The inhibition of fungi by caspofungin, an echinocandin, is attributed to interference with synthesis of the cell wall polymer β-1,3-glucan. The principal target of caspofungin is the protein Fks1, and binding leads to reduced activity of the enzyme glucan synthase and reduced formation of and extrusion of the glucan polymer through the cell membrane and into the wall (5). With Candida species, the result is a low MIC (typically <0.5 μg/ml), and fungicidal activity at or very close to the MIC (5). However, many isolates of Candida are able to grow at some caspofungin concentrations higher than the MIC (11), which we have termed the paradoxical effect. The frequency of this phenomenon is 21% of 76 Candida albicans isolates (4; D. A. Stevens, unpublished). The cause of the paradoxical growth was not mutations in resistance-associated regions of the β-1,3-glucan synthase complex, selection of a resistant subpopulation, or destruction of the drug, but the mechanism remains unelucidated (12). We proposed that the gene(s) is promptly activated by the presence of high caspofungin concentrations (11, 12), and speculation on the mechanism included “compensatory upregulation of synthesis of another wall component” (11).

A stimulus for the present studies was our observation (10) that an echinocandin and a chitin synthesis inhibitor acted in a highly synergistic manner in vitro, which was corroborated in vivo (3). This suggests that if one cell wall polymer is inhibited, the other might compensate, and that if both were blocked, the fungus would be defenseless.

The present studies examined cell wall content in an isolate (C. albicans strain 95-68) that we reported previously (11) as another wall component” (11).

Growth in the presence of high (12.5 μg/ml) concentrations of caspofungin and its absence was as described previously (11, 12). The cells were disrupted by >150 cycles of freezing and thawing followed by glass bead bombardment until no viable cells could be demonstrated by subculture, and the disruption was verified by microscopy (12). The cell walls were separated from cytosol and glass beads by centrifugation (12), and the walls were dried, lyophilized, and frozen. Two separate preparations were made, 7 months apart, in the presence and absence of caspofungin. Each preparation was studied, two or three 30-mg samples of each culture condition in each time period, and each sample was studied in duplicate. Student’s t test was used for comparison of caspofungin with no caspofungin for each carbohydrate group analyzed.

Glucan and chitin contents were determined by published methods (2, 9) with several modifications. Each sample analyzed was a 10-mg quadruplicate sample. Lyophilized cell wall (≥20 mg) was extracted three times with 500 μl of 3% NaOH at 75°C for 1 h. The supernatants from NaOH extraction were added to 2 volumes of ethanol and incubated at −20°C for >1 h. After centrifugation at 15,000 rpm for 5 min at 4°C, precipitates were dissolved in water, and hexose contents were measured by the borosulfuric acid method (1) (alkali-soluble glucans). The NaOH extraction pellets were washed once with 100 mM Tris-HCl and 10 mM Tris-HCl (pH 7.5). The pellets were digested with 4 mg/ml Zymolyase 100T (prepared from Arthrobacter luteus, primarily β-1,3-glucan hydrolase; Seikagaku Corporation, Tokyo, Japan) in 10 mM Tris-HCl overnight at 37°C and centrifuged for 10 min at 15,000 rpm at 4°C. The supernatants were divided into two portions. One was used for measurement of hexose content (alkali-insoluble β-1,3-glucans). The other was dialyzed against distilled water using a Spectra/Por membrane with a molecular weight exclusion of 6,000 to 8,000 (Spectrum Medical Industries, Inc., Rancho Dominguez, CA), and hexose contents were measured (alkali-insoluble β-1,6-glucans). Zymolyase-insoluble pellets were washed once with 10 mM Tris-HCl and water. The pellets were suspended in water and divided into two portions. One was used for measurement of hexose content (Zymolyase-indigestible pellets). The other was centrifuged, and the pellet was used for measurement of hex-
mannoproteins, with small amounts of glucan (those glucans not cross-linked to chitin).  P/H9262 hexosamine content was measured by colorimetry (13). D-Glu-ratation, the pellets were suspended in water, and the lets were digested in 6 N HCl at 100°C for 6 h. After evapo-

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paradoxical growth is consistent with inferences from gene regulation of the intracellular protein kinase C cell wall integ-

fungin (15) confirmed earlier observations that there is up-

upregulated. The depression of pressed by caspofungin, and the chitin content is significantly 

deglucans, and chitin. The glucan content is significantly de-

caspofungin on the cell wall content of all hexoses, including 

results (means of duplicate results/experiment; two ex-

were highly significant with respect to caspofungin effect in the two independent runs.

These results provide an explanation for the paradoxical effect described previously (11); namely, that those isolates which are capable of surviving in caspofungin at high concentrations do so by compensatorily increasing their cell wall chitin content. At these concentrations the glucan synthesis inhibition effect of caspofungin remains highly significant. This also confirms findings (12) with the enzyme itself that upregu-

lation of glucan synthase is not the mechanism of escape. Studies with C. albicans involving only brief exposure to caspo-

fungin (15) confirmed earlier observations that there is up-

regulation of the intracellular protein kinase C cell wall integ-

pathway. That compensatory chitin synthesis occurs in para-

differential growth is consistent with inferences from gene interaction studies of Saccharomyces cerevisiae of what occurs in the usual caspofungin growth inhibition (8).

We also demonstrate that caspofungin also inhibits synthesis of β-1,6-glucan, as well as β-1,3-glucan. β-1,6-Glucan synthesis and incorporation proceed through several enzyme steps (14), and Fks1p inhibition would affect a late stage. Our finding is consistent with suggestions from gene disruption studies (6) and studies of glucose linkages in Cryptococcus neoformans (7) and broadens our understanding of caspofungin antifungal mechanisms.

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eron Douglas for helpful suggestions and Marife Martínez for assis-

REFERENCES

1. Badin, J., C. Jackson, and M. Schubert. 1953. Improved method for deter-


Med. 84:288–291.


TABLE 1. Glucan and chitin content of cell walls in the presence and absence of caspofungin

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Presence of caspofungin</th>
<th>Hexose Content</th>
<th>Alkali insoluble</th>
<th>Chitin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>β-1,3-plus β-1,6-glucan</td>
<td>β-1,6-Glucan</td>
<td>β-1,3-Glucan</td>
</tr>
<tr>
<td>1</td>
<td>No</td>
<td>134.9 (8.7)</td>
<td>34.0 (5.4)</td>
<td>100.9</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>8.0 (4.3)</td>
<td>3.4 (0.7)</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>86.1 (6.5)</td>
<td>20.2 (2.8)</td>
<td>56.9</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>31.5 (10.5)</td>
<td>5.4 (5.4)</td>
<td>26.1</td>
</tr>
</tbody>
</table>

* Results are means (standard deviations) of quadruplicate assays in two separate experiments/row. The isolate was grown in the presence and absence of caspofungin twice (two separate preparations). Glucan and chitin contents are expressed as μg/ml dried cell wall. The hexose content of the Zymolyase-indigestible pellet is shown. The β-1,3-glucan value was derived by subtraction of β-1,6-glucan from the combination determined previously (14). Alkali-soluble glucan is almost completely mannoproteins, with small amounts of glucan (those glucans not cross-linked to chitin). P values compare data in the presence and absence of caspofungin.

P ≤ 0.0001.

P = 0.02.

P = 0.0002.