Structural and functional analysis of genes encoding fork head proteins in *Cryptococcus neoformans*

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The nucleotide sequences of *Cnfkh1* and *Cnfkh2* have been deposited in DDBJ/EMBL/GenBank databases under the Accession Numbers AB117520 and AB117521, respectively.

**Introduction**

*Cryptococcus neoformans* is a human pathogenic yeast causing a fatal disease, meningitis, not only in immunocompromised patients but also in apparently healthy individuals. It is considered to be important to identify genes involved in its pathogenicity in order to overcome the infection of the fungus. We report here structural analysis of two novel genes of *C. neoformans* homologous to *sep1* of *Schizosaccharomyces pombe*, which encodes a transcription factor with a so-called fork head domain. Four possible open reading frames homologous to Sep1p were identified in the genome, and two of them were detected to be expressed by RT-PCR. We cloned cDNAs for the genes to analyze their structures. Their structures in two different serotypes of *C. neoformans* were compared to detect the diversity and conservativeness in different portions of the genes. Heterologous expression study was also conducted in order to see if fork head domain protein genes identified in this study complement *sep1Δ* of *Sch. pombe*.

Key words: *Cryptococcus neoformans*, fork head domain proteins, gene structure, heterologous expression, RT-PCR, transcription factor.
has been almost revealed in *C. neoformans*, yet needs global functional analysis to find out possible drug targets (Heitman et al., 1999). For this purpose, we got interested in genes involved in cell growth and proliferation, because they are essential process of the fungus for its pathogenesis.

In a fission yeast *Schizosaccharomyces pombe*, one of the best genetically understood fungi, a transcription factor Sep1p has been identified and functionally analyzed (Ribar et al., 1997, 1999; Zilahi et al., 2000). It is involved in its normal septum formation and thus cell division of *Sch. pombe*, so the null mutant strain fails to separate from the daughter cells causing hyphal growth (Ribar et al., 1999). Sep1p has a conserved domain of about 100 amino acid residues, called a fork head domain, which is also known as a “winged helix” (Ribar et al., 1997, 1999; Zilahi et al., 2000), of which structure is highly conserved from yeast to human. It has been shown to localize in nuclei, most probably, to regulate the expression of downstream genes to control septum formation and/or cell separation (Zilahi et al., 2000).

Other fork head domain proteins to control cell separation include Fkh1 and Fkh2 in *Saccharomyces cerevisiae* (Hollenhorst et al., 2000; Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000). Among them, Fkh2 is considered to be a component of a ternary transcription factor controlling expression of *SWI5, CLB2* and *ACE2*, which are involved in cell cycle regulation (Pic et al., 2000). Fkh1 is shown to be involved in cell cycle and pseudohyphal growth with overlapping function to Fkh2 (Hollenhorst et al., 2000). *Candida albicans*, an important pathogen of immunocompromised patients, has recently shown to have a fork head domain protein, Fkh2p, regulating its morphogenesis (Bensen et al., 2002).

The accumulating data raised a speculation that fork head domain proteins might play an important role in regulating cell cycle, proliferation, and morphogenesis, which we consider important for pathogenicity of *C. neoformans*. Here, we identified expression of two genes encoding fork head domain proteins, designated as Cnfkh1 and Cnfkh2, and analyzed their structural characteristics. We also compared the structure of those genes from different serotypes of *C. neoformans*. In addition, we asked if those fork head domain proteins complement the developmental defect of *sep1* mutation in *Sch. pombe* by heterologous gene expression study.

**Material and methods**

**Strains and media**

*Cryptococcus neoformans* serotype D strain B-4500 was used in this study. The fungus was preincubated on a potato dextrose agar slant at 30°C before use. For DNA or RNA preparation from *C. neoformans*, YPG medium (1% yeast extract, 1% peptone and 1% glucose) was used, and cells were grown at 30°C with shaking at 150 rpm. For heterologous gene expression studies, *Saccharomyces pombe* strains 0-39 leu1-32 h+ (wild type) and 2-995 sep1::ura4- ura4-D18 leu1-32 h+ , a derivative of sep1 deletion mutant described in Zilahi et al. (2000), were used. After transformation, cells were grown on the solid medium EMMA (Mitchison, 1970) in the presence or absence of 5µg/mL thiamine.

**RT-PCR, cloning, nucleotide sequence analysis**

A total RNA was extracted from *C. neoformans* B-4500 with the TRIZOL LS Reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA was then used for RT-PCR and cloning by using the GeneRacer kit with SS II RT, TOPO Cloning (Invitrogen) according to the manufacturer’s protocol. Primers used for 5' RACE to identify the transcriptional starts of Cnfkh1 and Cnfkh2 were 238-5RACE (5'-TCGGGCTTCCTGACCGCTTTTT-3') and 240-5RACE (5'-AAGATAGACCGCCAAAGGAAGACG-3'), respectively. For 3' RACE, nested primers 238-3RACE-nested (5'-GCGGTAAAGGCGGTTGGTGGACAG-3') or 240-3RACE-nested (5'-AGGAACGAAAAAGGAGAAGAAAG-3') were used following the initial amplification with primers 238-3RACE (5'-GAAAACAGCCGTCAAGGAGCCCGA-3') or 240-3RACE (5'-GCTCTTTCTTGGCCGGCTCTATTT-3'), respectively. At least three individual clones were selected, and nucleotide sequences of the inserts were analyzed with BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (ABI) according to the manufacturer's protocol. Primer sets SEP238.UP (5'-CGGGATCCATGCTGGAGCTTTGACTAA-3') and SEP238.LP (5'-CGGGATCCATGCTGGAGCTTTGACTAA-3') were used to amplify deduced coding sequences of Cnfkh1 and Cnfkh2, respectively. After PCR amplification with the cDNA as template, the fragments were digested with BamHI, and cloned into the BamHI site of pK19 to create pKIS110 and pKIS111, respectively. The inserts were then sequenced to identify the distribution of exons and introns. The information for the structures of Cnfkh1 and Cnfkh2 is deposited in DDBJ/EMBL/GenBank databases.
(accession numbers AB117520 and AB117521). Genomic sequence data of C. neoformans B-4500 and H99 (serotype A) were obtained from C. neoformans Genome Project, Stanford Genome Technology Center (SGTC), funded by the NIAID/NIH under cooperative agreement AI47087, and The Institute for Genomic Research, funded by the NIAID/NIH under cooperative agreement U01 AI48594, and C. neoformans sequencing project, Duke Center for Genome Technology, and the Genome Sequence Centre, BC Cancer Research Centre, respectively. The coding and franking sequences of Cnfkh1 and Cnfkh2 were retrieved from those databases for precise comparison.

Test of effect of overexpression of Cnfkh1 and Cnfkh2 in sep1Δ cells

Cells of wild type 0-39 leu1-32 h+ and 2-995 sep1::ura4 +::ura4::D18 leu1-32 h+ strains were transformed with pREP3X (Forshbury et al., 1993), pKIS113 and pKIS114, according to a method adapted from Okazaki et al. (1990). pKIS113 and pKIS114 were respectively constructed by inserting BamHI fragments of pKIS111 (Cnfkh2) and pKIS110 (Cnfkh1) into the cloning site of pREP3X. Cells were spread out to thiamine (5 µg/mL) containing EMMA plates and incubated at 30°C. Ten to twenty colonies appeared on plates were transferred onto fresh EMMA plates with thiamine. After one day incubation, they were replica plated onto EMMA plates with or without thiamine. After incubation for three days at 30°C, the morphology of cells were visualised by Olympus BH-2 microscope and a DP-70 digital camera and images were captured using its integrated software package.

Results and discussion

The amino acid sequence of a transcription factor SEP1 of Sch. pombe (GenBank Acc. No. U88191) was used for BLAST homology search against C. neoformans genome database of SGTC. Four different parts of the genome were found to have homology to the fork head domain of SEP1. There have been identified four different fork head domain proteins within the genome database of S. cerevisiae (http://genome-www.stanford.edu/Saccharomyces/) and Sch. pombe (http://www.sanger.ac.uk/Projects/S_pombe/). Not only yeast species, filamentous fungi such as Neurospora crassa (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/) and Aspergillus nidulans (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/) are also considered to have four fork head domain proteins. They are all ascomycetes while C. neoformans belongs to basidiomycetes, thus the number of fork head domain proteins per genome might be conserved within fungal kingdom, which will be revealed as the genome sequence projects of other fungal species become completed.

Among four, primers 238-5RACE and 240-5RACE raised amplification of fragments indicating expression of the genes (Fig. 1), which were designated as Cnfkh1 (lane 1) and Cnfkh2 (lane 3), respectively. The other two did not show the amplification of the fragment (lanes 2 and 4). We also tried to amplify 3' ends of the transcripts with primers designed to detect their expression, but none of them gave amplification, however, for Cnfkh1 and Cnfkh2, with nested primers, single fragments were obtained as seen for the 5’-RACE (data not shown). At this moment, we are unable to conclude that the remaining two sep1 homologues are not transcribed at any stage of C. neoformans life cycle. In this study, we isolated RNA sample from only early stationary cells. But the transcription pattern might vary in different conditions, such as media, temperature, pH or growth phase, as morphological transition and cell cycle regulation in C. neoformans were reported to be influenced by various environmental shift (Ohkusu et al., 2001; Yoshida et al., 2001). Further examination with various growth conditions might allow the expression of those homologues.

The fragments amplified with primers 238-5RACE and 240-5RACE were then purified, clo-
ned, and the nucleotide sequences of at least three independent clones from each PCR fragment were determined. The nucleotide sequences were then compared to the genomic DNA sequence to find the transcription initiation points and the distribution of exons and introns. Translational initiation codons (ATG) were chosen to lead the transcription of exons and introns. Translational initiation codons for both genes were identified by 3'RACE. Then, based on the data, translational termination codons for both genes were identified. The sizes of the introns identified in this study vary from 29 to 819 bases, with a mean of 81 bases and 48 bases (WOOD et al., 2002), thus the sizes of the introns identified in this study are much larger than the average of those in Sch. pombe.

The other two within the coding region were 55 and 76 nucleotides, respectively. Cnfkh2 was also interrupted by two introns, which sizes were 79 and 52 nucleotides, respectively, and both were within the coding sequence.

We also identified the transcriptional termination sequences for both Cnfkh1 and Cnfkh2 genes by 3'RACE. Then, based on the data, translational termination codons for both genes were
the bottom of the alignment indicate identical, similar, and less similar residues, respectively, when compared (FHDS-II). CnFkh1 also has a conserved motif for CnFkh2 has two fork head domain signature 2 respectively. Tides consist of 789 and 597 amino acid residues, of which polypeptide deduced amino acid sequences of CnFkh1 and Cn- were performed to find out the structures of those decided. We then designed primers (see Materials and methods) in order to amplify the entire ORFs, and PCR to amplify the entire coding sequences were performed to find out the structures of those genes. Based on the nucleotide sequences of ORFs, deduced amino acid sequences of CnFkh1 and Cn- Fkh2 were identified (Fig. 2), of which polypeptides consist of 789 and 597 amino acid residues, respectively. The PROSITE database search (FALQUET et al., 2002) revealed that CnFkh1 has one, and CnFkh2 has two fork head domain signature 2 (FHDS-II). CnFkh1 also has a conserved motif for fork head domain signature 1 (FHDS-I), although it does not completely match the consensus pattern deposited in PROSITE database (FALQUET et al., 2002). Comparison of the amino acid sequence of fork head domain of CnFkh1 with those of other fork head domain proteins is shown in Figure 3. On the other hand, CnFkh2 has two separate FHDS-IIs but no FHDS-I-like sequence is identified. We surveyed the deposited amino acid sequences in the PROSITE database, but no protein was found to have more than two FHDS-IIs. Thus, this might be the first case to identify a protein with multiple fork head domains.

In addition to fork head domains, FHA (fork-head associated) protein interaction domains have been identified in several other fork head domain proteins. The alignment with multiple fork head domains. The deduced amino acid sequences are also indicated below the corresponding nucleotide sequences. Asterisks indicate positions of identified transcriptional initiation points. Introns are indicated by lower cases.
Fig. 4. Heterologous expression of Cnfkh1 and Cnfkh2 genes in *Schizosaccharomyces pombe*. Wild type strain 0-39 (A–F) and sep1Δ strain 2-995 (G–L) were transformed with pREP3X (A, D, G, J), pKIS113 (B, E, H, K) or pKIS114 (C, F, I, L), and grown under repressive condition (A–C, G–I) or inducing condition (D–F, J–L). Arrows in panels are explained in the text.
recently been recognized as a functionally important structure for a fork head domain protein (DUROCHER & JACKSON, 2002). In S. cerevisiae, it has been shown that the FHA domain of Fkh2p interacts with the phosphorylated Ndd1p to activate cell cycle progression (REYNOLDS et al., 2003). We thus searched through the amino acid sequences of CnFkh1 and CnFkh2 to identify FHA structures. CnFkh1 was found to possess a typical FHA motif, G78, R79, S86, N127 and G128. On the other hand, no conserved motif was identified in CnFkh2. Taken together, CnFkh2 seems to be a quite atypical FKH domain protein whereas CnFkh1 shows more typical structure as a FKH domain protein.

Cryptococcus neoformans is known to be classified into four serotypes, and the pathogenicity is often associated with serotype. Recently, genomes of both serotype A and D strains were sequenced. Based on the data, we compared nucleotide sequences of CnFkh1 and CnFkh2 of two different strains by the CLUSTAL W software (THOMPSON et al., 1994). We made five different comparisons: 1) one thousand nucleotides upstream of the transcriptional start; 2) the transcriptional start to the transcriptional termination point; 3) one thousand nucleotides downstream of the transcriptional termination point; 4) open reading frames; 5) translated amino acid sequences (Table 1). Non-coding regions (1 and 3) are more variable than coding sequences (2 and 4), and open reading frames are more conserved compared to non-coding sequences. The amino acid sequence of CnFkh1 is as well conserved as its open reading frame, while that of CnFkh2 is not, but when similarity of amino acid residues is considered, CnFkh2 is also more conserved in two strains (see 5, in parentheses), again suggesting that the protein sequence is more selected as coding sequence is. We are now underway to analyze the function of those genes in C. neoformans, and its possible involvement in morphogenesis.

We then asked if those fork head protein genes complement sep1 function of Sch. pombe, which also encodes a fork head protein of Sch. pombe. The morphology of cells was altered when pKIS114 was overexpressed in both wild type and sep1Δ cells. A significant portion of cells became extremely long in both cases (Fig. 4F,L). However, the cell shape was also influenced even under the repressive condition (Fig. 4C). The reason for this is still unclear, but it may be because of a strong promoter activity of pREP3X, which allows a little leak of CnFkh1 gene expression to cause this unusual morphological development. It is known (FORBURG, 1993) that the strong nmt1 promoter in pREP3 plasmids does not switch-off completely, and the expression of the cloned gene can provide a phenotype even under repressed conditions if the corresponding protein has a strong effect. Additional experiments using different promoters might answer the question. CnFkh2 gene was also expressed in Sch. pombe wild type and sep1Δ cells (Fig. 4E,K). Compared to CnFkh1, CnFkh2 raised a slighter effect, and only under inducing conditions, about 15% of cells seemed to be dead (see arrows in Fig. 4E,K), but under repressing conditions, there was no noticeable change in morphology (compare Fig. 4 A and B, or G and H). However, sep1Δ was not complemented by either CnFkh1 or CnFkh2, suggesting that the functions of those fork head proteins are different, at least in Sch. pombe.

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References


Table 1. Identity and similarity of nucleotide and amino acid sequences of CnFkh1 and CnFkh2 in two different strains of Cryptococcus neoformans.a

<table>
<thead>
<tr>
<th>Gene</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CnFkh1</td>
<td>80.9</td>
<td>91.3</td>
<td>79.1</td>
<td>93.1</td>
<td>95.6 (96.6)</td>
</tr>
<tr>
<td>CnFkh2</td>
<td>77.7</td>
<td>92.5</td>
<td>78.5</td>
<td>93.3</td>
<td>89.4 (98.1)</td>
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a Regions 1–5 used for comparisons are explained in the text.


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