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Expression of *Candida albicans SAP1* and *SAP2* in Experimental Vaginitis

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Several strains of *Candida albicans* were compared for their ability to cause vaginal infection in a rat model, and their vaginopathic potentials were correlated with the expression of two aspartyl proteinases genes (*SAP1* and *SAP2*) and adherence in vivo to the vaginal epithelium. Dot blot reactions and Northern blot analysis with RNA extracted from the vaginal fluid of rats infected with the highly vaginopathic strains H12 and 10261 demonstrated the expression of both *SAP1* and *SAP2* during the first week of infection. In contrast, neither gene was expressed during infection by a nonvaginopathic strain (N), even though the organism could be recovered during the first 24 h postinfection. A moderately vaginopathic strain (P) also expressed both genes, but the level of *SAP1* mRNA appeared to decrease prior to that of *SAP2*. Neither gene was expressed, even by the highly vaginopathic strains, after the first week of infection, concomitant with a decrease in the number of organisms recovered from the vaginas. Analysis of in vivo adherence showed that the nonvaginopathic strain (N) adhered to vaginal epithelial cells less readily than the highly vaginopathic strain (H12) and moderately vaginopathic strain (P). Thus, in addition to its inability to express *SAP1* and *SAP2* in vivo, the nonvaginopathic strain does not colonize host cells to the same extent as the other strains tested. Our results demonstrate the early in vivo expression of two aspartyl proteinase genes during candidal vaginitis and suggest its association with the establishment of a vaginal infection.

Mucosal candidiasis represents a frequent clinical problem, particularly in human immunodeficiency virus-infected patients who suffer from recurrent, severe forms of oro-esophageal and/or vaginal infections (19, 21, 22, 40). The mechanisms of pathogenesis of these infections have not been totally established, but the loss of host defense mechanisms is generally a prerequisite for infection to occur (4, 15, 33). In human immunodeficiency virus-infected subjects, in addition to the immunodepression of anticandidal T-cell-mediated immunity (15), a selection of particularly aggressive *Candida* strains has been reported (2, 7, 10). For *Candida albicans*, the most virulent and most frequently isolated species of *Candida*, the possible virulence attributes are dimorphism, adherence, enzyme secretion, phenotypic switching, antigen variation, and possession of complement-binding receptors (3, 7, 10, 12, 30, 40, 41). However, the actual contribution of each of these factors to the pathogenesis and severity of the disease awaits elucidation.

Secreted aspartyl proteinases (SAP) appear to be a virulence-associated attribute of *Candida* species. These enzymes can cleave several proteins which are important in host defenses, such as antibodies of both immunoglobulin G and A isotypes (35). Also, SAP may promote the colonization, penetration, and invasion by *C. albicans* (1, 9, 18, 28, 31, 32, 36).

Since the expression of these enzymes in systemic candidiasis may be less important then when the organism colonizes mucosal surfaces (5, 7, 36), we have been studying SAP secretion and activity in clinical and experimental vaginitis. With this infection, a clear correlation between the ability of strains to secrete aspartyl proteinase(s), both in vitro and in vivo, and to cause disease has been observed (5, 6, 9–11). Following this observation, we have now studied the expression of two aspartyl proteinase genes (*SAP1* and *SAP2*) in an experimental rat vaginitis model. We accomplished this objective using several strains of *C. albicans* which differ in virulence, according to previously published reports (8, 14, 34).

MATERIALS AND METHODS

Strains and growth conditions. Strain ATCC 10261 as well as clotrimazole-sensitive (H12) and derived clotrimazole-tolerant (P and N) strains of *C. albicans* were used throughout this study. Their sources, morphologies, and virulence characteristics have been described elsewhere (14, 34).

Escherichia coli was maintained in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl [pH 7.2]) supplemented with ampicillin (50 μg/ml). The details of the genomic constructs and cloned genes have been reported previously by Wright et al. (43) and Hube et al. (20). For aspartyl proteinase induction studies, the media used were YD, which contained 0.2% (wt/vol) yeast extract (Difco) and 2% (wt/vol) glucose, and YBD, which contained 0.2% yeast extract and 0.2% BSA (bovine serum albumin; Sigma Chemical Co., St. Louis, Mo.). Glucose (2%) was used to enhance proteinase secretion (9). BSA was added to a final concentration of 0.2% from a 2% filter-sterilized stock solution. YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used to grow cells for studies of the rat vaginitis model (see below). Sabouraud-dextrose agar (Difco) containing chloramphenicol (50 μg/ml) was used for the enumeration of CFU in vaginal fluid, as previously reported (8–10, 12).

Plasmids. pRW2 is a pUC19 derivative into which a *Pst*I-*Hin*dIII fragment of the *SAP2* gene was cloned, as described by Wright et al. (43). YRp7 contains a *Bam*HI-*Eco*RV fragment of the *SAP1* gene, as described by Hube et al. (20). pCa *Act1* is a pBR322 plasmid containing a *Sal*I fragment of the *C. albicans* β-actin gene (24).

Experimental vaginal infection. Ovariectomized, female, Wistar rats (80 to 100 g; Charles River, Wilmington, Mass.) were injected subcutaneously with 0.5 mg of estradiol benzoate (Benzatrone; Samil, Rome, Italy). Six days after the first estradiol treatment, the animals were inoculated intravaginally with 10^7 yeast cells per 0.1 ml of each strain tested. The inoculum was dispensed into the vaginal cavity through a syringe equipped with a multipurpose calibrated tip (Combitip; PBI, Milan, Italy).

The yeast cells had been previously grown in YDP at 28°C on a gyratory shaker (200 rpm), harvested by low-speed centrifugation (1,500 × *g*), washed, counted in a hemacytometer, and suspended to the required number in 0.86% NaCl.

Enumeration of *C. albicans* in the vaginal cavity was achieved by culturing 1-μl samples (by using a calibrated plastic loop, Disponoic; PBI), obtained from each animal every 2 days, on Sabouraud agar containing chloramphenicol. Cultures were incubated at 28°C for 48 to 72 h. Other samples were smeared on glass slides, fixed, and stained by the periodic acid-Schiff method for microscopic examination. For RNA detection, vaginal fluid was also collected by washing the vaginal cavity with 0.5 ml of a sterile saline solution.

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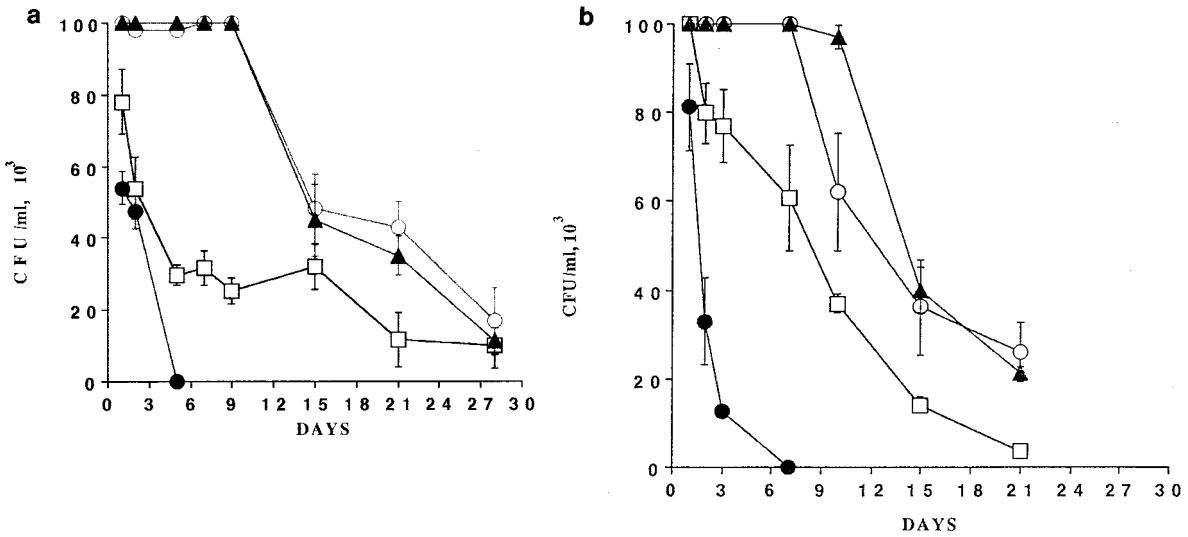

FIG. 1. Kinetics of vaginal infections and fungal clearance from the vaginas of oophorectomized, estrogen-treated rats challenged on day 0 with the indicated *C. albicans* strains, in two independent experiments (a and b). ▲, 10261; ○, H12; □, P; ●, N.

Adherence to vaginal epithelial cells. The adherence of *C. albicans* H12, N, and P to vaginal epithelial cells was measured at 4 and 24 h postinfection. For periodic acid-Schiff-stained smears of vaginal fluids prepared in triplicate, adherence of the organism was quantitated in 10 high-power fields; a total of 50 epithelial cells was counted for each strain per time interval on slides. Adherence was measured as the average number of blastoconidia per vaginal epithelial cell.

RNA isolation. RNA was isolated from yeast cells grown in YD or YBD. *C. albicans* cells were grown in YD and subcultured into fresh medium (to an optical density at 600 nm of 1.0). After 12 h of growth in a rotatory shaker at 30°C, BSA at a final concentration of 0.2% was added to some cultures. After 4 h of incubation with BSA, the cultures were centrifuged (1,500 × *g*), and the pellet was suspended in extraction buffer (0.1 M Tris [pH 7.5], 0.1 M LiCl, 0.01 M dithiothreitol). The cells were disrupted with glass beads (diameters of 425 to 600 μm; Sigma).

Total cellular RNA was isolated by sequential phenol-chloroform extraction and precipitated with 100% ethanol, according to established procedures (20, 24). The vaginal fluids taken from rats at different time intervals during the infection were centrifuged, and the cellular pellets were resuspended in extraction buffer. RNA was isolated as described above for in vitro cultures. Vaginal fluids from uninfected estrogen-treated rats were used as controls.

Dot blot assay. A dot blot assay for mRNA detection was performed by using the Bio-Dot microfiltration apparatus (Bio-Rad, Richmond, Calif.) and nylon membranes (Dupont, Boston, Mass.) according to the manufacturer's instructions (GeneScreen; Dupont). Briefly, filters were washed in TE buffer (10 mM Tris-HCl, 1 mM EDTA) and then placed in the apparatus. TE (100 μl) was added to the wells, and then 35 μl of each RNA sample was added. The samples were filtered by gravity for 2 h, and then 100 μl of TE was added sequentially, three times. Subsequently, each membrane was removed from the apparatus, fixed with UV light, baked at 80°C for 1 h, and hybridized with DNA probes as described for Northern (RNA) blots (see below).

Northern blot analysis. Total cellular RNA (40 μg) was ethanol precipitated prior to resuspension in loading buffer containing 30 μg of ethidium bromide per ml. The samples were heated at 60°C for 15 min. Samples were fractionated by electrophoresis in 1% agarose–2.2 M formaldehyde gel with 1× MOPS (morpholinepropanesulfonic acid) as a running buffer (5× MOPS is 0.2 M MOPS, 50 mM sodium acetate, and 5 mM EDTA [pH 7]). The agarose gel was soaked in distilled water for 5 min, incubated for 30 min in 0.5 N NaOH and for 30 min in 0.5 M Tris-HCl (pH 7.5), and then transferred to a nylon membrane (Dupont) by capillary blotting in 10× SSC solution (20× SSC is 3 M NaCl plus 0.3 M sodium citrate [pH 7]). Filters were UV cross-linked, baked at 80°C for 2 h, and prehybridized at 42°C for 4 h in a solution containing 5× SSPE (20× SSPE is 3 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, and 0.02 M EDTA [pH 7.4]), 50% formamide, 5× Denhardt's (50× Denhardt's is 1% Ficoll, 1% polyvinylpyrrolidone, and 1% BSA), 10% dextran sulfate, and 1% sodium dodecyl sulfate (26). Hybridization was done for 16 to 20 h in the same buffer containing ^{32}P-random-primed-labelled probes (Prime-it Random Primer Extension Kit; Stratagene, La Jolla, Calif.). The activity of each probe was approximately 10^6 cpm. The membranes were washed sequentially in 2× SSPE (two washes at room temperature for 20 min) and 0.2× SSPE at 60°C for 20 min and subjected to autoradiography (−70°C for 20 h) with reflection film (Dupont).

Statistical analyses. Differences in vaginal CFU and adherence to vaginal cells were statistically assessed by analysis of total variance and the Mann-Whitney U test.

RESULTS

Experimental vaginitis model. The ability of mutant strains P and N of *C. albicans* to colonize the vaginas of oophorectomized estrogen-treated rats was compared with that of the parental strain (*C. albicans* H12) as well as to that of a previously established vaginopathic strain (ATCC 10261) from our stock collection. The vaginal infection was assessed in two independent experiments involving five and four rats, respectively, infected with each strain. The results of the two experiments are shown in Fig. 1a and b, respectively. The kinetics of vaginal infection by *C. albicans* H12 was similar to those of other vaginopathic strains previously studied (8–12). Initial counts ($\geq 10^5$ CFU) were observed for approximately 1 week postinfection and were followed by a lower but persistent number of CFU until about 4 weeks postchallenge (Fig. 1a). The overall time course profile of CFUs for this strain was similar to that for strain 10261. Figure 1 also shows that the cell counts for strain P were lower than those for the parent strain, especially during the first week postchallenge. However, like H12, this strain was still present in the vagina at 21 to 27 days (Fig. 1a). In contrast, the number of CFUs of strain N rapidly decreased over time, and the organism was completely cleared from the rat vaginas by the end of the first week (Fig. 1). This mutant thus behaved like the nonvaginopathic yeasts documented elsewhere (8).

Vaginal scrappings from rats infected with *C. albicans* H12, N, and P for 4 h showed the typical yeast form for all strains (Fig. 2a to c). One day after challenge (Fig. 2d to f), each of the three strains developed hyphal filaments. Subsequently, the hyphal elements persisted until elimination occurred. In keeping with vaginal CFU counts, cells of strain N were not observed in vaginal scrappings taken on day 7 (Fig. 2g to i). Thus, the intravaginal morphology pattern exhibited by each of the strains was similar to those of all other previously tested germinative strains of the fungus in this animal model (8–10, 12).

Adherence to vaginal epithelial cells in vivo. The capacity of *C. albicans* H12, P, and N to adhere to vaginal epithelial cells

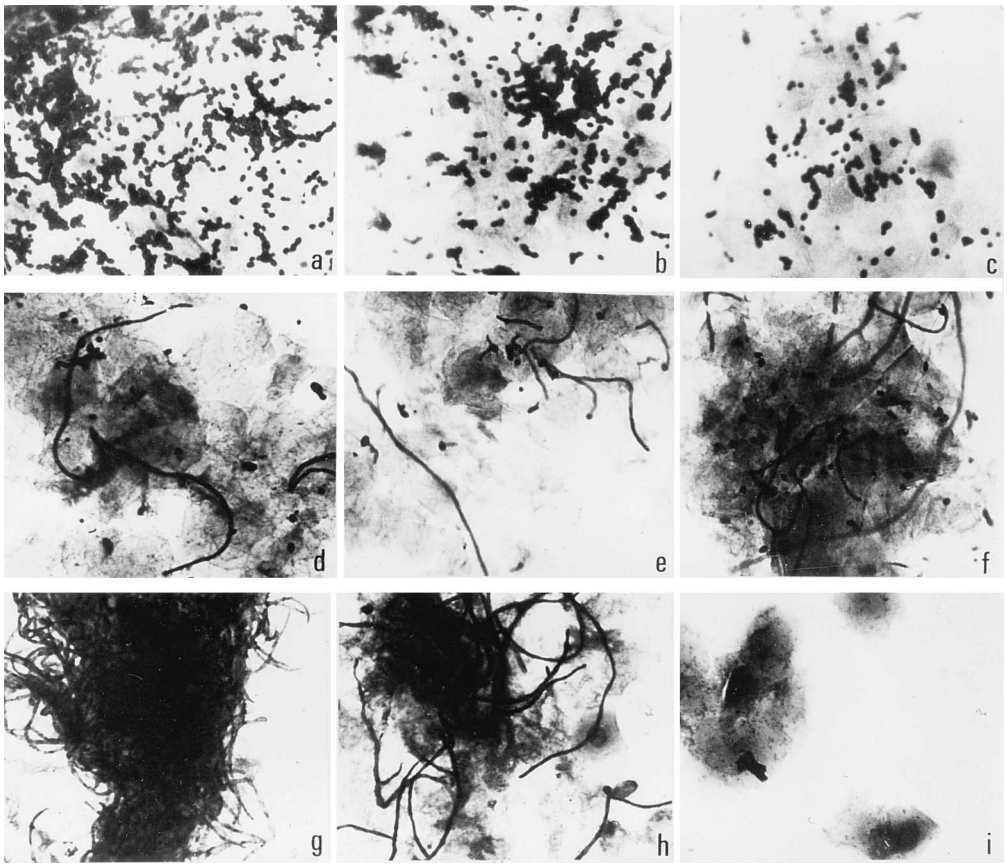

FIG. 2. Microscopic appearance of vaginal scrapings from rats infected on day 0 with strain H12 (a, d, and g), P (b, e, and h), or N (c, f, and i) of *C. albicans* taken at 4 h (a to c), 24 h (d to f), or 7 days (g to i) after challenge. The smears were stained by the periodic acid-Schiff method. Magnification, ×430.

was also assessed during rat vaginitis. Table 1 shows the results of an experiment in which the adherence to vaginal cells was determined at 4 and 24 h postinfection. The data show that strains H12 and P were equally adherent, whereas the adherence of strain N was reduced by approximately 58 and 44% at 4 and 24 h postinfection, respectively, compared with the adherence of strains H12 and P.

Aspartyl proteinase production in vitro. To determine the sensitivity of the dot blot assay in determining the expression of *SAP1* and *SAP2* transcripts, different amounts of purified RNA from in vitro cultures of *C. albicans* were hybridized with actin and *SAP1* probes (Fig. 3). The results showed that the assay could detect transcripts in 1 μg of total RNA. In Fig. 3, hybridization with the actin probe in all RNA samples is shown, whereas RNA only from BSA-induced cells gave a positive signal with the *SAP1* proteinase probe. The specificity of the hybridization in the bio-dot assay was subsequently confirmed by Northern blot for *SAP1* and also for the *SAP2* transcript (see below).

Expression of *SAP1* and *SAP2* during vaginal infection by different *C. albicans* strains. To monitor the expression of

TABLE 1. In vivo adherence of *C. albicans* strains (H12, N, and P) to vaginal epithelial cells

Strain	Avg no. of adherent organisms/vaginal cell postinfection[a]	
	4 h	24 h
H12	25.0 ± 2.4	27.0 ± 3.4
N	10.7 ± 1.7[b]	15.0 ± 4.4[b]
P	20.8 ± 1.7	30.0 ± 3.0

[a] Adherence is expressed as the average number of adherent *C. albicans* cells per vaginal epithelial cell ± standard error. As many as 10 high-power fields per strain for each time interval were examined so that adherence could be assessed for a total of 50 vaginal epithelial cells. Results were obtained from vaginal samples prepared in triplicate.

[b] Analysis of total variance (F ratio) showed significance (*P* values) of <0.01 and <0.05 for the 4- and 24-h assays, respectively. The Mann-Whitney U test gave a *P* value of <0.01 (two-tailed) for the comparisons between strains N and P or H12 at 4 h and a *P* value of <0.05 (two-tailed) for the comparison between strains N and P or H12 at 24 h.

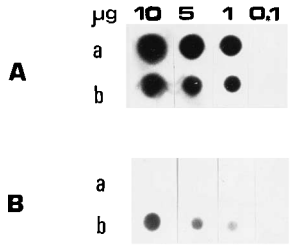

FIG. 3. Dot blot for mRNA detection in cultures of *C. albicans*. Various amounts of purified RNA (indicated above the lanes) from cultures of *C. albicans* 10261 (noninduced, rows a) and BSA-induced (rows b) were added to the membrane in the bio-dot microfiltration apparatus and then hybridized with the actin (A) or *SAP1* (B) probe. For methodological details and definitions, see the text.

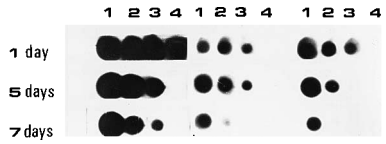

FIG. 4. Dot blot for *SAP1* and *SAP2* mRNA detection during infection. Pelleted vaginal fluids taken from rats at different time intervals (1, 5, or 7 days) after challenge with *C. albicans* 10261 (lanes 1), H12 (lanes 2), P (lanes 3), and N (lanes 4) were hybridized with actin (left panel), *SAP2* (center panel), or *SAP1* (right panel).

SAP1 and *SAP2* during vaginal infection, we extracted RNA from pelleted vaginal fluids collected at different times after infection by various strains of *C. albicans*. In these experiments, the high proteinase producer, vaginopathic strain ATCC 10261, was also used as a reference strain. The results of RNA hybridizations following infection by strain 10261 showed that both *SAP1* and *SAP2* were expressed during the first week of infection (Fig. 4). When the same approach was used to detect proteinase expression by other strains of *C. albicans*, *SAP1* transcription was detected in strains H12 and P at day 1 after vaginal infection, whereas the *SAP1* gene was detected at day 5 only in strain H12. In comparison, *SAP2* expression was detected at both 1 and 5 days postinfection by strains H12 and P. However, by day 14 postchallenge, no transcripts were detected as a decrease in the vaginal burden of *C. albicans* occurred (data not shown). Concomitantly, a loss of the actin gene signal was also observed, along with a decrease in vaginal burden of the organism (Fig. 4).

In contrast, the dot blot reactions with the RNA extracted from vaginal fluids of rats infected with the nonvaginopathic mutant strain N were negative with both proteinase probes (Fig. 4), even on day 1, when vaginal CFU counts, microscopy, and actin transcription documented the presence of N cells in the vaginas. Rapid elimination of N strain cells from the vagina (Fig. 1) paralleled the lack of actin message. Dot blot assays of vaginal fluids from estrogen-treated, noninfected animals were negative when RNA was probed with actin or *SAP1* and *SAP2* (data not shown).

Northern blot analyses. The specificity of the dot blot reaction in detecting *SAP1* and *SAP2* message was confirmed by Northern analysis (Fig. 5). RNA extracted from in vitro cultures and from vaginal fluids of *C. albicans*-infected rats which gave positive reactions by dot blot was analyzed after electrophoresis, transferred to nylon membranes, and hybridized with actin and proteinase probes. The RNA isolated from *C. albicans* 10261 grown in vitro under conditions of proteinase induction or noninduction or taken from vaginal fluids of infected rats contained a 1.4-kb transcript which hybridized to the actin probe (Fig. 5a). On the other hand, the Northern blots with *SAP1* or *SAP2* as a probe were positive with RNA only from *C. albicans* grown in the proteinase-induction medium (BSA + YD) (Fig. 5a) and with cells isolated from vaginal fluids of rats at 5 days postinfection. The 1.7-kb *SAP1* and 1.9-kb *SAP2* transcripts observed are in agreement with the data reported by Wright et al. (43).

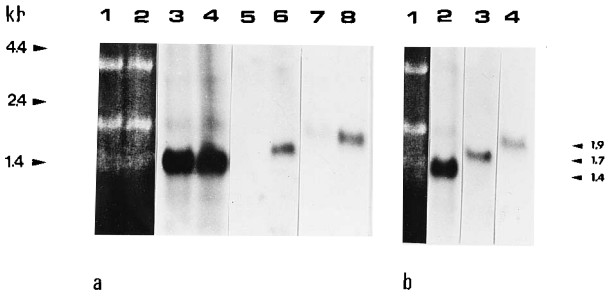

FIG. 5. (a) Northern blots with RNA isolated from *C. albicans* 10261 grown in vitro under conditions of noninduction (lanes 1, 3, 5, and 7) or induction of proteinase (lanes 2, 4, 6, and 8). RNA was separated by electrophoresis (ethidium bromide-stained gel; lanes 1 and 2) and hybridized with actin (lanes 3 and 4), *SAP2* (lanes 5 and 6), or *SAP1* (lanes 7 and 8). (b) RNA purified from vaginal fluid of rats infected with *C. albicans* 10261 was separated by electrophoresis (lane 1) and hybridized with actin (lane 2), *SAP2* (lane 3), or *SAP1* (lane 4). The positions of the transcripts (sizes in kilobases) are indicated on the left and right.

DISCUSSION

An understanding of the pathogenesis of candidiasis has been obtained in part by studies which define the virulence characteristics of *C. albicans*, the most virulent species of *Candida* (7, 30). Several factors may be involved in virulence, among which the aspartyl proteinases have been studied most extensively. They are represented by well-defined proteins with known enzymatic properties that are encoded by different, although closely related, genes, designated *SAP1* to *SAP7* (16, 20, 23, 25, 27, 28, 32, 42, 43). There is evidence that these genes are produced during infection (1, 9, 11, 36, 37); however, it has not been established that their expression is essential for disease development. In particular, it is unclear which enzyme is produced by a particular strain, in which type of disease (mucosal or invasive) the enzyme(s) is contributory, and when during the course of infection the SAP are produced. Equally important, the host target protein(s) of SAP is not completely known. The SAP proteins may also be differentially expressed. For instance, Soll and collaborators (29, 41) found that *SAP1* is transcribed by the opaque but not the white *C. albicans* phenotype; they suggest that the transcription of *SAP1* is controlled by phenotypic switching.

Most indirect evidence which links proteinase production and virulence in *C. albicans* has been obtained from systemic infection models (23). However, because SAP requires an acid pH for activity, a more relevant environment might be the skin or mucosal surfaces such as the vaginal mucosa (6, 7). In fact, secretion of active proteinase and tissue degradation have been amply demonstrated in *Candida* vulvovaginal and skin infections (36), and a strong correlation between clinical and experimental vaginitis and in vitro or in vivo proteinase secretion has been found (6, 9). Finally, anti-proteinase antibodies might contribute to protection from experimental candidal vaginitis (5).

In this study, we have focused on the contribution of *C. albicans SAP1* and *SAP2* to experimental vaginitis by using strains of *C. albicans* with similar growth rates in vitro but with different morphologies of hyphal forms and virulence (14). In this study the parent strain (H12) gave the most prolonged vaginal infection, comparable to that observed previously for strain 10261 (34). H12 was also more virulent than strains N and P in the systemic model of candidiasis (14). Of these two strains, strain P showed less vaginopathic potential than H12 but still caused a persistent vaginal infection, while strain N was rapidly cleared from the vagina, as is usually seen with avirulent *C. albicans* or *Saccharomyces cerevisiae* (5, 8, 12).

Interestingly, the difference in virulence between P and N in the vaginitis model is opposite that reported for the two strains in a murine systemic infection, in which N was more virulent than P (14), similar to a previous observation for a nongerminative strain of *C. albicans* (CA-2) (8, 34). Strain N was also

less adhesive to vaginal cells, but the adherence differences in general did not appear to influence the outcome of the vaginal infection, as H12 and P were similarly adhesive but not equally vaginopathic. On the other hand, if the virulence of *C. albicans* is multifactoral, then one could predict requirements for adherence and proteinase production in influencing infectivity; i.e., P could adhere but not express SAP to the same level as do parent cells, while N lacked both determinants and, consequently, had the lowest vaginopathic capabilities. Of interest is our observation that all strains converted to a hyphal morphology; therefore, the avirulence of N could not be associated with an inability to undergo morphogenesis in the vaginal canal.

Our data clearly indicate that both *SAP1* and *SAP2* are expressed early in the infection process by *C. albicans* 10261 and H12. Apparently, the two genes may be expressed less by strain P, with intermediate vaginal virulence, while the nonvaginopathic strain (N) did not express detectable levels of either gene, at any time examined. Northern analysis of RNA isolated from cells in the vaginal fluids of infected rats and hybridized with actin and proteinase probes showed strong positive reactions with transcripts similar in size to those reported in other studies (20, 43). Moreover, the bands detected with RNA extracted from the vaginas of infected rats, after hybridization with each probe, were the same as those obtained with RNA isolated from *C. albicans* grown in vitro and induced with BSA. In contrast, the reactions with RNA extracted from noninduced in vitro cultures as well as from vaginal fluids of noninfected rats were negative with all probes. Thus, on the basis of the transcript size and the specificity of induction, the Northern analysis confirmed that the proteinase messages were indeed expressed during infection.

As precise quantitation of *SAP1* and *SAP2* mRNA was not done in this study, no conclusions can be drawn from different intensities of the signals in the dot blot reaction. Nonetheless, the data indicate that transcripts of both genes tends to disappear with clearance of the organism from the vagina. Also, *SAP1* expression by P seems to decrease prior to *SAP2* expression, and this seems to occur well before the elimination of *C. albicans* from the vagina.

It is not clear whether the lack of expression of either SAP by the nonvaginopathic (N) mutant is a cause or a consequence of decreased vaginal counts. Apparently, proteinase genes are no longer expressed after the first week postinfection, even when the highly vaginopathic strains are cleared from the vagina. It might be relevant, however, that no proteinase expression by the low vaginopathic N cells was detected even at day 1 postinfection when the cell burden (and actin message) was apparently the same as the burden in those vaginopathic strains (P and H12) expressing elevated levels of proteinase. The interpretation of our findings needs to be weighed with our observation that strain N produces proteinase to some extent in vitro, although in an amount less than the amounts produced by strains 10621, H12, and P. This strain might express another gene among the SAP family not revealed by the *SAP* probes used in this study. If so, those *SAP* genes may or may not be relevant to vaginal infection. Our data demonstrate that the 43- to 44-kDa protein, which displays proteinase activity and has been identified serologically in experimental vaginitis (9, 10, 34, 36), is probably the result of (at least) two *SAP* genes. Our results make it very feasible to study the expression of null *SAP1* or *SAP2* mutants in a model of infection in which aspartyl proteinase is likely to play a role in disease development.

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