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Molecular cloning and characterization of *WdTUP1*, a gene that encodes a potential transcriptional repressor important for yeast-hyphal transitions in *Wangiella (Exophiala) dermatitidis*

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Abstract

The general transcriptional repressor Tup1p is known to influence cell development in many fungi. To determine whether the Tup1p ortholog (WdTup1p) of *Wangiella dermatitidis* also influences cellular development in this melanized, polymorphic human pathogen, the gene (*WdTUP1*) that encodes this transcription factor was isolated, sequenced and disrupted. Phylogenetic analysis showed that the WdTup1p sequence was closely related to homologues in other polymorphic, conidiogenous fungi. Disruption of *WdTUP1* produced mutants (*wdtup1Δ*) with pronounced growth and cellular abnormalities, including slow growth on various agar media and exclusively as a filamentous morphotype in liquid media. We concluded that WdTup1p represents an important switch regulator that controls the yeast-to-filamentous growth transition. However, detailed observations of the filamentous growth of the disruption mutant showed that the hyphae produced by the *wdtup1Δ* mutants, unlike those of the wild type, were arrested at a stage prior to the formation of true hyphae and subsequent conidia production.

Keywords

morphogenesis; gene disruption; pseudohyphae; moniliform hyphae

1. Introduction

Tup1 proteins are multifunctional transcription factors that have nuclear localization signals and bring about the repression of a wide-range of target genes (Neer et al., 1994; Van der Voorn and Ploegh, 1992). A model has been proposed suggesting that Tup1p, in a complex with Ssn6p, is recruited to the promoters of certain genes through interaction with specific DNA-binding sequences (Smith and Johnson, 2000). Molecular and biochemical studies document

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that a Tup1p has three functionally defined domains: an N-terminal domain involved in interactions with Ssn6p (Carrico and Zitomer, 1998; Zhang et al., 2002), a C-terminal seven repeated WD40 domain important for protein interactions and tetramerization (Sprague et al., 2000), and a central domain required for the repression activity of the complex (Fagerstrom-Billai and Wright, 2005; Tzamarias and Struhl, 1994). In spite of their similar structures, repressions of target genes by Tup1 proteins are reported to occur by different molecular mechanisms (Fagerstrom-Billai and Wright, 2005).

The Tup1p of *Saccharomyces cerevisiae* (ScTup1p) has been extensively studied. Numerous reports document that it functions as a general transcriptional repressor responsible for the regulation of diverse genes involved in a wide range of physiological processes, including genes that control glucose and oxygen repression, sporulation and flocculation (Courey and Jia, 2001; Smith and Johnson, 2000). In the dimorphic pathogen *Candida albicans*, CaTup1p also acts as a general transcriptional repressor that functions as an important regulator of filamentous growth (Braun and Johnson, 1997). In addition, CaTup1p is known to control the transcription of many genes involved in virulence, and inactivation of CaTup1p results in mutants that show constitutive filamentous growth and reduced virulence (Braun and Johnson, 1997, 2000). Although orthologs of Tup1p in other fungi have been studied to lesser degrees, most were also found to influence cellular development. For example, in *Neurospora crassa*, the Tup1p ortholog *rco-1* controls the mycelial repression of conidial gene expression, and mediates the repression of genes involved in several other processes (Yamashiro et al., 1996). Similarly, in *Aspergillus nidulans*, the Tup1p ortholog *rcoA* has pleiotropic effects on cellular development and controls asexual development (Hicks et al., 2001). Moreover, in *Penicillium marneffei*, TupA is involved in the repression of both spore and yeast morphogenesis (Todd et al., 2003). Finally, although disruption of *TUP1* does not affect the morphology of yeast and hyphal growth in *Cryptococcus neoformans*, it does reduce the mating frequencies in both *MATa* and *MATα* cells, thereby demonstrating its importance to mating type-dependent phenotypes (Lee et al., 2005).

Morphological transition plays an important role in the pathogenicity of many human fungal pathogens. In *Histoplasma capsulatum* and *Paracoccidioides brasiliensis*, the morphological change from filamentous forms to yeast forms is essential for their pathogenicity (Maresca and Kobayashi, 2000; Da Silva, 1999). However, in *C. albicans*, both yeast and filamentous forms are important for infection (Calderone and Fonzi, 2001; Mitchell, 1998). It is a general belief that yeast-to-hyphal transitions in *C. albicans* facilitate cell adhesion and the penetration of host cells, whereas the hyphal-to-yeast transitions facilitate more rapid growth and the expression of unique gene products that protect the fungus from host clearance mechanisms (Brown and Gow, 1999; Odds, 1994; Whiteway and Bachewitch, 2006). Thus understanding the mechanisms that influence morphotype transitions in fungal pathogens should also shed light on their pathogenesis.

The melanized pathogenic fungus *Wangiella (Exophiala) dermatitidis* has been increasingly detected as an infectious agent of phaeohyphomycosis (Chang et al., 2000; Matsumoto et al., 1994; Schnitzler et al., 1999). In vitro, this polymorphic fungus exists as a budding yeast morphotype when cultured in most rich media (Szániszlo, 2006). However, it can be easily manipulated in the laboratory to carry out cellular transitions to one of its alternate morphologies, which include both a variety of filamentous morphotypes, such as pseudohyphae, moniliform hyphae and true hyphae, and a number of isotropically enlarged cell types variously referred to as sclerotic cells, planate cells and sclerotic bodies (Szániszlo, 2006). Although both pseudohyphae and moniliform hyphae of *W. dermatitidis* have constrictions at cell junctions, the septa formed at those junctions in the latter do not allow cell separations and are identical to those found in true hyphae of ascomycetous molds, as evidenced by each septum having a central septal pore and nearby associated Woronin bodies (Oujezdzsky

et al., 1973). In contrast, the cells of the pseudohyphae of *W. dermatitidis* can be separated without harm by mild sonication or vortexing, because they are composed of end-to-end polarized yeast-like cells that are slow or fail to separate and are devoid of such obviously different septa (Cooper et al., 1984; Grove et al., 1973). In contrast, sclerotic cells of *W. dermatitidis* are isotropically enlarged, nonpolarized morphotypes that may become internally partitioned with one septum to produce planate cells or multiple intersecting septa to produce sclerotic bodies. Because all the growth forms of this polymorphic fungus have also been observed in infected host tissues, *W. dermatitidis* is increasingly used as a model for gaining insights into the biology of the more than one hundred other dematiaceous pathogenic fungi (Brush and Money, 1999; Matsumoto et al., 1994; Szanislo, 2006).

In this report, we describe the cloning of the *TUP1* homolog of *W. dermatitidis* (*WdTUP1*) and the phylogenetic relationship of its encoded ortholog to those of other fungi. Also, we report the results of functional studies that establish that WdTup1p is an important regulator of yeast-to-hyphal conversion in this medically important melanized fungus.

2. Materials and methods

2.1. Strains and media

The *W. dermatitidis* wild-type strain 8656 (ATCC 34100) and *wdtup1Δ* (*WdTUP1::hph*) strains used in this study were routinely cultured in the rich broth medium YPDB (1% yeast extract, 2% peptone, and 2% glucose). When required, agar (1.5% w/v) was added to produce solid medium (YPDA) for strain maintenance and mutant selection. The potato dextrose agar (PDA), and cornmeal agar (CMA) were both purchased from Difco Scientific (USA), whereas the CDNA-starch agar and the CDN and SD agars (CDNA & SDA, respectively) and broths (CDNB & SDB, respectively) were prepared as described previously (Cooper and Szanislo, 1993; Karuppayil and Szanislo, 1997; Ye and Szanislo, 2000). *E. coli* XL1-blue (Stratagen, USA) was grown in Luria-Bertani medium supplemented with 100 µg/ml ampicillin and was used for the subcloning and plasmid preparation. Drug selection plates for isolating *W. dermatitidis* transformants were made by adding hygromycin B (HmB; Sigma, USA) to YPDA at a final concentration of 50 µg/ml for selection of resistance conferred to transformants by the hygromycin phosphotransferase (*hph*) gene.

2.2. PCR amplification, RACE and plasmid construction

Degenerate primers for the amplification of the *WdTUP1* gene sequence were designed on the basis of two highly conserved regions identified from various cloned *TUP1* genes. The primer sequences were as follows: primer Tup1, 5'-ATGGNCAYGARCARGAYAT H-3', primer Tup2, 5'-CGNGARTTYTTRTGNCCYTG-3'. The PCR amplifications were performed as described previously (Liu et al., 2004) and the 600-bp PCR product was cloned into pGEM-T Easy vector (Promega, USA) to produce plasmid pLZ. Primer set: tup1upF, 5'-GGAGCCGAGGACAAGCGG-3' and tup1upR, 5'-TTTGCGGTGGACGG GATG-3' were used to amplify a 450-bp PCR product that was used for Northern analysis. To clone the cDNA of *WdTUP1*, a RACE (rapid amplification of cDNA ends) experiment was carried out using the SMARTTM RACE cDNA Amplification Kit (BD Biosciences, USA). Two overlapping primers pRaceT1, 5'-ACCATGGCAGCTAGAGG CGGTCCTG-3' and pRaceT2, 5'-GGGACCAGTCCGCTTGAGATGCTCT-3' were used to generate the 5'-RACE and 3'-RACE following the user manual provided by the manufacturer. The resulting PCR products were cloned into pGEM-T Easy Vector (Promega, USA) and subjected to sequencing. The final cDNA of *WdTUP1* was amplified using primer pTWF, 5'-GCGGCCTTTTTTCGCCTGTATCGC-3' and pTWR, 5'-GTCTCCCAACCAAAATCCAATTTT-3'. To construct the *WdTUP1* disruption vector, the plasmid pCB1004 (provided by J. Sweigard, DuPont Co), which contains the hygromycin B

(HmB) resistance gene *hph* from *E. coli* and the tryptophan synthase (*trpC*) promoter from *A. nidulans* (Carroll et al., 1994) was first digested with *EcoRV* and *SmaI* and then the linearized plasmid was self-ligated by blunt-end ligation to produce plasmid pHB1400. Subsequently, a 600-bp *XhoI/HindIII* fragment from pLZ, which contains part of the *WdTUP1* gene, was cloned into the corresponding sites of pHB1400 to produce the disruption vector pHB1500.

2.3. Transformations

Transformations of *W. dermatitidis* were carried out by electroporation of intact yeast cells as described previously (Wang et al., 1999). Prior to a transformation, the plasmid pHB1500 was linearized with *PstI*.

2.4. Preparation and analysis of nucleic acids

Isolation of *W. dermatitidis* genomic DNA and Southern blotting were carried out as described previously (Liu et al., 2004; Wang et al., 1999). For RNA isolation, *W. dermatitidis* cells were harvested, washed with deionized water, then dried thoroughly with filter paper and frozen in liquid nitrogen. After the frozen cells were ground thoroughly with a mortar and pestle, total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germany). Northern blotting was then performed by the methods described previously (Wang et al., 1999). DNA fragments used for probes were labeled with αP^{32} dATP by using the DECA prime II DNA labeling kit (Ambion, USA) and hybridizations were carried out using PerfectHym™ Plus Hybridization Buffer (Sigma, USA). The methods for the construction and screening of the cosmid library of *W. dermatitidis* were described previously (Feng et al., 2002; Liu et al., 2004).

2.5. Microscopy

Cellular morphology was documented by using either a Leica DM IRBE Inverted Microscope (Leica Microsystems AG, Wetzlar, Germany) or a Zeiss AX10 microscope (Carl Zeiss, Inc, Germany). The procedures for staining cell wall chitin with Calcofluor (Sigma) or for staining nuclei with DAPI (4', 6'-diamidino-2-phenylindole; Accurate Chemical, USA) have been described previously (Ye and Szanislo, 2000). Colony images were recorded using a Cannon G3 digital camera attached to an Olympus Model SZ dissecting microscope (Olympus Corporation, Japan).

2.6. Production of conidiophores and conidia

To compare conidiophore and conidium production, a thin layer of PDA (1 cm × 1 cm × 2 mm) applied to one surface of a glass slide was inoculated with each strain and then covered with a cover glass (2 cm × 4 cm). After the resulting slide cultures were incubated in moisturized chambers at 26 °C or 37 °C for 10 days, hyphal, conidiophore and conidial development was observed directly with a Zeiss AX10 light microscope (Carl Zeiss, Germany).

2.7. Induction of hyphal and sclerotic morphotypes

For induction of the hyphal morphotypes from yeast cells grown in YPDB, the wild-type and *wdtup1Δ* strains were plated onto CDNA-starch with or without the addition of 50 μM NH₄⁺ (or 0.1% yeast extract as the nitrogen source). Colony morphologies were then noted after 8 days of growth at 37°C. For sclerotic body induction, CDNB prepared with or without 10 μM Ca²⁺ and adjusted to pH 2.5 or 6.0, was first inoculated with either the wild type or *wdtup1Δ* strains at a final concentration of 1 × 10⁸ cells per ml. Then after incubation for 8 days, the resulting growth was microscopically observed and the morphotypes noted.

2.8. Sequence analyses

All clones were sequenced at the Institute of Cellular and Molecular Biology of the University of Texas at Austin. The nucleotide sequence of *WdTUP1* was assigned the GenBank accession number [AY608603](#). The deduced amino acid sequence of WdTup1p was used in conjunction with BLAST to query GenBank for homologous proteins. Selected peptide sequences from this search were phylogenetically analyzed. Briefly, after alignment of the selected sequences using ClustalX (version 1.81; Thompson, et al., 1997; Hall, 2001), parsimony analysis was performed employing PAUP* (version 4.0b10; Swofford, 2002).

Results

3.1. Cloning and sequencing of a *WdTUP1* gene fragment

A specific 600-bp PCR product was amplified from *W. dermatitidis* genomic DNA using degenerate primers with designs based on the conserved sequence of Tup1 homologs from *S. cerevisiae* and *C. albicans* (data not shown). After the product was cloned into the pGEM-T vector (Promega, USA), sequence analysis showed it encoded a peptide with high similarity to other Tup1 proteins. Therefore the gene was named *WdTUP1*. After Southern blotting experiments using the PCR product as a probe showed that *WdTUP1* was present as a single copy in the *W. dermatitidis* genome (data not shown) the same probe was used to identify two positive clones by screening a cosmid library. A series of subclonings and sequence analyses of one of them revealed that it contained the full-length *WdTUP1* gene consisting of a single open reading frame of 1,910 bp with a 53-bp putative intron at the 3'-end and encoding a putative protein (WdTup1p) having 619 amino acids with a calculated mass of 67.98 kDa and a pI of 7.2. Amplification of the *WdTUP1* cDNA by RACE, followed by cloning and sequencing, then revealed that the 5' end of the cDNA was present 381 bp upstream from the start codon and was preceded by a pyrimidine-rich sequence, whereas the 3' end consisted of a poly(A) tail that was 255 bp downstream of the stop codon (data not shown). The cDNA sequencing also confirmed the presence of the 53 bp intron in *WdTUP1*. The schematic structure of the deduced amino acid sequence of WdTup1 showed the expected putative N-terminal coiled coil (residues 23-49), the proline-rich region (residues 139-242) and seven WD40 repeats (Fig. 1A). Phylogenetic comparisons of the deduced amino acid sequence of WdTup1p with homologous sequences from a variety of other fungi, as well as with that of a Tup1p ortholog of the cellular slime mold, *Dictyostelium discoideum* used as an outgroup, showed that the Tup1p sequences clustered in accordance with current taxonomic concepts (Fig. 1B). For example, three distinct major groups were noted, in addition to the outlying taxon, *Mycetozoa*. As expected, one branch included the basidiomyceteous yeasts *Ustilago maydis* and *Cryptococcus neoformans*, whereas the other two groups divided the ascomyceteous fungi into clades of yeasts and molds. Curiously, the Tup1p sequence of the budding yeast *Yarrowia lipolytica* appeared less closely related to its orthologs in the members of the Saccharomycetes than to that of the fission yeast *Schizosaccharomyces pombe*. Moreover, the molds were divided between two discrete branches, one containing known perithecial fungi (Sordariomycetes) and the other comprising three different classes that include pathogenic members of the Eurotiomycetes (e.g., *Penicillium marneffei*, *Coccidioides immitis*, and *Aspergillus fumigatus*). Interestingly, *W. dermatitidis* fell within this branch.

3.2. Disruption of *WdTUP1*

The *WdTUP1* gene was inactivated in *W. dermatitidis* by the site-specific integration of the *Pst*I-linearized disruption vector pHB1500. Homologous recombination of pHB1500 in the *W. dermatitidis* genome was predicted to produce two truncated fragments of *WdTUP1* separated by the vector sequence and the *hph* gene (data not shown). Southern analysis, using the 600-bp *WdTUP1* fragment as a probe, of genomic DNA from 12 transformants obtained on YPDA-HmB selection plates and digested with *Bgl*II showed that three had expected band

shifts from 8 kb to 13.4 kb, which confirmed that *WdTUP1* was disrupted in these transformants (data not shown). Subsequent Northern analysis of RNA isolated from the three mutants showed none produced a signal when probed with the *WdTUP1* specific probe (Fig. 2A). In contrast, a *WdTUP1* transcript signal with the expected size of 2.5 kb was clearly detected in the RNA from the wild-type strain (Fig. 2A). Because the probe used in this study detects a sequence upstream of the site into which the disruption vector site-specifically integrated, the absence of the detection of the *WdTUP1* transcript confirmed that the integration had abolished the transcription of *WdTUP1*, thus eliminating the possibility for the translation of its corresponding protein product. The successful disruption of *WdTUP1* in the haploid background also suggested that *WdTUP1* is not essential for viability. However, all efforts to identify complemented mutants were not successful, even though numerous attempts were tried and different complementation vectors were used. We suspect that for unknown reasons the filamentous phenotypes of the resulting *wdtup1Δ* disruption mutants were resistant to transformation by electroporation.

3.3. Disruption of *WdTUP1* produced mutants with pronounced growth and cellular abnormalities

The *wdtup1Δ* mutants showed reduced growth at both 25°C and 37°C. For example, on YPDA, colonies formed by the three disruption mutants were smaller and generally darker than those of the wild-type (Fig. 3A and B). Because all three mutants had identical phenotypes, only the mutant *wdtup1Δ-1* was used in our subsequent and more-extensive studies, which clearly showed that the disruption of *WdTUP1* produced numerous profound effects. For example, stereomicroscopic observations demonstrated that on a variety of agar media, colonies formed by this mutant were consistently smaller and darker than those formed by the wild type at both 25°C and 37°C (Fig. 3). Also, on low NH₄, hyphae-inducing CDNA-starch, the colonies of the *wdtup1Δ-1* mutant (Fig. 4B), unlike those of the wild type (Fig. 4A), produced no aerial hyphae. More detailed microscopic observations revealed that the wild-type colonies mainly consisted of true hyphae as evidenced by the parallel cell wall (Fig. 4C), whereas, the colonies of *wdtup1Δ-1* mutant mainly consisted of yeast, short pseudohyphae, and possibly some moniliform hyphae (Fig. 4D). In addition, when the growth from liquid YPDB cultures was similarly observed, the *wdtup1Δ-1* cells were uniformly filamentous even at very early stages of cell growth (Fig. 5G to I). This filamentous growth then became even more pronounced with the passage of time. For example, by 72 h, almost all the growth of *wdtup1Δ-1* was filamentous and large hyphal microcolonies were very common (Fig. 5J to L). Furthermore, almost all the filamentous growth produced by *wdtup1Δ-1* was multibranching in a manner that suggested that each hyphal microcolony was formed by the growth and multiple branching of one or few starting cells. Although individual budding yeast of *wdtup1Δ-1* were very rare in the YPDB cultures and there was almost no increase of colony forming units, even after long term shaken growth and vigorous vortexing of the culture, the cell mass did increase over time as evidenced by the increased turbidity and more cell aggregates in the culture (data not shown). Eventually (after 7 days), cell lysis occurred and cell aggregates disintegrated and became cell debris (data not shown). Based on the above observation we determined that the hyphal units were of the moniliform type and were physically connected by simple septa and thus not separable at cell-cell junctions in the manner of cells of pseudohyphae. In spite of that, the filamentous growth in the liquid culture never advanced beyond the moniliform hyphal stage to form a true hyphal morphotype. DAPI staining showed that each individual cell in the hyphae had one nucleus (Fig. 5I and L) and Calcofluor staining for chitin showed that the primary staining sites were at septal regions, however, sometimes lateral cell wall areas were also inordinately stained (Fig. 5H and K). Taken together, these data suggested that the disruption of *WdTUP1* results in mutants that constitutively produce filamentous growth of the moniliform hyphal type, which does not progress to the production of true hyphae.

3.4. Disruption of *WdTUP1* abolished the mutant's ability to form conidiophores and conidia

Because the *wdtup1Δ* mutant remained in the moniliform hyphae stage and could not advance to true hyphae formation, one possible consequence of this would be its loss of the ability to form conidiophores and conidia. Support for this suggestion was provided by finding that even after 10 days of incubation at 25°C or 37°C on the conidia-inducing medium PDA, the *wdtup1Δ-1* mutant had produced no conidiophores and conidia (Fig. 6B and D), whereas the wild type had produced many of both (Fig. 6A and C).

3.5. Disruption of *WdTUP1* did not affect the mutant's ability to undergo yeast-sclerotic body transition

Because *W. dermatitidis* is also capable of nonpolarized growth, we investigated whether the *WdTUP1* disruption affected the production of the sclerotic morphotypes. The results showed that when the *wdtup1Δ-1* mutant was grown under the pH 2.5 condition that induces the sclerotic morphotypes in the wild type strain, it formed those morphotypes just as well as the wild-type did (Fig. 7).

3.6. Transcription analysis of *WdTUP1*

Northern blotting showed that under yeast growth conditions, an abundance of *WdTUP1* transcript was detected in the RNA from the wild-type *W. dermatitidis* (Fig. 2B), the level of which was reduced dramatically when *W. dermatitidis* was grown under a condition that induces hyphal growth (Fig. 2B). This result suggested that under this condition, less *WdTUP1* was transcribed and thus less WdTup1p was produced, thereby reducing the repression of hyphal growth. The net result was more hyphae formed under this condition. This notion is consistent with the proposed role of *WdTUP1* as a repressor of hyphal growth in *W. dermatitidis*. Moreover, the mRNA level of *WdTUP1* was reduced to an undetectable amount in the RNA of cells grown under conditions that induced the sclerotic morphotypes (Fig. 2B), indicating that *WdTUP1* was probably not directly involved in this process.

4. Discussion

The cloning and sequencing of the *WdTUP1* gene of *W. dermatitidis* showed that its deduced gene product had high identity with other Tup1 proteins of various fungi, and shared similar domain architecture. Considering that *W. dermatitidis* is a conidiogenous, filamentous mold, which is also vegetatively polymorphic, it was not surprising to find that WdTup1p showed closest phylogenetic relationships with the derived proteins of other filamentous, conidiogenous fungi.

The disruption of *WdTUP1* in *W. dermatitidis* resulted in mutants that grew less vigorously on various solid media and produced colonies that lacked aerial hyphal growth on hyphae-inducing agar media. However, in liquid media, the *wdtup1Δ* mutant produced exclusively filamentous and branching microcolonies. The appearance of the cells in the filaments of the microcolonies suggested that each probably developed from one or a few original cells. Furthermore, the resulting filamentous growth started at a very early stage of development and became dominant as cultures aged. We also found that when grown on either the hyphae-inducing agar media or in the liquid medium tested, the colonies of the *wdtup1Δ* mutant consisted of hyphae that more resembled moniliform hyphae than pseudohyphae. Although the moniliform morphotype appeared similar superficially to the pseudohyphae of the *C. albicans tup1Δ* mutant (Murad et al., 2001), because it too had constrictions at regions of septum formation, further study suggested that the *wdtup1Δ* filamentous morphotype more resembled moniliform hyphae than pseudohyphae because of its resistance to cell separations at septal regions by votexing. However, because the moniliform hyphae of *wdtup1Δ* did not subsequently produce true hyphae we concluded that the mutation led to a developmental arrest

that inhibited the transition of the moniliform hyphal segments to true hyphae in the manner which invariably occurs during yeast-to-hyphal transition in the wild-type strain of *W. dermatitidis* (Oujezdsky et al., 1973; Szaniszló, 2006).

As a potential general transcriptional regulator, WdTup1p may be involved in the regulation of many processes in *W. dermatitidis*. For example, the *WdTUP1* disruption mutant showed increased sensitivity to 0.8 M KCl compared with the wild-type *W. dermatitidis* (data not shown), which suggested that *WdTUP1* plays a role in a stress response pathway in a manner similar of that reported with *C. neoformans* (Lee et al., 2005). Despite its potential role in many processes, we decided during this study to focus on investigating the specific role of WdTup1p in controlling the morphological transitions of its many morphotypes because each may differentially enhance its pathogenicity. Our results suggested that under normal yeast growth conditions, WdTup1p specifically repressed the yeast-to-filamentous morphological transitions, as evidenced by the phenotype demonstrated by the disruption mutant that showed constitutive filamentous growth. Support for this concept was also provided by the fact that the transcription level of *WdTUP1* was maintained at a minimal level under the condition that induces hyphal growth, as indicated by our Northern analysis (Fig. 2). Our previous morphological studies showed that once *W. dermatitidis* cells passed a certain development stage, they commit to either filamentous or sclerotic form growth depending on the growth condition they encounter (Szaniszló, 2006). However, in this study, we found that WdTup1p probably was not involved in the yeast to sclerotic body transition because the disruption of *WdTUP1* did not affect this process. Furthermore, the disappearance of the *WdTUP1* transcript under the growth condition required for this transition suggested that once a cell was committed to that morphogenetic path, there was no further need to maintain the inhibition of the filamentous growth. This in turn suggested that WdTup1p was no longer needed with the result that its transcription decreased as shown by the Northern analysis (Fig. 2).

It is clear that a plethora of roles are played by Tup1p transcription factors in fungi, and both similarities and differences have been shown in terms of the cellular morphogenetic processes they regulate (Lee et al., 2005; Todd et al., 2003). Those roles in turn suggest that although the Tup1p proteins of different fungi have similar protein sequences, functionally they behave very differently. For example, two different models have been proposed in order to interpret the molecular function of Tup1p in control of cellular morphogenesis in *C. albicans* and *P. marneffei* (Braun and Johnson, 1997; Todd et al., 2003). In the case of *C. albicans*, it was proposed that filamentous growth is the default growth form and CaTup1p acts as a negative regulator that controls this process (Braun and Johnson, 1997). However, with *P. marneffei*, it is instead suggested that the yeast morphotype is the default growth form and PmTupA negatively controls this process (Todd et al., 2003). In contrast, our study of *W. dermatitidis* demonstrated that WdTup1p has even another role in control of morphogenesis and cellular growth. In this case, the *wdtup1Δ* mutant grew exclusively filamentous in a manner that resembles the situation associated with the disruption of the *CaTUP1* gene in *C. albicans* (Braun and Johnson, 1997), but the filamentous growth of *W. dermatitidis* stopped at a stage that did not allow the formation of true hyphae and conidia. In other words, the *wdtup1Δ* mutant was able to initiate the morphological switch from yeast-to- mold growth, but it could not progress from the moniliform hyphal stage to a true hyphal stage, which in turn may be required for the production of conidiophores and conidia.

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Abbreviations

WdTUP1

W. dermatitidis TUP1 gene

RACE

rapid amplification of cDNA ends

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Fig. 1.

Schematic representation of the predicted WdTup1p (A). The broken arrows indicate the positions of the degenerate primers used to amplify *WdTUP1*. The solid arrows indicate the positions of the primers used to amplify the DNA fragment used for the Northern analysis. The positions of the coiled-coil, proline-rich and the seven WD domains are shown. The location of the intron is also indicated. A phylogenetic tree derived from a bootstrapped heuristic analysis of selected sequences homologous to WdTup1p (B). The bootstrap analysis involved 3,000 replicates of sequences from 23 fungal taxa and one Mycetozoa taxon used as the outgroup. The shaded bars to the right of the tree denote the taxa of the various clades. A dotted box indicates the phylogenetic position of WdTup1p. The numbers at each node refer to the percentage of replicates in which all members of a clade descended from the same branch point. The following designations, and their corresponding GenBank number, depict each Tup-like protein from a particular organism used in this figure: Afum, *Aspergillus fumigatus* (XM747562); Aory, *Aspergillus oryzae* (BAE61783); Ater, *Aspergillus terreus* (EAU33421); Calb, *Candida albicans* (XP719068); Cgla, *Candida glabrata* (XP445334); Cglo, *Chaetomium globosum* (EAQ89004); Cimm, *Coccidioides immitis* (EAS37254); Cneo, *Cryptococcus neoformans* (XP570974); Ddis, *Dictyostelium discoideum* (XP640309); Dhan, *Debaryomyces hansenii* (XP456913); Egos, *Eremothecium gossypii* (NP986433); Enid, *Emmeriella nidulans* (AAG28504); Gzea, *Gibberella zeae* (XP382677); Klac, *Kluyveromyces lactis* (XP455547); Mgri, *Magnaporthe grisea* (XP363984); Ncra, *Neurospora crassa* (AAB37245); Pang, *Pichia*

angusta (AAR28449); Pmar, *Penicillium marneffei* (AAL99251); Pnod, *Phaeosphaeria nodorum* (EAT83532); Scer, *Saccharomyces cerevisiae* (AAA35182); Spom, *Schizosaccharomyces pombe* (NP592910); Umay, *Ustilago maydis* (XP759427); Wder, *Wangiella dermatitidis* (AY608603); and Ylip, *Yarrowia lipolytica* (XP500061).

Fig. 2.

Northern blot analyses of *WdTUP1* transcription. Northern blot analysis of wild type *W. dermatitidis* and *wdtup1Δ-1*, *-2*, *-3* disruption mutants (A). Total RNA isolated from the *W. dermatitidis* wild type and from the *wdtup1Δ-1*, *-2*, *-3* mutants, respectively, grown in YPDB at 25°C and hybridized with either *WdTUP1* specific probe or a *WdACT1* loading control. Northern blot analysis of *WdTUP1* transcription under different growth conditions (B). Total RNA was isolated from the wild type *W. dermatitidis* grown in YPDB medium (Yeast: Yeast form), hyphae inducing medium (HF: hyphal form), and sclerotic body inducing medium (SB: sclerotic body form) at 25°C and hybridized with either *WdTUP1* specific probe or a *WdACT1* loading control.

Fig. 3.

Colony morphologies of the wild type and the *wdtup1Δ* strains after incubation for 4 days on YPDA incubated at either 25°C or 37°C (A). Colony morphologies of the wild type and the *wdtup1Δ-1* strain after incubation for 4 days on YPDA, SDA and CDNA-starch at either 25°C or 37°C (B).

Fig. 4. Colony morphologies of the wild type (A) and the *wdtup1Δ-1* disruption mutant (B) grown on CDNA-starch hyphae-inducing medium at 37°C for 8 days. Cellular morphologies of the wild type and the *wdtup1Δ-1* disruption mutant from the above plate (C and D).

Fig. 5. Cellular morphologies and staining properties of the wild type and the *wdtup1Δ-1* disruption mutant cultured in liquid YPDB. The wild type grown at 37°C for 12 h (A to C) or for 72 h (D to F) and the *wdtup1Δ-1* disruption mutant grown at 37°C for 12 h (G to I) or for 72 h (J to L), then fixed with 5% formaldehyde, stained with Calcofluor or DAPI, and viewed by DIC or fluorescence microscopy. All cells are shown at the same magnification.

Fig. 6. Cellular morphologies of wild type and the *wdtup1Δ-1* disruption mutant grown in conidia-inducing PDA slide culture at 25°C (A and B) and 37°C (C and D) for 10 days. All pictures are shown at the same magnification. The arrows indicate conidiophores with conidia.

Fig. 7. Cellular morphologies of wild type (A) and the *wdtup1Δ-1* disruption mutant (B) grown in a sclerotic morphotype-inducing, pH 2.5 medium.