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A structural model for regulation of NHEJ by DNA-PKcs autophosphorylation

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Abstract

The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Ku heterodimer together form the biologically critical DNA-PK complex that plays key roles in the repair of ionizing radiation-induced DNA double-strand breaks through the non-homologous end-joining (NHEJ) pathway. Despite elegant and informative electron microscopy studies, the mechanism by which DNA-PK co-ordinates the initiation of NHEJ has been enigmatic due to limited structural information. Here, we discuss how the recently described small angle X-ray scattering structures of full-length Ku heterodimer and DNA-PKcs in solution, combined with a breakthrough DNA-PKcs crystal structure, provide significant insights into the early stages of NHEJ. Dynamic structural changes associated with a functionally important cluster of autophosphorylation sites play a significant role in regulating the dissociation of DNA-PKcs from Ku and DNA. These new structural insights have implications for understanding the formation and control of the DNA-PK synaptic complex, DNA-PKcs activation and initiation of NHEJ. More generally, they provide prototypic information for the phosphatidylinositol-3 kinase-like (PIKK) family of serine/threonine protein kinases that includes Ataxia Telangiectasia-Mutated (ATM) and ATM-, Rad3-related (ATR) as well as DNA-PKcs.

Keywords

DNA-PKcs; non-homologous end joining; DNA double strand break repair; SAXS; phosphorylation

Introduction

Cellular DNA is continually exposed to endogenous and exogenous agents that cause multiple forms of DNA damage. This damage must be faithfully and efficiently repaired in order to maintain the integrity of the genome and to ensure reliable duplication and inheritance of genetic material. One of the most deleterious forms of DNA damage is the double-strand break (DSB), which can arise through replication fork collapse or exposure to

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free radicals, reactive oxygen species, chemical agents such as chemotherapeutic drugs, UV and ionizing radiation (IR) [1].

IR-induced DSBs are formed when two single strand (ss) breaks occur in close proximity on opposite strands of the DNA. IR-induced DSBs are characterized by the presence of additional DNA damage, including base lesions and abasic sites within one to two helical turns of the DNA (so called clustered DNA damage), and frequently contain non-ligatable end groups such as 3' phosphate or 3'-phosphoglycolate groups that must be removed prior to ligation [2–5]. In mammalian cells, the majority of IR-induced DSBs are repaired by the non-homologous end-joining (NHEJ) pathway. NHEJ is also responsible for the repair of DSBs produced during V(D)J recombination and, to a lesser extent, class switch recombination, thus defects in NHEJ can lead to defects in DSB repair, increased radiation sensitivity and immune deficiencies [6–10]. Accordingly, the core NHEJ factors have largely been identified based on their requirement for cellular survival after IR and by their function in V(D)J recombination. These core factors include the Ku70/80 heterodimer, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Artemis, XRCC4, DNA ligase IV and XLF (also called Cernunnos).

NHEJ is thought to proceed through three key steps: recognition of the break, DNA processing to remove non-ligatable ends or other forms of damage at the termini, and finally ligation of the DNA ends (Figure 1). Recognition of the DSB is carried out by the Ku heterodimer (Step 1, Figure 1), which is also required for recruitment of DNA-PKcs, the XRCC4/DNA ligase IV complex and XLF, reflecting its essential role in NHEJ [11–16]. In current models, binding of Ku to DNA is followed by recruitment of DNA-PKcs, which causes the inward translocation of Ku, positioning DNA-PKcs at the extreme DNA termini [15,17] (Step 2, Figure 1). Formation of the DNA-PK holoenzyme is dependent upon DNA binding as in the absence of DNA the complex does not form [18].

The interaction of two DNA-PKcs molecules on adjacent sides of the DSB (a configuration often referred to as a synaptic complex) (Step 2, Figure 1) stimulates the protein kinase activity of DNA-PKcs, leading to DNA-PKcs autophosphorylation and dissociation (Steps 4 and 5, Figure 1). Depending on the complexity of the DSB and the nature of the break termini, different processing factors may also be recruited (Step 3, Figure 1). Potential processing factors include Artemis, an endonuclease which interacts with, and is activated by, DNA-PKcs [19–26], as well as the Mre11-Rad50-Nbs1 (MRN) exo/endonuclease complex [27–30]. DNA polymerases μ and λ are reported to fill-in missing nucleotides [31,32], and polynucleotide kinase/phosphatase (PNKP), which interacts with XRCC4, removes 3' phosphate groups and/or adds 5'-phosphate groups to DNA termini prior to ligation [33–35]. The final step in NHEJ is DNA ligation, which is carried out by the XRCC4/DNA ligase IV complex (Step 6, Figure 1). In addition, XLF interacts with XRCC4 and the XRCC4/ligase IV complex to stimulate end joining [36–39] by promoting re-adenylation of ligase IV [40]. In this review, we focus on the early events of NHEJ, in particular the key role DNA-PKcs and Ku play in coordinating the cellular response to IR-induced DSBs.

DNA-PKcs protein kinase activity is critical for NHEJ

DNA-PKcs is a member of the phosphatidylinositol-3 kinase-like (PIKK) family of serine/threonine protein kinases that also includes Ataxia Telangiectasia-Mutated (ATM) and ATM-, Rad3-related (ATR) [41]. DNA-PKcs is a large polypeptide of over 4000 amino acids and, like other PIKKs, is composed of a large N-terminal domain, predicted to be largely α -helical, and a C-terminal kinase domain flanked by FAT (FRAP, ATM, TRRAP) and FAT-C terminal (FAT-C) domains (Figure 2). DNA-PKcs is recruited to DNA ends through interaction with the Ku heterodimer [42]. Ku is composed of 70 and ~80 kDa

subunits that together form a basket shaped structure which encircles a molecule of dsDNA [43]. The asymmetrical ring allows the Ku70 subunit to bind in the major groove proximal to the DSB and the Ku80 subunit in the minor groove distal to the break site [15]. In addition, both Ku70 and Ku80 contain unique C-terminal regions. The C-terminal region of Ku70 contains a SAP (SAF-A/B, Acinus and PIAS) domain that has been proposed to interact with chromatin [44,45], while the C-terminal region (CTR) of Ku80 contains a globular helical domain suggested to be involved in protein-protein interactions [46,47] in addition to a region at the extreme C terminus that is required for interaction with DNA-PKcs [48–50].

Small angle X-ray scattering (SAXS) analysis [51] of the full length Ku heterodimer in solution reveals that the Ku80 CTR forms a flexible arm consistent with its ability to interact with and recruit DNA-PKcs to DSBs [52]. Furthermore, the dimensions of the Ku80 CTR are such that in addition to recruiting and positioning the DNA-PKcs molecule proximal to the Ku heterodimer, it may also help to stabilize the adjacent DNA-PKcs molecule on the opposite side of the DSB, hence stabilizing the overall synaptic complex [52]. Indeed, such tethering of the DNA-PK molecules across the break may promote *trans*-autophosphorylation, which facilitates dissociation of DNA-PKcs from the DSB (discussed below).

The protein kinase activity of DNA-PKcs is essential for NHEJ as small molecule inhibitors or mutations that inactivate DNA-PKcs kinase activity result in radiation sensitivity and defects in DSB repair [53–55]. Identification of the physiological substrates of DNA-PKcs is therefore critical for understanding its role in NHEJ. Like other PIKK family members, DNA-PKcs phosphorylates many substrates, including itself (Supplementary Table 1), Artemis and XLF on serines or threonines that are followed by glutamines, *i.e.* SQ or TQ motifs [26,56–61]. However, an increasing number of *in vitro* substrates of DNA-PKcs are phosphorylated on serines or threonines that are followed by other amino acids, frequently leucine or tyrosine. For example, non-SQ/TQ *in vitro* phosphorylation sites have been identified in DNA-PKcs itself [59] (Supplementary Table 1); Ku70 and Ku80 [62]; XRCC4 [63,64]; XLF [60]; Artemis [26,61], DNA ligase IV [65], the Werner syndrome helicase, WRN [66,67] and heterogenous nuclear ribonuclear protein, hnRNP-U (also known as scaffold attachment factor-A or SAF-A) [68,69]. Moreover, phosphorylation of non-SQ/TQ sites in DNA-PKcs [70,71], XLF [60] and hnRNP-U [68,69] are DNA-damage induced and DNA-PK-dependent *in vivo* suggesting that these motifs are *bona fide* DNA-PK targets.

Surprisingly, although DNA-PKcs phosphorylates multiple proteins *in vitro*, relatively few phosphorylation events have been shown to be required for NHEJ *in vivo*. For example, although DNA-PKcs phosphorylates Ku, XRCC4, XLF and DNA ligase IV *in vitro*, phosphorylation at these sites does not seem to be essential for DSB repair *in vivo* [60,64,65,72]. Similarly, although Artemis is highly phosphorylated by DNA-PK *in vitro* [26,61,73], phosphorylation is predominantly ATM-dependent *in vivo* [74]. Indeed, to date, the most established substrate of DNA-PKcs is DNA-PKcs itself. DNA-PKcs is highly phosphorylated *in vitro* [59,75] and is phosphorylated at many of the same sites *in vivo* in response to DNA damage [56,59,70,71,75–77] (Figure 2 and Supplementary Table 1). DNA damage-induced phosphorylation of DNA-PKcs has been reported to be DNA-PKcs-dependent, *i.e.* consistent with autophosphorylation [16,70,71,75,78] although DNA-PKcs can also be phosphorylated by the related PIKKs, ATM and ATR [76,77].

Several lines of evidence suggest that phosphorylation of the Thr2609/ABCDE cluster of phosphorylation sites (see Figure 2) plays a significant role in regulating the dissociation of DNA-PKcs from Ku and DNA. Purified DNA-PKcs and Ku proteins co-immunoprecipitate in the absence of ATP but DNA-PKcs dissociates from Ku when ATP is included in the

reaction [79–81] and phosphorylation-induced dissociation is reversed by protein phosphatases [82]. In contrast, purified DNA-PKcs containing serine/threonine to alanine mutations at six phosphorylation sites within the Thr2609 cluster is considerably less efficient at dissociation from Ku *in vitro* than is wild type DNA-PKcs [52,70,83]. Together, these studies support a model whereby DNA-PKcs is recruited to DNA-bound Ku, which stimulates the protein kinase activity of DNA-PKcs, resulting in autophosphorylation *in trans* across the DSB (Step 4, Figure 1) [71] and release of autophosphorylated DNA-PKcs from DNA-bound Ku (Step 5, Figure 1). Further support for this model comes from *in vivo* studies which have shown that wild type DNA-PKcs is released from sites of laser-induced damage more rapidly than either kinase-dead DNA-PKcs or DNA-PKcs containing alanine substitutions at Ser2056 and the Thr2609/ABCDE cluster [16,52]. Significantly, cells expressing DNA-PKcs in which the Thr2609/ABCDE cluster has been mutated to alanine are more radiation sensitive than cells lacking DNA-PKcs altogether [84]. Moreover, in these cells, the major alternative DSB repair pathway, homologous recombination, is suppressed [85], suggesting that autophosphorylation of DNA-PKcs may play a role in regulating pathway choice as well as NHEJ progression.

Although these studies point to a critical role for Thr2609/ABCDE phosphorylation in the regulation of NHEJ, the *in vitro* DNA-PKcs autophosphorylation sites identified to date (shown in Figure 2 and Supplementary Table 1) represent only a fraction of the total sites autophosphorylated [83] and the effects of additional autophosphorylation events on DNA-PKcs function *in vitro* and *in vivo* have yet to be determined. Moreover, phosphorylation at different sites within DNA-PKcs can have different biological outcomes [85,86], therefore the regulation of DNA-PKcs activity *in vivo* may be complex. Adding to this complexity, several recent proteomics studies have identified multiple additional *in vivo* phosphorylation [87–97] and acetylation sites [98,99] in DNA-PKcs (Figure 2 and Supplementary Table 1), and it will be interesting to determine both the enzymes responsible for these post-translational modifications as well as their effects on DNA-PK function.

Autophosphorylation-induced conformational plasticity in DNA-PKcs

Due in part to its large size (>4000 amino acids), characterization of DNA-PKcs has been difficult, however, elegant cryo-electron microscopy (cryo-EM) and negative stain EM studies have provided considerable information on the overall structure and dimensions of DNA-PKcs, albeit at low resolution [18,100–106]. These image-based structures consistently reveal a globular-shaped monomeric molecule with overall dimensions of approximately 70–120 × 130 × 150–160 Å [101,106–108]. The protein is composed of several recognizable regions: a head or crown domain and a palm or base as well as arms and a brow/forehead that surround a central intramolecular cavity or channel with dimensions suitable for binding single stranded and/or double-stranded (ds) DNA [101,102,106]. (Figure 3A). Indeed, dsDNA has been modeled into this large channel [106,107], whilst ssDNA has been modeled into a smaller channel within the head domain [102,106]. However, precise localization of the different domains of DNA-PKcs within these structures has proved challenging, with different models placing the kinase domain at opposite locations in the overall structure [103,104,106,109].

The recently reported X-ray crystallographic structure of DNA-PKcs (in complex with the Ku80 CTR) at 6.6 Å has helped resolve some of these important questions, and reveals additional clues as to the function of DNA-PKcs [110]. In keeping with the cryo-EM structures, the X-ray structure of DNA-PKcs has a head or crown domain atop a ring-shaped palm or base region composed of two arms (shown in green in Figure 3B) that encircle a central open cavity [110]. These arms (which correspond to the N-terminal ~2880 residues of the protein, shown in green in Figure 2) are composed of multiple anti-parallel HEAT repeats, and other α -helical structures, which fold back on themselves and reverse direction

creating a gap at the base of the molecule (Figure 3B). The arms thus form a pincer-like structure that encircles the putative dsDNA binding channel. Importantly, this structural arrangement places the FAT, kinase and FAT-C domains at the apex of the pincer arms [110] (magenta and yellow in Figure 3B). The regions at the top of the arms, abutting the FAT-kinase-FAT-C domains, are predicted to have considerable flexibility (indicated by blue arrows in Figure 3B), and may represent hinge regions that allow DNA-PKcs to undergo dynamic conformational changes [110].

The solution structure of DNA-PKcs obtained by SAXS reveals a similarly shaped molecule, with a recognizable head/crown and a large ring structure in the palm/base, corresponding to the central dsDNA binding channel (Figure 3C) that compares well with previous cryo-EM and low-resolution X-ray structures [52,111]. The SAXS structure also shows an enclosed cavity in the head region (indicated by the dark shading in Figure 3C) which may be analogous to the ssDNA binding channel proposed from EM structures [106].

Significantly, the solution structure of phosphorylated DNA-PKcs reveals that autophosphorylation produces a large conformational change and an increase in the D_{max} from 155Å to 180Å [52] (Figures 3C,D). As discussed above, the hinge regions identified in the crystal structure [110] (indicated by the blue arrows in Figure 3B) are in close proximity to the C-terminal FAT-kinase-FAT-C domains located at the apex of the ring (coloured magenta and yellow in Figure 3B). Since the FAT domain begins at residue 2883 (Figure 2), it seems likely that the cluster of *in vivo* phosphorylation sites, including the functionally significant Thr2609/ABCDE cluster, is located in close proximity to the hinge regions. Taken together the SAXS and crystallography data are consistent with autophosphorylation inducing a conformational change that increases the gap at the base of the pincers, opening the central DNA binding cavity and releasing DNA-PKcs from dsDNA [52,110,111]. Interestingly, DNA-PKcs also contains two caspase cleavage sites (Asp2713 and Asp2983) [112], suggesting that the hinge region is also proteolytically sensitive, allowing apoptotic cleavage of DNA-PKcs to separate the C-terminal FAT/kinase/FATC domain from the N-terminal DNA binding domain (Figure 2).

Conclusions and Perspectives

Recent structural data from crystallography and SAXS solution analyses have contributed significantly to our understanding of the initiating events of NHEJ and the dynamics of both assembly and disassembly of the DNA-PK complex. In particular, the recent X-ray structure provides the most detailed glimpse to date of the overall structure of DNA-PKcs [110]. This breakthrough crystal structure, in concert with SAXS structures of non-phosphorylated and autophosphorylated DNA-PKcs [52], provides a probable structural basis for the autophosphorylation-induced release of DNA-PKcs from DNA, which may be important not only for completion of NHEJ but also for regulation of pathway choice. Moreover, since other PIKK family members are also composed of an α -helical N-terminal domain and C-terminal FAT-kinase-FATC domains [41], the related protein kinases, ATM and ATR, will likely share a similar structure to that of DNA-PKcs. Indeed, the similarity in cryo-EM structures of DNA-PKcs and ATM, both of which exhibit a “pincer-arm” structure, supports this notion [113].

Together, these results provide a foundation for a more complete understanding of the structural dynamics of the DNA-bound DNA-PKcs-Ku complex, as well as the later steps in the repair of DSBs by NHEJ. However, the available structures are still of low resolution, preventing the assignment of individual residues, and further work is required to fully characterize the effects of phosphorylation on DNA-PKcs structure and function. Similarly, although low-resolution structures have been generated for the DNA-bound DNA-PKcs-Ku

complex [52,105], the precise positioning of both the DNA and Ku within these complexes has made interpretation difficult and further work will provide more detailed information on the precise geometry of the putative DNA-PK synaptic complex. Nevertheless, taken together these studies illustrate how biochemical, cellular and structural approaches integrate to further our understanding of the functional roles of DNA repair machines in the DNA damage response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-PK catalytic subunit
ds	double-stranded
DSB	DNA double strand break
IR	ionizing radiation
NHEJ	non-homologous end joining
ss	single-stranded
SAXS	small angle X-ray scattering
XLf	XRCC4 like factor
XRCC4	X-ray cross complementing gene 4

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Figure 1. A simple model for NHEJ

IR induces DNA ss breaks on opposite DNA strands resulting in a DSB with overhanging ends. In step 1 of NHEJ, the DSB is detected and bound by the Ku70/80 heterodimer (red). Once bound to the DSB, the flexible CTR of Ku80 recruits DNA-PKcs (blue) which induces inward translocation of Ku and positions DNA-PKcs at the extremity of the DSB (step 2). In step 3, the DNA ends are processed by one or more possible enzymes that include Artemis (yellow), PNKP (pink), DNA polymerases (not shown), or MRN (not shown). Either before or after end processing, DNA-PKcs undergoes autophosphorylation (step 4, indicated by arrows/dashed lines), resulting in a conformational change that opens the central DNA binding cavity, resulting in the release of autophosphorylated DNA-PKcs from DNA (step 5). In the final step (step 6), the XRCC4/DNA ligase IV complex (green/grey) ligates the DNA ends in a reaction that is stimulated by XLF (purple).

Figure 2. Domain map of DNA-PKcs showing identified post-translational modifications

Amino acid numbers in DNA-PKcs (accession number P78527) corresponding to approximate domain boundaries (determined from the UniProt protein database [114]) are shown in black. The N-terminal alpha-helical domain (amino acids 1–2882) is shown in green. Positions of the L-rich (Leucine-rich), FAT (pink), kinase (yellow) and FATC (magenta) domains were determined by UniProt and confirmed in NCBI [115]. Identified phosphorylation sites are indicated in blue and acetylation sites are shown in red with the approximate positions indicated by either a circle for individual sites or a line for clustered sites. *In vitro* sites are shown in italics and *in vivo* phosphorylation sites are highlighted in bold with DNA damage-induced phosphorylation sites indicated by asterisks. Phosphorylation sites within the ABCDE/Thr-2609 cluster are boxed. References for phosphorylation sites are provided in Supplementary Table 1. The acetylation site at amino acid 2 is from [99]; other acetylation sites are from [98]. Caspase cleavage sites (Asp2713 and Asp2983) are from [112] and are indicated by dashed lines.

Figure 3. Structural models of DNA-PKcs

(A) Cryo-EM structure of DNA-PKcs at ~ 7 Å showing the crown/head (red), arms (yellow), forehead/brow (purple), and base (blue), with the DNA binding cavity in the centre of the molecule. Reprinted with permission from [106]; (B) X-ray structure of DNA-PKcs in complex with the Ku80CTR at 6.6 Å from [110] (PDB ID 3KGV) showing the N-terminal HEAT/ α -helical domains or arms (green), the FAT and FATC domains (magenta), and the kinase domain in yellow. A putative DNA binding domain within the central cavity is shown in blue and the forehead in light green. The regions where the HEAT repeat/ α -helical arms abut the FAT/kinase/FATC domains (indicated by blue arrows) are predicted to exhibit conformational flexibility [110]. Phosphorylation within or close to this flexible region may induce conformational changes that regulate the interaction of DNA-PKcs with DNA (see text for details); (C) left - Single SAXS envelope for non-phosphorylated DNA-PKcs. Centre, superimposition of the DNA-PKcs crystal structure (coloured as in Fig 3B) on the average SAXS envelope. The enclosed cavity in the head region, observed in the EM structures, is observed in the SAXS data as dark shading (see text for details). Right, centre panel rotated by 90°; (D) left - Single SAXS envelope for autophosphorylated DNA-PKcs. Centre, average SAXS envelopes for autophosphorylated DNA-PKcs superimposed with the X-ray structure. Right, average SAXS envelope for autophosphorylated DNA-PKcs, rotated by 90° as in panel C.