β -Tubulin genes and the basis for benzimidazole sensitivity of the opportunistic fungus *Cryptococcus neoformans*

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The basidiomycete *Cryptococcus neoformans* causes life-threatening infections in immunocompromised patients, and available chemotherapeutic agents are potentially toxic or have limited efficacy. *In vitro*, *C. neoformans* is very sensitive to selected benzimidazole compounds (e.g. albendazole), which act by disrupting microtubules through binding to the β -tubulin subunit. To understand the basis for this benzimidazole sensitivity, we have characterized *C. neoformans* β -tubulin genes and their expression. Analysis of PCR amplification products, genomic and cDNA clones and Southern blots identified two β -tubulin genes. *TUB1* contains seven introns, including one that splits the start codon, and encodes a 447 amino acid protein with >80% identity to most other β -tubulins. A partial sequence of *TUB2* revealed a higher density of introns and a considerably more divergent β -tubulin. The relative expression of *TUB1* to *TUB2* determined by reverse-transcription PCR was about 3:1, consistent with a more limited role for the *TUB2* product. Comparisons of β -tubulin sequences from *C. neoformans* and from various benzimidazole-sensitive and -resistant organisms strongly suggest that the *TUB1* product represents the primary benzimidazole target. This was supported by the identification of a His6 to Gln change in *TUB1* from three independently isolated albendazole-resistant mutants.

Keywords: *Cryptococcus neoformans*, tubulin, benzimidazole, microtubule, drug resistance

INTRODUCTION

Cryptococcus neoformans is a yeast-like fungus distinguished by the presence of a capsule. Unlike the better-known ascomycetous yeasts (e.g. *Candida* and *Saccharomyces* species), *C. neoformans* is a basidiomycete. This group includes a wide range of fungi, including mushrooms and many plant pathogens. *C. neoformans* itself is the most common cause of systemic mycosis in patients with AIDS (Panther & Sande, 1990; Hay, 1991). Meningitis and, less frequently, pneumonia are the typical manifestations of cryptococcosis. *C. neoformans* infections can be effectively treated with amphotericin B; however, toxic reactions to this drug are common. Other agents, such as fluconazole, are

safer but less active (for reviews see Sugar *et al.*, 1990; Powderly, 1992). Thus, there is a need for new anti-cryptococcal agents with lower toxicity and higher efficacy.

Recently, we reported that *C. neoformans* is very sensitive *in vitro* to several derivatives of a group of drugs called the benzimidazoles, which are widely used to treat helminth infections in humans and animals and as systemic fungicides in agriculture (Cruz *et al.*, 1994). Benzimidazoles have been shown to disrupt mitosis in sensitive organisms through binding to the β -tubulin subunit of microtubules (for a review see Lacey, 1988). This interaction has been partially characterized at the molecular level through mutational analysis in model fungi (Thomas *et al.*, 1985; Ohrbach *et al.*, 1986; Jung & Oakley, 1990; Fujimara *et al.*, 1992; Jung *et al.*, 1992; Li *et al.*, 1996). However, the benzimidazole binding site, and the basis for the selective toxicity of these compounds, remain incompletely defined.

Abbreviation: RT-PCR, reverse-transcription polymerase chain reaction.
The GenBank accession numbers for the sequences reported in this paper are G5DB:S:1050831 and 1050832.

Fig. 1. PCR amplification of β -tubulin gene fragments from *C. neoformans* genomic DNA (lane 3). Lane 1, *Hind*III-cleaved lambda DNA size markers (with 0.56 and 2.0 kbp bands indicated). Lane 2, negative control that received H_2O in place of DNA.

Here we present the complete sequence of a *C. neoformans* β -tubulin gene (*TUB1*), a partial sequence of a second more divergent gene copy (*TUB2*), a comparison of their expression at the RNA level and the characterization of three benzimidazole-resistant isolates with mutations in *TUB1*. These data contribute to our understanding of benzimidazole- β -tubulin interaction, and furthermore to our general knowledge of *C. neoformans* molecular biology.

METHODS

Strains. *C. neoformans* strains used in this study were grown in YPD (1% yeast extract, 1% peptone, 2% dextrose) at 30 °C with aeration. *C. neoformans* 1KR is a clinical isolate from the blood of an AIDS patient and was kindly provided by M. Barlett (Indiana University, Indianapolis, IN, USA). *C. neoformans* 14116 was obtained from the American Type Culture Collection.

Isolation of DNA. Genomic DNA was prepared from *C. neoformans* by the procedure of Moore & Edman (1993). Briefly, cells were harvested by centrifugation after overnight growth in YPD and resuspended in a solution of 1 M sorbitol/1% β -mercaptoethanol/1 mg mureinase ml⁻¹ (US Biochemical). Cell lysis and protein precipitation were followed by precipitation of DNA with an equal volume of 2-propanol. DNA was resuspended in H_2O , treated with RNase, and precipitated again with ethanol and 7.5 M ammonium acetate.

Isolation of RNA. An overnight culture of *C. neoformans* 14116 was centrifuged, resuspended in fresh YPD medium and incubated at 30 °C for 3 h. Cells were harvested by centrifugation and total RNA was isolated by a modification of the procedure described by Tolkacheva *et al.* (1994). First, cells were resuspended in cold RNA buffer (0.5 M NaCl, 200 mM Tris/HCl, pH 7.5, 10 mM EDTA, 1 mg mureinase ml⁻¹), and broken by continuous vortexing with glass beads. The resulting lysate was extracted twice with phenol/chloroform/isoamyl alcohol and ethanol precipitated. Total RNA was twice precipitated with 4 M LiCl followed again by ethanol precipitation.

Southern blot analysis. Genomic DNA from strain 14116 was digested with *Eco*RI, *Pst*I and *Hind*III, electrophoresed through agarose and blotted to nitrocellulose (Davis *et al.*, 1986). The blot was hybridized under moderately stringent conditions to a gel-purified *TUB1* gene fragment labelled with ³²P by random priming. The *TUB1* fragment was obtained by PCR amplification of *C. neoformans* 14116 genomic DNA with BT107–BT261R primers (see below). After exposure, *TUB1* probe was eluted by addition of boiling H_2O , and the blot was hybridized to a *TUB2* probe using the same conditions.

Cloning of *C. neoformans* *TUB1* and *TUB2* genes. Amplification by PCR of genomic DNA was the initial approach used to characterize *C. neoformans* β -tubulin genes. Oligonucleotide primers (Katiyar & Edlind, 1994) were employed which correspond to highly conserved β -tubulin sequences adjacent to amino acid residues 107 and 261. PCR was performed as previously described (Katiyar & Edlind, 1994) with *C. neoformans* 14116 genomic DNA. Two products of approximately 0.6 kbp and 0.75 kbp were obtained and purified from a 1% agarose gel following electrophoresis (Fig. 1). These products were cloned into M13mp19 and sequenced. Both sequences were β -tubulin-gene-related, contained introns, and were clearly distinct from each other and from other tubulin genes in the GenBank database. The genes encoding the 0.6 and 0.75 kbp fragments were designated *TUB1* and *TUB2*, respectively. The complete sequence of *TUB1* was determined from a combination of genomic and cDNA clones isolated from lambda libraries by hybridization to a *TUB1* probe (Davis *et al.*, 1986). The genomic DNA library was kindly provided by E. Spitzer (State University of New York, Stony Brook, NY, USA) and the cDNA library by J. Edman (University of California, San Francisco, CA, USA). The sequence was subsequently confirmed from PCR-amplified DNA.

PCR analysis of *TUB1* and *TUB2* expression. The relative expression of *C. neoformans* β -tubulin genes was determined by PCR analysis of reverse-transcribed mRNA (RT-PCR). *TUB1* and *TUB2* cDNAs were prepared using Mo-MuLV reverse transcriptase (New England Biolabs), 3 μ g total RNA, and a primer complementary to nt 1191–1210 of *TUB1*, which are conserved in *TUB2* (Fig. 3). PCR was then performed using this primer in conjunction with primers specific for *TUB1* (nt 946–966) or *TUB2* (nt 51–69, not shown). Control experiments with cloned DNA demonstrated that the two primer pairs were equally specific and sensitive. Three sets of reactions were conducted for 20, 22 and 25 cycles to ensure that amplification during the exponential phase was obtained. Genomic DNA was amplified in parallel for comparison to the cDNA. PCR products were compared by agarose gel electrophoresis and ethidium bromide staining.

Isolation and characterization of benzimidazole-resistant mutants. Mutants were selected by plating 1×10^7 cells from a

fresh overnight culture of strain 1KR on YPD plates containing $2 \mu\text{g}$ albendazole ml^{-1} . Plates were incubated at 30°C for 72 h. Colony formation occurred at a frequency of 1×10^{-6} . Stability of the resistant mutants was tested by subculturing three times without the drug, followed by retesting for albendazole resistance. Genomic DNA was isolated from these mutants and their *TUB1* genes were amplified by PCR, cloned, and sequenced as described above.

RESULTS AND DISCUSSION

Amplification and copy number of *C. neoformans* β -tubulin genes

PCR of genomic DNA with primers representing highly conserved β -tubulin sequences (residues 100–107 and 260–266) generated two distinct fragments of approximately 0.6 and 0.75 kbp (Fig. 1). Cloning and sequencing revealed that both encoded β -tubulin-like proteins, and hence their corresponding genes were designated *TUB1* (0.6 kbp) and *TUB2* (0.75 kbp). To determine gene copy number, the two PCR fragments were used individually to probe a Southern blot of genomic DNA digested with three different restriction enzymes. A single major band was observed in all lanes (Fig. 2), suggesting the presence of only these two genes. In some lanes a second weak band was seen due to cross-hybridization of the *TUB1* probe to the *TUB2* gene, or vice versa.

Analysis of *TUB1* and *TUB2* gene sequences

The *TUB1* partial sequence demonstrated a high degree of similarity to β -tubulin genes from other fungi and animals, while the *TUB2* sequence was relatively divergent (see below). Consequently, *TUB1* was of more immediate interest, and its gene, including flanking regions, was cloned from lambda libraries and sequenced in its entirety (Fig. 3). Analysis of the *TUB1* sequence predicts a 447 amino acid protein. The coding sequence is interrupted by seven introns, at codons 1, 21, 90, 174, 308, 354 and 408. In comparison, the partial sequence of *TUB2* encoding amino acids 108 to 259 (not shown) revealed five introns, at codons 138, 203, 238, 242 and 258; i.e. four more than found in the comparable region of *TUB1*. All introns are only 50–57 nt long, except the first intron of *TUB1*, which is 196 nt. Furthermore, all introns include 5' GTNNGY and 3' YAG splice junction signals.

Analysis of the 5' flanking region of *TUB1* for potential transcriptional control elements revealed a canonical TATA box (TATAAA) 250 bp upstream of the coding sequence (i.e. –250; Fig. 3). Other *C. neoformans* genes have been reported to have TATA boxes at variable positions upstream of the start codon: *URA5* at –342, *MAT α* at –106, and *LAC1* at –539 (Edman & Kwon-Chung, 1990; Moore & Edman, 1993; Williamson, 1994). The wide range of TATA box locations suggests that these may not be true promoter elements. Comparison of the *TUB1* 5' flanking region to the corresponding regions of other *C. neoformans* genes

Fig. 2. Examination of β -tubulin gene copy number in *C. neoformans* by Southern blotting. Genomic DNA was cleaved with *Pst*I (lanes 1 and 4), *Eco*RI (lanes 2 and 5), or *Hind*III (lanes 3 and 6). The blot was hybridized under moderately stringent conditions to purified fragments of *TUB1* (lanes 1–3) or *TUB2* (lanes 4–6) and intentionally overexposed to reveal cross-hybridizing bands. DNA size markers are shown.

revealed a 13 bp sequence at –87 (Fig. 3) which is also present upstream of the dihydrofolate reductase gene (at –162) (Sirawaraporn *et al.*, 1993). Additionally, a 10 bp pyrimidine-rich sequence is directly repeated at –98 and –76. With regard to translational initiation, analysis of the sequences adjacent to the start codon of *TUB1* and 15 other *C. neoformans* genes revealed that 9 of 14 genes including *TUB1* have A at position –3, as in many eukaryotes (Kozak, 1986); the remaining genes have either C (3) or G (2). No polyadenylation signal was readily identifiable in the 3' flanking region of *TUB1*, consistent with earlier findings (Edman & Kwon-Chung, 1990).

Analysis of *TUB1* and *TUB2* protein sequences

The predicted amino acid sequences of *TUB1* and *TUB2* are aligned in Fig. 4 with representative β -tubulin sequences from other organisms. This alignment indicates that *TUB1* encodes a conserved β -tubulin, with 81, 82, and 84% identity to β -tubulins from the ascomycete *Aspergillus nidulans*, human, and the basidiomycete *Schizophyllum commune*, respectively. On the other hand, *TUB2* appears to encode a highly divergent β -tubulin with 71–73% identities to the tubulins above. The presence of a highly divergent β -tubulin along with a conserved β -tubulin has been reported in other fungi (May *et al.*, 1987). In the region of overlap (residues 108–259), there is 73% identity between *TUB1* and *TUB2*.

Both genes demonstrate similar codon usage. There is a strong bias for the use of C in the third position of Phe and Asn codons; specifically, only the AAC codon was used for Asn in both genes, 17 times in *TUB1* and 9 times in the partial *TUB2* sequence.

TyrArgArgLysAlaPheValHisTrpTyrThrGlyGluGlyMetAspGluLeuGluPhe	408
TACAGGCGAAAGGCTTTCGTACATTGGTACACAGGAGAGGGGTATGGATGAGCTTGAATTC	1989
	SerGluAla 411
GTAAGCATCTTGCCATTTTTCGCCGTAAACAGATAAGTAACAATAGTGGTTAGT TCTGAGGC	2049
GluSerAsnLeuGlnAspLeuValSerGluTyrMetGlnTyrGlnGluAlaGlyAlaAsp	431
CGAATCCAACTTCGAGACCTGGTTTCCGAGTACATCGAGTACCCAGGAGGCCCGAGCGGA	2109
AspGluIleTyrAspGluGluIleProIleGluGluGluGluMetEnd	247
AGCGAAATCTATGGTGATGAGGAGATCCCATCTCAGGAGGAGGAGATTAAGGTCTCTC	1169
CATCCCTAATGTTGCCCTTCTACCCACATTTACCCTTTGCATCTGTGCGACCCACCA	2229
CCACAGCGCATATAAGGTCGTTGGGTTTTCGAAGGTTTATCTTATTTCCCGCGCATAT	2289
ATATCCCCCCCCTTTCCTTTTGGCTCACCCGAGGTTGAGTTCTCCGATGTCTCGCTAG	2349
GAGACGGTGTCGTCATATCTGCACATTCITTTCAAGTATTGGTCTCTACAGTCTCTATG	2409
AACCTGTGAGGCGGATG (poly A)	2425

Fig. 3. Nucleotide and predicted amino acid sequences of *C. neoformans* TUB1. The coding region (bold) is interrupted by seven introns. In the 5' flanking region a TATA box and two 10 bp direct repeats are underlined; a 13 bp sequence also present in the promoter region of the *C. neoformans* DHFR gene is double-underlined.

↓

(Q)

TUB1 MREIVHLQTGCGQNGIGAKPWEVVSSEHGQIADGSGYKTTDTQLERINRVYNEAAAGKYV 60
 ScoD.....E.....L.....N.....Q.....S.....I.....G.....N.....
 AniA.....QTI.....G.....LDGS.....V.....N.....S.....L.....M.....F.....SGN.....
 HumA.....I.....D.....DPT.....T.....H.....DS.....L.....TG.....

TUB1 PRAVLVDLEPGTMDSIRGGPLGSLFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVV 120
 ScoI.....V.....S.....G.....A.....
 AniCV.....A.....F.....E.....N.....V.....
 HumV.....S.....F.....QI.....
 TUB2P.....L

↓ ↓

TUB1 RREAERCDLQCGFQITHSLGGTGAGMGTLLISKIREEPFDRMCTTSVVPVSPKVSPTVV 180
 ScoK.....GT.....Y.....
 AniG.....L.....A.....
 HumK.....S.....L.....S.....Y.....I.....N.....
 TUB2QQ.....NS.....S.....VL.....S.....LA.....LG.....Y.....LA.....IF.....E.....

↓ ↓

TUB1 EPYNATLSVHQLVENSEDETFCIDNEALYDICTRLTKLSTPTYGDLNHLVSVMSGVTTC 240
 ScoH.....F.....I.....S.....
 AniH.....M.....N.....S.....A.....
 HumT.....Y.....F.....T.....AT.....
 TUB2V.....T.....N.....I.....C.....N.....VSD.....IQS.....E.....K.....S.....IAK.....T.....F.....T.....

↓

TUB1 RFPGQLNSDLRKLAVNMVFPFRLHFMMVGFAPLTARGSASYFAVTVPELTQQMPDAKNMM 300
 ScoL.....T.....QQ.....
 AniW.....S.....AY.....P.....S.....P.....
 HumA.....P.....S.....QQ.....L.....
 TUB2V.....

TUB1 AASDRPHRGYLTVACYRRGVKSMKEVEDQIQSVQAKNSAYFVEWIPGNISAAQCIDIPPRG 360
 ScoAMP.....E.....M.....N.....N.....VL.....S.....A.....
 AniF.....N.....CSAIF.....MRNI.....S.....QS.....N.....QS.....LS.....
 HumC.....AVF.....RM.....DE.....MLN.....N.....S.....N.....VKT.....V.....

TUB1 LKMSSTFICNSTSIQSLFKRIGEQFSAMYRRKAFVHWYTGEGMDELFSEAESNLQDLVS 420
 ScoR.....V.....LG.....A.....E.....VSD.....T.....FK.....L.....Q.....M.....T.....M.....A.....
 AniG.....E.....V.....D.....T.....F.....L.....M.....T.....MN.....
 HumA.....G.....A.....E.....S.....T.....F.....L.....M.....T.....MN.....

TUB1 EYMQYQEAGADDEIYGDDEEPIEEEE 447
 ScoQ.....D.....TVEE.....GEYE.....VIEDQ.....
 AniQ.....D.....SISEGEEYEA.....EE.....M.....G.....E.....
 HumQ.....D.....T.....EE.....GEFE.....AEE.....VA.....

Fig. 4. Alignment of β -tubulin amino acid sequences from *C. neoformans* (TUB1, TUB2), *S. commune* (Sco; Russo *et al.*, 1992), *A. nidulans* benA (Ani; May *et al.*, 1987) and human (Hum; Lewis *et al.*, 1985). Residues identical to the TUB1 sequence are represented by dots. Arrows indicate residues implicated in benzimidazole activity (6, 165, 167, 198, 200 and 241). The His6 to Gln (Q) mutation identified in 3 *C. neoformans* albendazole-resistant mutants is noted.

Relative expression of *TUB1* and *TUB2*

Using RT-PCR with gene-specific primers, the relative levels of *TUB1* and *TUB2* mRNA were compared. As shown in Fig. 5, after 22 cycles (lanes 8–11) the amplification was still in linear phase; i.e. had not plateaued as in lanes 12–15. From a comparison of lanes 9 and 11, there appears to be approximately threefold less *TUB2* compared to *TUB1* transcript, while in lanes 13 and 15 both transcripts are readily detectable and the difference is approximately twofold. While *TUB2* is clearly transcribed, its lower level, along with its more divergent sequence, suggests a more limited and specialized role for its β -tubulin. Further evidence for this is provided by the analysis below.

Correlation with benzimidazole sensitivity

Mutational studies using model fungi have implicated six β -tubulin residues in benzimidazole activity: His6, Ala165, Phe167, Glu198, Phe200 and Arg241 (Thomas *et al.*, 1985; Ohrbach *et al.*, 1986; Jung & Oakley, 1990; Fujimara *et al.*, 1992; Jung *et al.*, 1992; Li *et al.*, 1996). Comparisons of the *C. neoformans* *TUB1* and *TUB2* sequences to β -tubulin sequences from benzimidazole-resistant and -sensitive organisms (such as those in Fig. 4) strongly suggest that *TUB1* represents the basis for benzimidazole sensitivity. Specifically, Phe200, a residue that has been shown to be predictive of benzimidazole sensitivity in helminths, fungi and protozoa (Katiyar *et al.*, 1994), is present in *TUB1* but not in *TUB2* β -tubulin. Glu198, also highly correlated with benzimidazole sensitivity, is only found in *TUB1*.

Mutations conferring benzimidazole resistance

The premise that β -tubulin, and specifically *TUB1* β -tubulin, represents the basis for *C. neoformans* benzimidazole sensitivity was supported by the identi-

fication of a His6 to Gln mutation in *TUB1* from three independently isolated albendazole-resistant mutants. This residue has been previously implicated in benzimidazole activity in three fungi: *A. nidulans* (Jung *et al.*, 1992), *Trichoderma viride* (Goldman *et al.*, 1993) and *Septoria nodorum* (Cooley & Caten, 1993). Furthermore, binding studies have shown that tubulin from an *A. nidulans* mutant with a His6 to Leu mutation has a lower binding affinity for carbendazim compared to wild-type tubulin (Davidse & Flach, 1977; Jung *et al.*, 1992). This suggests that the resistance conferred by this mutation results from a direct effect on benzimidazole binding and not, for example, an indirect increase in microtubule stability.

All previously characterized fungal mutants were selected for resistance to benzimidazole derivatives substituted at the 2-position with either carbamate (benomyl and carbendazim) or thiazol (thiabendazole) but unsubstituted at the 5-position. Albendazole, on the other hand, is an anthelmintic carbamate derivative with a 5-propylthio group. The fact that mutations in His6 were selected with derivatives both substituted and unsubstituted at the 5-position suggests that His6 does not interact with this side chain. Indeed, the relative effect of the *C. neoformans* mutation on sensitivity was greater for carbendazim (≥ 7 -fold increase in 50% inhibitory concentration) than for albendazole or fenbendazole (3.5-fold increase; data not shown). It would be of interest to further examine the role of β -tubulin residue 6 in benzimidazole binding by site-directed mutagenesis (Li *et al.*, 1996).

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Fig. 5. RT-PCR analysis of the relative expression of *TUB1* and *TUB2*. All lanes included the conserved downstream primer. *TUB1*-specific upstream primer was added to lanes 2, 4, 5, 8, 9, 12 and 13; *TUB2*-specific upstream primer was added to lanes 3, 6, 7, 10, 11, 14 and 15. Template cDNA was added to lanes 5, 7, 9, 11, 13 and 15; genomic DNA was added to lanes 4, 6, 8, 10, 12, and 14; lanes 2 and 3 received H₂O only. PCR was conducted for 20 (lanes 4–7), 22 (lanes 8–11) or 25 (lanes 12–15) cycles. DNA size markers are in lane 1, with selected bands indicated.

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