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Molecular cloning and characterization of chitinase genes from *Candida albicans*

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ABSTRACT **Chitinase (EC 3.2.1.14) is an important enzyme for the remodeling of chitin in the cell wall of fungi. We have cloned three chitinase genes (*CHT1*, *CHT2*, and *CHT3*) from the dimorphic human pathogen *Candida albicans*. *CHT2* and *CHT3* have been sequenced in full and their primary structures have been analyzed: *CHT2* encodes a protein of 583 aa with a predicted size of 60.8 kDa; *CHT3* encodes a protein of 567 aa with a predicted size of 60 kDa. All three genes show striking similarity to other chitinase genes in the literature, especially in the proposed catalytic domain. Transcription of *CHT2* and *CHT3* was greater when *C. albicans* was grown in a yeast phase as compared to a mycelial phase. A transcript of *CHT1* could not be detected in either growth condition.**

Chitinase (EC 3.2.1.14) is an essential component in the hydrolysis of chitin, the β-1,4-linked homopolymer of *N*-acetylglucosamine. Chitinase has a variety of roles in the biological world: bacteria produce chitinase for nutritive purposes—i.e., to assimilate chitin as a carbon and/or nitrogen source (1, 2). It is thought that plants produce chitinases as part of a repertoire of pathogenesis-related proteins, in response to fungal invasion (3). All chitin-containing organisms produce chitinase along with chitin synthase (EC 2.4.1.16) to mediate cell wall synthesis and growth (4). Fungi, in which chitin occurs often as a major component of the cell wall, will produce chitinase throughout the growth process. In the yeast *Saccharomyces cerevisiae*, chitin constitutes only about 1% of the cell wall but is found in rich deposits around the septum between mother and daughter cell (5). There is a clearly defined role for chitinase in *S. cerevisiae*, since disruption of its structural gene (*CTS1*) results in cell clumping and failure of the cells to separate after division (6). This phenomenon is also observed when demethylallosamidin, a specific inhibitor of chitinase, is added to growing cells of *S. cerevisiae* (7).

Candida albicans is a dimorphic fungus that can grow in a yeast phase or a filamentous (hyphal) phase depending on environmental conditions (8). Its genome is diploid (9), but a sexual cycle has still to be elucidated. *C. albicans* exists as a commensal of warm-blooded animals and humans but can act as an opportunistic pathogen in immunocompromised hosts (10, 11). It is proposed that the conversion of yeast to filamentous growth contributes to the virulence of this organism (12), but this assertion has been questioned, since yeast and hyphal forms (as well as an intermediate pseudohyphal phase) can be seen in infected tissue (13).

Chitinase activity has been demonstrated in preparations of *C. albicans*, both in soluble cytoplasmic extracts (14, 15) and in microsomal preparations (16). However, the enzyme(s) has yet to be purified and characterized in detail. Here we report the isolation and characterization of three chitinase genes (*CHT1*, *CHT2*, and *CHT3*) from *C. albicans*. We also present results on expression of these genes in yeast and hyphal forms of this organism.†

MATERIALS AND METHODS

Strains and Media. The *C. albicans* strains used in this paper were ATCC 10261, SGY243 (*ade2* Δ*ura3*::*ADE2*/*ade2* Δ*ura3*::*ADE2*) (17), and CAI-4 (Δ*ura3*::*imm434*/Δ*ura3*::*imm434*) (18). *C. albicans* was routinely grown in yeast phase with YPD medium (1% yeast extract/2% peptone/2% glucose) at 30°C. For dimorphic growth a temperature/pH regime was used for germ-tube formation (19). Hyphal formation was also induced by the addition of 20% (vol/vol) fetal bovine serum to YPD, with a shift up in temperature to 37°C (20).

Escherichia coli strain DH5αmcr was grown in LB (Luria–Bertani) medium containing 1% Bacto-tryptone, 0.5% Bacto-yeast extract, and 0.5% NaCl. LB was supplemented with ampicillin (100 μg/ml) when required.

DNA Extraction, Southern Blotting, and Library Construction. Plasmid DNA was prepared by alkaline lysis or, on a large scale, by cesium chloride centrifugation (21). Genomic DNA from *C. albicans* was prepared by the following method: cultures were grown to saturation in 500 ml of YPD (36 hr). After pelleting (1500 × *g*, 10 min) the cells were washed once in TSE buffer (50 mM Tris, pH 8.0/150 mM NaCl/100 mM EDTA) and lyophilized. The cells were then ground in a mortar briefly and added to a solution of TSE with 1% SDS (40 ml). This slurry was mixed gently for 24 hr at room temperature. Cell debris was removed by centrifugation and to the supernatant was added 0.1 volume of 5 M sodium perchlorate. The solution was extracted once with 0.5 volume of chloroform/isoamyl alcohol (24:1, vol/vol), and nucleic acids were precipitated with 2 volumes of ethanol. The pellet was resuspended in TE buffer (10 mM Tris, pH 8/1 mM EDTA) and subjected to two rounds of centrifugation in cesium chloride with ethidium bromide (100 μg/ml).

Genomic DNA (2.5 μg) was digested with a range of restriction enzymes and the fragments were separated by electrophoresis in 0.7% agarose. Alkaline Southern blotting was performed by standard techniques with transfer to Hybond N^+ (Amersham).

For library constructions, genomic DNA from strain ATCC 10261 was digested to completion with *Hin*dIII. After phenol extraction and ethanol precipitation the DNA was ligated to plasmid pUC18, which had been previously digested with *Hin*dIII and treated with phosphatase. After transformation the library was plated to give a cell density of about 10,000 colonies per plate (six plates). After 18 hr of growth, plates were overlaid with supported nitrocellulose filters (Schleicher & Schuell) for transfer. After transfer of colonies by standard procedures, filters were UV crosslinked (UV Stratalinker 2400; Stratagene) and air dried before hybridization. It was also found necessary to construct an *Eco*RI library as above (see *Results*).

DNA Sequencing. Sequencing of both strands was carried out by the chain-termination method of Sanger *et al.* (22) with the Sequenase 2.0 kit (United States Biochemical) and a cycle

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U15800 and U15801).

sequencing kit (Epicentre Technologies, Madison, WI). Nested deletions were constructed with exonuclease III (Exo-Size deletion kit; New England Biolabs) according to the manufacturer's instructions. In addition, restriction sites were exploited for subcloning, and any gaps were bridged by oligonucleotide primers (Biopolymer Laboratory, Massachusetts Institute of Technology).

RNA Extraction and Northern Blotting. For RNA extraction, *C. albicans* was grown overnight as a yeast (30°C). Cultures were then diluted 1:50 into the appropriate prewarmed medium and grown for a further 3 hr. Cells were collected and washed once in diethyl pyrocarbonate-treated ice-cold water. The pellet was resuspended in RNA extraction buffer (200 mM Hepes, pH 7.6/0.5 M NaCl/10 mM EDTA) and stored at −70°C if not used immediately. Acid-washed glass beads were added to just below the meniscus; 1 volume of phenol (equilibrated with water) and 0.1 volume of 20% SDS were added. Cells were vigorously vortexed for two 2-min intervals followed by 1 min on ice. The aqueous phase, after pelleting cell debris, was extracted twice with phenol and nucleic acids were precipitated with 2 volumes of ethanol. Most DNA was removed from the pellet by washing three times with 3 M sodium acetate (pH 6.0).

For Northern blotting, RNA was separated on formaldehyde agarose and transferred to Hybond N^+ (Amersham) in 20× standard saline citrate (SSC). The membrane was washed in 2× SSC for 15 min and fixed in dilute alkali (23).

Hybridization. Double-stranded DNA probes were labeled with [α-^{32}P]dCTP by random oligonucleotide priming according to the manufacturer's instructions (United States Biochemical). Filters and membranes were hybridized at 42°C with ^{32}P-labeled PCR products in a solution containing 50 mM Tris·HCl (pH 7.5), 1 M NaCl, 1% SDS, 10% dextran sulfate, 5× Denhardt's reagent, 50% formamide, and denatured calf thymus DNA at 50 μg/ml. Washings were carried out at 60°C with 2× SSC/1% SDS for 10 min (twice) followed finally with 0.1× SSC/0.1% SDS for 10 min.

Miscellaneous Procedures. Competent *E. coli* DH5αmcr cells were transformed by standard protocols (21). PCR amplification was performed in a Perkin-Elmer thermocycler with a GeneAmp kit (Perkin–Elmer/Cetus) as described (24). The primers were 5′-ATCATGRTITAYTGGGGICARAA-3′ and 5′-GAGCAR-TARTARTTRTTRTARAAYTG-3′ and were designed to include the conserved chitinase amino acid sequences Tyr-Trp-Glu-Gln-Asn and Gln-Phe-Tyr-Asn-Asn, respectively. The 600-bp amplification product was cloned into the *Sma* I site of pUC18 by standard methods (21). Chitinase activity was measured by a microtiter method (25) employing 4-methylumbelliferyl β-D-*N*-tetracetylchitotetraoside as substrate (Carbohydrates International, Arlo, Sweden).

RESULTS

Chitinase Activity Is Detected Throughout Growth of *C. albicans*. During normal growth of *C. albicans* in rich medium (YPD), chitinase activity can be detected both in the extracellular supernatant and in washed cell preparations (Fig. 1). When the soluble chitinase substrate 4-methylumbelliferyl β-D-*N*-tetraacetylchiotetraoside is used, measurement of washed cells reflects periplasmic and cell wall-bound activity (26). Chitinase production mirrored growth during exponential phase, in agreement with previous findings (14), and is analogous to chitinase production of *S. cerevisiae* (6). Another similarity with *S. cerevisiae* is that when *C. albicans* is grown in a minimal medium (SD), chitinase secretion is about 10% that obtained from growth in YPD.

Cloning of Chitinase Genes of *C. albicans*. By comparing a range of plant and fungal chitinases deposited in the databanks of the National Center for Biotechnology Information (NCBI, 1993), we designed degenerate PCR primers based on the highly conserved amino acid sequences encoded by these genes, Tyr-Trp-Gly-Gln-Asn and Gln-Phe-Tyr-Asn-Asn, which encompass the proposed catalytic domain (see *Discussion*). These primers amplified a 600-bp product from genomic DNA of *C. albicans* ATCC 10261. When labeled with ^{32}P and used to probe a Southern filter of *Candida* genomic DNA, a three-band pattern was consistently found with different restriction enzyme digests (Fig. 2). The PCR products were cloned into pUC18 and sequence analysis of individual clones revealed the presence of three genes. The inserts from these individual clones collectively yielded the hybridization pattern of Fig. 2 (results not shown). A *Hin*dIII genomic library was constructed in pUC18 and the 600-bp PCR mixture was used

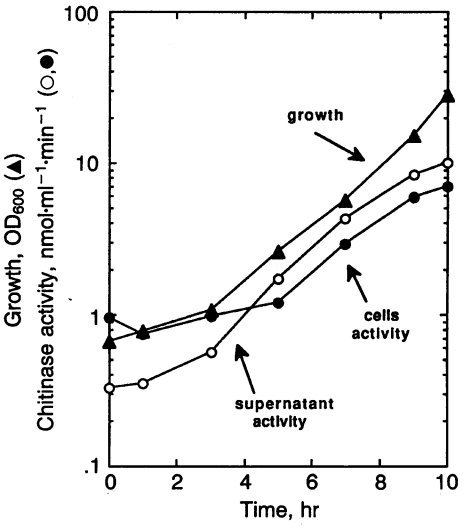

FIG. 1. Growth and chitinase production of *C. albicans* ATCC 10261 in YPD medium at 30°C. Cells from an overnight culture were diluted 1:100 into prewarmed medium and grown at 30°C with shaking. At intervals shown a sample was removed for growth estimation (▲) by OD measurement at 600 nm. For chitinase assay, washed cells and supernatant were used: cells were harvested by centrifugation from 1 ml of culture. The supernatant was saved and the cell pellet was washed twice in phosphate-buffered saline and resuspended to 1 ml in McIlvaine's buffer (pH 4.0). Cells (●) and supernatant (○) (20 μl) were added to 75 μl of buffer with 40 μM substrate. After 60 min at 37°C the reaction was stopped and the activity was calculated from duplicate wells.

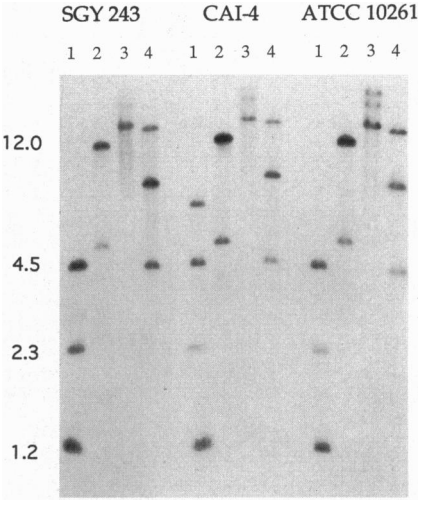

FIG. 2. Southern analysis of genomic DNA from three *C. albicans* strains (SGY 243, CAI-4, and ATCC 10261). DNA was digested with *Hin*dIII (lanes 1), *Eco*RI (lanes 2), *Bam*HI (lanes 3), or *Bgl* II (lanes 4). The blot was probed with a ^{32}P-labeled 600-bp PCR product mixture from genomic DNA. Molecular size markers (kbp) are shown at left.

to screen about 60,000 colonies. From this primary screen three clones were isolated: pCHTH1 (1.2 kb) (*CHT1*), pCHTH2 (4.5 kb) (*CHT2*), and pCHTH3 (2.3 kb) (*CHT3*). Sequence analysis with universal forward and reverse primers indicated that only pCHTH2 contained the whole gene (*CHT2*). We therefore constructed an *Eco*RI genomic library to retrieve a 4.9-kb fragment in pUC18 containing the whole *CHT3* gene (pCHTR3).

Analysis of Nucleotide and Derived Amino Acid Sequences of *CHT2* and *CHT3*. From plasmid pCHTH2, 2343 bases were sequenced in both orientations and an open reading frame of 1752 bp was found which predicts a polypeptide consisting of 583 aa with a calculated molecular mass of 60,856 Da. For plasmid pCHTR3, 2138 bases were sequenced in each orientation, encompassing an open reading frame of 1704 bp which predicts a polypeptide consisting of 567 aa with a calculated molecular mass of 60,092 Da. No intron sequences were found in either gene. The predicted isoelectric points of the two chitinase proteins are 4.56 and 4.65 for *CHT2* and *CHT3*, respectively.

The 5′ untranslated region of each gene contains both CAAT and TATA structures as well as a general pyrimidine-rich presence, as is common for many yeast and *Candida* genes (27–30). Comparison of the two *Candida* chitinases revealed a similarity of 36% over the whole protein; when both were compared with the *Saccharomyces* CTS1 protein, CHT2 was 36% similar and CHT3 was 38% similar (Fig. 3). However, when alignments were generated from the N-terminal regions of these chitinases and compared with other chitinase genes found in the databanks, homology was distinctly higher (Fig. 4). The three *Candida* partial gene products and CTS1 of *S. cerevisiae* were about 55–65% similar to each other in this N-terminal region. Similarity was slightly less (about 45%) when these chitinases were compared to the *Rhizopus* gene products (31).

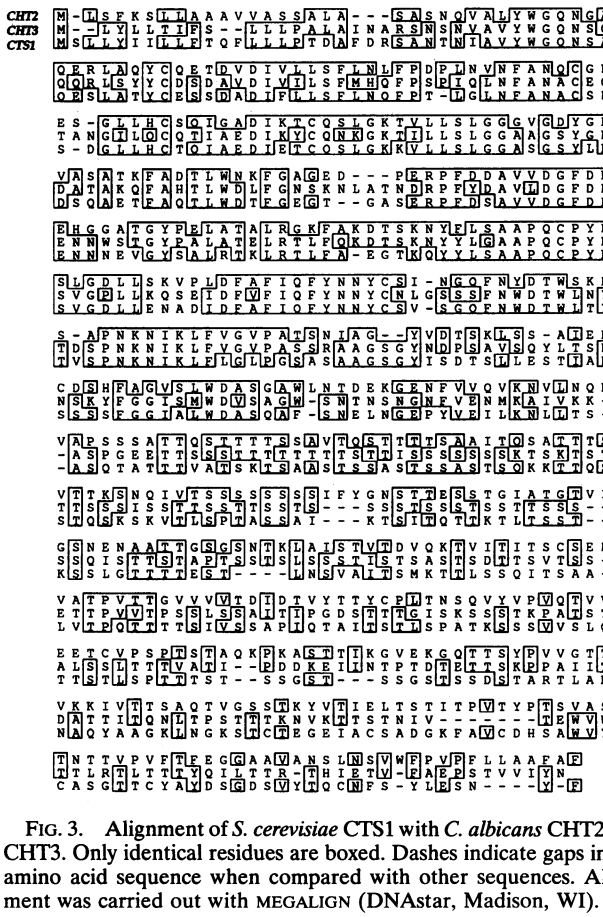

FIG. 3. Alignment of *S. cerevisiae* CTS1 with *C. albicans* CHT2 and CHT3. Only identical residues are boxed. Dashes indicate gaps in the amino acid sequence when compared with other sequences. Alignment was carried out with MEGALIGN (DNAstar, Madison, WI).

In CHT2 there are three potential N-glycosylation sites (Asn-Xaa-Ser/Thr), and in CHT3 there are four. Both genes have a region near the 3′ end in which >70% of the amino acids encoded are either serine or threonine; for CHT2 this region is aa 311–370 (72% serine/threonine) and in CHT3 this region is aa 319–426 (89% serine/threonine). These serine/threonine-rich domains can act as potential sites for O-mannosylation and are also found in *Saccharomyces* CTS1 and the chitinases from *Rhizopus*.

As seen in Fig. 4, an alignment of fungal chitinases reveals striking homology in a series of blocks throughout the N-terminal region. These highly conserved blocks contain invariant aspartic and glutamic residues which have been implicated in the catalytic mechanism of these enzymes (6, 32). Mileweski *et al.* (33), by chemical modification, provided indirect evidence that an aspartic/glutamic carboxyl group(s) was important for catalytic activity of a partially purified *C. albicans* chitinase. Watanabe *et al.* (34), by site-directed mutagenesis, provided strong evidence that aspartic and glutamic residues in a bacterial chitinase were important for catalysis. Also conserved are six invariant cysteine residues (Fig. 4) which may be involved in correct folding of the active domain.

In the chitinase CTS1 from *S. cerevisiae* there is a region after the serine/threonine-rich domain which codes for a cysteine-rich high-affinity chitin-binding polypeptide. In *Candida* CHT2 and CHT3 no homology to this region was found (Fig. 3). We have also been unable to bind extracellular chitinase to various forms of chitin (results not shown). Searches with the predicted amino acid sequences of the 3′ regions failed to detect significant homology to proteins in GenBank (release 85).

Northern Analysis of *C. albicans* Chitinase Expression. A pH and temperature regime (19) was chosen for gene expression studies in dimorphic forms, because only those two parameters are changed whereas the medium remains constant. At a pH of 4.5 and a temperature of 30°C, *C. albicans* grows purely as a yeast. When the pH and temperature are increased to 6.7 and 37°C respectively, the fungus germinates to eventually form hyphae. Expression of both *CHT2* and *CHT3* is slightly greater in the yeast phase of growth than in hyphae (Fig. 5). Expression of *CHT1* could not be detected in either phase, even after prolonged exposure of the hybridization filter to x-ray film. This result is perplexing and suggests the possibility of differential expression during chlamydiospore formation. Hyphal induction was also carried out in calf serum-enriched medium (20) and the pattern of preferential induction of *CHT2* and *CHT3* in the yeast phase was corroborated (results not shown).

Chromosomal Locations of *C. albicans* Chitinases. By using an *Sfi* I macrorestriction blot of *Candida* strain 1006 chromosomes (35), *CHT1* was mapped to chromosome R (*Sfi* I fragments M and S). *CHT2* was mapped to chromosome 5 (*Sfi* I fragment I), and *CHT3* was located on chromosome R (*Sfi* I fragment D). Mapping was carried out by B. B. Magee's group at the University of Minnesota (results not shown).

DISCUSSION

In this study a set of degenerate oligonucleotides directed to the consensus catalytic sequences of cloned fungal and plant chitinases were used in the PCR with genomic DNA from the human pathogen *C. albicans* as substrate. Examination of the cloned reaction products revealed three distinct chitinase sequences. Two of these genes (*CHT2* and *CHT3*) were sequenced and their deduced peptide structures were analyzed. *CHT1* has yet to be sequenced in full. Aside from the catalytic domain, which is homologous in CHT2 and CHT3, the only other region of similarity is a serine/threonine-rich

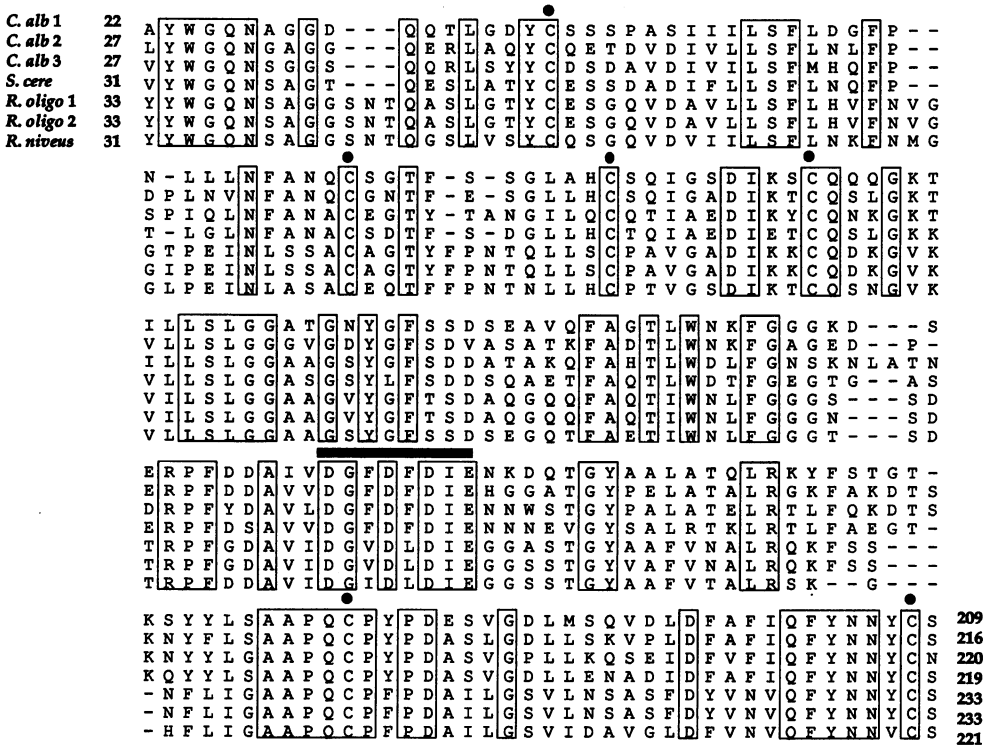

FIG. 4. Alignment of highly conserved, proposed catalytic regions present in fungal chitinases. Only identical residues have been boxed. Dashes indicate gaps in the amino acid sequence when compared with other sequences. Numbers refer to the position of selected peptide fragments in relation to their respective start codons. Invariant cysteine residues are indicated with a dot. The proposed aspartic and glutamic catalytic residues are highlighted with a bar. *C. alb* 1, *C. albicans* CHT1; *C. alb* 2, *C. albicans* CHT2; *C. alb* 3, *C. albicans* CHT3; *S. cere, S. cerevisiae* CTS1; *R. oligo* 1, *R. oligosporus* chi1; *R. oligo* 2, *R. oligosporus* chi2; *R. niveus, R. niveus* chitinase.

domain which occurs in both genes downstream of the catalytic domain. This is a potential site for O-linked glycosylation, and the extracellular location of chitinase activity throughout the growth phase (Fig. 1) suggests that at least one of the enzymes is secreted.

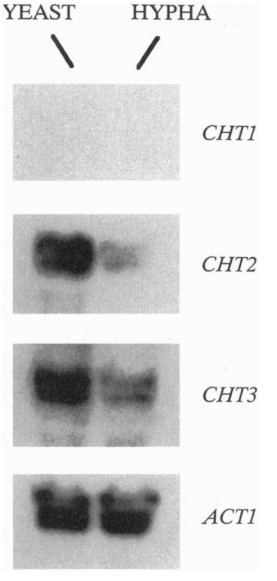

FIG. 5. Northern analysis of chitinase expression during yeast and hyphal development. *C. albicans* strain ATCC 10261 was grown overnight in Lee's medium at pH 4.5 and 30°C and diluted 1:50 into prewarmed medium at either pH 4.5 and 30°C or pH 6.7 and 37°C. Cells were allowed to grow for 3 hr before harvesting and extraction of total RNA. At this time >90% of yeast cells had germinated at pH 6.7 and 37°C. Equal amounts of RNA were loaded as judged by ethidium bromide staining. After Northern transfer the filter was probed with pCHT1, stripped, and reprobed with pCHT2, pCHT3, and *C. albicans ACT1* (actin gene), with sequential washing steps.

The function of the region after the serine/threonine domain for both CHT2 and CHT3 is not known. In CHT2 there is a similar number of cysteine residues as in the respective *S. cerevisiae* CTS1 region, and it has been pointed out that flanking cysteines are found in high-affinity chitin-binding regions in a number of chitinases (6, 36). We do not suggest that such a chitin-binding region is absent from CHT2, but we have not yet found high-affinity chitin-binding activity associated with the domain. In CHT3 there are no cysteines in this region (Fig. 3), although hydrophobicity plots for CHT2 and CHT3 are similar (results not shown). The possible function of these 3′ regions in *CHT2* and *CHT3* could be addressed by attaching a reporter gene 5′ of the region and investigating cellular localization of the expressed fusion protein.

Three genes encoding chitin synthases have been cloned from *C. albicans* (37–39). It is possible that both chitin synthase and chitinase act in concert to lay down chitin microfibrils during growth of this organism (40). One of these chitin synthase genes (*CHS2*), which is preferentially transcribed in hyphae, has been disrupted and the mutant was found to have a reduced chitin content in hyphae although the amount of chitin in yeast cultures was normal (20).

Preliminary results from disruption of *CHT2* suggest a role in cell separation, since washed cells from early exponential phase tend to aggregate in clumps or clusters. The rather poor expression of *CHT2* and *CHT3* in a *S. cerevisiae* chitinase deletion strain has led us to construct expression systems utilizing a stronger promoter (*GAL1* and *Saccharomyces CTS1*) which should aid in the purification of these enzymes. This work and results of *CHT2* and *CHT3* disruptions will be presented elsewhere.

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somal mapping of the *Candida* chitinase genes. We thank Dr. J. F. Ernst (Heinrich-Heine University, Dusseldorf) for the gift of the *C. albicans* actin gene. This work was supported by grants from the National Institutes of Health, GM31318 (to P.W.R.) and CA14051 (to R. O. Hynes).

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