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Secreted Aspartyl Proteinases and Interactions of *Candida albicans* with Human Endothelial Cells

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The endothelial cell interactions of homozygous null mutants of *Candida albicans* that were deficient in secreted aspartyl proteinase 1 (Sap1), Sap2, or Sap3 were investigated. Only Sap2 was found to contribute to the ability of *C. albicans* to damage endothelial cells and stimulate them to express E-selectin. None of the Saps studied appears to play a role in *C. albicans* adherence to endothelial cells.

In susceptible hosts, *Candida albicans* enters the bloodstream and causes deep-seated infection in target organs. Because the organisms must cross the endothelial cell lining of the blood vessels to enter these organs, the interaction between *C. albicans* and vascular endothelial cells is likely to be a critical step in the initiation of a disseminated infection.

Secreted aspartyl proteinases (Saps) are believed to be virulence determinants of *C. albicans* (6, 14). Recently, homozygous null mutants of *C. albicans* deficient in *SAP1*, *SAP2*, *SAP3*, or *SAP4* to *-6* were constructed by site-directed mutagenesis (7, 15). These *sap*-deficient mutants exhibit diminished virulence in the guinea pig and mouse models of hematogenously disseminated candidiasis compared to that of the parent strain, SC5314. To investigate potential mechanisms responsible for this diminished virulence, we compared strains lacking the ability to secrete Sap1, Sap2, or Sap3 with the parent strain in terms of their ability to adhere to, stimulate, and cause damage to endothelial cells in vitro.

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C. albicans SC5314, a clinical isolate (5), and homozygous null mutants of this strain deficient in Sap1, Sap2, or Sap3 have been described previously (7). *C. albicans* M40 and M41 are heterozygous *SAP2* revertants created from the *sap2* null mutant by the method described below. For use in the experiment, all organisms were grown in liquid YPD medium (1% yeast extract, 2% Bacto Peptone, 2% glucose [wt/vol]) for 16 h at 25°C. The cells were processed according to our previously described method (9) and suspended in RPMI 1640 containing 10% pooled human serum for endothelial cell studies.

All of the null mutants studied had growth rates similar to that of the parent strain when grown in YPD medium (7). We also determined that the extracellular phospholipase activity of the null mutants was not significantly different from that of the parent strain as determined by the egg yolk agar assay (data not shown) (9).

To create heterozygous *SAP2* revertants, protoplasts of a homozygous *sap2 ura3* null mutant were prepared as described by Hube et al. (7). Linearized plasmid DNA containing *SAP2* and *URA3* was introduced into protoplasts of the *sap2* null mutant. To screen for *SAP2* transformants, Ura^+ clones were transferred to microtiter plates containing YCB/BSA medium (1.17% yeast carbon base, 1% glucose, 0.5% bovine serum albumin). Because *sap2* null mutants do not grow in this medium (7), this procedure identified transformants that expressed the wild-type *SAP2* gene. Transformants with growth rates comparable to that of the wild-type strain, SC5314, were screened for the presence of *SAP2* by PCR with *SAP2*-specific primers (5′-TGATTGTCAAGTCACTTATAGT-3′ and 5′-CTTAGGTCAAGGCAGAAATACTG-3′). Seven positive transformants were further analyzed for secreted proteolytic activity by the method of Hube et al. (7). Only two of these transformants, M40 and M41, showed growth rates and proteolytic activity comparable to those of the parent strain, SC5314 (data not shown).

The ability of the different mutants to cause endothelial cell injury was determined by a ^{51}Cr-release assay as previously described (9). We used human umbilical vein endothelial cells, which were harvested and maintained by our previously described method (3). In these experiments, the inoculum was 10^5 blastospores per well of a 24-well plate. We first determined the amount of endothelial cell injury after a 3-h incubation. Of the mutants tested, only the *sap2* null mutant caused less endothelial cell injury than did *C. albicans* SC5314 (data not shown). Next, time course studies were performed with the *sap2* null mutant, the *SAP2* revertant, and the parent strain. The *sap2* null mutant consistently caused 31% less endothelial cell injury after 1.5 h than the parent strain ($P = 0.0001$) (Fig. 1). With longer incubation, this difference in endothelial cell injury decreased. After 3 and 4.5 h, the *sap2* null mutant caused 20 and 6% less endothelial injury than the parent strain, respectively ($P < 0.0001$ and $P < 0.01$). There was no difference in endothelial cell injury when the *sap2* null mutant and the parent strain were incubated with endothelial cells for 8 h ($P > 0.05$). In these experiments, the *SAP2* revertant caused an

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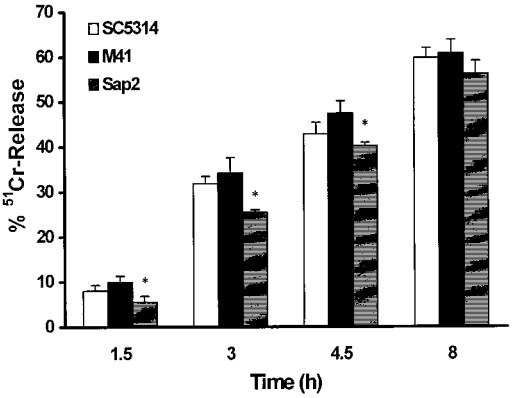

FIG. 1. Endothelial cell injury caused by *C. albicans* SC5314, a *sap2* null mutant (Sap2), and the *SAP2* revertant (M41). Results are the mean ± standard deviation of nine determinations. *, $P < 0.01$. The data were analyzed by analysis of variance and corrected for multiple comparisons with the Bonferroni correction.

amount of endothelial cell injury similar to that induced by the parent strain at all time points.

Because candidal germination is a prerequisite for the induction of endothelial cell injury (4, 8), we measured the extent of germination when the different strains were incubated with endothelial cells by microscopical examination (8). We found that the percentage of germinating organisms and average germ tube length of each null mutant were similar to those of the parent strain (data not shown).

Contact between *C. albicans* and endothelial cells is also required for endothelial cell injury to occur (3). Therefore, we measured the adherence of *C. albicans* to endothelial cells in six-well tissue culture plates by our standard procedure (9). The inoculum was 3.5×10^2 organisms per well. Adherence was determined by counting the number of adherent CFU and expressed as a percentage of the original inoculum. Deletion of *SAP1*, *SAP2*, or *SAP3* did not significantly affect the ability of *C. albicans* to adhere to endothelial cells after a 30-min incubation period (data not shown). Additionally, time course studies showed that the levels of adherence of the *sap2* null mutant and the parent strain were similar when the organisms were incubated with endothelial cells for 15 to 90 min (Fig. 2). In contrast, the *SAP2* revertant strain (M41) was almost twice as adherent as either the parent strain or the *sap2* null mutant at all time points ($P < 0.0001$). The endothelial cell adherence of a second *SAP2* revertant (M40) was also tested. As with M41, this revertant was twice as adherent as the parent strain (data not shown).

Next, the ability of the different mutants to stimulate endothelial cells to express E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) was determined by whole-cell enzyme-linked immunosorbent assay with endothelial cells grown in 96-well plates (11). The inoculum was 10^4 blastospores per well, and the organisms were incubated with endothelial cells for 8 h. Endothelial cells infected with the *sap2* null mutant showed a 28% decrease in E-selectin expression compared to cells infected with either the parent strain or the *SAP2* revertant ($P = 0.01$ for both comparisons) (Fig. 3). In other experiments, the *sap1* and the *sap3* null mutants induced the same level of endothelial cell E-selectin expression as that of the parent strain (data not shown). Additionally, there was no significant difference in endothelial cell ICAM-1 or VCAM-1 expression induced by any of the null mutants compared to that of the parent strain (data not shown).

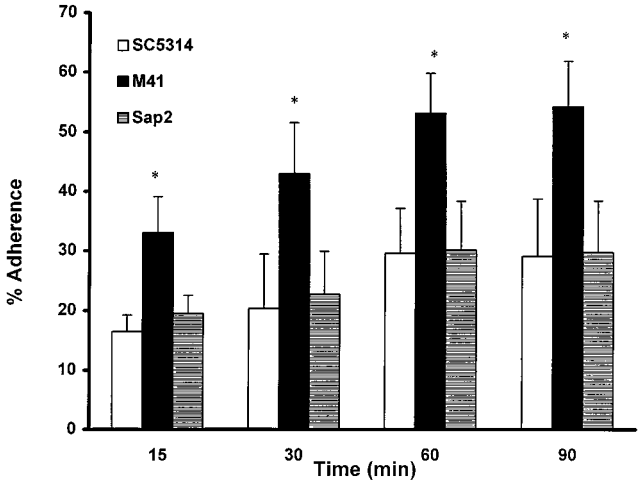

FIG. 2. Adherence of *C. albicans* SC5314, a *sap2* null mutant (Sap2), and the *SAP2* revertant (M41) to endothelial cells. Results are the mean ± standard deviation of nine determinations. *, $P < 0.0001$. The data were analyzed by analysis of variance and corrected for multiple comparisons with the Bonferroni correction.

Previously, we have found that null mutants of *C. albicans* deficient in secreting any of the enzymes Sap1, Sap2, Sap3, or Sap4 to -6 have diminished virulence in the guinea pig and mouse models of hematogenously disseminated candidiasis compared to that of the parent strain (7, 15). These findings suggest that these isoenzymes are virulence factors in *C. albicans.*

It is likely that endothelial cell injury caused by *C. albicans* in vivo enables the organism to escape from the blood vessels and cause disseminated infection. Therefore, we examined the ability of the *sap* null mutants to cause endothelial cell injury in vitro. We found that Sap2 likely contributes to endothelial cell injury in the initial period following contact between endothelial cells and *C. albicans*. The finding that the *sap2* null mutant caused as much endothelial cell injury as the parent strain after 8 h suggests that other candidal factors, such as secreted phospholipase(s), also contribute to this process. Alternatively, upregulation of other Sap isoenzymes may occur at later incubation periods.

It has been proposed that Saps may contribute to the virulence of *C. albicans* by enhancing its adherence to host tissues

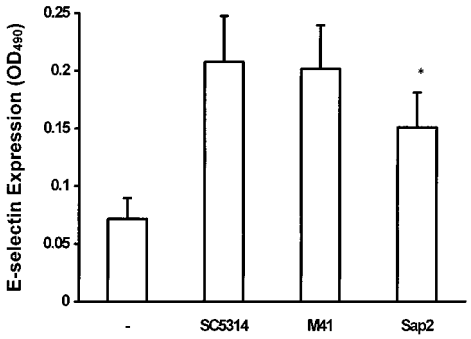

FIG. 3. Endothelial cell E-selectin expression (optical density at 490 nm [OD_{490}]) in response to medium alone (−), *C. albicans* SC5314, or the proteinase mutants. Results are the mean ± standard deviation of at least nine determinations. *, $P = 0.01$. The data were analyzed by analysis of variance and corrected for multiple comparisons with the Bonferroni correction.

(1, 2, 10, 12, 13). Our results obtained with the null mutants suggest that, individually, none of the Sap1 to -3 isoenzymes appears to contribute to the adherence of *C. albicans* to endothelial cells in vitro. It is still possible that Saps do play a role in *C. albicans* adherence to endothelial cells. For example, it is possible that in a null mutant deficient in one Sap isoenzyme, there is compensatory overproduction of other Saps. Alternatively, Saps other than the ones tested may enhance adherence to endothelial cells. Why the adherence of the *SAP2* revertants was greater than that of the parent strain is unclear. However, it is possible that the reintroduction of *SAP2* may have resulted in overproduction of the Sap2 isoenzyme and therefore increased adherence to endothelial cells.

In a previous study, we have determined that *C. albicans*-induced endothelial cell injury and leukocyte adhesion molecule expression appeared to be related, since both processes follow a similar time course, and both are induced only when endothelial cells phagocytize live, germinating *C. albicans* cells (4). However, in the present study, we were able to dissociate endothelial cell injury from endothelial cell stimulation. We found that although the *sap2* null mutant caused significantly less endothelial cell injury, this mutant induced the same level of expression of ICAM-1 and VCAM-1 as that of the parent strain. Only E-selectin expression was decreased in response to the *sap2* null mutant. These results suggest that *C. albicans* stimulates endothelial cells to express ICAM-1 and VCAM-1 by a mechanism that is independent of the early induction of endothelial cell injury. They also indicate that *C. albicans* stimulates endothelial cells to express E-selectin by a mechanism that is different from that of ICAM-1 and VCAM-1.

In summary, Sap2 contributes to endothelial cell injury induced by *C. albicans* and plays a role in the induction of endothelial cell E-selectin expression by *C. albicans*. However, we found no evidence that the Sap1 to -3, when tested individually, alter the adherence of *C. albicans* to endothelial cells. These in vitro findings may help explain the reduced virulence of the *sap2*-deficient mutant in experimental hematogenous candidal infections in vivo.

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REFERENCES

1. **Borg, M., and R. Rüchel.** 1988. Expression of extracellular acid proteinase by proteolytic *Candida* spp. during experimental infection of oral mucosa. Infect. Immun. **56:**626–631.
2. **El-Maghrabi, E. A., D. M. Dixon, and J. W. Burnett.** 1990. Characterization of *Candida albicans* epidermolytic proteases and their role in yeast-cell adherence to keratinocytes. Clin. Exp. Dermatol. **15:**183–191.
3. **Filler, S. G., J. N. Swerdloff, C. Hobbs, and P. M. Luckett.** 1995. Penetration and damage of endothelial cells by *Candida albicans*. Infect. Immun. **63:**976–983.
4. **Filler, S. G., A. S. Pfunder, B. J. Spellberg, J. P. Spellberg, and J. E. Edwards, Jr.** 1996. *Candida albicans* stimulates cytokine production and leukocyte adhesion molecule expression by endothelial cells. Infect. Immun. **64:**2609–2617.
5. **Hilenski, L. L., F. Naider, and J. M. Becker.** 1986. Polyxin D inhibits colloidal gold-wheat germ agglutinin labeling of chitin in dimorphic forms of *Candida albicans*. J. Gen. Microbiol. **132:**1441–1451.
6. **Hube, B.** 1996. *Candida albicans* secreted aspartyl proteinases. Curr. Top. Med. Mycol. **7:**55–69.
7. **Hube, B., D. Sanglard, F. C. Odds, D. Hess, M. Monod, W. Schäfer, A. J. P. Brown, and N. A. R. Gow.** 1997. Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* attenuates virulence. Infect. Immun. **65:**3529–3538.
8. **Ibrahim, A. S., S. G. Filler, M. A. Ghannoum, and J. E. Edwards, Jr.** 1993. Interferon-γ protects endothelial cells from damage by *Candida albicans*. J. Infect. Dis. **167:**1467–1470.
9. **Ibrahim, A. S., F. Mirbod, S. G. Filler, Y. Banno, G. T. Cole, Y. Kitajima, J. E. Edwards, Jr., Y. Nozawa, and M. A. Ghannoum.** 1995. Evidence implicating phospholipase as a virulence factor in *Candida albicans*. Infect. Immun. **63:**1993–1998.
10. **Klotz, S. A.** 1994. Adherence of *Candida albicans* to endothelial cells is inhibited by prostaglandin I_2. Infect. Immun. **62:**1497–1500.
11. **Noel, R. F., Jr., T. T. Sato, C. Mendez, M. C. Johnson, and T. H. Pohlman.** 1995. Activation of human endothelial cells by viable or heat-killed gram-negative bacteria requires soluble CD14. Infect. Immun. **63:**4046–4053.
12. **Ollert, M. W., R. Söhnchen, H. C. Korting, U. Ollert, S. Bräutigam, and W. Bräutigam.** 1993. Mechanisms of adherence of *Candida albicans* to cultured human epidermal keratinocytes. Infect. Immun. **61:**4560–4568.
13. **Ray, T. L., and C. D. Payne.** 1988. Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase. Infect. Immun. **56:**1942–1949.
14. **Rüchel, R.** 1992. Proteinase, p. 17–31. *In* J. E. Bennett, R. J. Hay, and P. K. Peterson (ed.), New strategies in fungal disease. Churchill Livingstone, Edinburgh, Scotland.
15. **Sanglard, D., B. Hube, M. Monod, F. C. Odds, and N. A. R. Gow.** 1997. A triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5*, and *SAP6* of *Candida albicans* causes attenuated virulence. Infect. Immun. **65:**3539–3546.