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DNA ligase III and DNA ligase IV carry out genetically distinct forms of end joining in human somatic cells

Sehyun Oh[1], Adam Harvey, Jacob Zimbric[2], Yongbao Wang[3], Thanh Nguyen, Pauline J. Jackson, Eric A. Hendrickson*

Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota Medical School, Minneapolis, MN 55455, United States

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ABSTRACT

Ku-dependent C-NHEJ (classic non-homologous end joining) is the primary DNA EJing (end joining) repair pathway in mammals. Recently, an additional EJing repair pathway (A-NHEJ; alternative-NHEJ) has been described. Currently, the mechanism of A-NHEJ is obscure although a dependency on LIGIII (DNA ligase III) is often implicated. To test the requirement for LIGIII in A-NHEJ we constructed a LIGIII conditionally-null human cell line using gene targeting. Nuclear EJing activity appeared unaffected by a deficiency in LIGIII as, surprisingly, so were random gene targeting integration events. In contrast, LIGIII was required for mitochondrial function and this defined the gene's essential activity. Human Ku:LIGIII and Ku:LIGIV (DNA ligase IV) double knockout cell lines, however, demonstrated that LIGIII is required for the enhanced A-NHEJ activity that is observed in Ku-deficient cells. Most unexpectedly, however, the majority of EJing events remained LIGIV-dependent. In conclusion, although human LIGIII has an essential function in mitochondrial maintenance, it is dispensable for most types of nuclear DSB repair, except for the A-NHEJ events that are normally suppressed by Ku. Moreover, we describe that a robust Ku-independent, LIGIV-dependent repair pathway exists in human somatic cells.

1. Introduction

A serious challenge to genomic integrity is the occurrence of a DNA DSB (double-strand break) [1]. To avoid the pathological outcomes that result from even a single unrepaired DNA DSB, all cells have developed efficient DSB repair pathways. In most organisms, there are two major pathways: HR (homologous recombination) and C-NHEJ (classic-non-homologous end joining) [2,3]. HR is preferentially used in lower organisms, however in mammals – and particularly in human cells – the majority of DSBs are repaired *via* C-NHEJ.

C-NHEJ facilitates the direct ligation of the broken ends of a DSB. Since the DNA termini formed at DSBs are, however, often complex and can contain non-ligatable end groups, the repair of such DNA lesions may require the processing of the ends prior to ligation [1,4]. This requirement often leads to the loss or addition of nucleotides from either side of the DSB, making C-NHEJ "error-prone". The mechanism of C-NHEJ-mediated DSB repair postulates that Ku (the Ku70/Ku86 heterodimer) binds to the DSB ends, where it recruits downstream C-NHEJ factors that facilitate processing [5]. Finally LIGIV (DNA ligase IV), in association with XRCC4 (X-ray-cross-complementation gene 4) and XLF (Cernunnos/XRCC4-like factor), performs the end ligation reaction [1]. This linear, stepwise model for C-NHEJ may be oversimplified as there is evidence that LIGIV, XRCC4 and XLF may perform roles both upstream and downstream in the repair process [6–8].

There is an additional EJing pathway present in higher eukaryotes. It has interchangeably been referred to as MMEJ (micro-homology-mediated end joining) [9], B-NHEJ (backup-NHEJ) [10] and A-NHEJ (alternative-NHEJ) [11], (hereafter, A-NHEJ). Unlike the HR and C-NHEJ pathways, which are conserved from bacteria to man, the A-NHEJ pathway has evolved in a somewhat checkered manner and can only be detected in about a third of eukaryotic genomes [12]. It is presumed that an end-binding factor besides Ku is required to bind onto the broken DNA ends, stabilize

* Corresponding author at: Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota Medical School, 6-155 Jackson Hall, 321 Church Street, SE, Minneapolis, MN 55455, United States. Tel.: +1 612 624 5988; fax: +1 612 624 0935.
E-mail addresses: so2411@columbia.edu (S. Oh), jzimbric@gmail.com (J. Zimbric), yongbao.x.wang@questdiagnostics.com (Y. Wang), hendr064@umn.edu (E.A. Hendrickson).
[1] Current address: Department of Microbiology and Immunology, Columbia University, 701 West 168th Street, HHSC 1208, New York, NY 10032, United States.
[2] Current address: 26193 Cedar Spring Road, Winona, MN 55987, United States.
[3] Current address: Cancer Diagnostics Service, Quest Diagnostics Nichols Institute, Chantilly, VA 20151, United States.

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them, protect them from random nuclease degradation and finally funnel the ends into the A-NHEJ pathway [13]. Then, because microhomology is frequently used to mediate the repair event, some end resection is required [14]. Alignment activities to bring the microhomologies into register are also needed, followed by the action of a flap-like nuclease to trim non-base paired regions and finally a ligation complex to covalently link the ends back together [15]. Because the pathway uses microhomology to mediate the repair event, deletions always accompany the repair event, as does loss of one of the blocks of microhomology [4].

Several laboratories have made dedicated attempts to identify A-NHEJ factors. In particular, a brute-force nuclear extract fractionation protocol identified LIGIII (DNA ligase III; [12]), heretofore known only for its role in BER (base excision repair), as the candidate ligase required for A-NHEJ [16]. Using guilt-by-association as a scientific rationale, PARP1 (poly (ADP-ribose) polymerase 1) and XRCC1 (X-ray cross complementing gene 1), two proteins known to interact with LIGIII during BER, were subsequently identified as also being involved in A-NHEJ [13,17,18]. PARP1 is presumed to compete with Ku for binding to broken DNA ends thereby dictating pathway choice [13,18] whereas XRCC1 appears to act as a chaperone for LIGIII [19]. Additional factors have also been implicated in A-NHEJ. Thus, CtIP (C-terminal interacting protein) and the MRN (Mre11:Rad50:Nbs1) complex – factors known to be involved in the end resection events required for HR – have also been implicated in the end resection steps of A-NHEJ [20–24].

If the factors needed for A-NHEJ are not completely defined and the A-NHEJ reaction mechanism nebulous, it is also fair to say that the biological role(s) of A-NHEJ is even more poorly understood. Most of the current interest in A-NHEJ, however, stems from its implicated use in the chromosomal translocations that are present in cancer cells. Sequencing of human cancer genomes has revealed that many [25–27], albeit not all [28] chromosomal translocations have microhomology at their breakpoint junctions, which implicates A-NHEJ in their genesis. This hypothesis gained support from work in which LIGIII conditionally-null murine cells showed decreased translocation frequencies and reduced microhomology usage [29–32]. An additional biological process where A-NHEJ has been implicated is in the random insertion events associated with rAAV (recombinant adeno-associated virus)-mediated gene targeting. Although rAAV can mediate high frequency gene targeting, the majority of the viral integration events still occur randomly [33]. Moreover, our laboratory has reported that a reduction in the C-NHEJ genes Ku70 [34] and LIGIV [35] had almost no impact on the random rAAV integration rate – implying that these events may be mediated instead by A-NHEJ. In summary, although A-NHEJ was a neglected subject for many years, in the past decade it has proven itself to be an increasingly interesting and biologically relevant topic.

A key feature of A-NHEJ is its dependence on LIGIII [16]. Unlike the other ligases, LIGIII is molecularly heterogeneous [12,36,37]. Thus, alternative translation initiation generates mitochondrial and nuclear forms of LIGIII, which either contain or lack a MLS (mitochondrial localizing sequence), respectively [36]. The existence of LIGIII isoforms implies diverse functional roles for LIGIII. One experimental approach to unraveling the complexity of LIGIII is to generate a LIGIII-deficient model system, which has already been accomplished in the chicken cell line, DT40 [38], and in the mouse [30,32,39]. In these systems the gene is essential due to its presumed requirement for mitochondrial DNA replication. Moreover, in LIGIII conditionally-null mice no obvious nuclear DNA repair phenotypes could be detected [30,32]. The extrapolation of these studies to humans is unfulfilled as neither LIGIII patients nor LIGIII-deficient human cell systems have been described.

In this study, we conditionally inactivated the LIGIII gene in the HCT116 human cell line and confirmed that the loss of LIGIII results in death due to mitochondrial dysfunction. We also constructed a cell line that exclusively expressed a mitochondrial-only form of LIGIII. The nuclear LIGIII deficiency in this cell line caused a growth retardation, but it did not affect the overall NHEJ repair activity nor did it result in hypersensitivity to DNA damaging agents. Unexpectedly, we also demonstrate that LIGIII-dependent A-NHEJ does not mediate rAAV random integration events. These findings were extended by constructing human cell lines that were doubly deficient for either Ku and LIGIII or Ku and LIGIV. These experiments demonstrated that LIGIII is required for the enhanced A-NHEJ activity that is observed in Ku-deficient cells and that the vast majority of repair events in a Ku-deficient cell are still LIGIV-dependent. In conclusion, human LIGIII has an essential function in mitochondrial maintenance, however it is dispensable for most nuclear DSB repair, except for the A-NHEJ that is normally suppressed by Ku. In addition, we demonstrate that human cells have a robust Ku-independent, but LIGIV-dependent EJing activity.

2. Materials and methods

2.1. Primers

The sequences for all primers referenced in this section can be found in Supplemental Table 1.

Supplementary Table 1 can be found, in the online version, at http://dx.doi.org/10.1016/j.dnarep.2014.04.015.

2.2. Construction of LIGIII-null cells

Conditional and non-conditional knockout LIGIII targeting vectors were constructed as described, with a few modifications [33,40]. For the conditional knockout vector, the left and right homology arms, the latter of which contained the floxed exon 5, were generated by PCR. For the left homology arm, Exon5_LARM_F1 and Exon5_LARM_SacR1 primers were used. For the right homology arm, the PCR products generated from Exon5_RARM_XhoF1 × Exon5_RARM_R1 primers and Exon5_KpnF1 × Exon5_XhoR1 primers were ligated after *XhoI* restriction enzyme digestion. The relevant homology arms and a NEO (neomycin-resistance) gene cassette were assembled together through ligations followed by unique restriction enzyme digestions, and then cloned into the pAAV-MCS vector. A non-conditional knockout targeting vector was generated in a similar way, but it did not include the floxed exon 5 sequences. To select for productively infected cells, the rAAV-infected cells were incubated in 1 mg/ml G418-containing media for approximately 2 weeks. At this time, genomic DNA was purified from all G418-resistant clones and PCR was used to screen for the subset of those in which correct targeting had taken place. Targeted clones were screened with Exon5_SC_F2 × NeoR2 primers, and retargeted clones were confirmed by LIG3_LArm_F3 × LIG3_RArm_R2 primers.

2.3. Construction of a $Ku86^{flox/-}$:$LIGIII^{mito/-}$ cell line

Conditional and knock-in LIGIII targeting vectors were constructed as described above. In the first round of targeting, $Ku86^{flox/-}$ cells [41] were infected with a conditional knockout (*i.e.*, "flox:NEO") vector, as described in the creation of the $LIGIII^{flox/-}$ cell line. After confirming correct targeting, clones were Cre-treated, and screened for the loss of the drug selection cassette (NEO), but retention of the conditional (floxed) exon, thus yielding a $Ku86^{flox/-}$:$LIGIII^{+/flox}$ cell line. In the second round of targeting, these cells were infected with a rAAV knock-in vector, which introduced ATC point mutations into the two closely spaced ATGs that enable nuclear LIGIII expression. For the rAAV LIGIII knock-in targeting vector construction, the left homology arm

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