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Role of the yeast DNA repair protein Nej1 in end processing during the repair of DNA double strand breaks by non-homologous end joining

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ABSTRACT

DNA double strand breaks (DSB)s often require end processing prior to joining during their repair by non-homologous end joining (NHEJ). Although the yeast proteins, Pol4, a Pol X family DNA polymerase, and Rad27, a nuclease, participate in the end processing reactions of NHEJ, the mechanisms underlying the recruitment of these factors to DSBs are not known. Here we demonstrate that Nej1, a NHEJ factor that interacts with and modulates the activity of the NHEJ DNA ligase complex (Dnl4/Lif1), physically and functionally interacts with both Pol4 and Rad27. Notably, Nej1 and Dnl4/Lif1, which also interacts with both Pol4 and Rad27, independently recruit the end processing factors to *in vivo* DSBs *via* mechanisms that are additive rather than redundant. As was observed with Dnl4/Lif1, the activities of both Pol4 and Rad27 were enhanced by the interaction with Nej1. Furthermore, Nej1 increased the joining of incompatible DNA ends in reconstituted reactions containing Pol4, Rad27 and Dnl4/Lif1, indicating that the stimulatory activities of Nej1 and Dnl4/Lif1 are also additive. Together our results reveal novel roles for Nej1 in the recruitment of Pol4 and Rad27 to *in vivo* DSBs and the coordination of the end processing and ligation reactions of NHEJ.

1. Introduction

DNA double strand breaks (DSB)s are cytotoxic and mutagenic lesions that must be repaired to maintain genomic integrity. Although multiple pathways have evolved to repair DSBs, these can be divided into two groups depending upon whether extensive DNA sequence homology is used to guide the repair. In mammalian cells, the majority of DSBs are repaired by non-homologous end joining (NHEJ) whereas this type of repair makes relatively minor contribution to DSB repair in the yeast, *Saccharomyces cerevisiae* [1–3].

The major NHEJ pathways in mammals and yeast share several functionally homologous components, including Ku70-Ku80 (yKu70-yKu80), DNA ligase IV-XRCC4 (Dnl4/Lif1) and XLF (Nej1) [1–3]. DSB repair by NHEJ is initiated by the binding of the Ku heterodimer to DNA ends followed by protein-mediated DNA end-bridging. In mammals, this is carried out by the catalytic subunit of the DNA-dependent protein kinase (DNA PKcs), which binds to Ku-bound DNA ends and then juxtaposes the ends *via* an interaction between DNA PKcs molecules bound to two different DNA ends [4]. End-bridging in yeast, which lacks a homolog of DNA PKcs, is carried out by the Mre11/Rad50/Xrs2 complex [5]. DNA end joining is carried out by DNA ligase IV/XRCC4 (Dnl4/Lif1) together with XLF (Nej1) [6–20]. While the binding of Ku to a DNA end serves as the signal for the recruitment of the other NHEJ factors [21–26], studies in yeast have shown that both Dnl4/Lif1 and Nej1 contribute to the stable association of yKu70-yKu80 with DSBs *in vivo* [15,26].

Most DSB ends generated by ionizing radiation and/or oxygen free radicals have termini that cannot be directly ligated. The processing of juxtaposed DNA ends by nucleases and Pol X family DNA polymerases to generate ligatable termini results in small deletions and insertions at the repair site [1–3]. Pol X family members, Pol mu, Pol lambda and terminal transferase in mammals and Pol4 in yeast,

Abbreviations: BRCT, breast cancer susceptibility protein 1 C-terminal domain; CBP, calmodulin binding peptide; ChIP, chromatin immunoprecipitation; DNA PKcs, DNA-dependent protein kinase catalytic subunit; DSB, DNA double strand break; GST, glutathione-S-transferase; NHEJ, non-homologous end joining; PCR, polymerase chain reaction.

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have been implicated in the addition of nucleotides to DSB termini [1–3,27]. The nuclease Artemis, which binds to DNA PKcs, plays a role in the processing of a subset of DSBs in mammalian cells [28]. Genetic studies in yeast have implicated a DNA replication protein, Rad27, a homolog of mammalian flap endonuclease 1 [29], in the processing of a subset of DSBs with incompatible DNA ends [30]. A later study using a different assay failed to reproduce these findings, suggesting that Rad27 either is not involved in end processing or is functionally redundant with other nucleases in the end processing step of this repair pathway [31]. In support of the idea that Rad27 participates in the end processing reactions, we previously characterized physical and functional interactions among Pol4, Rad27 and Dnl4/Lif1 that co-ordinate end processing and ligation [32,33]. Here we demonstrate that Nej1 also interacts with Pol4 and Rad27, modulating their DNA synthesis and nuclease activities, respectively. In addition, we show that interactions with both Nej1 and Dnl4/Lif1 contribute to the recruitment of Pol4 and Rad27 to *in vivo* DSBs.

2. Materials and methods

2.1. Yeast strains

The yeast strains SLY1A, SLY1A *lif1*Δ and SLY1A *nej1*Δ have been described previously [15,34]. To generate a strain lacking both Lif1 and Nej1, the plasmid pRS306 was used to generate *URA3* marked deletions of *LIF1* in the SLY1A *nej1*Δ strain.

A sequence encoding a 9× Myc tag was added to the C-termini of both Pol4 and Rad27 proteins by the polymerase chain reaction using primers flanking the 3′ end of either the *POL4 or RAD27* genes and plasmid pYM20-9Myc-hph as the template. The PCR fragments were used to replace the wild type *POL4* and *RAD27* genes of SLY1A with versions encoding Pol4 and Rad27 proteins with a C-terminal c-Myc tag.

2.2. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as described [26,35]. Briefly, 2% galactose was added to mid-log phase cultures grown in pre-induction medium (3% glycerol/2% lactic acid/0.05% glucose) at 30 °C to induce HO endonuclease expression. After *in vivo* cross-linking with 1% formaldehyde (v/v), cells were lysed and genomic DNA was sonicated to yield fragments with an average size of 500–700 base pair. For immunoprecipitation, the sonicated extracts were incubated with anti-Myc antibody (Millipore) at 4 °C overnight, followed by 1 h incubation with Protein A/G beads (Santa Cruz). After cross-link reversal, genomic DNA was purified and amplified by real-time quantitative PCR (StepOne, Applied Biosystems) using a primer set (5′ CCCTGGTTTTGGTTTTGTAG 3′ and 5′ CACATCTTCCCAATATCCGTCACC 3′) that annealed at a site adjacent to the HO-induced DSB and primers specific for the *PRE1* gene situated on chromosome V (5′ CCCACAAGTCCTCTGATTTACATTCG 3′, and 5′ GGAATTCACCGCATGGTTTTCATAAGAG 3′). The recruitment of protein to the HO break is expressed as relative immunoprecipitation, which represents the ratio of the specific signal at the HO break to the nonspecific signal at the *PRE1* locus, normalized to the value obtained from an uninduced sample.

2.3. Plasmids

Plasmids pET-28b-Pol4, pET-28b-Pol4ΔBRCT, pGST-Pol4, pET-Rad27 and pGST-Rad27 have been described previously [32,33]. DNA sequences encoding full length Nej1 and derivatives lacking either the N-terminal 129 amino acids (ΔN-Nej1) or the C-terminal 120 amino acids (Nej1-ΔC) were amplified by PCR from pYes2.1-Nej1 [15] and subcloned into the *Escherichia coli* expression plasmid pET28a(+) to generate pET28a-Nej1, pET28a-ΔN-Nej1, pET28a-Nej1-ΔC. The sequences of the plasmid inserts were verified.

2.4. Protein purification

Calmodulin binding peptide (CBP)-tagged Nej1, his-tagged Dnl4/Lif1 and his-tagged human XLF were purified as described previously [5,15,36]. To purify his-tagged versions of Pol4, Rad27 and Nej1, *E. coli* Rosetta cells (Novagen) harboring expression plasmids were grown at 37 °C in 2×YT medium (16 g tryptone, 10 g yeast extract and 5 g NaCl per liter) containing 100 μg/ml kanamycin and 34 μg/ml chloramphenicol. At an optical density of 0.5 at 600 nm, isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.2 mM and growth was continued overnight at 16 °C. Cells were harvested, resuspended in lysis buffer (50 mM Tris–HCl pH7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 0.2% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, 2 μg/ml aprotinin) and lysed by sonication. After centrifugation, his-tagged proteins were purified from the cleared lysate by phosphocellulose (P11, Whatman) and nickel-nitrilotriacetic acid-agarose (Qiagen). His-tagged versions of Pol4 and Rad27 were further purified by SP Sepharose FF (GE Healthcare) column chromatography whereas his-tagged versions of full length Nej1, and N- and C-terminal fragments of Nej1 were further purified by Mono Q (GE Healthcare) column chromatography. Dnl4/Lif1 and yeast Ku were purified from yeast cells as described previously [5].

To purify glutathione-S-transferase (GST)-tagged proteins, *E. coli* DH5 cells harboring either pGST-Pol4 or pGST-Rad27 were grown in 2xYT medium containing 100 μg/ml ampicillin and harvested as described above. Cell pellets were resuspended in GST binding buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors and 0.2% Triton X100 and then sonicated. After clarification, the lysate was incubated with Glutathione Sepharose beads that had been pre-equilibrated with GST binding buffer at 4 °C for 2 h. The beads were washed with GST binding buffer prior followed by elution of bound proteins with 10 mM glutathione in GST binding buffer. Protein concentrations were measured by the Bradford assay [37] using bovine serum albumin as the standard. Purified proteins were dispensed in small aliquots, flash-frozen in liquid nitrogen and stored at −80 °C.

2.5. Pull down assays

Purified his-tagged Pol4, Pol4ΔBRCT or Rad27 (1 μg) were immobilized on nickel beads and then incubated with extracts (200 μg) from yeast cells expressing CBP-tagged Nej1 [15] in 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 5% glycerol, 0.2% NP-40 containing a mixture of protease inhibitors. After washing with the same buffer containing 30 mM imidazole, proteins bound to the beads were eluted with SDS sample buffer. CBP-Nej1 was detected by immunoblotting with CBP antibody (Millipore). To determine the region of Nej1 that interacts with Pol4 and Rad27, nickel beads in the absence or presence of his-tagged full-length Nej1, Nej1 C-terminal domain (ΔN-Nej1), or Nej1 N-terminal domain (Nej1-ΔC) were incubated with 1 μg of purified GST-tagged Pol4 or GST-tagged Rad27 as described above. GST fusion proteins bound to the beads was detected by immunoblotting with GST antibody (Cell Signaling).

2.6. Gap-filling DNA synthesis assays

DNA polymerase assays with Pol4 were carried out as described previously with DNA substrates that contained either a 1-nucleotide gap within a DNA duplex or a 3-nucleotide gap generated by the annealing of the partially complementary single