Cernunnos Interacts with the XRCC4-DNA-ligase IV Complex and Is Homologous to the Yeast Nonhomologous End-joining Factor Nej1*

DNA double strand breaks are considered as the most harmful DNA lesions and are repaired by either homologous recombination or nonhomologous end joining (NHEJ). A new NHEJ factor, Cernunnos, has been identified, the defect of which leads to a severe immunodeficiency condition associated with microcephaly and other developmental defects in humans. This presentation is reminiscent to that of DNA-ligase IV deficiency and suggests a possible interplay between Cernunnos and the XRCC4-DNA-ligase IV complex. We show here that Cernunnos physically interacts with the XRCC4-DNA-ligase IV complex. Moreover, a combination of sensitive methods of sequence analysis revealed that Cernunnos can be associated with the XRCC4 family of proteins and that it corresponds to the genuine homolog of the yeast Nej1 protein. Altogether these results shed new lights on the last step, the DNA religation, of the NHEJ pathway.

DNA double strand breaks are caused by exposure to ionizing radiation or chemical agents. They also result from physiological DNA rearrangements occurring during meiosis or, in vertebrate cells, during specialized recombination events underlying the development and maturation of the adaptive immune system (1). Two main mechanisms were developed in eukaryotic cells for efficient DNA double strand break repair: the accurate homologous recombination and the nonhomologous end-joining (NHEJ) pathways (see Ref. 2 for review). Six mammalian factors constitute the core NHEJ apparatus: the Ku70/80 heterodimer, the DNA-PKcs kinase, the Artemis endonuclease, and the XRCC4-DNA-ligase IV complex responsible for the final ligation step (3). Additional NHEJ factors have been recognized in yeast, such as Nej1, which interacts with the yeast XRCC4 homolog Lvp1p (4–6). We recently identified a novel human NHEJ DNA repair factor, Cernunnos, the defect of which results in immune deficiency and microcephaly (7). One striking characteristic of Cernunnos patients is their clinical and biological resemblance with DNA-ligase IV patients (8–10). This includes microcephaly and immune deficiency, mostly characterized by a severely impaired development of B and T lymphocytes. Both conditions are caused by a faulty NHEJ, which translates into increased cellular sensitivity to DNA-damaging agents and the impaired capacity to join double-stranded DNA ends in vitro and in vivo. This parallel prompted us to hypothesize that Cernunnos may be acting at the same level as the XRCC4-DNA-ligase IV complex during DNA repair and may indeed incorporate into this complex.

EXPERIMENTAL PROCEDURES

Antibodies and Immunoprecipitation—The Cernunnos Orf, together with a C terminus V5 or myc epitope tag, was cloned into the pcDNA3.1 vector (7) and used to transfect 293T cells. Cells were lysed for 20 min on ice in 1 ml of lysis buffer (1× TNE) containing 50 mM Tris (pH 8.0), 2 mM EDTA, 0.5% Nonidet P-40, 1% phosphatase inhibitor cocktails (1 and 2, Sigma), and protease inhibitor (Roche Applied Science). One mg of cell lysate was first pre-cleared with rabbit or mouse IgG (Santa Cruz Biotechnology) and then incubated (1 h, 4°C) with 20 μl of prewashed protein A-Sepharose beads (Amersham Biosciences). Immunoprecipitations were performed on pre-cleared lysates using anti-V5 (Invitrogen), anti-DNA-ligase IV (Acris), and anti-XRCC4 (Serotec) rabbit polyclonal or murine monoclonal anti-myC (Santa Cruz Biotechnology) antibodies for 1 h at 4°C. Immune complexes were collected with protein A-Sepharose beads. Immunoprecipitations were analyzed by Western blotting with murine monoclonal anti-V5 (Invitrogen), anti-myC, or rabbit polyclonal anti-DNA-ligase IV and anti-XRCC4 antibodies.

In Silico Sequence Analysis: Molecular Modeling—PSI-BLAST (11) searches were performed at NCBI (www.ncbi.nlm.nih.gov/blast) using the nr database (2,920,020 sequences) (BLAST2.2.12, inclusion threshold E-value = 0.005). Guidelines to the use of hydrophobic cluster analysis (HCA) are given in Refs. 12 and 13. Modeling of three-dimensional structure was done using Modeller (14).

RESULTS AND DISCUSSION

Cernunnos Interacts with the XRCC4-DNA-Ligase IV Complex—We searched for a possible interaction of Cernunnos with the XRCC4-DNA-ligase IV complex by transfecting 293T cells with a V5 epitope-tagged form of Cernunnos. The XRCC4-DNA-ligase IV complex was immunoprecipitated using the V5 antibody, and immunoprecipitates were used to assay interaction with Cernunnos and XRCC4 by co-immunoprecipitation using the yeast Nej1 antibody. As shown in Fig. 4A, Cernunnos physically interacts with XRCC4 in NHEJ, we performed careful bioinformatics analysis of its protein sequence. Using HCA, a sensitive two-dimensional approach that is well suited to analyze the two-dimensional texture of proteins and compare their secondary structures (12, 13), we first identified the Cernunnos sequence a globular domain, ranging from amino acids 1–230. Using this sequence as query in PSI-BLAST (11) searches with default parameters, we detected different homologs of Cernunnos at convergence by iteration 4. A few of these sequences are shown in Fig. 2A (yellow box). A marginal similarity with a putative DNA double strand break repair protein from Arabidopsis thaliana was observed just above the threshold E-value (E-value = 0.28%, 16% of identity over 108 amino acids). This protein turned out to be the plant XRCC4 homolog (white box in Fig. 2A), sharing with the human protein 24% sequence identity over the head and stalk regions. The similarity reported by PSI-BLAST was supported by institutional grants from INSERM as well as grants from the Ministere de l’Education Nationale, de l’Enseignement Superieur, et de la Recherche. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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between human Cernunnos and Arabidopsis XRCC4 encompasses the globular “head” domain of XRCC4 and the initial portion of the helical stalk, the overall region that promotes XRCC4 dimerization (16–20). The similarity was supported at the two-dimensional level using HCA, revealing the conservation of the secondary structures forming the head domain, which contains a β-sandwich and two α-helices, as well as the helical stalk (Figs. 2A and 3A and data not shown). Most of the conserved features correspond to hydrophobicity (positions in which hydrophobicity is conserved are green in Fig. 2, with highly conserved aromatic residues in purple). These amino acids mainly participate in the head core structure or are involved in the contact with the initial portion of the stalk (e.g. Trp13 (W13) in Fig. 3A). Only a few surface-exposed residues are conserved, such as Phe117 and Trp119, which may be part of a potential interaction site (Fig. 3A). The finding of deleterious missense mutations (R57G and C123R in Fig. 3A) in the vicinity of these residues in patients (7) further strengthens the likely functional significance of this region of Cernunnos. Other PSI-BLAST searches were performed using Cernunnos homologous sequences as queries, which highlighted marginal similarities with additional XRCC4 proteins (E-value > 0.001), also supported at the two-dimensional level (data not shown). This first finding thus reveals a novel family of structurally similar proteins, gathering XRCC4 and Cernunnos. When our manuscript was under review, a similar conclusion was drawn by Ahnesorg et al. (15), who used fold recognition methods to unravel the structural relationship between Cernunnos/XLF and XRCC4. However, these automatic methods proposed two different alignments of Cernunnos/XLF with the two available experimental structures of XRCC4, which yet share an almost identical sequence (99% identity; see Fig. 1 of supplemental data in Ref. 15). Here, we propose a refined alignment of the two protein sequences, performed using the sensitive HCA methodology. This one benefited from the additional evolutionary information coming from the direct consideration of the whole Cernunnos and XRCC4 families. Accordingly, the alignment of the Cernunnos family sequences well conserved the structural invariants constituting the XRCC4 hydrophobic core (positions are shaded green and violet in Fig. 2A). These features were reinforced when considering the Nej1 sequences (see below).

Several reports, including structural studies, have proposed that XRCC4 forms dimers in association with DNA-ligase IV or tetramers, the DNA-ligase IV-interacting region, and the tetramer stabilization region overlapping in the helical tail of XRCC4 (Fig. 2B) (16–20). Equilibrium sedimentation analysis associated with in vitro mutagenesis suggested that these two forms of the XRCC4 protein (dimers with DNA-ligase IV versus tetramers) are mutually exclusive (18). Although more difficult to align in the helical tail, Cernunnos and XRCC4 share similarities, especially in the DNA-ligase IV binding region of XRCC4 (Fig. 2B). Lys188 (Cernunnos Lys187), one of the residues involved in a salt bridge with the DNA-ligase IV BRC1 C terminus domain linker, appears conserved in several sequences, as well as the F180I/XXF cluster (Cernunnos F199L/XXF), which makes hydrophobic contacts with DNA-ligase IV (20).

The finding that Cernunnos is related to XRCC4 on the one hand and the demonstration that Cernunnos interacts with the XRCC4/DNA-ligase IV complex on the other hand raises the interesting issue of the possible combinatorial associations of these two proteins within XRCC4/Cernunnos dimers and/or tetramers (Fig. 3B) and the possible functional consequences of these various combinations. As a first way to address this issue, we performed co-immunoprecipitation experiments following cotransfection of 293T cells with V5 epitope-tagged Cernunnos and myc epitope-tagged Cernunnos. As shown in Fig. 1C, immunoprecipitation with anti-V5 and anti-myc co-precipitated the Cernunnos-myc and Cernunnos-V5, respectively. This suggests that Cernunnos, as described for XRCC4, can form homoduplexes or at least that two Cernunnos molecules are part of the same complex as also demonstrated by Ahnesorg et al. (15). Although the definite answers to these questions need further in vitro and in vivo investigations, one can already postulate that, given the embryonic lethality of XRCC4 mutant mice (21) and the severe immunodeficiency and developmental anomalies of human Cernunnos-deficient patients (7), these two factors, although part of the same complex, are not redundant. Moreover, overexpression of XRCC4 in Cernunnos-deficient cells does not reverse the defective V(D)J recombination (supplemental Fig. S1) indicating that these two factors play specific parts during the NHEJ process.

Cernunnos Is Homologous to Yeast Nej1—Several putative partners of XRCC4 and DNA-ligase IV have been identified in mammals and yeast. One of these, Nej1/Lif2p, has been identified in Saccharomyces cerevisiae through its interaction with Lfs1p, the yeast homolog of XRCC4, and by virtue of the fact that it is transcriptionally repressed/expressed in yeast diploid and haploid cells respectively (4–6, 22). Nej1 thus appears as a critical regulator of NHEJ activity in yeast cells undergoing mating type switching. Moreover, ectopic expression of Nej1 restores NHEJ in MATa/MATα diploid cells to some extent (4–6). To date no homolog of Nej1 has been identified in other organisms, including the fission yeast Schizosaccharomyces pombe. Given their interaction with the XRCC4/DNA-ligase IV complex and their similar molecular weight, we tackled the idea that Cernunnos may represent the genuine mammalian Nej1 homolog.

Cernunnos was identified from human to several yeast species (S. pombe, Neurospora crassa, Gibberella zeae, Magnaporthe grisea, Aspergillus nidulans, Debaryomyces Hansenii, Yarrowia lipolytica) by data base mining (Fig. 2A, yellow and green boxes, and data not shown) but intriguingly could not be found within the PSI-BLAST significant results in the S. cerevisiae species. No candidate could be found within the non-significant alignments either. Taking
the opposite approach, using the S. cerevisiae Nej1 sequence as query in a PSI-BLAST search (same parameters as above) highlighted marginal similarities with two yeast sequences (Kluyveromyces lactis (E-value = 0.038) and Aphis gossypii (E-value = 0.14), Fig. 2A, blue box), which could be further supported at the two-dimensional level (data not shown). To further broaden the analysis, these loose similarities were quoted with significant E-values and extended to other yeast species in a reciprocal strategy. For example starting from the A. gossypii sequence, we highlighted significant similarities at convergence by iteration 4 with the C. albicans (E-value = 8 x 10^-62) and D. hansenii (E-value = 9 x 10^-69) sequences in Fig. 2, blue and green boxes. Surprisingly, the D. hansenii Nej1 sequence highlighted here (GenBank™ identifier 49657009) was identical to the D. hansenii Cernunnos sequence identified above, making this yeast species the critical intermediate protein, which allows revealing the relationship between Nej1 and Cernunnos. Indeed, the yeast Cernunnos homolog, Lif1, shares very low sequence similarity with human XRCC4, which cannot be detected using PSI-BLAST. Regarding this observation, one can hypothesize that the Cernunnos/XRCC4 couple has evolved jointly and led to similarly highly divergent sequences in human and yeast. This concerted evolution, together with the more pronounced conservation of the dimerization regions (the "head" regions) might reflect the physical interaction of the two proteins, which has been demonstrated for Nej1/Lif1.

Conclusion—We demonstrate here that the newly identified DNA repair factor, Cernunnos, interacts with the XRCC4-DNA-ligase IV complex, is the mammalian homolog of the yeast Nej1 factor, and that both belong to an extended XRCC4 family. The great variability of the Cernunnos/Nej1 sequences among species is reminiscent of that of the XRCC4 sequences, and we show that they are structurally related. Indeed, the yeast XRCC4 homolog, Lif1, shares very low sequence similarity with human XRCC4, which cannot be detected using PSI-BLAST. Regarding this observation, one can hypothesize that the Cernunnos/XRCC4 couple has evolved jointly and led to similarly highly divergent sequences in human and yeast. This concerted evolution, together with the more pronounced conservation of the dimerization regions (the "head" regions) might reflect the physical interaction of the two proteins, which has been demonstrated for Nej1/Lif1.

Altogether this analysis indicated that Cernunnos is one homolog of Nej1 and that both proteins belong to an extended XRCC4 family. The great variability of the Cernunnos/Nej1 sequences among species is reminiscent of that of the XRCC4 sequences, and we show that they are structurally related. Indeed, the yeast XRCC4 homolog, Lif1, shares very low sequence similarity with human XRCC4, which cannot be detected using PSI-BLAST. Regarding this observation, one can hypothesize that the Cernunnos/XRCC4 couple has evolved jointly and led to similarly highly divergent sequences in human and yeast. This concerted evolution, together with the more pronounced conservation of the dimerization regions (the "head" regions) might reflect the physical interaction of the two proteins, which has been demonstrated for Nej1/Lif1.

Conclusion—We demonstrate here that the newly identified DNA repair factor, Cernunnos, interacts with the XRCC4-DNA-ligase IV complex, is the mammalian homolog of the yeast Nej1 factor, and that both belong to an extended XRCC4 family. These findings have several important implications. As we discussed above, the similarity between XRCC4 and Cernunnos raises the question of the true nature of the XRCC4-Cernunnos/DNA-ligase IV complex.
Nej1 has been considered in yeast as a cell type NHEJ regulator based on its specific transcriptional down-regulation in MATα/MATα diploid cells (4–6, 22). Indeed NHEJ is influenced in yeast by the mating type status of the cells. Diploid MATα/MATα cells demonstrate lower NHEJ activity compared with haploid cells expressing either MATα or MATα (27–29). Ectopic expression of Nej1 in MATα/MATα diploid cells restores NHEJ in these cells to some extent (4–6). The exact role of Nej1 during NHEJ in yeast is unknown. One controversial study had suggested that Nej1 could facilitate the nuclear localization of Lif1 (4). However, we did not find any abnormal XRCC4 subcellular distribution in Cernunnos-deficient cells from a Cernunnos-deficient patient referenced as P2 in Buck et al. (7) (supplemental Fig. S2). Likewise, a normal nuclear localization of XRCC4 was noted in 2BN cells (15). This observation is in agreement with results described in another Nej1/Lif1 study in yeast (6). Whether the Cernunnos level could somehow regulate NHEJ in mammalian cells is another interesting issue. It would be of particular interest to assess the expression of Cernunnos during the various phases of the cell cycle or in cells known to preferentially use homologous recombination such as during meiosis.

Altogether, the link we made between Cernunnos and Nej1 should help design new experiments to clarify the functional role of both factors during NHEJ-mediated DNA repair.

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