Increases in SLT2 Expression and Chitin Content Are Associated with Incomplete Killing of Candida glabrata by Caspofungin

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Incomplete killing was observed for caspofungin against Candida glabrata, which was associated with increased SLT2 expression and elevated chitin content. In contrast, fungicidal activity and no chitin increase were observed in an isogenic Δslt2 strain, suggesting a role for SLT2 and chitin production in the response of C. glabrata to caspofungin.

Up-regulation of the cell wall integrity (CWI) pathway in Candida albicans upon damage to the cell wall and increases in chitin have correlated with the paradoxical attenuation of caspofungin activity at clinically relevant supra-MIC concentrations (21, 22). Although this effect has not been observed in Candida glabrata (4, 20), studies have reported incomplete killing against some isolates (2, 3, 5, 23). In Saccharomyces cerevisiae, SLT2 encodes a mitogen-activated protein kinase of the CWI pathway that confers protection from damage to the cell wall. An SLT2 homolog has been identified in the C. glabrata genome sequence (http://cbi.labri.fr/Genolevures/index.php). Our objective was to evaluate the potential role of SLT2 and increases in chitin content in the escape of C. glabrata from the fungicidal activity of caspofungin.

A C. glabrata Δslt2 strain and its isogenic parent, C. glabrata 200989, were used in all experiments (7). Stock solutions of caspofungin acetate (Merck & Co., Inc., Whitehouse, NJ) were prepared in sterile distilled water and further diluted in RPMI broth buffered with 0.165 M morpholinepropanesulfonic acid (pH 7). The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) colorimetric assay was conducted at caspofungin concentrations from 0 to 32 μg/ml as previously described (6, 13). The absorbance was read at 492 nm, and readings were converted to percent absorbance with a maximum 1.6-log10 CFU/ml reduction (Fig. 1B). Incomplete killing was observed for caspofungin against strain 200989, as measured by the XTT assay (Fig. 1A). Complete growth inhibition did not occur at any concentration tested against strain 200989, with a maximum 1.6-log10 CFU/ml reduction (Fig. 1B). In contrast, a 100% reduction in absorbance was observed in the Δslt2 strain at concentrations of ≥0.5 μg/ml, and fungicidal activity was observed at 1 and 16 μg/ml (maximum 4.2-log10 CFU/ml reduction) (Fig. 1C). In addition, caspofungin (0.125 μg/ml) had excellent activity, with a 2.6-log10 reduction in CFU/ml. Increased expression of SLT2 was also observed at each caspofungin concentration versus control expression following 10 min of exposure (>2-fold; P < 0.05), and this was most elevated after 180 min (∼3.8-fold;
$P < 0.05$ (Fig. 2A). However, SLT2 transcription was no longer increased at 12 h.

These phenotypic and genotypic data are consistent with findings in previous reports demonstrating a role of the CWI pathway in the coordination of the response to caspofungin-mediated cell wall damage. A rapid and transient induction of SLT2 transcription in S. cerevisiae following a brief exposure to caspofungin has previously been demonstrated (1, 17), while a 50% decrease in viable cells was observed in strains lacking Slt2p activity compared to the isogenic parent (17). Similarly, increased expression of the C. albicans homolog of SLT2, MKC1, has also been observed at elevated drug concentrations, where a paradoxical attenuation of caspofungin activity occurs, and this paradoxical phenotype was absent in a Δmkc1Δmkc1 homozygous mutant (22).

We also examined chitin content following a caspofungin challenge. In strain 200989, CHS3 transcript levels increased by >2-fold at each caspofungin concentration after 180 min ($P < 0.05$) (Fig. 2B), accompanied by a >3.3-fold increase in chitin content versus control levels ($P < 0.05$) (Fig. 2C). In contrast, CHS3 expression did not significantly increase in the Δslt2 strain, nor was chitin content elevated following caspofungin challenge. These data suggest a link between SLT2 and increased chitin content upon cell wall damage by caspofungin in C. glabrata. The increase in chitin content was not unexpected, as other investigators have also demonstrated significant increases in chitin following exposures to different concentrations of echinocandins (16, 21). A link between the CWI pathway and chitin synthesis has also been described for C. albicans. A recent study reported activation of Mkc1p upon exposure to cell wall damaging agents, resulting in an up-regulation in CHS3 transcription and an increase in cell wall chitin (14). However, with the deletion of MKC1, CHS3 expression and chitin content remained unchanged.

While these data indicate an apparent association between SLT2, chitin content, and the response to caspofungin challenge in C. glabrata, further studies involving additional isolates are warranted. Although the CWI pathway appears to be conserved among fungi, this pathway and chitin synthesis have not been fully described for C. glabrata. Additionally, other potential mechanisms for caspofungin tolerance, including up-regulation of 1,3- and 1,6-β-glucan synthesis, increased export of cell wall components for cell wall repair, and the calcineurin pathway, require further investigation (11, 12, 15, 21, 22, 24). These initial data invite further studies since these may lead to strategies that improve outcomes for patients with invasive candidiasis.

### FIG. 1

(A) In vitro XTT colorimetric assay. Percent absorbance (means ± standard deviations) for the parent C. glabrata strain, ATCC 200989 (●), and the corresponding Δslt2 (○) strain compared to results for the control are plotted on the y axis, and increasing concentrations of caspofungin (0 to 32 μg/ml) are plotted on the x axis. (B and C) Plots of log₁₀ CFU/ml (means ± standard deviations) versus time for the parent C. glabrata strain, ATCC 200989 (B), and the Δslt2 strain (C) at the following caspofungin concentrations: ■ and □, control; ▲ and △, 0.125 μg/ml; ◆ and ◇, 1.0 μg/ml; ♦ and ○, 16.0 μg/ml.
FIG. 2. (A) Relative expression of SLT2 in parent strain \textit{C. glabrata} ATCC 200989 (means ± standard deviations) following caspofungin exposure for 10 min (dark bars), 180 min (gray bars), or 12 h (white bars). (B) Relative expression of CHS3 in the parent \textit{C. glabrata} strain, ATCC 200989 (dark bars), or the corresponding Δslt2 strain (gray bars) following caspofungin exposure for 180 min. Expression levels were normalized using \textit{ACT1} as the housekeeping gene. (C) Changes in chitin content (means ± standard deviations) compared to control levels in the parent \textit{C. glabrata} strain, ATCC 200989 (dark bars), and the corresponding Δslt2 strain (gray bars) following exposure to 0.125, 1.0, or 16.0 \(\mu\)g/ml of caspofungin for 12 h.

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