JOURNAL OF BACTERIOLOGY, Sept. 1995, p. 5215–5221 Vol. 177, No. 18
0021-9193/95/$04.00+0

Candida albicans Secreted Aspartyl Proteinases: Isoenzyme Pattern Is Determined by Cell Type, and Levels Are Determined by Environmental Factors

THEODORE C. WHITE AND NINA AGABIAN*

Intercampus Program in Molecular Parasitology, University of California at San Francisco and Berkeley, and Department of Stomatology and the Oral AIDS Center, University of California at San Francisco, San Francisco, California 94143

Received 21 March 1995/Accepted 10 July 1995

For the pathogenic yeast *Candida albicans*, secreted aspartyl proteinase (Sap) activity has been correlated with virulence. A family consisting of at least eight *SAP* genes can be drawn upon to produce Sap enzymatic activity. In this study, the levels of Sap1, Sap2, and Sap3 isoenzymes were monitored under a variety of growth conditions for several strains, including strain WO-1, which alternates between two switch phenotypes, white (W) and opaque (O). When cultured under proteinase-inducing conditions, most strains and W cells produce Sap2, while O cells produce Sap1, Sap2, and Sap3. Both W and O cells of strain WO-1 produce Saps in enriched and defined media that do not induce Saps from other strains. The specific Sap isoenzyme that is produced is determined by the cell type, while the level of Sap production is determined by environmental factors. The levels and temporal regulation of the *SAP* mRNAs as determined by Northern (RNA) analysis were consistent with Sap protein levels and with previous results. S1 analysis showed that *SAP6* is the predominant *SAP* gene transcribed during hyphal induction at neutral pH. These studies define the culture conditions which control the levels of *SAP* mRNAs and Sap proteins, and they indicate that both the yeast/hyphal transition and phenotypic switching can determine which of the Sap isoenzymes is produced.

Candida albicans is a dimorphic yeast which exists as a commensal oral and/or vaginal colonization in most healthy individuals. Under certain circumstances usually associated with a compromised host immune system, *C. albicans* and related species can become pathogenic, causing oral, vaginal, and/or systemic candidiasis. While the regulatory processes which control the transition between nonpathogenic and pathogenic states have not been elucidated, several factors which contribute to the development of disease have been identified (2, 16; also see below).

C. albicans exists in two distinct forms, a spherical budding yeast form and a long, slender hyphal form (reviewed in reference 16). Both forms are present in the commensal and disease states of an infection. In addition to the yeast/hyphal transition, *C. albicans* displays phenotypic switching at detectable frequencies (10^{-2} to 10^{-5} per cell cycle) between alternate cell types that are manifest as alternate colony morphologies on agar plates (reviewed in reference 23). At least three independent switch phenotype systems have been described, including white/opaque, smooth/star/wrinkled, and petite. Each strain of *C. albicans* displays a particular switch phenotype system. For strain WO-1, the cells alternate between white (W) and opaque (O) colony phenotypes which differ with respect to a variety of characteristics, including lipid composition, drug sensitivity, and cellular morphology. This ability of *C. albicans* to switch among several different cell phenotypes may contribute to the virulence of the strain.

A potential virulence factor of *C. albicans* that was first identified by Staib (25, 26) and has been studied by a number of laboratories over the last 25 years is the secreted aspartyl proteinase (*SAP* gene; Sap protein). Sap enzymatic activity is routinely monitored in culture supernatants from cells grown in proteinase-inducing media containing bovine serum albumin (BSA) at pH 5, although Sap enzymatic activities have also been detected in culture supernatants from cells grown in media containing a variety of other substrates (reviewed in references 3 and 20). In *C. albicans* strains and other *Candida* species, including *C. tropicalis*, *C. parapsilosis*, and *C. krusei*, levels of Sap enzymatic activity correlate with the degree of virulence of a given isolate in animal models (21, 22). Sap antigen has been detected at lesional sites (18), and inhibitors of Sap enzyme activity reduce the adhesion of *C. albicans* to epithelial cells ex vivo (17), suggesting a role for Sap in adhesion or invasion.

Although Sap activity has been widely studied, the reported biochemical properties of Sap are often conflicting; the molecular weight, pI, inhibitor profile, substrate specificity, and N-terminal sequence of the protein vary between strains or laboratories (reviewed in references 3 and 20). One explanation for these discrepancies is suggested by the recent cloning and sequencing of seven distinct *SAP* genes (*SAP1* [4], *SAP2* [30], *SAP3* [29], *SAP4* [10], *SAP5*, *SAP6*, and *SAP7* [11]) and comparison of the N-terminal protein sequences, which suggests the existence of at least one more *SAP* gene, *SAP8* (13). The seven cloned genes each encode a mature protein which is highly conserved and a precursor peptide that contains the most evolutionarily divergent region of the *SAP* genes.

The steady-state levels of selected *SAP* mRNAs, which are controlled by transcription and RNA degradation, have been studied under certain defined conditions. In standard proteinase-inducing medium, selected laboratory strains contain high levels of *SAP2* mRNA and low levels of *SAP1* mRNA (30). For strain WO-1, O cells contain *SAP1* mRNA (referred to as *PEP1* by Morrow and coworkers), while W cells do not (15).

* Corresponding author. Mailing address: Intercampus Program in Molecular Parasitology, University of California at San Francisco, 3333 California St. Suite 150, San Francisco, CA 94118. Phone: (415) 476-6845. Fax: (415) 476-0664. Electronic mail address: agabian@itsa.ucsf.edu.

TABLE 1. Sap2 proteinase production[a]

Medium or media[b]	Sap2 proteinase in the following cell line[b] at:									
	25°C					37°C				
	10261	3153A	SS	W	O[c]	10261	3153A	SS	W	O[c]
Inducing media	2	2	2	2	2	2	2	2	2	2
Inhibitory media	2	(2)	(2)	2	(2)	2	−	−	(2)	−
Rich media										
YEPD	2	−	−	2	(2)	2	−	−	−	−
YEPD/BSA	2	2	2	2	−	2	−	−	−	−
YEPD/Sera	2	−	−	−	(2)	−, H	−, H	−, H	−, H	−, H
Defined media										
Lee's 4.5	−	−	−	−	−	−	−	−	−	−
Lee's 4.5/BSA	−	−	−	2	−	−	−	−	−	−
Lee's 6.7	−	−	−	−	−	−, H	−, H	−, H	−, H	−, H
Lee's 6.7/BSA	2	−	−	2	−	−, H	−, H	−, H	−, H	−, H
RPMI	−	−	−	−	−	−, H	−, H	−, H	−, H	−, H
RPMI/BSA	−	−	−	−	−	−, H	−, H	−, H	−, H	−, H

[a] 2, Sap2 protein present in culture supernatants; −, Sap2 not detected; H, hyphae induced during growth of the culture. Parentheses indicate that the protein was detected but at low levels.

[b] All media and cell lines are described in Materials and Methods. Results for inducing and inhibitory media are summaries of results obtained with each medium separately.

[c] Cultures were inoculated with O cells. These cultures grow at 25°C as O cells, and they grow at 37°C as W (yeast) cells.

SAP1 mRNA is rapidly lost when the O cell population converts to W cells as the temperature is raised to 37°C (14).

Hube et al. (5) recently showed that *SAP2* is the major *SAP* mRNA in many strains and that the levels of this mRNA are not affected by temperature or carbon source and are maximal during mid-log growth. Their analysis also showed that in O cells of strain WO-1, *SAP1*, *SAP2*, and *SAP3* mRNAs are detected during logarithmic growth, while *SAP3* mRNA is also present in stationary phase. *SAP* mRNAs were detected transiently during hyphal production at neutral pH; the DNA probe used in the analysis hybridizes with *SAP4*, *SAP5*, and *SAP6*. The data of these investigators suggest that peptides from BSA stimulate *SAP2* mRNA levels, while free amino acids inhibit expression.

While different levels of *SAP* mRNAs have been detected, a correlation with Sap protein levels has not been made. We have previously shown that protein products of three of the *SAP* genes (*SAP1*, *SAP2*, and *SAP3*) can be resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (29). In the present study, we determine the major Sap proteins secreted by laboratory strains under a variety of proteinase-inducing and proteinase-inhibiting conditions, including those used by Hube et al. (5). We conclude that the pattern of Sap isoenzymes in strain WO-1 is unique compared with those in other strains. While most strains, including W cells and many clinical isolates, primarily secrete Sap2, the major proteinases secreted by O cells are Sap1, Sap2, and Sap3. Northern analysis of total RNA using oligonucleotide probes specific for the *SAP* genes shows that RNA and protein levels are comparable under most conditions, and an mRNA specific for *SAP4*, *SAP5*, or *SAP6* is detected during hyphal formation at neutral pH (consistent with previous results [5]). Finally, by S1 nuclease analysis, we have determined that *SAP6* mRNA is the major *SAP* mRNA expressed in hyphae at pH 7.

MATERIALS AND METHODS

Strains. The *C. albicans* strains used included strains 3153a (ATCC 28367), WO-1 (28), ATCC 10261, and SS (3). Cultures were maintained at 25°C on YEPD agar plates (10 g of yeast extract, 20 g of peptone, 20 g of dextrose, and 15 g of Bacto Agar per liter) and subcultured weekly or were stored at −70°C in YEPD containing 10% glycerol. The WO-1 strain was maintained separately as both W and O phenotypes on YEPD plates containing phloxine B (5 μg/ml). Cell cultures were inoculated by using a single colony grown on a YEPD agar plate. Culture medium components were obtained from Fisher, and proteins used as nitrogen sources were obtained from Sigma.

Culture media. Several different media were tested for their effects on Sap isoenzymes.

(i) Inducing media. Inducing media (Tables 1 and 2) included the following: YCB-BSA (1.17% [wt/vol] yeast carbon base, 0.1% yeast extract, 0.2% BSA [pH 5.0]) (29), YNB-BSA (0.17% yeast nitrogen base, 2% glucose, 0.2% BSA [pH 5.0]) (1), 10× YNB-BSA (1.7% yeast nitrogen base, 2% glucose, 0.2% BSA [pH 5.0]), YBD (0.2% yeast extract, 0.2% BSA, 2% glucose) (19), and MMO (modified MacDonald/Odds [7]) (2% glucose, 0.2% BSA, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5× Basal Eagle Medium Vitamins, 400 ng of nicotinic acid per ml).

(ii) Inhibitory media. Inhibitory media (Tables 1 and 2) consisted of many of the inducing media listed above with the addition of NH_4^+ and included the following: YCB-BSA-NH_4 (YCB-BSA [see above] plus 0.1 M NH_4 tartrate), YNB-BSA-NH_4 (YNB-BSA plus 0.1 M NH_4 tartrate), 10× YNB-BSA-NH_4 (10× YNB-BSA plus 0.1 M NH_4 tartrate), and YCB-AS (1.17% yeast carbon base plus 0.5% ammonium sulfate [pH 5.0]).

TABLE 2. Sap1 and Sap3 proteinase production at 25°C[a]

Medium or media[b]	Sap1 and/or Sap3 proteinase production by the following cell line[b]:	
	W	O
Inducing media	−	1, 3
Inhibitory media	−	1
Rich media		
YEPD	−	1, 3
YEPD/BSA	(3)	1
YEPD/Sera	−	1, (3)
Defined media		
Lee's 4.5	−	1
Lee's 4.5/BSA	−	1, (3)
Lee's 6.7	−	1
Lee's 6.7/BSA	−	1, (3)
RPMI	−	−
RPMI/BSA	−	−

[a] 1, Sap1 protein present in culture supernatants; 3, Sap3 protein present in culture supernatants; −, neither Sap1 nor Sap3 detected. Parentheses indicate that the protein was detected but at low concentrations.

[b] All media and cell lines are described in Materials and Methods. Results for inducing and inhibitory media are summaries of results obtained with each medium separately.

(iii) Enriched media. Enriched media (Tables 1 and 2) included the following: YEPD (1% yeast extract, 2% peptone, 2% dextrose), YEPD/BSA (1% yeast extract, 2% peptone, 2% dextrose, 0.2% BSA), and YEPD/Sera (1% yeast extract, 2% peptone, 2% dextrose, 0.2% bovine serum).

(iv) Defined media. Defined media (Tables 1 and 2) included the following: Lee's 4.5 and Lee's 6.7 (modified Lee's medium at pH 4.5 or 6.7 as described previously [6]), Lee's 4.5/BSA and Lee's 6.7/BSA (Lee's medium at pH 4.5 or 6.7 plus 0.2% BSA), RPMI (RPMI 1640 with 2 g of glucose per liter, 0.3 g of L-glutamine per liter, and 2.0 g of $NaCO_3$ per liter), and RPMI/BSA (RPMI, 0.2% BSA).

Cultures were usually grown in these media for 48 h to late stationary phase. The cells were pelleted (12,000 × *g*, 10 min) in a microcentrifuge, and the culture supernatants were stored at −20°C.

SDS-PAGE and Western immunoblot analysis. Western analyses were performed as previously described with 8% polyacrylamide gels (29:1 acrylamide/bisacrylamide ratio [29]) under denaturing conditions. In all Western blots, the membranes were treated with periodate to remove carbohydrate epitopes that cause high backgrounds (see reference 29 for details).

Northern analysis. *C. albicans* cells were grown in either YEPD or YCB-BSA, monitored spectrophotometrically at 600 nm, and harvested at defined optical densities. RNAs were prepared at optical densities of 1 (6 to 7 h after inoculation; mid-log phase), 3 (8.5 to 11 h; mid-late log phase), 9 (11.5 to 16 h; late log phase), and 20 (14.5 to 22 h; stationary phase), depending on the growth of each cell type. Total RNA was prepared as previously described (10). Northern analyses were performed with 10 μg of total RNA as described previously (8). Four specific oligonucleotides were designed on the basis of the sequence of the precursor portion of the cloned gene. Because of the high degree of similarity among *SAP4*, *SAP5*, and *SAP6*, an oligonucleotide could not be designed to recognize each of the genes, and so an oligonucleotide that recognizes all three was designed. An oligonucleotide specific for the actin gene sequence was designed to monitor mRNA levels. All five oligonucleotides were used for Northern blot hybridization, and their sequences are as follows: *SAP1* specific, 5′ CCAG TAGCATTAACAGGAGTTTTAATGACA 3′; *SAP2* specific, 5′ TGACCATT AGTAACTGGGAATGCTTTAGGA 3′; *SAP3* specific, 5′ TTGATTTCACCT TGGGGACCAGTAACATTT 3′; *SAP4* to *SAP6* specific, 5′ TTTAACTTCGA CAGTTGGATCTTTTGGATC 3′; and actin specific, 5′ GGAGTTGAAAGT GGTTTGGTCAATACCAGCAGCTTCCAAACCTAAATCAG 3′. The oligonucleotides were labeled with polynucleotide kinase and [γ-^{32}P]ATP (12). Blots were prehybridized, hybridized, and washed at 55°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS as described previously (8).

S1 analysis. S1 analysis was performed as described previously (10). Gene-specific probes from *Bgl*II fragments of the coding regions were prepared by labeling with polynucleotide kinase and [γ-^{32}P]ATP. The fragments include the following: for *SAP4*, a 760-bp fragment from position 205 to position 964 of the coding region (10); for *SAP5*, a 682-bp fragment from position 416 to position 1097 of the coding region (11); and for *SAP6*, a 665-bp fragment from position 389 to position 1053 of the coding region (11). For controls, the three DNA fragments were cloned into pBluescript SK− (Stratagene). In vitro transcription with T3 or T7 polymerase produced RNA transcripts that were used to demonstrate that the three probes do not cross-react under the experimental conditions.

RESULTS

Saps and cell growth. As we showed previously, Sap1, Sap2, and Sap3 are resolved by 10% SDS–PAGE and detected by immunoblotting with a rabbit polyclonal antiserum (29). We then used N-terminal protein sequencing to identify the three protein bands as Sap isoenzymes and to demonstrate that comigrating bands from three different strains (3153A, SS, and WO-1) each represented a single Sap isoenzyme (Sap2) (29). We have improved the resolution by using 8% SDS–PAGE (Fig. 1) and have used these gel conditions to determine the major Sap isoenzymes that are secreted under a variety of growth conditions.

We have previously shown that Sap isoenzymes are produced beginning in mid-log phase and that Sap isoenzyme levels increase in the late log and stationary phases of cell growth (29). This pattern of Sap production is seen for W cells and O cells of strain WO-1 and for strains SS and 3153A. We have also reported that the levels of Sap isoenzymes persist in the media without significant degradation for up to 5 days. To ensure that each cell line was grown to stationary phase and that Sap isoenzyme levels were maximal, Sap isoenzyme levels were assayed routinely at 48 h.

Tables 1 and 2 tabulate the Sap isoenzymes secreted in culture supernatants of cells of laboratory strains 10261, 3153A, and SS and the W and O switch phenotypes of strain WO-1 grown under a variety of conditions. Each cell line (referring to strain or switch phenotype) was grown at both 25 and 37°C. O cells grown at 25°C remain O cells for the 48 h of incubation, while O cells grown at 37°C convert to W cells within 2 to 4 h (14, 24). The Sap isoenzymes produced by cultures grown at 37°C after inoculation with O cells (Tables 1 and 2) are the same as the Sap isoenzymes produced by W cells, demonstrating that the Sap protein pattern changes with the conversion of O cells to W cells, consistent with the results of previous Northern analyses (14, 24).

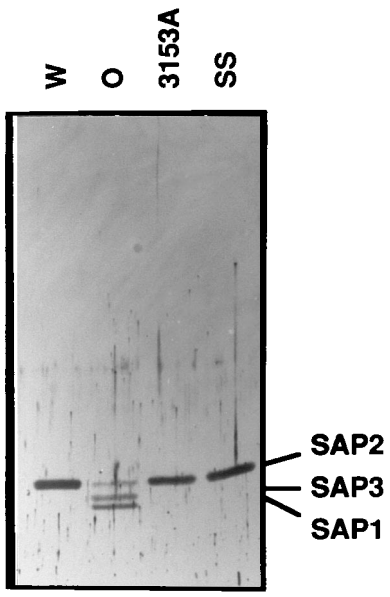

FIG. 1. Improved resolution of Sap isoenzymes. Culture supernatants from W and O cells of strain WO-1 and from strains 3153A and SS were electrophoresed by 8% SDS–PAGE (see Materials and Methods). After electrophoresis, the gel was blotted to nitrocellulose and probed with a polyclonal rabbit antiserum directed against Sap. Three bands with different apparent molecular weights were observed, and these have been identified as *SAP1*, *SAP2*, and *SAP3* by N-terminal sequencing (29).

Cell morphology of each cell line was assessed microscopically at 24 and 48 h during the growth of the cultures. At 25°C, most cell lines grow as yeast cells, with the exception of the O cells, which retain their distinctive large, oblong shape. At 37°C, cell lines, including those inoculated with O cells, grow as yeast cells, and hyphae are induced under the expected conditions, including Lee's medium at pH 6.7 and media containing serum (Table 1).

Sap2 isoenzyme. Sap2 is produced by all cell lines at both temperatures in proteinase-inducing media of various formulations (Table 1; see Materials and Methods). In addition, Sap2 is produced in over 40 clinical isolates from human immunodeficiency virus-infected patients when grown in proteinase-inducing medium (YCB-BSA) (27). Low levels of Sap3 are occasionally detected in culture supernatants from some of these isolates (see below).

Previous reports have suggested that the presence of NH_4^+ reduces Sap activity (1), potentially affecting the level of Sap protein or its enzymatic activity. We tested for inhibition of Sap levels by adding ammonium tartrate or ammonium sulfate to the proteinase-inducing media (producing the media designated as inhibitory media in the tables). NH_4^+ reduces Sap2 levels at 37°C in most cell lines and has a limited effect on Sap2 levels at 25°C (Table 1).

While Sap enzymatic activity has rarely been detected in enriched growth media such as YEPD, the Sap2 isoenzyme is produced under these conditions (Table 1), although the level of Sap2 depends on the cell line used. Levels of Sap2 in enriched media are reduced when cells are grown at 37°C, and they can be increased by the addition of BSA or serum for some cell lines.

Sap enzymatic activity in defined media (i.e., Lee's medium) has been studied by Morrow and coworkers (15), who showed that Sap enzymatic activity and *SAP1* mRNAs are found almost exclusively in O cells (see below). However, SDS-PAGE showed that Sap2 is produced in W cells in defined Lee's media after the addition of serum (Table 1) at either pH 4.5 or pH 6.7. The Sap2 isoenzyme is not produced in the defined medium RPMI from any cell line tested (see below).

Sap1 and Sap3 isoenzymes. Levels of Sap1 and Sap3 were monitored at 25 and 37°C for all cell types but were detected only in W and O cells grown at 25°C (Table 2) (27). Sap1 is produced by O cells under all growth conditions tested with the exception of the defined medium RPMI; its levels are not affected by NH_4^+ or the presence of BSA or serum. Sap3 is also produced by O cells, although low levels of Sap3 can occur in other cell lines (see below). Sap3 is eliminated by the addition of NH_4^+ to the culture, is induced in Lee's medium by the addition of BSA, and is reduced in enriched media by the addition of BSA or serum.

Effects of medium, pH, and substrate. Saps were not detected in any cell line grown in the defined medium RPMI 1640, while Saps were detected in O cells grown in the defined modified Lee's medium. When O cells were grown in 1:1 mixtures of RPMI medium and Lee's medium (which induces Sap1), Saps were not detected, while controls (1:1 mixtures of Lee's medium and water) produced Sap1, suggesting that a component of RPMI medium inhibits Sap1 production. RPMI-1640 medium (Gibco-BRL) contains 41 components, including six inorganic salts, 20 amino acids, 11 vitamins, D-glucose, glutathione, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and phenol red. Modified Lee's medium contains 20 components, including nine inorganic salts, nine amino acids, D-glucose, and D-biotin. Differences between the two media, in which RPMI contains a component that is not present in Lee's medium or in which the component is present at a higher concentration in RPMI medium, include five inorganic salts, 14 amino acids, 10 vitamins, glutathione, HEPES, and phenol red. Each of these 32 components was tested separately and in various combinations (modified Eagle's medium vitamin solution [Gibco-BRL], Casamino Acids, and inorganic salt mixtures) for its inhibition of Sap expression in Lee's medium. Components of RPMI medium were added to a base of Lee's medium either alone or in limited combination, and all failed to inhibit Sap1 production, whereas the addition of complete RPMI medium always inhibited Sap1 production (27), suggesting that a complex combination of RPMI medium components inhibits Sap production.

While Sap activity is known to have a pH optimum between 4 and 5 and *SAP2* mRNA levels have been shown to be maximal at pH 4.0 (5), pH regulation of Sap isoenzyme levels has not been investigated. Proteinase-inducing medium was buffered with sodium citrate (100 mM) at five incremental pH values between 3 and 7. The final pH after growth of the cultures to stationary phase ranged from 3.25 to 5.8. The pattern of Sap isoenzymes in W cells (Sap2) and O cells (Sap1, Sap2, and Sap3) was not affected by the pH of the growth medium (Fig. 2), although the levels of the Sap isoenzymes were affected by pH. As seen in Fig. 2, levels of Sap3 were highest at low pH (pH 3.2 [lane 1]), levels of Sap1 remained elevated at higher pH (i.e., pH 5.6 [lane 4] and pH 5.9 [lane 5]), and levels of Sap2 in both W and O cells were greatest at pH 4.1 (lane 2 for both W and O cells), although all three Sap isoenzymes were detected at every pH. This suggests that the levels of the three Sap isoenzymes are controlled by the pH of the medium. The optimum expression of Sap2 at pH 4 is consistent with the *SAP2* mRNA levels (5), although the detection of Sap isoenzymes in every sample may reflect a difference in timing of mRNA expression at different pHs that was not detected by Northern analyses.

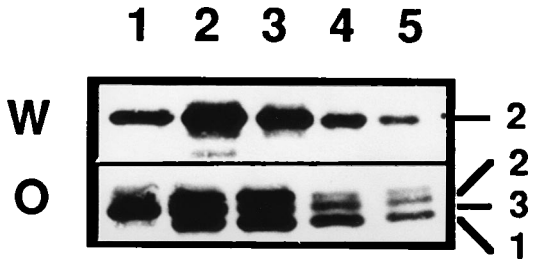

FIG. 2. Western analysis of culture supernatants at different pHs. W cells (W panel) and O cells (O panel) were grown in YCB-BSA buffered with 100 mM sodium citrate for 48 h. The final pHs of the buffered cultures for W cells were as follows: lane 1, 3.28; lane 2, 4.19; lane 3, 4.83; lane 4, 5.48; and lane 5, 5.68. Those for O cells were as follows: lane 1, 3.24; lane 2, 4.13; lane 3, 4.77; lane 4, 5.59; and lane 5, 5.86. Samples of the culture supernatants were analyzed by Western blot analysis (see Materials and Methods). This exposure of the data was chosen to detect the presence of all three Sap isoenzymes in the O cells (lane 5).

While Sap enzymatic activity degrades a variety of substrates, Sap isoenzymes have been monitored only in proteinase-inducing medium containing BSA as a substrate (12, 13, 29). Cell lines, including W and O cells and strains 3153A and SS, were grown in proteinase-inducing medium containing other proteins as nitrogen sources, including bovine serum, casein, and hemoglobin. In addition, two dye-linked proteins, azocasein and azokeratin, were tested. No change in Sap isoenzyme pattern was observed when cell lines were grown in these media compared with the pattern observed when they were grown in media containing BSA (27). Azocasein, BSA (filtered or autoclaved), bovine serum, and casein all induced equivalent amounts of Sap. Azokeratin did not induce significant amounts of Sap compared with a protein-minus control. Surprisingly, hemoglobin induced Sap at 25°C but did not induce it at 37°C.

***SAP* RNA levels.** Differences in Sap protein levels may be due to differences in transcription, RNA processing, protein translation, protein stability, or secretion. Northern analyses were performed (Fig. 3) to determine if Sap protein levels are strictly correlated with *SAP* mRNA levels. Total RNA from cell lines 3153A, SS, W, and O prepared at several points in the growth of the culture in proteinase-inducing medium was blotted to nitrocellulose and probed with four oligonucleotides that are directed at the precursor regions of the *SAP* genes. As this part of the gene is the most divergent, these oligonucleotides are specific for *SAP1*, *SAP2*, and *SAP3* genes. Because of the high degree of homology among *SAP4*, *SAP5*, and *SAP6*, the *SAP4-6* oligonucleotide recognizes all three sequences.

The Northern analyses (Fig. 3) were consistent with the Western blot analyses (Tables 1 and 2) and suggest that the *SAP* genes are regulated at the RNA level. *SAP2* mRNA was detected in all cell lines, while *SAP1* and *SAP3* mRNAs were detected in O cells and *SAP3* mRNA was detected at low levels in cell lines other than O cells (see lane 3 for each cell line in Fig. 3). No hybridization signal was observed when these

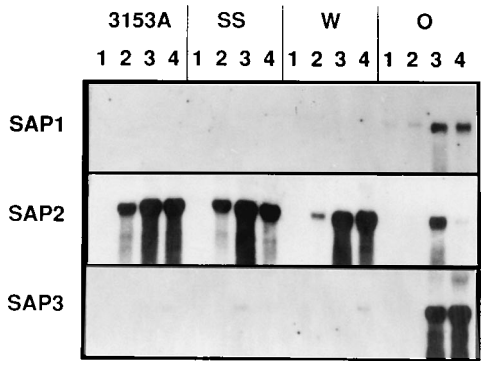

FIG. 3. Northern analysis of *SAP* mRNA levels. Total RNA from W cells and O cells of strain WO-1 and from strains 3153A and SS was prepared at several time points during the growth of the culture, and samples were taken at optical densities of 1 (mid-log phase; lanes 1), 3 (mid-late log phase; lanes 2), 9 (late log phase; lanes 3), and 20 (stationary phase; lanes 4). Identical blots were probed with oligonucleotides specific for *SAP1*, *SAP2*, *SAP3*, *SAP4* to *SAP6*, and actin. Blots were hybridized and washed at 40°C in 3× SSC–0.1% SDS. Panels for *SAP1*, *SAP2*, and *SAP3* are shown. The *SAP4-6* oligonucleotide did not hybridize with any RNA species in these blots. The actin oligonucleotide hybridized evenly across the entire blot, demonstrating that equal amounts of total RNA were loaded in each lane (27). The smears below the bands for each *SAP* mRNA may be the result of increased mRNA degradation as the cells reached stationary phase. The shadow bands above the *SAP* signals, especially in the *SAP2* panel, represent trapping of *SAP* mRNA in the rRNA bands of total RNA.

Northern blots were hybridized with the *SAP4-6* oligonucleotide (27), suggesting that *SAP4*, *SAP5*, and *SAP6* mRNAs are not present under these conditions. The results shown in Fig. 3 demonstrate that Sap isoenzyme levels are reflected in the amount of mRNA for each *SAP* gene. These results cannot distinguish between differential transcription, pre-mRNA processing, and mRNA decay as the factor(s) that controls steady-state mRNA levels.

The Northern analyses further show that *SAP* gene expression occurs relatively late in the growth of the culture, since the *SAP2* mRNA peaks in very late log phase (lane 3 for each cell line in Fig. 3) and the mRNAs for *SAP1* and *SAP3* peak in stationary phase (lane 4 for each cell line). The delayed expression of the *SAP* genes during growth is most probably due to the presence of small amounts of yeast extract in the culture medium (added as a vitamin supplement) which serve as a nitrogen source in the initial growth of the culture. Northern analysis shows that cultures grown in proteinase-inducing medium without yeast extract express the *SAP* genes earlier in the growth of the culture (27), as has been described previously (5).

***SAP* mRNAs during hypha formation.** Hube et al. detected an mRNA that hybridizes with a probe for *SAP4*, *SAP5*, and *SAP6* during hyphal cell production stimulated by serum at neutral pH (5). We extended these results by showing that under such conditions, the laboratory strain SS and a clinical isolate both produce mRNA which hybridizes with the *SAP4-6* oligonucleotide, while W and O cells do not produce such an mRNA (28), suggesting expression differences between W and O cells of strain WO-1 and yeast cells of other strains. Northern analysis of total RNA from a clinical isolate prepared at 2 and 5 h after hypha production in three different media (Fig. 4) showed that the mRNA recognized by the *SAP4-6* oligonucleotide is produced within the first 2 h after hyphal induction and that its levels are reduced by 5 h postinduction. We were able to detect transcripts during hypha formation in a defined medium (Lee's medium at pH 6.7); such transcripts have not been previously reported (5). mRNAs that hybridize to oligonucleotides specific for *SAP1*, *SAP2*, and *SAP3* were not detected under these conditions (27).

Culture supernatants from cells induced to form hyphae at neutral pH were sampled at 2 and 5 h and after overnight growth (the same cultures from which the total RNA was prepared [see above]). Western blot analysis of these supernatants probed with the polyclonal antisera did not detect any Sap protein. This may be due to the transient, low-level expression of *SAP*s under these conditions or to the possibility that the antiserum does not recognize Sap4, Sap5, or Sap6.

***SAP4*, *SAP5*, and *SAP6* expression.** To determine which of the *SAP* genes was expressed during hypha formation at neutral pH, DNA probes from the coding regions of *SAP4*, *SAP5*, and *SAP6* were end labeled, hybridized with total RNA of a clinical isolate prepared from the cells 2 h after hyphal induction, and digested with S1 nuclease, which does not digest labeled DNA fragments if they are hybridized with RNA. The results of the S1 analysis, shown in Fig. 5, indicate that the major *SAP* gene transcript during hyphal induction at neutral pH is *SAP6*, while a lower level of *SAP5* mRNA was detected. *SAP4* mRNA was not detected in several experiments.

DISCUSSION

Sap isoenzymes. The complex pattern of Sap enzymatic activity in *C. albicans* has been partially explained by the identification of the *SAP* multigene family. Northern analyses by several laboratories (5, 10, 15, 30; also our present results [see above]) have detected mRNA levels for specific *SAP* genes under a limited set of conditions, which most probably reflect differential transcription of the *SAP* genes but which may also be affected by RNA processing or control during pre-RNA processing. We have performed a comprehensive survey of Sap protein isoenzyme levels under a wide variety of conditions (Tables 1 and 2). Under standard conditions, RNA and protein

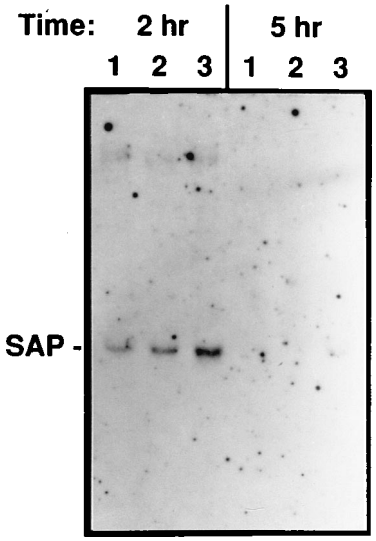

FIG. 4. Transient *SAP* mRNA levels in hyphal cells at neutral pH. An overnight culture of clinical isolate 92-4053 was washed in sterile water and suspended at an optical density of 0.75 in three different media: 10% serum, pH 7 (lane 1); 10% serum in Lee's medium, pH 6.7 (lane 2); and 100% Lee's medium, pH 6.7 (lane 3). Total RNA was prepared at 2 and 5 h after hyphal induction, by which time most cells had formed hyphae and germ tubes (precursors to hyphae). The Northern blot was hybridized with the *SAP4-6* oligonucleotide and washed at 40°C in 3× SSC–0.1% SDS. Faint hybridization at the top of the blot represents genomic DNA remaining in the total RNA preparations.

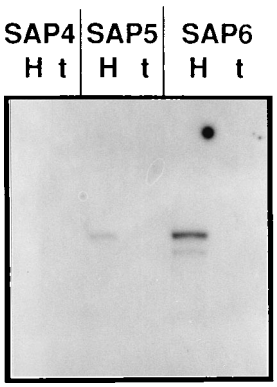

FIG. 5. S1 nuclease analysis of *SAP* mRNA levels during hypha formation. DNA probes were prepared from within the coding regions of *SAP4*, *SAP5*, and *SAP6* (labeled in the figure). Each probe was labeled, denatured, and hybridized overnight in the presence of total RNA from a clinical isolate prepared at 2 h after hyphal induction (H) or in the presence of carrier tRNA (t). After hybridization and S1 nuclease treatment, the samples were electrophoresed through a denaturing 7% polyacrylamide gel and the gel was exposed to X-ray film for autoradiography. The probes for *SAP5* and *SAP6* were approximately the same size, as can be seen in the figure. The probe for *SAP4* was slightly larger and was not detected in these analyses.

levels are comparable, suggesting that posttranscriptional regulatory mechanisms do not substantially regulate Sap levels.

Western blot analysis, which does not depend on enzymatic activity, was used in this study to rapidly screen for Sap isoenzymes in culture supernatants representing a large number of growth conditions and several strains and cell types (Tables 1 and 2). Stationary-phase culture supernatants were tested, since they contain all Saps accumulated during growth. Degradation of Saps in culture supernatants is not a major problem in these analyses, as we have monitored Sap isoenzyme levels for up to 5 days without a change in either the pattern or the level of Sap isoenzymes (27).

The antiserum that was used in much of this analysis is a polyclonal rabbit serum prepared against Sap2. As described previously (29), this antiserum reacts with Sap1, Sap2, and Sap3 (Fig. 1), which is not surprising given the high levels of homology among the *SAP* genes (11). The antibody does not detect Sap isoenzymes produced during hyphal growth at neutral pH. Similarly, two other polyclonal antibodies easily recognized Sap1, Sap2, and Sap3 in control culture supernatants but did not detect any proteins in hyphal culture supernatants. The levels of *SAP* mRNA detected during hypha formation are low and transient (Fig. 4) compared with the levels of *SAP1*, *SAP2*, and *SAP3* mRNA in yeast cells (Fig. 3), and this may explain our inability to detect hypha-specific Saps by Western analysis. At this time, the protein products of *SAP4*, *SAP5*, *SAP6*, and *SAP7* have not been detected under any conditions.

Saps and cell type. Different isoenzymes are produced by yeast cells and by hyphae and by cells exhibiting different switch phenotypes, suggesting that cell type controls the pattern of Sap isoenzyme expression. Yeast cells of laboratory strains and clinical isolates produce Sap2 (Table 1) and low levels of Sap3, which can be detected by Western analysis (27) or Northern analysis of total RNA (Fig. 4). Hyphal cells at neutral pH contain *SAP6* mRNA and lower levels of *SAP5* mRNA, as detected by Northern analysis of total RNA. In the W/O switch system, W cells produce Sap2 (Table 2) and O cells produce Sap1, Sap2, and Sap3, although the level of each of the Saps in O-cell supernatants depends on the growth conditions.

Saps and environmental control. The highest levels of Sap are found under proteinase-inducing conditions. In general, rich media and defined media do not induce Saps, although W and O cells continue to produce Saps in these growth media (Tables 1 and 2). The defined medium RPMI inhibits Sap production in O cells, although we have been unable to determine the combination of components in RPMI which causes this inhibition. Other growth conditions were tested for effects on Sap levels. The pH of the medium does not effect the pattern of expression but does have an effect on the levels of Sap isoenzymes, with high levels of Sap3 occurring at low pH (pH 3.2), higher levels of Sap1 occurring at higher pH (pH 5.5), and maximal levels of Sap2 occurring at intermediate pH (pH 4.1). The major effect of temperature was the conversion of O cells to W cells at 37°C. The presence of NH_4^+ reduced or eliminated the levels of Sap under proteinase-inducing conditions, with a more pronounced effect at 37°C. We have also shown that high concentrations of NH_4^+ inhibit the enzymatic activity of Saps (27).

Our Western analysis results (see Tables 1 and 2) are not completely consistent with previous Northern analysis results (5) with regard to the time of maximal Sap production during culture growth and the inhibition by amino acids and pH. These discrepancies are most likely the result of the methods used in each investigation. The level of mRNA detected by Northern analysis is dependent on the growth stage of the culture at the time the RNA is prepared (Fig. 3), while the amount of Sap protein detected by Western analysis represents the accumulation of Sap isoenzymes during 48 h of growth. Small differences in the constituents of the media may alter the timing of gene expression as well as the overall levels of Sap expression. Thus, mRNA levels monitored at one specific time point in the growth of the culture may or may not reflect final Sap protein levels.

Sap enzymatic activity and protein levels. Despite the identification of the *SAP* gene family and the characterization of *SAP* mRNAs and Sap isoenzymes, there remain clear discrepancies between enzymatic activities and the Sap protein levels for specific growth conditions. The best example is that of W and O cells, for which Morrow and coworkers monitored the Sap enzymatic activity levels and levels of *SAP1* mRNA (15). In their studies, high levels of Sap enzymatic activity correlated with high levels of *SAP1* mRNA in O cells, while in W cells (grown in defined medium or serum) and in two clinical isolates, low levels of Sap enzymatic activity were detected but were not associated with *SAP1* mRNA. The Western blot analysis whose results are shown in Fig. 1 and Tables 1 and 2 confirms that Sap1 is secreted by O cells, identifies Sap2 as the major Sap isoenzyme secreted by W cells, and identifies Sap3 as a major component of O-cell culture supernatants. The levels of total Sap protein, as determined by Western blot analysis, are roughly equivalent in W and O cells (Fig. 1) (29), in contrast to the large differences in Sap enzymatic activity described by Morrow et al. (15).

To reconcile the discrepancy between the Sap protein levels in YCB-BSA media (as determined by Coomassie stains and Western blots [28, 29]) and Sap activity in defined medium and serum (as determined by enzymatic assay [15]), we monitored the levels of Sap activity in culture supernatants of cell lines grown in YCB-BSA by the azocasein assay (15). The results were consistent with those of Morrow et al. (15). However, repeated assays of culture supernatants demonstrated that the assay is extremely sensitive to the pH of the culture medium, and the standard curve for the assay has a narrow linear range because of enzyme inhibition by the product of the reaction (9). The assay is most likely sensitive to the pH of the culture

supernatant because extremes of pH irreversibly inactivate Sap activity. These findings suggest that caution must be employed in interpreting the proteinase activity assays based on the currently used conditions and that important Sap isoenzymes may be overlooked because of low enzymatic activity in these artificial assays.

***SAP* mRNA levels.** Northern blot analysis (Fig. 4) detected mRNAs which hybridize with *SAP4*, *SAP5*, and *SAP6* between 2 and 5 h after hyphal induction at neutral pH, confirming the results of Hube et al. (5) and extending those results by showing that at least one clinical isolate expresses these mRNAs in a defined medium. This suggests that pH and hypha production alone are sufficient, and that other components of serum are not necessary, for the expression of these *SAP* mRNAs.

Of the three most similar *SAP* genes (*SAP4*, *SAP5*, and *SAP6*), expression of *SAP6* is the highest during hyphal induction at neutral pH, as determined by S1 nuclease analysis (Fig. 5). The *SAP5* mRNA is present at lower levels, while the *SAP4* mRNA is not detected. These signals are not due to contaminating levels of genomic DNA in the total RNA, which would result in equal signals for the three probes. In addition, DNA-DNA hybrids are not formed under these conditions (27). The *SAP5* signal shown in Fig. 5 is not the result of cross hybridization of the *SAP5* probe with *SAP6* RNA, as determined by control experiments with in vitro RNA transcripts of cloned *SAP* gene fragments.

These studies catalogue several factors, including cell type and environmental cues, that result in the selective production of Sap isoenzymes in *C. albicans*. These data suggest that Sap enzymatic activities are predominantly regulated by differential transcription of the *SAP* gene family. Future characterization of the regulatory regions in the *SAP* promoters during differential expression will help to determine how Sap activity is controlled in *Candida* virulence and disease.

ACKNOWLEDGMENTS

We thank David Soll, University of Iowa, for many helpful discussions and for providing strains WO-1 and 3153a; Remo Morelli, San Francisco State University, for providing strain SS; Ruth Greenblatt and the members of the Bay Area Research Consortium on Women with AIDS and John S. Greenspan, Deborah Greenspan, and the members of the Oral AIDS Center for providing clinical samples; Christine Morrison for the gifts of polyclonal antisera and culture supernatants; and our colleagues in the laboratory for many helpful comments.

This work was supported in part by NIH grants 1RO1AI-33317 and PO1-DE-O7946 to N.A. and by University of California University-Wide AIDS Research Program grant K92-SF-011 from the state of California and NIH grant RO1DE-11367 to T.C.W.

REFERENCES

1. **Banerjee, A., K. Ganesan, and A. Datta.** 1991. Induction of secretory acid proteinase in *Candida albicans*. J. Gen. Microbiol. **137:**2455–2461.
2. **Cutler, J. E.** 1991. Putative virulence factors of *Candida albicans*. Annu. Rev. Microbiol. **45:**187–218.
3. **Douglas, L. J.** 1988. *Candida* proteinases and candidosis. Crit. Rev. Biotechnol. **8:**121–129.
4. **Hube, B., C. J. Turver, F. C. Odds, H. Eiffert, G. J. Boulnois, H. Kochel, and R. Ruchel.** 1991. Sequence of the *Candida albicans* gene encoding the secretory aspartate proteinase. J. Med. Vet. Mycol. **29:**129–132.
5. **Hube, H., M. Monod, D. A. Schofield, A. J. P. Brown, and N. A. R. Gow.** 1994. Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. Mol. Microbiol. **14:**87–99.
6. **Lee, K. L., H. R. Buckley, and C. C. Campbell.** 1975. An amino acid liquid synthetic medium for development of mycelial and yeast forms of *Candida albicans*. Sabouraudia **13:**148–153.
7. **Macdonald, F., and F. C. Odds.** 1980. Inducible proteinase of *Candida albicans* in diagnostic serology and in the pathogenesis of systemic candidosis. J. Med. Microbiol. **13:**423–435.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **McKerrow, J.** Personal communication.
10. **Miyasaki, S. H., T. C. White, and N. Agabian.** 1994. A fourth secreted aspartyl proteinase gene (*SAP4*) and a *CARE2* repetitive element are located upstream of the *SAP1* gene in *Candida albicans*. J. Bacteriol. **176:**1702–1710.
11. **Monod, M., G. Togni, B. Hube, and D. Sanglard.** 1994. Multiplicity of genes encoding secreted aspartic proteinases in Candida species. Mol. Microbiol. **13:**357–368.
12. **Morrison, C. J., S. F. Hurst, S. L. Bragg, R. J. Kuykendall, H. Diaz, D. W. McLaughlin, and E. Reiss.** 1993. Purification and characterization of the extracellular aspartyl proteinase of Candida albicans: removal of extraneous proteins and cell wall mannoprotein and evidence for lack of glycosylation. J. Gen. Microbiol. **139:**1177–1186.
13. **Morrison, C. J., S. F. Hurst, S. L. Bragg, R. J. Kuykendall, H. Diaz, J. Pohl, and E. Reiss.** 1993. Heterogeneity of the purified extracellular aspartyl proteinase from *Candida albicans*: characterization with monoclonal antibodies and N-terminal amino acid sequence analysis. Infect. Immun. **61:**2030–2036.
14. **Morrow, B., T. Srikantha, J. Anderson, and D. R. Soll.** 1993. Coordinate regulation of two opaque-phase-specific genes during white-opaque switching in *Candida albicans*. Infect. Immun. **61:**1823–1828.
15. **Morrow, B., T. Srikantha, and D. R. Soll.** 1992. Transcription of the gene for a pepsinogen, *PEP1*, is regulated by white-opaque switching in *Candida albicans*. Mol. Cell. Biol. **12:**2997–3005.
16. **Odds, F. C.** 1988. Candida and candidosis: a review and bibliography. Bailliere Tindall, Philadelphia.
17. **Ray, T. L., and C. D. Payne.** 1988. Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase. Infect. Immun. **56:**1942–1949.
18. **Ray, T. L., C. D. Payne, and B. J. Morrow.** 1991. *Candida albicans* acid proteinase: characterization and role in candidiasis. Adv. Exp. Med. Biol. **306:**173–183.
19. **Ross, I. K., F. De Bernardis, G. W. Emerson, A. Cassone, and P. A. Sullivan.** 1990. The secreted aspartate proteinase of *Candida albicans*: physiology of secretion and virulence of a proteinase-deficient mutant. J. Gen. Microbiol. **136:**687–694.
20. **Ruchel, R., F. de Bernardis, T. L. Ray, P. A. Sullivan, and G. T. Cole.** 1992. Candida acid proteinases. J. Med. Vet. Mycol. **1:**123–132.
21. **Ruchel, R., R. Tegeler, and M. Trost.** 1982. A comparison of secretory proteinases from different strains of *Candida albicans*. Sabouraudia **20:**233–244.
22. **Ruchel, R., K. Uhlemann, and B. Boning.** 1983. Secretion of acid proteinases by different species of the genus *Candida*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **255:**537–548.
23. **Soll, D. R., B. Morrow, and T. Srikantha.** 1993. High-frequency phenotypic switching in Candida albicans. Trends Genet. **9:**61–65.
24. **Srikantha, T., and D. R. Soll.** 1993. A white-specific gene in the white-opaque switching system of Candida albicans. Gene **131:**53–60.
25. **Staib, F.** 1965. Serum-proteins as nitrogen source for yeastlike fungi. Sabouraudia **4:**187–193.
26. **Staib, F.** 1969. Proteolysis and pathogenicity of *Candida albicans* strains. Mycopathol. Mycol. Appl. **37:**345–348.
27. **White, T., and N. Agabian.** Unpublished data.
28. **White, T. C., G. A. Kohler, S. H. Miyasaki, and N. Agabian.** Expression of virulence factors in *Candida albicans*. Can J. Bot., in press.
29. **White, T. C., S. H. Miyasaki, and N. Agabian.** 1993. Three distinct secreted aspartyl proteinases in *Candida albicans*. J. Bacteriol. **175:**6126–6133.
30. **Wright, R. J., A. Carne, A. D. Hieber, I. L. Lamont, G. W. Emerson, and P. A. Sullivan.** 1992. A second gene for a secreted aspartate proteinase in *Candida albicans*. J. Bacteriol. **174:**7848–7853.