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Candida albicans-Secreted Aspartic Proteinases Modify the Epithelial Cytokine Response in an In Vitro Model of Vaginal Candidiasis

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Secreted aspartyl proteinases (Saps) are important virulence factors of *Candida albicans* during mucosal and disseminated infections and may also contribute to the induction of an inflammatory host immune response. We used a model of vaginal candidiasis based on reconstituted human vaginal epithelium (RHVE) to study the epithelial cytokine response induced by *C. albicans*. In order to study the impact of the overall proteolytic activity and of distinct Sap isoenzymes, we studied the effect of the proteinase inhibitor pepstatin A on the immune response and compared the cytokine expression pattern induced by the wild-type strain SC5314 with the pattern induced by Sap-deficient mutants. Infection of RHVE with the *C. albicans* wild-type strain induced strong interleukin 1α (IL-1α), IL-1β, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor, gamma interferon, and tumor necrosis factor alpha responses in comparison with cytokine expression in noninfected tissue. Addition of the aspartyl proteinase inhibitor pepstatin A strongly reduced the cytokine response of RHVE. Furthermore, *SAP*-null mutants lacking either *SAP1* or *SAP2* caused reduced tissue damage and had a significantly reduced potential to stimulate cytokine expression. In contrast, the vaginopathic and cytokine-inducing potential of mutants lacking *SAP4* to *SAP6* was similar to that of the wild-type strain. These data show that the potential of specific Saps to cause tissue damage correlates with an epithelium-induced proinflammatory cytokine response, which may be crucial in controlling and managing *C. albicans* infections at the vaginal mucosa in vivo.

A characteristic feature of vaginal candidiasis is a chronic inflammation of the mucosa. *Candida albicans*, normally a commensal habitant of mucosal surfaces, is the most frequent cause of this type of infection (9). In a recent study using an in vitro vaginal model, we have shown that a distinct set of *SAP* genes is expressed during infection of vaginal epithelial tissue and that secreted aspartic proteinase 1 (Sap1) and Sap2 in particular, but not Sap3 to Sap6, seem to contribute to tissue damage (13). Furthermore, evidence for the expression of *SAP1* and *SAP2* and their dominant role in an experimental rat vaginitis model was reported previously (3) and supports the view that these Saps act as key virulence factors for this type of infection (10). Previous studies have shown that *C. albicans* has the ability to induce an epithelial cytokine response (14, 15) and that Saps contribute to an inflammatory mucosal response by the activation of interleukin 1β (IL-1β) (1). Therefore, we predicted that Saps may have a distinct role in the induction of other proinflammatory and chemoattractive cytokines. We studied the epithelial expression of cytokines using the *C. albicans* wild-type strain SC5314, Sap-deficient mutants, and the proteinase inhibitor pepstatin A and compared the pattern and level of cytokine expression to those for noninfected tissue. The cytokine response was studied in an in vitro model of vaginal candidiasis based on reconstituted human vaginal epithelium (RHVE) by quantitative reverse transcription-PCR (RT-PCR) and fluorescence-activated cell sorter (FACS) analyses. Our data suggest that distinct Saps play a crucial role in the induction of a chemokine response during *C. albicans* vaginal infections.

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MATERIALS AND METHODS

***Candida* strains.** The clinical *C. albicans* wild-type strain SC5314 (5), the *SAP*-null single-mutant *sap1*, *sap2*, and *sap3* strains (6), the *sap4* to *sap6* triple mutant (12), the *SAP2* reconstituted strain M40 (7), and a *SAP1* reconstituted strain (13) were used in the study. For further comparisons, we also used the Ura$^-$ *sap1* mutant carrying the empty pCIp10 plasmid (13).

Culture media and growth conditions. For the infection of the reconstituted vaginal epithelium, inocula were prepared by culturing *C. albicans* for 24 h at 37°C on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.). Cells were washed three times in 0.9% NaCl, and approximately 2×10^6 cells were suspended in 10 ml YPG (1% yeast extract, 2% peptone, 2% glucose) medium (Difco). The suspension was cultured for 16 h at 25°C with orbital shaking. A suspension of 4×10^6 cells from this culture was incubated with shaking in fresh medium for a further 24 h at 37°C. After three washes with phosphate-buffered saline (PBS), the final inoculum was then adjusted to the desired density with PBS solution.

RHVE and model of vaginal candidiasis. The human epithelium for the in vitro model of vaginal candidiasis was supplied by Skinethic Laboratory (Nice, France). It was obtained by culturing transformed human keratinocytes of the cell line A431, derived from a vulval epidermoid carcinoma (11). Keratinocytes were incubated in serum-free conditions in a defined medium, based on the MCDB-153 medium (Clonetics, San Diego, Calif.), containing 5 μg/ml insulin, on a 0.5-cm^2 microporous polycarbonate filter for 7 days at the air-liquid interface in six-well plates. A431 cells form a three-dimensional epithelial tissue resembling human vaginal mucosa in vivo. The in vitro model and all culture media were prepared without antibiotics and antimycotics.

Triplicate infection experiments were performed for each *C. albicans* strain. RHVE was infected with 2×10^6 *C. albicans* cells of the SC5314 parental strain, the *sap1*, *sap2*, *sap3*, and *sap4* to *sap6* mutants, and the *SAP1* and *SAP2* revertant strains in 50 μl PBS for 12 and 24 h. Controls contained 50 μl PBS alone.

To investigate the mechanism of cytokine stimulation, time course experiments were repeated with heat-inactivated *C. albicans* SC5314 cells. Killed yeast

cells and killed serum-induced hyphae were prepared by heat inactivation at 90°C for 30 min. Plating on Sabouraud's agar confirmed that no *Candida* cells survived this treatment.

For inhibition of Sap activity, pepstatin A (Sigma, Deisenhofen, Germany) was dissolved in methanol and added to 50 μl PBS containing 2×10^6 *C. albicans* SC5314 cells at a final concentration of 15 μM pepstatin A. The same concentration of the inhibitor was also applied for the maintenance media (1 ml) of the epithelial cultures (final methanol concentration, 0.7%). To exclude an inhibitory effect of the proteinase inhibitor on cytokine regulation, we analyzed controls containing noninfected RHVE stimulated with 5 μg tumor necrosis factor alpha (TNF-α) (Sigma) in 50 μl PBS in the presence and absence of pepstatin A. All tissue cultures were incubated at 37°C with 5% CO_2 at 100% humidity for 12 and 24 h.

Three independent experiments were performed. For controls, tissues were inoculated with 50 μl PBS only.

RNA isolation and cDNA synthesis (RT). For the detection of mRNA, samples were collected and shock-frozen in liquid nitrogen. Total RNA from shock-frozen samples was isolated using RNAPureTM (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. RNA concentrations were measured by absorbance at 260 nm. cDNA synthesis was performed using Superscript II reverse transcriptase (Gibco, Eggenstein, Germany) following the manufacturer's instructions.

Quantitative RT-PCR. To quantify cytokine gene expression, 20 ng of cDNA was used to amplify the transcripts of selected genes in "real time" in a LightCycler (Roche, Grenzach-Wyhlen, Germany) with the "FastStart DNA Master SYBR Green I kit" (Roche) at 3 mM Mg^{2+} final concentration. Data were monitored and analyzed with LightCycler software, version 3.5. Annealing temperature and elongation time were optimized for each primer pair. Primer pairs used to amplify genes encoding aldolase, IL-1α, IL-1β, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFN-γ), and TNF-α have been published recently (14). The corresponding DNA amplicon for each primer pair was serially diluted (6 log units). Aliquots of these dilution series were used to generate standard curves in the same LightCycler PCR run that analyzed the cDNAs studied. Absolute relative quantification of these cDNAs was achieved with the LightCycler software.

Quantification of cytokine secretion by epithelial cells infected with *C. albicans*. Epithelial tissues were infected either with the PBS-washed *C. albicans* wild-type strain in the presence and absence of 15 μM pepstatin A or with Sap-deficient mutants and revertants or were treated with PBS only. After 12 h and 24 h, samples of the maintenance medium surrounding the epithelial tissues were collected and centrifuged to determine the cytokines secreted into the supernatant. Amounts of IL-1β, IL-6, IL-8, IL-10, IFN-γ, and TNF-α were measured by FACS analysis. Flow cytometric data were acquired on a Becton Dickinson FACScan using the Human Inflammation Kit (Becton Dickinson, Heidelberg, Germany). Data were analyzed using Cellquest software (Becton Dickinson).

Statistical analysis. The statistical significance of expression levels was determined using the least-significant-difference test. A P value of 0.05 or less was considered significant.

RESULTS

Basal expression levels of cytokine mRNA. Basal expression levels of cytokine mRNA in vaginal keratinocytes in the absence of *C. albicans* were monitored 12 and 24 h after incubation with PBS only. Quantitative RT-PCR of these samples demonstrated constant levels of mRNA expression for aldolase and for the cytokines IL-1α, IL-1β, IL-8, GM-CSF, IFN-γ, and TNF-α (Fig. 1).

The viability of *C. albicans* is essential for the induction of cytokine mRNA expression. Real-time RT-PCR demonstrated a significant increase in gene expression for IL-1α, IL-1β, IL-8, GM-CSF, IFN-γ, and TNF-α 12 h and 24 h after stimulation with viable wild-type *C. albicans* in comparison with PBS-treated keratinocytes. To analyze whether the induction requires the physiological activity of the fungus, we repeated the experiment with killed *C. albicans* hyphae and killed yeast cells. Stimulation with either growth form of heat-inactivated *C. albicans* cells failed to induce significant cytokine up-regulation at both time points (Fig. 1).

The proteinase inhibitor pepstatin A abolishes the induction of cytokine mRNA expression. One possible effector of cytokine induction may be the extracellular proteinases of *C. albicans*. To investigate whether Sap activity contributed to the expression of cytokines during the in vitro infection of vaginal epithelium, we analyzed the effect of the proteinase inhibitor pepstatin A. The inhibitor significantly reduced the gene expression levels of IL-1β, IL-8, GM-CSF, IFN-γ, and TNF-α 12 h and 24 h after infection (Fig. 1).

We concluded that the repression of cytokine induction was due to the inhibition of either epithelial or fungal aspartyl proteinases. To exclude the possibility that the proteinase inhibitor had any effect on epithelial cells which in turn reduced cytokine expression, we stimulated the RHVE with recombinant human TNF-α in the presence and absence of pepstatin A. Cytokine expression was clearly induced by TNF-α but was not significantly influenced by pepstatin A (Fig. 1).

Specific Saps affect cytokine mRNA expression. The fact that the aspartyl proteinase inhibitor reduced RHVE cytokine expression during infection indicated that Saps are likely to be involved in this process. We analyzed the contribution of distinct Sap isoenzymes by studying the cytokine profile elicited by *sap1*, *sap2*, and *sap3* single mutants and a *sap4* to *sap6* triple mutant in the RHVE model (Fig. 2). Only mutants lacking *SAP1* and *SAP2*, which were both attenuated in their potential to cause tissue damage, had a strongly reduced ability to induce the gene expression of IL-1β, IL-8, GM-CSF, IFN-γ, and TNF-α compared with the wild-type strain SC5314 (Fig. 2). Reintroduction of plasmids (pCIp10) carrying the *SAP1* and *SAP2* genes into the Ura^- *sap1* and *sap2* mutants reversed this effect for IL-1β, IL-8, and GM-CSF (Fig. 3). A mutant carrying only the empty plasmid pCIp10 behaved like the mutants carrying the disruption cassette (not shown).

Mutants lacking *SAP3* or *SAP4* to *SAP6* stimulated expression of IL-1α, IL-1β, IFN-γ, and TNF-α to a similar extent as the wild-type cells. In contrast, the *sap4* to *sap6* triple mutant stimulated stronger IL-8 and GM-CSF mRNA levels than did SC5314 (Fig. 2).

FACS analysis confirmed proteinase-dependent secretion of cytokines during experimental vaginal *C. albicans* infection. Real-time RT-PCR indicated that *C. albicans* induced the up-regulation of specific cytokines during infection of vaginal epithelium. To confirm that these cytokines are secreted into the surrounding maintenance medium on the protein level, we used FACS analysis (Fig. 4). Noninfected mucosae secreted only low levels of IL-10, IFN-γ, and TNF-α and moderate levels of IL-1β and IL-6 but considerable amounts of IL-8. In response to infection with *C. albicans* wild-type cells, the epithelial cells produced significant, increasing amounts of IL-1β, IL-6, IL-8, IL-10, IFN-γ, and TNF-α. Addition of pepstatin A significantly decreased IL-1β, IL-6, IL-8, IL-10, and TNF-α production (Fig. 4).

The cytokine protein levels also differed depending on the *SAP* mutant. Mutants lacking *SAP3* or *SAP4* to *SAP6* induced secretion of IL-1β, IL-6, IL-8, IL-10, and TNF-α at levels similar to or higher than those with the wild-type strain. In contrast, production of these cytokines was clearly reduced in tissues infected with the *sap1* or *sap2* mutant (Fig. 5). Rein-

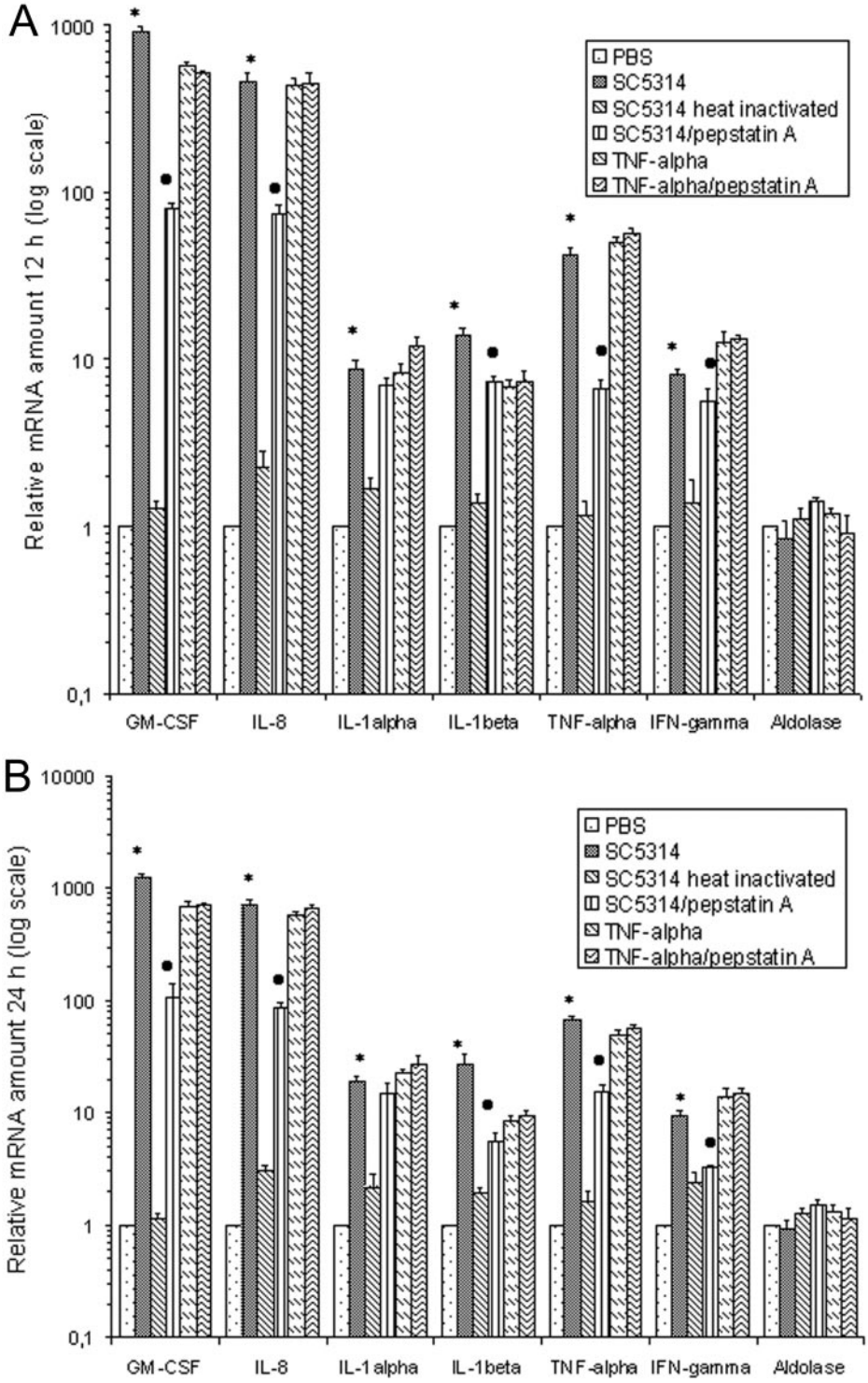

FIG. 1. Epithelial cytokine gene expression in the RHVE model 12 h (A) and 24 h (B) after stimulation with heat-inactivated or viable *C. albicans* SC5314 cells or 200 U/ml TNF-α in the presence or absence of pepstatin A. Quantitative analysis in triplicate of GM-CSF, IL-8, IL-1α, IL-1β, TNF-α, IFN-γ, and aldolase mRNA levels relative to those in noninfected tissue (PBS). Expression values of cytokines (means ± standard deviations) stimulated by *Candida* or TNF-α were related to the levels in noninfected epithelia 12 h after incubation with PBS (taken as 1.0) and separated into classes by the least-significant-difference test ($P < 0.05$). Asterisks indicate significantly higher levels of cytokine expression induced by viable *C. albicans* cells than by heat-inactivated *Candida* cells or PBS treatment. Solid circles indicate significant inhibition of cytokine expression by pepstatin A. TNF-α-induced cytokine levels in the presence of pepstatin were not significantly reduced from those in the presence of TNF-α alone. Combined data from three independent experiments performed in triplicate are shown.

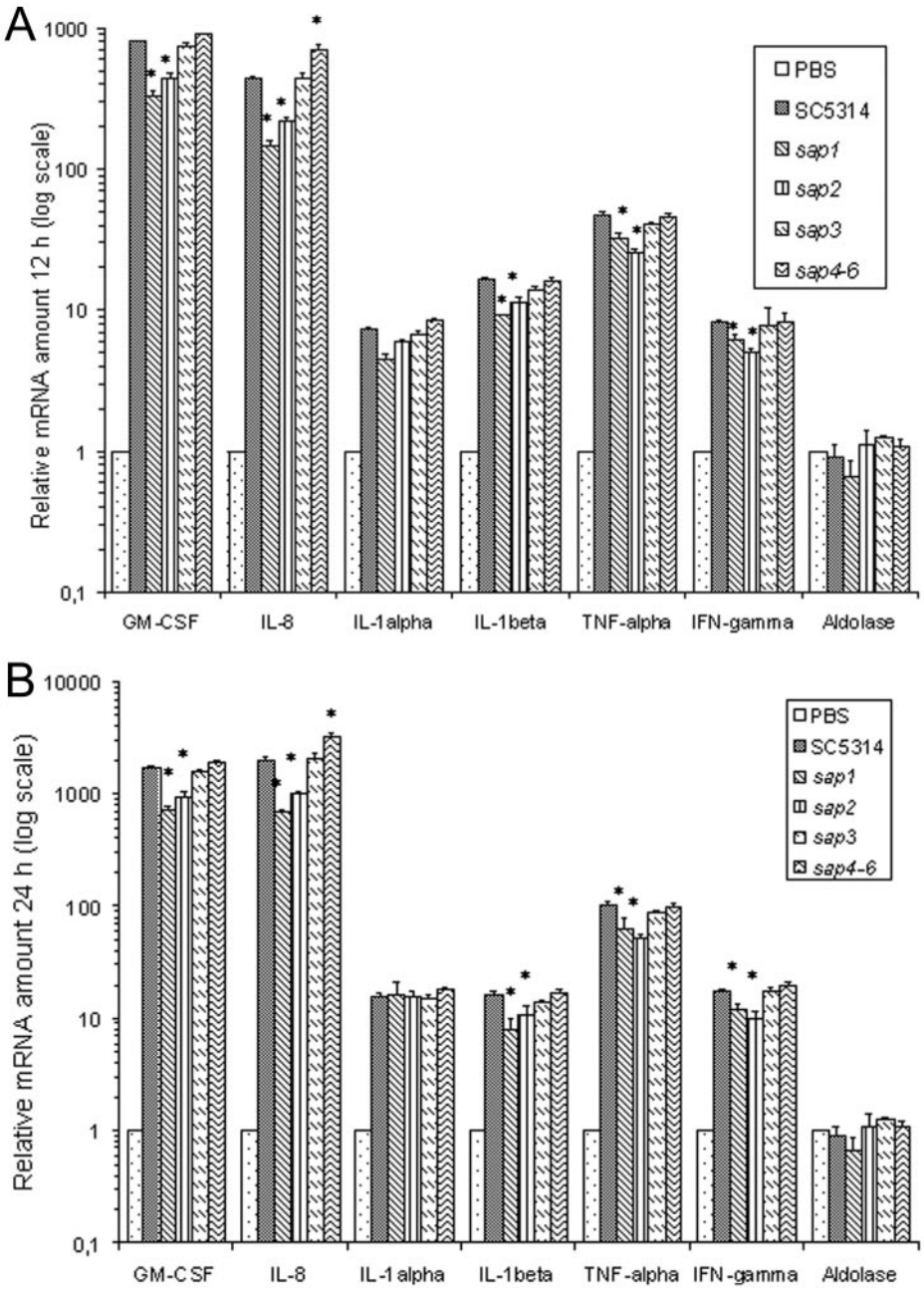

FIG. 2. Induction of epithelial cytokine gene expression 12 h (A) and 24 h (B) after infection of the RHVE with SC5314 wild-type cells or *sap1*, *sap2*, *sap3*, or *sap4* to *sap6* mutant cells. Asterisks indicate cytokine expression levels significantly different ($P < 0.05$) from the response to the wild-type strain SC5314. Combined data from three independent experiments performed in triplicate are shown.

troduction of the *SAP1* and *SAP2* genes into the *sap1* and *sap2* mutants, respectively, reconstituted the levels of IL-8 and IL-6 secreted by the epithelial tissue (Fig. 5).

DISCUSSION

Both host and fungal attributes affect the development of mucosal *C. albicans* infections. In recent years some studies have explored the host response during mucosal infections by studying the cytokine expression of epithelial cells stimulated by *C. albicans* (14, 15). On the pathogen side, the role of virulence attributes for localized candidiasis has been intensively investigated (reviewed in reference 10). However, it is not clear which fungal factors can directly affect the epithelial immune response of the host and whether specific virulence factors such as the *C. albicans* secreted proteinases can contribute to the development of an inflammatory reaction at the site of infection. For example, Beausejour et al. (1) demonstrated a direct activation of the IL-1β precursor by *C. albicans* Sap2. We postulated that in addition to the activation of certain cytokine precursors, Saps may also trigger immune responses by inducing the expression of inflammatory cytokines.

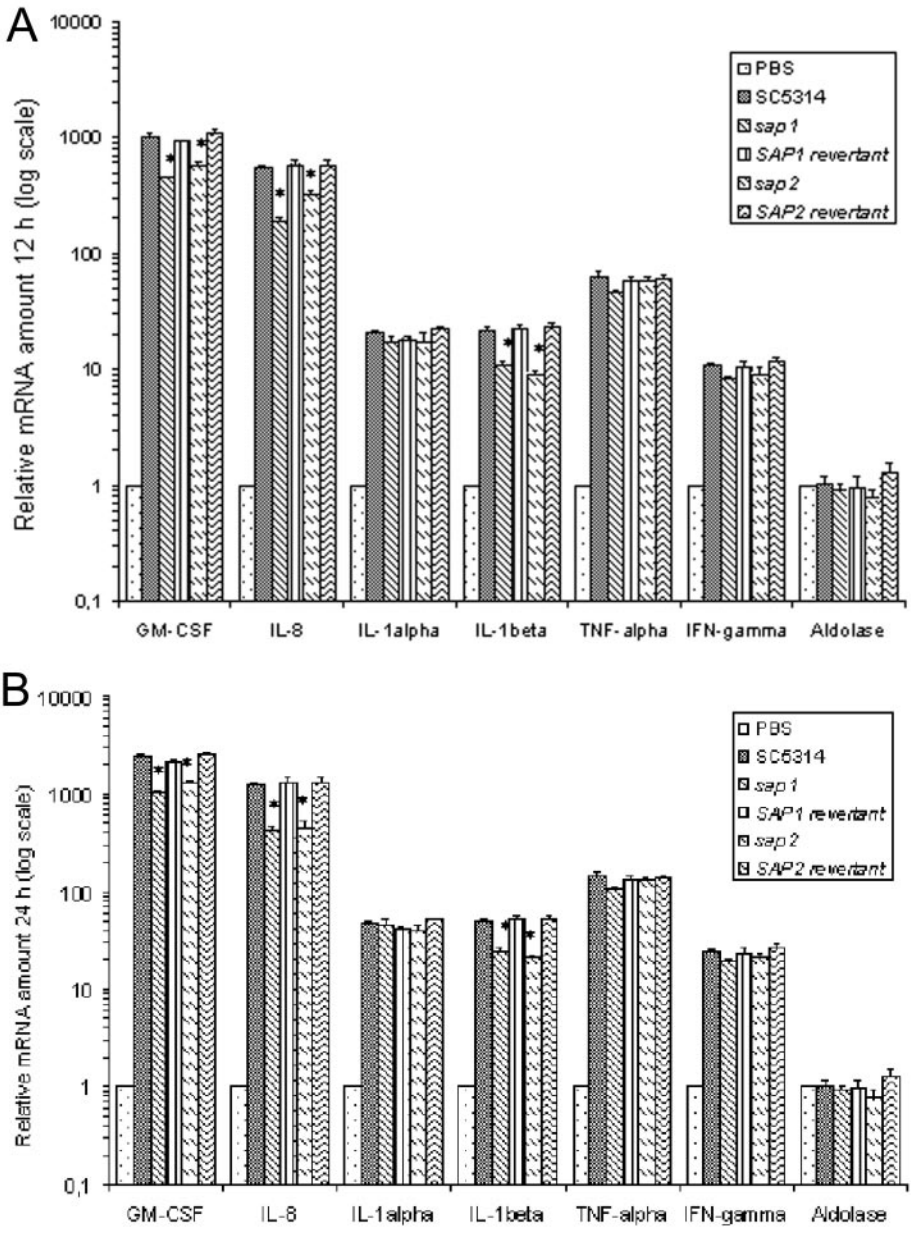

FIG. 3. Induction of epithelial cytokine gene expression 12 h (A) and 24 h (B) after infection of RHVE by *C. albicans* SC5314, *sap1* mutant, *SAP1* revertant, *sap2* mutant, and *SAP2* revertant cells. Asterisks indicate cytokine expression levels induced by the mutants which are significantly different ($P < 0.05$) both from those with the wild-type strain SC5314 and from those with their respective revertant strains. Quantitative analysis of each experiment was performed in triplicate. Combined data from three independent experiments performed in triplicate are shown.

Several cytokine genes are known to be linked with a protective Th1 response, and chemotaxis and activation of macrophages, neutrophils, and lymphocytes in vivo are known to be up-regulated during experimental infection of mucosal surfaces by *C. albicans* (14–16). This includes the expression of IL-1α, IL-1β, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, and TNF-α, which were all up-regulated in this study, suggesting that vaginal mucosa is an important source of proinflammatory cytokines in response to *C. albicans*.

The complete failure of heat-killed *C. albicans* blastospores and hyphae to induce a proinflammatory cytokine response strongly indicates that fungal factors must be actively expressed, released, or modified by viable, physiologically active *C. albicans* cells in order to stimulate epithelial cytokine gene expression. Since extracellular Saps are known fungal virulence factors of mucosal infections (10) and since *C. albicans* elicits a strong immune response compared with less-proteolytic *Candida* species such as *Candida glabrata* in an oral model of candidasis (14), we predicted that the secreted fungal proteinases may play a crucial role in the induction of epithelial cytokines.

Evidence obtained in this study supported our hypothesis. Firstly, Sap inhibition by pepstatin A clearly altered the cytokine expression of RHVE, indicating that the immune re-

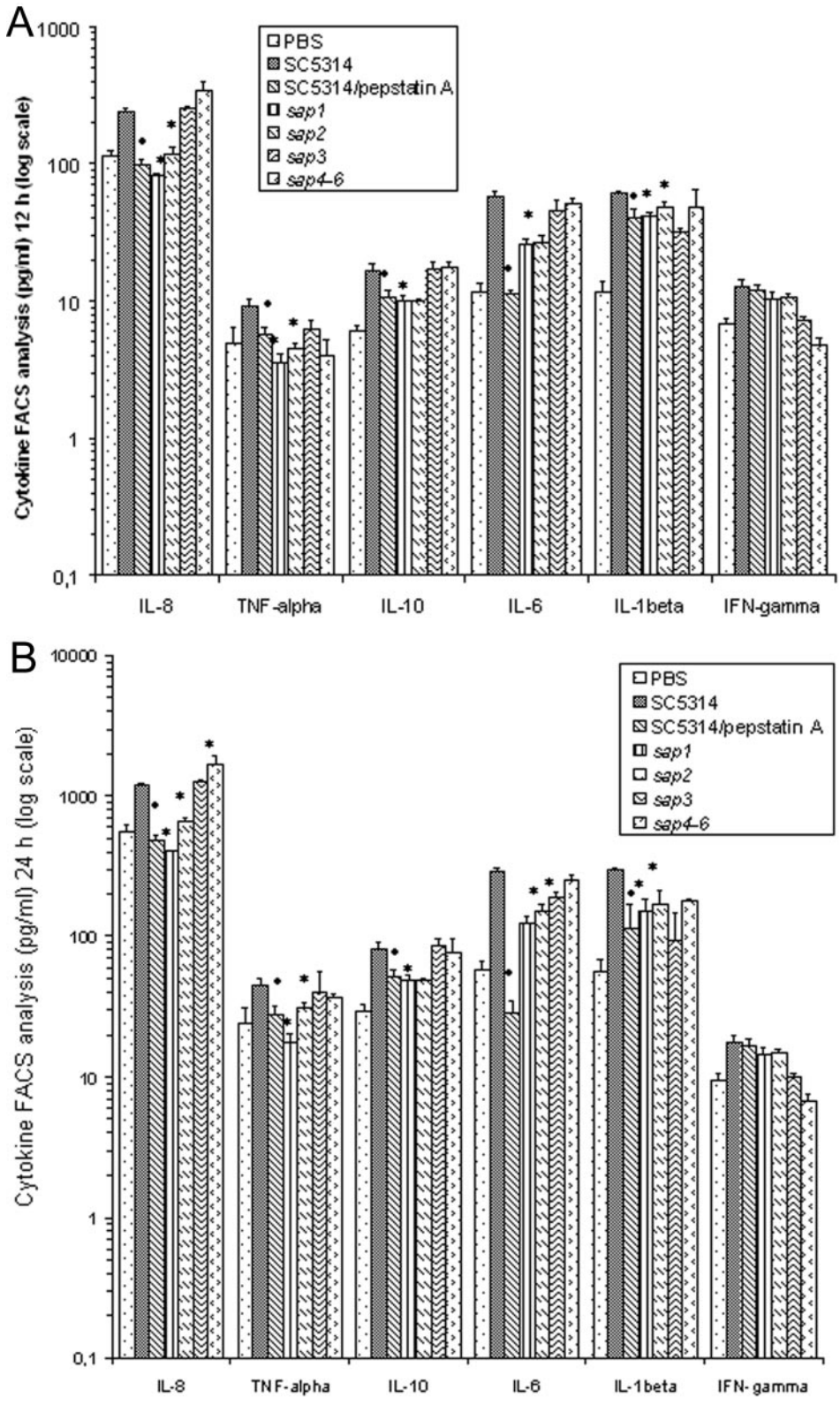

FIG. 4. Secretion of cytokines 12 h (A) and 24 h (B) after stimulation by viable *C. albicans* SC5314 in the presence and absence of pepstatin A or by *sap1*, *sap2*, *sap3*, or *sap4* to *sap6* mutant cells. Solid circles indicate significant inhibition of cytokine levels in the presence of pepstatin A compared to levels for wild-type infection in the absence of pepstatin A ($P < 0.05$). Asterisks indicate cytokine expression levels induced by *sap1* and *sap2* mutants that are significantly different ($P < 0.05$) from the response to the wild-type strain SC5314. Quantitative analysis of each experiment was performed in triplicate. Combined data from three independent experiments are shown.

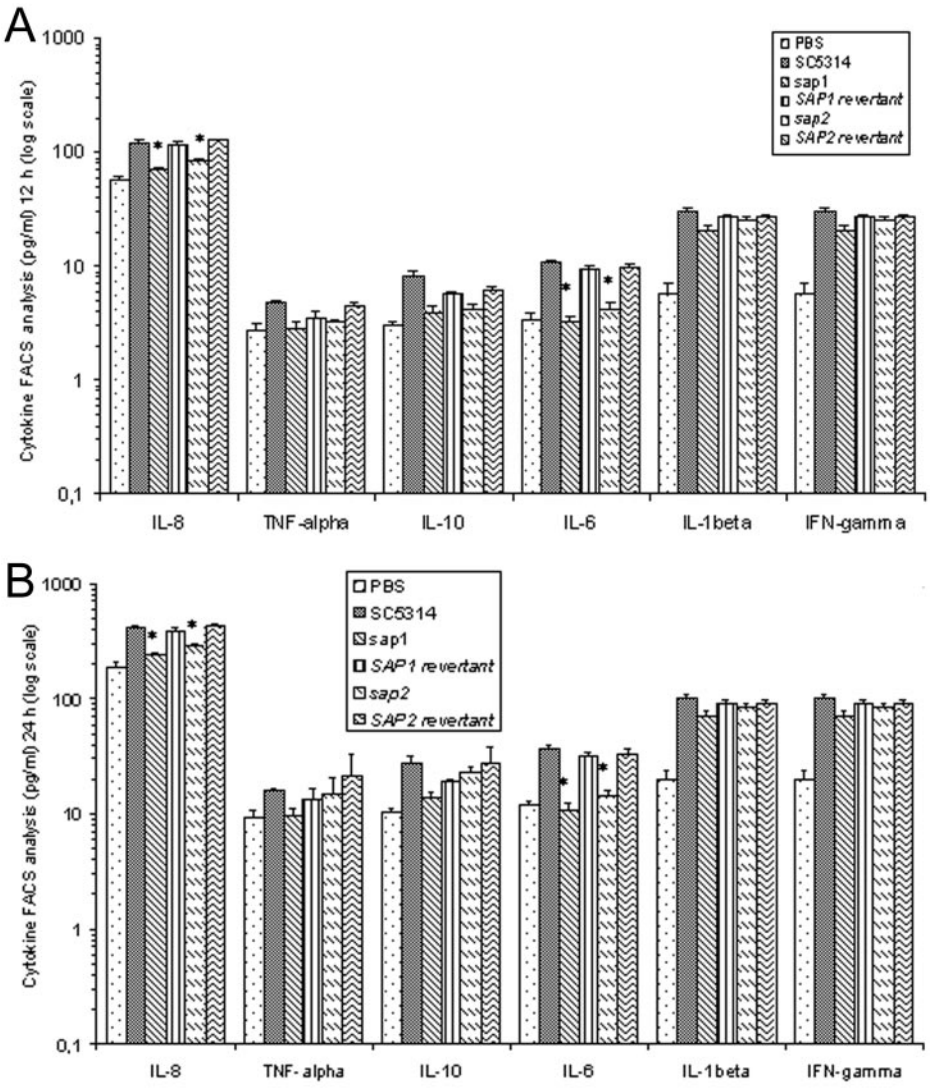

FIG. 5. Epithelial secretion of cytokines 12 h (A) and 24 h (B) after stimulation by *C. albicans* SC5314, *sap1* mutant, *SAP1* revertant, *sap2* mutant, or *SAP2* revertant cells. Asterisks indicate cytokine levels induced by the *sap1* and *sap2* mutants that are significantly different ($P < 0.05$) from the responses to SC5314 and their respective revertant strains. Combined data from three independent experiments performed in triplicate are shown.

sponse is affected by the proteolytic activity of the pathogen. The most striking inhibition of gene expression was that for the chemoattractive cytokines IL-6, IL-8, and GM-CSF, which are known to be important in the recruitment of inflammatory cells (4, 17). Pepstatin A, however, did not reduce cytokine expression down to basal levels, suggesting that Saps are probably not the only mechanisms by which *C. albicans* stimulates RHVE.

The data therefore indicate that Saps may contribute to the pathogenesis of inflammatory mucosal lesions by inducing the up-regulation of epithelial proinflammatory cytokines. A similar effect has been seen in previous studies with serine proteinases secreted by *Aspergillus fumigatus*. These proteinases stimulated the expression of IL-6 and IL-8 in airway epithelial cells, and the immune response was blocked in the presence of proteinase inhibitors (2, 8, 18).

Secondly, *C. albicans* mutants lacking specific *SAP* genes lost their potential to induce epithelial expression of distinct cytokines. Interestingly, IL-1α mRNA expression was not affected by pepstatin A or the *sap* mutants. This result suggests that *C. albicans* stimulates the expression of IL-1α by a mechanism different from that for the other cytokines. Previously we demonstrated that Sap1 and Sap2, but not Sap3 to Sap6, contribute in causing tissue damage in the vaginal model (13). In this study, we observed that the cytokine expression levels seem to correlate with tissue damage and that Sap1 and Sap2, but not Sap3 to Sap6, were necessary for stimulating this chemoattractive cytokine gene expression profile. Interestingly, the histological damage caused by the *sap4* to *sap6* triple mutant seemed to be greater than that with the wild type (13). Accordingly, the cytokine gene expression level was higher in epithelial tissue infected with the *sap4* to *sap6* triple mutant. This might be explained at least partially by a compensatory up-regulation of *SAP1* to *SAP3* expression in cells lacking *SAP4* to *SAP6* (M. Schaller, unpublished data), which would in

turn enhance epithelial tissue damage and induce stronger stimulation of cytokine expression.

Differences in gene (TNF-α, IFN-γ) or protein (TNF-α, IL-10, IL-1β) expression levels induced by the *sap1* and *sap2* mutants compared with SC5314 in Fig. 2 and 3 or Fig. 4 and 5, respectively, were rather minor and varied between experiments, which suggests that these cytokines might be stimulated mainly by mechanisms that do not involve Saps.

In general, we could demonstrate a good correlation between the mRNA and protein expression data. The discrepancy seen for IL-1β and TNF-α (Fig. 2 and 4) may be explained by the fact that the real-time PCR analysis monitored the gene expression only at a distinct time point, e.g., 12 h, while the FACS analysis done at the same time point reflected the total (accumulated) amount of protein secreted into the medium from the beginning of the experiment to 12 h.

In summary, we provide evidence that proteinases secreted by *C. albicans*, in particular Sap1 and Sap2, can induce a proinflammatory cytokine response in the RHVE model. These results reflect the capacity of epithelial cells to respond to the virulence activities of *C. albicans* (and other pathogens) by secreting a Th1-response-like cytokine pattern, which may initiate a chemoattractive and protective immune response in the presence of effector cells such as neutrophils and lymphocytes in vivo (13).

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