Transcription of the Gene for a Pepsinogen, *PEP1*, Is Regulated by White-Opaque Switching in *Candida albicans*

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Cells of *Candida albicans* WO-1 spontaneously switch between a white and opaque CFU, and this phase transition involves a dramatic change in cellular phenotype. By using a differential hybridization screen, an opaque-specific cDNA, *Op1a*, which represents the transcript of a gene regulated by switching, has been isolated. The gene for *Op1a* is transcribed by opaque but not by white cells. The nucleotide sequence of the *Op1a* cDNA reveals over 99% base homology with an acid protease gene of *C. albicans*, and the predicted amino acid sequence demonstrates that the product of this gene is a member of the family of pepsinogens, which possess a hydrophobic leader sequence for secretion and two catalytic aspartate domains. Southern blots of both genomic DNA digested with 14 different endonucleases and electrophoretically separated chromosomes were probed with the *Op1a* cDNA. No polymorphisms were detected in either case between white and opaque cells, suggesting that no genomic reorganization occurs in the proximity of the gene during the white-opaque transition. Although transcription of *Op1a* correlates with the high levels of extracellular protease activity in opaque cell cultures and the absence of activity in white cell cultures, stimulation of extracellular protease activity by addition of serum albumin is not accompanied by *Op1a* transcription in cultures of WO-1 white cells or cultures of two additional clinical isolates of *C. albicans*, suggesting that expression of one or more other protease genes is stimulated in these cases. The results demonstrate that transcription of the *Op1a* gene is under the rigid control of switching in strain WO-1.

Many infectious microorganisms generate population variability by switching spontaneously and at high frequency between two or more phenotypes (6, 15, 19, 51), and *Candida albicans* is no exception (43, 44). *C. albicans* and related species are capable of switching between a number of phenotypes usually distinguishable by colony morphology (32, 40, 41, 47). In the case of strain WO-1, switching involves a phase transition between white and opaque colony formation, and this transition includes a dramatic change in cellular phenotype (2–4, 35, 41, 45). In the budding white phase, cells are round to slightly ovoid and are indistinguishable from the standard budding phenotype of most other strains of *C. albicans*. However, in the budding opaque phase, cells are ellipsoidal and twice the size of budding white cells. Opaque cells exhibit a unique pimpled wall morphology with channels traversing pimples and membrane-bound blebs protruding from pimple apices (2–4). They also have altered permeability (35), altered sterol and lipid composition (18), an enlarged cytoplasmic vacuole (41), altered drug sensitivity (48), and altered sensitivity to neutrophils and in vitro oxidants (22). The mechanism of switching in *C. albicans* has not been elucidated (43, 44), and to date there have been only indirect indications that it involves differential gene expression (2–4). To test whether the white-opaque transition is accompanied by differential gene expression, we have performed a differential hybridization screen to identify cDNA sequences representing genes transcribed exclusively in the opaque phenotype in defined nutrient medium. Here we report the identification of the first gene regulated by switching in *C. albicans*. This gene, *Op1a*, is transcribed exclusively in the opaque phenotype of strain WO-1. The nucleotide sequence of the cDNA reveals

that it represents the transcript of an acid protease gene of *C. albicans*, a member of the pepsinogen family.

MATERIALS AND METHODS

Growth and development. Cells from *C. albicans* WO-1 were stored on agar slants in capped tubes. For experimental purposes, cells from a storage slant were clonally plated on agar containing the nutrient composition of Lee's medium (27) supplemented with 70 μ g of arginine per ml and 0.1 μ M ZnSO₄ (7), which we will refer to as modified Lee's medium. Cells from white and opaque colonies were independently diluted into 25 ml of liquid modified Lee's medium, and cultures were rotated at 200 rpm at 25°C as previously described (7, 46). The method of pH-regulated dimorphism (11, 42) was used to generate semisynchronous cultures of budding or hypha-forming cells. Micrographs of white and opaque colonies were taken through a Wild M8 dissection microscope, and micrographs of individual cells were taken through a Zeiss ICM 405 inverted microscope equipped with differential interference contrast optics. Kodak T-MAX 100 film was used in all cases.

RNA and DNA preparation. For RNA isolation, populations of white or opaque cells were disrupted in a Mini-Beadbeater (Biospec Products) with glass beads of 425- to 600- μ m diameters (Sigma). Total cellular RNA was isolated by using guanidinium isothiocyanate extraction as described by Chomczynski and Sacchi (12). Polyadenylated RNA was isolated by using a microcrystalline grade of oligo(dT) cellulose (New England BioLabs) as recommended by the manufacturer. Total cellular DNA was isolated from pure populations of budding white and opaque cells essentially by the method described for *Saccharomyces cerevisiae* (5), except that spheroplasts were prepared by using Novozyme (Novo BioLabs).

Synthesis of cDNA probes. White and opaque cell cDNA

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probes were synthesized from approximately 3 to 4 μ g of polyadenylated RNA. For each reaction, 2.5 μ g of oligo(dT) primer was added to each poly(A)⁺ preparation and incubated at 65°C for 5 min. Reverse transcription reactions were performed at 40°C for 1 h in a final volume of 50 μ l of a solution containing 0.5 mM deoxynucleoside triphosphates without dCTP, 100 μ Ci of [³²P]dCTP (>3,000 Ci/mmol), 500 U of mouse mammary leukemia virus reverse transcriptase lacking RNase H activity (Superscript, Bethesda Research Laboratories), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, and 10 mM dithiothreitol. After cDNA synthesis, the RNA was removed by treatment with NaOH at 90°C for 5 min and then by neutralization with HCl. The labelled cDNA was purified on a G-50 spin column (5 Prime-3 Prime).

Isolation of opaque-specific cDNA clones. An opaque-specific cDNA library was constructed in λ gt10 by the methods of Maniatis et al. (30). The library was plated on five 150-mm plates with approximately 20,000 plaques per petri plate. Duplicate nitrocellulose filters were prepared, and the first filter of each set was hybridized with white cDNA probe while the second filter was hybridized with opaque cDNA probe. Hybridization was carried out at 65°C in a solution of 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.4), 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS), and 100 μ g of sonicated, denatured calf thymus DNA per ml. Filters were washed first in 2 $\times$ SSC containing 0.5% SDS and then in 0.5 $\times$ SSC at 65°C. For each positive plaque, an agarose plug was taken and the phage were eluted in buffer consisting of 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM MgSO₄, and 0.01% gelatin. Phage DNA was prepared from small-scale liquid lysates (30) of phage eluted from each positive plaque. Approximately 1 μ g of phage DNA was digested with *Eco*RI, and the DNA fragments were resolved on duplicate 0.7% agarose gels. The gels were blotted onto Zetabind (Cuno) and hybridized with either white or opaque cDNA probes. Clones exhibiting a positive signal exclusively with the opaque cDNA probe in Southern blots were chosen for further characterization. The differentially positive clones were plaque purified by repeating the differential hybridization screen as described above.

A Northern (RNA) blot of opaque RNA probed with the positive cDNA clone, Op1, indicated that the transcript was 1.7 kb long. Op1 was incomplete, containing only 350 bp. To isolate a longer sequence, Op1 cDNA was used as a probe to screen a second opaque cDNA library constructed in the bacteriophage vector λ gt11 according to the methods of Maniatis et al. (30). The longest cDNA insert isolated, Op1a, contained approximately 1,300 bp. The intact cDNA could not be released from the cloning site of the bacteriophage vector as a contiguous sequence because of an internal *Eco*RI site. Therefore, plaque-purified phage was used as a template for the polymerase chain reaction (PCR) (16, 38). For PCR, 20-mer primers that were complementary to either arm of λ gt11, 5'-CACCAGACCAACTGGTAATG-3' and 5'-GCGACGACTCCTGGAGCCCG-3', were synthesized. Phage was diluted into water and heated at 70°C for 5 min to disrupt phage particles, and the liberated phage DNA was used as a template for PCR. The conditions used for primer annealing and extension were 51°C for 1 min and 72°C for 3 min, respectively, with a total of 35 cycles. Amplified Op1a cDNA was purified from a TAE agarose gel by using GeneClean (Bio 101). PCR-amplified cDNA was blunt ended by using T4 DNA polymerase (5) and ligated into the *Eco*RV site of pGEM5zf(+) (Promega).

Northern and Southern blot analysis. For Northern blot analysis, total cellular RNA was fractionated on a 1.25% agarose-formaldehyde gel, transferred to Zetabind (Cuno), UV cross-linked, and hybridized at 65°C in a solution containing 0.5 M NaHPO₄ (pH 7.2), 1 mM EDTA, 7% SDS, 1% bovine serum albumin, and 100 μ g of sonicated, denatured calf thymus DNA per ml (13). DNA probes were labelled with [³²P]dCTP by nick translation (Bethesda Research Laboratories). Filters were routinely hybridized for 16 to 20 h and washed sequentially at 65°C, first in a solution containing 40 mM NaHPO₄ (pH 7.2), 5% SDS, 1 mM EDTA, and 0.5% BSA, and then in a solution containing 40 mM NaHPO₄, 1 mM EDTA, and 1% SDS. For Southern blot analysis, DNA was digested to completion with the appropriate restriction endonuclease, size fractionated on a 0.7% agarose gel, and blotted to Zetabind (Cuno) by the method of Southern (49). DNA filters were hybridized by using the conditions described above for Northern blot analysis. Autoradiography was performed at -70°C with one intensifying screen for 18 to 20 h for Southern blots or at room temperature for 1 to 2 h for Northern blots with Kodak XAR film.

DNA sequence determination. The cDNA clone was sequenced by the dideoxy-chain termination method by using a modified form of T7 polymerase (Sequenase version 2.0; U.S. Biochemicals). The cDNA sequence was determined from the PCR-amplified cDNA as well as from various subclones generated by directional cloning of appropriate restriction fragments into pGEM vectors (Promega). Sequence reactions were performed with CsCl-purified plasmid templates (30). Sequencing was performed in both directions. Sequences were analyzed with the Microgenie Sequence Analysis Program (Beckman Instruments) (33). The GenBank DNA and the NBRF protein data bases were searched with the Microgenie Sequence Analysis Program (version 7).

Separation of *C. albicans* chromosomes by transverse alternating field electrophoresis. White and opaque cells were grown to mid-log phase in modified Lee's medium at 25°C. Agarose chromosome plugs were prepared according to the method of Vollrath and Davis (55). Protoplasts were generated within agarose plugs by using a 1:1 mixture of Zymolase 20T (ICN Biomedicals) and Novozyme (NovoBio Laboratories). Chromosome-sized DNA molecules were resolved on a 0.65% agarose gel by using transverse alternating field electrophoresis (GeneLine; Beckman). The gel was run in 0.5 $\times$ TBE (1 $\times$ TBE is 50 mM Tris, 50 mM boric acid, and 1 mM EDTA) at 100 V with 1-min pulses for the first 6 h, with 2-min pulses for the next 12 h, with 4-min pulses for the next 16 h, with 7-min pulses for the next 20 h, and finally, at 80 V with 10-min pulses for 18 h. The running buffer was changed every 24 h. Two-centimeter plugs of white and opaque DNA preparations were run to improve resolution of the chromosome bands. The gel was blotted and hybridized by using the conditions described above for Southern blot hybridization analysis.

Assay for protease activity in culture supernatants. Cells were grown to stationary phase at 25°C in 25 ml of either modified Lee's medium (pH 6.8) or serum medium (2% glucose, 7.3 mM KH₂PO₄, 4.1 mM MgSO₄, 1 μ g of biotin per ml, and 5% goat serum [GIBCO BRL]), pH 5.0. In one noted experiment serum medium was supplemented with vitamin mixture modified Eagle medium (MEM) (GIBCO BRL) at a final concentration of 1 $\times$ by the method of Staib (50). Cells were collected after 36 h by centrifugation, and the culture supernatants were directly assayed for protease activity by using the chromogenic substrate azocasein (54). Serial dilu-

FIG. 1. The white-opaque transition at the colony and cellular level. (A) Clonal colonies generated by cells from a white colony in which one cell switched to opaque; (B) clonal colonies generated by cells from an opaque colony in which one cell switched to white; (C) differential interference contrast images of cells from a white colony dome (note the spherical morphology of individual cells); (D) differential interference contrast images of cells from an opaque colony dome (note the elongate shape of individual cells). Bars in panels C and D, 10 μ m. w, White colony; op, opaque colony.

tions of pepsin A from porcine stomach mucosa (Sigma) were assayed as standards in a range of 0 to 450 U. A 200- μ l volume of culture supernatant or pepsin A was added to 1 ml of 0.5% azocasein (Sigma) in citrate phosphate buffer (24 mM citric acid, 50 mM Na_2HPO_4), pH 5.0. Reaction mixtures were incubated at 37°C for 1 h. Undigested substrate was precipitated with 2 ml of 10% trichloroacetic acid and removed from solution by centrifugation. The supernatant was neutralized with 2 ml of 0.5 M NaOH, and the A_{440} was determined. All assays were performed in duplicate. One unit of pepsin A was defined as the amount of activity which resulted in a positive A_{280} change of 0.001 per min at 37°C, pH 2.0, of trichloroacetic acid-soluble product from hemoglobin (Sigma). Units of activity were normalized to 10^7 cells by using the cell concentration at the time of assay.

RESULTS

Cells of strain WO-1 switch frequently and reversibly between the white and opaque phase. When cells of *C. albicans* WO-1 were plated from a storage slant onto nutrient agar, the majority of subsequent colonies exhibited the white hemispherical dome characteristic of the white phenotype, while a minority exhibited the slightly larger, flat, grey morphology of the opaque phenotype. When cells of a single white colony were in turn plated, more than 99% gave rise to white colonies and less than 1% to opaque colonies (Fig. 1A), and when cells of an opaque colony were in turn plated, more than 99% gave rise to opaque colonies and less than 1% gave rise to white colonies (Fig. 1B). When cells from a

white colony dome were examined with differential interference contrast microscopy, the majority (>99%) exhibited the round shape characteristic of the white cell phenotype (Fig. 1C), and when cells from an opaque colony dome were examined in a similar fashion, the majority (>99%) exhibited the elongate, asymmetrical morphology characteristic of the opaque cell phenotype (Fig. 1D).

Selection of the opaque-specific cDNA clone Op1a. In order to obtain cDNA sequences of transcripts expressed exclusively in opaque cells, a cDNA library in λ gt10 vector was constructed from poly(A)⁺ RNA of opaque cells. Duplicate filters were hybridized with radiolabelled single-stranded cDNA of either white or opaque cells. Thirty-five of 100,000 plaques yielded positive hybridization signals with opaque but not white cDNA probe. The 35 putative clones were further analyzed by screening duplicate Southern blots of phage DNA with white or opaque cDNA probes. Two of the 35 putative clones yielded positive hybridization signals exclusively with the opaque cDNA probe. The two clones, Op1 and Op2, were determined to be identical since they (i) hybridized to transcripts of equivalent size in Northern blots, (ii) displayed identical hybridization patterns in Southern blots of genomic DNA, and (iii) displayed cross hybridization in Southern blots (data not shown). Both Op1 and Op2 were significantly smaller than the transcript to which they hybridized on a Northern blot. Therefore, a longer, 1.3-kb cDNA was isolated from a second opaque-specific cDNA library by using Op1 as a probe, and this cDNA, Op1a, was chosen for further characterization.

Op1a is expressed exclusively in opaque cells. Northern

FIG. 2. Northern blots of white and opaque cell RNA probed with Opl1 (upper gel) and the constitutively expressed *ADE2* gene (lower gel). Lanes 1 and 3, individual white cultures grown at 25°C; lanes 2 and 4, individual opaque cultures grown at 25°C; lanes 5 and 6, white budding and white hypha-forming cultures, respectively, grown at 37°C under the regime of pH-regulated dimorphism. Note that Opl1 transcript is observed only in opaque cell RNA.

blots of RNA prepared from opaque cells and white cells were probed with Opl1. Opl1 cDNA was complementary to a single 1.7-kb transcript present in two independent opaque clones (Fig. 2, lanes 2 and 4), but absent in two independent white clones (Fig. 2, lanes 1 and 3) grown at 25°C in modified Lee's medium. A complementary transcript was absent in both white budding and white hypha-forming cells (Fig. 2, lanes 5 and 6, respectively) generated semisynchronously at 37°C in modified Lee's medium under the regime of pH-regulated dimorphism (11, 42). Reprobing of Northern blots with the constitutively expressed *ADE2* gene (23) indicated that intact *ADE2* transcript was present in white and opaque cell RNA preparations.

Southern blot hybridization of electrophoretically separated chromosomes with Opl1. Transverse alternating field electrophoresis resolved 10 chromosome-size DNA molecules in strain WO-1 and revealed no polymorphism between the white and opaque phenotypes (Fig. 3A). When blotted and probed with Opl1, hybridization was evident in 2 of the 10 bands of both white and opaque phenotypes (Fig. 3B). Since *C. albicans* is diploid (31, 34) and contains eight pairs of chromosomes (26), several of the bands resolved in strain WO-1 probably represent electrophoretically separable, rearranged homologs (39). Therefore, the two bands which hybridized with Opl1 cDNA probably represent alleles on electrophoretically separated homologs.

Absence of genomic reorganization in the vicinity of the Opl1 gene during the white-opaque transition. DNA from white and opaque cells was digested individually with 14 different restriction endonucleases and probed with Opl1 cDNA. These restriction enzymes were chosen such that they represented a wide array of recognition motifs varying both in base composition and in DNA sequence. In all cases, the hybridization patterns of white and opaque DNA were identical (Fig. 4), indicating that there is no gross genomic reorganization in the vicinity of the Opl1 gene during the white-opaque transition. In addition, the methylation-sensitive isoschizomers *MspI* and *HpaII* did not demonstrate differences in the methylation of cytidine residues in their

FIG. 3. Southern blot of electrophoretically separated chromosomes of white (W) and opaque (O) cells probed with Opl1 chromosomes were separated by transverse alternating field electrophoresis. (A) Ethidium bromide-stained gel; (B) autoradiogram of Southern blot.

respective recognition sequences in white and opaque DNA (Fig. 4, lanes 8 and 9).

The Opl1 gene is present but unexpressed in the o-smooth phenotype of strain 3153A. *C. albicans* 3153A predominates in the o-smooth phenotype (40), which is analogous to the white phase of strain WO-1 (41). Although strain 3153A is capable of high-frequency switching (40, 43), it does not possess in its switching repertoire the opaque colony-forming phenotype. Southern blot hybridization of *HindIII*-digested WO-1 and 3153A DNA with Opl1 cDNA resulted in identical patterns (Fig. 5A, lanes 2 and 4, respectively), and Southern blot hybridization of *EcoRI*-digested WO-1 and 3153A DNA (Fig. 5A, lanes 1 and 3, respectively) resulted in similar patterns differing in a band present in WO-1 and absent in 3153A. These results demonstrate that the Opl1 gene is present in strain 3153A. However, Northern blot hybridization with Opl1 cDNA revealed no Opl1 transcript in either budding or hypha-forming 3153A cells (Fig. 5B, lanes 3 and 4, respectively) generated semisynchronously at 37°C in modified Lee's medium under the regime of pH-regulated dimorphism (11, 42).

Sequence analysis: Opl1 cDNA represents the transcript of a pepsinogen. The Opl1 cDNA clone isolated from an opaque cDNA library by a differential hybridization screen is 1,283 nucleotides in length. The entire DNA sequence is virtually identical to a recently reported *C. albicans* aspartyl protease gene (20), with a base homology greater than 99%. The sequenced cDNA contains nearly the entire hydrophobic signal sequence including a lysine at position 36, a characteristic of many secreted aspartyl proteases (14). The cDNA contains a glutamine codon, which is the first amino-terminal amino acid of the mature enzyme (20). The nucleotide sequence indicates that the enzyme coded by this transcript contains two catalytic aspartyl residues within domains that are highly conserved among aspartyl proteases (14, 52). These two catalytic domains are separated by 185 amino acids. Figure 6 shows that the two catalytic sites are highly conserved not only in the primary amino acid sequence, but also in their respective positions within the active form of the enzyme between Opl1 and several members of the pepsinogen family (52, 53). The crystal structures of many pepsinogen family members reveal a high degree of

FIG. 4. Op1a-probed Southern blots of opaque (O) and white (W) cell DNA digested with 14 different endonucleases. Molecular masses of standards are presented to the left of the figure. Lanes: 1, *EcoRV*; 2, *EcoRI*; 3, *KpnI*; 4, *HaeIII*; 5, *SacII*; 6, *XhoI*; 7, *BamHI*; 8, *MspI*; 9, *HpaII*; 10, *HpaI*; 11, *MboII*; 12, *RsaI*; 13, *BglII*; 14, *HindIII*.

similarity in conformation, in that the two aspartyl catalytic sites are connected to one another through the formation of hydrogen bonds in the mature secreted form of the enzyme (14). Since the size of the transcript identified by Northern analysis was approximately 1.7 kb, the Op1a cDNA is presumably lacking more than 400 nucleotides of the mature transcript.

The entire Op1a nucleotide sequence did not exhibit significant homology with any sequences in the nucleotide data base, with the exception of the *C. albicans* gene

encoding for aspartyl protease. However, comparison of the entire Op1a predicted amino acid sequence with the protein data base revealed a high degree of homology with several members of the pepsinogen family. The predicted amino acid sequence of Op1a exhibited 45% homology with porcine pepsin, including conservative changes, spanning a region of 260 amino acids which includes both aspartyl catalytic sites. This homology was not previously noted for this gene (20).

Opaque but not white cells secrete acid protease under noninducing conditions. The cell-free medium of stationary-phase cultures of opaque cells grown in modified Lee's medium for 36 h contained 88.6, 36.3, and 17.4 U of protease activity per 10^7 cells, respectively, in three independent experiments (Table 1, Experiments 1, 2, and 3). However, little or no acid protease activity was detected in the cell-free medium of stationary-phase cultures of white cells grown under the same conditions (Table 1, experiments 1, 2, and 3). The protease activity in the medium of opaque cell cultures was at least 886, 363, and 58 times those in the medium of white cell cultures in the respective experiments.

FIG. 5. Southern (A) and Northern (B) blots of strains 3153A and WO-1 probed with Op1a. (A) Lanes 1 and 3, *EcoRI*-digested WO-1 and 3153A DNA, respectively; lanes 2 and 4, *HindIII*-digested WO-1 and 3153A DNA, respectively. Molecular masses of standards are presented to the left of the panel. (B) Lanes 1 and 2, RNA of white and opaque cells, respectively, of strain WO-1; lanes 3 and 4, RNA of budding and hypha-forming cultures, respectively, of strain 3153A, developed under the regime of pH-regulated dimorphism. At the bottom of panel B, the Northern blot was reprobed with the constitutively expressed *ADE2* gene.

A. N-terminal Domain

<i>Op1a(PEP1)</i>	(32)	Val	Ile	Val	Asp	Thr	Gly	Ser	Ser
<i>Penicillopepsin</i>	(33)	Leu	Asn	Phe	Asp	Thr	Gly	Ser	Ala
<i>Porcine Pepsin</i>	(32)	Val	Ile	Phe	Asp	Thr	Gly	Ser	Ser
<i>Human Pepsin</i>	(31)	Val	Val	Phe	Asp	Thr	Gly	Ser	Ser

B. C-terminal Domain

<i>Op1a(PEP1)</i>	(217)	Val	Leu	Leu	Asp	Ser	Gly	Thr	Thr
<i>Penicillopepsin</i>	(208)	Ser	Gly	Ile	Asp	Thr	Gly	Thr	Thr
<i>Porcine Pepsin</i>	(215)	Ala	Ile	Val	Asp	Thr	Gly	Thr	Ser
<i>Human Pepsin</i>	(214)	Ala	Ile	Val	Asp	Thr	Gly	Thr	Ser

FIG. 6. A comparison of the N-terminal and C-terminal aspartyl domains of the catalytic site of Op1a (*PEP1*) and three other members of the pepsinogen family. The numbers in parentheses refer to the amino acid number for the aspartyl residue within the mature form of the enzyme. Residues in bold print are highly conserved and characteristic of pepsins.

TABLE 1. Extracellular protease activity and presence of the Op1a (*PEP1*) transcript in growth cultures of WO-1 white and opaque and two clinical isolates

Expt and culture ^a	Medium ^b	Protease activity ^c (U/10 ⁷ cells)	Difference (fold) in activity	Op1a (<i>PEP1</i>) gene expression ^d
1				
W	Lee's	0.0		–
O	Lee's	88.6	>886	+
W	Serum	9.6		–
O	Serum	98.3	10	+
2				
W	Lee's	0.0		–
O	Lee's	36.3	>363	+
W	Serum	1.9		–
O	Serum	70.0	37	+
3				
W	Lee's	0.3		–
O	Lee's	17.4	58	+
W	Serum	1.2		–
O	Serum	77.9	65	+
Hp33	Lee's	0.4		–
Hp33	Serum	0.6	1.5	–
RP1-I3	Lee's	0.0		–
RP1-I3	Serum	2.0	>20	–

^a In experiment 3, vitamins were added to growth media by the method of Staib (50). Hp33 is a commensal strain of *C. albicans* isolated from the vaginal canal of a healthy woman; RP1-I3 is an infectious strain of *C. albicans* isolated from the vaginal canal of a recurrent vaginitis patient. W, white; O, opaque.

^b Lee's, modified Lee's medium; Serum, serum medium (see Materials and Methods).

^c Each activity is the average of duplicate measurements.

^d The presence (+) or absence (–) of the Op1a transcript was assessed by Northern blot hybridization with Op1a cDNA.

Since it has been reported that excretion of acid protease in test strains can be stimulated by the addition of serum proteins (28, 37), white and opaque cell cultures were grown to stationary phase in media containing 5% goat serum, and the cell-free media were assayed for protease activity. Although activity was detected in white cell cultures containing serum in three independent experiments (two without and one with vitamin supplement), the stimulated levels were still 10-, 37-, and 65-fold less than parallel opaque cell cultures containing serum.

To test whether the small but significant stimulation by serum of secreted protease activity in white cell cultures correlated with stimulated transcription of the Op1a gene, Northern blots of whole cell RNA were probed with Op1a cDNA. The level of Op1a transcript in opaque cells grown in the presence of serum proteins was slightly elevated over that in parallel cultures of opaque cells grown in the absence of serum proteins (Fig. 7), which correlated with the small difference in the level of excreted protease activity assayed in the culture supernatant (Table 1, experiment 2). However, there was no detectable Op1a transcription in white cells grown in the presence of serum proteins (Fig. 7), even though there was a low but significant level of secreted protease activity (Table 1, experiment 2). This experiment was repeated by culturing white cells at 37°C in medium containing bovine hemoglobin which induced extracellular

FIG. 7. Northern blot of whole cell RNA from unstimulated and serum-stimulated cultures of white and opaque cells probed with Op1a. Lane 1, unstimulated white cells; lane 2, stimulated white cells; 3, unstimulated opaque cells; 4, stimulated opaque cells. At the bottom, the Northern blot was reprobed with the *ADE2* gene.

protease activity (25). No Op1a transcript was detectable in the white cell culture (data not shown).

The results with strain WO-1 suggest that the Op1a gene is under the rigid regulation of the white-opaque transition and that the stimulation of secreted protease activity in white cell cultures is the result of a protease other than the gene product of Op1a. To test whether serum stimulation in other strains involved Op1a or an alternative protease gene, two clinical isolates of *C. albicans* Hp33 and RP1-I3, both isolated from the vaginal canal, were grown to stationary phase in either modified Lee's medium or serum-containing medium supplemented with vitamins. Both the level of extracellular protease and the level of Op1a transcript were measured. In the case of strain Hp33, there was slight stimulation of extracellular protease activity by serum, but no detectable Op1a transcript in the absence or presence of serum (Table 1, experiment 3). In the case of RP1-I3, there was greater than a 20-fold stimulation of extracellular protease activity by serum, but no detectable Op1a transcript in the absence or presence of serum (Table 1, experiment 3). These results demonstrate that in WO-1 white cells, Hp33, and RP1-I3, serum stimulation of extracellular protease activity does not involve Op1a gene transcription.

DISCUSSION

Op1a expression is regulated by switching. Most strains of *C. albicans* and related species are capable of switching spontaneously and reversibly at high frequency among a number of phenotypes distinguishable by colony morphology (10, 32, 40, 43, 44). In the case of *C. albicans* WO-1, it has been demonstrated that switching between the white and opaque phases affects not only colony morphology, but also a number of basic physiological and structural characteristics of the budding cell (2–4, 17, 21, 22, 35, 41, 45). In spite of the pleiotropic effect switching exerts on phenotypes, there has been relatively little evidence that switching involves differential gene transcription. By using two-dimensional polyacrylamide gel electrophoresis, one minor white-specific and two minor opaque-specific polypeptides were identified (45), and by using a rabbit antiserum developed against opaque cells, a 14.5-kDa opaque-specific polypeptide was identified and localized at the apex of the unique opaque

cell wall pimple (3). However, in neither case was the level of regulation determined. Here, we have described the isolation of an opaque-specific cDNA, *Op1a*, by differential hybridization of an opaque cDNA library. The gene for *Op1a* is transcribed by budding cultures of opaque but not white cells grown in the same defined liquid medium. We assume that the *Op1a* message is abundant in opaque cells since (i) *Op1a* cDNAs have been isolated repeatedly in independent screens; (ii) the opaque cDNA library was not subjected to subtraction hybridization, making it more likely that only abundant messages were represented; and (iii) the autoradiography time necessary to identify the *Op1a* transcript in Northern blots probed with the *Op1a* cDNA was unusually short.

Since a hyphalike intermediate phenotype is involved in the transition from white to opaque (8) and since opaque budding cells but not white budding cells express a surface antigen also found on the surface of hyphae (2, 3), we examined whether the putative opaque-specific gene was expressed during hypha formation. No *Op1a* transcript was observed in cultures of white cells induced to form hyphae en masse under the regime of pH-regulated dimorphism, supporting the conclusion that *Op1a* gene expression is opaque specific under the conditions employed. Since most other strains of *C. albicans* are capable of switching but in most cases do not exhibit an opaque phenotype in their switching repertoire, we tested whether the *Op1a* gene was present in the genome of strain 3153A and whether it was expressed by cells in the basic o-smooth phenotype of its switching repertoire (40). Southern blot hybridization demonstrated that the *Op1a* gene is present in the genome of strain 3153A, but Northern blot hybridization demonstrated that the *Op1a* gene is not transcribed in either budding or hypha-forming cells of that strain. However, the *Op1a* gene may be expressed in one of the six other, more unusual switch phenotypes in the switching repertoire of strain 3153A (40), and this possibility is now being tested.

No genomic reorganization occurs in the vicinity of the *Op1a* gene. Since it has been suggested that switching in *C. albicans* may involve reversible genomic rearrangement (9, 40, 43), we examined whether genomic reorganization occurred in the vicinity of the *Op1a* gene. Digestion with 14 different endonucleases resulted in no polymorphism between opaque and white cells, indicating that there is no gross genomic rearrangement in the vicinity of the *Op1a* gene. Additionally, the methylation-sensitive isoschizomers *MspI* and *HpaII* did not reveal methylation differences in the *Op1a* gene between white and opaque cells. This is not surprising since we have now identified additional opaque-specific genes by using the same differential screen employed to identify *Op1a* (30a), and a white-specific gene by using a differential screen of a white cDNA library after subtraction hybridization (49a). Therefore, it is likely that switching from white to opaque and from opaque to white involves coordinated multigene activation and repression, in both directions, and regulation therefore probably involves sites unlinked to the gene(s) involved in the primary switching event.

Southern blot hybridization of electrophoretically separated chromosomes revealed two separable chromosomes hybridizing to *Op1a*. Since *C. albicans* contains eight chromosomes (26) and transverse alternating field electrophoresis separated 10 high-molecular-weight molecules in strain WO-1, we have assumed that the two hybridizing chromosomes represent electrophoretically separable rearranged homologs (39). Since both the chromosome separation pat-

terns, including intensities, and the hybridization patterns with *Op1a* cDNA are identical between white and opaque, it seems unlikely that chromosomal reorganization is the basis for either the white-opaque transition or the regulation of the *Op1a* gene.

The *Op1a* cDNA represents the transcript of a pepsinogen. Sequence analysis has demonstrated that the *Op1a* cDNA is more than 99% homologous to the acid protease gene of *C. albicans* isolated and sequenced by Hube et al. (20). The *Op1a* cDNA begins at base 6 of the open reading frame and ends 112 bases after the termination codon. Hube et al. (20) had determined the sequence of the 21 N-terminal amino acids of the purified enzyme and were therefore able to identify the N-terminal glutamine codon of the mature enzyme in the gene sequence. Our results suggest that the entire reading frame and subsequent 112 bases are transcribed intact. However, the *Op1a* cDNA contains only 1,283 bases and the single transcript identified on Northern blots contains roughly 1,700 bases, leaving approximately 400 bases of the transcript unsequenced. The predicted amino acid sequence of the *Op1a* cDNA shows significant homology to the family of pepsinogens. The *C. albicans* enzyme contains a hydrophobic leader sequence with a characteristic lysine at position 36 and two catalytic aspartyl domains which combine to form the active site, characteristic of the pepsinogen family (14, 52). Including conservative changes, the amino acid sequence of the mature protein deduced from the *Op1a* cDNA exhibits 38% homology with porcine pepsin and 45% for the sequence containing both catalytic domains. We therefore suggest that the gene for this acid protease of *C. albicans* be renamed *PEP1*, in accordance with the convention for naming *C. albicans* genes (24).

Regulation of *PEP1* transcription and acid protease secretion. We have demonstrated that in defined nutrient medium containing sugar, salts, thiamine, and amino acids (7), opaque cells but not white cells secrete copious amounts of acid protease and that this difference correlates with the presence and absence of *PEP1* transcript in the respective phenotypic phases. However, it was previously demonstrated that the addition of serum proteins to the supporting medium stimulates the level of secreted protease in some strains of *C. albicans* (28, 37). We have also found that the addition of serum to growth cultures of opaque and white cells stimulates the extracellular levels of secreted protease. However, in three independent experiments, stimulated levels of extracellular protease in white cells were still only 1/10, 1/37, and 1/65, respectively, those levels in parallel opaque cell cultures, and stimulation of extracellular protease activity was not accompanied by detectable transcription of the *Op1a* gene. Stimulation of secreted protease levels in two additional strains of *C. albicans* was also not accompanied by detectable transcription of the *Op1a* gene. These results suggest that serum stimulation of acid protease secretion involves an acid protease other than *PEP1*, a possibility now under investigation.

Switching and pathogenesis. It has been suggested that switching may provide *C. albicans* and related species with variability within a population for responding rapidly to a change in the environment (43, 44). In the case of *C. albicans* WO-1, it has been demonstrated that the white-opaque transition affects a number of putative virulence traits, including adhesion to epithelium (21), sensitivity to antifungal agents (48), sensitivity to erythrocytes and in vitro oxidants (22), and changes in surface antigenicity (2, 17). Here, we have provided evidence that the first gene to be

identified which is regulated by the white-opaque transition at the level of transcription codes for a pepsinogen, and secreted acid protease has been implicated as a virulence factor in *C. albicans* pathogenesis (1, 25, 29, 36). This provides further evidence that switching may coordinately regulate expression of virulence traits, adding to the pathogenic success of *C. albicans* (44).

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