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Basidiomycete *Lentinula edodes* CDC5 and a novel interacting protein CIPB bind to a newly isolated target gene in an unusual manner

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ABSTRACT

We isolated a target gene for the *Lentinula edodes* putative transcription factor Le.CDC5 that contains a c-Myb-type DNA-binding domain. The gene, termed *ctg1*, encodes a novel protein (159 amino acid residues) with a leucine zipper-like sequence and contains a 7-bp Le.CDC5-binding sequence, 5′GCAATCT3′, in its transcribed region downstream of the start codon. Chromatin immunoprecipitation analysis strongly suggested that intracellular Le.CDC5 binds to this 7-bp sequence on *L. edodes* chromatin. Binding was most efficient on chromatin from the stipes of mature fruiting bodies. Two Le.CDC5-interaction partners were identified in *L. edodes* and named CIPA and CIPB. The CIPB protein (127 amino acid residues) binds to a 6-bp sequence with the consensus sequence 5′CAACAC/T/G3′. The *ctg1* gene contains nine 6-bp consensus (or consensus-like) sequences, six are in the 5′-upstream region and three in the transcribed region downstream of the start codon. At least two each of the upstream and downstream sequences appear to bind CIPB *in vitro*. We suggest that Le.CDC5 and CIPB can cooperatively regulate the expression of *ctg1*.

1. Introduction

The CDC5 protein, a *Schizosaccharomyces pombe cdc5*$^{+}$ gene product (Sp.cdc5p), harbors a c-Myb-type DNA-binding domain at the N-terminus and is an essential factor for cell-cycle promotion (Ohi et al., 1994). Sp.cdc5p-related proteins (SPCDC5RPs) with highly conserved c-Myb-type DNA-binding domains have been isolated from various eukaryotes including cells from the plant *Arabidopsis thaliana* (Hirayama and Shinozaki, 1996), human (Bernstein and Coughlin, 1997; Ohi et al., 1998), and the fly *Drosophila melanogaster* (Ohi et al., 1998). The observation that the c-Myb-type DNA-binding domain is essential for the function of Sp.cdc5p (Ohi et al., 1994) and that human CDC5 has a transactivation domain (Bernstein and Coughlin, 1997) led to the hypothesis that Sp.cdc5p and SPCDC5RPs play a role as transcription factors that regulate the expression of genes essential for G2/M cell-cycle promotion (Bernstein and Coughlin, 1998). *Arabidopsis thaliana* CDC5 and human Cdc5 proteins bind to a 7-bp sequence with the consensus sequence 5′CTCAGCG3′ (complementary 5′CGCTGAG3′) (Hirayama and Shinozaki, 1996) and to a 12-bp sequence with the consensus sequence 5′GATTTAACATAA3′ (complementary 5′TTATGTTAAATC3′) (Lei et al., 2000), respectively, suggesting that they are transcription regulators. However, no target gene for Sp.cdc5p and SPCDC5RPs was identified. Sp.cdc5p, *Saccharomyces cerevisiae* Cef1 (Sp.cdc5p homologue), and human Cdc5L were later found to be essential for pre-mRNA splicing and to represent a component of large splicing complexes (McDonald et al., 1999; Burns et al., 1999; Tsai et al., 1999; Ajuh et al., 2000). Therefore, subsequent studies of Sp.cdc5p and SPCDC5RPs, especially in yeast and human, tended to analyze pre-mRNA splicing complexes. At present, Sp.cdc5p and SPCDC5RPs are considered to be splicing rather than transcription factors.

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We investigated the molecular mechanism underlying the fruiting development of the basidiomycetous mushroom *Lentinula edodes* (shiitake), which exhibits a dramatic morphological differentiation from vegetative mycelia to huge fruiting bodies in which a large number of basidiospores are produced. A variety of genes (or cDNAs) specific for fruiting development have been isolated from *L. edodes* and their properties and functions have been analyzed. One of these, *Le.cdc5* (Miyazaki et al., 2004), is a homologue of the *Sp.cdc5*$^{+}$ gene (Ohi et al., 1994). The deduced *Le.cdc5* product (Le.CDC5) consists of 842 amino acid residues. In order, starting from the N-terminus, it possesses a c-Myb-type DNA-binding domain, several putative nuclear localization signals, putative transactivation domains such as proline-rich and acidic amino acid-rich regions, two phosphorylation sites for A kinase, and a leucine zipper $LX_6LX_6LX_6L$ between the A-kinase-phosphorylation sites (Miyazaki et al., 2004). The *Le.cdc5* gene is most abundantly transcribed in primordia and small immature fruiting bodies (Miyazaki et al., 2004). On the other hand, Le.CDC5 is present in similar amounts in primordia and immature- and mature fruiting bodies, indicating that the Le.CDC5 protein molecules synthesized in the beginning and early stage of fruiting-body formation remain in mycelial tissues even after the progression of small immature fruiting bodies to maturity (Nakazawa et al., 2006).

The N-terminal 185-amino acid peptide of Le.CDC5 (named Le.CDC5 (1–185) peptide) with the c-Myb-type DNA-binding domain binds to a 7-bp sequence with the consensus sequence $5'G_{17}C_{18}A_{27}A_{31}T_{28}G_{25}T_{19}3'$ (the numbers to the right of bases indicate how many of 33 bases in the cloned binding-sites are identical in the consensus sequence) (Miyazaki et al., 2004). To demonstrate that the Le.CDC5 protein functions as a transcription factor, we attempted to isolate its target gene(s). A genomic binding site (GBS) cloning experiment (Inoue et al., 1991) with the Le.CDC5 (1–185) peptide and an inverse PCR assay resulted in the isolation of a target gene designated *ctg1* (Le.CDC5 target gene). It contains a 7-bp consensus-like sequence in its transcribed region downstream of the start codon to which the Le.CDC5 (1–185) peptide binds. We also identified two proteins that interact with Le.CDC5 through the C-terminus-proximal leucine zipper of Le.CDC5; we named these proteins CIPA and CIPB (CDC5 interaction partner A and B). We found that the CIPB protein, homologues of which appear to be present in other basidiomycetous fungi, binds to a 6-bp sequence with the consensus sequence 5′CAACAC/G/T3′. The 5′-upstream and transcribed regions of the *ctg1* gene contain nine such 6-bp consensus (or consensus-like) sequences. We posit that CIPB binds to at least four of these nine sequences *in vitro*. Here, we suggest that Le.CDC5 and CIPB regulate *ctg1* expression cooperatively.

2. Materials and methods

2.1. Genomic binding-site (GBS) cloning

We performed the GBS cloning experiment of Inoue et al. (1991) using the method reported by Miyazaki et al. (2004) and employed chromosomal DNA of *L. edodes* FMC2 (Katayose et al., 1990).

2.2. Electrophoretic mobility-shift analysis (EMSA)

Six DNA fragments (probes 1–6) with *Mlu*I-cohesive ends, prepared from the promoter region and the 5′-half of the *ctg1* gene by PCR, were end-labelled with [α^{32}P]dCTP and Klenow fragment as described by Miyazaki et al. (2004). The 147-bp fragment (nucleotides (nt) 100–246, probe 1), 134-bp fragment (nt 199–332, probe 2), 117-bp fragment (nt 251–367, probe 3), 221-bp fragment (nt (−)208 to 13, probe 4), 231-bp fragment (nt (−)285 to (−)55, probe 5), and 127-bp fragment (nt (−)103 to 13, probe 6) were prepared by *ctg1* forward primer 4 and *ctg1* reverse primer 3, *ctg1* forward primer 6 and *ctg1* reverse primer 4, *ctg1* forward primer 7 and *ctg1* reverse primer 5, *ctg1* forward primer 2 and *ctg1* reverse primer 2, *ctg1* forward primer 1 and *ctg1* reverse primer 1, and *ctg1* forward primer 3 and *ctg1* reverse primer 2, respectively (Fig. 1B and C). GST-Le.CDC5 (1–185) was prepared according to Miyazaki et al. (2004). GST-CIPA (6–320) and GST-CIPB (1–127) were prepared as follows: The *cipA* cDNA sequence (nt 16–960) encoding CIPA (6–320), prepared by PCR using *cipA* forward primer 1 and *cipA* reverse primer 1 (see Table 1), and the *cipB* cDNA sequence (nt 1–381) encoding CIPB (1–127), prepared by PCR using *cipB* forward primer 1 and *cipB* reverse primer 1 (Table 1), were inserted into the expression vector pGEX-2TK (GE Healthcare, Piscataway, NJ). The resulting recombinant plasmids were introduced into *Escherichia coli* BL21, producing GST-CIPA (6–320) and GST-CIPB (1–127). EMSA was performed according to Miyazaki et al. (2004). Where necessary, non-labelled DNA fragments were also incubated as a competitor in the amounts indicated in the legend to Fig. 2.

2.3. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out basically as described (Orlando et al., 1997; Kuo and Allis, 1999). DNA cross-linking and immunoprecipitation in preprimordial aggregated mycelium, primordia, immature fruiting bodies, and stipes and pilei of mature fruiting bodies of *L. edodes* FMC2 (Katayose et al., 1990) were as follows: For DNA-protein fixation, samples were put into fixation buffer (25 mM Hepes–KOH (pH 8.0), 50 mM KCl, 1 mM EDTA, 0.5 mM EGTA) containing 1% formaldehyde. Vacuum was applied for about 10 min, the samples were rotated at 4 °C for 2 h in the same buffer, and then transferred to phosphate-buffered saline (PBS) (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) containing 0.3 M glycine. Vacuum was again applied for 10 min, the samples were incubated again for 30 min in the same buffer, dried with paper towels, frozen, and stored in −80 °C until further processing. Samples (0.8 g each) were suspended in 200 μl lysis buffer (50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% SDS, protease inhibitor cocktail (Nakarai Tesque, Japan)), glass beads (1 g) were added and then the samples were homogenized by Micro Smash (TOMY, Japan) and centrifuged. To the resulting supernatants, we added 1 ml each of ChIP dilution buffer (25 mM Tris–HCl (pH 8.0), 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 5% glycerol) and then the mixtures were repetitively sonicated (6×, 10 s each). Chromatin samples were sheared to an average size of 400–500 bp by the sonication. The samples were centrifuged to remove cell debris and the resulting supernatants were suspended in 50 μl ChIP dilution buffer containing protein A/G agarose beads (Santa Cruz, USA) (50% slurry; the beads were pretreated with a sufficient amount of single-stranded DNA). The mixtures were rotated at 4 °C for 30 min and then centrifuged. To the resulting supernatants, we added anti-CDC5 rabbit IgG antibody (Nakazawa et al., 2006) or preimmune rabbit IgG; this was followed by 4-h rotation at 4 °C. Then we added 50 μl of the above ChIP dilution buffer containing protein A/G agarose beads to the reaction mixture and after 1-h rotation at 4 °C the mixture was centrifuged. Immunoprecipitates were washed 4 times with the ChIP dilution buffer, treated overnight with proteinase K at 37 °C, and incubated at 65 °C for 7 h to unlink DNA cross-links. The eluted DNA samples were subjected to quantitative PCR analysis using the 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The amount of chromatin-derived DNA seg-