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DNA-PK and ATM phosphorylation sites in XLF/Cernunnos are not required for repair of DNA double strand breaks

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ABSTRACT

Nonhomologous end joining (NHEJ) is the major pathway for the repair of DNA double strand breaks (DSBs) in human cells. NHEJ requires the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), Ku70, Ku80, XRCC4, DNA ligase IV and Artemis, as well as DNA polymerases μ and λ and polynucleotide kinase. Recent studies have identified an additional participant, XLF, for XRCC4-like factor (also called Cernunnos), which interacts with the XRCC4–DNA ligase IV complex and stimulates its activity *in vitro*, however, its precise role in the DNA damage response is not fully understood. Since the protein kinase activity of DNA-PKcs is required for NHEJ, we asked whether XLF might be a physiological target of DNA-PK. Here, we have identified two major *in vitro* DNA-PK phosphorylation sites in the C-terminal region of XLF, serines 245 and 251. We show that these represent the major phosphorylation sites in XLF *in vivo* and that serine 245 is phosphorylated *in vivo* by DNA-PK, while serine 251 is phosphorylated by Ataxia-Telangiectasia Mutated (ATM). However, phosphorylation of XLF did not have a significant effect on the ability of XLF to interact with DNA *in vitro* or its recruitment to laser-induced DSBs *in vivo*. Similarly, XLF in which the identified *in vivo* phosphorylation sites were mutated to alanine was able to complement the DSB repair defect as well as radiation sensitivity in XLF-deficient 2BN cells. We conclude that phosphorylation of XLF at these sites does not play a major role in the repair of IR-induced DSBs *in vivo*.

1. Introduction

In human cells, DNA double strand breaks (DSBs) are repaired by either the homologous recombination pathway or by non-homologous end joining (NHEJ). Proteins required for NHEJ include the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), the Ku70/80 heterodimer and the XRCC4-DNA ligase IV complex. Absence or inactivation of any of these factors leads to radiation sensitivity and defects in DNA DSB repair and V(D)J recombination [1–3]. Additional proteins such as Artemis, polynucleotide kinase (PNK) and DNA polymerases of the Pol X family are also required for NHEJ under some circumstances (reviewed in [4]). In 2006, another factor, named XLF for XRCC4-like factor (also called Cernunnos), was identified. XLF interacts with XRCC4 and cells that lack XLF are radiation sensitive and defective in DSB repair and V(D)J recombination [5–7]. XLF is similar in structure to XRCC4 in that the first ~233 amino acids adopt a globular head domain

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that is followed by a long, α-helical stalk [5,8,9]. However, unlike XRCC4, the C-terminal stalk in XLF doubles back on itself to form a cone-shaped structure [8,9]. The structure of the C-terminal ~50 amino acids of XLF is not known, although it has been predicted to facilitate DNA binding *in vitro* [8]. Several recent studies have shown that XLF stimulates the activity of the XRCC4–DNA ligase IV complex *in vitro* [10–12]. However, its precise role in NHEJ remains to be determined.

One of the important players in NHEJ is DNA-PKcs. DNA-PKcs is a member of the phosphatidylinositol-3 kinase-like (PIKK) family of protein kinases which includes Ataxia-Telangiectasia Mutated (ATM) and ATM- and Rad3-related (ATR) [13]. Given that the protein kinase activity of DNA-PK is required for NHEJ [14–16], one of the major challenges to understanding its function in DSB repair is to identify its physiologically relevant substrates. Like ATM and ATR, DNA-PKcs phosphorylates its substrates on serines or threonines that are followed by glutamine (*i.e.* SQ/TQ sites), however, it can also phosphorylate substrates on serines and threonines that are followed by other amino acids (reviewed in [3]). *In vitro*, DNA-PK substrates include many of the proteins involved in NHEJ including DNA-PKcs itself, Ku70, Ku80, XRCC4, DNA ligase IV and Artemis (reviewed in [4]). However, the *in vitro* DNA-PK phosphorylation sites in Ku70, Ku80 and XRCC4 are not required for NHEJ [17–19]. DNA-PKcs is also autophosphorylated *in vitro* and is phosphorylated on multiple sites *in vivo*. Phosphorylation of DNA-PKcs likely regulates access of other repair factors to DSBs and is required for NHEJ (reviewed in [4,20]). XLF, the newest member of the NHEJ pathway, contains several SQ/TQ motifs (S55, S132, T223 and S251), and therefore we considered that it might be a substrate for DNA-PK.

Here, we show that XLF is phosphorylated *in vitro* by DNA-PK on multiple sites including serines 245 and 251 and that these sites are phosphorylated *in vivo* in irradiated human cells. Using phosphospecific antibodies, we show that IR-induced phosphorylation of serine 245 is largely DNA-PK-dependent, while IR-induced phosphorylation of serine 251 is ATM-dependent. The major phosphorylation sites are located in the extreme C-terminal region of XLF, which we show directly binds DNA *in vitro*. However, phosphorylation by DNA-PK did not have a major effect on the ability of XLF to interact with DNA. XLF has recently been shown to be recruited to sites of UV laser-induced DNA damage *in vivo* [21]. We show that XLF in which six of the identified *in vivo* phosphorylation sites have been mutated to alanine (6A-XLF) is still recruited to sites of UV laser-damaged DNA. Moreover, 6A-XLF is still able to complement the repair defect in XLF-deficient 2BN cells [22,23] with respect to kinetics of histone H2AX phosphorylation as well as survival after IR. Therefore, we conclude that although XLF is phosphorylated in a PIKK-dependent manner *in vivo*, phosphorylation at these sites is unlikely to be required for repair of IR-induced DSBs *in vivo*.

2. Materials and methods

2.1. Cloning and expression of human XLF in bacteria

Full-length human XLF (hXLF) cDNA (Genebank accession number: NM_024782) was cloned from a human cDNA library as described previously [18]. The sequences of the primers used (GST-hXLF) are provided in Supplementary methods.

The PCR product was subcloned into the pGEX6P1 vector (GE Healthcare) at BamHI/XmaI sites to create a GST-fusion protein, or into pQE30 (QIAGEN) vector at BamHI/XmaI sites for a His-tagged protein, respectively. DNA sequences were confirmed by the University of Calgary DNA Services Facility. The pGEX6P1-XLF plasmid was introduced into *Escherichia coli* BL21 for protein expression and GST-tagged XLF protein was purified over glutathione-conjugated sepharose (GE Healthcare). The pQE30-XLF plasmid was introduced in *E. coli* M15 for expression and the His-XLF protein was purified over Ni-NTA agarose (Qiagen). The conditions used for expression and purification of GST- and His-tagged proteins in bacteria are provided in Supplementary methods.

2.2. Mutational analysis of XLF

N- and C-terminal deletions of XLF were generated by PCR using oligonucleotide 5′-GST-hXLF and the primer 3′-hΔ239, or oligonucleotide 3′-GST-hXLF and primer 5′-hΔ240, respectively (see Supplementary methods for details). These constructs were expressed in bacteria to generate a C-terminal deletion mutant corresponding to amino acids 1–239 (GST-XLF$_{1–239}$) and an N-terminal deletion mutant corresponding to amino acids 240–299 (GST-XLF$_{240–299}$). Conditions for the expression and purification of XLF are described in Supplementary methods. Site direct mutagenesis was used to generate single serine or threonine to alanine mutations of hXLF at serines 55, 132, 203, 245, 251 and 263 and threonines 223 and 266. PCR was performed for each designed mutation with the following sets of complementary primers: 5′-h55S > A, 3′-h55S > A, 5′-h132S > A, 3′-h132S > A, 5′-h203S > A, 3′-h203S > A, 5′-h223T > A, 3′-h223T > A, 5′-h245S > A, 3′-h245S > A, 5′-h251S > A, 3′-h251S > A, 5′-h263S > A, 3′-h263S > A, 5′-h266T > A, 3′-h266T > A (see Supplementary methods for details). The same primers were used to generate mutations in pGEX6P1 (for bacterial expression), and pIRES-hrGFP-1a-hXLF and pEGFP-C2-hXLF for expression in mammalian cells (see below).

2.3. Generation of vectors for transient expression of XLF in human cells

hXLF DNA was amplified using pGEX6P1-XLF as a template and the PCR product was subcloned into either the mammalian expression vector pIRES-hrGFP-1a (Stratagene) to generate XLF containing a C-terminal FLAG-tag or pEGFP-C2 (BD Biosciences) to generate XLF containing an N-terminal GFP fusion. The primers used (5′-hXLF-FLAG, 3′-hXLF-FLAG, 5′-EGFP-hXLF and 3′-EGFP-hXLF) are shown in Supplementary methods. The quadruple phosphorylation mutant (4A; S132/203/245/251A) and the sextuple mutant (6A; S132/203/245/251/S263/T266A) were generated in pIRES-hrGFP-1a and/or pEGFP-C2 using the primers described above.

2.4. Immunoblotting

Samples were subjected to SDS PAGE, transferred to nitrocellulose and probed as described previously [18]. The