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Effects of amino-acid substitutions in β tubulin on benomyl sensitivity and microtubule functions in *Coprinus cinereus*

Tadashi Matsuo, Yuki Yamamoto, Hajime Muraguchi and Takashi Kamada*

Department of Biology, Faculty of Science, Okayama University, Okayama 700–8530, Japan

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The sensitivity of the homobasidiomycete *Coprinus cinereus* to the benzimidazole fungicide benomyl allowed us to isolate β-tubulin mutants as strains resistant to benomyl. To understand the molecular basis for the interaction between benomyl and β tubulin and for cellular defects in the β-tubulin mutants, we first analyzed the wild-type β1-tubulin gene (*benA*) of *C. cinereus*, revealing that *benA* contains eight introns and encodes a 445 amino-acid protein. We then characterized 16 β1-tubulin mutants. The 16 mutations involved 11 different amino-acid substitutions at 10 different residues in β1 tubulin. The mutated residues were widely distributed along the primary sequence of β1 tubulin, from residue 3 in the N-terminal domain to residue 350 in the intermediate domain, but half of them appeared to be close to the αβ intradimer interface in an atomic model determined by electron crystallography. The benomyl resistant strain BEN193, which exhibits clear heat sensitivity for hyphal growth and defects in various cellular processes, had a novel mutation, i.e., the Leu to Phe substitution at residue 350. Benomyl resistance and the heat sensitivity in BEN193 were suppressed by additional amino-acid substitutions at various residues in β1 tubulin, suggesting that conformational changes of β1 tubulin are involved in the alterations.

Key Words——benomyl resistance; *Coprinus cinereus*; microtubule; β tubulin.

Microtubules are involved in a variety of cellular processes in eukaryotic cells, including mitosis, the movement and positioning of organelles, and cell morphogenesis. They play a diversity of roles by controlling polymerization and depolymerization of the α- and β-tubulin heterodimers, in association with various microtubule-associated proteins.

Antimicrotubule drugs, which affect the dynamics of microtubules, have been invaluable research tools. These drugs have been used during screens for mutants defective in microtubule functions, which are useful both for studies of microtubule-related cellular processes and investigations of the interactions between microtubules and the antimicrotubule drugs. In fungi, the benzimidazole fungicide benomyl has often been used for screens for mutations in tubulin. Molecular analysis of the mutations isolated revealed that several amino-acid residues in β tubulin were crucial for benomyl sensitivity in a number of ascomycete fungi including *Aspergillus nidulans* (Jung and Oakley, 1990; Jung et al., 1992), *Neurospora crassa* (Orbach et al., 1986; Fujimura et al., 1992, 1994), and *Venturia inaequalis* (Koenraadt et al., 1992). However, the molecular basis for the interaction between benomyl and β tubulin and for cellular defects in the β-tubulin mutants is incompletely defined.

It is generally believed that basidiomycete fungi are resistant to benomyl. However, we found that the homobasidiomycete *Coprinus cinereus* is sensitive to benomyl, which allowed us to isolate mutants resistant to the drug (Kamada et al., 1989a). We have shown that some of the benomyl-resistant strains isolated have mutations in the structural gene for β1 tubulin (Kamada et al., 1989b). We have also shown that some of the β1-tubulin mutants exhibit, in addition to benomyl resistance, heat sensitivity for hyphal growth, and defects in various cellular processes unique to homobasidiomycetes, such as transhyphal migration of nuclei in dikaryosis, the pairing of the two nuclei in the dikaryon, and/or fruit-body morphogenesis from the dikaryon (Kamada et al., 1989a, 1989b). In the present study, to understand the molecular basis for the interaction between benomyl and β1 tubulin, and for defects in cellular processes in the β1-tubulin mutants, we first cloned and sequenced the wild-type β1-tubulin gene of *C. cinereus*. We then characterized β1-tubulin mutations in benomyl-resistant strains isolated previously (Kamada et al., 1989a, 1989b) and those identified during the present study as extragenic suppressors of a mutation in a gene (*mipA*) whose product appears to interact with β1 tubulin (Kamada et al., 1990). We also analyzed intragenic suppressor mutations of the heat-sensitive, benomyl-resistant β1-tubulin mutant, BEN193, in order to get an insight into the interaction between benomyl and β1 tubulin.

The DDBJ/GeneBank/EMBL accession number for the sequence reported in this paper is AB000116.

* To whom correspondence should be addressed.

Materials and Methods

Fungal strains and culture conditions Strains of *Coprinus cinereus* (Schaeff. ex Fr.) S. F. Gray *sensu* Konr. listed in Table 1 were used. MY medium (Rao and Niederpruem, 1969) solidified with 2% (w/v) agar in Petri dishes 9 cm in diam was used for routine mycelial cultures, and the same medium without agar in 9 cm Petri dishes for mycelial cultures for extraction of DNA. MY agar plates supplemented with 100 mg/l of tryptophan were used for production of oidia from a strain requiring tryptophan, and those with 10 or 30 μg/ml of benomyl for screening for mutants and tests of sensitivities to benomyl, respectively. Stock solution of benomyl was 20 mg/ml in DMSO and was added to the medium maintained at 60°C just before pouring. Benomyl (99% pure) was a kind gift of Du Pont Japan (Tokyo). Cultures were maintained at 28°C unless otherwise stated.

Mutagenesis and isolation of mutants Oidia of the wild-type homokaryon, 5302, were mutagenized by UV as described previously (Kamada et al., 1984). For isolation of suppressor mutants of the *mipA* mutant (BEN193R24), we took advantage of the fact that this mutant exhibits cold sensitivity at 20°C and supersensitivity to benomyl at 10 μg/ml (Kamada et al., 1990): mutagenized oidia were incubated at 20°C on MY agar plates without benomyl, or at 28°C on MY plates with 10 μg/ml benomyl. After incubation in the dark for about two weeks, colonies with larger diameters among survivors were isolated.

Hybridization DNA was blotted to Hybond-N (Amersham, Arlington Heights, IL). Hybridization was carried

Table 1. Strains of *Coprinus cinereus* used in the present study.

Strain	Genotype	Source
5302	*A2B2*	This laboratory
292	*A3B1 trp1-1,1-6*	P. J. Pukkila
BEN193R24F$_1$No.4	*A2B2 mipA-24*	Kamada et al. (1990)
BEN8F$_1$No. 45	*A1B1 benA-8*	Kamada et al. (1989)
BEN49F$_1$No. 30	*A1B1 benA-49*	Kamada et al. (1989)
BEN82F$_1$No. 41	*A1B1 benA-82*	Kamada et al. (1989)
BEN120F$_1$No. 62	*A1B1 benA-120*	Kamada et al. (1989)
BEN140F$_1$No. 30	*A1B1 benA-140*	Kamada et al. (1989)
BEN150F$_1$No. 31	*A1B1 benA-150*	Kamada et al. (1989)
BEN154F$_1$No. 52	*A1B1 benA-154*	Kamada et al. (1989)
BEN193F$_1$No. 42	*A1B1 benA-193*	Kamada et al. (1989)
BEN215F$_1$No. 35	*A1B1 benA-215*	Kamada et al. (1989)
BEN330F$_1$No. 34	*A1B1 benA-330*	Kamada et al. (1989)
BEN193R1	*A2B2 benA-193R1*	Kamada et al. (1990)
BEN193R2	*A2B2 benA-193R2*	Kamada et al. (1990)
BEN193R5	*A2B2 benA-193R5*	Kamada et al. (1990)
BEN193R11	*A2B2 benA-193R11*	Kamada et al. (1990)
BEN193R14	*A2B2 benA-193R14*	Kamada et al. (1990)
BEN193R15	*A2B2 benA-193R15*	Kamada et al. (1990)
BEN193R17	*A2B2 benA-193R17*	Kamada et al. (1990)
BEN193R20	*A2B2 benA-193R20*	Kamada et al. (1990)
BEN193R45	*A2B2 benA-193R45*	Kamada et al. (1990)
BEN193R56	*A2B2 benA-193R56*	Kamada et al. (1990)
BEN193R73	*A2B2 benA-193R73*	Kamada et al. (1990)
BEN193R78	*A2B2 benA-193R78*	Kamada et al. (1990)
BEN193R84	*A2B2 benA-193R84*	Kamada et al. (1990)
BEN193R100	*A2B2 benA-193R100*	Kamada et al. (1990)
BEN193R106	*A2B2 benA-193R106*	Kamada et al. (1990)
BEN193F$_1$No. 32	*A3B2 benA-193 trp1-1,1-6*	This study
MIPA-R1F$_1$No. 31	*A2B2 benA-MR1*	This study
MIPA-R2F$_1$No. 44	*A2B2 benA-MR2*	This study
MIPA-R3F$_1$No. 60	*A2B2 benA-MR3*	This study
MIPA-R4F$_1$No. 49	*A2B2 benA-MR4*	This study
MIPA-R5F$_1$No. 18	*A2B2 benA-MR5*	This study
MIPA-R6F$_1$No. 34	*A1B1 benA-MR6*	This study

out at 42°C in 50% formamide in 6×SSPE (0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, pH 7.4) overnight, then the membrane was washed twice in 5×SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0) plus 0.5% SDS at 65°C for 5 min and once in 0.1×SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.5) plus 1% SDS at 50°C for 30 min. BioPrime DNA Labeling System (Gibco BRL, Gaithersburg, MD) and Photogene Nucleic Acid Detection System (Gibco BRL) were used for preparation of biotinylated probes, and for detection of biotinylated probes hybridized to nucleic acids, respectively.

For screening for a β-tubulin gene, we probed dot-blotted DNAs from a cosmid library of *C. cinereus* (May et al., 1991) with a 1.6 kb *Eco*RI fragment from a β-tubulin cDNA clone of *C. cinereus*. The cDNA was kindly provided by L. A. Casselton of the University of Oxford.

Nucleotide sequencing To sequence the wild-type β1-tubulin gene on both strands, we recovered nested deletions of pBI204KS (see Results) using a Double-Stranded Nested Deletion Kit (Pharmacia). The deletion products thus obtained and the PCR products (see below) were sequenced using a model 373A DNA sequencer (Applied Biosystems, Foster City, CA).

PCR The PCR was carried out on genomic DNAs of β1-tubulin mutants to sequence their *benA* alleles. We designed six pairs of sense and antisense primers (BT1 to BT6), which cover the entire length of the ORF of the β1-tubulin gene (Fig. 1). Each primer had a 19 or 20-bp-long sequence which was complementary to the sense or antisense sequence of the β1-tubulin gene. In order to sequence the PCR products directly, we added -20M13 primer sequence (17 bp) to the 5′ end of each designed sense primer sequence and M13 reverse primer sequence (16 bp) to that of each designed antisense primer sequence. Twelve primers (each 35 to 37-bp-long) were purchased from Cruachem, Kyoto, Japan or Gibco BRL. The optimal results were obtained when 100 ng of genomic DNA and primers (300 nM each) were subjected to PCR cycles using Expand Long Template PCR System (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Following denaturation of the template at 94°C for 3 min, 30 PCR cycles were performed of 30 s at 94°C, 30 s at 55°C, and 45 s at 68°C. After amplification, the samples were electrophoresed using a 1.5% agarose gel, the bands of amplified products were excised from the ethidium bromide-stained gel, and the DNAs were purified from the gel using Geneclean II Kit (Bio 101, Inc., Vista, CA).

Extraction of DNA from *C. cinereus* DNA was extracted from the mycelium of *C. cinereus* according to Zolan and Pukkila (1986) except that, after treatment with SEVAG (chloroform - isoamyl alcohol, 24:1 (v/v)) , samples were further purified using phenol.

Transformation Protoplasts were prepared from ungerminated oidia and transformed as described by Binninger et al. (1991) except that 10 mg/ml Novozyme 234 (Novo Nordisk, Bagsvaerd, Denmark) instead of Cellulase Onozuka R-10 was used for production of protoplasts.

CHEF electrophoresis Plugs of intact *C. cinereus* chromosomes for CHEF (clamped homogeneous electric fields) electrophoresis were prepared according to the procedure of Zolan et al. (1992) except that the final concentration of low melting agarose in the plug was 0.5%, and plugs in NDS (0.5 M EDTA, 10 mM Tris-HCl pH 8.0, 1% sodium lauryl sarcosinate) were incubated at 37°C. CHEF electrophoresis was done using a CHEF-DR II apparatus (Bio-Rad, Hercules, CA) as described by Zolan et al. (1992) with a modification that electrophoresis was performed in the following four successive phases: the first phase was 115 h at 50 V with ramping of the pulse time from 3600 s to 1800 s during the time; the second phase, 24 h at 50 V with the ramping from 1800 s to 1300 s; the third phase, 30 h at 60 V with the ramping from 1300 s to 800 s; the last phase, 30 h at 80 V with the ramping from 800 s to 600 s.

Results

Isolation of β1-tubulin mutants as extragenic suppressors of the *mipA* mutation (*mipA-24*) β1-Tubulin mutants of

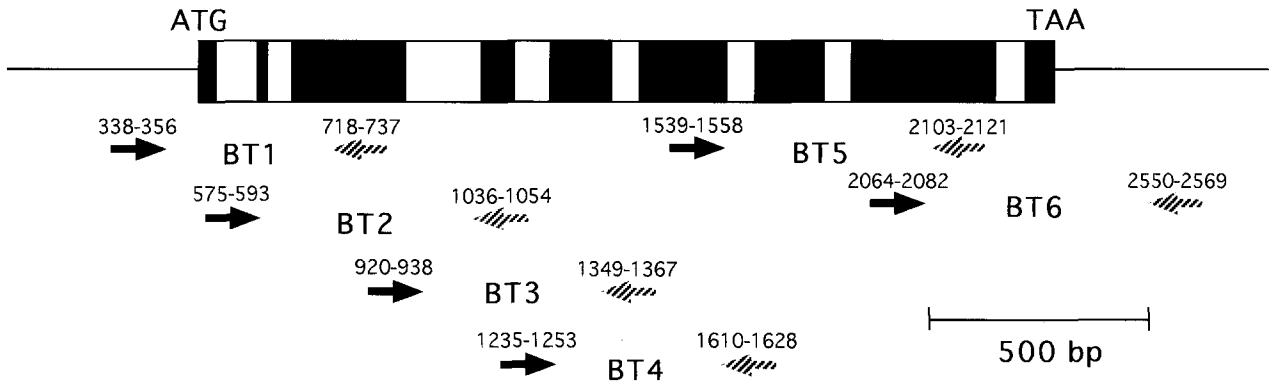

Fig. 1. DNA sequencing strategy for mutant alleles of the β1-tubulin gene of *C. cinereus*. Black boxes correspond to exons of the gene and white ones to the intervening introns. Six pairs of sense and antisense PCR primers (BT1 - BT6) were designed to cover the entire length of the ORF: solid arrows indicate sense primers, and striped arrows antisense primers. The numbers on each arrow indicate the ends of each designed primer, corresponding to the nucleotide numbers in Fig. 2. PCR products were sequenced directly as described in Materials and Methods.

C. cinereus have already been obtained as benomyl-resistant strains (Kamada et al., 1989a, 1989b). In an attempt to obtain other types of β1-tubulin mutants than those obtained as benomyl-resistant strains, we isolated extragenic suppressors of the *mipA* mutation, *mipA-24*. Since the *mipA* mutant was obtained as an extragenic suppressor of a β1-tubulin mutation, *benA-193* (Kamada et al., 1990) , the product of *mipA* seems likely to interact with β1 tubulin.

We isolated 70 revertants of the *mipA* mutant (BEN193R24), by taking advantage of the fact that this mutant shows cold-sensitivity for growth and supersensitivity to benomyl, and genetically analyzed 56 of the 70 revertants isolated. Of the 56 revertants analyzed, 7 were due to extragenic suppressors of *mipA*, while the remaining 49 were due to intragenic suppressors of *mipA* (data not shown). The extragenic suppressors were all due to single-gene mutations (data not shown). Intercrosses between the extragenic suppressor mutations indicated that they comprised two groups in terms of genetic linkage. Furthermore, analysis of linkage between the loci for suppressor mutants and the β1-tubulin gene suggested that suppressor mutations in one of the two groups, to which six of the seven mutations belonged, are in the β1-tubulin gene (data not shown). Thus, six potential β1-tubulin mutants were obtained as suppressors of the *mipA* mutation, which were named MIPA-R1–MIPA-R6 (Table 1).

Cloning and analysis of the wild-type β1-tubulin gene We screened a cosmid library of *C. cinereus* (May et al., 1991) for a β-tubulin gene as described in Materials and Methods, and obtained two positive clones, named 204-H-7 and 219-K-6. Digestion of these two clones with *Xba*I, *Pst*I, *Eco*RI, or *Hin*dIII followed by Southern analysis using the β-tubulin cDNA as the probe showed that the two clones overlap each other in the sequence of the genomic DNA (data not shown). With these clones, we transformed the double mutant (BEN193F_1No. 32) of the heat-sensitive β1-tubulin mutant (BEN193) and the tryptophan-requiring mutant (#292). We found that the heat sensitivity was rescued at a rate of 88% (38/43) of trp^+ transformants by 204-H-7, and at a rate of 84% (32/38) by 219-K-6, indicating that both clones carry the β1-tubulin gene.

We performed Southern analysis of the genomic DNA of the wild-type strain of *C. cinereus*, 5302, and the β1-tubulin clone (219-K-6) after digestion with *Xba*I, *Pst*I, *Eco*RI, or *Hin*dIII, using the β-tubulin cDNA as the probe. We found that the band patterns of the digests of the genomic DNA were the same as those of 219-K-6 (data not shown). Even though the hybridization was done at low stringency (50% formamide, 35°C), no bands other than those from the β1-tubulin clone were detected in the blot of the genomic DNA. These results suggest that *C. cinereus* has only one β-tubulin gene.

We separated the chromosomes from the wild-type strain, 5302, by CHEF electrophoresis and performed Southern analysis using the β-tubulin cDNA as probe. We found that the β1-tubulin gene is located on chromosome XIII (the smallest chromosome of this fungus, Pukkila, 1993) (data not shown).

We subcloned the 3.2 kb *Hin*dIII-*Sal*I fragment of 204-H-7 into the *Hin*dIII-*Sal*I site of pBluescriptII KS, confirmed that the resulting subclone (pBI204KS) rescued the β1-tubulin mutation, then sequenced the 3.2 kb fragment as described in Materials and Methods. Fig. 2 shows the nucleotide sequence and the amino-acid sequence of the β1-tubulin gene, which was deduced based on the *C. cinereus* nucleotide sequence and the amino-acid sequences of the β tubulins of another homobasidiomycete, *Schizophyllum commune* (Russo et al., 1992), of the ascomycete fungi *A. nidulans* (May et al., 1987) and *N. crassa* (Orbach, 1986), and of the yeast *Saccharomyces cerevisiae* (Neff et al., 1983). The ORF of 1937 nucleotides was interrupted by eight introns. The sequences at the 5′ and 3′ boundaries of the eight introns were quite similar to the consensus 5′-GTRNGT ... YAG-3′ sequences for splicing of introns in filamentous fungi (Gurr et al., 1987).

Amino-acid substitutions in β1-tubulin mutants and their effect on sensitivity to benomyl We examined the sequences of 16 β1-tubulin mutants obtained as benomyl-resistant strains or as extragenic suppressors of the *mipA* mutation (Table 2). We found that the mutants examined all had missense mutations in the β1-tubulin ORF, which resulted in 11 different amino-acid substitutions at 10 different residues in β1 tubulin (Table 2). The mutated residues were widely distributed along the primary sequence of β1 tubulin, from residue 3 in the N-terminal domain to residue 350 in the intermediate domain. At residue 200, two different amino-acid substitutions were found: one was from Phe to Leu and the other from Phe to Ser (Table 2).

All the β1-tubulin mutations, including those in strains isolated as extragenic suppressors of the *mipA* mutant, exhibited resistance to benomyl, although the level of benomyl resistance differed between mutants (Fig. 3).

Amino-acid substitutions in intragenic suppressors of the β1-tubulin mutant (BEN193) and their effect on sensitivity to benomyl BEN193 carrying the Leu to Phe substitution at residue 350 in β1 tubulin (Table 2) exhibits clear heat sensitivity for hyphal growth (Kamada et al., 1990). In a previous study, intragenic suppressor mutants of this mutation had been isolated by taking advantage of the sensitivity (Kamada et al., 1990). We analyzed 15 of the intragenic mutants (Table 1). The 15 mutations all involved additional amino-acid substitutions (Table 3). The locations of suppressor mutations were distributed widely from residue 87 to 358 in the primary sequence of β tubulin. All the suppressor mutations increased or decreased sensitivity to benomyl (Fig. 4). Mutations at residues 171, 173, and 358 were conspicuous in their effects on the sensitivity to benomyl. They caused supersensitivity to benomyl: the mutants were sensitive to 10 μg/ml of benomyl, to which the wild type was resistant (data not shown).

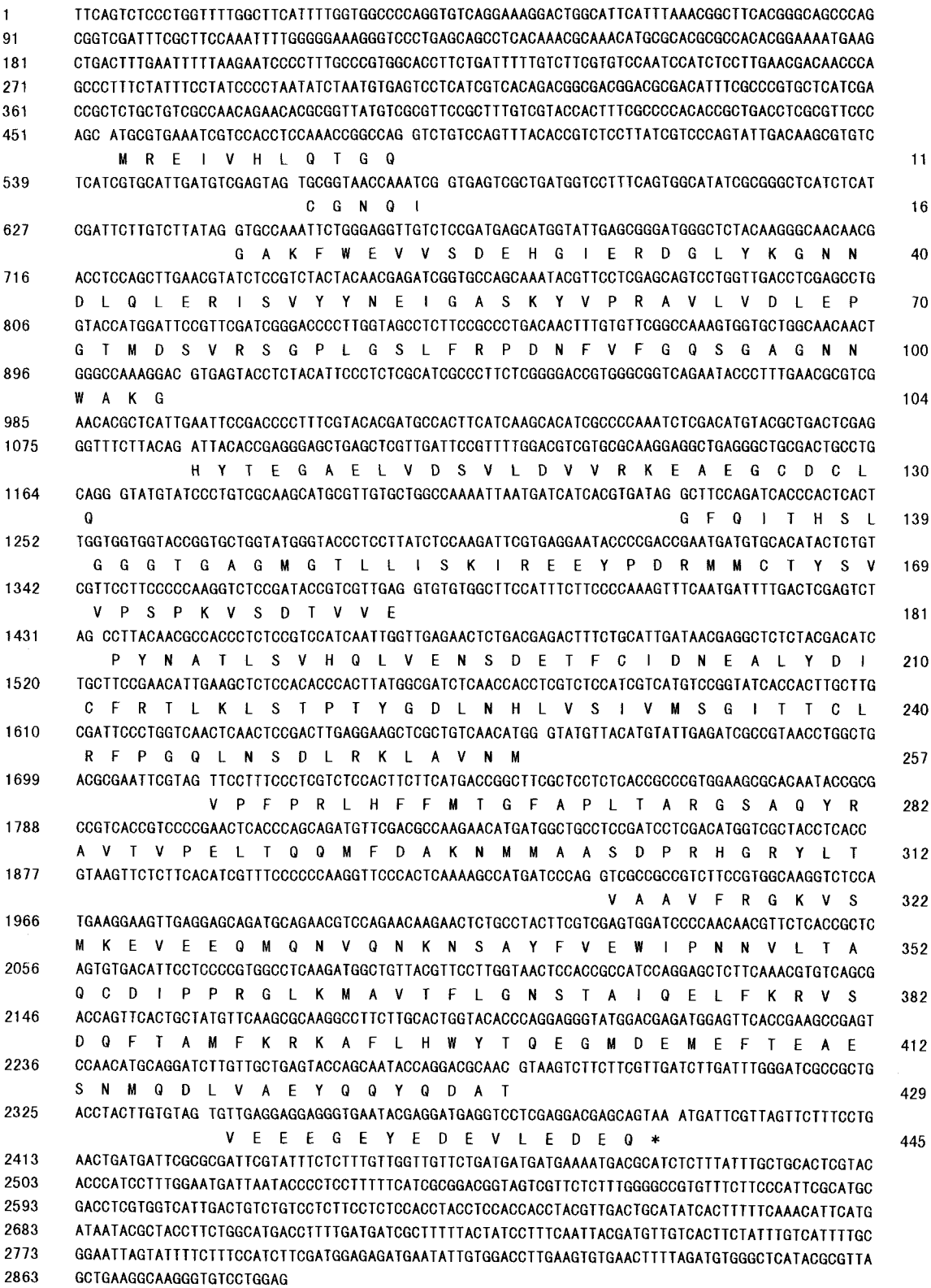

Fig. 2. Nucleotide and predicted amino-acid sequence of the wild-type β1-tubulin gene of *C. cinereus*.

Table 2. Amino-acid substitutions in 16 β1-tubulin mutants.

Allele	Mutation	Amino-acid alteration	Amino-acid residue altered
benA-8	TTC→CTC	Phe→Leu	200
benA-49, 120, 140	TTC→TCC	Phe→Ser	200
benA-82	ACC→ATC	Thr→Ile	107
benA-150	TTC→TTA	Phe→Leu	20
benA-154	CAC→CTC	His→Leu	6
benA-193, MR3, MR4	CTC→TTC	Leu→Phe	350
benA-215	GAG→AAG	Glu→Lys	198
benA-330	TAC→TGC	Tyr→Cys	50
benA-MR1	CAG→AAG	Gln→Lys	134
benA-MR2	TAC→CAC	Tyr→His	36
benA-MR5, MR6	GAA→GAT	Glu→Asp	3

Table 3. Amino-acid substitutions in 15 intragenic suppressor mutants of BEN193.

Allele	Mutation	Amino-acid alteration	Amino-acid residues altered
benA-193R1, 193R5	GAT→GAA	Asp→Glu	177
benA-193R2	TTG→TCG	Leu→Ser	215
benA-193R11	CTC→CCC	Leu→Pro	228
benA-193R14	TTC→TCC	Pro→Ser	341
benA-193R15, 193R106	CCC→TCC	Pro→Ser	358
benA-193R17	CCC→CTC	Pro→Leu	173
benA-193R20	CTC→CCC	Leu→Pro	273
benA-193R45	CCC→TTC	Pro→Phe	173
benA-193R56	AAC→ATT	Asn→Ile	89
benA-193R73	CCT→CTT	Pro→Leu	182
benA-193R78	CCT→TTT	Pro→Phe	87
benA-193R84	CCT→CTT	Pro→Leu	87
benA-193R100	CCT→TCT	Pro→Ser	171

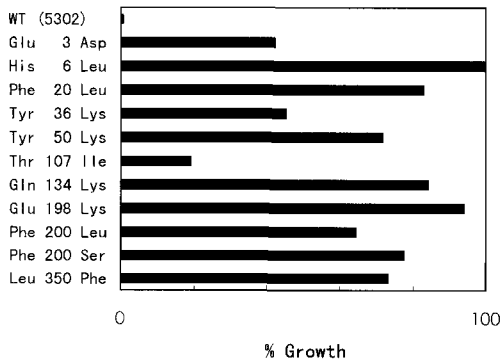

Fig. 3. Effect of amino-acid substitutions in the sequence of β1 tubulin on sensitivity to benomyl. The mutants were examined for growth on 30 μg/ml of benomyl. Abscissa indicates percentages of the colony diameter on agar plates containing benomyl compared to the colony diameter on plates without benomyl. Values are means of three measurements.

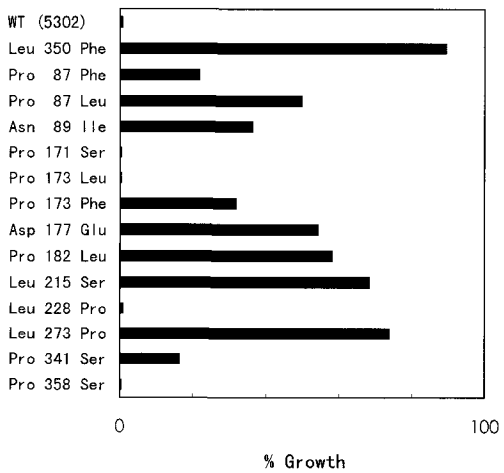

Fig. 4. Effects of additional amino-acid substitutions in the β1-tubulin mutant BEN193, carrying the Leu to Phe amino-acid substitution at residue 350, on sensitivity to benomyl. Abscissa is as in Fig. 3. Values are means of three measurements.

Discussion

In the present study, we have identified 11 different amino-acid substitutions at 10 different residues along the primary sequence of β1 tubulin by analysing 16 β1-tubulin mutants. Of the 10 residues, 5 (6, 50, 134, 198 and 200) are known to be able to confer altered sensitivity to benzimidazole, when substituted, in several fungi including model fungi such as *A. nidulans* (Jung and Oakley, 1990, Jung et al., 1992; Jung et al., 1998) and *N. crassa* (Fujimura et al., 1992), a plant pathogen *V. inaequalis* (Koenraadt et al., 1992), and the basidiomycete yeast *Cryptococcus neoformans* (Cruz and Edlind, 1997). Substitutions at the remaining 5 residues (3, 20, 36, 107 and 350) were found to give rise to altered benzimidazole sensitivity for the first time during the present study. Five (residues 3, 36, 50, 198, 350) of the 10 residues are close to the αβ intradimer interface in an atomic model of the αβ-tubulin dimer determined by electron crystallography (Nogales et al., 1998). This suggests the possibility that benomyl may bind to the αβ intradimer interface like colchicine (Shearwin and Timasheff, 1994). In addition to the 10 residues found in *C. cinereus*, 6 residues, i.e., residues 165 and 257 in *A. nidulans* (Jung and Oakley, 1990; Jung et al., 1998), residues 167, 237 and 250 in *N. crassa* (Fujimura et al., 1994; Orbach et al., 1986) and residue 241 in *S. cerevisiae* (Thomas et al., 1985), have been reported to be able to confer altered sensitivity to benzimidazole when substituted. Four (residues 165, 237, 241, 250) of these 6 residues also appear to be close to the αβ intradimer interface in the atomic model, supporting the above hypothesis. The hypothesis is also consistent with the facts that some of the benomyl-resistant mutants carry mutations in α1 tubulin in *C. cinereus* (Kamada et al., 1990) and that some extragenic suppressors of the benomyl-resistant, β1-tubulin mutant (BEN193) were α1-tubulin mutants (Kamada et al., 1990). In this context, it is interesting to note that missense mutations at residue 350, which is located at the interface, in β2 tubulin of *Chlamydomonas reinhardtii* confer resistance to colchicine (Lee and Huang, 1990).

Six of the 11 different amino-acid substitutions found in β1 tubulin of *C. cinereus* involve gain or loss of an aromatic amino acid (phenylalanine or tyrosine), and three (residues 36, 50, 350) of the six residues are close to the αβ intradimer interface (Table 2). This suggests the possibility that the arrangement of aromatic amino acids in the αβ intradimer interface may be of importance for benomyl to bind. Alternatively, gain or loss of the aromatic amino acids may alter the conformation of β1 tubulin more severely than gain or loss of the other amino acids because of the bulkiness of the side chains of the aromatic amino acids.

It seems unlikely that all of the residue substitutions that confer benomyl resistance lie so close as to interact directly with benomyl, considering the relatively small size of benomyl (MW 290). Some of the mutations may alter the accessibility and/or affinity of benomyl to the hypothetical binding domain(s) by affecting three-dimensional structure of the hypothetical binding domain(s) distantly rather than changing the hypothetical amino-acid residues interacting directly with benomyl. This explanation might be supported by the fact that benomyl resistance in the missense mutation at residue 350 was variously suppressed by mutations at different residues of the β tubulin. Eleven of the 13 suppressor mutations involve gain or loss of proline, which has high potential to cause conformational changes of proteins.

In previous papers, we reported that some β1-tubulin mutations of *C. cinereus* cause, in addition to benomyl resistance, heat sensitivity for hyphal growth and also

Table 4. Phenotypic effects of the 11 amino-acid substitutions in β1 tubulin.

Amino-acid residue altered	Amino-acid alteration	Heat[1] sensitivity	Astral[2] microtubules	Nuclear[1] migration in dikaryosis	Nuclear[3] pairing in dikaryon	Fruiting[1]
3	Glu→Asp	+	ND	+	ND	ND
6	His→Leu	−	+−	−	+−	Eln
20	Phe→Leu	+	ND	+	+	ND
36	Tyr→His	−	ND	−	ND	−
50	Tyr→Cys	+	+−	+	+−	+
107	Thr→Ile	+	+−	+	+−	+
134	Gln→Lys	−	ND	−	ND	−
198	Glu→Lys	+	+	+	+−	+
200	Phe→Leu	+	ND	+	−	+
200	Phe→Ser	−	−	−	ND	+
350	Leu→Phe	−	−	−	−	−

ND, not determined; +, normal; +−, weakly inhibited; −, inhibited; Eln, stipe elongation in fruit body maturation was inhibited.

1) Data on heat sensitivity for hyphal growth, nuclear migration in dikaryosis, and fruiting are from Kamada et al. (1989a).

2) Data on the formation of astral microtubules are from Tanabe and Kamada (1994).

3) Data on nuclear pairing in the dikaryon are from Kamada et al. (1993).

various defects in cellular processes unique to homobasidiomycetes, such as nuclear migration in dikaryosis, nuclear pairing in the dikaryon, the formation of astral microtubules in the spindles during conjugate division in the dikaryon and/or the formation of fruit bodies from the dikaryon (Kamada et al., 1989a, 1989b, 1993; Tanabe and Kamada, 1994). The present study revealed that different amino-acid substitutions at different residues in β1 tubulin cause different cellular defects (Table 4), and that two different amino-acid substitutions at the same residue (residue 200) cause different phenotypes. Among the mutants tested, BEN193 exhibits the most remarkable phenotypes. This mutant exhibits the greatest heat sensitivity for hyphal growth (Kamada et al., 1990), the most severe disturbance of the pairing of the two nuclei (Kamada et al., 1993), and complete inhibition of nuclear migration in dikaryosis and fruiting from the dikaryon (Kamada et al., 1989a). BEN193 is able to assemble spindle microtubules but lacks astral microtubules in the dikaryon (Tanabe and Kamada, 1994). In the present study, it was found that these strong defects are caused by a novel mutation, i.e., Leu to Phe at residue 350.

Inhibition of aster formation seems not to be residue-specific, but it could be caused by different missense mutations in different residues in β tubulin. In *C. cinereus*, aster formation is inhibited in BEN120 and BEN140 (Tanabe and Kamada, 1994), which both carry the Phe to Ser substitution at residue 200 (Table 2), as well as in BEN193 carrying the Leu to Phe substitution at residue 350. It has been reported in *S. cerevisiae* that aster formation is strongly inhibited in the strain carrying three missense mutations at residues 233, 239 and 245 of the β tubulin (Huffaker et al., 1988; Sullivan and Huffaker, 1992).

The alterations in the structure and/or dynamics of microtubules that cause the respective cellular defects in the mutants remain to be clarified.

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