Identification of a CAP (adenylyl-cyclase-associated protein) homologous gene in *Lentinus edodes* and its functional complementation of yeast CAP mutants

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The adenylyl-cyclase-associated protein, CAP, was originally identified in yeasts as a protein that functions in both signal transduction and cytoskeletal organization. This paper reports the identification of a cDNA and genomic DNA that encodes a CAP homologue from the mushroom *Lentinus edodes*. The *L. edodes cap* gene contains eight introns and an ORF encoding a 518 amino acid protein. The *L. edodes* CAP is 35.5% and 40.9% identical at the amino acid level with *Saccharomyces cerevisiae* CAP and *Schizosaccharomyces pombe* CAP, respectively. The C-terminal domain shows greater homology (39–46% identity) with yeast CAPs than does the N-terminal domain (27–35% identity). Southern blotting and Northern blotting results suggest that *L. edodes cap* is a single-copy gene and uniformly expressed. Expression of the *L. edodes* CAP in both *Schiz. pombe* and *Sacch. cerevisiae* complemented defects associated with the loss of the C-terminal domain function of the endogenous CAP. By using a yeast two-hybrid assay, an interaction was demonstrated between the *L. edodes* CAP and *Schiz. pombe* actin. This result and the functional complementation test indicate that CAP from *L. edodes* has a conserved C-terminal domain function.

Keywords: actin, adenylyl cyclase, cAMP, Ras

INTRODUCTION

The adenylyl-cyclase-associated protein (CAP) was simultaneously identified in the budding yeast *Saccharomyces cerevisiae* as an adenylyl-cyclase-binding protein with a molecular mass of 70 kDa, and as a mutated protein that suppresses the heat-shock-sensitive phenotype resulting from the activated *RAS2*^{val19} mutation (Fedor-Chaiken *et al.*, 1990; Field *et al.*, 1990). Although genetic experiments implicate a function for CAP in RAS regulation of adenylyl cyclase *in vivo*, CAP is not necessarily required for the activation of adenylyl cyclase by RAS *in vitro* (Mintzer & Field, 1994; Wang *et al.*,

1992). However, CAP may be involved in facilitating this interaction *in vivo* (Yu *et al.*, 1994).

Deletion analysis has revealed that *Sacch. cerevisiae* CAP has two distinct functional domains. The N-terminal domain (amino acids 1–168) is required and sufficient for the *RAS2*^{val19} responsiveness of adenylyl cyclase, while the widely conserved C-terminal region (amino acids 368–526) appears to play a role in the nutritional response and serves an important cytoskeletal regulatory function through the binding of monomeric actin (Gerst *et al.*, 1991; Vojtek *et al.*, 1991; Shima *et al.*, 1997). Deletion of the C-terminal region results in abnormal phenotypes, including nutritional and temperature sensitivity, grossly enlarged cells, abnormal shape, random budding and a disorganized cytoskeleton (Field *et al.*, 1990; Gerst *et al.*, 1991). Expression of the C-terminal domain of CAP is sufficient to suppress these defects (Field *et al.*, 1990; Vojtek *et al.*,

Abbreviations: SH3, Src homology 3; CAP, adenylyl-cyclase-associated protein.

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1991). These deficiencies could also be partially complemented by the overexpression of profilin in *Sacch. cerevisiae* (Vojtek *et al.*, 1991). CAP and profilin can compensate some of each other's functions in *Dictyostelium discoideum* (Gottwald *et al.*, 1996). The deletion of the centrally located, proline-rich domain (amino acids 169–367) of *Sacch. cerevisiae* CAP is phenotypically silent, but recently, the central region was identified as the SH3 (Src homology 3) binding region and was found to modulate cytoskeletal localization (Freeman *et al.*, 1996). This region was also thought to be related to the localization of CAP in yeast (Zelicof *et al.*, 1996).

Following the identification of CAP in *Sacch. cerevisiae*, CAP homologues were also identified in other eukaryotes, ranging from the fission yeast *Schizosaccharomyces pombe* to humans; two CAP homologues were found in humans and rat respectively (Kawamukai *et al.*, 1992; Fenger *et al.*, 1994; Vojtek & Cooper, 1993; Zelicof *et al.*, 1993; Gieselmann & Mann, 1992; Matviw *et al.*, 1992; Marcus *et al.*, 1993; Yu *et al.*, 1994; Gottwald *et al.*, 1996; Swiston *et al.*, 1995). *Schiz. pombe* CAP, which was the second CAP to be identified, has a similar function to *Sacch. cerevisiae* CAP. The N-terminal domain is associated with adenyl cyclase binding, while deletion of the C-terminal domain results in morphological and nutritional defects (Kawamukai *et al.*, 1992). All of the homologous proteins identified share structural similarity and functional complementation. Structural similarity is greater in the C-terminal domains and central domains of CAPs than in the N-terminal domains. This is consistent with the observation that the expression of all tested CAP homologues in *cap* yeast strains can complement the defects resulting from the deletion of the C-terminal domain of endogenous CAP, but not those resulting from the deletion of the N-terminal domain (Kawamukai *et al.*, 1992; Fenger *et al.*, 1994; Vojtek & Cooper, 1993; Zelicof *et al.*, 1993; Gieselmann & Mann, 1992; Matviw *et al.*, 1992; Gottwald *et al.*, 1996; Swiston *et al.*, 1995). To investigate whether CAP and its functions have been conserved in mushrooms, we screened for a CAP homologue in the mushroom *Lentinus edodes*, strain FMC2. This mushroom is known to require high intracellular levels of cAMP activity for the onset of fruiting (Takagi *et al.*, 1988) and to contain a homologue of the *ras* gene (Hori *et al.*, 1991). Here we report the identification of the *cap* gene of *L. edodes* and show that it encodes a protein structurally and functionally homologous to yeast CAPs.

METHODS

Strains and media. *Schiz. pombe* strain MK1818d [*h90 leu1-32 ade6-210 ura4-d18 cap::ura4 (cad11)*] and *Sacch. cerevisiae* strain SKN32 [*MATa leu2 ura3 trp1 ade8 can1 cap::HIS3*] were used for the functional complementation test. *Sacch. cerevisiae* strain L40 [*MATa his3 trp1 leu2 ade2 LYS2::lexA-HIS3 URA3::lexA-lacZ*] was used for the two-hybrid assay. *Schiz. pombe* was grown in either YEA rich medium (0.5% yeast extract, 3% glucose, 75 mg adenine l⁻¹)

or PMA (pombe minimal medium with 75 mg adenine l⁻¹) synthetic medium supplemented, when necessary, with 75 mg leucine l⁻¹ (Mitchison, 1970). *Sacch. cerevisiae* was grown in YPD rich medium (1% yeast extract, 2% peptone, 2% glucose), or SC (synthetic complete) medium with appropriate auxotrophic supplements (Rose *et al.*, 1990). The lithium acetate method was used for yeast transformation (Ito *et al.*, 1983). The *L. edodes* strain FMC2 (Takagi *et al.*, 1988) was used. Mycelia were grown in SMY medium (1% sucrose, 1% malt extract, 0.4% yeast extract, pH 5.6). Fruiting bodies were formed according to the method previously described (Takagi *et al.*, 1988).

DNA manipulation and analysis. General procedures for DNA manipulation and analysis were as described by Sambrook *et al.* (1989). *L. edodes* FMC2 genomic DNA was extracted from mycelia at the vegetative growth stage, as described by Zolan & Pukkila (1986). The PCR kit was purchased from TaKaRa Biomedicals and was used as recommended by the supplier. Reaction mixtures in a total volume of 100 µl were incubated for 30 cycles (a typical cycle was for 30 s at 94 °C, 60 s at 52 °C and 120 s at 72 °C). The annealing temperature and duration of each step varied slightly depending on the primers and the length of fragments to be amplified. The sequences of both strands of the DNA from the clones were determined by a modified dideoxy chain-termination method (Sanger *et al.*, 1977) using the ABI sequencer Prism 377 and the Perkin Elmer Cycle-Sequencing kit. For amplification of a part of the *cap* gene, degenerate oligonucleotides complementary to peptides PPPPP and THKNP, which are two well-conserved motifs in most CAP homologues (amino acids 278–282 and amino acids 333–337 in *Sacch. cerevisiae* CAP), were designed and used as PCR primers. Using three forward primers and three reverse primers, nine possible primer combinations were tried in order to amplify the *cap* fragment by PCR. The primers used were as follows (N represents any one of the four nucleotides). (1) Primers encoding THKNP (reverse primers): THK1, 5'-GGGCGGCCGC(AC)GG(AG)TT(TC)TT(GA)TG(AG)GT-3'; THK2, 5'-AAGCGGCCGCACGTAAANNNGG(AG)TT(TC)TT(GA)TGNGT-3'; THK3, 5'-AAGCTT(AG)TT(TC)TT(GA)TG(ATG)GT-3'. (2) Primers encoding PPPPP (forward primers): P1, 5'-AAGCGGCCGC(AT)CC(AT)-CC(AT)CC(AT)CC(AT)CCT-3'; P2, 5'-GGATCCNCCNC-C(TA)CC(TA)CC-3'; P3, 5'-AAGGATCCNCCNCC(TAC)-CC(CG)CC-3'.

To amplify full-length *cap* from genomic DNA, primers were designed from the *cap* cDNA sequence (Fig. 2) and used in PCR. The forward and reverse primers were: Pfg (forward primer), TAAGCTTCGGCAGGATGTCCT; Prg (reverse primer), TGGATCCGATAATATGCATGT.

An *L. edodes* cDNA library was constructed by inserting the cDNA synthesized on poly(A)⁺ RNA into an *Eco*RI-cut and dephosphorylated λ gt10 vector (Huynh *et al.*, 1985) according to the procedures previously described (Hori *et al.*, 1991). Poly(A)⁺ RNA was prepared from primordia and immature fruiting bodies.

Plasmids. Plasmid pMf9-6 was constructed by inserting a 196 bp *Not*I–*Not*I PCR fragment containing the *cap* sequence from the genomic DNA of *L. edodes* FMC2 into the *Not*I site of pBluescript II SK+. pMfcCAP was constructed by inserting a 1.7 kb *Eco*RI–*Eco*RI fragment containing *cap* cDNA into pBluescript II SK+ (Fig. 1). The cDNA was derived by screening a λ gt10 *L. edodes* cDNA library using plaque hybridization and the PCR fragment as a probe. pMfgCAP was constructed by inserting a 2.1 kb PCR fragment containing *cap* genomic DNA into the pT7-Blue T-vector

Fig. 1. Restriction maps of the plasmids used in this study. The construction of all plasmids is described in Methods. Open boxes indicate coding sequences of *cap* or other genes; stippled boxes represent the non-coding sequence flanking *cap* or *actin* genes derived from the cDNAs. The hatched boxes indicate the yeast *ADH1* promoter. The solid lines indicate the vector sequences: the thinner lines represent those derived from expression vectors or two-hybrid vectors, and the thicker lines represent those derived from the vector. Abbreviations for restriction enzymes are: B, *Bam*HI; Cl, *Cla*I; El, *Eco*RI; EV, *Eco*RV; H, *Hind*III; Nd, *Nde*I; N, *Not*I; P, *Pst*I; Sac, *Sac*I; Sal, *Sal*I; Sm, *Sma*I; Xb, *Xba*I; Xh, *Xho*I.

(Novagen). pPT1CAP was constructed by inserting the *Eco*RV–*Sma*I fragment derived from pMfcCAP, which contains the *cap* cDNA, into the *Sma*I site of the *Schiz. pombe* expression vector pART1 (McLeod *et al.*, 1987). The fragment was placed in the correct direction for expression. The same fragment was inserted into the *Sma*I site of the *Sacch. cerevisiae* expression vector pAD5 (Kawamukai *et al.*, 1992) to construct the plasmid pPD5CAP. Plasmid pCGE2 expresses *Schiz. pombe* CAP (Kawamukai *et al.*, 1992). pADHCAP contains the *Sacch. cerevisiae* CAP coding sequence cloned into the *Sma*I site of pAD4, a plasmid similar to pADANS (Ballester *et al.*, 1989; Field *et al.*, 1990). For the two-hybrid assay, pBTMCAP was constructed by inserting the *Eco*RV–*Sma*I fragment containing *cap* cDNA into the *Sma*I site of pBTM116 (Bartel *et al.*, 1993). The fragment was inserted in the correct frame so as to allow expression of a LexA–CAP fusion protein. Plasmid pGDAC was obtained from another two-hybrid screening; it contained the *Schiz. pombe* actin gene cloned into the *Eco*RI–*Xho*I site of pGADGH. The inserted fragment was confirmed to be in the correct frame for expression of the Gal4-fused protein. The plasmids are illustrated in Fig. 1.

Southern hybridization. Using the insert containing *cap* cDNA from plaque hybridization as a probe, we conducted Southern hybridization with *L. edodes* genomic DNA. The genomic DNA was first digested with several restriction enzymes, and then hybridization was performed under low-stringency conditions by using a washing buffer containing $0.5 \times$ SSC.

Northern-blot analysis. Total cellular RNA was isolated from primordia, immature fruiting bodies (stage I: just after formation of the pileus and stipe), immature fruiting bodies (stage II: fruiting bodies enlarged but gill tissues not yet visible), and mature fruiting bodies of *L. edodes* according to the method of Han *et al.* (1987). The RNA samples (20 μ g each) were size-fractionated, transferred to a nylon filter, hybridized with 32 P-labelled probes under stringent conditions according to the method previously described (Hori *et al.*, 1991), and autoradiographed.

Yeast two-hybrid assay. *Sacch. cerevisiae* strain L40 was co-transformed with pBTMCAP or its derivatives along with pGDAC or its derivatives, spread onto SC-Leu-Trp synthetic medium and grown for 2 d at 30 °C. The transformants were then plated onto a new SC-Leu-Trp plate covered with a piece of sterilized filter, further incubated for 1–2 d and tested for β -galactosidase activity by a filter assay (Fields & Song, 1989).

RESULTS

Cloning of *L. edodes* cDNAs encoding a CAP-homologous protein

Part of the *cap*-homologous gene from *L. edodes* genomic DNA was amplified by PCR. Fragments of about 200 bp or larger (considering the possibility of the existence of introns) were chosen, cloned into pBluescript II SK+, and their nucleotide sequences were determined. Among approximately 40 sequenced clones, one clone designated pMf9-6, derived from the primer combination THK2 and P1, was found to encode an amino acid sequence highly homologous to the corresponding region of other CAP proteins. The 196 bp *Not*I–*Not*I fragment was labelled with 32 P and used as a probe to screen, by plaque hybridization, a *L. edodes* cDNA library previously constructed in the phage vector λ gt10 using the *Eco*RI site (Hori *et al.*, 1991). One positive clone designated G3 was obtained after screening about 2×10^5 plaques. The G3 insert was cut out by *Eco*RI digestion and subcloned into pBluescript II SK+ to construct pMfcCAP (Fig. 1). The nucleotide sequence of G3 was determined. It contained an ORF encoding a protein of 518 aa that was structurally homologous to yeast CAP proteins and other homologues; therefore we designated the isolated gene *cap*. The cDNA and predicted amino acid sequence of *L. edodes cap* is shown in Fig. 2 (the nucleotide sequence of the *L. edodes cap* gene has been submitted to DDBJ/ GeneBank/EMBL: accession number AB001578).

Sequence comparison between *L. edodes* CAP and its homologues

The *L. edodes* CAP shares high homology with other CAP homologues. It is 35.5% and 40.9% identical with *Sacch. cerevisiae* CAP and *Schiz. pombe* CAP, respectively, at the amino acid level. The identity between the C-terminal domain of *L. edodes* CAP and those of *Sacch. cerevisiae* and *Schiz. pombe* CAP is 39.0% and 46.2%, respectively, while the N-terminal domains of *L. edodes* CAP are 26.8% and 35.1% identical with the corresponding regions of *Sacch. cerevisiae* and *Schiz. pombe* CAP, respectively. So the C-terminal domain of

<i>S. c</i> CAP	1	MPDSKYTMG-YNVKKLKRIFETARLQSVT-----
<i>L. e</i> CAP	1	MS-----EGLHSKLTIRKLAATSRIDIAASWPT-----SKPETS
<i>S. p</i> CAP	1	ISDMINIRETGYNFTLLKRLAATSRIDIVESBKPLPNMRRPSRDSN
<i>S. c</i> CAP	32	-----IQEGYIQNKLEKSKNNKES--DSGADANTNEPSAENPEVE
<i>L. e</i> CAP	40	EATPSSSSAAAASSDPTDSTDAIA-----ASTMR
<i>S. p</i> CAP	51	SQNNITFNIGTPPATFTSTGSPAVASLHDQVAAAIAPRNRSLTTSAVE
<i>S. c</i> CAP	73	QDELCITAPQSYIGEN-IDLLERESGKIDTV-GLDALQQLKGGQSQTLF
<i>L. e</i> CAP	73	ETKSSVVVDFVVIDGKVKFKKITSFAASVIVGLLPPQKKEEL
<i>S. p</i> CAP	101	AVDASISAYDFCSK-ILSKYMESSKIGEL-TAQSEHNEANLQOV
<i>S. c</i> CAP	121	LRAVRSRFPFYSSQTHADSRFINENIKLGQLDSINQSK-YEAYSA
<i>L. e</i> CAP	123	INTASCVFSD--DPTQSLDTFPHSTEARNRAPASKDRDWTFBHV
<i>S. p</i> CAP	149	LSVLLKAOQFMDSELELLEFKRIQSELLTINIRDEHTAPE-ENQIST
<i>S. c</i> CAP	170	LSGAPLFSYAVD-TIVSMVTDFTDAQFWTFLILTYRSTNAVEVA
<i>L. e</i> CAP	171	ISASFPVAVLNSKSPHIAITDQLYTCRRIRISLGLGRBAKAV
<i>S. p</i> CAP	198	VMSGIILGKGVTPPFTLSFMSMSSSQFAARUMEPFPGSLQLIQEV
<i>S. c</i> CAP	218	KKKLSFDNLKAIKIEYHTVSND--EKDMDFADAMASTKNTGATSSP
<i>L. e</i> CAP	220	SSSASAPETRKIVLFTHTGSLNSN--EIV--GAY--AASSP--A
<i>S. p</i> CAP	248	RSYLLTLTLITVKTBFKDTSTODAVELKTAALNL--SASSTQAP
<i>S. c</i> CAP	268	SPASAAAPAPFFPFFPAPASVFESNDTFTSSDANKSGIGSVAPLHQ
<i>L. e</i> CAP	260	NISGAILDQFPFFPFFFPAPAA---PPVTAPATTPADTAAYFAETUR
<i>S. p</i> CAP	296	SSGDSANGLQFPFFPFFPSSNDF--WKDSNEPAPADNKSOMGSAFABTHK
<i>S. c</i> CAP	317	ENIKKSKKVKVCKQOQHNNPESQSTSTSTGSKSGP-PPRKSTSTK
<i>L. e</i> CAP	306	ADVVRKSKVKVCKQOQHNNPESQSTSTSTGSKSGP-PPRKSTSTK
<i>S. p</i> CAP	344	REGISLKKVKVCKQOQHNNPESQSTSTSTGSKSGP-PPRKSTSTK
<i>S. c</i> CAP	366	TRRIRKEIVNKKFIDNENETESLVIANK-DESIFIGKISQVLVTE
<i>L. e</i> CAP	356	GKTKKFAEKKAKNNT-LKEEKSVVVGQVQVNLGKSKSVSTP
<i>S. p</i> CAP	392	PVTRIRIEENTKRVY-NVYNHSLILLSNEHSSQIICSSCTIIE
<i>S. c</i> CAP	415	EVYVVISSETESCSVLVLSIGMDLANKGIVNHLQASIKSKS
<i>L. e</i> CAP	405	EEPAHTVILKIQELSLIVSISISTSPSAALITGAALQOQSSIS
<i>S. p</i> CAP	441	GRNDTSMSSKRRISVVVITLAAFDIACSNGGVNMEVQVYVQV
<i>S. c</i> CAP	465	GNNYLSKESNTLTYSCSTA-SVNLIGEDDD--YVEFPIQMKHSF
<i>L. e</i> CAP	455	TSNYYLSQASIHATTHAACSSNVNIVEGEFVVEFQNLMLRTCL
<i>S. p</i> CAP	491	GSSVYLSKSSSSSVVSSSTLIVV--NEEDYALRIVSIIKHK
<i>S. c</i> CAP	513	AL--KPKSAMFPAK
<i>L. e</i> CAP	505	KL--KSLVTTVVFVE
<i>S. p</i> CAP	538	NEKSLVSEIQR-E

Fig. 3. Alignment of the amino acid sequences of *L. edodes* (*L. e*) CAP (accession number AB001578) with *Sacch. cerevisiae* (*S. c*) CAP (accession number P17555) and *Schiz. pombe* (*S. p*) CAP (accession number P36621). *L. edodes* amino acid residues identical to the corresponding residues of either of the yeast CAPs together with the residues of the yeast CAPs are highlighted with a black background. Amino acid residues are numbered on the left. The spaces were manually introduced to maximize the alignment.

Expression of the *cap* gene during the development of *L. edodes* from a mycelium into a fruiting body

Total cellular RNA was isolated from primordia, immature stage I fruiting bodies, immature stage II fruiting bodies and mature fruiting bodies, and was subjected to Northern blot analysis using *cap* cDNA as a probe. As shown in Fig. 5, a clear hybridization signal of 1.7 kb (*cap* band) was detected in all the RNA blots with comparable intensity. To ensure equal loading and transfer of RNA, the radioactivity was removed from the blots and rehybridized with the cDNA clone (1.2 kb) of *L. edodes ras*, which has been shown to be transcribed at a constant level during development of the mycelium into a fruiting-body (Hori *et al.*, 1991). As shown in Fig. 5, a 1.2 kb *ras* signal was detected in all the RNA blots, again with equal intensity. These results show that the *cap* gene is also transcribed at a constant level during development of the mycelium into a fruiting body.

Fig. 4. A phylogenetic tree of the amino acid sequences of *L. edodes* CAP and other CAP homologues generated using the DNASIS software (Hitachi Software Engineering). The scale indicates the percentage identity as calculated from multiple alignments. The DNA or protein sequence accession numbers are: mouse CAP, P40124; rat CAP1, Q08163; human CAP1, Q01518; human CAP2, P40123; rat CAP2, P52481; hydra CAP, P40122; *Caenorhabditis elegans* (*C. e.*) CAP, AF000265; *D. discoideum* (*D. d.*) CAP, P54654; *L. edodes* (*L. e.*) CAP, AB001578; *Schiz. pombe* (*S. p.*) CAP, P36621; *Sacch. cerevisiae* (*S. c.*) CAP, P17555.

Fig. 5. Transcriptional expression of the *cap* gene during development of the mycelium into a fruiting body in *L. edodes*. Total cellular RNAs isolated from the primordia (lane 1), immature fruiting bodies [stage I: just after the formation of pileus and stipe], (lane 2), immature fruiting bodies [stage II: fruiting bodies are enlarged but gill tissues are not yet visible] (lane 3), and mature fruiting bodies (lane 4) were subjected to Northern-blot analysis using the ³²P-labelled *cap* cDNA fragment as a probe (top). The label was removed from the blots by boiling in 0.1% SDS. After confirming the removal of radioactivity, the resulting blots were rehybridized with ³²P-labelled *L. edodes ras* cDNA (Hori *et al.*, 1991) (bottom).

Complementation of *Cap*⁻ phenotypes in yeast by expression of the *L. edodes* CAP

In *Sacch. cerevisiae*, loss of the C-terminal functional domain of CAP results in abnormal cellular morphology (round and enlarged cells), and growth impairment on rich medium (YPD), on synthetic medium (SC) containing 6.5 mM valine and at 37 °C (Gerst *et al.*, 1991).

Fig. 6. Expression of *L. edodes* CAP suppresses the temperature-sensitive phenotype of the *Schiz. pombe cap* strain MK1818d. Cells were grown on PMA plates, replica-plated onto fresh PMA plates, and then incubated at 30 °C or 37 °C. A, *Schiz. pombe* strain MK1818d transformed with pART1 (expression vector); B, MK1818d transformed with pPT1CAP, a plasmid expressing *L. edodes* CAP; C, MK1818d transformed with pCGE2, a plasmid expressing *Schiz. pombe* CAP. Three independent transformants are shown for each treatment.

In *Schiz. pombe*, deletion of the C-terminal functional domain also results in abnormal cellular morphology, growth impairment at 37 °C and abnormalities in mating and sporulation. To see if it is possible to functionally complement a *Schiz. pombe cap* mutant with *L. edodes* CAP, we constructed the plasmid pPT1CAP and expressed it in the *Schiz. pombe cap* strain MK1818d (see Methods). Expression of the *L. edodes* CAP in *Schiz. pombe* restored its ability to grow at 37 °C (Fig. 6), restored normal cellular morphology, and restored the capability of cells to sporulate when grown on minimum medium (PMA plates) (Fig. 7).

To investigate the possibility of functional conservation between *L. edodes* CAP and *Sacch. cerevisiae* CAP, we

Fig. 8. Expression of *L. edodes* CAP suppresses the temperature-sensitive phenotype and inability to grow on rich medium of the *Sacch. cerevisiae cap* strain SKN32. Cells were grown on SC plates containing appropriate auxotrophic supplements and replica-plated onto various media. For each treatment, three independent transformants are shown. (a) SKN32 transformed with pAD5 (control vector; A), pPD5CAP (a plasmid expressing *L. edodes* CAP; B), or pADHCAP (a plasmid expressing *Sacch. cerevisiae* CAP; C), was replica-plated onto SC-Leu plates and incubated at 30 °C or 37 °C. (b) SKN32 transformed with the same three plasmids as in (a) was replica-plated onto SC-Leu medium containing 6.5 mM valine (left) or YPD rich medium (right) and incubated at 30 °C.

constructed the plasmid pPD5CAP and expressed the *L. edodes* CAP in the *Sacch. cerevisiae cap* strain SKN32 (see Methods). The expression of *L. edodes* CAP

Fig. 7. Expression of *L. edodes* CAP restores the cellular morphology and sporulation of the *Schiz. pombe cap* strain MK1818d. Cells grown for 2 d on plates of minimal medium PMA were dispersed in distilled water and photographed. (a) MK1818d, in which the *cap* gene has been replaced with the *ura4* gene; (b) MK1818d transformed with the control plasmid, *Schiz. pombe* expression vector pART1; (c) MK1818d transformed with pPT1CAP, a plasmid expressing *L. edodes* CAP; (d) MK1818d transformed with pCGE2, a plasmid expressing *Schiz. pombe* CAP. In (a) and (b), enlarged and round cells (black arrows) are abundant, and the cells do not sporulate. In (c) and (d), normal-sized and sporulating cells (open arrowheads) are abundant. Bar 10 µm.

Fig. 9. Cellular morphology of the *Sacch. cerevisiae* cap strain SKN32 and transformants expressing various plasmids. Cells grown for 2 d on SC plates without the selective supplements were dispersed in water and photographed. (a) SKN32, in which the *cap* gene has been replaced with the *HIS3* gene; (b) SKN32 transformed with the control plasmid, *Sacch. cerevisiae* expression vector pAD5; (c) SKN32 transformed with pPD5CAP, a plasmid expressing *L. edodes* CAP; (d) SKN32 transformed with pADHCAP, a plasmid expressing *Sacch. cerevisiae* CAP. In (a) and (b), enlarged and round cells with a disordered surface (black arrowheads) are abundant. In (d), the cell shape and size are restored (open arrowhead). In the cells expressing *L. edodes* CAP (c), the cell shape is restored to some extent (cells are not so round and do not appear to have a disordered surface as in a and b) (black arrow). Bar 10 μ m.

restored the ability to grow on rich medium (YPD) and on SC medium containing a high concentration of valine, and produced normal growth at 37 °C. The degree of restoration of the normal phenotype was similar to that seen in the mutant cells transformed with the plasmid expressing *Sacch. cerevisiae* CAP (Fig. 8). The cell shape was restored to some extent even though there were still some abnormal cells (Fig. 9). This might be due to the low expression level of *L. edodes* CAP in SKN32 cells.

Yeast two-hybrid assay

Previous results from biochemical and yeast two-hybrid assays have indicated that yeast and human CAP interact with actin *in vivo* (Amberg *et al.*, 1995; Freeman *et al.*, 1995; Hubberstey *et al.*, 1996). The *L. edodes* CAP can complement the morphological function of *Schiz. pombe* CAP. We therefore speculated that the *L. edodes* CAP should be able to interact with *Schiz. pombe* actin *in vivo*, thus enabling the normal formation of *Schiz. pombe* actin filaments. We performed the yeast two-hybrid system assay to test this hypothesis. We first constructed pBTMCAP, an expression plasmid that expresses a LexA-fused *L. edodes* CAP protein (see Methods), and used this plasmid to co-transform *Sacch. cerevisiae* strain L40 with plasmid pGADAC, which has the *Schiz. pombe* actin gene cloned into pGADGH, where it is orientated in the correct frame to allow expression. As negative controls, the cells were simultaneously co-transformed with combinations of pGADAC and pBTMmsa2 along with pBTMCAP and pGADGb. The *msa2* gene encodes an RNA-binding

protein and the *gbd1* gene encodes G β ; these two proteins are unrelated to either CAP or actin. As a positive control, CAP and actin of *Schiz. pombe*, two proteins known to interact with each other *in vivo*, were similarly co-expressed in *Sacch. cerevisiae* strain L40 and β -galactosidase activity was measured. The *L. edodes* CAP was found to interact with *Schiz. pombe* actin as indicated by the appearance of blue-coloured colonies. However, judging by the intensity of the blue colour, the interaction seemed to be weaker than that of the positive control (results not shown).

DISCUSSION

CAP is an interesting protein that is involved in signal transduction and cytoskeleton formation, which are two important processes in a wide range of organisms. We have identified a CAP homologue from the mushroom *L. edodes* and analysed its characteristics. Comparison of the amino acid sequence of *L. edodes* CAP with those of other CAP homologues indicated that it is nearly equidistantly related to homologues from other organisms, and that the homology with other CAPs is about 30%. The homology among mammalian CAPs is extremely high (Fig. 4), in contrast to CAP homologues from *Sacch. cerevisiae* and *Schiz. pombe*, which share little homology. This is consistent with the notion that these two yeast species are not as closely related as their common name – yeast – would appear to suggest. The *L. edodes* CAP homologue displays the highest degree of homology with *Schiz. pombe* CAP, with an identity of 40.9%, while it shows 35.5% identity with *Sacch.*

cerevisiae CAP. The C-terminal domain and the central domain also tended to be well-conserved between *L. edodes* CAP and the two yeast CAPs compared to the degree of conservation between the N-terminal domains of yeast and mushroom CAPs. The RLE repeat localized in *Sacch. cerevisiae* CAP at amino acids 19–21 and 26–28, which is conserved in most homologues, can potentially form an amphipathic helix suitable for protein–protein interaction (Vojtek & Cooper, 1993). This repeat was altered to RLE/RIE (aa 15–17 and 22–24) in *L. edodes* CAP. The proline-rich stretch, which is presumed to be the SH3 protein binding motif (Freeman *et al.*, 1995), contained 11 consecutive proline residues from amino acids 269 to 279 in mushroom CAP. This is the longest stretch of proline among all the CAP homologues identified so far. We were unable to detect another CAP homologue in *L. edodes* by low-stringency Southern blotting. In addition, the *cap* gene is likely to be transcribed at similar levels during the development of *L. edodes* from a mycelium into a fruiting-body. From the genomic sequences, eight introns were identified. This is the first time that introns have been identified in a CAP-homologous gene. The fact that CAP proteins have been conserved during the evolution from single-cell eukaryote yeasts to humans implies that CAP fulfils essential cellular functions. The CAP of *L. edodes* is more homologous to *Schiz. pombe* CAP than to *Sacch. cerevisiae* CAP. Expression of the *L. edodes* CAP in the *Schiz. pombe cap* strain MK1818d fully complemented the defects resulting from the loss of the C-terminal domain of the endogenous CAP. The expression of *L. edodes* CAP in the *Sacch. cerevisiae cap* strain SKN32 suppressed some of the defects related to the deletion of endogenous CAP, but the restoration of cell size and morphology was incomplete. We speculate that this may be due to the insufficient level of expression of *L. edodes* CAP.

An interaction between CAP and actin has been suggested by previous genetic studies on yeast CAP (Vojtek *et al.*, 1991; Amberg *et al.*, 1995) and by the co-purification of porcine CAP with actin (Gieselman & Mann 1992). Yeast CAP was suggested to be required for normal actin filament formation. Recent studies show that yeast CAP interacts with monomeric actin and thus prevents it from polymerizing *in vitro* (Freeman *et al.*, 1995). This interaction requires the C-terminal domain, but not the N-terminal domain, of CAP (Hubberstey *et al.*, 1996). Using the yeast two-hybrid system, the conservation of the interaction between CAP and actin in yeast and mammal was verified (Amberg *et al.*, 1995; Freeman *et al.*, 1995; Hubberstey *et al.*, 1996). Our functional complementation analysis result showed that *L. edodes* CAP can fully complement the morphological defects of *Schiz. pombe cap* strains, and the results of the two-hybrid assay indicate that *L. edodes* CAP interacts with *Schiz. pombe* actin *in vivo*. These results provide convincing evidence that CAP homologues from other organisms can suppress the morphological defects associated with the *cap* mutation in yeast cells.

Although many CAP homologues have been cloned, only yeast CAPs have been functionally identified as adenylyl-cyclase-associated proteins; the identification of others has only been based on their homology and conservation of the C-terminal domain functions with yeast CAPs. It will be interesting to see if *L. edodes* CAP does indeed function as an adenylyl-cyclase-associated protein.

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