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Characterization of Interaction of C- and N-Terminal Domains in *LIM15/DMC1* and *RAD51* from a Basidiomycetes, *Coprinus cinereus*

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Both *LIM15/DMC1* and *RAD51* are thought to be essential for meiosis in which homologous chromosomes pair and recombine. The primary purpose of the present study was to investigate the homotypic and heterotypic interactions among their terminal domains. We prepared cDNAs and recombinant proteins of the full-length, N-terminal, and the C-terminal domains of *LIM15/DMC1* (*CoLIM15*) and *RAD51* (*CoRAD51*) from the basidiomycete *Coprinus cinereus*. In both two-hybrid assay *in vivo* and pull-down assay *in vitro*, either CoLim15 or CoRad51 interacted homotypically between the C-terminal domains, respectively, but no heterotypic interaction was observed between CoLim15 and CoRad51. The N-terminal domain of CoLim15 bound to ssDNA and dsDNA, while the C-terminal domain of CoRad51 appeared to interact weakly with ssDNA. Based on these results, the interaction among the strand-exchange proteins and meiosis was discussed.

***Key Words: Coprinus cinereus; LIM15/DMC1* (*CoLIM15*); *RAD51* (*CoRAD51*); homotypic interaction; C- and N-terminal interaction.**

We investigated meiosis-related proteins and their relationship to meiotic pairing and meiotic recombination using meiotic cells in a basidiomycete, *Coprinus cinereus* (1–10). This organism is especially well suited for studies of meiosis, because its meiotic cycle is long and naturally synchronous (9–12). We could, therefore, precisely characterize each of the meiosis-related proteins in relation to each of the meiotic events. In this connection, we have been interested in the roles of RecA homologs such as *DMC1* and *RAD51* in *Coprinus* meiosis, because they have been to report to interact in meiotic events prior to meiotic chromosome synapsis during prophase (13–20).

Both *DMC1* and *RAD51* are required for normal meiotic recombination, for the formation of the synaptonemal complex and for normal progression from meiotic prophase (21, 22). *DMC1* and *RAD51* appear to have both distinct and overlapping roles in meiosis (21–23), and immunoprecipitation studies indicated that human *DMC1* interacted heterotypically and directly with human *RAD51* (24). On the other hand, Dresser *et al.* (23) reported that *DMC1* functioned in a *Saccharomyces cerevisiae* meiotic pathway that is largely independent of the *RAD51* pathway. Aihara *et al.* reported recently that human *RAD51* bound to DNA via its N-terminal domain (25). The structural interactions of RecA homologs should be investigated in more detail.

We searched for a meiotic RecA homolog, *LIM15/DMC1*, of *C. cinereus*, and succeeded in cloning the gene, *CoLIM15* (10). On the other hand, another RecA homolog, *CoRAD51*, was identified in *C. cinereus* by Stassen *et al.* (26), indicating that the basidiomycete produces typical two meiosis-related RecA-like proteins. Like human and yeast, sufficient amounts of the recombinant proteins of *CoLIM15* and *CoRAD51* from the basidiomycete, can be obtained for analysis of structural interactions. The primary purpose of the present study was to investigate the interaction of the C- and N-terminal domains of the *Coprinus* strand-exchange proteins, and to discuss the relationship among these proteins and *Coprinus* meiosis. We prepared cDNA molecules and recombinant peptides of full-length, N-terminal and C-terminal domains of CoLim15 and CoRad51, and test their interaction by two-hybrid assay and pull-down assay, and gel-shift mobility assay. There has been only one previous report that the N-terminal region of Rad51 mediated both self-association and Rad51–Rad52 interaction in the yeast *Saccharomyces cerevisiae* (27). Unexpectedly,

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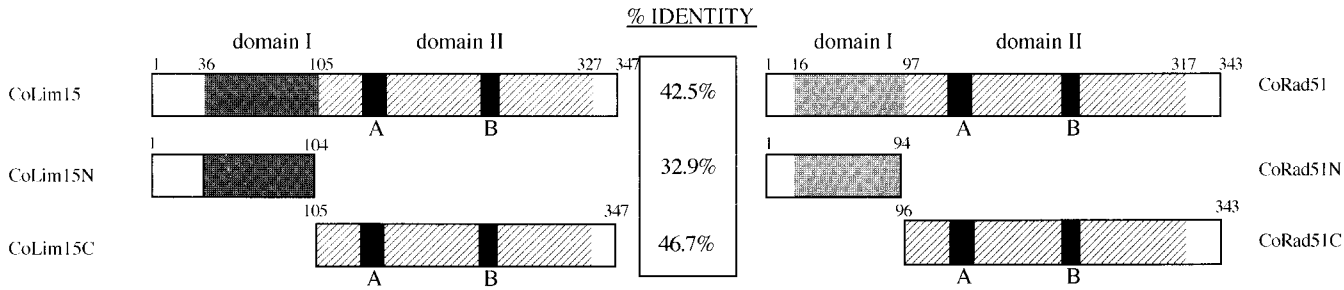

FIG. 1. Truncation derivatives of the *Coprinus* Lim15 and Rad51 proteins used in this experiment. Identities of full-length, N-terminal, and C-terminal region of the two proteins are shown. The black boxes depict nucleotide binding motifs (10, 26).

homotypic interactions of either CoLim15 or CoRad51 occurred between their C-terminal domains, and CoLim15 did not interact heterotypically with CoRad51. The N-terminal domain of CoLim15 bound to both ssDNA and dsDNA, but CoRad51 appeared to bind only weakly to DNA at the C-terminal domain.

MATERIALS AND METHODS

Yeast two-hybrid analysis. Full length *Coprinus LIM15* and *RAD51* cDNAs were amplified by PCR and inserted into pBS. The cDNA fragments encoding N- or C-terminal domains of *CoLIM15* (*CoLIM15N* or *CoLIM15C*) were also amplified and inserted into pBS. The *CoLIM15*, *CoRAD51*, *CoLIM15N* and *CoLIM15C* genes were cloned into pGADT7 and pGBKT7 to produce fusions to the GAL4 DNA-binding and activation domains. To obtain cDNA fragments encoding N- or C-terminal domains of *RAD51* (*CoRAD51N* or *CoRAD51C*), the full-length *CoRAD51* gene was digested with *Apa*LI and blunted with the Klenow fragment. Then, *CoRAD51N* or *CoRAD51C* DNA fragments were inserted into pGADT7 and pGBKT7. All constructs were sequenced to verify the correct open reading frames. The GAL4 fusion constructs were transformed into the yeast strain AH109 and cells were plated on synthetic media lacking adenine, histidine, leucine and tryptophan or on media lacking leucine and tryptophan. Transformation, colony lift β-galactosidase assays were carried out according to Matchmaker kit manual (Clontech Laboratories). The truncation derivatives are shown in Fig. 1.

Protein expression and purification. The recombinant CoLim15, CoRad51, CoLim15N, CoLim15C, CoRad51N and CoRad51C proteins were isolated as follows. The cDNA fragments were subcloned into the bacterial expression vector pET28b or pET32b (Novagene), and the plasmid constructs were introduced into E. coli strain BLR21 (DE3). These E. coli were grown at 30°C in 2XYT medium with 2% glucose and 50 μg/ml kanamycin (pET28b) or carbenicillin (pET32b). At OD600 = 0.7, Recombinant protein synthesis was induced by the addition of 1 mM IPTG, and after 5 h the cells were harvested by centrifugation, frozen in liquid N_2 and stored at −80°C. The cell pellets were resuspended in Lysis buffer (20 mM Tris-HCl, pH 7.9, 10% glycerol, 500 mM NaCl, 5 mM imidazole), containing 5 mM 2-melcapto ethanol and the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), leupeptin (1 μM) and pepstatin A (1 μM). Cells were lysed by addition of 1 mg/ml of lysozyme and stirred on ice for 30 min, then sonicated and Triton X-100 was added to 0.1%. Insoluble material was removed by centrifugation at 25000 r.p.m. for 30 min in a Beckman 70 Ti rotor. Proteins were loaded onto a 1 ml HiTrap Chelating column (Amersham Pharmacia Biotech). The column was washed successively with T buffer (20 mM Tris-HCl pH 8.0, 10% glycerol, 500 mM NaCl, 0.02% NP-40) containing 10 and 50 mM imidazole, respectively. The bound proteins were eluted with a 20 ml linear gradient of 50–600 mM imidazole in T buffer. Fractions of proteins were identified by SDS-PAGE, pooled and dialyzed against storage buffer (20 mM Tris-HCl pH 8.0, 10% glycerol, 1 mM EDTA, 0.5 mM DTT, 200 mM NaCl) and stored in aliquots at −80°C. The protein concentrations were determined using a Bio-Rad protein assay kit with γ-globulin as the standard.

Pull-down assay of CoLim15 and/or CoRad51, and their C- and N-terminal domains in vitro. The pull-down assay was performed by essentially the same method as described by Kovalenko *et al.* (30). The purified proteins (1 μg each) were incubated in binding buffer (200 μl) contained 25 mM NaHEPES, pH 7.2, 200 mM NaCl, 10% glycerol, 0.5 mg/ml BSA, 0.5 mM DTT, 0.1% Triton X-100, for 30 min at room temperature. Binding complex were precipitated with 10 μl of S-protein Agarose (Novagene). After incubation of the binding reaction at RT for 60 min, the bound proteins were washed five times with binding buffer and eluted by incubation with 3 M $MgCl_2$. The eluted proteins were separated by SDS–PAGE and western blotting was carried out using a rabbit anti-CoLim15 polyclonal antibody.

Binding of CoLim15, CoRad51, CoLim15N, CoLim15C, CoRad51N, and CoRad51C to DNA. Binding of the CoLim15, CoRad51, CoLim15N, CoLim15C, CoRad51N or CoRad51C protein to DNA was studied by gel mobility shift assay essentially as described by Kovalenko *et al.* (30). Single-stranded ϕX174 DNA (15 μg) or *Eco*RV-linealized pBS DNA were incubated with each protein in 20 μl of reaction buffer containing 25 mM NaHEPES, pH 7.2, 5% glycerol, 2 mM ATP, 15 mM $MgCl_2$, 100 μg/ml BSA, 1 mM DTT. After 30 min at RT, samples were analyzed by electrophoresis through 0.8% agarose gels in 0.5× TBE buffer. DNA and DNA-protein complexes were visualized by ethidium bromide staining. All DNA concentrations are expressed in moles of nucleotides.

RESULTS AND DISCUSSION

Specific interactions between CoLIM15, CoRAD51, or their truncation derivatives. *CoLIM15, CoRAD51* and their truncation derivatives were used in two-hybrid and *in vitro* binding studies. The truncation derivatives of *CoLIM15* and *CoRAD51*, i.e. *CoLIM15N*, *CoLIM15C*, *CoRAD51N* and *CoRAD51C* are shown in Fig. 1. *CoLIM15* and *CoRAD51* showed 42.5% amino acid sequence identity. The identity between their truncated N-terminal domains was lower than that of their C-terminal domains (Fig. 1).

Table I shows specific protein-protein interactions between *CoLIM15*, *CoRAD51*, *CoLIM15N*, *CoLIM15C*, *CoRAD51N* and *CoRAD51C* determined using the plasmids described above. Homotypic interactions were detected for both *CoLIM15* and *CoRAD51* (Table

TABLE I

Protein-Protein Interactions Analyzed in the Two-Hybrid System

BD \ AD	CoLim15	CoLim15N	CoLim15C	CoRad51	CoRad51N	CoRad51C
CoLim15	++	−	++	−	−	−
CoLim15N	−	−	−	−	−	−
CoLim15C	+	−	++	−	−	−
CoRad51	−	−	−	++	−	++
CoRad51N	−	−	−	−	−	−
CoRad51C	−	−	−	++	−	++

++: Indicates a strong interaction, *i.e.*, Ade^+ and His^+ phenotypes were detected within 3 d of growth at 30°C on synthetic media lacking Ade, His, Leu, and Trp, and a $LacZ^+$ phenotype was detected within 1 h of incubation at 30°C in the presence of X-gal.

+: Indicates a weak interaction, *i.e.*, under the conditions described above, the Ade^+ and His^+ phenotypes were detectable after 7 d of growth at 30°C and detection of the $LacZ^+$ phenotype required 3 h incubation.

I), as reported previously for yeast and mammalian homologues (23, 27, 30). We also tested heterotypic interactions with each protein expressed either as a fusion to the GAL4 activation domain or to the GAL4 DNA-binding domain. In contrast to their ability to self-interact, heterotypic interactions between *CoLIM15* and *CoRAD51* were not observed (Table I). Amino acid sequence homology between *CoLIM15* and *CoRAD51* was more than 40%. Human *DMC1* was reported previously to interact directly with human *RAD51* (24), and similarly heterotypic interaction, albeit weak, is known to occur between mouse *DMC1* and *RAD51* (23). Moreover, both of the *Coprinus* proteins were expressed at the meiotic prophase stage, zygotene, at which the homologous chromosomes pair (10), and our unpublished data). Although these observations led us to speculate that heterotypic interaction would occur between *CoLIM15* and *CoRAD51*, especially in meiosis, the results of this study were unexpectedly negative (Table I). At least in *Coprinus*, *CoLIM15* and *CoRAD51* must work independently in the homologous chromosome pairing process in meiosis.

Next, to determine which regions of CoLim15 and CoRad51 proteins were essential for homotypic interactions, we used the plasmids described above expressing for their terminal domains. Two-hybrid assay showed that the C-terminal domain fragment of CoLim15 (CoLim15C) containing amino acids 105 to 347 interacted with the full-length protein (CoLim15), while the N-terminal domain fragment (CoLim15N) containing amino acids 1 to 104 did not (Table I). CoLim15C could also interact only with CoLim15C (Table I), indicating that the C-terminal region, or at least a portion of it, was required for the homotypic interaction. Similarly, the C-terminal domain fragment of CoRad51 (CoRad51C) mediated CoRad51 self-association (Table I). The N-terminal domains of these proteins were not related to the homotypic interactions.

Pull-down assay for interaction of C- and N-terminal domains in CoLim15 and/or CoRad51 in vitro. The interaction between CoLim15 and CoRad51 was also tested by pull-down assay as reported by Kovalenko *et al.* (30). We first expressed two forms of full-length CoLim15 in *E. coli* and then highly purified: (i) His-tag-CoLim15, and (ii) Thioredoxin-His-tag-S-tag-CoLim15 (Trx-CoLim15). The first form designated as His-CoLim15, which contained a tag of six histidine residues in its N-terminus, was purified by chelate column chromatography as described under Materials and Methods. Similarly, His-forms and Trx-forms of CoRad51 were also made. His-forms of CoLim15N, CoLim15C, CoRad51N and CoRad51C were made. The purification results are shown in Fig. 2A. Each of the Trx-forms was bound to S-protein agarose, and then each of the His-forms was pulled down. CoLim15, CoRad51, CoLim15N, CoLim15C, CoRad51N and CoRad51C could not be pulled down by the Trx-tag alone. As shown in Fig. 2B, His-CoLim15 and His-CoLim15C showed substantial retention to Trx-CoLim15, and also His-CoRad51 and His-CoRad51C showed retention to Trx-CoRad51, while none of the N-terminal fragments reacted to any of the Trx-forms. These *in vitro* results coincided well with those of the two-hybrid analysis *in vivo* (Table I).

These observations concerning the homotypic interactions at the C-terminal domains *in vivo* and *in vitro* were consistent with the results of mammalian *DMC1* and *RAD51* reported recently (22), although in *Coprinus* the strand-transfer proteins did not interacted heterotypically. *Coprinus* belongs to the fungi, and is closer to yeasts than mammals. Nevertheless, in *Saccharomyces cerevisiae* these proteins were reported to show self-association via the N-terminal domains (27). The meiotic prophase of *S. cerevisiae*, especially at zygotene and pachytene, is exceptional (31). Since the yeast homologous chromosome pairs synapse properly with the formation of the synaptonemal complex only after they have been tightly aligned by recombination

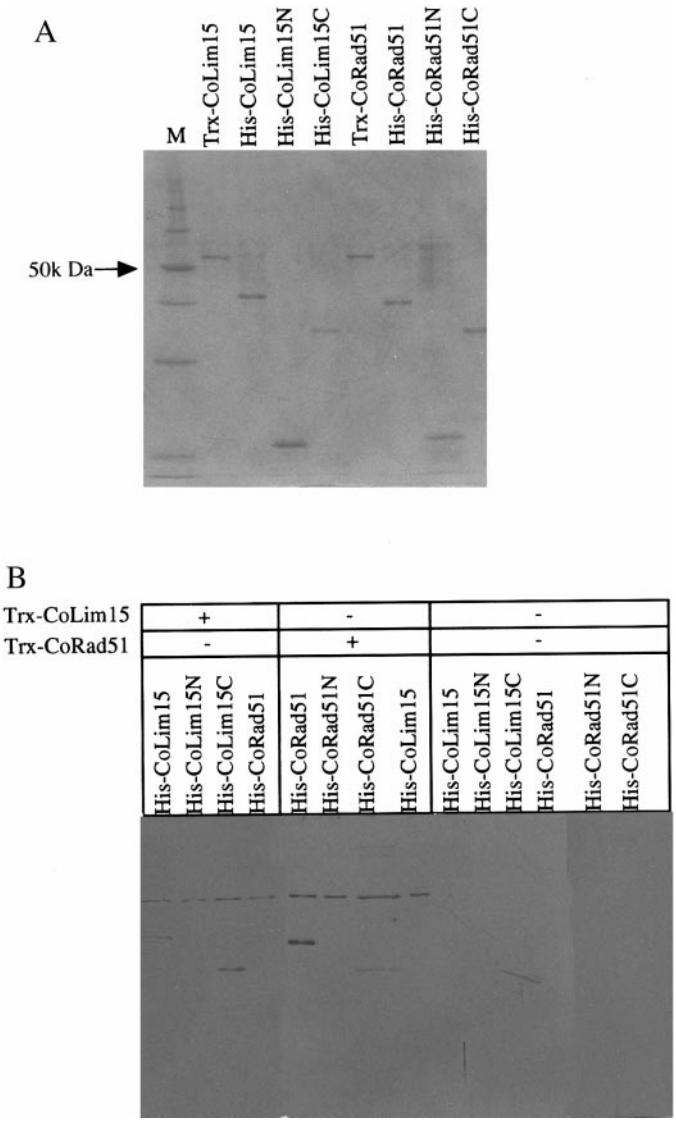

FIG. 2. Protein-protein interactions determined *in vitro* by pull-down assay. (A) Purification of proteins. Proteins were separated by SDS–PAGE and visualized with Coomassie Blue. M, Precision-weight marker (Bio-Rad). (B) Pull-down assays under the presence of Trx-CoLim15 or Trx-CoRad51, or under the absence of Trx-CoLim15 or Trx-CoRad51.

(31), the stages of zygotene and pachytene are not morphologically discernible from each other. On the other hand, *Coprinus* as well as mammals and plants require homologous chromosome pairing at zygotene and their recombination at pachytene (10). The differences in the domains for the homotypic interaction might reflect those of meiotic systems, irrespective of their phylogenetic distances. As described above, there was no heterotypic interaction between *CoLIM15* and *CoRAD51*. Shinohara *et al.* (21) observed no heterotypic interaction between *DMC1* and *RAD51* in *S. cerevisiae*, and concluded that unlike mammals they have both distinct and overlapping roles in meiosis in yeast. The phylogenetic distance may be reflected in the heterotypic interaction between *DMC1* and *RAD51*.

Gelshift assay for interaction of C- and N-terminal domains in CoLim15 and/or CoRad51. The observation that CoLim15 or CoRad51 could interact homotypically at the C-terminal domain, and the fact that they are DNA-binding recombination enzymes, prompted us to examine which domains bind to DNA. A gel shift assay was used for this purpose. The regions encoding *CoLIM15*, *CoRAD51* and their C- and N-terminal domains were each amplified by PCR, cloned into the pET28b expression vector and transformed into *E. coli* for protein induction. Extracts prepared from the cells induced for 5 h were shown to contain six histidine N-terminal-tagged fusion proteins (data not shown). Each of the proteins was purified to near homogeneity as shown by SDS-PAGE analysis of column fractions obtained by Hi-trap chromatography.

Two types of DNA substrates were utilized: single-stranded ϕx174 virion DNA (ssDNA), and double-stranded *Eco*RV-linearized pBS DNA (dsDNA). As shown in Fig. 3, we found that both CoLim15 and CoRad51 bound to both ssDNA and dsDNA in our assay. The binding to ssDNA or dsDNA of CoRad51 was observed in a dose dependent manner (left panels in Fig. 3A or 3B). The CoLim15-ssDNA complex was also in a dose dependent manner, and with increasing amount of the protein, the complex band was shifted to the low-mobility region in the gel (left panel in Fig. 3A). In the case of CoLim15, a high-molecular weight protein-dsDNA complex was formed that barely moved into the 0.8% agarose gel and the density of the band left at the well was dose-dependent (left panel in Fig. 3A). CoRad51 similarly bound to dsDNA in a dose-dependent manner (left panel in Fig. 3B).

Figure 3 also shows the binding to ssDNA or dsDNA of each domain, CoLim15N, CoLim15C, CoRad51N, or CoRad51C. Interestingly, CoLim15 bound to ssDNA via the N-terminal domain (middle panel in Fig. 3A), while CoRad51 appeared to interact with ssDNA via the C-terminal domain (right panel in Fig. 3A). CoLim15N also bound to dsDNA (middle panel in Fig. 3B), but CoRad51C did not (right panel in Fig. 3B). The binding of the C-terminus of CoRad51 to DNA may be weak. In 1999, Aihara *et al.* reported that human Rad51 protein bound to DNA via its N-terminal domain (25). Our results concerning the DNA-binding site of CoRad51 were different from those obtained with the human counterpart, and the DNA-binding site of CoLim15, the mushroom homolog of Lim15/Dmc1, was the N-terminus.

As described previously (10), *CoLIM15* might be required mainly for strand arrangement between homologous DNA molecules at zygotene. Terasawa *et*

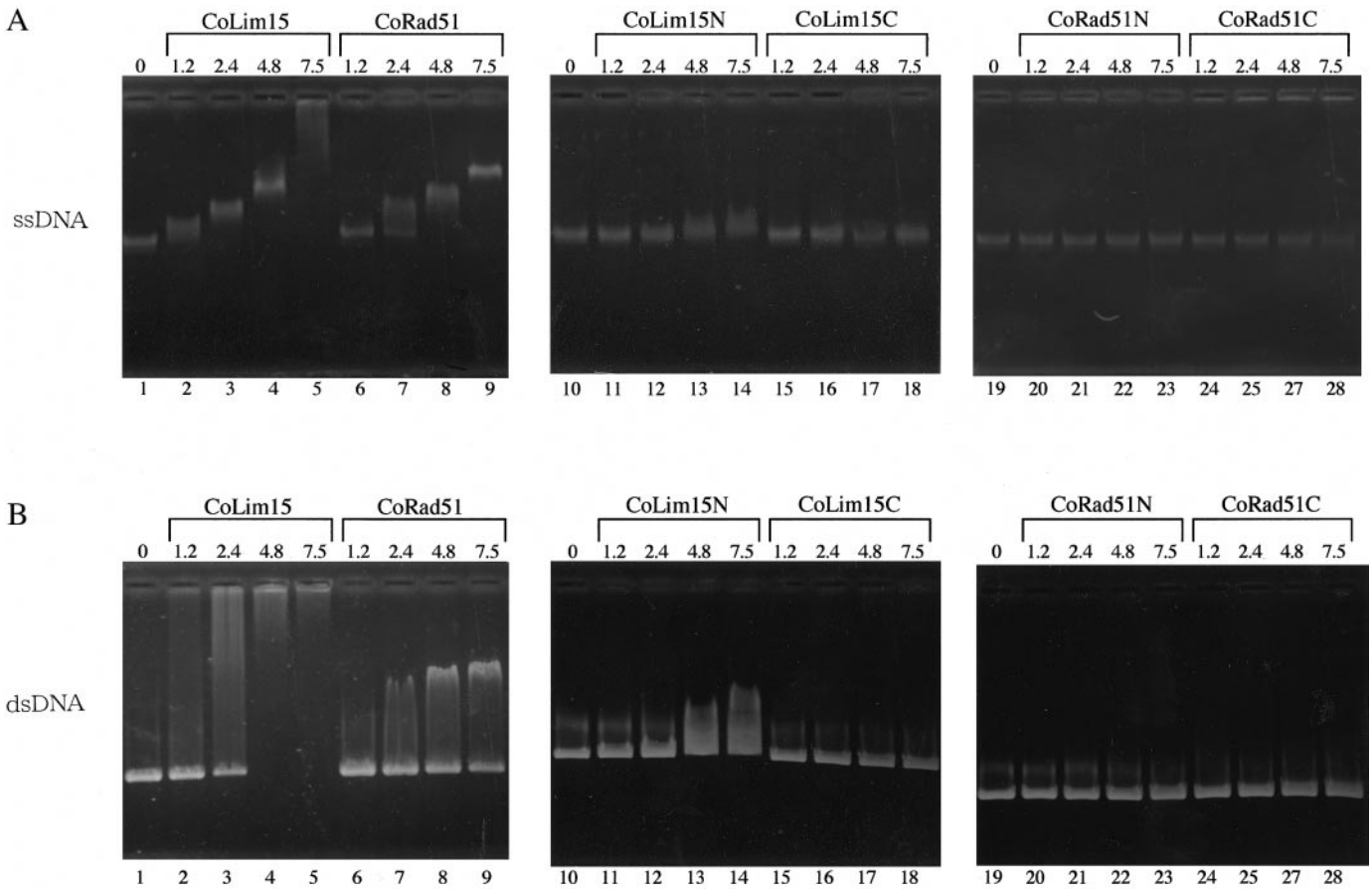

FIG. 3. Binding of CoLim15 and CoRad51 to single- and double-stranded DNA. Indicated amounts of full-length CoLim15 (lanes 2–5), CoRad51 (lanes 6–9), CoLim15N (lanes 11–14), CoLim15C (lanes 15–18), CoRad51N (lanes 20–23), and CoRad51C (lanes 24–28) with (A) ssDNA or (B) dsDNA. Lane 1, 10, 19 no protein. Complexes were analyzed by agarose gel electrophoresis.

al. (32) reported that *RAD51* was expressed at leptotene to zygotene in lily meiosis, suggesting that at least it is not required for recombination at pachytene. The present results indicated clearly not only that *CoLIM15* and *CoRAD51* interact homotypically at their C-terminal domains, but also that they do not interact heterotypically with each other. CoLim15 binds to DNA via its N-terminal domain, and CoRad51 interacts with DNA via the C-terminal domain. These observations suggested that these molecules do not work together at the same site(s) on DNA. These *Coprinus* strand-transfer proteins may have both distinct and overlapping roles in *Coprinus* meiosis, although they are simultaneously present at zygotene at which stage the homologous chromosomes pair. Although it has been suggested that there are two possible meiotic events that may require RecA-like protein, i.e., homologous chromosome pairing at zygotene and their recombination at pachytene, the strand arrangement between homologous DNA molecules at zygotene may be separated into two events requiring distinct RecA-like proteins.

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