SI-3

Regulation of Pseudohyphal Growth in *Candida albicans*

1. Introduction

Candida albicans is a frequently encountered fungal pathogen in humans. The virulence properties of this fungus have been attributed to a number of factors including adherence to host cells, secretion of hydrolytic enzymes, and the capacity of morphological transition (*1*). *C. albicans* assumes reversible morphological transitions among budding yeast, thread-like hyphae, and pseudohyphae. Although pseudohyphae vary in shape from attached strings of yeast like cells to long filaments with constriction at the septa and were considered as intermediates between the morphological extremes represented by yeast and true hyphal form (characterized by elongated cells in filaments that lack constrictions at the septa), the developmental process has not been established (*1*). Recently a surprising negative regulator of hyphal growth was discovered by the deletion of *TUP1*, whose disruption led to constitutive hyphal growth in *C. albicans* (*6*). In *S. cerevisiae*, Tup1p acts as a global transcription repressor forming a complex with Ssn6p, and either *tup1* or *ssn6* mutant shows pleiotropic phenotype such as flocculation, temperature-sensitive growth, defects in sporulation, lack of glucose repression and the loss of mating type in α strains, *etc* (*7*). Target of a Tup1p-Ssn6p complex included glucose-repressed genes, a-specific mating genes, hypoxia-induced repressible genes, DNA-damage inducible genes, haploid specific genes and flocculation genes. A distinct upstream DNA-binding protein regulates each these sets of genes, recruited to the promoter with a complex Tup1p-Ssn6p (*7*). Ssn6p, a counterpart of Tup1p, is a representative protein containing tetratricopeptide repeats (TPR) motif of 34 amino acids residues with the consensus $WX_2LGX_2YX_8AX_3FX_2AX_4P$ (X, any amino acids) (*8*). The proteins containing TPR motif like Ssn6p have been found in variety of organisms, may interact with particular target protein and play a wide range of roles in cell cycle regulation, transcription, splicing or protein import (*8*).

2. Results

Since Tup1p functions as a repressor of filamentous growth (*6*), we intended to investigate Ssn6p homolog in *C. albicans* and its relevance to morphogenesis, and isolated a full-length gene, designated *PRF1* (pseudohypha regulating factor), from a *C. albicans* ATCC 10231 genomic library by means of polymerase chain reaction (PCR) product based on a sequence trace of *SSN6* homolog from Standford *C. albicans* sequencing project. The open reading frame of *PRF1* encoded a polypeptide of 1085 amino acids with a calculated molecular mass of 120,403 kDa, and contained 6 CUG codons, which encodes serine in *C.*

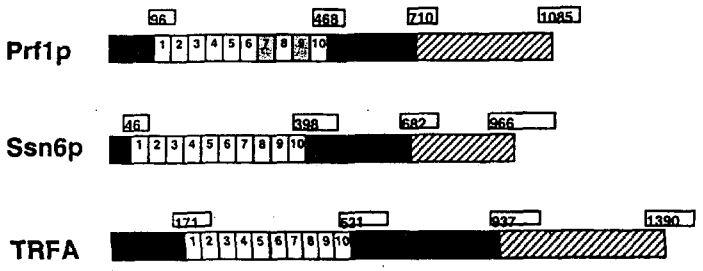

Fig 1. Schematic representation of the primary structures of the Prf1p, Ssn6p and TRFA. Each TPR unit is numbered. TPR-7 and TPR-9 within Prf1p counters to the rules and are somewhat elongated. Glutamine-rich region (Q), proline- and glutamine-rich regions (P, Q), arginine-rich region (N) are represented and putative cAMP- and cGMP-dependent phosphorylation regions are hatched.

albicans but leucine in *S. cerevisiae* and elsewhere (*9*). The predicted amino acid of *PRF1* showed 39% and 34% identities with those of *S. cerevisiae* Ssn6p and *Dictyostelium discoideum* TRFA over entire amino acid sequence, respectively (*10*, *11*). Like Ssn6p and TRFA, Prf1p contained 10 copies of TPR units in tandem. The amino acid sequences of the 10 TPR units within Prf1p were 67% and 54% identities with those of Ssn6p and TRFA, respectively. Outside TPR region, Prf1p contained glutamine-rich and proline-rich regions (frequently observed in transcription factors), and cAMP- or cGMP-dependent phosphorylation sites (Fig. 1).

To see whether the *C. albicans* Prf1p was able to functionally substitute for *S. cerevisiae* Ssn6p, Prf1p was expressed in *S. cerevisiae ssn6* mutant strains. *S. cerevisiae ssn6* mutant cells carrying *C. albicans PRF1* partially restored a repression of glucose, showed a reduced flocculence, grew rapidly, were not temperature sensitive at 37°C, and showed an restored growth in glycerol, indicating that several phenotypic characteristics in *S. cerevisiae ssn6* mutant had been partially complemented by the *C. albicans PRF1*. Furthermore, the overexpressed *C. albicans PRF1* restored repression of a genomic a-specific gene reporter, *STE6-PHO5*. This reporter encodes acid phosphatase under the control of the a-specific *STE6* promoter, which is not expressed in α cells but normally in **a** cells. Consequently, functional equivalence as well as structural similarity between Prf1p and Ssn6p indicates that Prf1p apparently has the possibility of the same molecular function as Ssn6p recruited to DNA by various DNA binding proteins to repress transcription.

To elucidate the roles of *PRF1* in *C. albicans*, two copies of *PRF1* were disrupted by using homologous recombination . Deletion of *PRF1* in *C. albicans* did not affect the viability but promoted pseudohyphal growth in response to temperature. The *prf1/prf1* strains favored

to grow as a yeast form regardless of medium composition below 30°C, but above that temperature, they showed a promoted pseudohyphal growth without transition to true hyphae. Especially, on solid YEPD (yeast extract-peptone-dextrose) medium at 37°C for 3 days, *prf1/prf1* strain changed colony morphology from normal smooth type to rough scallop type, whereas wild-type strain maintained a uniform smooth form. When the *prf1/prf1* mutant and wild-type strains were further incubated on the same condition for 8 days, colonies from wild-type strain developed radial filaments emerging from the edge of them, but the *prf1/prf1* strain showed no emerging filaments and a severely scallop colony morphology (scallop type) (Fig. 2A). The *prf1/prf1* strain reintroduced with one copy of wild-type *PRF1* regained the ability of developing filaments at the edge of colonies. This defect in emerging filaments of *prf1/prf1* cells was also confirmed on solid Spider, Lee's, corn meal agar + 0.33% tween 80, and 10% serum medium. In addition, the *prf1/prf1* cells grown in various liquid media (particularly in YEPD medium) at 37°C favored to exclusively grow as pseudohyphae rather than true hyphae (Fig. 2B).

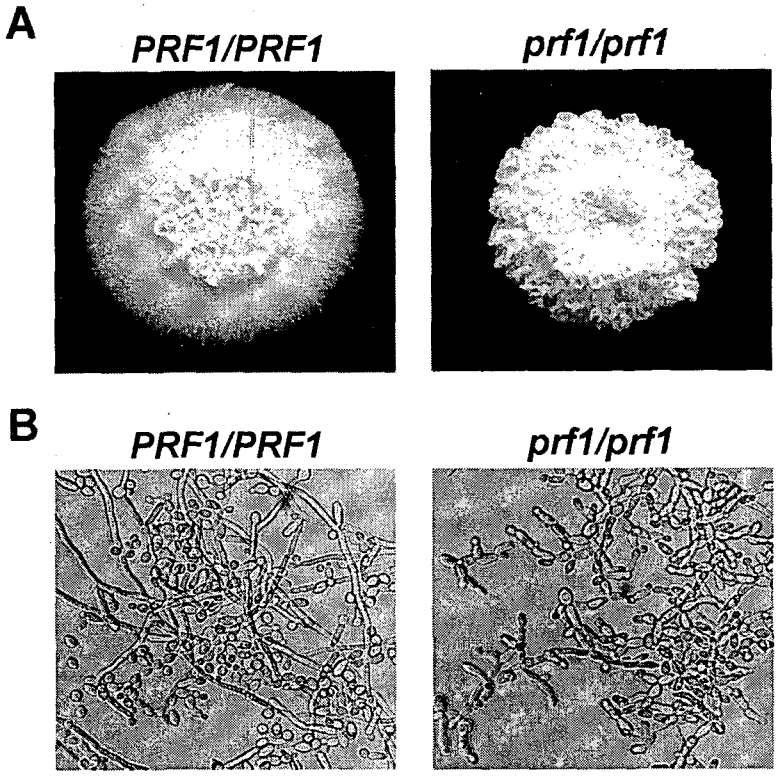

Fig. 2. Colony and cell morphologies of wild-type or *prf1/prf1* cells.

(A) Colonies were grown solid YPD medium at 37°C for 8 days. (B) Wild-type (SC5314) and *prf1/prf1* cells (CH403) were grown in YPD for 12 hours at 37°C, and then were microscopically observed and photographed at 40x with phase optics.

C. albicans morphological transition is controlled by at least two regulatory proteins, Cph1p and Efg1p (*2*, *5*). The mutant in either *CPH1* or *EFG1* shows a suppressed filamentous growth and the mutant in both factors grew exclusively as a yeast form in various conditions (*5*). Thus we constructed a double or triple mutant to investigate the epistatic relations of Efg1p, Cph1p, and Prf1p. Although *cph1/cph1 prf1/prf1* strain was indistinguishable in its morphology characteristics from *prf1/prf1* strain during the incubation on YEPD medium at 37°C for 8 days, the absence of Prf1p in the *efg1/efg1* strain led to a marked reduction in wrinkled colony morphology and pseudohyphal formation. Furthermore, the *cph1/cph1 efg1/efg1 prf1/prf1* triple mutant more detectably decreased the degree of wrinkled colony morphology and pseudohyphae than *efg1/efg1 prf1/prf1* strain. This result suggested that Cph1p, Efg1p and Prf1p pathway make additive contribution to pseudohyphal growth.

A restricted concentration of oxygen or an embedded culture in agar stimulates filamentous growth of *C. albicans*. Even *cph1/cph1 efg1/efg1* double mutant, reported to grow as yeast on conditions almost tested, occurred emerging filaments from each colony under those conditions (*12*). Thus, it seems likely that there is another pathway for filamentous growth of *C. albicans*, which is operative in response to oxygen or physical contact. Very interestingly, the alternative filamentous growth pathway partially depends upon Prf1p, because *prf1/prf1* strains showed the remarkably suppressed filamentous growth under anaerobic or embedded condition. An anaerobic or embedded condition allows wild-type cells to form filaments, which is even more enhanced in *efg1/efg1* strains. The enhanced filamentation in *efg1/efg1* strains is presumably involved in Prf1p, because *efg1/efg1 prf1/prf1* strain generates the slightly winkled colony without emerging filaments. Although *prf1/prf1* strains form filaments to some degrees under anaerobic condition, *efg1/efgl prf1/prf1* cells rarely exhibit any filament and mostly grew as yeasts or some pseudohyphae. To a lesser degree, Cph1p is related to pseudohyphal formation of *efg1/efg1 prf1/prf1* cells even in anaerobic condition, which was confirmed by *cph1/cph1 efg1/efg1 prf1/prf1* mutation. These results indicate that Prf1p is an important contributor to filamentous growth under anaerobic or embedded culture conditions.

The importance of Prf1p in virulence of *C. albicans* is supported by experiments that the *prf1/prf1* cells are avirulent in mouse model system despite their facility of pseudohyphal growth and resistance against oxidative stress. All of mice injected with *prf1/prf1* strain (CH403; *prf1/prf1*) survived to the 30-day end point. Other mice injected with wild-type (SC5314; *PRF1/PRF1*) or revertant (CH404R; *PRF1/prf1*) strain died by 15 day (Fig. 3). The loss of virulence in the *prf1/prf1* strains seemed to result from a defect of growth in serum at 37°C where the cells rarely grew and aggregated, although *prf1/prf1* cells grew almost as rapidly as wild-type cells below 25°C. Thus it seemed likely that Prf1p plays crucial roles in

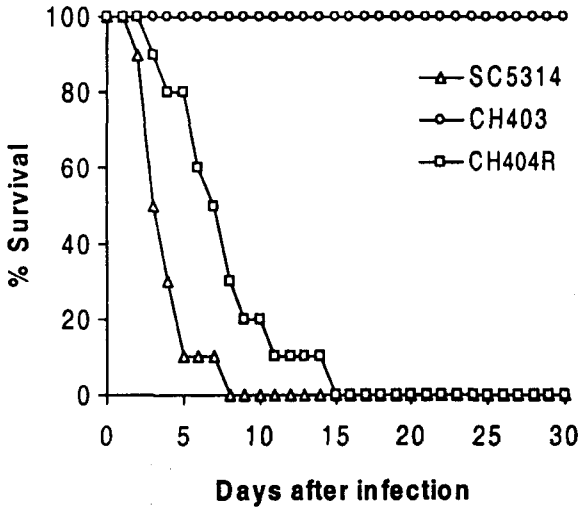

Fig 3. Virulence assay. Each *Candida* strain SC5314 (wild-type, *PRF1/PRF1*), CH403 (*prf1/prf1*) or CH404R (revertant; *PRF1/prf1*) was tested for virulence by injecting 0.1 ml of cell suspension (10^6 cells) into BALB/c mice. Statistical analysis showed survival difference ($p<0.01$) by the Wilcoxon rank sum test.

growth and virulence in blood stream of host cells where temperature was constantly adjusted at 37°C.

3. Discussion

Comparison of *prf1/prf1* cells with *tup1/tup1* cells displayed significantly different characteristics that the *tup1/tup1* cells, for instances, always grew filaments consisting of true hyphae and pseudohyphae regardless of conditions and severely penetrated into agar and develop into long chain filaments under anaerobic or embedded conditions, but the *prf1/prf1* mutant cells promoted pseudohyphal growth and inhibited transition to true hyphae depending on temperature, were more less invasive, and did not form almost emerging filament under anaerobic or embedded condition. However as Ssn6p in *S. cerevisiae*, Prf1p also has a possibility of forming a complex with *C. albicans* Tup1p and of acting as a general repressor controlling morphogenesis, stress response, and metabolic process. This inference is due to the fact that *C. albicans* Prf1p had a degree of amino acid conservation with Ssn6p, the complementation for *S. cerevisiae ssn6* mutant, and some characteristics of *prf1/prf1* cells such as the enhanced growth in glycerol and growth arrest at 42°C, which are shown in *tup1/tup1* cells. The finding that deletion of *PRF1* promoted pseudohyphal growth depending upon condition such as temperature but that of *TUP1* constitutively grew as pseudohypha and true hypha regardless of temperature, indicates that Prf1p and Tup1p each will repress a

specific genes for true hyphae or pseudohyphae. Additionally, it has been reported that filaments of *tup1/tup1* cell are additively affected by Cph1p and Efg1p (*13*). Likewise, pseudohyphal of *prf1/prf1* cells was additively done by them. On the basis of these results, we addressed a simple model for morphological transition in *C. albicans*, which was involved in Cph1p, Efg1p, Tup1p and Prf1p (Fig. 4). According to our presumptive model, Prf1p together with Tup1p represses genes whose expression is required for pseudohyphal growth and Tup1p alone may do genes for true hyphal growth. In contrast, Cph1p and Efg1p additively activated the expression of the genes required for true hyphal and pseudohyphal growth. Efg1p, Cph1p and Tup1p each had independent effect on filamentous growth, regardless of other genes and whether the pathway are entirely separate is not known, but inducible gene expression from several filament-specific genes indicates that the pathways represented by these genes are not arranged in a dependent, linear way (*13*). Thus a putative Prf1p-Tup1p complex is brought to DNA upstream of these genes by postulated DNA-binding proteins whose synthesis or activity is down regulated by hypha- or pseudohypha-inducing environmental conditions. The conditional pseudohyphal growth of *prf1/prf1* cells indicates that Prf1p will not directly interact with the putative DNA-binding proteins but Tup1p will do with them. In absence of Prf1p, a Prf1p-Tup1p complex was not formed and its target genes were expressed, leading to pseudohyphal growth, while Tup1p still repressed genes required for hyphal growth.

Prf1p might take part in one of several redundant pathways through which genes required for pseudohyphal growth can be repressed. After all, this lack of Prf1p seems to promote pseudohyphal growth and block true hyphal growth. It is, however, entirely possible that the pseudohyphal growth of *prf1/prf1* cells may be mediated indirectly via disturbance of

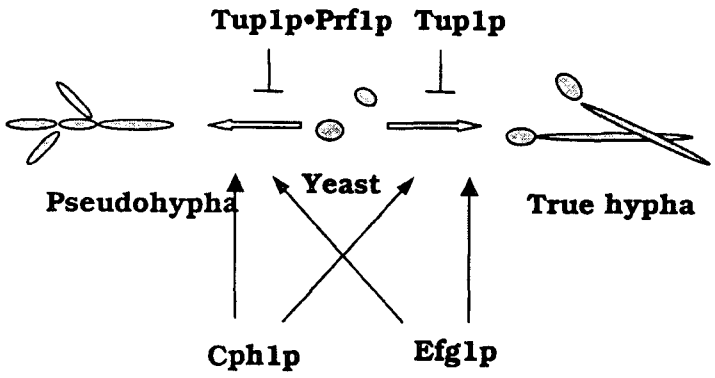

Fig. 4. Model for direction of morphological transition controlled by Prf1p in *C. ablbicans*. A putative Prf1p-Tup1p complex repress pseudohyphal growth but Tuplp alone does true hyphal growth. Cphlp and Efglp may additively activate the morphological transition from yeast to true hypha or to pseudohypha.

nutrient metabolism, alternation of local chromatin structure and genetic machinery abnormality. There have been many efforts to elucidate the precise molecular mechanism of morphological transition in *C. albicans*, but only a few definitive answers are available until now. The presence of Prf1p as a regulator of pseudohyphal growth is thought to be significant, because the lack of Prf1p in *C. albicans* loses the direction of morphological transition of *C. albicans* from yeast to true hyphae, and derived the yeast cells to pseudohyphae. Therefore, the investigation of the functional roles of Prf1p will provide us with many significant clues for unveiling the morphogenesis of *C. albicans*, which are still poorly understood. The finding that the *PRF1* deletion lead to the loss of pathogenicity will finally permit a detailed analysis of virulence determinant as well as the precise identification of putative drug targets in *C. albicans*.

4. Reference

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