DNA topoisomerase II interacts with Lim15/Dmc1 in meiosis

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ABSTRACT

Lim15/Dmc1 is a meiosis specific RecA-like protein. Here we propose its participation in meiotic chromosome pairing-related events along with DNA topoisomerase II. Analysis of protein–protein interactions using *in vitro* binding assays provided evidence that *Coprinus cinereus* DNA topoisomerase II (CcTopII) specifically interacts with *C.cinereus* Lim15/Dmc1 (CcLim15). Co-immunoprecipitation experiments also indicated that the CcLim15 protein interacts with CcTopII *in vivo*. Furthermore, a significant proportion of CcLim15 and CcTopII could be shown to co-localize on chromosomes from the leptotene to the zygotene stage. Interestingly, CcLim15 can potently activate the relaxation/catenation activity of CcTopII *in vitro*, and CcTopII suppresses CcLim15-dependent strand transfer activity. On the other hand, while enhancement of CcLim15's DNA-dependent ATPase activity by CcTopII was found *in vitro*, the same enzyme activity of CcTopII was inhibited by adding CcLim15. The interaction of CcLim15 and CcTopII may facilitate pairing of homologous chromosomes.

INTRODUCTION

In meiosis, 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 then followed by the formation of single-stranded DNA (ssDNA). The single-stranded ends then invade regions of homology in the corresponding allele. Following the initial repair synthesis, the crossover and the non-crossover pathways diverge (1,2). These reactions are mediated by RecA-like recombinase, Lim15/Dmc1 and Rad51 which play a central role in the strand exchange reaction (3,4). While Rad51 also participates in homologous recombination and DNA repair in mitosis, Lim15/Dmc1 function is restricted to meiotic cells (3,5).

From cytological observations, homologous chromosomes start to associate with each other in the zygotene stage and synapse to form synaptonemal complexes (SCs) during the pachytene stage of meiosis. During these periods, two types of recombination nodules (early and late) appear as spherical proteinaceous structures (6). At zygotene stage, early recombination nodules are observed at the convergence point between axial elements of homologues (7,8). They are thought to be involved in homology searching and synapsis. The late nodules are larger and more densely stained and appear from the early pachytene chromosomes, and are probably to be the sites of crossover recombination (9,10). Rad51 and Lim15/Dmc1 co-localize as numerous foci at the zygotene stage in yeast and lily (11,12), and localize in the early recombination nodules of the lily (13). These observations suggest that Rad51 and Lim15/Dmc1 may be involved in recombination-related homology searching in early recombination nodules. In yeast and mice, deficiency of Lim15/Dmc1 results in asynapsis or synapsis between non-homologous regions, indicating that Lim15/Dmc1 is required for the establishment of axial connections between homologues (14,15).

Recent findings imply that Rad51 interacts with various nuclear factors such as RP-A (16–18), Rad52 (19–21), Rad54 (22–24) and Rad55–Rad57 heterodimers (25). However, only a few proteins are known to interact with Lim15/Dmc1. Rad54 homologue protein, Rdh54/Tid1, interacts with both Rad51 and Lim15/Dmc1, and is involved in crossover interference in *Saccharomyces cerevisiae* (26,27). Meiosis specific proteins Mei5 and Sae3 form a ternary complex with Lim15/Dmc1 to ensure interhomologue recombination through single strand invasion (28,29). Biochemical analyses

revealed that the Human form of Dmc1 promotes ATP-dependent interhomologue DNA strand exchange (30). These observations demonstrate that Lim15/Dmc1 may be a key regulator of interhomologue recombination during meiosis. Identification of additional proteins that interact with Lim15/Dmc1 will shed more light on their functional roles.

We reported previously molecular cloning of Lim15/Dmc1 (CcLim15) from a basidiomycete, *Coprinus cinereus*. This organism is especially well suited for studies of meiosis, because its meiotic cell cycle is long and naturally synchronous (31–34). Each fruiting cap is extremely rich in meiotic cells at the same stage, and the nuclear numbers are readily discernible. And documented here, we have found an interaction of CcLim15 and *C.cinereus* DNA topoisomerase II (CcTopII) on screening in a yeast two-hybrid system using CcLim15 as the bait with a cDNA library established from *Coprinus* meiotic cell lysate. Basic role of DNA topoisomerase II in cells is to catalyse the transport of one DNA double helix through a transient double strand break in another DNA molecule. Hitherto, it has only been elucidated that the role of DNA topoisomerase II in meiosis is to untie entangled chromatin, mainly in the M_1 phase (35,36). The purpose of the present study was, taking advantage of the available material, to focus on interactions of CcLim15 with CcTopII and to determine their relation to meiotic development. Our data suggest that CcTopII is directly involved in meiotic chromosome pairing-related events via CcLim15.

MATERIALS AND METHODS

Culture of *C.cinereus* and collection of fruiting bodies

The basidiomycete *C.cinereus* (ATCC #56838) was used in this study. The culture methods used and meiotic stage definition were as described previously (37).

Yeast two-hybrid screening and molecular cloning of CcLim15 and CcTopII

Yeast two-hybrid screening was performed with MATCHMAKER Two-Hybrid System 3 (CLONTECH). The cDNA of *C.cinereus* Lim15 (*CcLim15*) was amplified by PCR and the product was cloned into the EcoRV-digested pBluescript II SK (+) vector (pBSK+) (Stratagene) before subcloning into NdeI and XhoI sites of the GAL4-binding domain plasmid, pGBKT7. Total RNA was isolated (TRIzol; Invitrogen) from *Coprinus* meiocytes and reversed transcribed using a TimeSaver cDNA synthesis kit (Amersham Phamacia). cDNA was subsequently cloned into the EcoRI-linearized GAL4 activation domain vector, pGADT7. Positive colonies were screened for β-galactosidase activity using a filter-lift assay. Activation domain plasmids, pGADT7, were isolated from yeast colonies displaying a positive phenotype and transformed into bacteria to obtain plasmids suitable for sequencing reactions.

Molecular cloning of *CcLim15* was performed as described previously (37). For the *C.cinereus* DNA topoisomerase II gene, the inserted DNA fragment in the pGADT7 clone was excised and used as a probe to screen the full length of the DNA topoisomerase II gene by the plaque hybridization method. Screening of a *Coprinus* meiocyte cDNA library

resulted in isolation of a clone, designated as *CcTopII* (*C.cinereus Topoisomerase II*).

Two-hybrid and β-galactosidase assays

Full length and cDNA fragments encoding N- or C-terminal domains of *CcLim15* (*CcLim15$_{1-345}$*, *CcLim15NT$_{1-104}$* or *CcLim15CT$_{104-345}$*) and cDNA fragments encoding N-terminal, middle or C-terminal domains of *CcTopII* (*CcTopIINT$_{1-750}$*, *CcTopIIMD$_{751-1065}$* or *CcTopIICT$_{1066-1569}$*) were amplified by PCR and inserted into the EcoRV-digested pBSK+ vector. The cDNAs of *CcLim15$_{1-345}$*, *CcLim15NT$_{1-104}$* and *CcLim15CT$_{104-345}$* were subcloned into NdeI and XhoI sites of pGADT7 and pGBKT7, to produce fusions to the GAL4 DNA-binding and activation domains. *CcTopIINT$_{1-750}$*, *CcTopIIMD$_{751-1065}$* and *CcTopIICT$_{1066-1569}$* were also subcloned into NdeI and BamHI sites. GAL4 fusion constructs were simultaneously co-transformed and plated with established methodology and β-galactosidase reporter gene expression of individual yeast colonies was monitored by CPRG-based liquid culture assay (yeast protocols handbook; Clontech). At least four individual colonies were assayed for each transformation.

Production of recombinant proteins and antibodies

Overexpression and purification of CcLim15 protein were accomplished as reported previously (38) and anti CcLim15 rabbit polyclonal antibodies were raised as detailed earlier (37). Histidine-tagged full-length CcTopII protein was expressed for purification using BAC-TO-BAC HT Baculovirus Expression System (Invitrogen) as described previously (39). CcTopII recombinant protein was then injected into a rabbit and a rat. Non-immune sera gave no staining when tested on *Coprinus* meiotic tissues.

To construct bacterial expression plasmids for glutathione *S*-transferase (GST) fusion proteins with the CcLim15 fragments, PCR was performed using the CcLim15 cDNA as a template. The following primer pairs were used for the PCRs in combination: for GST-CcLim15$_{1-345}$, 5′ primer, 5′-GGAATTCATGCCTCCTCGCCGTGCT-3′, and 3′ primer, 5′-AATCTCGAGCTAAACGTCAGCCCAACC-3′; for GST-CcLim15NT$_{1-104}$, 5′ primer, 5′-TAACCCGGGAATGCCTCCTCG-CCGTGCT-3′, and 3′ primer, 5′-AATCTCGAGGCGTTTAT-CCTGAATCTC-3′; for GST-CcLim15CT$_{104-345}$, 5′ primer, 5′-TAACCCGGGAAAGCGAGTGCTTGTTATC-3′, and 3′ primer, 5′-AATCTCGAGCTAAACGTCAGCCCAACC-3′. PCR fragments were double digested with EcoRI–XhoI (GST-CcLim15$_{1-345}$) or SmaI–XhoI (GST-CcLim15NT$_{1-104}$ and GST-CcLim15CT$_{104-345}$) and inserted between EcoRI and XhoI or SmaI and XhoI sites of the pGEX-6p-1 vector (Amersham Bioscience), respectively. GST-fusion proteins were expressed in *Escherichia coli* and purified as described (40). For construction of the His-tagged CcTopIICT$_{1066-1569}$, the following primer pair was used for the PCR in combination: 5′ primer, 5′-GGAATTCCATATGCTTGTCGAGTTC-TTCGG-3′, and 3′ primer, 5′-CCCAAGCTTCTCATCATCA-ACGAACATCGA-3′. Resulting PCR fragments were double digested with NdeI–HindIII and inserted between NdeI and HindIII sites of pET21b (Novagen). His-tagged recombinant proteins were also expressed and purified with a kit according to the manufacturer's protocol (Novagen).

Co-immunoprecipitation

Rabbit anti-CcTopII polyclonal antibodies, rabbit anti-CcLim15 polyclonal antibodies and control rabbit serum were coupled with CNBr-activated sepharose beads according to the manufacturer's instructions (Amersham Pharmacia). 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 phenyl-methylsulfonyl 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 of the pH by adding 2 M Tris–HCl (pH 8.8), proteins were separated by SDS–PAGE. Western blotting was carried out using a rat anti-CcTopII polyclonal antibody, or a rabbit anti-CcLim15 polyclonal antibody.

In vitro binding assays

Purified GST or GST-fusion fragments of CcLim15 (100 μg) were incubated with His-tagged CcTopIICT$_{1066-1569}$ recombinant proteins (100 μg) for 1 h at room temperature (25°C) with 5 ml of GST pull-down buffer [50 mM Tris–HCl (pH 8.0), 350 mM NaCl and 0.1% Triton X-100]. The mixture was then added to a 500 μl glutathione 4B-Sepharose (Amersham Bioscience) column and then washed with 20 vol of pull-down buffer. Proteins were eluted with 1 ml of GST elute buffer [50 mM Tris–HCl (pH 8.0), 350 mM NaCl, 0.1% Triton X-100 and 10 mM reduced glutathione], boiled for 5 min after addition of 5× Laemmli sample buffer, resolved by SDS–PAGE and transferred to a nitrocellulose membrane (BioRad). Similarly, His pull-down was performed with His pull-down buffer [Tris–HCl (pH 8.0), 350 mM NaCl, 0.1% Triton X-100, 10 mM imidazole and 5 mM β-mercaptoethanol] and His elute buffer [Tris–HCl (pH 8.0), 350 mM NaCl and 250 mM imidazole] using Ni-NTA agarose resin. Samples were detected by immunoblotting using a rabbit polyclonal antibody against GST or a mouse monoclonal antibody against His-tag.

RNA extraction and northern hybridization analysis

Total RNA was prepared from caps of *C.cinereus* at meiotic prophase by TRIzol according the manufacturer's protocol. RNA samples were separated on 1.2% agarose-formaldehyde gels, blotted to membrane and hybridized with a ^{32}P-labelled probe as described by Nara *et al.* (37).

Immunostaining of nuclei of *C.cinereus* meiotic cells

Immunostaining of nuclei of *C.cinereus* meiotic cells was carried out as described previously (41). The slides were treated with either anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes) for anti-CcLim15 (1:1000) or anti-rat IgG conjugated with Alexa Fluor 568 (Molecular Probes) for anti-CcTopII (1:1000). Slides were subsequently stained with a solution of TOTO-3 (Molecular Probes) and examined under a fluorescence microscope (BioRad Radiance 2000 laser scanning confocal microscope).

D-loop assays

D-loop reactions were performed essentially as described previously (42). Standard reactions (25 μl) contained 5′-^{32}P-end-labelled 83mer ssDNA, 5′-TTGATAAGAGGT-CATTTTTGCGGATGGCTTAGAGCTTAATTGCTGAAT-CTGGTGCTGTAGCTCAACATGTTTTAAATATGCAA-3′ (1.5 μM), CcLim15 (0.5 μM; hexamer) and various concentrations of CcTopII (dimmer) protein in standard buffer [50 mM Tris–HCl (pH 8.0), 15 mM MgCl$_2$, 2 mM ATP, 2 mM DTT, 100 μg/ml BSA, 20 mM creatine phosphate and 2 U/ml creatine phosphokinase]. After 10 min at 30°C, excess supercoiled M13 mp18 RF DNA (25 μM) was added and incubation was continued for 4 min for CcLim15. Reaction products were deproteinized by the addition of one-fifth volume of stop buffer (final 0.5% SDS and 1 mg/ml proteinase K) followed by 15 min incubation at 37°C. Labelled DNA products were analysed by electrophoresis through 0.8% agarose gels run in 1× TAE buffer at 4 V/cm for 3 h, dried onto filter paper and visualized by autoradiography. All DNA concentrations were determined in moles of nucleotides.

Topoisomerase assay

Relaxation and catenation activities of DNA topoisomerase II were assessed by detecting the conversion of pBSK+ plasmid to its relaxed or catenated forms (43). DNA topoisomerase II reactions were performed in 20 μl of reaction mixture containing 50 mM Tris–HCl buffer (pH 8.0), 120 mM KCl, 10 mM MgCl$_2$, 1 mM ATP, 0.5 mM DTT, 0.5 mM EDTA, 30 μg/ml BSA, pBSK+ plasmid DNA (200 ng), 3.75 nM CcTopII (dimmer) and various concentrations of CcLim15 (hexamer) protein. Incubation was at 30°C for 30 min and terminated by adding 2 μl of loading buffer consisting of 5% sarkosyl, 0.0025% bromophenol blue and 25% glycerol. The mixtures were then subjected to 1% agarose gel electrophoresis in TAE running buffer, followed by staining and detection with SYBR gold (Molecular probe).

ATPase assays

ATPase activities of CcLim15 and CcTopII were measured as described previously (38), using [γ-^{32}P]ATP (Amersham bioscience). Nanomolar concentrations of CcLim15 or CcTopII, 400 nCi of [γ-^{32}P]ATP and 100 μM cold ATP were incubated with 2.5 ng/μl of single-stranded or double-stranded M13 DNA in buffer containing 25 mM Tris–HCl (pH 8.0), 5 mM MgCl$_2$, 100 μg/ml BSA, 1 mM CaCl$_2$ and 1 mM DTT at 30°C, and aliquots of the reactions were analysed by thin layer chromatography. The amounts of ATP and Pi were quantified from the autoradiograms using an image analyzer (BAS 3000, Fuji Film).

RESULTS

CcLim15 interacts with CcTopII

The *C.cinereus* cDNA library in the two-hybrid vector pGADT7 and CcLim15, expressed as a translational fusion with the Gal4 DNA-binding domain in pGBKT7 as the bait, were simultaneously co-transformed into the yeast strain, AH109, and positive colonies were screened for

β-galactosidase activity using a filter-lift assay. Plasmid inserts were sequenced and compared against the GenBank™ database by BLAST search analysis. The screening of yeast transformants revealed clones containing cDNA encoding the DNA topoisomerase II homologue of *C.cinereus* (*CcTopII*).

The insert DNA of a pGADT7-CcTopII clone was then excised and used to screen the *Coprinus* meiocyte cDNA library by the plaque hybridization method. Screening of a *Coprinus* meiocyte cDNA library with the fragment as a probe resulted in isolation of a 4716 bp clone designated as CcTopII. The open reading frame encoded a predicted product of 1572 amino acid residues with a molecular mass of 180 kDa, similar in size to other eukaryotic CcTopII forms. Southern blotting analysis revealed the *CcTopII* gene to exist as a single copy per genome (data not shown). The nucleotide sequence data of CcTopII reported in this paper appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number (AB185148).

Then we overexpressed and purified both recombinant full length CcTopII and CcLim15 proteins, and generated rabbit and rat polyclonal antibodies. We succeeded in making polyclonal antibodies against CcTopII and CcLim15. Figure 1A shows the results of western blotting analysis of the crude extract from *C.cinereus* meiotic tissues and the purified recombinant full-length proteins. A 180 kDa and a 37 kDa protein band, corresponding to CcTopII and CcLim15, respectively, were detected. No significant staining was detectable with the pre-immune sera. The migration differences between the endogenous and recombinant proteins in Figure 1A are the result of a His-tag present on the recombinant protein. We found anti-CcLim15 and anti-CcTopII immunoprecipitate CcTopII and CcLim15 from crude extracts *Coprinus* meiotic tissue, while the control serum did not (Figure 1B and C), the results indicating that CcLim15 specifically interacts with CcTopII *in vivo*. To our knowledge, this is the first report of such an interaction between CcLim15 and CcTopII.

Specific interactions of CcTopII, CcLim15 or their truncation derivatives, and results of *in vitro* pull down assays

To further define intramolecular regions responsible for the interaction, successive deletion constructs of CcLim15 and CcTopII were tested by yeast two-hybrid analysis. Figure 2A shows the truncation constructs of CcLim15 and CcTopII that were cloned into the bait vector pGBKT7 and prey vector pGADT7, respectively. These were co-transformed into the yeast strain AH109 for quantitative β-galactosidase assay. Activation of the reporter gene occurred with CcTopIICT$_{1066-1569}$ as prey but not CcTopIINT$_{1-750}$ and CcTopIIMD$_{751-1065}$ against CcLim15$_{1-345}$ (full length). Furthermore, in place of CcLim15$_{1-345}$, CcLim15CT$_{104-345}$ could moderately interact with CcTopIICT$_{1066-1569}$ (Table 1). Transfection of the CcTopIICT$_{1066-1569}$ construct with empty prey plasmid, pGBKT7, prevented the positive interaction observed in the β-galactosidase liquid assay.

In order to substantiate the interaction of CcLim15CT$_{104-345}$ and CcTopIICT$_{1066-1569}$, an *in vitro* pull-down assay was carried out as reported previously (44). First, we expressed each form of recombinant protein, GST-CcLim15$_{1-345}$, GST-CcLim15NT$_{1-104}$, GST-CcLim15CT$_{104-345}$ and 6× His-tag

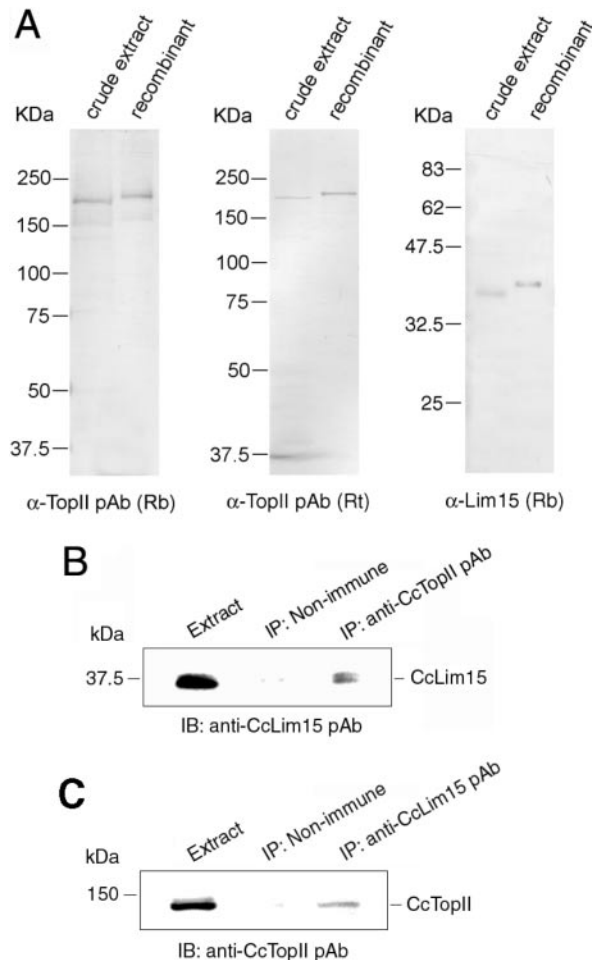**Figure 1.** CcLim15 and CcTopII interact *in vivo*. (**A**) Anti-TopII and anti-Lim15 antibody were generated and tested for their specificity using western analysis of crude extracts of meiotic tissues and the purified recombinant full-length proteins. Numbers indicate the positions and sizes of the protein standards. Crude extracts of fruiting body tissues were immunoprecipitated (IP) with a polyclonal antibody (pAb) against (**B**) CcTopII or (**C**) CcLim15. Proteins were separated by SDS–PAGE and visualized by alkaline phosphatase after western blot analysis. For controls, crude extracts were incubated with a mixture of non-immune sera. The positions of molecular weight markers (in kDa) are indicated. The immunoblots (IB) were probed with (**B**) CcLim15 or (**C**) CcTopII pAbs.

Table 1. Results of the liquid β-galactosidase assay with CPRG as substrate to demonstrate CcLim15 and CcTopII interactions

No.	DNA-binding domain plasmid	Activation domain plasmid	Activity
1	pGBKT7-CcLim15	pGADT7-CcTopIINT 1-750	<1
2	pGBKT7-CcLim15	pGADT7-CcTopIIMD 751-1065	<1
3	pGBKT7-CcLim15	pGADT7-CcTopIICT 1066-1569	16.5
4	pGBKT7-CcLim15NT 1-104	pGADT7-CcTopIICT 1066-1569	<1
5	pGBKT7-CcLim15CT 104-345	pGADT7-CcTopIICT 1066-1569	3.49
6	pGBKT7	pGADT7-CcTopIICT 1066-1569	<1

β-galactosidase activity was determined by CPRG-based liquid culture assay for the 3-day-old AH109 yeast transformants containing the indicated plasmids. At least four individual colonies were assayed for each transformation.
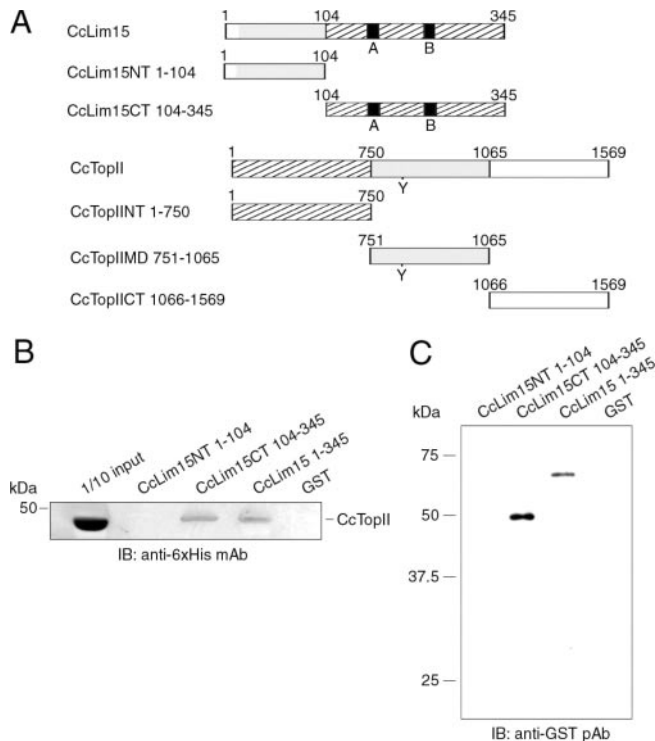
Figure 2. *In vitro* interaction between CcLim15 derivatives and CcTopIICT. (**A**) Diagrammatic representation of intact and truncated CcLim15 and CcTopII proteins. The conserved domain of CcLim15 including walkerA and walkerB motifs is indicated by black and striped boxes. The character 'Y' in the shaded box of CcTopII represents the active site of the protein. Open boxes indicate non-conserved regions. 6× His-tag-CcTopIICT$_{1066-1569}$ was mixed with GST-CcLim15$_{1-345}$, GST-CcLim15NT$_{1-104}$ or GST-CcLim15CT$_{104-345}$ at 25°C for 1 h, and then mixed either with (**B**) GST Sepharose-4B beads or with (**C**) His-bind resin beads, poured into columns, then washed with 20 vol of each buffer. The amount of protein present in the affinity column was quantified by western blotting. The immunoblots were probed either with (**B**) anti-His tag monoclonal antibody (mAb) or with (**C**) anti-GST tag pAb.

(6His)-CcTopIICT$_{1066-1569}$, in *E.coli* and highly purified them.

6His-CcTopIICT$_{1066-1569}$ and GST-CcLim15 proteins were mixed *in vitro*, and the fraction eluted with imidazole on His-bind resin beads was collected and electrophoresed and stained with an antibody against the GST tag. As shown in Figure 2B and C, CcLim15$_{1-345}$ and CcLim15CT$_{104-345}$ appeared to bind to CcTopIICT$_{1066-1569}$ *in vitro*. In order to rule out the possibility that co-precipitation was due to unspecific binding between CcTopIICT$_{1066-1569}$ and the GST tag, we mixed them and confirmed that no staining was observed. Similarly, the GST-CcLim15 proteins and 6His-CcTopIICT$_{1066-1569}$ were mixed *in vitro*, and the fraction eluted with glutathione by the GST Sepharose-4B beads was collected. CcTopIICT$_{1066-1569}$ also appeared to bind to CcLim15 full length and CcLim15CT$_{104-345}$ *in vitro*. When we mixed only the 6His–CcTopIICT$_{1066-1569}$ and the GST tag, no staining was observed. These observations coincided well with the findings of the two-hybrid analysis *in vivo* described above, indicating that the interaction between the two proteins did not occur because of nonspecific binding of tags.
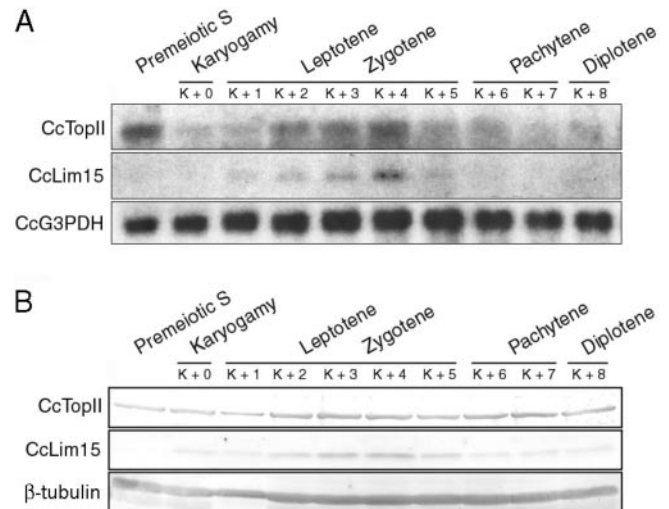

Figure 3. Expression of CcTopII and CcLim15 during meiotic development. (**A**) Northern blot analysis of *CcTopII* and *CcLim15* gene expression at each stage during the meiotic cell cycle. Each lane contained 20 µg of total RNA isolated from caps of *C.cinereus* at premeiotic S phase, karyogamy (K + 0), leptotene/zygotene (from K + 1 to K +5) and pachytene (K + 6, K + 7). The blot was hybridized with CcTopII (upper panel) and CcLim15 (middle panel) fragments, then reprobed for the *C.cinereus* glyceraldehyde 3-phosphate dehydrogenase (G3PDH) message as a loading control (bottom panel). (**B**) CcTopII and CcLim15 protein existences at various stages of meiotic tissues. Each lane contained 15 µg of crude extract from *C.cinereus* meiotic tissues. Proteins were separated by SDS–PAGE and visualized by alkaline phosphatase after western blot analysis. The immunoblots were probed with CcTopII (top panel) and CcLim15 (middle panel) pAbs. Anti-β-tubulin mAb (Chemicon) was used as a loading control (bottom panel).

Expression of CcLim15 and CcTopII during meiosis

Since the biochemical specificity pointed to some unknown role(s) in meiosis, next, the expression and distribution of the molecules were studied with reference to the timing of meiotic events. As described in the Introduction, the meiotic cycle in fruiting caps is separated into dikaryonic and monokaryonic stages. Dikaryonic cells are in premeiotic stages from the S phase to leptotene, and the beginning of the karyogamy stage, at which the two nuclei are fused, is the start of the zygotene stage in which homologous chromosomes pair. In the monokaryonic cells immediate after fusion, the paired chromosomes recombine. Meiotic cells at the same stage are easily observable and collectable.

For the determination of whether the *CcLim15* and *CcTopII* genes are expressed at the time of zygotene, pachytene or both, total RNA was extracted from basidia taken from synchronous cultures every hour after the induction of meiosis, and analysed by hybridization with *CcLim15* or *CcTopII* probes. Transcripts of *CcLim15* or *CcTopII* were observed mostly at zygotene, although *CcTopII* was expressed also in the premeiotic S phase during which the genomic DNA replicates (Figure 3A). As shown in Figure 3A, *CcTopII* transcripts began to accumulate immediately after the light period began (K + 1) and, slightly earlier than the *CcLim15* transcripts, becoming most abundant at 3.0–4.0 h after karyogamy (K + 3, K + 4). The signals had faded by 5.0 h after karyogamy (within zygotene, K + 5) and completely disappeared at pachytene (K + 7). *CcLim15* transcripts began to accumulate

at K + 2, and became most abundant 4.0 h after karyogamy (K + 4), then disppappearing by pachytene (K + 6).

Also, we analysed the existence of CcLim15 and CcTopII proteins during each meiotic stage by western blot (Figure 3B). CcLim15 protein was begun to accumulate after karyogamy and became most abundant at 3.0–4.0 h (K + 3, K + 4), result which was paralleled by the expression pattern of *CcLim15*. Although the transcript of CcLim15 was immediately disappearing by pachytene (K + 6), CcLim15 protein was left at low level (Figure 3B). However, CcTopII protein was found through out the meiotic stage. Since DNA topoisomerase II was also known as a chromosome scaffold protein, our data supported the previous study (45). Nonetheless, as shown in the Figure 3A, it is noteworthy that the transcript of CcTopII was found at the stage from leptotene to zygotene.

Since the majority of the basidia were in zygotene, rather than pachytene, as judged by fluorescent microscopy of the monokaryonic nuclei and electron microscopy to determine the presence of SCs, it was concluded that both *CcLim15* and *CcTopII* are expressed mostly at zygotene, well coinciding with previous data (37). Therefore, the interaction probably occurs at the stage when synaptinemal complexes are formed between homologous chromosomes. Meiotic expression of DNA topoisomerase II and a contribution to meiotic prophase events have hitherto been unclear. We therefore confirmed and characterized the interaction more precisely by immunostaining *in vivo*.

Nuclear localization of CcLim15 and CcTopII during the meiotic cell cycle

Chromosomes in meiotic cells exhibit a unique organization in nuclei. Upon entry into the leptotene stage of the meiotic prophase, chromosomes that are initially diffused in nuclei form long and thin thread-like structures and each acquires a proteinaceous axial core to which the two sister chromatids are attached. In the next stage, zygotene, homologous chromosomes become aligned, forming SCs. During the following two pachytene and diplotene stages, homologous chromosomes that are aligned form intermediate structures called chiasmata. The subsequent two cell divisions occur in order to achieve the correct and reductive distribution of the chromosomes into four gametes.

In the basidiomycete *C.cinereus* these events can be readily captured under light microscopy. In *C.cinereus* fruiting caps, the meiotic cell cycle occurs synchronously so that large amounts of cells at each stage can be easily obtained. Dikaryotic cells correspond to the premeiotic S phase stage. In the leptotene and zygotene stages, homologous chromosome pairing occurs. At the pachytene stage, chromosome pairs appear thicker. This is followed by the diplotene and diakinesis stages when chromosome condensation occurs and then two rounds of cellular division take place. As shown in Figure 4, the CcLim15 protein was found to be distributed on chromosomes in the nuclei from the leptotene (K + 3) to the zygotene stages (K + 5). CcLim15 signals are not detected after the pachytene (K + 7) stage (Figure 4). However, the CcTopII protein was localized on the chromosomes in nuclei during premeiotic S phase but also throughout the meiotic divisions, although most prominent from leptotene to pachytene (Figure 4). These observations are all consistent with the results of northern analysis. Importantly, as shown in Figure 4, significant proportions of CcTopII and CcLim15 could be shown to co-localize on chromosomes during the leptotene and zygotene stages, suggesting a physical association *in vivo*, consistent with their specific binding *in vitro*. This co-localization proved to be much less significant in the
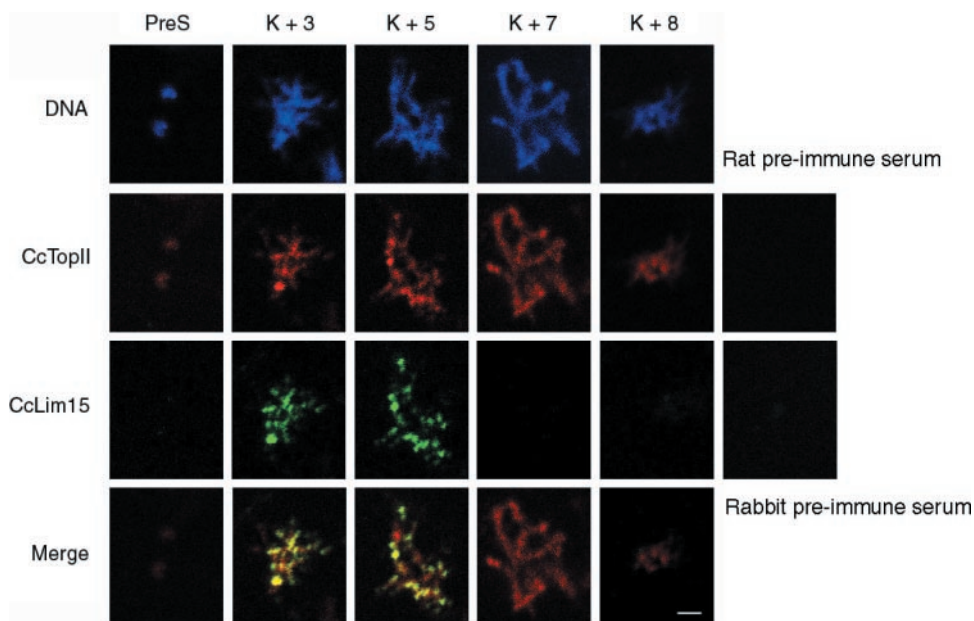

Figure 4. Nuclear localization of CcLim15 and CcTopII in the nuclei of *C.cinereus* meiotic cells. Spreads from fruiting body cells were stained blue with TOTO3. Double labelling with rat anti-CcTopII antibodies (red) and with rabbit anti-CcLim15 antibodies (green) was also performed. Merged images (yellow) 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 used did not cross-react with the meiotic tissues (data not shown) or with other primary antibodies (data not shown).

pachytene stage (Figure 4) and CcLim15 signals faded in nuclei after the diplotene stage, so that the two factors no longer associated. These results suggest that the interaction between CcTopII and CcLim15 is temporally regulated and is related to specific events in early stages of meiosis.

Influence of CcLim15 on CcTopII-dependent activity and of CcTopII on CcLim15-dependent activity

How does the complex of CcTopII and CcLim15 function in leptotene and zygotene? To answer this question, we next tested whether the two molecules affect each others biochemical activities. It is well known that CcLim15 exerts strand exchange and DNA-dependent ATPase activities, while CcTopII functions in DNA relaxation and catenation as a DNA-dependent ATPase (43).
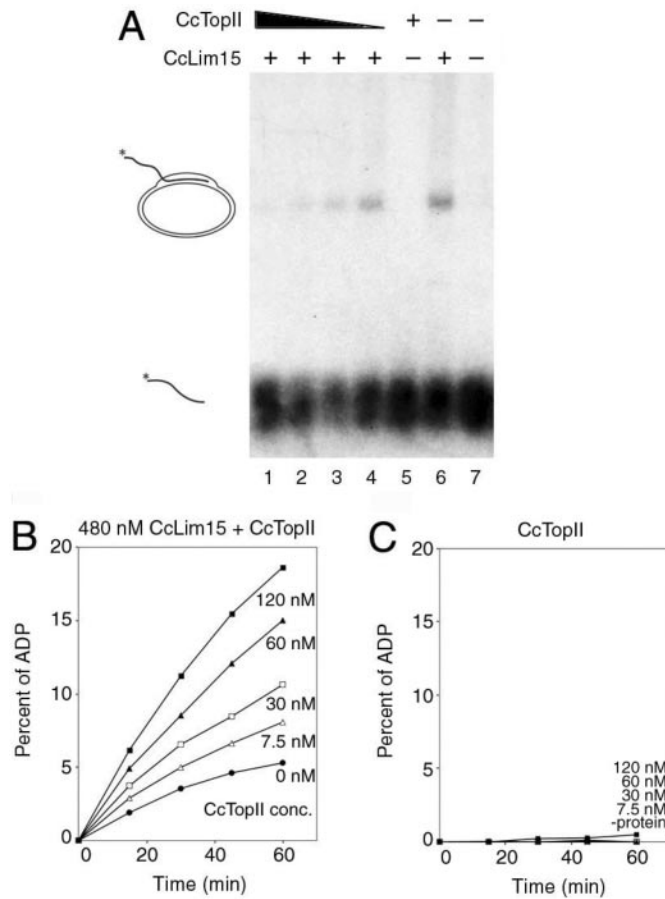
We performed D-loop assays using an end-labelled 83mer single-stranded oligonucleotide and a double-stranded M13 mp 18 RF containing the same sequence. D-loop formation with the recombinant CcLim15 protein was detected in the presence of ATP. This was suppressed by increasing amounts of CcTopII (Figure 5A). Furthermore, CcLim15's DNA-dependent ATP digestion potential was strongly enhanced by the CcTopII protein with ssDNA (Figure 5B). As shown in Figure 5B, the activity increased ∼4-fold in the presence of 120 nM CcTopII. Although DNA topoisomerase II also possesses ATPase activity, this was not seen when using ssDNA as the cofactor (Figure 5C). Prior to D-loop formation, Lim15/Dmc1 becomes activated upon binding to ATP (30). D-loop formation and ATPase activity are known to be a two-step mechanism for Lim15/Dmc1 enzymes (30). Given this enzymatic feature of Lim15/Dmc1, these results suggest that interaction between CcLim15 and CcTopII may facilitate the formation of the ATP–Lim15 complex, but not stimulate D-loop formation.
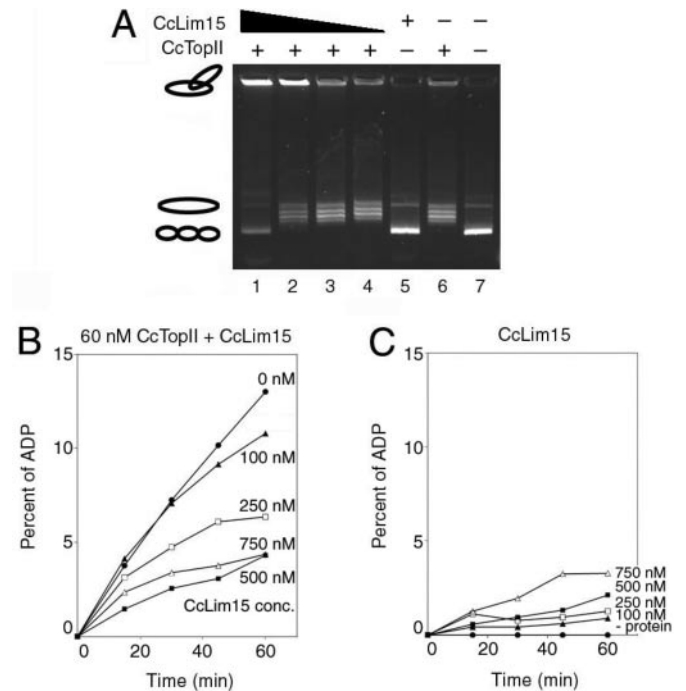

Figure 5. Effects of CcTopII on CcLim15 D-loops and ATPase activity. (**A**) The D-loop reaction was carried out in the presence of CcLim15 protein (0.5 μM; hexamer) and various concentrations of CcTopII protein(dimmer): 120 nM (Lanes 1 and 5), 60 nM (Lane 2), 30 nM (Lane 3) and 7.5 nM (Lane 4). After 8 min incubation, the DNA was deproteinized and analysed by agarose gel electrophoresis. (**B**) CcLim15 ATPase was measured. The reaction mixture contained single-stranded M13 DNA (2.5 ng/μl) as a co-factor. CcLim15 (480 nM) was mixed with various concentrations of CcTopII protein: 0 nM (closed circles), 7.5 nM (open triangles), 30 nM (open squares), 60 nM (closed triangles) and 120 nM (closed squares) of CcTopII protein. The reaction was initiated by addition of ATP. (**C**) ATP hydrolysis by various concentrations of CcTopII alone was examined in the absence of CcLim15 protein. Closed circles in (**C**) indicate ATPase activity without protein. The averages of three independent experiments are presented.

Figure 6. Effects of CcLim15 on CcTopII relaxation/catenation and ATPase activity. (**A**) Relaxation/catenation reactions were performed in a total volume of 20 μl and analysed on 1% agarose gels, as described under Materials and Methods. The positions of supercoiled DNA, relaxed DNA and catenated DNA are illustrated. CcLim15 was titrated against a fixed amount of CcTopII. Relaxation/catenation reaction mixtures contained 200 ng of negatively super-coiled pBluescript, 3.75 nM CcTopII (dimmer) and the various concentrations of CcLim15 (hexamer): 240 nM (Lanes 1 and 5), 60 nM (Lane 2), 15 nM (Lane 3) and 3.75 nM (Lane 4). Catenated DNA formation is efficient in the presence of CcLim15. (**B**) CcTopII ATPase was measured in reaction mixtures containing double-stranded M13 DNA (2.5 ng/μl) and 1 mM Ca^{2+} as a cofactor. CcTopII (60 nM) was mixed with the identified concentrations of CcLim15 protein: 0 nM (closed circles), 100 nM (closed triangles), 250 nM (open squares), 500 nM (closed squares) and 750 nM (open triangles). (**C**) ATP hydrolysis by the indicated concentrations of CcLim15 alone was also examined in the absence of CcTopII protein. Closed circles in (**C**) indicate ATPase activity without protein. The averages of three independent experiments are presented.

In order to examine the effect of CcLim15 on the enzymatic activity of CcTopII, we measured CcTopII activity in the presence or absence of CcLim15. In relaxation/catenation assay, CcLim15 could effectively enhance the catenation activity of CcTopII (Figure 6A). Relaxation was slightly stimulated, although the relaxed form may be increased in the intermediate processed prior to formation of catenation chains. We also measured DNA-dependent ATPase activity of CcTopII using double-stranded M13 DNA as a cofactor. Although CcLim15 itself had subtle DNA-dependent ATPase activity in the presense of 1 mM Ca^{2+} (Figure 6C), the ATPase activity of CcTopII was significantly inhibited by addition of CcLim15 in the presense of 1 mM Ca^{2+} (Figure 6B). These results suggest that interaction between CcLim15 and CcTopII may facilitate the catenation of CcTopII while inhibiting the DNA-dependent ATPase activity of CcTopII.

Our results raise the possibility that CcTopII is involved in meiotic recombination via CcLim15, by stimulating not strand exchange, but rather other chromosome pairing-related events.

DISCUSSION

In the present studies using a basidiomycete, *C.cinereus*, we clarified that a meiotic recA-like protein, Lim15/Dmc1 (CcLim15), interacts with DNA topoisomerase II (CcTopII), and that the bound pair probably functions in the meiotic prophase during which homologous chromosomes juxtapose and pair. Homologues of Lim15/Dmc1 and DNA topoisomerase II are universally distributed in eukaryotes, but to our knowledge this is the first report to document involvement of DNA topoisomerase II in the meiotic chromosome pairing process, and interaction of the enzyme with a meiosis-specific recA-like protein. The hitherto proposed role of DNA topoisomerase II in meiosis is to untie entangled chromatin, mainly in the M_1 phase (35,36). We would here like to focus on the significance of the CcLim15 and CcTopII interaction.

In the present study, transcripts of *CcLim15* or *CcTopII* were expressed mostly in the basidia at leptotene and zygotene, although *CcTopII* appeared to be upregulated slightly earlier. Immunostaining using the antibodies, the CcLim15 is distributed on the chromosomes in the nuclei from the leptotene to the zygotene stages. After the pachytene stage, the CcLim15 signals quickly fade. These expression patterns of CcLim15 were consistent with previous studies that demonstrated the presence of Lim15/Dmc1 in meiotic prophase nuclei at the leptotene to zygotene stage in yeast, lily and mouse (11,12,15). However, the CcTopII was detectable on the chromosomes on nuclei throughout the meiotic divisions, but very prominent from leptotene to packytene. Importantly, the expression and the co-localization of the two factors were consistent with the observation that CcLim15 interacts with CcTopII both *in vitro* and *in vivo*.

The Lim15/Dmc1 protein, unlike another eukaryotic recA-like protein, Rad51, shows only low strand exchange activity in many classical assays using naked DNA templates that were originally developed for bacterial RecA (3,37). This suggests that co-factors are required to stimulate the strand exchange activity of Lim15/Dmc1. The question therefore arises of whether CcTopII might be one such co-factor. However, the present investigation unexpectedly demonstrated suppression of the CcLim15-dependent strand exchange reaction by CcTopII using a naked DNA template *in vitro*. In a study of recA protein, it was found that the formation of D-loops does not require negative superhelicity but is accelerated by superhelicity >50-fold (46). However, DNA topoisomerase II is able to relax negative supercoiled DNA (47). Therefore, the fact that the CcTopII protein suppressed the D-loop formation by CcLim15 strongly suggests that this involved its relaxation of the double-stranded DNA (dsDNA) substrate. Indeed, CcTopII dose-dependently relaxed negative supercoils (data not shown). Since the ATPase activity of recA is thought to have a role in releasing the protein from ssDNA (48), CcTopII lowering of the affinity of CcLim15 for the DNA might also contribute to its suppression of D-loop formation.

Conversely, the CcLim15 protein could activate the DNA relaxation/catenation activity of CcTopII *in vitro*. In the presence of DNA-condensing agents such as supermidine and histone H1, DNA topoisomerase II can produce DNA catenates (49) and the N-terminal region (residue 1–104) of CcLim15 is rich in basic amino acids, and the isoelectric point is pH 10. The region has DNA-binding activity, and is capable of condensing DNA molecules. CcLim15 also shows high affinity for dsDNA (50). From these findings, we speculate that CcLim15 condenses dsDNA by binding to supercoils, and subsequently, could enhance the catenation activity of CcTopII, because the distance between the DNA molecules would be closer. At present we have no explanation for the unexpected inhibition of DNA-dependent ATPase activity of CcTopII by CcLim15.

How might CcTopII be involved in the CcLim15-dependent pathway? Of course, the reaction may be stimulated by CcTopII in the presence of additional co-factors, or the DNA templates for the *in vitro* strand exchange assay may need to possess a nucleosome structure in order to detect CcTopII-dependent stimulation. Several groups recently found this to be true for Rad51 and Rad54-dependent strand transfer activity (24,51–53). Further biochemical analysis of Lim15 and TopII proteins are needed to test this hypothesis.

The results presented here can be summarized by the following model. Early meiotic pairing may involve the formation of unstable side-by-side (paranemic) joints between intact DNA duplexes (54,55). Such reversible associations would provide a mechanism to deal with the interchromosomal tangles that are expected to result when uncondensed chromosomes, meandering through the nucleus, initiate pairing at multiple sites. Lim15 and TopII are probable to resolve these tangles more efficiently while assisting with chromosome pairing. Furthermore, unstable interactions would not deter chromosomal alignment because homologues would be held together at multiple sites along their entire length. Interactions between ectopic repeats would usually be displaced by homologous interactions as paranemic joints are broken and reformed. Based on the results presented here, we suggest that interaction between Lim15 and TopII serves to catenate the chromosomes and transiently stabilize the interchromosomal association, allowing for Lim15 to search and align the homologous chromosomes.

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