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Strand Exchange Reaction *in Vitro* and DNA-Dependent ATPase Activity of Recombinant *LIM15/DMC1* and *RAD51* Proteins from *Coprinus cinereus*

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We previously cloned recA-homolog genes from a basidiomycete, *Coprinus cinereus,* and obtained the recombinant proteins (Nara *et al., Mol. Gen. Genet.* 262, 781–789, 1999, see Ref. 1; Nara and Sakaguchi, *Biochem. Biophys. Res. Commun.* 275, 97–102, 2000, see Ref. 2). The primary purpose of the present study was to characterize the biochemical properties of the recombinant *LIM15/DMC1* (*CoLIM15*) and *RAD51* (*CoRAD51*) proteins. We purified the recombinant proteins, and their molecular masses were 37 and 35 kDa, respectively. Both enzymes showed DNA-dependent ATPase activity and ATP-dependent strand exchange reaction *in vitro.* CoRad51 was a five- to sixfold stronger DNA-dependent ATPase and showed greater dependency on single-stranded DNA than CoLim15. In meiosis, both enzymes were highly accumulated in the meiotic tissue at leptotene and zygotene stages at which the homologous chromosomes pair, but disappeared just before the pachytene stage at which they recombine. From these and the previously reported results, we discuss here the relationships between the enzymes and meiosis.

Key Words: Coprinus cinereus; LIM15/DMC1* (*CoLIM15*); *RAD51* (*CoRAD51*); DNA-dependent ATPase activity; strand exchange reaction *in vitro.

Eukaryotic organisms are known to have, two homologs of *Escherichia coli* recA protein, Lim15/Dmc1 and Rad51. The recA-like genes are thought to be essential for homologous recombination and DNA repair (3, 4). Stassen *et al.* reported that (5), phylogenetic analyses of eukaryotic RecA homologs revealed gene duplication early in eukaryotic evolution, which gave rise to two putatively monophyletic groups of RecA-like genes. One group of eleven genes, designated as the *RAD51* group, is orthologous to the *Saccharomyces RAD51* gene, and the other group of seven genes, designated as the *DMC1* group, is orthologous to the *Saccharomyces DMC1* gene (5). However, with the exception of the human and yeast Rad51 homologs, there have been no report studies to test whether these recombinant proteins possess DNA-dependent ATPase activity and mediate strand exchange reactions *in vitro,* although they high degrees of sequence homology have been reported among these proteins. One reason for the difficulty in functional characterization of these molecules may be that the recombinant proteins are liable to aggregate and precipitate in the host cells.

We investigated meiosis-related proteins and their relationships to meiotic pairing and meiotic recombination using meiotic cells in a basidiomycete, *Coprinus cinereus* (1, 2, 6–14). Previously, we reported the cloning and the characterization of the recA-like genes, *LIM15/DMC1* (*CoLIM15*) and *Rad51* (*CoRAD51*), in this organism (1). In both two-hybrid assay *in vivo* and pull down assay *in vitro,* CoLim15 or CoRad51 homotypic interactions between the respective C-terminal domains (2). In the present study, we purified and characterized the recombinant *CoLIM15* and *CoRAD51* proteins, and determined the precise relationships of these proteins to the meiotic process. Although they showed no heterotypic interactions among their terminal domains (2), they were accumulated at the same meiotic prophase stages. The N-terminal domain of CoLim15 bound to ssDNA and dsDNA, while the C-terminal domain of CoRad51 might interact weakly with ssDNA (2). Recombinant *CoLIM15* or *CoRAD51* proteins were biochemically different from each other. The interactions among the strand-exchange proteins and meiosis are discussed here based on these results.

MATERIALS AND METHODS

Preparation of recombinant CoLIM15 and CoRAD51 proteins. The *CoLIM15* and *CoRAD51* were amplified by PCR from *Coprinus* meiotic cDNA and cloned into pET28b (Novagene). The plasmid

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constructs were introduced into *E. coli* BLR (DE3). These *E. coli* were grown at 30°C in 3 l of 2× YT medium with 1% glucose and 50 mg/ml kanamaycin. At OD600 = 0.7, recombinant protein synthesis was induced by the addition of 1 mM IPTG and the cells were harvested after 5 h by centrifugation, frozen in liquid N_2, and stored at −80°C. The cell paste containing CoLim15 was resuspended in 70 ml of lysis buffer (0.1 mM Tris–HCl pH 8.0, 5% glycerol, 800 mM NaCl, 5 mM 2-mercaptoethanol) containing the protease inhibitor phenylmethylsulfonylfloride (1 mM), leupeptin (1 mM) and pepstatin A (0.1 mM). 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 25,000 rpm for 30 min in a Beckman 70 Ti rotor. Proteins were precipitated from the clarified supernatant using 55% ammonium sulfate, and the pellet was resuspended in lysis buffer and dialyzed against 31 of spermidine buffer (10 mM Tris–acetate pH 7.5, 7 mM spermidine-NaOH, 0.5 mM DTT) twice for 12 h. The precipitate containing CoLim15 was recovered by centrifugation and resuspended in 40 ml of buffer T (20 mM Tris–HCl, pH 8.0, 10% glycerol, 500 mM NaCl, 0.02% NP-40) containing 5 mM imidazole. Insoluble material was removed and the supernatant was loaded onto 10 ml HiTrap Chelating columns (Amersham Pharmacia Biotech). The column was washed successively with buffer T containing 10 and 80 mM imidazole, respectively. The bound proteins were eluted with a 100 ml linear gradient of 50–600 mM imidazole in buffer T. Fractions of CoLim15 were identified by SDS–PAGE, pooled and dialyzed against 3 l of buffer A (20 mM K-phosphate buffer, pH 6.8, 1 mM EDTA, 10% glycerol, 0.5 mM DTT) containing 200 mM KCl, twice for 12 h and loaded onto 10 ml HiTrap heparin columns (Amasham Pharmacia Biotech). The columns were washed with 50 ml of buffer A containing 200 mM KCl. The bound proteins were eluted with a 100 ml linear gradient of 0.2–2 M KCl in buffer A. The fractions containing CoLim15 were pooled, dialyzed against 31 of buffer B (10 mM K-phosphate pH 6.8, 10% glycerol, 350 mM KCl, 0.5 mM DTT) twice and loaded onto a CHT II cartridge column (BIO-RAD). The column was washed with 50 ml of buffer B and CoLim15 was eluted with a linear gradient of 10–800 mM K-phosphate in buffer B. The peak fraction containing CoLim15 was dialyzed against storage buffer (20 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 mM KCl, 50% glycerol, 0.5 mM DTT) and stored in aliquots at −80°C. The final yield of CoLim15 was ~1.25 mg as determined using a Bio-Rad protein assay kit with γ-globulin as the standard.

The cell paste containing CoRad51 was treated by the same method as used for CoLim15. The paste was loaded onto 10 ml Hi-Trap Chelating column and the fractions containing CoRad51 were collected and dialyzed against 3 l of buffer C (20 mM Tris–HCl, pH 8.0, 10% glycerol, 1 mM EDTA, 0.5 mM DTT) containing 100 mM KCl twice for 12 h and loaded onto 5 ml Hi-Trap heparin columns twice. The columns were washed with 50 ml of buffer C containing 100 mM KCl. The bound proteins were eluted with a 100 ml linear gradient of 0.1–2 M KCl in buffer C. The fractions containing CoRad51 were pooled, dialyzed against storage buffer (20 mM Tris–HCl pH 8.0, 1 mM EDTA, 200 mM KCl, 50% glycerol, 0.5 mM DTT) and stored in aliquots at −80°C. The final yield of CoRad51 was ~1.5 mg.

ATPase assay. ATPase activities of CoLim15 and CoRad51 were measured by the method described previously (15), using [γ-^{32}P]ATP (Amersham Pharmacia Biotech). Two micromolars of CoLim15 or CoRad51, 400 nCi of [γ-^{32}P]ATP and 100 μM cold ATP were incubated with or without 2.5 ng/μl of single-stranded or double-stranded ϕX174 DNA in buffer containing 25 mM K–Hepes, pH 7.2, 5 mM $MgCl_2$, 100 μg/ml BSA and 1 mM DTT at 37°C, and aliquots of the reactions were analyzed by thin layer chromatography. The amounts of ATP and Pi were quantified from the autoradiograms using an image analyzer (BAS 2000, Fuji Film).

Strand exchange assay. Strand exchange assays were performed by as described by Maeshima *et al.* (16). CoLim15, CoRad51 or RecA (Promega) at 2 μM was preincubated at 37°C for 5 min with 6 μM ^{32}P-labeled 55-mer oligonucleotides in buffer containing 25 mM K–Hepes pH 7.2, 5 mM MgCl2, 100 μg/ml BSA and 1 mM DTT. After preincubation, the concentration of $MgCl_2$ was increased to 15 mM, followed by addition of homologous duplex oligonucleotide at 4 μM. Incubation was continued at 37°C, and the aliquots were deproteinized in 1 mg/ml proteinase K and 0.5 mg/ml SDS.

Antibodies. CoLim15 and CoRad51 were injected into a rabbit and a rat, respectively. The preimmune sera gave no staining when tested on *Coprinus* meiotic tissues. The polyclonal anti-CoLim15 antibody was depleted of anti-CoRad51 cross-reactive components by absorption on Sepharose-bound (Amersham Pharmachia Biotech) CoRad51. Similarly, the polyclonal anti-CoRad51 serum was depleted of anti-CoRad51 cross-reactive components by absorption on CoLim15. This procedure abolished the cross-reactivity with other proteins observed on Western blotting.

Detection of CoLim15 and CoRad51 by Western blotting. About 100 mg of mycelium and meiotic tissue were homogenized with extraction buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol, 15% glycerol, 1 M NaCl, 0.1% NP-40) containing protease inhibitor phenylmethylsulfonylfluoride (1 mM), leupeptin (1 mM) and pepstatin A (0.1 mM). Then, glass-beads were added, vortexed for 10 × 1 min and clarified by centrifugation. Aliquots of 30 μg of each protein were separated by 12.5% SDS–PAGE (17). Proteins were transferred onto PVDF membranes using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad). The membranes were incubated in TBS-T containing 5% nonfat dry milk, then incubated with the anti-CoLim15 antibody or anti-CoRad51 serum at 1:200 dilution in TBS-T containing 1% BSA. The membranes were washed three times in TBS-T, incubated with anti-rabbit or rat IgG conjugated with AP (New England Biolabs) at 1:1000 dilution in TBS-T containing 1% BSA. Immunoreactive bands were visualized with NBT and BCIP in AP buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

Immunocytochemistry of CoLim15 and CoRad51 proteins. Hand-cut sections (0.5 mm thick) of fruiting bodies were fixed for at least 2 h at 4°C in fixative (4% paraformaldehyde, 50 mM phosphate buffer, pH 6.5, 5% PEG6000, 5 mM $MgCl_2$, 5 mM EGTA) and embedded in paraffin by standard procedures. Sections (5 μm) were deparaffinized and rehydrated through a graded ethanol series. Slides were incubated in PBS containing 1% BSA and 10% normal goat serum, then incubated with anti-CoLim15 antibody for 16 h at 4°C. Slides were washed with PBS containing 1% BSA, and incubated with anti-rabbit IgG conjugated with Alexa fluoro 568 (Molecular Probe) for 3 h at room temperature. The same slides were incubated with anti-CoRad51 serum and with anti-rat IgG conjugated with Alexa fluoro 488, then counterstained with DAPI. Immunofluorescence was observed using an Olympus BH2 microscope, and pictures were taked at ×400 magnification.

RESULTS AND DISCUSSION

Overexpression and isolation of recombinant LIM15/DMC1 (CoLIM15) and RAD51 (CoRAD51) homologous proteins. The recombinant *CoLIM15* and *CoRAD51* proteins were isolated as described under Materials and Methods. The cDNA fragments were subcloned into the bacterial expression vector pET28b, and the plasmid constructs were introduced into *E. coli* strain BLR21(DE3). The bacteria were cultured and homogenized, and the proteins were purified through a HiTrap Chelating column. Both of the proteins were rapidly and selectively precipitated from the cell-free extracts, but we obtained small amounts of each in the soluble fractions. As shown in Fig. 1A, the proteins were purified to near homogeneity suitable for enzy-

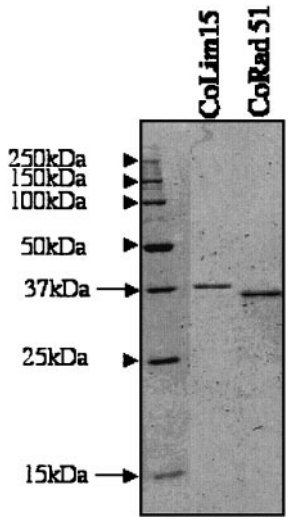

FIG. 1. Purified recombinant *CoLIM15* and *CoRAD51* proteins. Purified proteins were fractionated by SDS–PAGE and stained with CBB.

matic and physical studies. The molecular masses of the CoLim15 and CoRad51 were 37 kDa and 35 kDa, respectively, as expected from the cDNA sequences (Fig. 1A). To our knowledge, this represents the first report of a method to obtain large amounts of recombinant *LIM15/DMC1* homolog protein in the soluble state, which can be used for biochemical analyses. We failed to purify these proteins on such a large scale previously (2). Biochemical characterization of recombinant *RAD51* homolog protein has been performed previously using proteins from human (18) and *Saccaromyces* (19).

DNA-dependent ATPase activities of CoLIM15 and CoRAD51 proteins. The ATPase activities of CoLim15 and CoRad51 were examined in the presence or absence of excess single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) (Fig. 2). CoLim15 and CoRad51 were found to hydrolyze ATP in a concentration-dependent and time-dependent manner. As shown in Fig. 2, the rates of ATP hydrolysis exhibited by CoLim15 was significantly (five- to sixfold) lower than at observed with CoRad51. Although both of the recA-like proteins were DNA-dependent, CoRad51 was more dependent on ssDNA than on dsDNA (Fig. 2B). For CoLim15, the DNA species was a weak enhancer and was not necessarily important for the ATPase activity, indicating that the DNA dependency was not strict (Fig. 2A).

In the presence of ATP, *E. coli* recA protein bound to ssDNA to form nucleoprotein filaments. It also exhibited low affinity for dsDNA. This difference in affinity was important for homologous pairing reactions, since pairing occurs between a nucleoprotein filament and naked duplex DNA, rather than between two recA-coated DNA molecules (18). We previously reported that CoLim15 or CoRad51 interacted homotypically between the C-terminal domains, but no heterotypic interaction was observed between CoLim15 and CoRad51 (2). The N-terminal domain of CoLim15 was the region that bound to ssDNA and dsDNA, while the C-terminal domain of CoRad51 weakly interacted with ssDNA (2). The present results concerning the DNA-dependent ATPase activities might reflect the relationships between the N-terminal domain of CoLim15 and ssDNA and dsDNA, and also between the C-terminal domain of CoRad51 and ssDNA.

Strand exchange reaction of the CoLim15 and CoRad51 in vitro. Having established conditions under which CoLim15-ssDNA filament or CoRad51-ssDNA filaments and dsDNA interact, we next investigated whether they were capable of promoting homologous pairing reactions *in vitro.* The assay was performed using the methods described by Baumann *et al.* (18). Unlabeled ϕX174 ssDNA and the ^{32}P-5′-end-labeled linear duplex DNAs were utilized. However, CoLim15 did not show any activity in the mixture (data not shown). Therefore, we used another assay method with two synthetic linear complementary 55-mer sequences (16). Homologous pairing reactions were set up as fol-

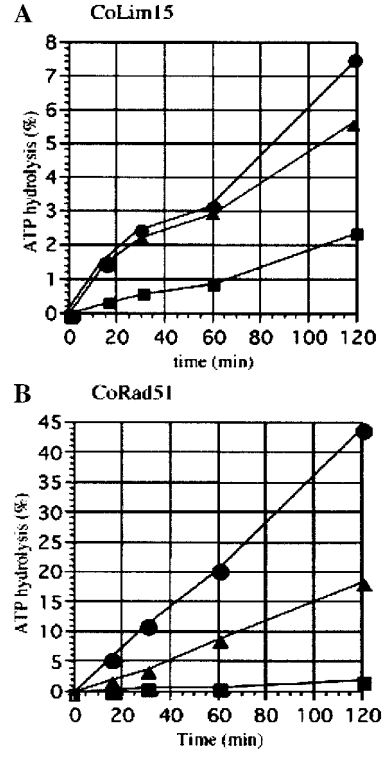

FIG. 2. ATPase activities of Colim15 and CoRad51. CoLim15 or CoRad51, [γ-^{32}P]ATP (400 nCi), and 100 μM of ATP were incubated with or without DNA at 37°C for various times, and aliquots were analyzed by thin layer chromatography. (■) Without DNA; (●), with ssDNA; (▲), with dsDNA.

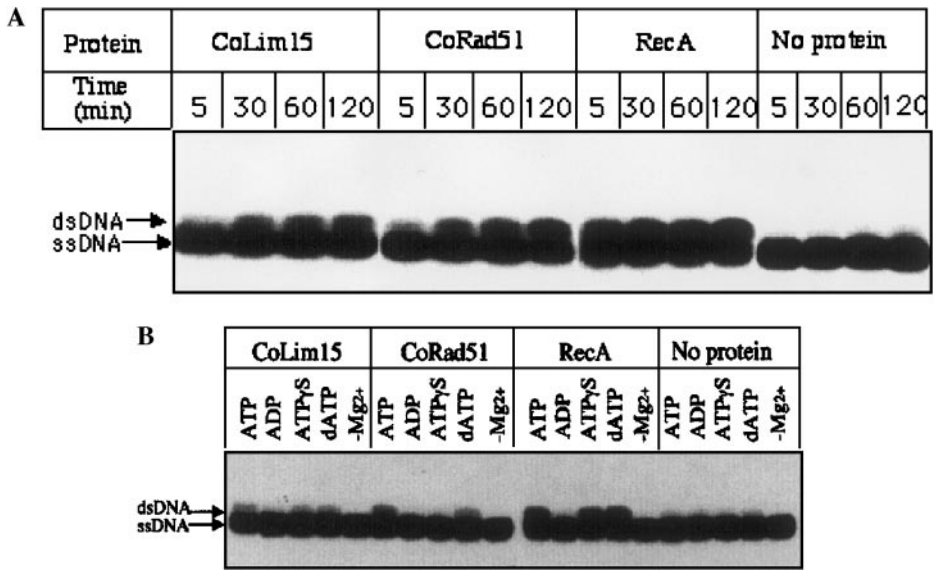

FIG. 3. Strand exchange activities of the CoLim15 and CoRad51. (A) Time courses of CoLim15 and CoRad51 promoted strand exchange reactions. (B) Cofactor dependence of CoLim15 or CoRad51 mediated strand exchange. Reactions were carried out for 1 h in standard buffer or in buffer in which ATP was replaced with ADP, ATP-γS or dATP or without Mg^{2+}. *E. coli* recA protein was used as a control.

lows. First, CoLim15 or CoRad51 was incubated at 37°C for 5 min with ^{32}P-5′-end-labeled 55-mer single-stranded sequences, followed by a further 5 min incubation. The complimentary duplex sequences were then added, and the complete reaction mixture was incubated for at most 2 h. The resulting DNA products were analyzed by polyacrylamide gel electrophoresis and visualized by autoradiography. Neither CoLim15 nor CoRad51 showed nuclease activity. As shown in Fig. 3A, both CoLim15 and CoRad51 possessed strand exchange activity, but like their DNA-dependent ATPase activities, the activity of CoLim15 was weaker than that of CoRad51. Their reactions seemed to be slower than that of reaction by *E. coli* recA protein (Fig. 3A). No signals were observed in the absence of the proteins (Fig. 3A).

Mg^{2+} and ATP were necessary for strand exchange by both of CoLim15 and CoRad51 as well as *E. coli* recA protein. The role of ATP in *E. coli* recA-promoted strand exchange reaction has been the subject of considerable discussion (20). Compared with *E. coli* recA protein, even CoRad51 had very weak DNA-dependent ATPase activity. CoLim15, like *E. coli* recA protein, promoted strand exchange in the presence of the non-hydrolyzable analog ATP-γS, but ADP did not promote strand exchange (Fig. 3B). dATP could substitute for ATP in the case of both CoLim15 and CoRad51 (Fig. 3B). The structure of ATP must be important to promote the active CoLim15 conformation that is essential for binding with the substrate 55-mer sequence. Since the N-terminal domain of CoLim15 was the region that bound to ssDNA and dsDNA, while the C-terminal domain of CoRad51 might weakly interact with ssDNA (2), the conformations of CoLim15 and CoRad51 activated by ATP must be biochemically different from each other.

Western blotting analysis and in vivo immunostaining using antibodies against CoLim15 and CoRad51. As meiotic prophase stages are biochemically separated into two different major RecA-requiring periods, i.e. strand arrangement at zygotene between homologous DNA molecules and recombination at pachytene between the arranged strands (21–23) each period requires a different type of RecA-like protein. In this connection, our previous finding that the *CoLIM15* and *CoRAD51* were stage-dependently transcribed at leptotene to zygotene (1) is of interest. We also reported previously (2) that CoLim15 or CoRad51 interacted homotypically between the C-terminal domains, but they had no heterotypic interactions among their terminal domains. Both the CoLim15 and CoRad51 were enzymatically different from each other as described in this report. They may be related only to two different events in the strand arrangement at zygotene between homologous DNA molecules, and not to recombination at pachytene between the arranged strands. However, this speculation was based on the results of Northern blotting analysis, and should be confirmed by Western blotting because the proteins may be very stable and persist beyond these stages. Therefore, we should next investigate the presence of the CoLim15 and CoRad51 in relation to homologous chromosome pairing and recombination in meiosis using in *C. cinereus*, in part because its meiotic cycle is long and naturally synchronous.

To determine whether the genes are specifically translated in meiosis, we raised polyclonal antibodies

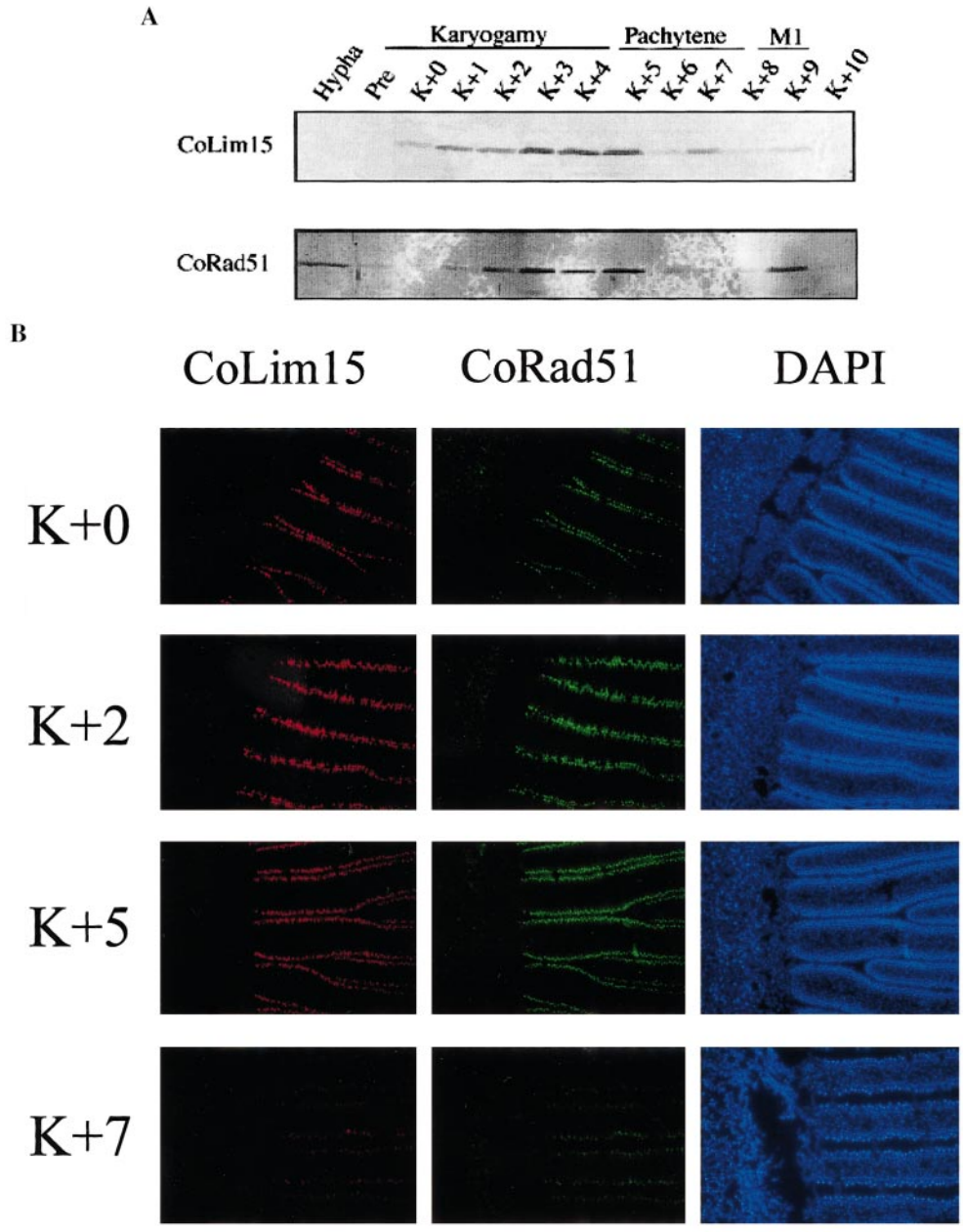

FIG. 4. (A) Western blotting analysis of CoLim15 and CoRad51. Samples were taken at each time point, and the CoLim15 and CoRad51 were detected by Western blotting. (B) Immunocytochemical analysis of the CoLim15 and CoRad51. Sections from the fruiting body tissues were stained with anti-CoLim15 (red) or anti-CoRad51 antibody (green). The nuclei were counterstained with DAPI.

against the CoLim15 and CoRad51, and accumulation of the proteins at the meiotic stages were analyzed by Western blotting and immunostaining. To determine whether the *CoLIM15* and *CoRAD51* are expressed at zygotene and/or pachytene, total protein was extracted from the basidia taken from the synchronous culture every 1 h after induction of meiosis, and immunostaining with each of the antibodies was performed (Figs. 4A and 4B). We detected signals of the CoRad51 in the mycelia (hyphae), and signals of both CoLim15 and CoRad51 in the meiotic cell cycle (Fig. 4A). Both of the proteins were observed only in the basidia at late leptotene to zygotene. As shown in Fig. 4A, both of the proteins began to accumulate markedly immediately after the beginning of the light period (K + 1) and became most abundant 2 to 4 h after karyogamy (K + 3 to K + 5). The signals had faded by 6 h after karyogamy (K + 6), although a weak CoLim15 signal appeared at middle pachytene (K + 7) (Fig. 4A). Then, a trace of the CoRad51 signal was again observed at

the M1 to tetrad stages (K + 9). Since the majority of the basidia were in zygotene, not pachytene, during this period as judged by fluorescent microscopic observation of the monokaryonic nuclei and from electron microscopic observation of the presence of synaptonemal complex, it was concluded that both of the proteins were present mostly at middle to late zygotene. These results were coincided well with the stages of expression of their transcripts (see Fig. 6 in Ref. 1), suggesting that the CoLim15 and CoRad51 in the meiotic cell cycle were produced at the correct time and rapidly metabolized after fulfilling their roles. To our knowledge, this is first report indicating that the *CoLIM15* and *CoRAD51* were expressed mainly at the zygotene stage, and that the proteins rapidly faded away at the transition between the zygotene and pachytene stages.

Figure 4B shows the subcellular localizations of the CoLim15 and CoRad51 in the meiotic cell cycle. The results of Western blotting clearly indicated that CoLim15 and CoRad51 were accumulated at the meiotic prophase stages. However, since the fruiting caps used as the meiotic tissues contained some somatic cells, it was possible that CoLim15 and CoRad51 were present in the somatic cells. Therefore, to confirm that the majority of the CoLim15 and CoRad51 were present in the meiotic cells, the in distributions were investigated by *in situ* immunofluorescence staining using the antibodies. Fluorescent images of their distributions during meiotic division by *in situ* indirect immunofluorescence staining and standard epifluorescence microscopy were shown in Fig. 4B. The tissues with dense DAPI staining on the surface of the gillus indicated the *Coprinus* meiotic tissues (Fig. 4B). As the important meiotic stages, the dense DAPI staining tissues from leptotene (K + 0), early to late zygotene (K + 2 and K + 5) and pachytene (K + 7) were selected. Using the antibody described above for Western blotting analysis, intense signals for the CoLim15 and CoRad51 were detected mainly at leptotene to zygotene, and faded away at pachytene similar to the results of Northern hybridization described previously (1) and those of Western blotting analysis in the present study. The majority of the CoLim15 and CoRad51 were present in the cells at meiotic prophase stages, and the meiosis-related events in which homologous DNA molecules pair require these proteins. The meiotic cells after the zygotene stage are capable of rapidly metabolizing both CoLim15 and CoRad51.

CoLim15 (or CoRad51)-deficient mutants are required for the further clarification of the roles of CoLim15 and CoRad51, and detailed investigations of the phenotypes of such mutants are necessary, including studies of genetic recombination frequency and the morphology of the synaptonemal complex formation. Attempts to knock out the *CoLIM15* and *CoRAD51* are currently underway in our laboratories.

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