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DNA polymerase mu interacts with a meiosis-specific RecA homolog Lim15 during meiosis in *Coprinus cinereus*

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ABSTRACT

Meiosis is a fundamental process in eukaryotes. Homologous chromosomes are paired and recombined during meiotic prophase I, which results in variation among the gametes. However, the mechanism of recombination between the maternal and paternal chromosome is unknown. In this study, we report on the identification of interaction between *Coprinus cinereus* DNA polymerase mu (CcPol mu) and CcLim15/Dmc1, a meiosis-specific RecA-like protein, during meiosis. Interaction between these two proteins was confirmed using a GST-pull down assay. A two-hybrid assay revealed that the N-terminus of CcPol mu, which includes the BRCT domain, is responsible for binding the C-terminus of CcLim15. Furthermore, co-immunoprecipitation experiments indicate that these two proteins also interact in the crude extract of the meiotic cell. A significant proportion of CcPol mu and CcLim15 is shown to co-localize in nuclei from the leptotene/zygotene stage to the early pachytene stage during meiotic prophase I. Moreover, CcLim15 enhances polymerase activity of CcPol mu early in the reaction. These results suggest that CcPol mu might be recruited by CcLim15 and elongate the D-loop structure during homologous recombination in meiosis.

Introduction

Meiosis combines two successive rounds of nuclear division, meiosis I and meiosis II, with a single round of DNA replication to produce haploid gametes from diploid cells in eukaryotes [1]. Meiotic homologous recombination occurs between homologous chromosomes during cell division at meiosis I. This recombination between homologous chromosomes ensures their correct segregation at meiosis I through the formation of chiasmata, which physically connect homologous chromosomes [1–3].

Homologous chromosomes are paired and recombined during meiotic prophase I, and then segregated into tetrads. Meiotic DNA recombination comprises several steps. First, meiosis-specific double-strand breaks are introduced followed by the formation of single-stranded DNA (ssDNA). The single-stranded ends then invade regions of homology in the corresponding allele. These reactions are mediated by RecA-like recombinase Lim15/Dmc1 and Rad51, which play a central role in the strand-exchange reaction [4,5].

After single-strand invasion, some DNA synthesis events occur. However, the mechanism for this initial repair synthesis is not fully understood. In a previous study, gene expression analysis showed that in *Coprinus cinereus* meiocytes, *CcPol mu* was expressed during meiotic prophase [6]. CcPol mu locates at the nucleus throughout zygotene and diplotene [6]. Furthermore CcPol mu is able to elongate the D-loop structure, which is thought to be formed by Lim15 [6]. Therefore we focused on CcPol mu and CcLim15.

Pol mu is known to be involved in DJH rearrangements [7], and Non-Homologous End Joining repair (NHEJ) [8]. However, it has not been established how CcPol mu operates during meiosis. CcPol mu comprises two domains: (i) N-terminal BRCT domain that mediates interaction with other protein factors required for recognition and binding to broken DNA ends and complexes, and (ii) polymerase X domain.

By contrast, Lim15 binds to ssDNA produced at DSB sites, and forms extensive helical nucleoprotein filaments along the DNA [9–12], like the RecA protein [13,14]. These nucleoprotein filaments capture intact dsDNA, and form the ternary complex containing ssDNA and dsDNA within the filament. The complex is a D-loop structure where homologous-pairing and strand-exchange reactions take place. Therefore, the ability to form a filament along the DNA is an important property for a RecA-type recombinase, such as Rad51 and Lim15 proteins.

Rad51 is expressed in both meiotic and mitotic cells [4,15], whereas Lim15 is only present in meiotic cells [16,17]. This observation suggests that Lim15 functions as a specific factor for meiotic homologous recombination. The Lim15 was first discovered in

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yeast [16], and has subsequently been found in many mammals [17] and plants [18]. Knockout of the *Lim15* gene in mice causes asynapsis and sterility [5,19], as in *Lim15*-deficient yeast [15]. In Arabidopsis, mutations of the *Lim15* gene result in meiotic defects [20]. These observations indicate that Lim15 is an essential factor for meiotic recombination. Further studies on Lim15 interactors should reveal mechanistic aspects of meiotic recombination.

The basidiomycete *C. cinereus* has been used as a genetic tool for mating type and sexual development genes [21–23] and is well suited to such studies on meiosis because of its synchronous meiotic cell cycle in the fruiting cap. Meiotic prophase I is particularly synchronous [24,25]. As reported previously, we succeeded in cloning the gene for *CcLim15* and found that CcLim15 expression is restricted to meiotic prophase I [26,27]. Because *C. cinereus* can be induced to synchronously enter the meiotic cell cycle upon exposure to light, we were able to specifically construct a meiotic prophase I stage cDNA library.

In this study, we characterize the interaction between CcPol mu and Lim15. Furthermore, we demonstrate that CcLim15 effects polymerase activity of CcPol mu. Our results help clarity the mechanisms controlling meiotic recombination.

Materials and methods

Construction of bacterial expression vector and purification of GST-fused CcPol mu from Escherichia coli. A construct encoding a fusion of glutathione S-transferase (GST) with CcPol mu was engineered for expression. The coding region of *CcPol mu* was amplified using the following primers: CcPol mu F, CGC*GGATCC*GCGATGACCCCCA AGAGACCA; and CcPol mu R TCC*CCCGGG*GGACTAGACGTCCGCGTTC (SmaI and BamHI recognition sites used for further cloning are highlighted in italics). The amplified product was digested with BamHI and SmaI and cloned into the corresponding sites of pGEX 6p-1 (GE Healthcare, Piscataway, NJ). This strategy generated a GST tag at the N-terminus of the recombinant protein. Overexpression and purification of CcLim15 protein was accomplished as reported previously [25]. Recombinant CcPol mu was produced in *E. coli* strain BL21(DE3) and purified using glutathione Sepharose 4B (GE Healthcare). Aliquots of recombinant CcPol mu were dialyzed against PBS containing 0.5 M NaCl and flash-frozen in liquid nitrogen, and then stored at −80 °C.

In vitro binding assay. GST-fused CcPol mu or GST (each at 3 nmol) as control were incubated with 50 μl of glutathione Sepharose 4B beads for 1 h at 4 °C in 1 ml of PBS. The beads were washed twice with 0.5 ml of PBS and then incubated with 1 ml of PBS containing 3% BSA for 1 h at 4 °C. After washing three times with PBS, the beads were incubated with CcLim15 (3 nmol) in 1 ml of PBS o/n at 4 °C. Beads were then washed five times with wash buffer [PBS, 1 M NaCl, 0.1% NP40] and bound proteins eluted into sample buffer. Samples were detected by Western blot using a rabbit monoclonal antibody against GST or the polyhistidine tag.

Co-immunoprecipitation. Rabbit anti-CcLim15 polyclonal antibodies and control rabbit serum were coupled with CNBr-activated Sepharose beads according to the manufacturer's instructions (Amersham Pharmacia, Uppsala, Sweden). Aliquots of 20 mg of crude extract from meiotic tissues were prepared in buffer A [50 mM Tris–HCl, pH 7.5, 0.01% Triton X-100 and 0.5 mg/ml BSA containing 0.35 M NaCl, 5 mM β-mercaptoethanol and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin and 1 mM pepstatin A)] and incubated with 0.3 ml of the beads for 1 h at 4 °C, then washed two times with buffer A and eluted with 20 μl of 50 mM glycine–HCl (pH 2.5). After neutralization with 2 M Tris–HCl (pH 8.8), proteins were analyzed by SDS–PAGE. Western blotting was carried out using either a rat anti-CcPol mu polyclonal antibody or a rabbit anti-CcLim15 polyclonal antibody.

Yeast two-hybrid screening and molecular cloning of CcLim15 and CcPol mu. To ascertain any direct interaction between CcPol mu and CcLim15 we used a yeast two-hybrid system. Yeast two-hybrid analysis was performed with MATCHMAKER Two-Hybrid System 3 (Clontech, Palo Alto, CA). The full length and cDNA fragment encoding the N- or C-terminal domain of CcLim15, CcPol mu was amplified by PCR and inserted into pGADT7 and pGBKT7 between the NdeI–SmaI or NdeI–BamHI restriction recognition sites. The vector pairs were co-transformed into the yeast strain AH109. Controls for self activating fusion protein were carried out in each of these assays by transformation of the specific expression construct with pGBKT7 and pGADT7 empty vector. Transformants were plated onto SD3, -His/-Leu/-Trp and incubated at 30 °C for 3 days. The colonies were streaked onto SD4, -His/-Leu/-Trp/-Ade and then checked by β-galactosidase assay according to Yeast Protocols Handbook (Clontech).

Immunostaining of nuclei of C. cinereus meiotic cells. Immunostaining of *C. cinereus* meiotic cells was performed as described previously [27]. A 1:100 dilution was used for both rabbit anti-CcLim15 and rat anti-CcPol mu primary IgGs. We also employed a 1:1000 dilution of both anti rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA) for anti-CcLim15 and anti rat IgG conjugated with Alexa Fluor 568 (Invitrogen) using anti-CcPol mu as secondary antibody.

DNA polymerase assay. A DNA polymerase assay was performed using the methods described by Sakamoto et al. [6]. *CcPol mu* and *CcLim15* were cloned into pET28a and the recombinant proteins were purified to homogeneity. Synthetic substrates (Fig. 4A) were made by annealing oligonucleotides 1 (5′-GCCAGGGACGGGGTGA ACCTGCAGGTGGGCGGCTGCTCATCGTAGGTTAGTATCGACCTATTGG TAGAATTCGGCAGCGTCATGCGACGGC-3′) and 2 (5′-AAGATGTCC TAGCAAGGCACCCTAGTAGC-3′). For DNA synthesis, a 20 μl reaction solution containing ^{32}P-labeled synthetic double-stranded substrate (0.4 pmol) with CcPol mu (15 pmol) and CcLim15 (60 pmol) or BSA (60 pmol) in reaction buffer (50 mM Tris/HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM dATP, dCTP, dGTP and dTTP, 0.5 mM dithiothreitol and 15% glycerol) was incubated for 30, 60 or 90 s at 37 °C.

Results

Identification of the interaction between CcPol mu and CcLim15 both in vitro and in vivo

To verify the direct interaction between CcPol mu and CcLim15, we expressed each form of recombinant protein, GST-CcPol mu and His-tagged CcLim15, in *E. coli* and then purified them to homogeneity (Fig. 1A). SDS–PAGE of the purified proteins gave bands of 95 kDa and 37 kDa, corresponding to the anticipated molecular mass of GST-CcPol mu and His-CcLim15, respectively. GST-CcPol mu and His-CcLim15 were mixed *in vitro*, and the fraction eluted with glutathione by the GST Sepharose 4B beads was also collected. His-CcLim15 co-precipitated, as detected by Western blot analysis with anti-GST IgG. His-CcLim15 was detected by Western blot using anti-His-tag IgG (see Fig. 1B). The bands highlighted by an asterisk in Fig. 1B suggest GST-CcPol mu was digested during the assay. Despite the partial digestion of GST-CcPol mu, His-CcLim15 protein was efficiently pulled-down in this assay (Fig. 1B). This result shows that CcPol mu directly interacts with CcLim15. No staining was observed for the GST tag. This observation indicates that the interaction between the two proteins did not occur due to non-specific binding of the tags.

Next, to confirm the interaction between CcPol mu and CcLim15 *in vivo*, we generated rabbit and rat polyclonal antibodies against CcPol mu and CcLim15. Each antibody showed specific recognition of its target protein [6,26].

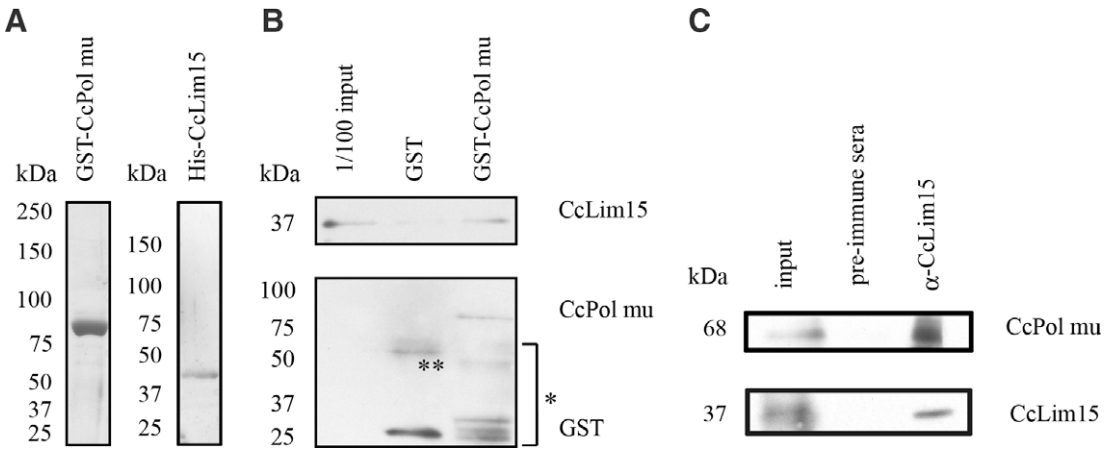

Fig. 1. Protein–protein interaction between CcPol mu and CcLim15. (A) Purification of GST-CcPol mu and His-Lim15. SDS–PAGE analysis of purified recombinant proteins overexpressed in *E. coli*. The proteins were run on a 10% and 12.5% SDS–polyacrylamide gel and stained with CBB. (B) 0.2 nmol His-T7-CcLim15 and glutathione Sepharose beads were incubated with 0.2 nmol of GST alone (lane 2) or GST-CcPol mu (lane 3). After washing the beads, bound proteins were eluted and analyzed by Western blotting using anti-His-tag (upper) and anti-(GST-tag) (lower) IgGs. The asterisk indicates digested products of GST-CcPol mu were detected by cross-reactivity with anti-GST-tag IgGs. A double asterisk indicates GST dimer. (C) CcPol mu and CcLim15 interact *in vivo*. Crude extracts of fruiting body tissues were immunoprecipitated (IP) with a polyclonal antibody (pAb) against CcLim15. Proteins were separated by SDS–PAGE and visualized by HRP after Western blot analysis. For controls, crude extracts were incubated with a mixture of pre-immune sera.

Fig. 1C shows the results of *in vivo* co-immunoprecipitation analysis using the crude extract from *C. cinereus* meiotic tissue in the stage of early pachytene. The crude extracts were co-immunoprecipitated with polyclonal antibodies against CcLim15. CcLim15 and CcPol mu were detected, indicating that CcPol mu specifically interacts with CcLim15 *in vivo*. For controls, crude extracts were incubated with a mixture of pre-immune sera. No significant staining was detectable with the pre-immune sera.

Interactions between CcPol mu and CcLim15, or their truncated derivatives, using yeast two-hybrid assays

Next, we confirmed which region of CcPol mu was responsible for binding to CcLim15 by using a two-hybrid assay. Fig. 2A shows the truncation construction of CcPol mu and CcLim15 that were cloned into the bait vector pGBKT7 and prey vector pGADT7, respectively. *CcPol mu* has two domains. Thus, we divided *CcPol mu* into two constructs, namely the N-terminus and C-terminus. The N-terminus of *CcPol mu* (CcPol mu N; amino acids 1–175) contains the BRCT domain. The C-terminus (CcPol mu C; amino acids 176–608) contains the polymerase X domain (Fig. 2A). By contrast, *CcLim15* is divided into two functional domains. The N-terminal part (CcLim15 N; amino acids 1–104) is required for both ssDNA and dsDNA binding, whereas the C-terminal part (CcLim15 C; amino acids 105–327) contains an ATPase domain that is conserved with prokaryotic RecA [28,29].

Activation of the reporter gene occurred with CcLim15 (full length) and the CcLim15 C as prey against the C-terminus of CcPol mu (Fig. 2B). The two-hybrid assay demonstrated that the N-terminus of *CcPol mu* interacts with the C-terminus of *CcLim15*. Co-transformation using empty vector against each construct (a control) did not show any colony formation on selective medium.

The interaction between the truncated mutants of CcPol mu and CcLim15 was confirmed by β-galactosidase assay. Colonies co-transformed with full length CcLim15 and CcLim15 C against CcPol mu C grown in SD/-Ade/-His/-Leu/-Trp were re-plated on SD/-Ade/-His/-Leu/-Trp using X-β-Gal as substrate for galactosidase (Fig. 2C). This result suggests the N-terminus of CcPol mu interacts with the C-terminus of CcLim15. Co-transfection of the pGBKT7–p53 construct and pGADT7–T7 antigen was used as positive control.

Nuclear localization of CcLim15 and CcPol mu during the meiosis cell cycle

Next, we examined the distribution of CcPol mu and CcLim15 in the meiotic nuclei by immunostaining. As described above, we raised specific antibodies against CcPol mu and CcLim15. These antibodies were then used in combination with immunofluorescence staining to investigate the intracellular distribution of CcPol mu and CcLim15.

Chromosomes in meiotic cells exhibit a unique organization in the nuclei. Dikaryotic cells correspond to the premeiotic S phase stage. Upon entry into the leptotene stage of meiotic prophase, chromosomes that are initially diffused in nuclei form long and thin thread-like structures. In the next stage, zygotene, homologous chromosomes become aligned, forming synaptonemal complexes. During the following two pachytene and diplotene stages, homologous chromosomes that are aligned form intermediate structures called chiasmata. Then the single-stranded ends are thought to invade regions of homology in the corresponding allele. The subsequent two cell divisions occur in order to achieve the correct and reductive distribution of chromosomes into four gametes. This is followed by the metaphase stages when chromosome condensation occurs and then two rounds of cellular division take place.

As shown in Fig. 3, CcLim15 was found to be distributed on chromosomes in the nuclei from the leptotene to the early pachytene stages. CcLim15 signals are not detected after the metaphase stage (Fig. 3). On the other hand, CcPol mu also localized to nuclei from leptotene/zygotene to early pachytene. Importantly, a significant proportion of CcPol mu and CcLim15 could be shown to co-localize on chromosomes during the leptotene/zygotene and early pachytene stages, suggesting a physical association *in vivo*, consistent with their specific binding *in vitro* (Figs. 1 and 2). Furthermore, CcLim15 and CcPol mu signals faded in nuclei after the metaphase stage, suggesting that the two factors were no longer associated. These results indicate that the interaction between CcPol mu and CcLim15 is temporally regulated and is related to specific events in the early stages of meiosis. To our knowledge, this is the first report to indicate CcPol mu and CcLim15 are co-located during the meiosis cell cycle.

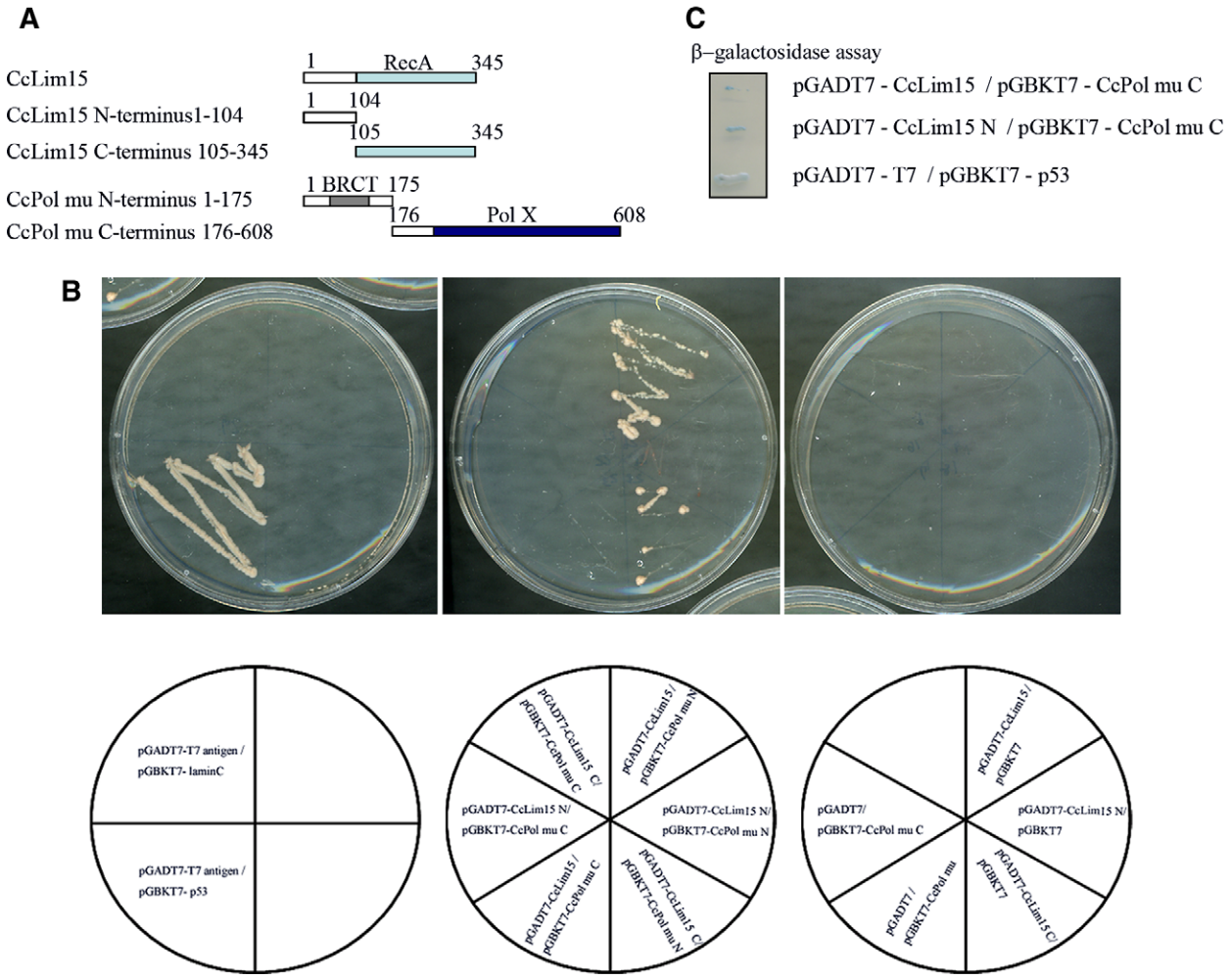

Fig. 2. CcPol mu and CcLim15 interact in the yeast two-hybrid system. (A) The indicated plasmid pairs were co-transformed into the AH109 yeast strain. (B) Transformants were plated on selective medium lacking adenine, histidine, leucine and tryptophan (SD/-Ade/-His/-Leu/-Trp). The interaction between p53 and the simian virus 40 T antigen was used as a positive control. Lamin C and the simian virus 40 T antigen were used as a negative control. (C) Galactosidase activity assay. Colonies grown in SD/-Ade/-His/-Leu/-Trp were re-plated on SD/-Ade/-His/-Leu/-Trp with b-Gal as the substrate for galactosidase.

CcLim15 enhances polymerase activity of CcPol mu

Our results indicated that CcPol mu interacts with CcLim15 both *in vivo* and *in vitro*. Next, we attempted to investigate the effect of CcLim15 on CcPol mu using a synthetic double-stranded DNA structures that serve as model substrates for DNA polymerase. The priming strands for extension by DNA synthesis were γ-^{32}P labeled (Fig. 4A). For control, we used BSA instead of CcLim15. CcPol mu with either CcLim15 or BSA was incubated for various times, 30, 60 or 90 s. Comparison of the results for each reaction time indicates CcLim15 activates the initial rate of CcPol mu polymerase activity. By contrast, BSA did not enhance the polymerase activity of CcPol mu. Thus, CcLim15 certainly enhances the initial rate of polymerase activity (Fig. 4B). Next, we repeated the polymerase assay described above a total of three times. We then quantified the DNA products that were elongated more than five nucleotides by using ImageJ application software. As shown in Fig. 4C, the polymerase activity of CcPol mu reproducibly increased in the presence of CcLim15.

Discussion

In this study, we demonstrated the interaction between CcLim15 and CcPol mu. We also confirmed that CcLim15 and CcPol mu localize to chromatin from leptotene/zygotene to early pachytene by fluorescent immunostaining, indicating that CcPol mu might interact with CcLim15 during meiosis. At this stage a single-strand overhang is formed by exonuclease activity, which is coated by Lim15. This overhang then invades the homologous double-stranded region of the other allele to generate a D-loop structure. Using a yeast two-hybrid assay, we determined that the BRCT domain of CcPol mu is responsible for binding the C-terminus of CcLim15. The BRCT domain is found within many DNA damage repair proteins (Ku70/80 and XRCC1) [30,31]. The unique diversity of this domain superfamily allows BRCT modules to interact and form homo/hetero BRCT multimers, BRCT–non-BRCT interactions or interactions within DNA strand breaks [32].

Intriguingly, there is a relationship between the BRCT domain and homologous recombination. BRCA1, which has a BRCT domain repeat, makes a complex with BRCA2, which is responsible for the location of Lim15 within the nuclei [33,34]. BRCA1/BRCA2 complex have established roles in genome integrity maintenance and in the control of homologous recombination by interacting with CcLim15 [34]. Whereas, the C-terminus of Lim15, which is a conserved domain in various species, has recombinase activity and DNA binding activity. The C-terminus of Lim15 may be responsible for binding proteins such as topoisomerase II, Ubc9 or BRCA2 [33,35,36]. Therefore Lim15 might be regulated by interaction through the C-terminus. Furthermore, from this study, CcLim15 seems to activate the initial rate of polymerase activity

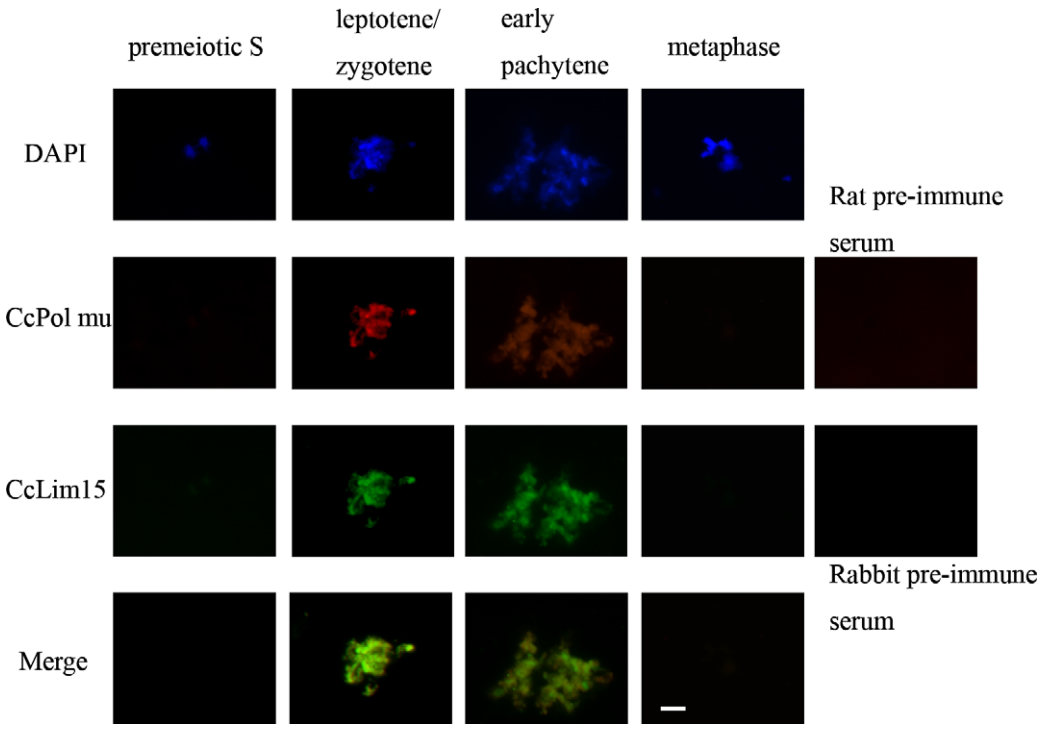

Fig. 3. Nuclear localization of CcPol mu and CcLim15 in the nuclei of *C. cinereus* meiotic cells. Spreads from fruiting body cells were stained blue with DAPI. Double labeling with rat anti-CcPol mu antibodies (red) and with rabbit anti-CcLim15 antibodies (green) was also performed. Merged images (yellow) show co-localization of the two proteins in discrete nuclear speckled structures. The scale bar equals 1 μm. No significant staining was detectable with the pre-immune sera. The secondary antibodies did not cross-react with the meiotic tissues or with other primary antibodies (data not shown).

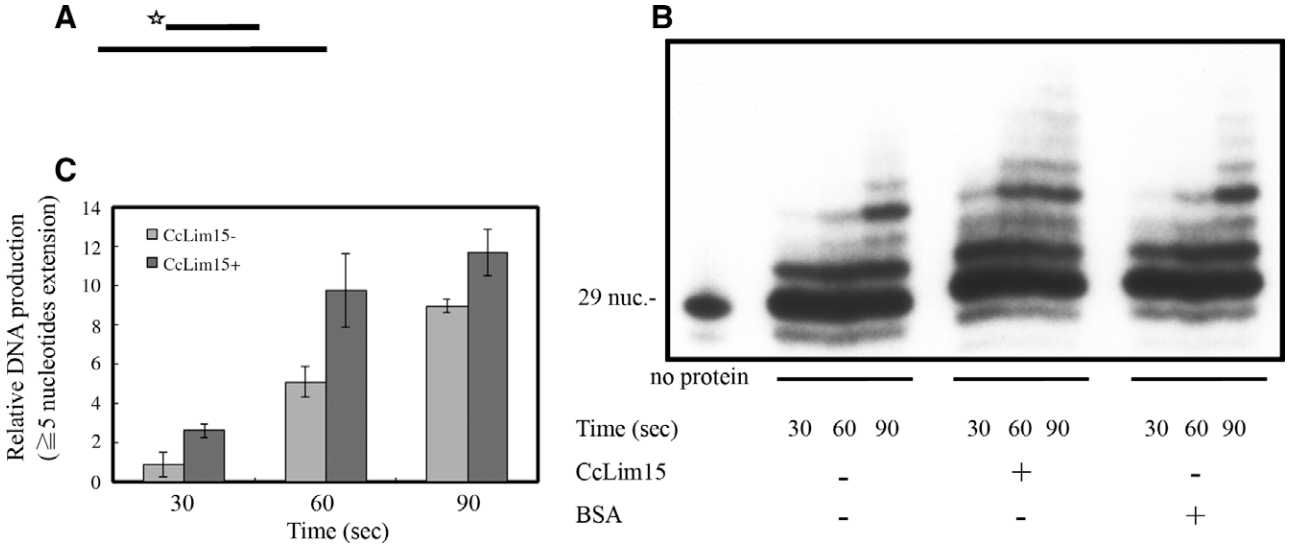

Fig. 4. DNA synthesis on a substrate mediated by CcPol mu. (A) A schematic representation of the synthetic substrate is shown. (B) DNA synthesis reactions were carried out on ^{32}P-labeled substrate with CcPol mu. The reaction products were then analyzed by denaturing PAGE. (C) Products elongated more than five nucleotides were quantified by ImageJ. The DNA production is compared with that seen with control (30 s in the absence of CcLim15). Average values from three independent experiments are shown with the standard error values.

of CcPol mu. In view of these findings, we propose that CcLim15 recruits CcPol mu and that CcPol mu polymerase activity is increased by CcLim15 elongating the D-loop structure, which is an intermediate of homologous recombination. The role of the interaction between CcPol mu and CcLim15 has not been elucidated yet. Further experiments may reveal the importance of this interaction during meiosis.

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