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The Tlo Proteins Are Stoichiometric Components of *Candida albicans* Mediator Anchored via the Med3 Subunit

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The amplification of the *TLO* (for telomere-associated) genes in *Candida albicans*, compared to its less pathogenic, close relative *Candida dubliniensis*, suggests a role in virulence. Little, however, is known about the function of the Tlo proteins. We have purified the Mediator coactivator complex from *C. albicans* (caMediator) and found that Tlo proteins are a stoichiometric component of caMediator. Many members of the Tlo family are expressed, and each is a unique member of caMediator. Protein expression analysis of individual Tlo proteins, as well as the purification of tagged Tlo proteins, demonstrate that there is a large free population of Tlo proteins in addition to the Mediator-associated population. Coexpression and copurification of Tloα12 and caMed3 in *Escherichia coli* established a direct physical interaction between the two proteins. We have also made a *C. albicans med3Δ/Δ* strain and purified an intact Mediator from this strain. The analysis of the composition of the *med3Δ* Mediator shows that it lacks a Tlo subunit. Regarding Mediator function, the *med3Δ/Δ* strain serves as a substitute for the difficult-to-make *tloΔ/Δ C. albicans* strain. A potential role of the *TLO* and *MED3* genes in virulence is supported by the inability of the *med3Δ/Δ* strain to form normal germ tubes. This study of caMediator structure provides initial clues to the mechanism of action of the Tlo genes and a platform for further mechanistic studies of caMediator's involvement in gene regulatory patterns that underlie pathogenesis.

Candida species are opportunistic pathogens that mainly exist in harmony with the human host as part of the commensal flora and cause infection only when host deficiencies permit. *Candida albicans* and *Candida dubliniensis* are two highly related pathogenic yeast species. *C. albicans*, however, has been shown to be more pathogenic in a wide range of infection models and is far more prevalent in human infection (28). In epidemiological studies, *C. albicans* has been found to be the most common cause of superficial and systemic infections. The comparison of the genomes of the two species has revealed that they are very similar, although there are some significant differences (20). Determination of the particular molecular pathways affected by these genomic differences and revealing their mechanism of action will lead to a better understanding of why *C. albicans* has evolved to be a successful pathogen. The majority of the genomic differences observed between the *C. albicans* and *C. dubliniensis* species can be accounted for by the expansion of gene families in *C. albicans* (20). The greatest difference in gene family size between the two species is the *TLO* family, which is comprised of 14 genes in *C. albicans* but only two genes in *C. dubliniensis*.

The *TLO* genes in *C. albicans* (ca*TLO*) are a family of 14 closely related paralogs (average nucleotide identity, 96.5%) primarily located subtelomerically. The two *TLO* paralogs in *C. dubliniensis* are relatively well diverged (nucleotide identity, 74.9%), while *Candida tropicalis* only has one (20). The *TLO* genes can be subdivided into three clades (α, β, and γ), based largely on the presence of indels and the alternative splicing of a subset of sequences that contain an intron (γ) (M. Anderson and J. Berman, unpublished data). Due to the large number of *TLO* genes, a *C. albicans tloΔ/Δ* strain is challenging to construct. Hence, the first insight into *TLO* gene function came from null mutants for *C. dubliniensis TLO1* that showed a major reduction in hypha formation in response to serum (20). The ca*TLO* genes *TLOγ11* and *TLOα12* appear to have similar functionality in that they are both able to complement the phenotype of the *C. dubliniensis tlo1* null (20). A clue to a potential mechanistic role for the *TLO* genes was revealed using a bioinformatics approach that suggested that the *TLO* genes were a distantly related ortholog of a subunit of the *S. cerevisiae* Mediator complex (6).

Mediator is a conserved interface between gene-specific regulatory proteins and the general transcription apparatus of eukaryotes. Transcription in purified and extract-based systems, *in vivo* studies, and cell-based assays have revealed Mediator to be a critical functional target for a wide variety of activators (9). The analysis of purified Mediator has demonstrated that the core of the *Saccharomyces cerevisiae* complex is composed of 21 polypeptides (14, 31). Biochemical (3) and structural (8) studies have allowed the assignment of subunits to structurally distinct modules of the Mediator complex, referred to as tail, middle, and head. The Cdk8 module is a separate subset of proteins that is variably associated with the core Mediator subunits (5, 36). Parallel biochemical and genetic experiments have demonstrated that certain Mediator subunits are critical for the activation of specific sets of genes (31, 45). Transcriptional profiling *in vivo* shows that other Mediator subunits are essential for the transcription of virtually all genes in *S. cerevisiae* (18), suggesting that the complex was also a general transcription factor. Additional studies of *S. cerevisiae* have also pointed to a role for some Mediator subunits in transcriptional

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repression and silencing that likely involves chromatin (19, 21, 38, 39, 49, 50).

A majority of *S. cerevisiae* Mediator subunits have clear orthologs in virtually all eukaryotes (6, 10, 37). The approximately half of *S. cerevisiae* Mediator subunits that are encoded by essential genes have amino acid identity levels as high as 20 to 30% with their mammalian counterparts. Med2 and Med3 are two subunits of *S. cerevisiae* Mediator that have no obvious orthologs in metazoans and have clear orthologs in only a small group of closely related fungi. *S. cerevisiae* Med2 (scMed2) and scMed3 are encoded by nonessential genes, occupy the extreme end of the tail module, and stabilize each other's presence in Mediator (25, 30). BLAST searches find clear orthologs of scMed2 and scMed3 in closely related fungi such as *Kluyveromyces*, but none in most other fungi, including *C. albicans* and *C. dubliniensis*. Using short motifs, however, a bioinformatics approach predicted the Tlo proteins to be the *C. albicans* orthologs of scMed2 (6). This prediction raised the question of whether one or more of the *C. albicans* Tlo proteins were subunits of *C. albicans* Mediator (caMediator). If Tlo proteins are subunits of the complex, it also begged the question of whether they play a structural and functional role in the complex that lends insight into this unusually large set of paralogs and their potential roles in virulence. Our characterization of the composition of *C. albicans* Mediator has shown that several Tlo proteins are members of Mediator. Our biochemical characterization of the interaction between the Tlo proteins and the caMed3 subunit of Mediator, as well as the analysis of a *med3* null mutant, suggests that the Tlo proteins participate in processes that affect virulence through their membership in the Mediator complex.

MATERIALS AND METHODS

Tagging plasmids. The plasmid pURA-GSCP (43) was used for C-terminal tagging with a modified TAP tag, and the plasmids pFA-HA-HIS1 and pFA-HA-ARG4 (24) were used for C-terminal tagging with a hemagglutinin (HA) epitope tag as previously described. To construct the tagging vectors pFA-FLAG-HIS1 and pFA-FLAG-ARG4, the 3×FLAG epitope was amplified from the strain yLM40 (1) using the primer pair LM010/LM009. This product was cleaved with KpnI and AscI and ligated into KpnI/AscI-cut pFA-HA-HIS1 and pFA-HA-ARG4 vectors (24), and the constructs were verified by sequencing. To construct the pFA-6His-FLAG-HIS1 vector for adding a dual 6-histidine (6His)/3×Flag tag to the C termini of proteins, the primer pair SRP017/SRP016 was used to amplify a PCR product from pFA-HA-FLAG that added a 6His tag in frame with the 3×Flag. This PCR product was cleaved with AatII and KpnI and ligated into the AatII/KpnI-cut vector, and the construct was verified by sequencing. To make versions of pFA-HA and pFA-6His-FLAG that have nourseothricin (ClonNAT) resistance rather than histidine prototrophy as the selectable marker, we amplified the ClonNAT resistance gene from the SAT1 marker in pSFS2 (35) using the primer pair SRP011/SRP012, cut this product with AscI and PmeI, and ligated it into AscI/PmeI-cut pFA-HA-HIS1 and pFA-6His-FLAG-HIS1. The sequences of all primers described are listed in Table S2 in the supplemental material.

Strains. The genotypes of all strains used in this work are listed in Table S1 in the supplemental material. In this paper, the caMediator subunits are assigned to specific open reading frames (ORFs) in the *C. albicans* database as shown in Fig. 2, and the *TLO* designations are assigned to specific ORFs in the *C. albicans* database as previously described (20). All tagged strains were made by transformation of *C. albicans* strains using a PCR product with at least 70 bp of homology, derived from the primer, to each region targeted. The appropriate targeting in positive colonies was monitored by PCR of the flanking regions (primer sequences are available upon request) and Western blotting of the tagged protein in whole-cell extracts. Since the DNA sequence of the *TLO* genes is nearly identical in both the ORF and 3′-untranslated region (UTR), all tagged *TLO* constructs were further verified by the complete sequencing of the ORF to confirm that the correct gene was tagged. To construct the Med8-6His-Flag-tagged strain (yLM112) for Mediator purification, the 6His-Flag cassette was amplified from pFA-6His-FLAG-SAT1 using the primer pair LM025/LM014 and used to transform KPC1 (BWP17) (47). A second strain for Mediator purification (yLM83), in which both copies of Med15 were Flag tagged, was constructed by amplifying the Flag cassette from pFA-FLAG-HIS1 and pFA-FLAG-ARG4 with the primer pair LM024/LM012 and sequentially transforming KPC1 (BWP17). To construct KPC3, in which a single copy of *MED8* is tagged at the C terminus with the GSCP tag, the GSCP tagging cassette was amplified from plasmid pURA-GSCP with the primer pair KPP004/KPP005 and transformed into KPC1 (BWP17). To construct the HA-tagged Tlo and Mediator strains, the following primer pairs were used to amplify the HA-HIS1 cassette from pFA-HA-HIS1 and transform KPC3: KPC6 *TLOα12-HA* (KPP035/KPP037), KPC9 *TLOα3-HA* (KPP035/KPP039), KPC11 *TLOα34-HA* (KPP035/KPP041), KPC22 *TLOα1-HA* (KPP035/KPP055), KPC23 *TLOα10-HA* (KPP035/KPP057), KPC28 *MED19-HA* (KPP074/KPP075), and KPC29 *MED16-HA* (KPP078/KPP079). To make the strains in which TLOα12 was Flag tagged and either one or two copies of *MED3* were HA tagged, the primer pair KPP035/KPP37 was first used to amplify the FLAG-HIS1 cassette from pFA-FLAG-HIS1 and transform KPC1 (BWP17). To HA tag the first copy of *MED3* in the TLOα12-Flag strain, the primer pair LM017/LM018 was used to amplify the HA-ARG4 cassette from pFA-HA-ARG4 and transform the TLOα12-Flag strain to obtain yLM107. To HA tag the second copy of *MED3* in yLM107, the primer pair LM017/LM018 was used to amplify the HA-SAT1 cassette from pFA-HA-SAT1 and transform yLM107 to obtain yLM109. The heterozygous and homozygous *med3* null strains were made using a previously described method (34) and the gene-specific primers MED3-P1, MED3-P3, MED3-P4, and MED3-P6. The first copy of *MED3* in yLM92, a *MTL***a**/**a** derivative of SN152 (34) selected on sorbose, was replaced by *HIS1* to generate the *med3* heterozygous null strain (yLM93). The second copy of *MED3* in yLM93 was replaced by *LEU2* to generate the homozygous *med3* null strain yLM96. A 6His-Flag tag was placed on the C terminus of Med8 for the purification of Mediator from a homozygous *med3* null strain. yLM96 was transformed with a PCR product containing the 6His-Flag cassette, amplified from pFA-6His-FLAG-SAT1 using the primer pair LM025/LM014, to generate yLM114. For the phenotypic analysis of the *med3* null strain, a *MED3* complemented strain (AZC53-3) and complementation control strain (AZC54-1) were constructed. Briefly, the ORF of *MED3* and about 400 bp of its upstream region were amplified from *C. albicans* genomic DNA by the primer pair AZP139/AZP140 and digested by KpnI and BamHI. In addition, a 400-bp region immediately 3′ to the end of the *MED3* ORF was amplified from genomic DNA by primer pair AZP141/AZP142 and digested by NotI and ApaI. Plasmid pAZE030 was generated by inserting these two DNA fragments sequentially into plasmid pSN69 (34). The plasmid for generating *med3* null complemented control strain pAZE029 was constructed in a similar way, except that only the ~400-bp region upstream of *MED3* was amplified, using primer pair AZP139/AZP144, and inserted into pSN69. The complemented and control strains were constructed by transforming yLM96 with KpnI-linearized pAZE030 and pAZE029. Positive colonies were tested by PCR for correct integration. Five complemented strains confirmed by PCR tests were carried out for further phenotypic analysis, and all of them showed phenotypes similar to those of *MED3* heterozygotes. Two complemented control strains had phenotypes that were similar to each other and to a *med3* null mutant.

Purification of native proteins from *C. albicans*. Mediator was purified from *C. albicans* strains containing Mediator subunits tagged at their C terminus with either a Flag tag or a combined 6His-Flag tag at their native loci. The procedures were identical for each strain, except that the final IMAC (immobilized metal affinity chromatography) purification step was skipped for the strains lacking a 6His tag. All chromatographic

steps were at 4°C unless otherwise stated. Tagged *C. albicans* strains were grown at 30°C to mid-log phase in yeast extract-peptone-dextrose (YPD) plus uridine, harvested by centrifugation, and washed once with cold double-distilled water, followed by one wash with 1× lysis buffer (180 mM Tris-acetate [OAc] [pH 7.8], 400 mM potassium acetate [KOAc], 1.2 mM EDTA, 12% glycerol) with 1 mM dithiothreitol (DTT) and 1× proteinase inhibitor cocktail (PIC) (32). The cell pellet was extruded through a syringe and flash frozen in liquid N_2. Cells (~25 g) were lysed under liquid N_2 by manual grinding with a mortar and pestle. The ground, frozen powder was allowed to slowly thaw and was resuspended in 2 volumes of 1.5× lysis buffer with 0.5 mM DTT and 1× PIC. The resuspended cells were sonicated (4 cycles [45 s on, 45 s off] at 20% power with a Sonic Dismembrator Model 500 [Fisher Scientific]) on ice. MgOAc was added to the sonicated lysate to a final concentration of 2.5 mM, followed by the addition of 200 U of benzonase (EMD). After stirring for 15 min, the lysate was centrifuged for 2 h at 36,000 rpm in a Ti45 rotor. The lysis supernatant was adjusted to 150 mM KOAc with buffer F-0 (25 mM HEPES-KOH [pH 7.6], 0.01% NP-40, 10% glycerol, and the concentration of KOAc in mM after the dash [e.g., F-0 indicates 0 mM KOAc]) with 0.5 mM DTT and PIC. The adjusted lysate was applied at 1 column volume (CV) per h to heparin Sepharose (GE Healthcare) equilibrated in buffer F-150 (with 0.5 mM DTT and PIC) using ~1 ml of resin per 10 mg of total protein loaded. The application of the lysate was followed by 1 CV of F-150 (with 0.5 mM DTT and PIC), and the unbound protein was saved for further analysis (see below). The column was washed with 2 CV of F-260 (with 0.5 mM DTT and PIC), and the intact Mediator was eluted with 2 CV of F-500 (with 0.5 mM DTT and PIC). Mediator-containing fractions, judged by Western blotting with an anti-Flag antibody, were pooled, incubated with 200 μl of M2 Flag agarose (Sigma), and equilibrated in F-300 (with 0.25 mM DTT and PIC) for 4 h with rotation. The amount of heparin F-500 eluate loaded was adjusted according to the Flag signal. The unbound protein was drained from the column, and the resin was washed twice with 10 ml F-300 (with 0.25 mM DTT and 1× PIC) followed by one wash with 10 ml F-300 (with 0.25 mM DTT, PIC, and 1 mM MgOAc). The resin was resuspended in 500 μl F-300 (with 0.25 mM DTT, PIC, 1 mM MgOAc, and 100 U benzonase) and incubated at room temperature for 30 min. Two 10-ml F-300 (with 2.5 mM β-mercaptoethanol [β-ME] and PIC) washes were followed by five 250-μl elutions with F-300 (with 2.5 mM β-ME, PIC, and 50 μg/ml 3×Flag peptide). Mediator containing Flag elution fractions were pooled, incubated with 125 μl of TALON (Clontech), and equilibrated with F-300 (with 2.5 mM β-ME plus PIC) for 2 h with rotation. After draining the unbound protein and five 2-ml F-300 (with 2.5 mM β-me and PIC) washes, Mediator was eluted with F-300 (with 2.5 mM β-ME, PIC, and 100 mM imidazole). To purify Flag-tagged Tloα12 from the heparin flowthrough, the unbound protein heparin fractions were adjusted to 300 mM KOAc and the Flag-agarose purification was run as described above.

Mass spectrometry. Talon elutions from parallel preparations from an untagged and a Med8-6His-Flag-tagged strain were trichloroacetic acid (TCA) precipitated, and the precipitates were washed twice with high-performance liquid chromatography-mass spectrometry (HPLC-MS)-grade acetone (Burdick and Jackson), dried by vacuum centrifugation, and treated with 20 μl of a 5-ng/μl trypsin solution (MS sequencing grade; Promega) overnight at 37°C. The resulting digest was desalted using an OASIS HLB solid-phase extraction plate (Waters) and analyzed by microcapillary LC-Orbitrap-MS/MS essentially as described previously (22, 23) using a long (90-min) analytical gradient. Tandem mass spectra were data searched using the SEQUEST algorithm (13) and the UniProt complete proteome set for *C. albicans* (www.uniprot.org; accessed on 17 February 2010), and peptide spectral matches were filtered to an estimated false discovery rate of <1% at the peptide level using the target-decoy strategy (12) and reported. To determine the identity of individual bands, the Med8-6His-Flag-tagged sample was subjected to SDS-PAGE in 7.5% and 12.5% gels and stained with colloidal Coomassie dye. Individual bands were excised and analyzed by LC-MS/MS at the Proteomics Core Facility of the Vermont Genetics Network as previously described (40). A band was assigned to a particular Mediator subunit based on peptides from that protein being the principal component of that sample versus other bands. Tables of the raw mass spectroscopy data are available upon request.

Antibodies. Recombinant ca*MED1* (amino acids 440 to 618) was fused to glutathione-*S*-transferase (GST), overexpressed in *E. coli* Rosetta 2 (Novagen) cells, and subsequently purified using glutathione Sepharose 4B (GE Healthcare) according to the manufacturer's recommendations. The pure recombinant protein was supplied as an antigen to Rockland Immunochemicals Inc. for the immunization of rabbits. For the so-called pan-Tlo antibody, the peptides ENLQTRLHNSLDEILK and DQNRKQS NVITSPNNELIQK were coinjected into rabbits for antibody production (Open Biosystems, Inc.). HA-tagged proteins were detected via Western blotting with a monoclonal anti-HA antibody (MMS-101R; HA.11; Covance). As a loading control in whole-cell extract Western blot analyses, the membrane was stripped and reprobed with rat α-Tub1 raised against *S. cerevisiae* Tub1 (no. ab1616; Abcam, Cambridge, MA). Western blots were performed using alkaline phosphatase-conjugated secondary antibodies and a fluorescent substrate (ECF; GE Healthcare) that could be quantified by imaging.

Binding assays. To analyze C-terminal domain (CTD) binding, 10 μg purified GST-CTD (15) or GST was bound to 5 μl of glutathione MagBeads (Genscript). Unbound proteins were removed by washing the beads in F-300 buffer containing 0.5 mM DTT. Equimolar amounts of purified *S. cerevisiae* Mediator (1) and *C. albicans* Mediator were added to the beads and incubated for 2 h. The supernatant was saved after incubation as the flowthrough fraction representing the unbound Mediator. Bound Mediator complex was eluted by boiling the beads in SDS-PAGE loading dye. To analyze activation domain binding, 10 μg purified GST-VP16 or GST-VP16Δ456 (17) was bound to 5 μl of glutathione MagBeads (Genscript). Unbound proteins were removed by washing the beads in F-300 buffer containing 0.5 mM DTT. Equimolar amounts of purified *C. albicans* Mediator were added to the beads and incubated for 2 h. The supernatant after incubation was saved as the flowthrough fraction representing the unbound Mediator. Bound Mediator complex was eluted by boiling the beads in SDS-PAGE loading dye.

Purification of the recombinant Tloα12/Med3 cocomplex. To construct a Tloα12/Med3 bicistronic *E. coli* expression vector with a 6His tag on the C terminus of Med3, the *MED3* gene was first amplified from *C. albicans* genomic DNA by PCR using the primer pair EHP001/EHP002. The PCR product was cleaved with restriction enzymes SalI and NotI and inserted into the pET21b vector (Novagen). To first isolate *TLOα12* from other *TLO* genes, a *TLOα12*-containing fragment was amplified from *C. albicans* genomic DNA using the primer pair EHP003/EHP004. The product was cleaved by XhoI and NotI and inserted into the pBSKS vector (Stratagene/Agilent). An individual clone was confirmed to be *TLOα12* by DNA sequencing. The primer pair EHP005/EHP006 was used to amplify the *TLOα12* ORF from this vector, and the product was cleaved by NheI and EcoRI and inserted into the pET21b-MED3-6His vector upstream of the Med3 gene. The two CUG codons in *TLOα12* and the one in *MED3* were replaced with UCG using site-directed mutagenesis (QuikChange lightning multisite-directed mutagenesis kit) to create the final bicistronic expression vector, pET21b-TLOα12-MED3-6His, which was confirmed by sequencing. *E. coli* BL21(DE3) competent cells (Stratagene) were transformed with the pET21b-TLOα12-MED3-His_6 plasmid, and positive colonies were inoculated in LB medium (plus Amp). The cells were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.5 to 0.6, induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and harvested after 4 h of shaking at 25°C. The harvested cells were resuspended in IMAC buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl) and lysed in a French pressure cell. After centrifugation, Tloα12 and Med3-6His were found to be in the soluble fraction and were subjected to purification by IMAC using Talon resin (Clontech) according to the manufacturer's protocols. Tloα12 and Med3-6His were both depleted from the load and recovered in the elution. Ion-exchange

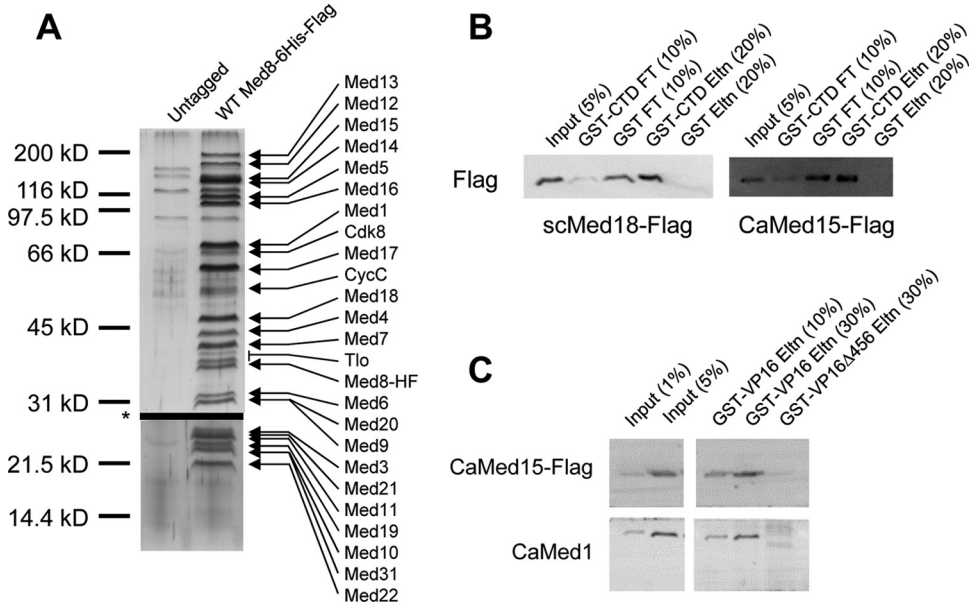

FIG 1 Composition and functional characterization of *C. albicans* Mediator. (A) Lysates from an untagged and a Med8-6His-Flag-tagged strain were subjected in parallel to multiple chromatographic separations. The elutions from the IMAC (Talon) step were analyzed by 7.5% (results above the asterisk) and 12.5% (below the asterisk) SDS-PAGE. The two gels were run on identical samples to ensure adequate resolution in both the high- and low-molecular-mass ranges. Proteins were revealed by staining with silver. The annotated identity of each band was determined by excising the band from another SDS-PAGE gel run in parallel and using mass spectrometry to analyze the protein content of each band. (B) Purified scMediator and caMediator bind to the CTD of RNA Pol II. Either GST or GST-CTD was prebound to magnetic glutathione beads, and a standardized amount of purified scMediator or caMediator was added (Input). The samples were monitored by Western blotting using the anti-Flag antibody against a Flag tag on scMed18 or caMed15. Percentages represent the percentages of the total sample at each stage that was loaded onto the gel. The flowthrough (FT) shows that GST-CTD, but not GST, depleted both scMediator and caMediator from the input. The bound scMediator and caMediator were recovered in the elution (Eltn) from the GST-CTD beads, while no detectable signal was recovered from the GST beads. (C) Purified caMediator binds to the activation domain of VP16, not an activation-deficient mutant (VP16Δ456). Either GST-VP16 or GST-VP16Δ456 was prebound to magnetic glutathione beads, and a standardized amount of purified caMediator was added (Input). The samples were monitored by Western blotting using the caMed1 antibody and the anti-Flag antibody against a Flag tag on caMed15. The percentages are of the total sample at each stage that was loaded onto the gel. Bound caMediator was recovered in the elution (Eltn) from the GST-VP16 beads, while little detectable signal was recovered from the GST-VP16Δ456 beads.

chromatography separated the TLOα12/Med3-6His complex from excess Med3-6His. The Talon elution fractions containing Tloα12 and Med3-6His were loaded onto a Mono Q HR 5/5 column (GE Healthcare) equilibrated in buffer A-100 (25 mM Tris-Cl [pH 7.5], 10 mM β-ME, and the mM amount of NaCl after the dash [e.g., A-100 indicates 100 nM NaCl]), washed with 5 column volumes of A-100, and eluted with a 20-ml linear gradient from A-100 to A-600. Fractions containing stoichiometric amounts of Tloα12 and Med3-6His were eluted at ~260 mM NaCl. These fractions were analyzed further by size-exclusion chromatography on Superose 6 (GE Healthcare) equilibrated in 50 mM sodium phosphate (pH 7.8), 300 mM NaCl, and 10 mM β-ME.

Germ tube formation assays. Cultures were allowed to grow overnight in YPD medium at 30°C to saturation. They were then reinoculated into 3 ml YPD medium supplemented with 2.5 mM *N*-acetylglucosamine (GlcNAc) (Sigma) at an OD_{600} of 0.2. After growing at 37°C on a roller drum for a certain amount of time, cells were harvested and stained with 50 μg/ml Blankophor (Valeant Pharmaceuticals) and analyzed using a DeltaVision Imaging System (Applied Precision) and the light field polarized differential interference contrast (POL DIC) or fluorescence (4′,6-diamidino-2-phenylindole [DAPI]) channel. Cell morphology was assessed in repeats on multiple days.

RESULTS

Purification and characterization of *C. albicans* Mediator reveals presence of Tlo proteins. To purify the native *C. albicans* Mediator, we devised new tagging constructs based on the pFA series of tagging vectors for *C. albicans* created by Whiteway and colleagues (24). Various tagging vectors with the 3×Flag (Sigma) and/or 6His tags and an expanded set of selectable markers were constructed for use in purification. Our experience purifying *S. cerevisiae* Mediator suggested that the 3×Flag tag would be an excellent tag for efficient recovery of large amounts of intact complex. For many *S. cerevisiae* Mediator subunits, there are clear *C. albicans* orthologs with 20% or greater amino acid identity (6). Among these well-conserved subunits, we found several that were suitable for tagging and purification. For the work described in this paper, however, we settled on placing a 6His tag followed by a 3×Flag tag on the C terminus of caMed8. Since the M2-Flag antibody has several cross-reactivities in *C. albicans*, we included the 6His tag to clean up any major impurities left after Flag-agarose. For the purification of caMediator, we prepared whole-cell lysates from tagged *C. albicans* strains and separated the lysate into bound and unbound fractions using heparin Sepharose. Intact Mediator binds heparin and can be eluted with 500 mM KOAc. This preparatory step served to increase the recovery of the primary affinity chromatography step and also to separate tagged Mediator subunits that were not incorporated into the larger complex away from intact Mediator (see below). Heparin-processed whole-cell lysate from an untagged and a Med8-tagged strain were further processed in parallel by Flag-agarose and Talon chromatography, and the elutions were analyzed by silver stain (Fig. 1A). Comparison of the elutions from the tagged and un-

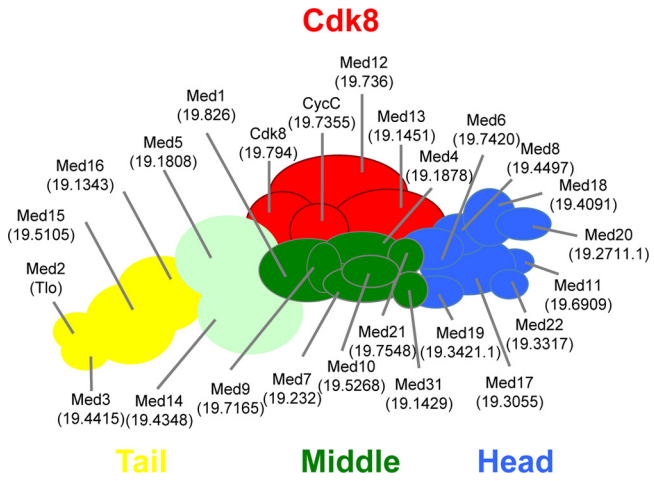

FIG 2 Assignment of *C. albicans* Mediator subunits to ORFs and model of its structure. This figure shows a model of the *C. albicans* Mediator based on an updated version of a model of the modular topology of the *S. cerevisiae* Mediator (14). We have colored Med5 and Med14 lime green, since they have characteristics of both tail and middle module subunits. The standardized Mediator subunit name is followed by the ORF designation in the *C. albicans* database in parentheses. The Tlo proteins can serve as orthologs for scMed2. Elsewhere in the manuscript we discuss the particular Tlo proteins that we have found can form a subunit of Mediator.

tagged strains reveals that the major bands in the Med8-6His-Flag elution are specific to the tagged sample, although some minor impurities do survive the tandem affinity chromatography approach. To determine the composition of the purified complex, we took two distinct mass spectrometry approaches. First, we TCA precipitated the tagged and untagged elution fractions (Fig. 1A) and subjected them directly to tandem mass-spectroscopic analysis. After subtracting the proteins found in the untagged strain from those found in the tagged strain, we were basically left with the composition of caMediator. This analysis showed that all orthologs, including the Tlo proteins, predicted by the bioinformatics approach (6) were present in the sample (Fig. 2). Since the Tlo proteins have very high amino acid sequence identity, it was difficult to distinguish individual Tlo proteins by mass spectrometry, although we did find a peptide specific to Tloα12. Aside from the predicted Mediator subunits, the only other proteins specific to the tagged sample were the two large subunits of RNA polymerase II (Pol II). The presence of small amounts of RNA Pol II was not unexpected, given that Mediator is known to interact with RNA Pol II through the CTD of the large subunit (31). A second mass spectrometry approach was used to assign specific bands from SDS-PAGE to specific subunits. We ran the TCA precipitate of the tagged sample on SDS-PAGE, excised individual bands, and subjected them to tandem mass spectrometry analysis. Using this data, it was relatively straightforward to assign subunits to individual bands, as shown in Fig. 1A. It is interesting that the subunits of the Cdk8 module, which do not copurify with the core scMediator using a similar protocol, are present in the caMediator complex. The subunits of the Cdk8 module, however, may be present on a subset of the Mediator complexes isolated, since the bands stain less intensely than their core subunit counterparts. This analysis also revealed more unique Tlo peptides originating from Tloα9 and Tloβ2. To verify that the purified caMediator possessed activities characteristic of functional, purified *S. cerevisiae* Mediator, we performed CTD and activation domain binding assays. In the CTD binding assay, a GST-CTD fusion protein was added to purified Mediator, and glutathione-Sepharose was used to pull down GST-CTD-associated proteins. Using this assay, we demonstrated that an equimolar amount of wild-type (WT) caMediator binds to a peptide containing CTD repeats of *S. cerevisiae* RNA Pol II with equal affinity to scMediator (Fig. 1B). Activation domain binding assays using a GST-VP16 activation domain fusion protein and a GST fusion with a VP16 activation domain mutant with attenuated activity (GST-VP16Δ456) showed that, similarly to its scMediator counterpart (17), caMediator bound specifically to VP16 (Fig. 1C). The composition and functional capabilities of caMediator indicate that we have purified an intact and active complex.

Multiple Tlo proteins are expressed and are present in super-stoichiometric ratios to other caMediator subunits. The mass spectrometry of purified caMediator indicated the presence of at least several Tlo proteins, but it did not quantify the stoichiometry of the Tlo proteins to other subunits or reveal whether Tlo proteins exist in a non-Mediator-associated form. To assess the total amount of Tlo protein in the cell compared to that of other Mediator subunits, we tagged a variety of Tlo and Mediator subunits with the HA tag. The HA tag was ideal, because the anti-HA antibody has no apparent cross-reactivities with native *C. albicans* proteins. Since ~600 bp of sequence 3′ of the stop codons of the Tlo genes has to be eliminated to find differing DNA sequence that will allow for the specific tagging of a certain *TLO*, only a subset of *TLO* genes was suitable for tagging. We chose Tloα1, Tloα3, Tloα10, Tloα12, and Tloα34 for our initial round of HA tagging. As a comparison, we chose two other tail module Mediator subunits (Med16 and Med3) and one head module subunit (Med19) for HA tagging. To analyze the relative expression levels of these proteins, cells from the tagged strains were grown to mid-log phase, lysed, and analyzed by Western blotting (Fig. 3). The amount of total protein loaded was normalized such that an anti-Tubulin antibody gave an equal amount of signal for all samples loaded. The Western blot shows that all five Tlo proteins tagged are expressed and that there is some variability in expression level. The high expression level for the α clade of *TLO* genes is consistent with mRNA measurements (Anderson and Berman, unpublished). The most important observation is that the expression of individual Tlo proteins either equals or substantially exceeds the expression of the core Mediator subunits (Fig. 3). If the expression levels of all of the Tlo proteins are added together, they are present in vast excess above the levels of the other Mediator subunits. Since the subunits of *S. cerevisiae* Mediator are present in a stoichiometric ratio (29), this observation suggested that there is a large population of non-Mediator-associated Tlo proteins in the cell. One other notable feature is that HA-tagged Tloα34 protein runs at a molecular mass close to that of the other Tlo proteins. The current annotation of the *C. albicans* genome predicts that Tloα34 has an extended N terminus (~80 amino acids) despite the existence of a second ATG start codon that aligns with the ATG start codons in the rest of the *TLO* paralogs. The observed mobility of Tloα34 does not support the existence of the predicted extended N terminus, but it shows that the ATG aligned with the rest of the paralogs is the true start codon.

The Tlo proteins are a stoichiometric component of purified caMediator. To further test the relationship of the Tlo proteins to

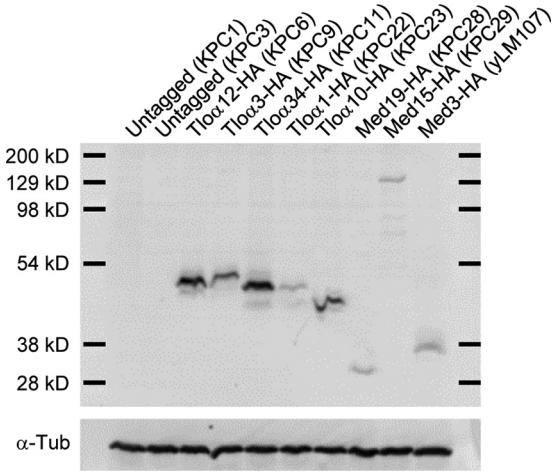

FIG 3 Expression analysis of Tlo proteins and Mediator subunits in whole-cell extracts. Whole-cell extracts were made from strains that were untagged or had a single copy of a Tlo or other Mediator subunit tagged with the HA epitope. The strain used for each extract is listed in parentheses. (Upper panel) A Western blot is shown that used an anti-HA antibody for detection. The total amount of protein loaded in each well was roughly normalized by cell OD, followed by a precise normalization with an anti-tubulin antibody. (Lower panel) The blot in this figure was reprobed with the anti-tubulin antibody, and each lane was shown to have an equal amount of tubulin.

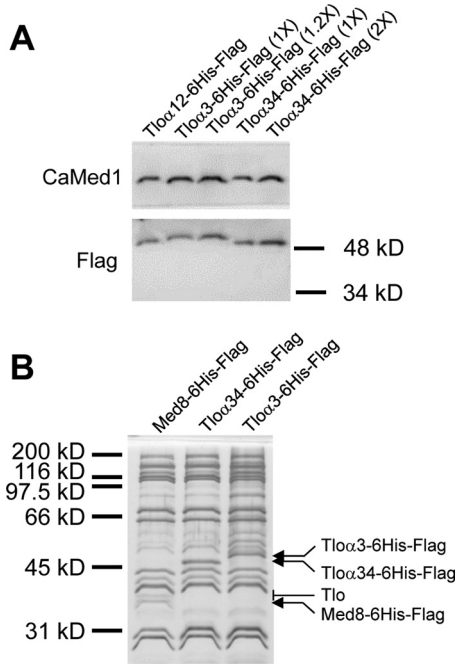

FIG 4 Tloα3, Tloα12, and Tloα34 are Med2 orthologs of caMediator. Whole-cell lysates from Tloα3-6His-Flag, Tloα12-6His-Flag, and Tloα34-6His-Flag strains were subjected to our Mediator purification protocol and analyzed by Western blotting and silver staining. (A) Western blot of various amounts of protein from the Flag-agarose elutions. One quantity of Tloα12-6His-Flag and two quantities of Tloα3-6His-Flag and Tloα34-6His-Flag elutions were loaded. The number in parentheses is the quantity of a sample relative to the lower quantity. The samples were probed with the caMed1 and Flag antibodies. The ratio of the Flag signal to the caMed1 signal is comparable between all three samples, indicating that each of the three Tlo proteins in the heparin elution was able to pull down a proportional amount of intact Mediator. (B) Comparison of equal amounts of the Med8-6His-Flag, Tloα34-6His-Flag, and Tloα3-6His-Flag Talon elutions by SDS-PAGE and silver staining of a 10% gel. The general pattern of silver stain bands indicates that both Tloα34-6His-Flag- and Tloα3-6His-Flag-purified Mediator are largely identical to the Med8-6His-Flag pure Mediator. Tloα34-6His-Flag and Tloα3-6His-Flag protein appear to be the only detectable Tlo component of each population of purified Mediator. The Tlo band observed in the Med8-6His-Flag Mediator is missing from the Tloα34-6His-Flag and Tloα3-6His-Flag samples, and a new band appeared at a molecular size that corresponds to the Tloα34-6His-Flag and Tloα3-6His-Flag signals detected by Western blotting in the corresponding samples. The molecular mass range occupied by the Tlo band in the Med8-6His-Flag Mediator is also left absent by the decrease in the molecular size of Med8, which is untagged in the Tlo-tagged samples.

caMediator, we raised a pan-Tlo polyclonal antibody by immunizing rabbits with two peptides from the N termini of the Tlo proteins that are 100% identical among all *C. albicans* paralogs. Since the mass spectrometry had indicated the presence of at least several different Tlo proteins in the purified Mediator, we used the pan-TLO antibody to determine the total amount of Tlo protein relative to other Mediator subunits in purified caMediator. The total amount of Tlo protein in the purified Mediator was calibrated using purified recombinant Tloα12, quantified by Bradford assay, as a standard. Analysis of 300 fmol of native *C. albicans* Mediator by Western blotting with the pan-Tlo antibody gave a signal that could be quantified by integrating the intensity of the fluorescent band and comparing it to the intensities of the recombinant Tloα12 standards (see Fig. 7B). This process showed that there is an amount of Tlo protein that is consistent with it being present in a one-to-one ratio with other subunits in the complex. This result indicates that the Mediator-associated Tlo proteins are present in roughly equimolar amounts to other Mediator subunits and is consistent with the Tlo proteins being the structural ortholog of scMed2, which is present in a 1:1 stoichiometry with other members of the *S. cerevisiae* complex (29).

The multiple bands in the pan-Tlo-N Western blot and the multiple Tlo proteins identified by mass spectrometry of purified caMediator support the idea that many different Tlo proteins can be Mediator subunits. To test this hypothesis directly with several Tlo proteins, we made three strains that each had a 6His-Flag tag on a different Tlo protein, and we followed the caMediator purification protocol. Equal amounts of tagged Tloα3, Tloα12, and Tloα34 were able to pull down an equal amount of Med1 (Fig. 4A), and the composition of the complex pulled down by Tloα3-6His-Flag and Tloα34-6His-Flag is similar to that of the Mediator pulled down by Med8-6His-Flag as judged by silver staining (Fig. 4B). There is no untagged Tlo band in the complexes pulled down by Tloα3-6His-Flag and Tloα34-6His-Flag detectable by silver staining (Fig. 4B) or Western blotting (data not shown). Combining these data with those from mass spectrometry, we have direct evidence that Tloβ2, Tloα3, Tloα9, Tloα12, and Tloα34 can associate with Mediator. Although we have no additional direct evidence, it is likely that other expressed members of the Tlo family, such as Tloα1 and Tloα10, can also associate with Mediator. In total, the data suggest that there are several different populations of Mediator within a *C. albicans* cell, each with a unique Tlo protein component.

A majority of Tlo protein in *C. albicans* is in a non-Mediator-associated form. Our expression data (Fig. 3) suggested that there is a substantial population of non-Mediator-associated, or free, Tlo protein in the cell. During our purification of the Tlo-6His-Flag proteins, we observed that most of the tagged protein was in the heparin flowthrough (data not shown), while a smaller frac-

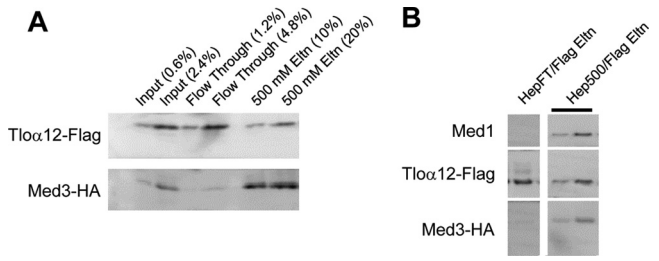

FIG 5 Majority of Tloα12 protein is in the heparin unbound protein fraction and is not associated with Mediator subunits. Lysate from a Tloα12-Flag-Med3-HA-tagged strain was separated on heparin Sepharose into unbound and bound fractions. Material from both fractions was purified on Flag-agarose. (A) Western blot analysis (using an anti-Flag and anti-HA antibody) of the input, flowthrough, and 500 mM KOAc elution (Eltn) of the heparin column shows that a majority of Med3 binds but that a majority of Tloα12 flows through. The number in parentheses represents the percentage of 1/1,000 of the total amount of each fraction loaded on the gel. (B) Western blot of the Flag elution from the heparin flowthrough (HepFT/Flag Eltn) and two amounts of the Flag elution from the heparin elution (Hep500/Flag Eltn) probed with the caMed1, Flag, and HA antibodies. The Flag signal from the HepFT/Flag elution sample exceeds that from the Hep500/Flag elution samples, yet the HepFT/Flag elution contains no detectable Mediator as monitored by the caMed1 and HA (Med3) antibodies.

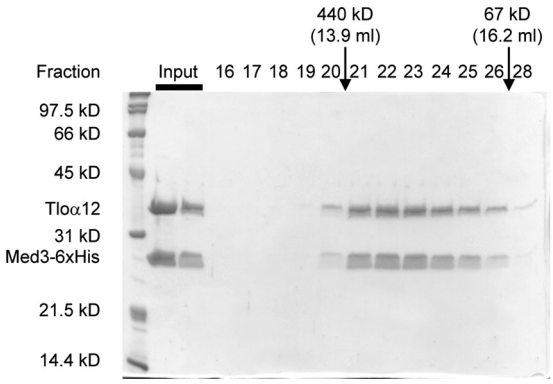

FIG 6 Size-exclusion chromatography shows that recombinant *C. albicans* Tloα12 and Med3 form a cocomplex. IMAC quantitatively depletes coexpressed Tloα12 and Med3-6His from *E. coli* lysates. The IMAC elution was separated by ion exchange followed by size exclusion. Input and fractions from the size-exclusion column (Superose 6) were analyzed by SDS-PAGE on a 12% gel, followed by staining with Coomassie blue. The relative migrations of two molecular size standards are marked with respect to the fractions collected.

tion was in the heparin elution from which we had previously isolated Mediator. To determine whether the Tlo protein in the heparin flowthrough was associated with other Mediator subunits, we fractionated lysates from a *TLOα12*-Flag strain that had an HA tag on both copies of Med3. A substantial amount of Tloα12-Flag protein was unable to bind the resin when the lysate of the *TLOα12*-Flag cells was applied to heparin Sepharose, as opposed to Med3-HA, which almost entirely bound to the heparin (Fig. 5A). When Flag-agarose was used to purify the Tloα12-Flag protein from the heparin flowthrough and elution, we observed that the heparin-bound Tloα12-Flag pulled down Med1 and Med3-HA, while Tloα12-Flag did not (Fig. 5B). This finding is consistent with earlier expression data (Fig. 3) and indicates that there is a large cellular population of Tlo protein that is not associated with even its closest structural binding partner, Med3, in the Mediator complex. Our estimate for individual Tlo proteins is that the free form is at least 10-fold more abundant in cells than the Mediator-associated form.

Since the heparin purification step cleanly separates each Tlo protein into a Mediator-associated and non-Mediator-associated form (Fig. 5), the relative amounts of a Tlo protein in the heparin input and elution are a metric of the affinity of that particular Tlo protein, versus other Tlo proteins, for Mediator. Although expressed at different levels, a similar fraction of Tloα12-6His-Flag, Tloα3-6His-Flag, and Tloα34-6His-Flag protein is bound to heparin (see Fig. S1 in the supplemental material). The similar affinity of these three Tlo proteins for Mediator leads to the conclusion (at least for Tloα12, Tloα3, and Tloα34) that the population of a Mediator complex containing a particular Tlo protein will be proportional to the total expression of that Tlo protein.

The Tlo proteins are structurally anchored to Mediator complex through Med3. Work in *S. cerevisiae* indicates that Mediator purified from either a *med2*Δ or a *med3*Δ strain lacks Med2, Med3, and Med15 (25, 30), and that recombinant versions of scMed2 and scMed3 expressed in insect cells can form a heterodimer (2). The connection between Med2 and Med3 in *S. cerevisiae* extends *in vivo*, where the impact of *med2* or *med3* deletion on global gene regulation is highly correlated (45). To investigate whether a similar relationship between the Tlo proteins and Med3 exists in *C. albicans*, we used strategies utilizing both recombinant and native proteins. First, we made a bicistronic expression vector in which Tloα12 and Med3 with a C-terminal 6His tag were coexpressed in *E. coli* (termed rTloα12). rTloα12 was completely depleted from cell lysates during purification by IMAC. After IMAC, equimolar amounts of rTloα12 and rMed3-6His comigrate exactly by both Mono Q ion-exchange resin (data not shown) and size exclusion (Fig. 6). Tloα12, and presumably other Tlo proteins, can form a tight, one-to-one complex with Med3. The cocomplex runs at a position on size exclusion that is much larger than what would be expected if one simply adds the molecular sizes of the two proteins. It is unclear whether this results from an unusual shape or from the oligomerization of the cocomplex.

Our second approach to probe the relationship between the Tlo proteins and Med3 was to purify caMediator from a *med3* null strain and determine whether Med3 was required to retain the Tlo proteins, and perhaps other subunits, in the native complex. The *med3* null strain is viable and grows with a doubling time similar to that of the WT at 30°C in YPD. Since Med8 is topologically distant from proteins likely to be affected by the absence of Med3, we used a 6His-Flag tag on Med8 to purify the *med3*Δ Mediator. The Med8-6His-Flag *med3*Δ Mediator was purified by following the same protocol as that for the wild type. In the first step of the purification, the level of recovery of Med8-6His-Flag signal in the heparin elution was lower than that of the wild type, indicating general instability of the complex in the *med3*Δ/Δ strain. A largely intact *med3*Δ complex, however, could be purified from the heparin elution. Using Western blotting to normalize the amount of Flag and Med1 signal, equimolar amounts of WT and *med3*Δ Mediator were analyzed by SDS-PAGE and silver staining (Fig. 7A). The silver-stained gel shows the *med3*Δ Mediator to be similar to wild-type Mediator, as most bands from the wild type are found in the mutant. Med5 and Med16, two tail module subunits, are clearly depleted in the mutant Mediator. Signal in the region for Med3 and Tlo proteins also appear to be depleted, but it is more difficult to tell due to the presence of overlapping bands from

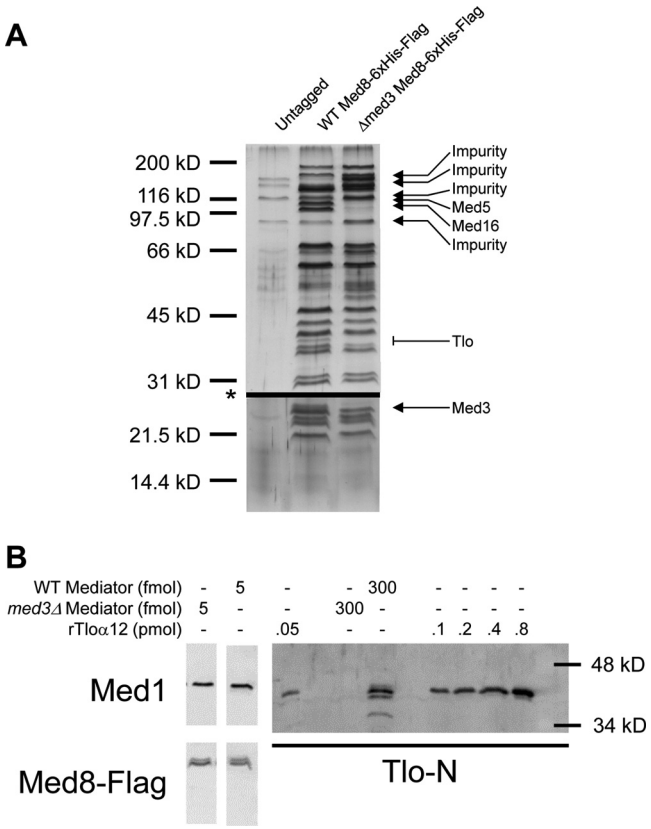

FIG 7 caMediator purified from a *med3* null strain lacks Tlo proteins and other tail module subunits. (A) Lysate from an untagged, Med8-6His-Flag-tagged WT strain and a Med8-6His-Flag-tagged *med3* null strain were subjected, in parallel, to multiple chromatographic separations. The elutions from the IMAC (Talon) step were analyzed by 7.5% (results above the asterisk) and 12.5% (below the asterisk) SDS-PAGE. The two gels were run on identical samples to ensure adequate resolution in both the high- and low-molecular-mass ranges. Proteins were revealed by staining with silver. The positions of bands identified in Fig. 1, which differ in the *med3*Δ Mediator, are marked with arrows. (B) Western blotting comparing various amounts of purified WT and *med3*Δ Mediator and recombinant Tloα12/Med3-6His complex. The amount of WT Mediator was estimated by comparison of band intensity on a Coomassie gel to a known standard (data not shown). caMed1 and Flag antibodies were used to demonstrate a normalized amount of Med1, and Med8-6His-Flag was present in both the purified WT and *med3*Δ Mediator. The pan-Tlo-N antibody was used to detect Tlo proteins in normalized amounts of the WT and *med3*Δ Mediator. The sensitivity of the pan-Tlo-N antibody was calibrated by adding known amounts of our recombinant Tloα12/Med3-6His complex. These standards show that the amount of Tlo protein in the WT Mediator is roughly equimolar to the total amount of Mediator loaded, and that the depletion of Tlo protein in the *med3*Δ Mediator at least exceeds ~85%.

other Mediator proteins. Given the structure of the tail module of scMediator, it seems likely that Med15 is also missing from the *med3*Δ Mediator. Since the position of the Med15 band overlaps that of Med14 and an impurity, the absence of Med15 is difficult to determine by silver stain. The amount of some bands seems to be enhanced in the *med3*Δ Mediator. These mostly appear, however, to be impurities that are more abundant due to the greater amount of total protein from the heparin elution that was applied to the Flag agarose relative to the wild-type sample and the untagged control. The larger amount was loaded due to the smaller amount of Flag signal relative to total protein in the *med3*Δ heparin elution. The Cdk8 module may be present in a larger fraction of total Mediator, as those bands stain more intensely than their wild-type counterparts. To more rigorously assess the presence or absence of the Tlo proteins in the *med3*Δ Mediator, we performed Western blotting with the pan-TLO-N antibody. Using equimolar amounts of purified wild-type and *med3*Δ Mediator, normalized by their Med1 and Flag signals (Fig. 7B), we compared the amounts of Tlo protein in the two samples. There is no detectable Tlo protein in the Δ*med3* Mediator (Fig. 7B). Our purified rTloα12/rMed3-6His cocomplex standards show that down to 1/6 of the amount of the Tlo protein in 300 fmol of wild-type Mediator can be detected. This puts the lower bound of the depletion at ~85% in the *med3*Δ Mediator. The depletion of Tlo protein does not seem to be a result of decreased Tlo expression, as tagged Tloα12 is expressed in comparable amounts in whole-cell extracts from wild-type and *med3*Δ/Δ strains (data not shown).

The *med3* null strain exhibits a defect in germ tube morphology. The primary approach in the molecular genetic analysis of a new gene is to make a null strain. The challenge to taking this approach with the *C. albicans TLO* genes is the daunting task of deleting both copies of all 14 *TLO* paralogs to make a clean null mutant. Our data presented here and previous results for *S. cerevisiae* suggest that Med2 and Med3 work together through Mediator. Although it is likely that ca*MED3* has functions that are independent of the *TLO* genes, we hypothesize that many of the effects of caMed3 deletion on Mediator function are similar to the effect of *TLO* deletion on Mediator function. Hence, we have begun to characterize the phenotype of the *med3* null mutant. To perform this analysis, we have created several independently derived *med3* null strains, a complemented *med3* null in which *MED3-ARG4* is introduced at the native *MED3* locus, and a complementation control in which *ARG4* is introduced at the native *MED3* locus. All strains were viable and grew with similar doubling times in YPD at 30°C and at 37°C degrees, and they had similar morphology under these conditions (data not shown). The analysis of a *C. dubliniensis TLO1* (cd*TLO1*) null mutant indicated that there was a defect in hyphal formation that could be complemented by the *C. albicans TLOγ11* and *TLOα12* genes, as well as the *C. dubliniensis TLO1* gene (20). Hence, we sought to determine the extent to which *MED3* is required for hyphal filament formation in liquid medium under GlcNAc- and temperature-induced conditions. We performed GlcNAc and temperature induction time-course experiments in which cells from wild-type, *med3*Δ/Δ, and various control strains were harvested after 1, 2.5, and 3.5 h and examined by Nomarski/DIC and fluorescence microscopy after staining with Blankophor (see Fig. S2 in the supplemental material). In contrast to the wild type, the *med3*Δ/Δ mutant exhibited a clear defect in hyphal filament morphology. Filaments formed in the *med3*Δ/Δ mutant by 2.5 h; however, they are much shorter and do not share similar morphology with cells that have a wild-type copy of *MED3* (Fig. 8). In the *med3*Δ/Δ strain, staining chitin with Blankophor (16) showed that a septal ring remained at the point of emergence of the filament from the blastospore and at points of constriction along the filament. In the strains with a wild-type copy of *MED3*, the filaments are longer and have properties typically associated with germ tubes, such as parallel cell walls in which the first septum forms within the germ tube (42). The GlcNAc- and temperature-induced filaments in the *med3*Δ/Δ strain exhibit many hallmarks consistent with their classification as pseudohyphae (42). Using serum and temperature to induce filamentation,

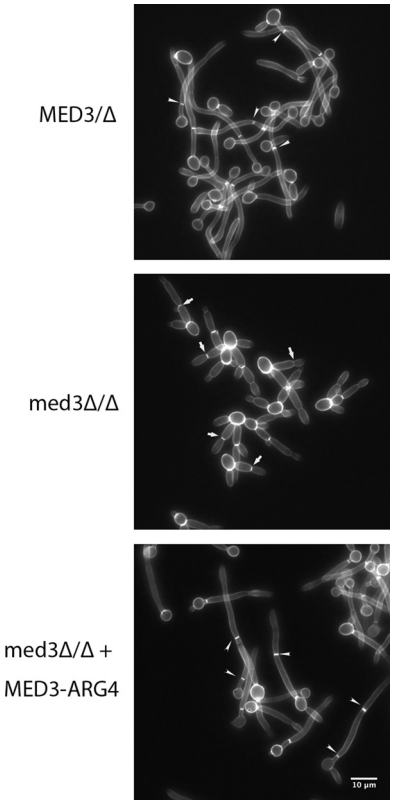

FIG 8 Morphology of *MED3*/Δ (yLM93), *med3*Δ/Δ (yLM96), and *med3*Δ/Δ plus *MED3-ARG4* (AZC53-3) cells undergoing the blastospore-to-filament transition. All strains were grown under non-filament-inducing conditions and were diluted into prewarmed YPD medium at 37°C in the presence of 2.5 mM GlcNAc. Cells were removed at various time points and stained with Blankophor. Each panel shows a separate strain after 2.5 h of induction. Arrows point to the location of septa formed along the filament. A complete set of strains from an entire time course is shown in Fig. S2 in the supplemental material. Bar at bottom left, 10 μm.

or performing the analysis in an *MTL***a**/α strain background, resulted in identical phenotype differences between the wild type and *med3* mutant (data not shown).

DISCUSSION

This study reports the first biochemical characterization of Mediator from *C. albicans*. Our analysis has revealed that the composition of the complex is identical to that of its *S. cerevisiae* counterpart, with no additional subunits, providing a sound basis for extending the findings for *S. cerevisiae* to *C. albicans*. Given the central role of Mediator in transcriptional regulation, this methodology provides a new vehicle for precise parallel biochemical and molecular genetic studies of caMediator in the complex regulatory patterns that underlie *C. albicans* virulence. A recent genetic analysis of the highly conserved *MED20*, *MED31*, and *MED13* genes in *C. albicans* showed that caMediator has roles in the expression of genes associated with virulence, such as genes related to morphogenesis and gene families enriched in pathogens (44). The purification of the Cdk8 module with caMediator provides opportunities for more directed studies of the components of this module that are frequently being identified as critical players in signaling pathways associated with virulence in fungal pathogens. *med13* null mutants have been reported to exhibit increased filamentous growth and abnormal cell wall morphology (46), and *Med12* null mutants exhibit abnormal colony morphology (33). Although the subunit composition of caMediator and scMediator is similar, the subunits and modules may functionally participate in completely different pathways and be part of the large-scale rewiring of transcriptional networks that has been demonstrated for DNA-bound transcription factors in *C. albicans* (4). Our discovery that the Tlo proteins are subunits of Mediator and the structural ortholog of scMed2 provides the greatest point of departure between caMediator and Mediator in other species, fungal or otherwise.

The combination of the direct interaction between Tloα12 and Med3, the finding that Tlo proteins are anchored within the tail module of Mediator through Med3, and the previously observed distant sequence similarity between scMed2 and the Tlo proteins (6) all point to the Tlo proteins being the true structural *C. albicans* orthologs of scMed2. In *S. cerevisiae*, Med2, Med3, and Med15 form a trimeric complex within the tail module (3). Mediator purified from either a Δ*med2*, Δ*med3*, or Δ*med15 S. cerevisiae* strain lacks these three subunits but is otherwise intact (25, 30). Med3, and presumably the Tlo proteins, plays an even greater role in *C. albicans* tail module stability, as our work shows that Med5 and Med16 are also missing from the *med3*Δ Mediator. The stability of purified protein complexes, however, can differ from their stability *in vivo*. This leaves open the possibility that a more intact tail module exists in *med3* null cells. Although other tail module subunits, such as Med15 and Med16, are well conserved in *S. cerevisiae* and *C. albicans*, the sequences for Tlo/Med2 and Med3 have diverged substantially between the two fungi. This divergence is reflected in the finding that Tloα12 is unable to complement a Δ*med2 gal* phenotype in *S. cerevisiae* (data not shown). The functional role of Tlo/Med2 and Med3 in the tail module of Mediator may be fungus specific. Among metazoan Mediator subunits, there are two that have been proposed to be distant scMed2 and scMed3 sequence-based orthologs. These subunits, however, have been characterized as head rather than tail module subunits and may play a decidedly different functional/structural role than their proposed fungal counterparts (6).

We have found that at least 5 of the 14 *C. albicans* Tlo paralogs can be incorporated into the caMediator complex. These individual Tlo subunits are present in a percentage of Mediator complexes in the cell that appears to be proportional to their expression, with no detectable sequence preference. Our data also suggest that there are several different Mediator complexes in *C. albicans* cells, each differing only in the identity of their Tlo protein subunit. Although we only have definitive data for 5 Tlo proteins, it is expected that all expressed Tlo proteins can associate with Mediator. The expression of multiple Tlo paralogs is striking compared to all other Mediator subunits in *C. albicans* and, to the best of our knowledge, all Mediator subunits in virtually all eukaryotes which are only present as a single ortholog. Although *C. dubliniensis* has two genomic Tlo paralogs (*TLO1* and *TLO2*), it appears that the expression of only one is detectable (L. C. Myers, unpublished data). A second striking difference between the *TLO* genes and genes encoding other Mediator subunits, within *C. albicans* and otherwise, is their varying levels of expression under different conditions (11, 48). This, of course, begs the question as to what advantage the expression of multiple paralogs confers upon *C. albicans* that has resulted in the maintenance of the *TLO* family throughout evolution. The two most straightforward rea-

sons are to provide some necessary functional variation that could arise from the sequence variation and to simply make more protein. Since there does not appear to be any sequence discrimination between Tlo proteins in regard to Mediator binding, sequence variation could affect Mediator interactions with other proteins. Alternatively, the primary function of the multiple Tlo paralogs could be to produce more protein. We have observed that there is Tlo protein in excess of what is required for Mediator. When epitope tagging is used to quantitate protein expression, there is the possibility that the tag or the removal of the native 3′-UTR could affect expression. We do not believe tagging is causing the observed Tlo abundance, since we find that the tagged Tlo proteins only pull down a small fraction of the total Tlo-containing Mediator in the cell. It unlikely that large amounts of Tlo proteins are necessary to drive their association with Mediator, since our data show that the Tlo proteins are tightly associated with the rest of the Mediator complex and copurify over several chromatographic steps. Another possibility is that excess Tlo protein is important for some additional function besides its direct association with Mediator. The primary function of the Med2/Med3/Med15 submodule in *S. cerevisiae* appears to be transcriptional activation. Δ*med2*, Δ*med3*, and Δ*med15* Mediator mutants in *S. cerevisiae* lose their ability to respond to a number of transcriptional activators *in vivo* and in purified transcription systems (3). Within this trimeric complex, Med15 appears to be the primary direct target of interactions with activation domains (7); however, one could envision a role of excess Tlo protein in attenuating the ability of the tail module to respond to activation by a squelching mechanism.

The complex transcriptional program that underlies hypha formation in *C. albicans* can respond to varied signals and involves many different sequence-specific DNA-bound transcription factors (41). A considerable amount is known about these sequence-specific DNA-bound transcription factors, but much less is known about their downstream targets. Recently, chromatin modification and remodeling has been revealed to play an important role in facilitating the action of these DNA-bound transcription factors (26, 27), but this is certainly only one aspect of a complex mechanism. Given the well-established role of Mediator in facilitating the regulation of specific gene expression programs, the demonstration that caMed3 and the Tlo proteins influence hyphal morphology suggests that these subunits work directly with DNA-bound factors to regulate genes involved in the yeast-to-hypha transition. This hypothesis will need to be tested in future work. Among all of the coactivators and corepressors that may influence the hyphal transcriptional program, caMed3 and the Tlo proteins are particularly intriguing due to their fungus- and *C. albicans*-specific properties that would differentiate the pathogens from the human host.

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