PKcs (top panel). A Ponceau-stained SDS-PAGE gel of the recombinant fusion proteins employed in the binding assay is shown at the bottom. The arrows highlight the position of migration of GST-Artemis, GST-Artemis$_{\Delta 445\text{-}647}$ and GST, respectively. Numbers correspond to the relative levels of ^{32}P incorporation corrected for Artemis level.