Tcc1p, a Novel Protein Containing the Tetratricopeptide Repeat Motif, Interacts with Tup1p To Regulate Morphological Transition and Virulence in Candida albicans

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The transcriptional factor CaTup1p represses many genes involved in intracellular processes, including the yeast-hypha transition, in the human fungal pathogen Candida albicans. Using tandem affinity purification technology, we identified a novel protein that interacts with CaTup1p, named Tcc1p (Tup1p complex component). Tcc1p is a C. albicans-specific protein with a 736-amino-acid polypeptide with four tetratricopeptide repeat (TPR) motifs in the N-terminal portion. Tcc1p formed a protein complex with CaTup1p via the TPR domain of Tcc1p, independently of CaSsn6p-CaTup1p. The tcc1Δ disruptant showed filamentous growth under conditions inducing the yeast form, as is true of the Catup1Δ mutant. Consistent with this result, the common set of hypha-specific genes was negatively regulated by both TCC1 and CaTUP1. These observations will provide new insights into CaTup1p-dependent transcriptional gene regulation in C. albicans.

Gene-specific transcriptional repression plays an important role in gene regulation of a broad range of organisms, from prokaryotes to higher eukaryotes. For example, gene repression is involved in timely regulation of growth, spatial restriction in differentiation, or responses to environmental changes. In the gene repression system conserved from lower to higher eukaryotes, the assembly of a multiprotein complex termed a “repressosome” has been under a great deal of study (reviewed in reference 9). In the Saccharomyces cerevisiae model, a central core complex contained in a typical repressosome comprises ScTup1p and ScSsn6p (Cyc8), orthologs of which have been found in humans, flies, worms, slime molds, and fungi (reviewed in reference 26).

ScTup1p and ScSsn6p form a protein complex to act as a global repressor in S. cerevisiae. This complex is targeted to promoters by DNA-binding proteins specific for the different classes of repressed genes (26). ScTUP1 was first identified as a mutant that was able to incorporate deoxythymidine (32). Subsequently, a number of distinct phenotypes of the Scsnp1 mutant have been observed, including slow growth, flocculation, loss of mating in alpha strains, poor sporulation, and loss of some aspects of glucose repression. Scsnn6 was first identified as a suppressor mutation of the snf1 mutant: Snf1p is required to derepress the expression of many glucose-repressible genes, including the SUC2 invertase gene, and the Scsnn6 mutation causes constitutive invertase synthesis (8). The Scsnn6 mutations are allelic to the cyc8 mutation (8), which causes increased production of iso-2-cytochrome c (23). Deletion of the ScSsn6 gene results in many phenotypes, most of which are identical to those of the Scup1 mutant. From the viewpoint of protein structure, ScTup1p contains seven copies of a WD40 repeat, named after two amino acids, tryptophan and aspartic acid, commonly found in the repeat and its length. The seven repeats fold into a propeller-like structure, which is hypothesized to bind the homeodomain protein α2 (17). ScSsn6p includes 10 copies of the tetratricopeptide repeat (TPR), comprising the 34 amino acids that make up the basic repeat (10), which is related to the interaction of ScSsn6p-ScTup1p (29) or ScSsn6p-α2 (27). Generally, TPR motifs have been found in a wide variety of proteins from all organisms, from humans to prokaryotes. They mediate molecular recognition and protein-protein interactions. While 22 proteins containing the TPR motif have been found encoded in the yeast genome, only three proteins are involved in transcriptional regulation: Ctr9p, Tfc4p, and ScSsn6p (10). Of the 10 copies of TPRs in ScSsn6p, the first to the third TPR motifs are known to be responsible for ScTup1p binding, whereas combinations of the other TPRs mediate interactions with different repressor proteins specific for each gene family regulated by the ScTup1p-ScSsn6p complex (29). Recently, studies of Tup1-dependent gene repression in Candida albicans have been undertaken by many scientists. C. albicans is an opportunistic fungal pathogen in humans and can cause either systemic or mucosal infection. In immunocompromised patients, infection with this organism can progress to severe systemic invasion, leading to life-threatening circumstances (20, 21). C. albicans is a polymorphic fungus capable of converting its cell shape from budding yeast to a filamentous form, including pseudohyphae and true hyphae. This morphological transition has been strongly associated with pathogenicity (6).

The C. albicans TUP1 gene was first isolated and disrupted by Braun and Johnson (4). Since then, several research groups have reported that Candida Tup1p represses hypha-specific...
genes (HSGs) under conditions inducing the yeast form, as suggested by the exclusive filamentation of the gene disruptant. CaTup1p may require the DNA-binding protein CaNrg1p for the repression of hypha-specific genes in a pathway that promotes yeast form growth, because the 

\[ \text{C. albicans} \]

\text{CaSSN6} with respect to morphogenesis regulation. the existence of a CaTup1p-binding partner other than any genes that were upregulated by Ca

\[ \text{excellent study based on DNA microarray analysis by Garcia-} \]

\text{gene disruptants are definitely different (12, 14). A recent, \}

\text{digm. However, the phenotypes of the CaSSN6 homozygous strains used in this study. Cells were grown in yeast-peptone-dextrose (YPD; \}

\[ \frac{1}{2} \text{M} \text{yeast nitrogen base without amino acids [Difco], } 2\% \text{ glucose, CSM-Ura [Qiagen Inc.], or SD-AU} \]

\text{(the same as SD-Ura except using CSM-Arg-Ura [Qiagen Inc.] instead of CSM-Ura) with shaking to induce the yeast form or in YPD (adjusted to pH 7.2) plus 10\% serum or Spider medium (1\% mannitol, 1\% Difco nutrient broth, 0.2\% K}_2\text{HPO}_4} \]

\text{at 37°C with shaking to induce hyphae. The rate of growth was measured by determining the optical density at 600 nm using a model TN-1500 Biophotorecorder (Advantec, Japan). For filamentous growth on the solid medium, strains were grown for 7 days at 37°C on 10\% cell serum solidified with the addition of 2\% agar or at 30°C on Spider medium solidified with the addition of 1.4\% agar. Escherichia coli XL1-Blue and cloning vectors pUC18 and pUC19 were used for DNA manipulation. General recombinant DNA procedures were performed as described by Sambrook and Russell (24). C. albicans was transformed by the method described by Umeyama et al. (30). An Applied Biosystems model 3100 automated capillary sequencer was used for nucleotide sequencing.}

\text{Northern hybridization and quantitative real-time reverse transcription (RT)-PCR were performed as previously described (13). All primers used for the amplification of Northern probes and real-time PCR are listed in Table S1 in the supplemental material.}

\text{Immunostaining was performed as described previously (30). Microscopic observation was performed using a conventional fluorescence microscope (model IX81; Olympus, Japan) equipped with a model DP70 digital camera (Olympus, Japan).}

\text{Animal experiments were performed as described previously (30). For each group, five male CD-1 (ICR) mice aged 4 weeks (Charles River, Japan) were inoculated with 10^3 CFU by intravenous injection. Kaplan-Meier survival curves were compared using the log rank test. A } P \text{ value of } <0.05 \text{ was considered significant.}

\text{Plasmid and strain construction. All primers used in this study are listed in Table S1 in the supplemental material. (i) p3HA-ARG4 vector. The p3HA-ARG4 plasmid was designed to fuse a protein with three tandem repeats of the hemagglutinin (3xHA) tag at the C terminus and to contain the ARG4 marker. A KpnI-SacI fragment digested from plasmid pRS-Arg4Spc1 (32) was cloned into the KpnI-SacI sites of pUC18 to yield pUC18-ARG4. Then, a 3.8-kb HindIII-PvuI fragment containing the ARG4 marker of pUC18-ARG4 and a 3.2-kb HindIII-PvuI fragment containing the 3xHA tag and the ACT1 terminator of p3HA-ACT1 were ligated to yield p3HA-ARG4. (ii) pMyC-SAT1 and p3HA-SAT1 vectors. The p3MyC-SAT1 plasmid was designed to facilitate the constitutive expression of a protein fused with a three tandem repeat of the Myc (3xMyc) tag at the C terminus and to contain a nourseothricin resistance marker, SAT1. To generate the DNA fragment 3xMyc-A, which encodes the N-terminal portion of the tag sequence, two oligonucleotides, 3xMyc-1 and 3xMyc-4, were annealed by boiling them for 3 min and then allowing them to cool to room temperature. To generate the DNA fragment 3xMyc-B, which encodes the C-terminal portion of the tag sequence, two oligonucleotides, 3xMyc-2 and 3xMyc-3, were annealed by the above-described method. The two DNA fragments 3xMyc-A and 3xMyc-B were then ligated, gel purified, and inserted into the Xhol-Sph1 sites of pFLAG-AC1T (31) to yield p3MyC-AC1T (30). A HindIII-Pst fragment digested from plasmid pSF51A (22) was cloned into the HindIII-Pst sites of pUC18 to yield pUC18-SAT1. Then, a 2.9-kb HindIII-PvuI fragment containing an SAT1 marker of pUC18-SAT1 and a 3.2-kb HindIII-PvuI fragment containing a 3xMyc tag and an ACT1 terminator of p3MyC-AC1T were ligated to yield p3MyC-SAT1. p3HA-\}

\text{ACT1 was used instead of p3MyC-AC1T to yield p3HA-SAT1. (iii) p6HF-Met3 vector. The p6HF-Met3 plasmid was designed to facilitate the conditional expression of a protein fused with a six-histidine–FLAG (HF) tag at the C terminus. A 5.6-kb Xhol-EcoRI fragment containing the MET3 promoter of p3HA-MET3 (30) and a 1-kb Xhol-EcoRI fragment containing the HF tag and the ACT1 terminator of p6HF-AC1T (16) were ligated to yield p6HF-Met3. (iv) TCC1 disruption. Gene disruption of TCC1 was performed using a method similar to that described previously (13). Briefly, two fragments, disTCC1-A and disTCC1-B, were amplified using primers disTCC1-1 and -2 and disTCC1-3 and -4, respectively, and used as a flanking homology region for a gene disruption cassette. The PCR-amplified disruption cassette containing an hph200-URA3-hph200 or ARG4 marker was transformed into the TUA4 arg4-\}

\text{ura3} \text{ strain (hph200 represents a 200-bp portion of hph). Finally, both alleles of the TCC1 locus were replaced with hph200 and ARG4, yielding strain TCC03. Direct- colony PCR and genomic PCR were performed to verify the strain construction in each step. (v) TCC1 revertant. For a complementation test, the DNA fragment TCC1comp was amplified from TUA4 chromosomal DNA using primers disTCC1-1 and TCC1comp-C. A mixture of the amplified DNA fragments TCC1comp and fragTCC1-HF (used for HF tagging, as described below) was introduced into TCC103 to generate TCC107, in which one allele has the wild-}

\text{type open reading frame tagged with His6-FLAG at the C terminus and the other allele is replaced by an ARG4 marker. At first, as a negative control, the DNA fragment TCC1comp-nega-C was amplified using primers TCC1comp-nega5' and TCC1comp-C and TUA4 chromosomal DNA as a template. Next, the DNA fragment TCC1comp-nega was amplified using primers disTCC1-1 and TUA4arg4. TCC1comp-A and TUA4arg4, and genomic PCR was performed to verify the strain construction in each step. (vi) TUP1, SSN6, and NRG1 disruptions. Gene disruption of TUP1, SSN6, and NRG1 was performed in a manner similar to that described above. A primer set consisting of disTUP1-1, -2, -3, and -4 or disNRG1-1, -2, -3, and -4 for the TUP1 or the NRG1 gene, respectively, was used for the creation of a disruption cassette. A disruption cassette for SSN6 was amplified using 120-mer-long primers, disSSN6-5’ and SSN6-3’. Both alleles of each of the TUP1, SSN6, or NRG1 genes were replaced with the ARG4 marker and a Ura-blaster cassette, hph200-URA3-hph200 (for SSN6) or his200-URA3-his200 (for TUP1 and NRG1), in strain TUA4, to generate the homogenous mutant TUP102, SSN602, or NRG102, respectively. SSN602 cells were plated on 5-fluoroorotic acid-containing medium to isolate the ura-\}

\text{segregants (SSN603). Direct-colony PCR and genomic PCR were performed to verify the strain construction in each step. (vii) HGC1 disruption. Single or double disruptions of HGC1 and/or TCC1 were performed using a method similar to that described above. Briefly, for the first allele, the two fragments disHGC1-A and disHGC1-B were amplified using primers disHGC1-1 and -2 and disHGC1-3 and -4, respectively, and used as flanking homology regions for a gene disruption cassette with p220-URA3 (30). The PCR-amplified disruption cassette containing the C220-URA3 marker was transformed into the TUA4 arg4-\}

\text{ura3} \text{ strain or TCC103 (tcc1A), yielding HGC101 or HGC111, respectively. Then, strain HGC101 and HGC111 were}
| Strain   | Parent | Genotype                                      | Reference |
|----------|--------|-----------------------------------------------|-----------|
| CAI4     | SC5314 | ura3Δ::imm434::ura3Δ::imm434                  | 11        |
| TUA4     | CAI4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 | 16        |
| TUA6     | TUA5   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/ARG4 RP10::p3HA-ACT1 | 13        |
| TCC103   | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 tcc1Δ::hph200/tcc1Δ::ARG4 | This study |
| TCC106   | TCC103 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TCC1ΔMeta-T7094::tcc1Δ::ARG4 | This study |
| TCC107   | TCC103 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TCC1-HF::tcc1Δ::ARG4 | This study |
| TUP102   | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 Catup1Δ::hisG200-URA3-hisG200/Catup1Δ::ARG4 | This study |
| NRG102   | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 Catrg1Δ::hisG200-URA3-hisG200/Catrg1Δ::ARG4 | This study |
| SSN602   | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 Cassn6Δ::hph200-URA3-hph200/Cassn6Δ::ARG4 | This study |
| SSN603   | SSN602 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 Cassn6Δ::hph200/Cassn6Δ::ARG4 | This study |
| HGC101   | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 lgc1Δ::C220-URA3/HGC1 | This study |
| HGC102   | HGC101 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 lgc1Δ::C220/HGC1 | This study |
| HGC104   | HGC102 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 lgc1Δ::C220-URA3 | This study |
| HGC111   | TCC103 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 tcc1Δ::hph200/tcc1Δ::ARG4 lgc1Δ::C220-URA3/HGC1 | This study |
| HGC112   | HGC111 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 tcc1Δ::hph200/tcc1Δ::ARG4 lgc1Δ::C220/HGC1 | This study |
| HGC114   | HGC112 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 tcc1Δ::hph200/tcc1Δ::ARG4 lgc1Δ::C220-URA3 | This study |
| iTUP1-HF  | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 iTUP1/TUP1-His6p-FLAG | This study |
| iTUP1-HA-ARG4 | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TUP1/TUP1-3×HA-ARG4 | This study |
| iTCC1-HF | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TCC1/TCC1-His6p-FLAG | This study |
| iTCC1-HA | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TCC1/TCC1-3×HA | This study |
| iSSN6-HF | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 SSN6/SSN6-His6p-FLAG | This study |
| iSSN6-HA | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 SSN6/SSN6-3×HA | This study |
| iSSN6-Myc | TUA4   | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 SSN6/SSN6-3×Myc | This study |
| 2TAG-TT  | iTUP1-HF | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TUP1/TUP1-His6p-FLAG TCC1/TCC1-3×HA | This study |
| 3TAG-TTS | 2TAG-TT | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TUP1/TUP1-His6p-FLAG TCC1/TCC1-3×HA SSN6/SSN6-3×Myc | This study |
| D2T05    | SSN603 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 ssn6Δ::hph200/ssn6Δ::ARG4 TUP1/TUP1-3×HA-ARG4 | This study |
| D2T06ACT1 | D2T05 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 ssn6Δ::hph200/ssn6Δ::ARG4 TUP1/TUP1-3×HA-ARG4 RP10::p3HA-SATI-TCC1 | This study |
| DeITCC1VEC | iTUP1-3HA-ARG4 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TUP1/TUP1-3×HA-ARG4 RP10::p6HF-ACT1 | This study |
| DeITCC1-W | iTUP1-3HA-ARG4 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TUP1/TUP1-3×HA-ARG4 RP10::p6HF-MET3-TCC1 | This study |
| DeITCC1-N1 | iTUP1-3HA-ARG4 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TUP1/TUP1-3×HA-ARG4 RP10::p6HF-ACT1-TCC1-N | This study |
| DeITCC1-N2 | iTUP1-3HA-ARG4 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TUP1/TUP1-3×HA-ARG4 RP10::p6HF-ACT1-TCC1-N2 | This study |
| DeITCC1-C1 | iTUP1-3HA-ARG4 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TUP1/TUP1-3×HA-ARG4 RP10::p6HF-MET3-TCC1-C1 | This study |
| DeITCC1-C2 | iTUP1-3HA-ARG4 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TUP1/TUP1-3×HA-ARG4 RP10::p6HF-MET3-TCC1-C2 | This study |
| iTCC1-HA-ARG3 | TUA4 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TCC1/TCC1-3×HA-ARG4 Catup1Δ::SATI1/Catup1Δ::SATI1/Catup1Δ::MET3-Catup1Δ | This study |
| TUP111   | iTCC1-HA-ARG4 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TCC1/TCC1-3×HA-ARG4 Catup1Δ::SATI1/Catup1Δ::MET3-Catup1Δ | This study |
| TUP112   | TUP111 | ura3Δ::imm434::ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 TCC1/TCC1-3×HA-ARG4 Catup1Δ::SATI1/Catup1Δ::MET3-Catup1Δ | This study |
plated on a medium containing 5-fluoroorotic acid to isolate HGC102 and HGC112, respectively. For another allele of HGC1, the DNA fragment containing the 3′ region of TUP1, DNA fragment TUP1-A was amplified with primers TUP1-N and TUP1-SacI-5′, and each DNA fragment was gel purified. A 400-bp portion of CaTUP1 containing an artificial SacI site was amplified by mixing the two DNA fragments (TUP1-A and TUP1-B) and the two primers (TUP1-N and TUP1-400-3′), digested by BamHI and SphI, and then cloned into the BamHI-SphI sites of pCaDis (7). The SacI-digested pCaDis-TUP1 was transformed into a heterozygous strain, TUP111, to generate TUP112. To verify the strain construction, direct-colony PCR was performed, after which the nucleotide sequence of the PCR fragment was confirmed.

**Yeast two-hybrid assay.** The MATCHMAKER two-hybrid system 3 (Clontech) was used for the yeast two-hybrid assay. A DNA fragment corresponding to amino acid positions 1 to 130 of CaTup1p was amplified by primers THTUP1-5′ and THTUP1-3′, using TUA4 chromosomal DNA as a template, digested with BamHI and PstI, and cloned into the BamHI-PstI sites of pGADT7 (Clontech), yielding pGADT7-TUP1-N.

A DNA fragment corresponding to amino acid positions 1 to 475 of the Tcc1p codon, optimized for S. cerevisiae, where two CUG codons were changed to TCG, was obtained by a three-step PCR. First, three DNA fragments were amplified using p6HF-TCC1-N as a template with primers THTCC1-5′ and THTCC1-1-mut-3′, primers THTCC1-1-mut-5′ and THTCC1-1-mut-2′, and primers THTCC1-1-mut-2′ and THTCC1-1-mut-3′, respectively. Each fragment was purified by agarose gel electrophoresis to prevent contamination of the template DNA. A second PCR was performed with two DNA fragments, THTCC1-NT-B and THTCC1-NT-C, and two primers, THTCC1-5′ and THTCC1-1-mut-2′, in the same tube to generate THTCC1-NT-AB. A third PCR was performed with two DNA fragments, THTCC1-NT-AB and THTCC1-1-mut-3′, respectively, to confirm the expected size. The amplified DNA fragments were cloned into the BamHI-PstI sites of pGKT7 (Clontech) to generate pGKT7-TUP1-AB.

As shown in Fig. 4, both pGKT7 and pGADT7 derivative plasmids were simultaneously introduced into S. cerevisiae AH109 (Clontech), and the transformants were checked for the expression of MEL1, which encodes α-galactosidase, according to the manufacturer’s protocol. Plasmids pGADT7-T and pGKT7-53 were supplied as positive controls in the MATCHMAKER two-hybrid system 3 (Clontech).

**Preparation of total cell lysates, purification, and Western blotting.** Cells were collected and disrupted with glass beads in NP-40 buffer (10 mM Tris HCl [pH 8], 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1% NP-40) using Bead Shocker (Yasui Kikai, Japan). After centrifugation at 10,000 × g for 10 min, the supernatant was extracted for Western blotting and purification. Tandem affinity purification was performed as described by Kaneko et al. (16). Western analysis was performed as described by Umezawa et al. (30). Anti-FLAG M2 monoclonal antibody and agarose were purchased from Sigma. Anti-HA F-7 (monoclonal; horseradish peroxidase conjugate), anti-Myc 9E10 (monoclonal; horseradish peroxidase conjugate), and anti-PSTAIRE (polyclonal) antibodies were purchased from Santa Cruz. Anti-HA and anti-Myc agarose were purchased from Sigma, and anti-histone H4 polyclonal antibody was purchased from Upstate.

**Subcellular fractionation.** Cells were harvested and treated to obtain spheroplasts with Zymolyase 100T in Zymolyase buffer (50 mM Tris HCl [pH 7.5], 10 mM MgCl2, 1 M sorbitol, 1 mM dithiothreitol) at 30°C for 40 min with mild shaking. The spheroplast suspension was introduced drop by drop into a beaker containing Ficoll buffer (18% [wt/vol] Ficoll-400, 10 mM Tris HCl [pH 7.5], 20 mM KCl, 5 mM MgCl2, 3 mM dithiothreitol, 1 mM EDTA). The diluted solution was centrifuged at 20,000 × g for 20 min at 4°C, and the supernatants and pellets were used for Western analysis as a cytoplasmic fraction. The resultant pellets were resuspended in Ficoll buffer in a volume equal to the supernatant and used for Western analysis as a nuclear fraction.

**Nucleotide sequence accession number.** The newly determined sequence for TCC1 was deposited in GenBank under accession number AB252688.

**RESULTS**

**Identification of proteins interacting with CaTup1p.** In the budding yeast, S. cerevisiae, Tup1p forms a protein complex with Snf1p to act as a global repressor. Until a few years ago,
It had been believed that *C. albicans* Sn6p would form a complex with CaTup1p to regulate its morphogenesis, on the basis of the *S. cerevisiae* paradigm. However, recent reports (12, 14) have shown that morphological phenotypes and morphology-related gene regulation of the Ca*sn6* mutant barely overlap those of the Ca*tp1* mutant, indicating that CaTup1p and CaSn6p act independently on morphological transition. Therefore, to identify a CaTup1p-binding partner involved in transcriptional repression for a hyphal program other than CaSn6p, we performed TAP. Crude extracts prepared from a strain in which CaTup1p was tagged with the His6-FLAG epitope sequence were subjected to TAP procedures, leading to the detection by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins composing a CaTup1p complex (Fig. 1A). We identified three major proteins of the purified protein complex by peptide mass fingerprinting using matrix-assisted laser desorption ionization–time of flight mass spectrometry. Two proteins approximately 60 kDa and 160 kDa in size were expected to be CaTup1p and CaSn6p, respectively. There is the possibility that a gel band corresponding to CaTup1p includes tagged or native protein. Peptide fingerprinting of the 80-kDa protein demonstrated that this band was presumed to be a novel protein corresponding to CaO19.6734 and Ca19.14026 (http://www-sequence.stanford.edu/group/candida). Since no protein homologous to this novel protein was found in the *S. cerevisiae* genome database (http://www.yeastgenome.org/) or other fungal genome databases, we called Tcc1p the *Tup1p*-complex component. Reciprocally, TAP procedures with strain iTCC1-HF or iSSN6-HF, in which a single genomic locus for *TCC1* or *CaSSN6* was tagged with His6-FLAG, identified CaTup1p as a binding protein (data not shown). At the start, we used the database sequence of CaO19.6734 to construct a C terminus-tagged protein. However, we could not detect a tagged protein by Western blotting. In fact, experimental nucleotide sequencing of a PCR-amplified DNA fragment corresponding to CaO19.6734 revealed that the sequence that we had determined had one base pair insertion compared to the coding sequences of CaO19.6734 in the *Candida* genome database; this frameshift leads to different lengths of the deduced polypeptides (Fig. 1B). Since we were able to detect an appropriate size for the Tcc1p protein epitope tagged at the C terminus based on the newly determined sequences (accession no. AB252688), we used this sequence for further analysis.

On the basis of the nucleotide sequences that we determined, the deduced open reading frame encoded a polypeptide of 736 amino acids with a calculated molecular mass of 80,177 Da. Tcc1p contains four copies of the TPR motif in the N-terminal portion and glutamine-rich regions in the C-terminal portion (Fig. 1C). In general, the TPR motif is involved in conservation of CaSn6p and Tcc1p. (D) Sequence alignment of the TPR motifs of CaSn6p and Tcc1p. The numbering on the right side refers to the position of each sequence in the protein. The TPR consensus sequence (3) is shown below the alignment, with the motif numbering indicated below the sequence. Eight amino acid residues (W/L/F, I/M, G/A/S, Y/L/F, A/S/E, F/Y/L, A/S/L, and P/K/E) show a high frequency of conservation. Boxed residues in the alignment indicate amino acids matching the consensus sequence shown below.

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**FIG. 1.** Identification of a protein encoded by Orf19.6734 as a component of the CaTup1p complex. (A) The CaTup1p complex was purified by tandem affinity purification (− or + TAP-tag) using anti-FLAG and Ni-nitrilotriacetic acid (Ni-NTA) agarose. Protein fractions of crude extracts (lanes 1 to 4) and purified samples (lanes 5 to 8) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by silver staining. Open arrowheads indicate the component of the CaTup1p complex identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry. Yeast cells (Y) of strains CAF2-1 (lanes 1 and 5) and iTUP1-HF (lanes 3 and 7) were grown at 30°C for 4 h in YPD medium (pH 5.6). Hyphal cells (H) of strains CAF2-1 (lanes 2 and 6) and iTUP1-HF (lanes 4 and 8) were grown at 37°C for 4 h in YPD medium (pH 7.2) containing 10% calf serum. M, molecular size marker. (B) Differences in nucleotide sequences between the database sequence (19.6734) and the analyzed sequence (AB252688). The altered nucleotide is indicated as boxed characters. The nucleotide positions of each open reading frame are indicated on the right. (C) Schematic diagram showing the sequence
Tcc1p and CaTup1p were tagged with disparate epitope tags (His6-CaSsn6p, we constructed a strain in which CaTup1p, Tcc1p, and CaSsn6p were expressed under the control of each native promoter in one cell of the 3TAG-TTS strain. Blots for total proteins (a, c, and i) and immunoprecipitated fractions (b, d, f, g, h, j, k, and l) were probed with anti-FLAG antibody (lower panels). Blots for total proteins (upper panels) and purified fractions (middle panels) were probed with anti-HA antibody. Blots for purified fractions were probed with anti-FLAG antibody (lower panels). protein-protein interaction (10). C. albicans Ssn6p also has nine copies of the TPR motif; the amino acid alignment of the TPR motif in the sequences of Tcc1p and CaSsn6p is shown in Fig. 1D with TPR consensus sequences. In addition, although Tcc1p was predicted to be a nuclear protein because it interacted with CaTup1p, it was found to contain neither nuclear localization signals nor nuclear exporting signals. Thus, Tcc1p, which was identified as a CaTup1p-binding partner, is a novel C. albicans-specific protein with a TPR motif.

**Tcc1p and CaSsn6p interact independently with CaTup1p.** To confirm that CaTup1p and Tcc1p bind to each other and to analyze the relationships among CaTup1p, Tcc1p, and CaSsn6p, we constructed a strain in which CaTup1p, Tcc1p, and CaSsn6p were tagged with disparate epitope tags (His6-FLAG, 3×HA, and 3×Myc, respectively) and performed immunoprecipitation, followed by Western blotting. We also constructed three strains, in each of which His6-FLAG-tagged CaTup1p, 3×HA-tagged Tcc1p, or 3×Myc-tagged CaSsn6p (iSSN6-Myc) was expressed under the control of each native promoter. Western analysis confirmed that each protein was expressed with each epitope tag as expected (Fig. 2A, lanes 1 to 4). Cell extracts from each strain were then subjected to tandem affinity purification. Western blotting using anti-FLAG, anti-HA, and anti-Myc antibodies with the immunoprecipitate from each strain demonstrated that Tcc1p-HA and CaSsn6p-Myc were expressed under the control of each native promoter. Western analysis confirmed the expression of each protein with each epitope tag (Fig. 2A, lanes 1 to 4). Cell extracts from each strain were then subjected to tandem affinity purification. Western blotting using anti-FLAG, anti-HA, and anti-Myc antibodies with the immunoprecipitate from each strain demonstrated that Tcc1p-HA and CaSsn6p-Myc were detected in the fraction of strain 3TAG-TTS (Fig. 2A, lane 5), indicating that the CaTup1p binds to Tcc1p and CaSsn6p. When a similar immunoprecipitation using anti-HA antibody agarose was performed, only CaTup1p-HF and Tcc1p-HA were detected and CaSsn6p-Myc was below the detectable level (Fig. 2A, lane 9). Similarly, an immunoprecipitation experiment using anti-Myc antibody agarose showed interaction between CaTup1p-HF and CaSsn6p-Myc and no interaction of Tcc1p-HA (Fig. 2A, lane 13). These reciprocal experiments indicated that CaTup1p-Tcc1p and CaTup1p-CaSsn6p were independent complexes.

To verify this independence more clearly, we investigated Tcc1p-CaTup1p interaction in a Cass6 deletion mutant background. For this purpose, tandem affinity purification was performed using the wild type and Cass6Δ cells expressing Tcc1p-HA and CaTup1p-HF (Fig. 2B). Western blotting using anti-HA antibody revealed that Tcc1p-HA was copurified with CaTup1p-HF, even in the absence of CaTup1p-HF, suggesting that CaSsn6p might have no effect on CaTup1p-Tcc1p complex formation.

The TPR domain of Tcc1p contributes to interaction with CaTup1p. To determine which region in Tcc1p is necessary for CaTup1p binding, we used an immunoprecipitation experiment and a yeast two-hybrid system. For immunoprecipitation, a series of His6-FLAG-tagged deletion mutants of the Tcc1p protein were expressed from the ACT1 or MET3 promoter in the C. albicans cells that contain HA-tagged CaTup1p. By performing TAP, the Tcc1p mutant that contains TPR motifs corresponding to the amino acid positions 1 to 475 (Tcc1-N1) or 1 to 250 (Tcc1-N2) was detected as a protein interacting with HA-tagged CaTup1p (Fig. 3B, lanes 3 and 4, respectively). CaTup1p-HA was not detected in the immunoprecipitated complex with the C-terminal domains of Tcc1p, corresponding to amino acid positions 251 to 736 (Tcc1-C1) and 476 to 736 (Tcc1-C2), although only a small amount of the Tcc1p mutant was present (Fig. 3B, lanes 5 and 6). Even when three times as much crude extract as indicated in Fig. 3B was used for purification, no signals of CaTup1p-HA were detected (data not shown). The faint signals of the Tcc1-N2, Tcc1-C1, and Tcc1-C2 deletion mutants could possibly be due to protein instability. These results suggest that the first two TPR domains of Tcc1p might serve an important role in binding to CaTup1p, although the possibility that the C-terminal glutamine-rich region can bind to CaTup1p could not be denied. To confirm the importance of the TPR domain in the CaTup1p-Tcc1p interaction in vivo, we designed a yeast two-hybrid system. The effect of the repressive activity of CaTup1p was avoided by using the portion of CaTup1p comprising amino acids 1 to 130, which contains no WD repeats, because...
the full-length CaTup1p fused to the Gal4 DNA-binding domain itself could repress the reporter activity. The CaTup1p mutant without this N-terminal portion could not bind to Tcc1p in the above-described immunoprecipitation experiment (our unpublished results). It is important to note that TCC1 contained two CUG codons, which encode serine in C. albicans but leucine in S. cerevisiae (25), in the sequence coding for amino acid positions 1 to 475. To express TCC1 functionally in S. cerevisiae, we changed both these codons into TCG by PCR-mediated mutagenesis, and the N-terminal portion of codon-optimized Tcc1p was fused to the Gal4-activating domain. When the Gal4-binding-domain-fused N-terminal portion of CaTup1p (amino acids 1 to 130) and the Gal4-activation domain-fused N-terminal portion of Tcc1p (amino acids 1 to 475) were simultaneously expressed in a host strain, AH109, that possesses an α-galactosidase MEL1 gene as a reporter, the constructed strain took on a blue color on agar medium containing X-Galactosidase (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Fig. 3C), indicating an in vivo protein-protein interaction. Integrating the results of immunoprecipitation and the yeast two-hybrid assay suggests that the TPR domain of Tcc1p and the N-terminal glutamine-rich domain of CaTup1p contribute to Tcc1p-CaTup1p binding.

Expression profile and nuclear localization of Tcc1p. To determine whether Tcc1p expression alters in response to nutrient depletion, cell cycle toxins, or hyphal induction and whether the expression profile overlaps that of CaTup1p, crude extracts were prepared from yeast or hyphal cells of strain 3TAG-TTS in which CaTup1p, Tcc1p, or CaSsn6p was separately tagged in one cell for Western blotting analysis. Western blotting using anti-FLAG antibody demonstrated that CaTup1p was expressed under all conditions examined (Fig. 4A). Tcc1p-HA was not detected in the G1 phase but was expressed under both yeast and hyphal growth conditions. The expression levels reached a peak when cells were arrested with nocodazole at the G2/M phase. Analysis by anti-Myc antibody Western blotting showed that the profile of the CaSsn6p-Myc expression was similar to that of Tcc1p-HA. Furthermore, we analyzed the CaTup1p protein complex immunoprecipitated from the samples under each condition. Western blotting with Tcc1p-HA and CaSsn6p-Myc showed that the alterations of protein interacting with CaTup1p were similar to the profiles of expression (data not shown). That is, the independent complexes CaTup1p-Tcc1p and CaTup1p-CaSsn6p could exist in similar stages of the cell cycle.

To examine the cellular localization of Tcc1p, CaTup1p, or CaSsn6p, each protein was tagged with three tandem repeats of HA at the C terminus, and cells expressing HA-tagged proteins were immunostained with anti-HA antibody. CaTup1p-HA and CaSsn6p-HA localized in the nucleus as expected (Fig. 4B). Immunostaining with anti-HA antibody also demonstrated nuclear localization of Tcc1p (Fig. 4B). Under conditions that induce hyphal, while obvious nuclear localization of CaTup1p-

FIG. 3. TPR domain within Tcc1p is necessary for binding to CaTup1p. (A) Diagram of Tcc1p TPR motif in the wild type and the deletion derivatives used for interaction assays. (B) Immunoprecipitation of CaTup1p-HA by the Tcc1p deletion mutant. Cells from strains DelTCC1-W (Tcc1-W; lane 1), DelTCC1VEC (vector; lane 2), DelTCC1-N1 (Tcc1-N1; lane 3), DelTCC1-N2 (Tcc1-N2; lane 4), DelTCC1-C1 (Tcc1-C1; lane 5), and DelTCC1-C2 (Tcc1-C2; lane 6) were cultured at 30°C for 4 h in YPD medium (pH 5.6). Crude extracts (lanes 1 to 3, 300 μg; lane 4, 600 μg; lanes 5 and 6, 1,000 μg each) were subjected to tandem affinity purification using anti-FLAG agarose and Ni-NTA agarose. Total protein (upper panel) and purified fractions (middle panel) were probed with anti-HA antibody. The same purified fractions were immunoblotted (IB) with anti-FLAG antibody (lower panel). The signals for the deletion mutants (W, N1, N2, C1, and C2) are indicated by arrowheads, while the 75-kDa nonspecific signal is indicated by an asterisk. (C) The interaction between the N-terminal portion of the codon-optimized Tcc1p and the N-terminal portion of CaTup1p was detected in the yeast two-hybrid system. Cell suspensions of strains harboring pGAD7 vector and pGBKT7 vector plasmids were spotted onto an X-Gal plate assay. Tup1-N is a pGAD7 derivative plasmid that contains the DNA fragment corresponding to amino acid positions 1 to 130 of CaTup1p. Tcc1-N is a pGBKT7 derivative plasmid that contains the codon-optimized DNA fragment corresponding to amino acid positions 1 to 475 of Tcc1p. The pGAD7-T and pGBKT7-53 plasmids, which encode the simian virus 40 large T antigen and the murine p53 protein, respectively, served as positive controls.
HA and CaSsn6p-HA was observed, no signal of Tcc1p-HA was detected (data not shown), suggesting that the accumulation of Tcc1p in the nucleus might be dependent on morphology. To confirm the nuclear localization biochemically, subcellular fractionation was performed. A successful fractionation was verified by Western blotting probed with anti-histone H4 antibody. Tcc1p-HA was detected in both the nuclear fraction and the cytoplasmic fraction (Fig. 4C). To investigate whether the accumulation of Tcc1p in the nucleus depends on CaTup1p, HA-tagged Tcc1p was expressed in a CaTUP1 conditional mutant, in which CaTUP1 lies under the regulation of the MET3 promoter, and subcellular fractionation was performed. The repression of CaTUP1 in the presence of methionine and cysteine was verified by microscopic observation and quantitative RT-PCR of CaTUP1 and ECE1 mRNA (data not shown). In the presence of methionine and cysteine (with the MET3 promoter off), CaTUP1 expression was 3% of that of the wild type and, as a consequence, a hypha-specific gene, HYR1, was elevated significantly. Even if the expression of CaTup1p was depressed, Tcc1p-HA was detected in both the nucleus and the cytoplasm (data not shown), indicating that the nuclear localization of Tcc1p may not depend on CaTup1p.

tcc1Δ disruptant shows cell elongation phenotype. To investigate the cellular functions of Tcc1p in C. albicans, we deleted both copies. If Tcc1p functions as a CaTup1p-mediated transcriptional repressor, phenotypes of the tcc1Δ mutant would at least partly overlap those of the Catup1Δ mutant. The two copies of TCC1 were sequentially replaced with ARG4 and a 200-bp portion of hph (hph200) in strain TUA4. The ability to generate a viable tcc1Δtcc1 null mutant strain indicates that TCC1 is not an essential gene in C. albicans. There are no significant differences between the wild type and the tcc1Δ mutant in their susceptibilities to fluconazole, calcofluor white, sodium chloride, or hydrogen peroxide (data not shown), indicating that TCC1 might not play an important role in drug or stress resistance. To confirm that the loss of the TCC1 function was responsible for any of the observed phenotypes, a PCR-amplified fragment containing a TCC1 complete open reading frame was used to replace the hph200 locus of the tcc1Δ mutant TCC103 to generate the reconstituted strain TCC107, in which Tcc1p is tagged with His6-FLAG at the C terminus. As a negative control strain, a PCR-amplified fragment containing only a 100-bp C-terminal portion of the TCC1 gene was used to replace the hph200 locus of TCC103 to generate null mutant TCC106. Both the null mutant TCC106 and the reconstituted strain TCC107, which have a single copy of URA3 at their respective TCC1 loci, were used for the following experiments to compare phenotypes of morphology and virulence.

The effect of the TCC1 deletion on the phenotype of the tcc1Δ mutant was studied under conditions that promote both yeast and hyphal growth in C. albicans (Fig. 5A). On a serum agar medium that induces hyphal growth, there were no significant differences among the wild type, the tcc1Δ null mutant, and the revertant. On a YPD agar medium adjusted to pH 5, under conditions in which the wild type and the revertant exhibit smooth colonies and the Catup1Δ and Caarg1Δ mutants should show filamentous growth, as reported previously (4, 5, 19), the TCC1 disruptant exhibited rough colonies, indicating more filamentous growth in the disruptant. The filamentous phenotype of the tcc1Δ mutant was enhanced by an alkaline

FIG. 4. Expression profile and nuclear localization of Tcc1p. (A) Western blotting for the detection of Tcc1p-HA. 3TAG-TTS cells expressing CaTup1p-HF, Tcc1p-HA, or CaSsn6p-Myc from each native promoter were used. Each lane was processed using a total cell extract with the following culture conditions: AS, asynchronous cells; Glc, unbudded cells collected in yeast nitrogen base medium (without glucose); HU, synchronized cells with 0.1 M hydroxyurea at the G1/S phase; NOC, synchronized cells with 20 μg/ml nocodazole at the G2/M phase; Ser, hyphal cells cultured in YPD containing 10% serum for 1 or 3 h (represented by 1 and 3); Spi, hyphal cells cultured in Spider medium for 1 or 3 h (represented by 1 and 3). After cells were grown under each condition, Western blotting was performed using anti-FLAG, anti-HA, or anti-Myc antibody. Tcc1p-HA was detected in both the nuclear fraction (lanes 1 and 3) and nuclear fractions (lanes 2 and 4).

(B) Fluorescence microscopy for the detection of Tcc1p-HA. TUA4 cells expressing HA-tagged Tcc1p, CaTup1p, and CaSsn6p were grown overnight at 30°C, inoculated into fresh YPD containing 10% serum for 1 or 3 h (represented by 1 and 3); Spi, hyphal cells cultured in Spider medium for 1 or 3 h (represented by 1 and 3). After cells were grown under each condition, Western blotting was performed using anti-FLAG, anti-HA, or anti-Myc antibody. Tcc1p-HA was detected in both the nuclear fraction (lanes 1 and 3) and nuclear fractions (lanes 2 and 4).

(C) Subcellular fractionation was analyzed by Western blotting using anti-PSTAIRE antibody. Tcc1p-HA was detected in both the nuclear fraction (lanes 2 and 4). To investigate whether the accumulation of Tcc1p in the nucleus depends on CaTup1p, HA-tagged Tcc1p was expressed in a CaTUP1 conditional mutant, in which CaTUP1 lies under the regulation of the MET3 promoter, and subcellular fractionation was performed. The repression of CaTUP1 in the presence of methionine and cysteine was verified by microscopic observation and quantitative RT-PCR of CaTUP1 and ECE1 mRNA (data not shown). In the presence of methionine and cysteine (with the MET3 promoter off), CaTUP1 expression was 3% of that of the wild type and, as a consequence, a hypha-specific gene, HYR1, was elevated significantly. Even if the expression of CaTup1p was depressed, Tcc1p-HA was detected in both the nucleus and the cytoplasm (data not shown), indicating that the nuclear localization of Tcc1p may not depend on CaTup1p.

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We then observed cell morphology in liquid yeast- and hypha-inducing media (Fig. 5B). In the YPD medium that supports yeast growth, cell elongation was observed with tcc1Δ cells, whereas the length of tcc1Δ cells was shorter than the previously reported lengths of Catup1Δ and Canrg1Δ cells (4, 5, 19). Calculation of the axial growth rates (length/width) supported the fact that tcc1Δ cells exhibited the cell elongation phenotype during yeast growth (TUA6, 1.18 ± 0.31 \(n = 204\); TCC106, 2.47 ± 0.85 \(n = 221\); and TCC107, 1.44 ± 0.26 \(n = 206\)). Additionally, tcc1Δ cells did not exhibit a Catup1Δ-like constitutive filamentation without constriction. There were no significant differences between the wild type and the tcc1Δ mutant grown in Spider medium or in serum medium, which induces hyphal growth. The phenotypes described above were restored in the reconstituted strain TCC107, indicating that filamentous phenotypes were caused solely by the deletion of the TCC1 gene.

Effects of TCC1 disruption on the transcription of hypha-specific genes. To determine whether the transcription of HSGs is induced under yeast growth conditions by TCC1 disruption as well as by CaTUP1 or CaNRG1 disruption, we compared the expression levels of HSGs such as HWP1 (28), ECE1 (2), HYR1 (1), and HGC1 (34) by Northern hybridization and quantitative real-time RT-PCR. If Tcc1p served as a transcriptional repressor for HSGs, as does CaTup1p, HSGs would be upregulated by Tcc1p deletion. The wild type, the disruptant, and the reconstituted strain were cultured under yeast or hyphal growth conditions and subjected to Northern blotting analysis (Fig. 6A). A comparison of yeast growth (Fig. 6A, lane 1) and hyphal growth (Fig. 6A, lane 2 or 3) confirmed the successful detection of HSGs. There were no significant differences between the wild type and the tcc1Δ disruptant in the HWP1, the ECE1, or the HGC1 gene expression levels, based on Northern analysis. The expression of HYR1 was decreased by TCC1 deletion under hyphal growth conditions, consistent with previous data indicating that HYR1 mRNA in the Catup1Δ mutant under such conditions was lower than that in the wild type (15). This implies that the Catup1p-Tcc1p complex might regulate the activation or repression of a transcriptional repressor in the HYR1 gene during filamentous growth. However, Northern analysis indicated that the yeast growth conditions did not seem to induce any significant HSGs.

As described above, the filamentous phenotype of the tcc1Δ mutant was less than that of the Catup1Δ or Canrg1Δ mutant (Fig. 5). Therefore, since it remains a possibility that derepression by TCC1 deletion might be weaker than that by CaTUP1 or CaNRG1 deletion, we attempted to detect the expression of HSGs by quantitative real-time RT-PCR, which has a higher sensitivity than Northern analysis. Compared to the results reported so far, the HWP1 and ECE1 mRNA were derepressed in Catup1Δ and Canrg1Δ cells but not in the Cassn6 mutant (Fig. 6B), consistent with the report by Garcia-Sanchez et al. (12). Interestingly, HYR1 mRNA and HGC1 mRNA were derepressed in Catup1Δ and Canrg1Δ cells and even in the Cassn6Δ cells. However, Garcia-Sanchez et al. (12) reported that HYR1 mRNA was not elevated in Canrg1Δ and Cassn6Δ cells and that HGC1 mRNA was not elevated in Cassn6Δ cells. These discrepancies are probably caused by an experimental difference between microarray analysis and quantitative RT-
PCR. These results indicate that \textit{HWP1}/\textit{ECE1} and \textit{HYR1}/\textit{HGC1} might be transcriptionally regulated in a different manner and that CaSn6p may regulate \textit{HYR1} and \textit{HGC1} negatively. Moreover, all HSGs tested were derepressed by the deletion of \textit{TCC1}, but the expression level of the \textit{TCC1} mutant was lower than that of the Ca\textit{tup}1/H9004 or Ca\textit{nrg}1/H9004 mutant (Fig. 6B). Therefore, these results further support that Tcc1p, accompanied by CaTup1p, might function as a transcriptional repressor involved in the morphological transition of \textit{C. albicans}.

The filamentous phenotype of the \textit{tcc1}Δ mutant is restored by \textit{HGC1} disruption. In the previous paragraph, we demonstrated that Tcc1p might repress the expression of the hypha-specific \textit{G}3 cyclin Hgc1p under conditions that induce yeast growth. The concept that \textit{HGC1} acts downstream of CaTup1p regulation has been supported by the previous report that constitutive filamentation is blocked by the deletion of \textit{HGC1} (34). To examine whether Tcc1p is also linked to the function of \textit{HGC1}, we deleted \textit{HGC1} together with \textit{TCC1}. The observation that the \textit{hgc1}Δ single mutant was deficient in hyphal formation (Fig. 7) was consistent with a previous report (34). The double mutant did not show filamentation on a serum medium (Fig. 7), in that the deletion of \textit{TCC1} did not induce filamentation of \textit{hgc1}Δ cells and the deletion of \textit{HGC1} reduced filamentation of \textit{tcc1}Δ cells. On a YPD agar medium, the deletion of \textit{HGC1} eliminated the filamentous phenotype of \textit{tcc1}Δ cells as well as that of Ca\textit{tup}1Δ cells. This reinforced the idea that CaTup1p and Tcc1p might coordinate in regulating the function of \textit{HGC1}, followed by the repression of hyphal formation.

Attenuated virulence in the \textit{tcc1}Δ disruptant. Ca\textit{TUP1} or Ca\textit{NRG1} disruption causes reduced virulence in a mouse model.
of systemic candidiasis (19). To determine whether Tcc1p plays an important role in pathogenicity, mice were intravenously injected with the wild type (TUA6), the tcc1Δ null mutant (TCC106), and the revertant (TCC107) and monitored for survival. We found that mice infected with 10^6 CFU of the tcc1Δ mutant did not die until 31 days postinfection, with a sustained-survival curve, whereas the same-size inocula of the wild-type or the reconstructed strain killed all infected mice within 11 days (Fig. 8). The mean survival times for the mice infected with the wild type, the null mutant, and the revertant were 7.5 ± 3.78 days, 21.2 ± 8.07 days, and 9 ± 2.35 days, respectively (TCC106 versus TUA6, P < 0.01; TCC106 versus TCC107, P < 0.02). This demonstrates that TCC1 is involved in C. albicans virulence and supports, in part, the concept that Tcc1p might function with CaTup1p in C. albicans.

**DISCUSSION**

In this study, we identified proteins that form complexes with CaTup1p, a global transcriptional repressor. Analogous to the S. cerevisiae homolog, CaSsn6p has long been regarded as a CaTup1p-binding partner for mediation of the negative regulation of yeast-hypha morphological transition in C. albicans. However, whether CaSsn6p actually interacts with CaTup1p remains unproven. Furthermore, a recent report (12) in which DNA microarray analysis was performed to compare the wild type and the Cassn6 mutant indicates that genes repressed by CaTup1p do not necessarily correspond to those repressed by CaSsn6p. In other words, it is highly possible that CaSsn6p may not be a CaTup1p-binding partner, at least in the morphological transition of C. albicans. In order to isolate a real binding partner that chiefly regulates morphogenesis, we used the so-called TAP technique, which we previously used for the purification of a septin complex (16). We found that complexes purified by the TAP technique contained CaSsn6p (Fig. 1). Moreover, immunoprecipitation/Western analysis of strains expressing CaTup1p and CaSsn6p tagged with a different epitope demonstrated that CaSsn6p interacted with CaTup1p (Fig. 2). Therefore, we were able to provide evidence that CaTup1p and CaSsn6p of C. albicans formed a protein complex, as did those of S. cerevisiae.

To identify a binding partner that actually regulates the morphology of C. albicans together with CaTup1p, we purified a protein complex including CaTup1p. One of the components contained in the CaTup1p complex was identified as TCC1, a Tup1p-complex component. In addition, we demonstrated that CaTup1p independently interacted with Tcc1p or CaSsn6p. However, Tcc1p and CaSsn6p share common properties and behaviors as described below. Their first similarity is the existence of a TPR domain: Tcc1p contains four copies of the TPR motif, and nine TPRs are located within the C. albicans Snfn6 polypeptide. Since both Tcc1p and CaSsn6p possess the TPR motif, Tcc1p probably interacts with CaTup1p in a mode similar to that of CaSsn6p. Actually, we demonstrated that the TPR domains of Tcc1p are responsible for CaTup1p binding by using immunoprecipitation and yeast two-hybrid analysis (Fig. 3). Their second behavior in common is their expression profiles. The expression levels of Tcc1p and CaSsn6p, which were tagged with different epitopes in one cell, were altered in response to cell cycle toxins and glucose depletion: the expression levels reached a peak when cells were arrested at the G2/M phase and decreased to an undetectable level in the unudded G1 cells (Fig. 4). In addition, the complex formation with CaTup1p in response to cell cycle toxins was no different between Tcc1p and CaSsn6p (data not shown). Their third similarity is localization to the nucleus. Immunostaining with anti-HA antibody demonstrated that Tcc1p and CaSsn6p localize to the nucleus (Fig. 4B), although both were predicted to contain no nuclear localization signals. Whereas subcellular fractionation further supports the nuclear localization of Tcc1p, Tcc1p was also detected in the cytoplasmic fraction. Tcc1p localization to the nucleus was not altered in a CaTUP1-depleted cell, suggesting that Tcc1p localization may not depend on CaTup1p. Since the nuclear localization of Tcc1p was not observed in hyphal cells, shuttling between the cytoplasm and the nucleus would be regulated by some signaling associated with morphology. Despite these common characteristics, Tcc1p and CaSsn6p seem to function differently: the phenotypes of the mutants and the transcriptional control of HWP1 and ECE1 were contradictory between Tcc1p and CaSsn6p. One of the reasons that the functions of Tcc1p and CaSsn6p have different effects is assumed to be because they might require disparate sequence-specific DNA-binding proteins. Nevertheless, derepression of HYR1 and HGC1 mRNA in tcc1Δ and Cassn6a cells might indicate the existence of a common DNA-binding protein. Future global transcriptional analysis using a DNA microarray might give clues for solving the question of why the depletion of each protein resulted in opposing phenotypes, despite the common binding partner, the similar expression profiles, and the nuclear localization. Also, study of a double deletion of TCC1 and CaSSN6 would explain how the two products share roles.

The Catup1Δ null mutant demonstrates constitutive filamentation even under conditions that induce the yeast form (4). The Cassn6Δ homozygous mutant shows a higher rate for phenotype switching (12) and no filamentation on serum or Spider agar medium (data not shown). When both alleles of TCC1 identified in this study were deleted, the disrupted grew in a pseudohyphal form under conditions that induce the yeast form. Obviously, the morphological phenotype of tcc1Δ is more similar to that of Catup1Δ than to that of Cassn6a from the viewpoint of the cell elongation phenotype. Taken together with the results of the immunoprecipitation experiment and morphological observation, it is highly possible that the protein complex consisting of CaTup1p and Tcc1p might behave independently of CaTup1p-CaSsn6p. However, the filamentous phenotype of the tcc1Δ mutant was not as severe as that of the Catup1Δ or Cassn6aΔ mutant, and the tcc1Δ mutant showed a relatively mild cell elongation. This reduced severity is probably associated with the degree of HSG repression. For example, the elevation of derepressed HSG transcription by the deletion of TCC1 was not stronger than that in the Catup1Δ or Cassn6Δ mutant (Fig. 6). In addition, although we did not compare them directly, the virulence demonstrated by the tcc1Δ, Catup1Δ, or Cassn6Δ mutant indicates a decrease or attenuation: all tcc1Δ mutant-infected mice were killed within 31 days (Fig. 8), while almost no mice inoculated with the strain from which CaTUP1 or CaNRG1 was deleted were dead within the time period examined (4, 5, 19). These results suggest that the degree of cell elongation, the increased quantity of HSG transcription, and the attenuated virulence, which are caused by gene disruption, are closely connected to each other. While the Catup1Δ mutant was demonstrated to be more sensitive to hydrogen peroxide
than the wild type (18), the tcc1Δ mutant did not show significant differences in drug or stress resistance (data not shown), enhancing the significance of the effect of Tcc1p on morphogenesis. Moreover, deletion of the CaTup1p-binding partner gene, TCC1, did not result in more severe filamentation than did deletion of TUP1, suggesting that a relationship of CaTup1p and Tcc1p is not as essential for transcriptional repression but that Tcc1p would play a supportive role for the CaTup1p function.

We have identified Tcc1p as a novel factor that interacts with CaTup1p. No homolog of Tcc1p could be identified in S. cerevisiae, suggesting the possibility that Tcc1p has C. albicans-specific functions. Considering the phenotypes of the tcc1Δ mutant, it would not be unreasonable to think about a relationship with CaNrg1p, a DNA-binding protein that represses hypha-specific genes during the yeast phase. Evidence that proves direct binding between Tupilp and Nrg1p in C. albicans has not yet been reported. Based on genes regulated by TCC1, a hypothesis is proposed that DNA-binding protein CaNrg1p recruits a Tcc1p-CaTup1p protein complex to promoter regions of hypha-specific genes, including HGC1, to repress transcription of these genes. A mechanical relationship among CaTup1p, Tcc1p, and CaNrg1p genes, including HGC1, 1905

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