Differential Expression of *Candida albicans* Secreted Aspartyl Proteinase and Phospholipase B Genes in Humans Correlates with Active Oral and Vaginal Infections

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The in vivo expression of *Candida albicans* secreted aspartyl proteinase *(SAP1–SAP8)* and phospholipase B *(PLB1* and *PLB2)* genes was analyzed in 137 human subjects with oral and vaginal candidiasis or carriage. Total RNA was isolated from whole unstimulated saliva or vaginal swabs, and the expression of *SAP1–8* and *PLB1–2* was evaluated by reverse-transcriptase polymerase chain reaction using specific primer sets. A spectrum of *SAP* gene expression profiles was obtained from different *C. albicans* strains during symptomatic disease and asymptomatic carriage. *SAP2* and *SAP5* were the most common genes expressed during both infection and carriage. *SAP1, SAP3, SAP4, SAP7, SAP8,* and *PLB1* expression was correlated with oral disease, whereas *SAP1, SAP3,* and *SAP6–SAP8* expression was correlated with vaginal disease. Furthermore, *SAP1, SAP3,* and *SAP8* were preferentially expressed in vaginal, rather than oral, infections. This study demonstrates the differential expression of the hydrolytic enzyme genes in humans and correlates the expression of specific *Candida* species virulence genes with active disease and anatomical location.

Candida species infections of mucosal surfaces are common, debilitating, and often recurring diseases. Approximately three-quarters of all healthy women of fertile age suffer from vaginal candidiasis, with significant associated physical and psychological morbidity [1, 2]. *Candida albicans,* the most virulent of the *Candida* species, can cause severe oral infections in even mildly immunocompromised hosts. Attributes that putatively contribute to *C. albicans* virulence include adhesion, hyphal formation, phenotypic switching, and extracellular hydrolytic enzyme production (reviewed in [3–7]). The extracellular hydrolytic enzymes, including the secreted aspartyl proteinase *(SAP)* and phospholipase *(PLB)* genes, are among the few gene products that have been shown to directly contribute to *C. albicans* virulence.

Four types of phospholipases have been reported in *C. albicans*—phospholipase A [8], B [9, 10], C [11], and D [12]—but only the *PLB1* and *PLB2* gene products have been detected extracellularly. Although *PLB1* is thought to account for most of the secreted phospholipase B activity in *C. albicans*, *PLB2* contributes in a minor way, because a *PLB1*-deficient strain still produces residual amounts of phospholipase B activity [13]. Phospholipase B1 is secreted during invasion of the gastrointestinal tract of mice [13], and *C. albicans* mutants lacking *PLB1* are significantly attenuated in virulence in both an intragastric infant-mouse model [13] and an intravenous model of murine candidiasis

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[14]. Serum samples from patients with invasive *Candida* species infections reacted with purified phospholipase B, which indicates that phospholipase B is produced in humans [13] and that blood isolates of *C. albicans* produce more phospholipase B activity in vitro than oral isolates from carriers of *Candida* species [15]. Although these studies implicate the phospholipase B enzyme family in *C. albicans* virulence, no studies have yet analyzed the expression of the *PLB1* and *PLB2* genes in human mucosal infections.

The *C. albicans* Saps are, by far, the best characterized members of the hydrolytic enzymes secreted by *C. albicans* and are encoded by 10 *SAP* genes (reviewed in [16–18]). Two distinct groups are clustered within the family; Sap1–Sap3 are ~67% identical, and Sap4–Sap6 are ~89% identical, with Sap7 being the most divergent, with only 20%–27% sequence homology to the other Saps [19]. The proteinase family is differentially regulated and expressed under a variety of laboratory growth conditions [20, 21], during experimental *C. albicans* infections using reconstituted human oral epithelium (RHE) [22], and in vivo [23]. Different *SAP* genes appear to be essential for mucosal *(SAP1–SAP3)* [24–27] and systemic *(SAP4–SAP6)* [28] infections and also are involved in *C. albicans* adherence [29], tissue damage [24, 30], and evasion of host immune responses [31]. The proteinases possess distinct differences in pH optima, with Sap1–Sap3 (yeast associated) having optimum activity at lower pH values, and Sap4–Sap6 (hyphal associated) having optimum activity at higher pH values [31].

C. albicans SAP gene expression in oral or vaginal infections has been studied in vitro and in vivo in animal models and in patient isolates. Using infected oral RHE, Schaller et al. [22] detected a specific order of *SAP* gene expression by reverse-transcriptase polymerase chain reaction (RT-PCR); *SAP1* and *SAP3* were expressed in the initial stages (possibly reflecting colonization), followed by *SAP6* (concomitant with germ tube formation and severe lesions), and *SAP2* and *SAP8* were expressed in late phases of infection. Of interest, a different *SAP* expression profile was observed using an RHE model of vaginal candidiasis; initially *SAP2, SAP9,* and *SAP10* transcripts were detected; followed by *SAP1, SAP4,* and *SAP5* (concomitant with lesions); and, finally, by *SAP6* and *SAP7* (M. Schaller, M. Bein, H. Korting, et al., personal communication). By use of *SAP*-deficient mutants, the *SAP1–SAP3* subfamily was shown to contribute to tissue damage in both oral and vaginal RHE models [24] (M. Schaller, M. Bein, H. Korting, et al., personal communication). Together, these 2 studies support a role for *SAP1–SAP3* in establishing *C. albicans* infections at human mucosal surfaces.

In a murine model of oropharyngeal candidiasis, using both normal and transgenic mice expressing human immunodeficiency virus (HIV) type 1, *SAP1–SAP6* and *SAP9* transcripts were detected continuously throughout the course of infection, with *SAP5* and *SAP9* being the most highly expressed; *SAP7* and *SAP8* were the only genes expressed transiently during infection [32]. Finally, by use of in vivo expression technology (IVET), as well as in a mouse model of esophageal candidiasis, transcription of *SAP5* and *SAP6* predominated [23], indicating a possible role for the *SAP4–SAP6* subfamily in mouse mucosal infections.

We have been interested in determining whether *SAP* gene expression patterns observed in vitro and in animal models are representative of those expressed in the context of human disease. We previously have described a radiation-based RT-PCR protocol that allowed us to assay the expression of individual *SAP* genes directly in the oral cavity without intermediate laboratory cultivation of the clinical isolates [33]. Using RT-PCR, we were able to amplify *SAP* mRNA from as little as 1 pg of total RNA and from <10 cfu of *C. albicans* in a patient sample. We analyzed the expression of *C. albicans SAP1–SAP7* in a small number of patients presenting with oral disease, as well as in asymptomatic carriers, and obtained data that suggested differential expression of individual members of the *SAP* gene family in humans [33]. In the present study, to determine whether the expression of individual extracellular hydrolytic enzyme genes correlates with oral or vaginal candidiasis, as opposed to carriage, and to discover whether certain *SAP* and *PLB* genes are specifically or selectively expressed during oral or vaginal disease, *SAP1–SAP8* and *PLB1* and *PLB2* gene expression were analyzed in 137 clinical samples obtained from patients colonized or infected either orally or vaginally with *C. albicans.*

MATERIALS AND METHODS

Strains, media, and culture conditions. The following *Candida* species and strains were used in this study: *C. albicans* NCPF 3156, *C. dubliniensis* NCPF 3949, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019, *C. guilliermondii* NCPF 3099, and *C. glabrata* ATCC 90030. Strains were maintained on Sabouraud (SAB) dextrose agar (Oxoid) stab cultures at room temperature. For DNA isolation, all cultures were grown in 10 mL of SAB dextrose broth (Oxoid) for 2 days at 27°C on an orbital incubator. *C. albicans* NCPF 3156 was used as a positive control for all optimization experiments.

Selection of primers and specificity of RT-PCR. In our initial study [33], a single set of PCR primers was used to amplify *SAP4–SAP6* together, which are highly homologous in nucleotide sequence [19]. However, in the present study, individual primer sets were designed to amplify *SAP4–SAP6* separately. One primer pair each for *ACT1* (actin), *SAP1–SAP8,* and *PLB1* and *PLB2* were designed (table 1). None of the primer sets amplified regions containing introns. The primer sets were tested against *C. albicans* DNA to check for accurate amplifi-

Table 1. Primer sets detecting *Candida albicans* secreted aspartyl proteinase genes *SAP1–SAP8,* phospholipase B genes *PLB1* and *PLB2,* and *ACT1* genes.

Gene [reference], primer	Sequence	Product, bp
SAP1 [34]		
Forward	5′-TCA ATC AAT TTA CTC TTC CAT TTC TAA CA-3′	161
Reverse	5′-CCA GTA GCA TTA ACA GGA GTT TTA ATG ACA-3′	
SAP2 [35]		
Forward	5′-AAC AAC AAC CCA CTA GAC ATC ACC-3′	178
Reverse	5′-TGA CCA TTA GTA ACT GGG AAT GCT TTA GGA-3′	
SAP3 [36]		
Forward	5′-CCT TCT CTA AAA TTA TGG ATT GGA AC-3′	231
Reverse	5′-TTG ATT TCA CCT TGG GGA CCA GTA ACA TTT-3′	
SAP4 [37]		
Forward	5′-TTA TTT TTA GAT ATT GAG CCC ACA GAA A-3′	171
Reverse	5′-GCC AGT GTC AAC AAT AAC GCT AAG TT-3′	
SAP5 [19]		
Forward	5′-AGA ATT TCC CGT CGA TGA GAC TGG T-3′	277
Reverse	5′-CAA ATT TTG GGA AGT GCG GGA AGA-3′	
SAP6 [19]		
Forward	5′-CCC GTT TTG AAA TTA AAT ATG CTG ATG G-3′	187
Reverse	5′-GTC GTA AGG AGT TCT GGT AGC TTC G-3′	
SAP7 [19]		
Forward	5′-GAA ATG CAA AGA GTA TTA GAG TTA TTA C-3′	196
Reverse	5′-GAA TGA TTT GGT TTA CAT CAT CTT CAA CTG-3′	
SAP8 [38]		
Forward	5′-GCC GTT GGT GCC AAA TGG AAT AGT TA-3′	256
Reverse	5′-ATT TGA CTT GAG CCA ACA GAA TGG T-3′	
PLB1 [10]		
Forward	5′-CCT ATT GCC AAA CAA GCA TTG TC-3′	179
Reverse	5′-CCA AGC TAC TGA TTT CAC CTG CTC C-3′	
PLB2 [39]		
Forward	5′-GTG GGA TCT TGC AGA GTT CAA GC-3′	270
Reverse	5′-CTC AAA GCT CTC CCA TAG ACA TCT G-3′	
ACT1 [40]		
Forward	5′-GAT TTT GTC TGA ACG TGG TAA CAG-3′	271
Reverse	5′-GGA GTT GAA AGT GGT TTG GTC AAT AC-3′	

cation of the correct genes, as well as against a panel of genomic DNA isolated from different *Candida* species to test for cross-reactivity. All the amplified gene products were purified by use of the QIAquick PCR purification system, according to the manufacturer's instructions (Qiagen), and were sequenced at the Advanced Biotechnology Centre (London, UK) to confirm the specificity of the reaction.

Study populations and type of* Candida *species infection. Patients were selected as they presented to the clinic by a single experienced clinician at each of the Oral Medicine and Genitourinary Medicine clinics. Two populations were used to obtain clinical samples for the analysis of *SAP* and *PLB* expression. The first population included patients with active *Candida* species infection, with clinical signs and symptoms of oral or vaginal candidiasis. Patients presenting with oral candidiasis were infected with $>2 \times 10^3$ *C. albicans* cfu/mL of saliva ($n = 40$; 25 men and 15 women; mean age, 47 ± 13 years). Of these 40 patients, 24 with oral candidiasis were HIV infected, and 16 were HIV uninfected. None of these 40 patients had been treated with anti-HIV proteinase drugs before or at the time of sample collection. The majority of patients presented with a single clinical form of *C. albicans* infection—that is, pseudomembranous (thrush) candidiasis (PC; $n = 14$) or erythematous candidiasis (EC; $n = 9$)—although some presented with

both types ($n = 4$). Six patients suffered from chronic atrophic candidiasis (CAC), which also can result from wearing dentures, and 7 patients with Sjögren's Syndrome, an autoimmune disease characterized by xerostomia (dry mouth), suffered from chronic candidiasis (CC). Vaginal infection was diagnosed primarily on clinical signs and symptoms and cultures positive for *C. albicans* ($n = 40$; mean age, 26.0 ± 7.2 years; *Candida* species colony-forming unit count range, 2 to $>10^4$ cfu). Of the 40 patients, the majority presented with PC or EC, although the clinical diagnosis of PC or EC for vaginal infection is not as explicit as it is for oral infection, probably due, in part, to physical, mechanical, and immunological [41, 42] differences between the vaginal lumen and the oral cavity. Positive diagnosis of vaginal infection met the following criteria: culture positive and the presence of at least 1 sign and 1 symptom of infection. Signs included edema (swelling), erythema, pseudomembranous plaques, and discharge; and symptoms included pruritus (itch), pain, and soreness. None of the patients presenting with vaginal disease was tested for HIV infection. In addition, none of the patients with oral or vaginal infection was treated with either antibiotic or antimycotic drugs for at least 1 month before sampling.

The second population included subjects who were asymptomatic carriers (*Candida* species carrier group)—that is, without any clinical signs or symptoms of infection. Oral *Candida* species carriers harbored 50–800 *C. albicans* cfu/mL of saliva ($n = 29$; 2 men and 27 women; mean age, 29 ± 11 years), and vaginal carriage status was based on cultures positive for *C. albicans,* regardless of colony-forming unit count (range, 4–550 cfu/swab; $n = 28$; mean age, 26 ± 5 years). All *Candida* carriers were either laboratory volunteers or individuals attending the Oral Medicine clinic at Guy's Hospital or the Genitourinary Medicine Clinic at St Thomas' Hospital. None of the individuals who comprised the *Candida* species carrier population was tested for HIV infection. Informed consent was obtained from all patients regarding the nature of the study. The collection of clinical samples was conducted according to the rules of the Guy's and St Thomas' Hospital Trust ethical review board.

Sample collection and preparation. Specimens were collected as either whole unstimulated saliva or vaginal swabs, because these provided reliable and consistent mRNA detection (data not shown). Duplicate samples of whole unstimulated saliva or vaginal swabs were collected at the same visit from each subject. The vaginal swabs were taken from the lateral vaginal wall; for patients presenting with infection, swabs were taken directly from areas of infection. One of the whole saliva samples was immediately frozen on dry ice at −70°C, and one of the vaginal swabs was immediately suspended in 1 mL of RNAlater (Ambion) and then frozen on dry ice. The purpose of each procedure was to preserve the integrity of RNA for the subsequent analysis of *SAP* and *PLB* gene expression in the clinical samples. The second saliva and vaginal swab samples were used to determine *Candida* colony-forming unit counts and for yeast identification, using the API 32C AUX system (BioMerieux), CHROMagar (CHROMagar), germ tube formation, and growth at 42°C [33].

Two hundred five individuals were enrolled in the oral study during a 2-year period. After *Candida* species identification, 64 matched the criteria of having clinical signs and symptoms of infection and of harboring $>2 \times 10^3$ *C. albicans* cfu/mL of saliva, and 38 were designated as asymptomatic *Candida* carriers harboring 50–800 *C. albicans* cfu/mL of saliva. For the vaginal study, 202 patients were enrolled during an 18-month period. Of these, 42 matched the criteria of having symptomatic *C. albicans* vaginal infection, and 34 were carriers. Total RNA was isolated from all qualifying samples. RT-PCR and PCR (without avian myeloblastosis virus [AMV] reverse transcriptase [RT]), using *ACT1* primers, were performed on each sample, to determine whether sufficient *C. albicans* total RNA was isolated for mRNA detection and whether the total RNA samples contained contaminating DNA, respectively. *ACT1* mRNA was successfully and reproducibly amplified from samples obtained from 40 patients with oral infections, 29 oral carriers, 40 patients with vaginal infections, and 28 vaginal carriers. Transcription of *SAP1–8* and *PLB1–2* genes was assayed in each of these samples by RT-PCR.

DNA and RNA isolation. Genomic DNA from *Candida* species was isolated by use of a phenol/chloroform-based protocol [33]. Total RNA from clinical samples was prepared using the RNeasy RNA isolation system (Qiagen), incorporating glass beads (400–600 μmol, Sigma). The QIAshredder unit (Qiagen) was used to homogenize the cell lysates, and, at the end of the procedure, the total RNA was treated overnight with DNAse (Qiagen), to allow the complete digestion of any contaminating DNA. Purified RNA from each sample was confirmed to be DNA-free by the absence of an amplified product after PCR (without RT) by use of *ACT1* (actin) primers.

RT-PCR analysis of* C. albicans SAP *and* PLB *mRNA expression. Thirteen RT-PCRs were performed for each RNA sample prepared from each clinical isolate. These included the 10 experimental reactions *(SAP1–8* and *PLB1–2),* and 3 different control reactions: an *ACT1* control to demonstrate the presence or absence of *Candida* species, a negative (water) control, and a positive control using genomic DNA isolated from *C. albicans* NCPF 3156 cells. Each RNA sample was analyzed in duplicate and, in many cases, in triplicate to verify the hydrolytic gene expression data. Complete congruence in gene expression was required in 2 separate analyses using the same RNA sample. RT-PCR conditions were optimized by use of modified Taguchi methods [43] and included a touchdown and

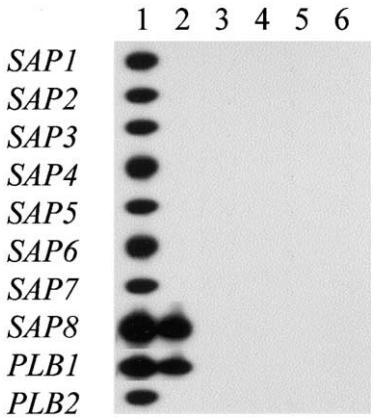

Figure 1. Reactivity of secreted aspartyl proteinase genes *SAP1–SAP8* and phospholipase B genes *PLB1* and *PLB2* primer sets with genomic DNA isolated from different *Candida* species: *C. albicans* NCPF 3156 (1), *C. dubliniensis* NCPF 3949 (2), *C. tropicalis* ATCC 750 (3), *C. parapsilosis* ATCC 22019 (4), *C. guilliermondii* NCPF 3099 (5), and *C. glabrata* ATCC 90030 (6). The expected *SAP* and *PLB* gene products were amplified from *C. albicans. SAP1–SAP7* and *PLB2* primers were specific for *C. albicans,* whereas the *SAP8* and *PLB1* primers amplified a polymerase chain reaction (PCR) product from *C. dubliniensis,* which was identical in size to *C. albicans.*

hot start protocol, as described elsewhere [33]. RT-PCR experiments using the *ACT1, SAP,* and *PLB* primers were performed by use of the Access RT-PCR system (Promega). Template RNA was added to the RT-PCR mix containing 1× AMV/Tfl buffer (Promega; proprietary information), 1 mmol $MgSO^4$, 0.1 mmol dNTPs, 0.6 μmol primers, 3.75 U AMV RT, and 1 μCi ^{32}P-dCTP (ICN Pharmaceutical). Radioactive-labeling was used to maximize sensitivity. After RT (48°C for 45 min), the sample was denatured at 94°C for 3 min, and 2.5 U of Tfl DNA polymerase was added to the reaction (hot start). Cycling times were as follows: denaturation at 94°C, annealing at 60°C, and extension at 72°C, each for 30 s. A final extension at 72°C for 10 min followed cycling. All radiolabeled RT-PCR products were electrophoresed through a 7% denaturing 7-mol urea polyacrylamide gel, exposed to autoradiography film at −70°C, and developed [33].

Statistical analysis. Fisher's exact test (2-tailed) was used to determine statistical differences in hydrolytic enzyme gene expression between patients with oral or vaginal infection and carrier subjects. The number of individuals used in this study was based on power calculations from our previous study, which showed a difference in proportions of the "indicator variable" of 0.0 and 0.6 [33]. Eighty percent power to show a statistically significant difference between proportions of subjects with an "indicator variable" of 0.2 versus 0.6 can be achieved with 28 subjects in each of our 4 main groups. Therefore, between 28 and 40 subjects were recruited in each group.

RESULTS

ACT1, SAP1–8, *and* PLB1–2 *primers.* To control for the presence of *Candida* species in the absence of *SAP* gene expression, primers were designed to detect *Candida* actin *(ACT1)* gene expression [33]. *ACT1* could be amplified from *Candida* species samples obtained from carriers that contained <10 cfu. The *SAP1–SAP8* and *PLB1* and *PLB2* primers amplified small PCR fragments, ranging in size from 161 to 277 bp (table 1). The specificity of each primer pair was determined by PCR using *C. albicans* genomic DNA, as described elsewhere [33], and the identity of the *SAP1–SAP8* and *PLB1* and *PLB2* PCR products was confirmed by DNA sequencing at the Advanced Biotechnology Centre (London; data not shown).

The *SAP1–SAP7* and *PLB2* primer sets were *C. albicans* specific and did not amplify any gene products from *C. dubliniensis, C. tropicalis, C. parapsilosis, C. guilliermondii,* or *C. glabrata* (figure 1). However, the *SAP8* and *PLB1* primers did amplify gene products identical in size in both *C. albicans* and its closely related species *C. dubliniensis* (figure 1), indicating that *C. dubliniensis* may possess homologues of *SAP8* and *PLB1.* However, we were able to ascertain that the expression of *SAP8* and *PLB1* detected in our experiments was attributable to *C. albicans,* because each of the clinical samples were plated and identified as monocultures of *C. albicans.*

To control for cross-reactivity with microbial flora or host RNA present in each sample, RT-PCR was performed using *SAP* and *PLB* primer sets and RNA isolated from oral (n = 7) and vaginal (n = 7) samples that were culture negative for *Candida* species, as described elsewhere [33]. No RT-PCR amplification products were detected under any of these conditions, demonstrating the specificity of the primer sets for *C. albicans* gene expression (data not shown).

RNA preparation from patient samples. The RNA and *Candida* colony-forming unit concentration means and ranges for the 4 study populations are displayed in table 2. It should be noted that only a small fraction of the total RNA is attributable to *C. albicans,* because the majority of RNA present in the entire sample is derived from variable amounts of host and bacterial RNA. RNA isolated from patient samples with as few

Table 2. Total RNA and *Candida albicans* colony-forming unit means and ranges in the 4 study populations.

Study population	RNA concentration mean ± SEM (range), μg/mL	Total *C. albicans* count, cfu
Oral infection (n = 40)	111 ± 91 (13–414)	2880 to >10^4
Oral carriage (n = 29)	89 ± 75 (15–352)	50–780
Vaginal infection (n = 40)	99 ± 57 (8–274)	2 to >10^4
Vaginal carriage (n = 28)	76 ± 48 (11–298)	4–550

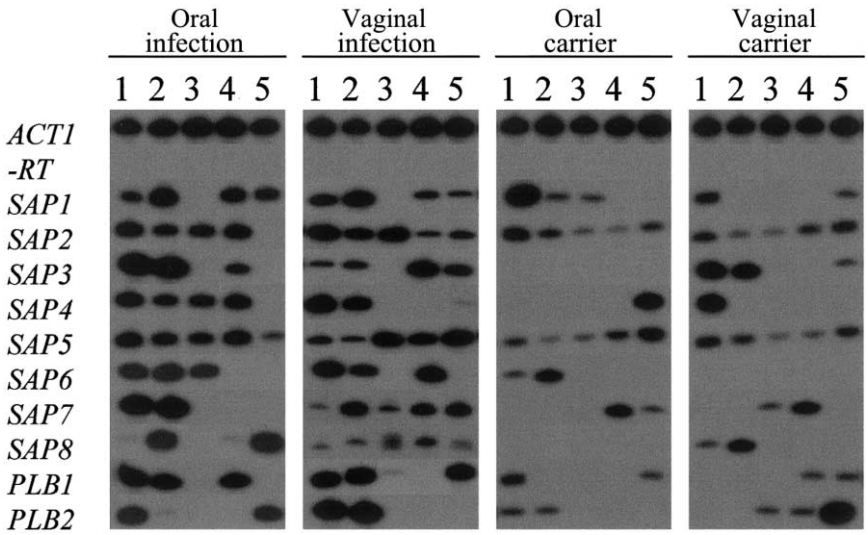

Figure 2. Detection of *Candida albicans ACT1* (actin), secreted aspartyl proteinase genes *SAP1–SAP8,* and phospholipase B genes *PLB1* and *PLB2* mRNA expression from clinical saliva and vaginal wash samples. Five (1–5) representative samples are presented for each study group: patients with oral infection, patients with vaginal infection, asymptomatic oral *Candida* carriers, and asymptomatic vaginal *Candida* carriers. The presence of actin signals *(ACT1)* indicates the presence of *C. albicans* in the clinical samples. The exclusion of avian myelobastosis virus reverse transcriptase (−RT) resulted in the absence of any polymerase chain reaction (PCR) signals using *ACT1* primers, confirming the absence of *C. albicans* DNA in the clinical samples. A spectrum of *SAP* and *PLB* gene expression profiles was obtained from different *C. albicans* clinical strains during symptomatic disease and asymptomatic carriage. Two patients with oral infection and 2 patients with vaginal infection (1 and 2) harbored *C. albicans* expressing the full repertoire of all 10 hydrolytic enzyme genes tested, and 3 patients (3–5) possessed *C. albicans* expressing fewer genes. The 5 oral and vaginal asymptomatic carriers harbor *C. albicans* expressing fewer hydrolytic enzyme genes than the oral and vaginal infected groups. *SAP2* and *SAP5* are the most common genes expressed during both infection and carriage.

as 50 cfu/mL in saliva or 2 cfu/mL in vaginal swabs was sufficient to detect *C. albicans*–specific gene expression. There was no observable difference in the colony-forming unit counts between patients with different types of infection (i.e., PC or EC). All the total RNA preparations were confirmed to be *Candida* DNA free by the lack of an *ACT1* amplification product by PCR (figure 2).

In our previous study [33], we showed that differences in *SAP* gene expression profiles between the oral infection and asymptomatic carrier groups were unlikely to be due to the greater numbers of *C. albicans* cells present in the infected patient group (i.e., that detection of *SAP* genes was independent of *C. albicans* RNA in the sample). This study confirms our initial assertion, because many samples from patients with oral and vaginal disease with greater *C. albicans* colony-forming unit counts expressed fewer hydrolytic enzyme genes than many oral and vaginal carriers who harbored lower *C. albicans* colony-forming unit counts. Furthermore, the detection of *C. albicans* actin *(ACT1)* from <10 *C. albicans* cfu in vaginal swab samples indicated that the methods employed were sufficiently sensitive to also detect *SAP* and *PLB* expression. The sensitivity of detection of *SAP* and *PLB* expression (independent of initial RNA concentration) also was assayed by diluting samples containing high numbers of *C. albicans* colony-forming units to levels of colony-forming units typically found in samples obtained from carriers (50–150 cfu/mL) and assaying gene expression in the diluted and undiluted samples. In samples obtained from 5 different patients, we found identical gene expression profiles in both the diluted and undiluted samples (data not shown), indicating that any differences in gene expression profiles between the disease and carrier populations were unlikely to be due to the greater numbers of *C. albicans* cells present in the infected patient groups.

C. albicans SAP *and* PLB *expression in samples from patients with oral candidiasis.* The pattern of *SAP1–SAP8* and *PLB1* and *PLB2* expression obtained from RT-PCR assay of patient saliva samples is shown in table 3 (and representative samples are illustrated in figure 2). *SAP2* and *SAP5* are the most common transcripts in both *C. albicans*–infected patients (90% and 95%, respectively) and carriers (79% and 93%, respectively). *SAP3* is expressed with the least frequency; only 35% of samples from infected patients and 14% of samples from carriers were positive for *SAP3* transcripts. Although none of the samples obtained from carriers expressed all of the genes assayed, 10 samples obtained from *C. albicans*–infected patients expressed the full repertoire of *SAP1–SAP8* and *PLB1* and *PLB2* genes (representative samples in figure 2). In general, the autoradiographic profiles indicate that *C. albicans* from patients with oral infection express a greater repertoire of hydrolytic enzyme genes (6–7 genes/patient) than did those from oral

Table 3. *Candida albicans* secreted aspartyl proteinase *(SAP)* and phospholipase B *(PLB)* gene expression in oral infection and carriage.

Group	*SAP1*	*SAP2*	*SAP3*	*SAP4*	*SAP5*	*SAP6*	*SAP7*	*SAP8*	*PLB1*	*PLB2*
Oral infection (n = 40)	28 (70)	36 (90)	14 (35)	28 (70)	38 (95)	32 (80)	31 (78)	19 (48)	23 (58)	19 (48)
Oral carrier (n = 29)	9 (31)	23 (79)	2 (14)	12 (41)	27 (93)	20 (69)	15 (48)	5 (17)	4 (14)	10 (34)
P[a]	**.002**	.302	**.008**	**.026**	1.00	.397	**.038**	**.011**	**<.001**	.202

NOTE. Data are no. subjects (%) who were positive for the expression of the particular gene.

[a] Probability values between the no. of subjects who were positive for the expression of a particular gene in the oral infection and oral carrier groups were determined by use of the 2-tailed Fisher's exact test. Significant differences in the frequency of detection are in boldface type.

carriers (4–5 genes/carrier). However, there were 4 instances when only ⩽3 genes were expressed in samples from infected individuals and 9 instances when ⩾7 genes were expressed in samples from carriers. When comparing *SAP* and *PLB* expression between patients with oral infection and carriers, we found that *SAP1, SAP3, SAP4, SAP7, SAP8,* and *PLB1,* are preferentially expressed in *C. albicans* infection (table 3).

C. albicans SAP *and* PLB *expression in samples from patients with vaginal candidiasis.* *SAP1–SAP8* and *PLB1* and *PLB2* expression by *C. albicans* in swab samples from vaginal infection and carriage is shown in table 4 (and representative samples in figure 2). *SAP2, SAP4,* and *SAP5* are the most common transcripts detected in both vaginal infections (100%, 88%, and 98%, respectively) and carrier states (89%, 75%, and 89%, respectively). A high proportion of samples from patients with vaginal infections (n = 17), compared with 1 sample from the carriers, harbored *C. albicans* expressing the full repertoire of all 10 genes (*SAP1–SAP8* and *PLB1* and *PLB2;* representative samples in figure 2). In general, *C. albicans* from vaginal infections express more hydrolytic enzyme genes (7–8 genes/patient) than did the vaginal carriers (~5 genes/carrier). When comparing *C. albicans SAP* and *PLB* expression between patients with vaginal infection and carriers, *SAP1, SAP3,* and *SAP6–SAP8* were preferentially expressed to a significant level in *C. albicans* infection (table 4).

Comparison of* C. albicans SAP *and* PLB *gene expression patterns from oral and vaginal samples. Similarities in the overall pattern of *SAP* gene expression are evident between *C. albicans* assayed in oral and vaginal sites. *SAP2* and *SAP5* are expressed with the greatest frequency in both the oral and vaginal infection and carrier groups; notably, 1 gene each from the *SAP1–SAP3* and *SAP4–SAP6* subfamilies (tables 3 and 4). Four genes, *SAP1, SAP3, SAP7,* and *SAP8* are preferentially expressed to a significant level during infection rather than carriage in both the oral and vaginal study groups (tables 3 and 4). The main differences between the oral and vaginal populations is that both *SAP4* (P = .026) and *PLB1* ($P \leqslant .001$) are preferentially expressed during oral infection versus oral carriage (table 3), but not in vaginal infection versus vaginal carriage (table 4). In addition, *SAP6* ($P \leqslant .001$) is preferentially expressed during vaginal infection versus vaginal carriage, but not in oral infection versus oral carriage. A comparison of the oral and vaginal *C. albicans*–infected groups alone reveals that *SAP1, SAP3,* and *SAP8* are expressed significantly more frequently during vaginal infection than during oral infection (table 5).

SAP *and* PLB *expression in HIV-infected and HIV-uninfected patients with oral candidiasis and in different clinical forms of* C. albicans *infection.* No statistical differences in the *SAP* or *PLB* expression profiles between HIV-infected and HIV-uninfected patients (table 6) or between the various clinical forms of disease (data not shown) were evident.

DISCUSSION

This study analyzed *C. albicans SAP1–SAP8* and *PLB1* and *PLB2* gene expression during oral and vaginal infection and colonization in 137 samples from separate individuals, and the results provide insight with regard to patterns of extracellular

Table 4. *Candida albicans* secreted aspartyl proteinase *(SAP)* and phospholipase B *(PLB)* gene expression in vaginal infection and carriage.

Group	*SAP1*	*SAP2*	*SAP3*	*SAP4*	*SAP5*	*SAP6*	*SAP7*	*SAP8*	*PLB1*	*PLB2*
Vaginal infection (n = 40)	36 (90)	40 (100)	27 (68)	35 (88)	39 (98)	31 (78)	24 (60)	30 (75)	25 (63)	25 (63)
Vaginal carrier (n = 28)	12 (43)	25 (89)	7 (25)	21 (75)	25 (89)	9 (32)	8 (29)	9 (32)	12 (43)	14 (50)
P[a]	**<.001**	.065	**.003**	.211	.298	**<.001**	**<.010**	**.001**	.140	.330

NOTE. Data are no. subjects (%) who were positive for the expression of the particular gene.

[a] Probability values between the no. of subjects who were positive for the expression of a particular gene in the vaginal infected and carrier groups were determined by use of the 2-tailed Fisher's exact test. Significant differences in the frequency of detection are in boldface type.

Table 5. *Candida albicans* secreted aspartyl proteinase *(SAP)* and phospholipase B *(PLB)* gene expression in oral and vaginal infection.

Group	*SAP1*	*SAP2*	*SAP3*	*SAP4*	*SAP5*	*SAP6*	*SAP7*	*SAP8*	*PLB1*	*PLB2*
Oral infection (n = 40)	28	36	14	28	38	32	31	19	23	19
Vaginal infection (n = 40)	36	40	27	35	39	31	24	30	25	25
P[a]	**.048**	.116	**.007**	.099	1.000	1.000	.094	**.021**	.820	.261

NOTE. Data are no. of subjects who were positive for the expression of the particular gene.

[a] Probability values between the no. of subjects who were positive for the expression of a particular gene in the oral and vaginal infection groups were determined by use of the 2-tailed Fisher's exact test. Significant differences in the frequency of detection are in boldface type.

hydrolytic enzyme gene expression in the context of human disease. The data show that all members of the *SAP* gene family can be expressed by *C. albicans* strains in vivo during the course of the same disease presentation (i.e., oral or vaginal, PC or EC); not only are individual *SAP* and *PLB* gene transcripts more frequently detected during active *C. albicans* infection compared with carriage (tables 3 and 4; figure 2), but there also is differential expression of certain hydrolytic enzymes during infection in the oral cavity and vaginal lumen (table 5). This study expands our initial analysis [33] by comparing *PLB1* and *PLB2,* as well as *SAP1–SAP8* gene expression, and by inclusion of *C. albicans* obtained not only from oral infections and carriage but also from vaginal infections and carriage.

This study did not analyze the expression of *SAP9* and *SAP10,* because there is good evidence to suggest that Sap9 and Sap10 both have C-terminal consensus sequences typical for glycophosphotidylinositol (GPI)–anchored proteins and, therefore, may not be secreted from the cell. In addition, Sap9 and Sap10 might be regulatory proteinases that may play a role in the cell surface integrity of the cell, which is in contrast to the putative functions of the other Saps (A. Albrecht, I. Pichova, M. Monod, and B. Hube [principal investigator], personal communication).

Many laboratory experiments have been performed to assess the expression patterns of genes in the *SAP* gene family. In general, Sap2 is the predominant *C. albicans* proteinase secreted in protein containing medium [20, 21, 35], a finding that agrees well with our own that *SAP2* is a predominant proteinase gene expressed by *C. albicans* isolated from colonization and infection of the oral cavity (100%) and vagina (90%) (table 3 and 4; figure 2). *SAP5* (like *SAP2*) is also universally expressed in samples obtained from oral and vaginal infections and colonization (tables 3 and 4; figure 2). This "dominant" expression of *SAP5* also has been observed in murine models of oropharyngeal candidiasis using IVET [23] and RT-PCR [32]. However, *SAP5* gene expression was not detected in an RHE model of oral candidiasis [22], a finding which contrasts with our data showing that *SAP5* is the most commonly expressed *SAP* gene at human mucosal surfaces. Because we find both *SAP2* and *SAP5* expressed in nearly every patient sample, whether from active disease or colonization, this suggests that individual members of the 2 main subfamilies *(SAP1–3* and *SAP4–6)* of the *SAP* gene superfamily are required by *C. albicans* to fulfill basic functions in relation to survival and proliferation in the human host. In support of this conclusion is the broad substrate specificity of Sap2 (which can degrade many proteins found in the oral cavity and vaginal lumen [17]), and its role in causing tissue damage as assessed in an RHE oral model [24] and virulence in a rat vaginitis model [25].

In comparisons of symptomatic disease and asymptomatic carriage, *SAP1* and *SAP3* are preferentially expressed in active oral and vaginal candidiasis (tables 3 and 4) but are more frequently found in association with vaginal infections (table 5). In laboratory settings, *SAP1* and *SAP3* gene expression are tightly coupled and appear to be regulated by phenotypic switching [20, 21, 44], a phenomenon that is thought to allow *C. albicans* to adapt to different host environments during the

Table 6. *Candida albicans* secreted aspartyl proteinase *(SAP)* and phospholipase B *(PLB)* gene expression in human immunodeficiency virus (HIV)–infected and HIV-uninfected patients with oral candidiasis.

Status	*SAP1*	*SAP2*	*SAP3*	*SAP4*	*SAP5*	*SAP6*	*SAP7*	*SAP8*	*PLB1*	*PLB2*
HIV infected (n = 24)	16	21	6	18	23	19	18	11	13	10
HIV uninfected (n = 16)	12	15	8	10	15	13	13	8	10	9
P[a]	.729	.638	.176	.490	1.000	1.000	.717	1.000	.747	.520

NOTE. Data are no. of subjects who were positive for the expression of a particular gene.

[a] Probability values between the no. of subjects who were positive for the expression of a particular gene in the HIV-infected and HIV-uninfected groups with oral candidiasis were determined by use of the 2-tailed Fisher's exact test.

course of an infection. Therefore, we did not expect to observe an uncoupling of *SAP1* and *SAP3* gene expression; *SAP1* was expressed in 70% and 90% of the samples obtained from patients with oral and vaginal infections, respectively, whereas *SAP3* was expressed in only 35% and 68% of samples, respectively (tables 3 and 4). The association of these switch phenotype-related members of the *SAP* gene family with active disease at both oral and vaginal sites is consistent with a role for phenotypic switching in the disease process [33] and supports observations that *C. albicans* isolated from patients with active oral candidiasis exhibit higher rates of phenotypic switching than similarly localized commensal strains [45].

Although *SAP3* is expressed at higher overall frequency in active mucosal infections, compared with asymptomatic colonization, it is not one of the more frequently detected members of the *SAP* gene family (tables 3 and 4). Within that context, however, *SAP3* expression is ~2-fold more prevalent in both carriers and patients suffering from vaginal rather than oral carriage and disease, raising the possibility that *SAP3* expression could facilitate *C. albicans* colonization and infection of the vaginal mucosa. Our data from *C. albicans* infections in humans also are consistent with those of Ripeau et al. [32], who showed that *SAP3* expression in the oral cavity of mice is down-regulated during persistent colonization. In summary, the universal expression of *SAP2* during colonization and infection and the preferential expression of *SAP1,* in particular, during active human disease supports the current evidence that the Sap1–Sap3 subfamily contribute to the pathogenesis and virulence of *C. albicans* at mucosal surfaces.

Perhaps one of our more surprising results was the detection of transcripts from genes in the *SAP4–SAP6* subfamily in all but 1 of the *C. albicans*–infected patients ($n = 79$) and all but 5 carriers ($n = 52$). Most studies using either *SAP*-deficient mutants or proteinase inhibitors indicate that *SAP4–SAP6* do not contribute to tissue damage and pathogenesis at mucosal surfaces [24, 25]. Rather, *SAP4–SAP6* are thought to promote systemic disease, because *SAP4*-, *SAP5*-, and *SAP6*-deficient mutants are more attenuated in virulence in systemic models of *C. albicans* infections [28, 31, 46, 47]. Our results that *SAP4* and *SAP6* are expressed more frequently during oral and vaginal infections, compared with carriage ($P = .026$ and $P \leq .001$, respectively; tables 3 and 4) are the first to indicate that the *SAP4–SAP6* subfamily also is involved in human mucosal disease.

The expression of *C. albicans SAP7* mRNA in samples from both oral and vaginal infections extends the findings of our initial study, where the expression of this gene was first reported [33]. *SAP7* transcripts have since been detected during late stages of in vitro vaginal infections in an RHE model (M. Schaller, M. Bein, H. Korting, et al., personal communication) and transiently expressed in a murine model of oropharyngeal candidiasis [32]. Unexpectedly, *SAP7* expression correlates with both oral and vaginal infection rather than carriage ($P = .038$ and $P \leq .010$, respectively; tables 3 and 4), which is particularly noteworthy, because *SAP7* is the most divergent of all the *SAP* genes [19]. Moreover, *SAP7* transcripts are not detected in a systemic murine model of intraperitoneal infection [47], leading to the possibility that *SAP7* may be expressed only when *C. albicans* is located at mucosal surfaces. Although the functions of Sap7 are completely unknown, its apparent preferential expression during oral and vaginal infections tentatively supports some role for this proteinase during human mucosal infections.

Similarly, *SAP8* is expressed in *C. albicans* isolated from active oral and vaginal infections rather than carriage ($P = .011$ and $P = .001$, respectively; tables 3 and 4). This is the first study to associate *SAP8* expression with human mucosal infections and corroborates the work of Ripeau et al. [32], who showed transient expression of *SAP8* in a murine model of oropharyngeal candidiasis. Little is known about Sap8 except that it is most similar to the *SAP1–SAP3* subfamily (~50%) and is temperature regulated in vitro [38]. Thus, the mechanism by which Sap8 contributes to human mucosal infections is still unclear and requires more functional data based on *SAP8*–deficient mutants and biochemical characterization of the Sap8 protein.

PLB1 expression correlates with oral infection ($P \leq .001$; table 3) but not vaginal infection. *PLB1* transcripts also have been detected in the oral cavity of mice [32], and using *PLB1*-deficient mutants *PLB1* has been shown to contribute to an intragastric model [13] and an intravenous model of murine candidiasis [14]. However, our study is the first, to our knowledge, to specifically correlate *PLB1* expression with human oral infections, rather than carriage. Because *PLB1* transcripts were detected in only 14% of oral carriers (table 3), our results are in agreement with a previous study that demonstrated little phospholipase activity in *C. albicans* strains isolated from the oral cavity of carriers [15]. It is possible that *PLB1* expression may be regulated by factors that also regulate the expression of hyphal morphology [10]; however, since hyphae are probably present in the vast majority of the samples examined (concluded from the widespread expression of *SAP4–SAP6,* which are coordinately expressed with hyphal development in laboratory settings [20, 21]), the infrequent detection of *PLB1* transcripts in comparison with *SAP4–SAP6* transcripts suggests that *PLB1* expression in vivo does not correlate with the presence of hyphae. *PLB2* was expressed in a similar number of subjects as *PLB1* but was not associated with either oral or vaginal infections (tables 3 and 4). Although *PLB2* transcripts are found in many samples in which *PLB1* transcripts are absent, any major functional role for *PLB2* during colonization and infection in humans may be limited considering that disruption of the *PLB1* gene results in a loss of most of the detectable extracellular phospholipase activity of *C. albicans* [13].

No correlations between the expression of specific *SAP* or *PLB* genes with either the HIV status (table 6) or the different clinical forms of candidiasis were evident. With respect to the different clinical forms of infection, the number of samples in each group (see Materials and Methods) was not sufficiently large to draw any firm conclusions. However, the former observation was perhaps surprising because there is a selection of *C. albicans* strains in HIV infection that appears to be associated with increased Sap2 production [48, 49]. Although no qualitative differences in *SAP* gene expression were found in this study to support those conclusions, it is possible that quantitative differences between these groups existed, which could be addressed by real-time RT-PCR.

In summary, the present study together with our initial study [33] are the only reports to address the expression of the *C. albicans* hydrolytic enzyme genes in humans, and have demonstrated differences in *SAP1–SAP8* and *PLB1* and *PLB2* expression during colonization and active disease in both the oral cavity and vaginal lumen. However, because this is a point prevalence study, it remains to be confirmed whether repeated samples from the same subject would consistently give the same profile of gene expression. In addition, it is likely that *SAP* or *PLB* expression varies with time and with different stages and severity of diseases in the same subject, regardless of whether being colonized or infected with *C. albicans*. It is also possible that some of the described differences in *SAP* and *PLB* expression that did not quite reach statistical significance might reach the predefined significance level ($P = .05$) if more subjects are analyzed. Furthermore, it cannot be ruled out that some hydrolytic enzymes might be expressed at a very low level under the detection limit for the assay, which may nonetheless be biologically relevant or may contribute to the regulation of expression of other genes. Finally, *SAP* gene expression and protein secretion are tightly coupled for Sap2 [50], although there is no solid experimental evidence to demonstrate a similar phenomenon for the other Saps, or whether all *SAP* or *PLB* mRNAs are equally translated with the same efficiency. Nevertheless, although there is an absence of proteomic tools to discriminate fully among the *SAP* genes, the differential expression data described in this study are suggestive for a differential virulence role of the respective enzymes at the oral and vaginal sites and between colonization and infection.

Because *Candida* species are able to colonize and infect essentially every tissue in the human host, it may be crucial for the fungus to possess a number of similar but independently regulated, functionally distinct hydrolytic enzyme genes (which can be expressed under a variety of environmental conditions) to provide sufficient flexibility to survive and promote infection at different niche sites [17]. However, the precise regulatory factors that control or influence these expression profiles in vivo remain to be elucidated. With access to the recently completed genome sequence of *C. albicans* (http://www-sequence.stanford.edu/group/Candida) and with the development of DNA microarrays specifically for *Candida* research, our understanding of *C. albicans SAP* gene regulation and their interactions with the human host should soon rapidly increase.

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