DNA ligase IV from a basidiomycete, *Coprinus cinereus*, and its expression during meiosis

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Received 19 February 2003
Revised 29 April 2003
Accepted 8 May 2003

DNA ligase IV is thought to be involved in DNA double-strand break repair and DNA non-homologous end-joining pathways, but these mechanisms are still unclear. To investigate the roles of DNA ligase IV from a biologically functional viewpoint, the authors studied its relationship to meiosis in a basidiomycete, *Coprinus cinereus*, which shows a highly synchronous meiotic cell cycle. The *C. cinereus* cDNA homologue of DNA ligase IV (*CcLIG4*) was successfully cloned. The 3-2 kb clone including the ORF encoded a predicted product of 1025 amino acid residues with a molecular mass of 117 kDa. A specific inserted sequence composed of 95 amino acids rich in aspartic acid and glutamic acid could be detected between tandem BRCT domains. The inserted sequence had no sequence identity with other eukaryotic counterparts of DNA ligase IV or with another aspartic acid and glutamic acid rich sequence inserted in *C. cinereus* proliferating cell nuclear antigen (*CcPCNA*), although the length and the percentages of aspartic and glutamic acids were similar. In addition, the recombinant *CcLIG4* protein not only showed ATP-dependent ligase activity, but also used (dT)$_{16}$/poly(dA) and (dT)$_{16}$/poly(rA) as substrates, and had double-strand ligation activity, like human DNA ligase IV. Northern hybridization analysis and in situ hybridization indicated that *CcLIG4* was expressed not only at the pre-meiotic S phase but also at meiotic prophase I. Intense signals were observed in leptotene and zygote. Based on these observations, the possible role(s) of *C. cinereus* DNA ligase IV during meiosis are discussed.

INTRODUCTION

DNA ligase IV (EC 6.5.1.1) is reportedly required for the DNA non-homologous end-joining (NHEJ) pathways, including recombination of the V(D)J immunoglobulin gene segments in cells of the mammalian immune system, and the enzyme is exclusively nuclear (see reviews by Featherstone & Jackson, 1999; Martin & MacNeill, 2002; Timson et al., 2000). NHEJ is the principal mechanism by which mammalian cells repair DNA double-strand breaks (DSBs) caused by exposure to ionizing radiation or certain classes of chemical mutagens. In the NHEJ pathway, broken DNA ends are rejoined, a process requiring the Ku proteins and DNA ligase IV (Jeggo, 1998). Mice lacking DNA ligase IV display embryonic lethality, implying that the enzyme has an essential function during early development (Barnes et al., 1998; Frank et al., 1998). Mutation of DNA ligase IV in humans may confer a predisposition to leukaemia (Riballo et al., 1999). DNA ligase IV is also known to form a complex with Xrcc4 (Critchlow et al., 1997; Grawunder et al., 1997; Herrmann et al., 1998; Modesti et al., 1999; West et al., 2000), suggesting that the enzyme works in the *in vivo* process of XRCC4 functions. The biologically functional roles of DNA ligase IV are still a long way from being clearly understood.

We shall consider roles of DNA ligase IV from a slightly different point of view. Yeasts lacking DNA ligase IV and LIF1, which share sequence identity with mammalian XRCC4, are viable but their sporulation efficiency is reduced (Herrmann et al., 1998; Schar et al., 1997). DNA ligase IV may play a role in meiosis.

During meiotic prophase I, chromosomes begin to condense,
and they show thin thread-like structures in leptotene. Homologue paring is initiated in leptotene, and continues in zygotene. Fully synapsed homologues are observed in pachytene as thick thread-like structures. During meiotic prophase I, several DNA metabolic processes are associated with meiotic recombination (Allers & Lichten, 2001a; Hunter & Kleckner, 2001; Paques & Haber, 1999; Villeneuve & Hillers, 2001). Meiotic recombination in yeast starts from meiosis-specific DSBs with formation of single-stranded DNA by exonuclease digestion. The single-strand portion invades the region having homologous sequences in the other allele. After single-ended strand invasion and initial repair synthesis, crossover and non-crossover pathways diverge (Allers & Lichten, 2001b). In both crossover and non-crossover pathways, repair-type DNA synthesis occurs with the action of DNA polymerase and ligation by DNA ligase (Paques & Haber, 1999; Villeneuve & Hillers, 2001). DNA ligase IV may be an important element in the coordinated multi-enzymatic processes of meiotic DNA metabolism.

For the purpose of this study, we chose to investigate the meiocytes of a basidiomycete, Coprinus cinereus. C. cinereus has been used as a genetic tool for studying mating type and sexual development (Brown & Casselton, 2001; Casselton, 2002; Casselton & Olesnicky, 1998; Kamada, 2002; Kues, 2000) and is well suited for studies on meiosis because of its synchronous meiotic cell cycle in the fruiting cap (Li et al., 1999; Nara et al., 1999; Pukkila et al., 1984; Raju & Lu, 1970). In this study, we cloned the DNA ligase IV cDNA of C. cinereus and characterized the recombinant protein, and then investigated its expression during meiotic development in detail by Northern blotting analyses, and by in situ hybridization of C. cinereus meiotic tissues. Based on these observations, the possible role(s) of C. cinereus DNA ligase IV during meiosis are discussed.

**METHODS**

**Culturing of Coprinus cinereus and collection of the fruiting bodies.** The basidiomycete C. cinereus ATCC 56838 was used in this study. The culture method was described previously (Nara et al., 1999). Cultures were incubated from day 0 to day 7 at 37 °C in total darkness and from day 7 onward at 25 °C under a 12 h light/12 h dark cycle to allow photoinduction of fruiting body formation. A series of meiotic events occurred synchronously in all the fruiting bodies under the proper light cycles as described previously (Nara et al., 1999). Typical procedures of photoinduction of meiosis were as follows. Karyogamy, which is defined as the time at which 5% of all basidia had fused nuclei, began at 04:00 (karyogamy +0 h, K+0). 1 h before the light was turned on. Photoinduction started at 05:00 (K+1). Fruiting caps containing meiotic cells at the leptotene to the zygote stages were observed between 04:00 (K+0) and 09:00 (K+5). Cells at the pachytene stage were observed between 10:00 (K+6) and 11:00 (K+7). Meiosis II cells were observed between 12:00 (K+8) and 14:00 (K+10).

cDNA cloning of C. cinereus DNA ligase IV. In order to isolate cDNA clones of C. cinereus DNA ligase IV (CcLIG4), two primers were synthesized corresponding to the amino acid motifs conserved among species: sense primer (5’-TGAGTGGAGAGAGGGTGAGG-3’) and anti-sense primer (5’-TCAGGAATCGGGGCTTATTG-3’) (N=A, C, G or T; Y=C or T, M=A or C; D=A, G or T). These primers were used for PCR of cDNA generated from total RNA isolated from meiotic tissues of C. cinereus. The PCR product was used to screen the C. cinereus junction ZAP II cDNA library as described previously (Nara et al., 1999). The DDBJ/EMBL/GenBank accession number of the nucleotide sequence reported in this paper is AB098474 for CcLIG4.

**Search for the three-dimensional structure of C. cinereus DNA ligase IV.** A modelled 3D structure for CcLIG4 was calculated by the Kabsch–Sander method and visualized using Insight II (Molecular Simulation) (Guex & Peitsch, 1997; Peitsch, 1996; Peitsch et al., 1995).

**Bacterial expression and purification of CcLIG4.** The coding region of CcLIG4 (residues 1–1025) was expressed as thioredoxin-tagged fusion protein using expression vector Novagen at Nco and XhoI sites. Recombinant CcLIG4 thioredoxin-tagged (Trx) fusion protein (Trx-CcLIG4) was produced in Escherichia coli BL21 (DE3) (Novagen) by growing the E. coli in 100 ml 2×YT medium containing 50 μg ampicillin ml⁻¹. The cells were grown to an OD600 of 0.6 at 37 °C, then IPTG was added to a final concentration of 1 mM. The cells were harvested after 20 h at 16 °C by centrifugation at 5000 r.p.m. for 10 min. The cell pellets were resuspended in 5 ml ice-cold binding buffer (20 mM Tris/HCl pH 7.9, 50 mM NaCl, 1 mM imidazole, 0.1% NP-40) and sonicated five times (20 kHz, 30 s). The supernatant was loaded on a Ni⁺-NTA column and eluted with 1 M imidazole in lysis buffer. The fraction containing Trx-CcLIG4 was dialysed against buffer A (50 mM Tris/HCl pH 7.5, 1 mM EDTA, 10%, v/v, glycerol, 0.1% NP-40) and used in a subsequent experiment.

**Enzyme assays.** Assays of DNA ligase activity were performed as described previously (Matsuda et al., 1996; Teraoka et al., 1993) with minor modifications. Adenylation was performed in a 20 μl reaction mixture containing 60 mM Tris/HCl (pH 7.8), 10 mM MgCl₂, 5 mM DTT, 50 μg BSA ml⁻¹ and 1 μg [γ⁻³²P]ATP (3000 Ci mmol⁻¹; 110 TBq mmol⁻¹) and 2 μl of the ligase fraction. NaPP, treatment was carried out in the presence of 0.05 mM NaPP, in the reaction buffer. Reactions were incubated for 10 min at room temperature, and then terminated by the addition of SDS-PAGE sample buffer. Adenylated proteins were resolved by SDS-PAGE.

Nick-lickigation reactions were performed using a (dT)₁₆ substrate, which was 5’-end-labelled using T₄ polynucleotide kinase and [γ⁻³²P]ATP (6000 Ci mmol⁻¹; 220 TBq mmol⁻¹). The radiolabelled (dT)₁₆ was hybridized with poly(dA) or poly(rA) (Amersham) as described previously (Tomkinson et al., 1991). Ligation reactions were performed in a 10 μl volume containing 1 ng DNA substrate, 60 mM Tris/HCl (pH 7.8), 10 mM MgCl₂, 5 mM DTT, 50 μg BSA ml⁻¹ for 10 min at 37 °C, and then the mixture was rapidly chilled to 0 °C. The ligation products were separated on denaturing 20% polyacrylamide 8 M urea gel. NaPP, treatment was performed in the presence of 0.5 mM NaPP, in the reaction buffer.

DSB ligation reactions were performed using a linearized pUC119 DNA with BamHI or Smal as described previously (Matsuda et al., 1996).

**Northern analysis.** RNA was prepared from C. cinereus using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The RNA samples were separated on 1.2% agarose-formaldehyde gels; 20 μg total RNA sample was loaded in each lane. The agarose gel was stained with ethidium bromide and blotted overnight in 20× SSPE to a Hybond-N⁺ membrane (Amersham). The DNA samples were separated on 1.2% agarose-formaldehyde gels; 20 μg total RNA sample was loaded in each lane. The agarose gel was stained with ethidium bromide and blotted overnight in 20× SSPE to a Hybond-N⁺ membrane (Amersham).
membrane was fixed with alkali and carefully rinsed with 2 × SSPE, and RNA was immobilized to the membrane and hybridized with 32P-labelled probe as described previously (Hamada et al., 2002).

**Effect of MMS on vegetative mycelium.** For examination of CcLIG4 mRNA levels, 300 ml aliquots of liquid YMG medium were inoculated with small pieces of *C. cinereus* mycelium tissue. After 2 days of growth at 37 °C with shaking, the tissue was harvested onto filter paper by filtration through a Buchner funnel. Untreated samples (after 0 h MMS treatment) were immediately frozen in liquid N2, and the rest of the tissues were returned to 500 ml YMG medium containing 0-01 % MMS. Total RNA was isolated from vegetative dikaryotic mycelium after 0–6 h of MMS treatment and analysed by Northern hybridization as described above.

**Preparation of riboprobes and in situ hybridization.** In situ hybridization of meiotic *C. cinereus* tissues was performed as described previously (Hamada et al., 2002). Riboprobes for *in situ* hybridization were labelled with digoxigenin-11-rUTP using a DIG RNA Labelling Kit (Boehringer Mannheim) according to the manufacturer’s protocol. The riboprobes were made using the cDNAs corresponding to amino acids 1–626 for CcLIG4 shown in Fig. 1(A).

Riboprobes were subjected to mild alkaline hydrolysis by heating at 60 °C for 3 min in 0-2 M carbonate/bicarbonate buffer and used at a concentration of 2 mg ml−1. The fruiting caps were fixed overnight at 4 °C with a mixture of 4 % (v/v) parafomaldehyde and 0-25 % (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 7-2). The fixed tissues were dehydrated in a series of xylene and ethanol and embedded in paraffin. The embedded tissues were sectioned at a thickness of 5 μm, and placed on microscope slides precoated with poly-l-lysine. The sections were deparaffinized with xylene and rehydrated through a graded ethanol series. They were subsequently pretreated with 10 mg proteinase K ml−1 in 100 mM Tris/HCl pH 7-5 and 50 mM EDTA at 37 °C for 30 min, dehydrated in a graded ethanol series, and dried under vacuum for 2 h. Hybridization and detection of the hybridized riboprobes were performed as described previously (Hamada et al., 2002).

**Fig. 1.** Amino acid sequence of *C. cinereus* DNA ligase IV. (A) Amino acid sequence alignment of CcLIG4, human (Hs) and *S. cerevisiae* (Sc) DNA ligase IV isoforms. Asterisks indicate amino acid identity common to all three sequences, and dots indicate amino acid identity between two of the sequences. Motifs were identified by comparison with previously identified conserved sequences in DNA ligases. A pair of degenerate primers was designed in the motifs I and V (indicated by arrow). CcLIG4 has an inserted sequence between tandem BRCT domains. DDBJ/EMBL/GenBank accession numbers for the sequences shown are: NM002312 (H. sapiens), CAA99193.1 (*Sacch. cerevisiae*). (B) A phylogenetic tree of eukaryotic DNA ligases, constructed by the UPGMA method. Horizontal distance is proportional to evolutionary divergence expressed as substitutions per site.
RESULTS

cDNA cloning of *C. cinereus* DNA ligase IV

We designed a set of degenerate primers, and isolated a 600 bp fragment of cDNA from *C. cinereus* meiosis-specific mRNA by RT-PCR amplification using these primers (see Methods). Screening of a meiotic cDNA library with the fragment as probe resulted in the isolation of a 3-2 kb clone designated as CcLIG4 (*C. cinereus* DNA ligase IV). The ORF of CcLIG4 encoded a predicted product of 1025 amino acid residues with a molecular mass of 117 kDa (Fig. 1A). The deduced amino acid sequence of CcLIG4 was compared with human and budding yeast (*Saccharomyces cerevisiae*) DNA ligase IV homologues (Fig. 1A). The CcLIG4 protein showed 26-3 % sequence identity with human DNA ligase IV, and 20-5 % sequence identity with *Sacch. cerevisiae* DNA ligase IV. The ORF was analysed for motifs of interest by comparing with previous alignments of ATP-dependent DNA ligases and RNA capping enzymes (Shuman *et al*., 1994). Fig. 1(B) shows a phylogenetic tree of eukaryotic DNA ligases, which was drawn based on alignment by the UPGMA method. All known eukaryotic DNA ligases have sequence identity to each other, and vertebrates only have three species of DNA ligase (I, III and IV). DNA ligase III is known to be a specific isoform in vertebrates (Martin & MacNeill, 2002). CcLIG4 was closely related to the fission yeast (*Schizosaccharomyces pombe*) DNA ligase IV (Fig. 1B). We failed to find a *C. cinereus* homologous cDNA or the gene of DNA ligase III, suggesting that only two species of DNA ligase, CcLIG1 and CcLIG4, exist in *C. cinereus*. We also succeeded in cloning the *C. cinereus* DNA ligase I cDNA (CcLIGI) (Namekawa *et al*., 2003).

The two carboxyl-terminal tandem BRCT domains, widely preserved in DNA ligase IV among eukaryotes, were also present in CcLIG4 (Fig. 1A). CcLIG4 had an inserted sequence, located between the tandem BRCT domains, which showed no sequence identity to the other eukaryotic counterparts of DNA ligase IV. The inserted sequence was composed of 95 amino acids from amino acid residues 810 to 904 (Fig. 1A). Interestingly, the features of the inserted sequence in CcLIG4 were very similar to those of CcPCNA described previously (Hamada *et al*., 2002). CcPCNA had an inserted sequence of 110 amino acids from amino acid residues 195 to 305 (Fig. 2A). Although the inserted sequences of CcLIG4 and CcPCNA had no sequence identity, they shared some common features: the length (about 100 amino acids), the amino acid composition (rich in aspartic acid and glutamic acid), and the isoelectric point (about 4) (Fig. 2B). The percentages of aspartic and glutamic acids in the inserted sequences of CcLIG4 and CcPCNA were 20-0 % and 19-0 %, and 21-4 % and 22-3 %, respectively. The inserted sequence of CcPCNA was rich in lysine (20-5 %), but that of CcLIG4 was not (5-3 %) (Fig. 2B).

To determine the copy number of the CcLIG4 gene in the genome, Southern hybridization was performed. Digested genomic DNA was hybridized with the CcLIG4 cDNA probe under high-stringency conditions. A single band was detected under these conditions in...
independent digests of *C. cinereus* genomic DNA, suggesting that *CcLIG4* exists as a single copy per genome (data not shown).

**Modelling of the three-dimensional structure of CcLIG4**

The three-dimensional structure of the CcLIG4 catalytic core and that of two BRCT domains were obtained by computer analysis. The crystal 3D structure of the bacteriophage T7 DNA ligase catalytic core (Subramanya *et al.*, 1996) and that of human DNA ligase III x BRCT domain (Krishnan *et al.*, 2001) have been reported. These enabled us to predict the 3D structures of CcLIG4 using the Kabsch–Sander method and Insight II (Molecular Simulation). The crystal 3D structure of the bacteriophage T7 DNA ligase catalytic core and human DNA ligase III a BRCT domain (Subramanya *et al.*, 1996) and that of human DNA ligase III a BRCT domain (Krishnan *et al.*, 2001) have been reported. These enabled us to predict the 3D structures of CcLIG4 using the Kabsch–Sander method and Insight II (Molecular Simulation). Fig. 3 shows the computer-simulated possible structure of CcLIG4 catalytic core and that of two BRCT domains. The catalytic core (I258 to A584) and two BRCT domains (V667 to K759, A931 to E1021) were very similar to their eukaryotic counterparts. The sequence of 810–904 amino acid residues (DE-rich peptide site), which was present between two BRCT domains, must protrude beyond the tandem BRCT domains, although we could not simulate this because the other DNA ligase IVs have no such DE-rich peptide site. In humans, the region between the tandem BRCT domains has been shown to bind to XRCC4 (Grawunder *et al.*, 1998).

**Catalytic and molecular properties of recombinant CcLIG4 protein**

As described in Methods, recombinant CcLIG4 thioredoxin tag (Trx) fusion protein (Trx-CcLIG4) was produced in *E. coli* BL21 (DE3). Trx-CcLIG4 was purified through a Ni⁺-NTA column. The purified Trx-CcLIG4 was separated on a 7-5% SDS-polyacrylamide gel and was visualized using Coomassie brilliant blue staining (Fig. 4A). The molecular mass was 145 kDa (Fig. 4A). The deduced amino acid sequence of Trx-LIG4 was calculated as 137 kDa. The reason for this discrepancy might be that the enzyme is rich in acidic amino acids. CcPCNA was also larger than expected (Hamada *et al.*, 2002).

Next, adenylation of CcLIG4 was tested, because DNA ligase reportedly forms a ligase–AMP complex as the reaction intermediate in the presence of ATP. The DNA ligase IV–AMP complex is known to be very stable and pre-adenylated (Grawunder *et al.*, 1997). ‘Pre-adenylated’ means that the ligase–AMP complex is formed beforehand by the endogenous ATP, and exists in a stable complex. The ligase–AMP complex is dissociated by NaPPi. As shown in Fig. 4(B), the Trx-CcLIG4–AMP complex was also pre-adenylated. NaPPi was required to do some dissociation before a new label.

**Fig. 3.** A modelled three-dimensional structure of CcLIG4. The catalytic core (I258 to A584) and tandem BRCT domains (V667 to K759, A931 to E1021) were modelled by the Kabsch–Sander method and Insight II (Molecular Simulation) using the crystal 3D structure of the bacteriophage T7 DNA ligase catalytic core and human DNA ligase III x BRCT domain. Red tubes represent α helix, and yellow arrows represent sheet structure. The blue dashed line represents the position of the inserted sequence. The Protein Database number of bacteriophage T7 DNA ligase is 1A0I; that of human DNA ligase III x is 1IMO.

**Fig. 4.** Purification of CcLIG4 thioredoxin tag fusion protein (Trx-CcLIG4). (A) SDS-PAGE analysis of Trx-CcLIG4 purified by Ni⁺-NTA column chromatography. The purified Trx-CcLIG4 was fractionated by 7-5% SDS-PAGE. The gel was stained with Coomassie brilliant blue. (B) Adenylation of recombinant Trx-CcLIG4. Trx-CcLIG4 (1 μg) was adenylated with [α-32P]ATP, in the presence or absence of 0-05 mM NaPPi in the reaction buffer. In vitro-adenylated T4 DNA ligase (4 x 10⁻³ mU) was used as a positive control. After 10% SDS-PAGE, the signals were detected by autoradiography.
could be added, although the Trx-tag was not adenylated (data not shown).

Fig. 5 shows the substrate-specificities for the nick-ligation. Equal amounts of purified Trx-CcLIG4 (100 ng) were assayed for ligation of (dT)16/poly(dA) (panel A) and (dT)16/poly(rA) (panel B) in the presence or absence of 0.5 mM NaPPi, and in the presence or absence of 1 mM ATP (as indicated) in the reaction buffer. Ligation products were separated on denaturing 20% acrylamide 8 M urea gels and detected by autoradiography. T4 DNA ligase (4 x 10^{-2} mU) was used as a positive control (lanes 1 and 7). The sizes of the ligation products are indicated on the left. Lanes 2 and 8 contained substrate only.

Tissue-specific expression of CcLIG4 in C. cinereus and induction by an alkylating reagent

The tissue-specific expression of the CcLIG4 transcript in the C. cinereus fruiting bodies was analysed by Northern blotting analysis. CcLIG4 was expressed in all the C. cinereus tissues studied (Fig. 7B), with the highest levels of transcript expression in the gill and top stipe at 4 h after karyogamy (K+4), and in vegetative mycelium. The gill is meiotic tissue, but other tissues are mitotic tissues and CcLIG4 was expressed not only in meiotic tissue, but also in mitotic tissues.

To determine the relationship of CcLIG4 to DNA repair in C. cinereus, we treated the mycelia with an alkylating agent, MMS, and analysed the expression pattern of CcLIG4 by Northern blotting. We detected strong expression signals of CcLIG4 transcripts shortly after MMS induction (Fig. 8). The induction of gene expression by DNA damage suggested that CcLIG4 has roles in somatic DNA repair as described for other eukaryotic DNA ligases IV.

Meiotic expression of CcLIG4 transcript

To determine how DNA ligase IV is regulated during meiosis, we obtained total RNA from a synchronous culture extracted at various periods after the induction of meiosis,
and analysed it by Northern hybridization for CcLIG4 (Fig. 9A). Interestingly, intense signals were observed in leptotene and zygotene, although the transcripts were detected in the premeiotic S-phase when genomic DNA replicates. According to Lu & Jeng (1975), premeiotic S phase occurs before the onset of karyogamy in C. cinereus. It takes 8 h under control conditions (25°C with a 16 h light/8 h dark regime). As shown in Fig. 9(A), after the premeiotic S phase, the signal began to accumulate slightly 0 h after lights on (K+0) and became most abundant 2 h after karyogamy (K+2) at the leptotene and zygotene stages, and then gradually faded away 7 h after karyogamy (within pachytene, K+7). To our knowledge, this is the first report to indicate that the DNA ligase IV gene was expressed mainly at the leptotene and zygotene stages, and that expression gradually faded away at the boundary between zygotene and pachytene. This expression pattern was very similar to the appearance of CcLIG1 (Namekawa et al., 2003).

To study the expression pattern further, the spatial CcLIG4 expression in the meiotic tissues was investigated by in situ hybridization using digoxigenin-labelled antisense CcLIG4 RNA as a probe. In situ hybridization was performed on paraffin sections of the cap tissues which were fixed for 16 h

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Fig. 7. Tissue-specific expression of CcLIG4 transcript in the C. cinereus fruiting bodies. (A) Tissues in C. cinereus fruiting bodies used for Northern blotting analysis are illustrated. Slices of each tissue were carefully cut off with a razor. (B) Northern blotting analysis of CcLIG4 expression in the fruiting bodies. Each lane contained 20 μg of total RNA isolated from the gill (lane 1), veil (lane 2), top stipe (lane 3), middle stipe (lane 4), base stipe (lane 5) and mycelium (lane 6). The blot was hybridized with either CcLIG4 cDNA (upper panel) or C. cinereus glyceraldehyde 3-phosphate dehydrogenase (CcG3PDH) cDNA (lower panel).

Fig. 8. Increase of CcLIG4 transcript following MMS treatment: Northern blotting analysis of CcLIG4 expression following MMS treatment. Each lane contained 20 μg of total RNA isolated from vegetative dikaryotic mycelium after 0–6 h of MMS treatment. The blot was hybridized with either CcLIG4 cDNA (upper panel), or C. cinereus glyceraldehyde 3-phosphate dehydrogenase (CcG3PDH) cDNA (lower panel).

Fig. 9. Meiotic expression of CcLIG4 transcript. (A) Northern analysis of CcLIG4 expression at various stages of meiosis. Each lane contained 20 μg of total RNA isolated from fruiting caps of C. cinereus at premeiotic S phase (lane 1), karyogamy (K+0), leptotene/zygotene (K+2 and K+5) and pachytene (K+7) stages. The blot was hybridized with either CcLIG4 cDNA (upper panel), or C. cinereus glyceraldehyde 3-phosphate dehydrogenase (CcG3PDH) cDNA (lower panel). (B) Localization of CcLIG4 transcript by in situ hybridization. The fruiting body tissues were sectioned and probed with CcLIG4 antisense riboprobes labelled with digoxigenin-UTP. The tissue was probed with CcLIG4 sense riboprobes labelled with digoxigenin-UTP as a negative control (NC).
Meiotic expression of CcLIG4

Northern blotting showed that CcLIG4 is expressed significantly in the leptotene and zygotene stages of the meiotic cell cycle. Although DNA ligase IV is an important element of DNA NHEJ pathways, it has been suggested that the NHEJ pathway is inhibited during the meiotic cell cycle. In mammalian meiotic cells, the Ku protein levels are much lower than in somatic cells, apparently reducing the capacity of the cells to carry out NHEJ and alternatively promoting homologous recombination (Goedecke et al., 1999). Taking this report into consideration, CcLIG4 may be involved not only in the NHEJ pathway of DSB repair, but also in meiotic recombination.

In many organisms, Spo11-dependent DSBs are required for meiotic recombination pathways. In yeast and mouse, Spo11-dependent DSBs are formed in the leptotene stage (Roeder, 1997; Mahadevaiah et al., 2001). Therefore, Spo11-dependent DSBs are expected in the leptotene stage in C. cinereus. After DSB formation, single-stranded DNA is generated by exonuclease digestion with Mre11 and Rad50. The single-strand portion invades the region having homologous sequences in the other allele (single-ended invasion, SEI) (Paques & Haber, 1999; Villeneuve & Hillers, 2001). The crossover and non-crossover pathways diverge soon after SEI. In the crossover pathway, double Holliday junction (DHJ) intermediates followed by SEI give rise mainly to crossover recombinants. Most noncrossover recombinants arise earlier via different pathways without any DHJ intermediate (Allers & Lichten, 2001b). In the meiotic chromosome structure, recombination nodules (early and late) are observed as dense structures associated with synaptonemal complexes. It has been suggested that early nodules which are present during the zygotene and early pachytene stages mark the site of SEI, and mostly lead to noncrossover recombinants. Late nodules are thought to contain DHJ intermediates, subsequently resolving as crossover recombinants during mid and late pachytene stages (Zickler & Kleckner, 1999; Allers & Lichten, 2001b). In many organisms, Spo11-dependent DSBs are required for meiotic recombination pathways. In yeast and mouse, Spo11-dependent DSBs are formed in the leptotene stage (Roeder, 1997; Mahadevaiah et al., 2001). Therefore, Spo11-dependent DSBs are expected in the leptotene stage in C. cinereus. After DSB formation, single-stranded DNA is generated by exonuclease digestion with Mre11 and Rad50. The single-strand portion invades the region having homologous sequences in the other allele (single-ended invasion, SEI) (Paques & Haber, 1999; Villeneuve & Hillers, 2001). The crossover and non-crossover pathways diverge soon after SEI. In the crossover pathway, double Holliday junction (DHJ) intermediates followed by SEI give rise mainly to crossover recombinants. Most noncrossover recombinants arise earlier via different pathways without any DHJ intermediate (Allers & Lichten, 2001b). In the meiotic chromosome structure, recombination nodules (early and late) are observed as dense structures associated with synaptonemal complexes. It has been suggested that early nodules which are present during the zygotene and early pachytene stages mark the site of SEI, and mostly lead to noncrossover recombinants. Late nodules are thought to contain DHJ intermediates, subsequently resolving as crossover recombinants during mid and late pachytene stages (Zickler & Kleckner, 1999; Allers & Lichten, 2001b). Northern blotting showed CcLIG4 to be expressed significantly in the leptotene and zygotene stages. In the current meiotic recombination model described above, we speculate that DNA ligase IV may ligate the synthesized strand to complete the noncrossover pathway in the zygotene and...
early pachytene stages, before crossover recombination is consummated. On the other hand, there is also a possibility that DNA ligase IV relates to crossover recombination in mid and late pachytene. Mammalian DNA ligase IIIβ is thought to have a role in meiotic recombination in the latter part of the pachytene stage (Chen et al., 1995). In C. cinereus, predicted to lack DNA ligase III, CcLIG4 may play a similar role during meiosis, although there is no direct evidence of any relation to crossover or noncrossover pathways at this time.

CcLIG4-deficient mutants are required for further information, and more detailed investigation of the phenotype of the mutants is necessary, including studies on the genetic recombination frequency and morphology of the synaptinemal complex. A project to knock out the gene has been tried and further studies on conditional mutants of CcLIG4 would directly address the question of how CcLIG4 plays roles during meiosis.

ACKNOWLEDGEMENTS

We thank Dr M. Moore and Mr R. Sakaguchi for critical reading of our manuscript, and Dr S. Kimura, Mr T. Ishizaki, Ms S. Ishii, Mr T. Yamaguchi, Ms A. Koshiyama, Ms H. Sugawara for technical assistance and other help. S. Namekawa was supported by Research Fellowship of the Japan Society for the Promotion of Science for Young Scientist.

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