Roles of Cellular Respiration, CgCDR1, and CgCDR2 in *Candida glabrata* Resistance to Histatin 5

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Histatin 5, a human salivary protein with broad-spectrum antifungal activity, is remarkably ineffective against *Candida glabrata*. Fluconazole resistance in this fungus is due in most cases to upregulation of CgCDR efflux pumps. We investigated whether the distinct resistance of *C. glabrata* to histatin 5 is related to similar mechanisms.

In the past decade, *Candida glabrata* has emerged as the second most common fungal pathogen in opportunistic fungal infections in humans. This increase is likely related to the widespread use of the antimycotic drug fluconazole, to which *C. glabrata* is inherently less sensitive than *C. albicans*, *C. parapsilosis*, and *C. tropicalis* (18). Resistance to azoles has been associated with the upregulation of the genes CgCDR1 and CgCDR2 (also designated *PDH1*) which encode proteins belonging to the ATP binding cassette family of multidrug resistance membrane-associated efflux pumps (1, 2, 12, 19, 20, 23). Other reported mechanisms of azole resistance in *C. glabrata* which may actually be related to CDR upregulation involve the generation or selection of respiratory-deficient petite mutants (3, 4). In the quest to develop new antimycotics with possibly different cellular targets, histatins, a group of salivary cationic proteins, have received special attention (5, 8, 16, 17, 21). While histatins are highly effective against diverse classes of fungi, they are significantly less effective against *C. glabrata* (9, 24). Furthermore, other, structurally unrelated, cationic antifungal peptides, belonging to the magainin and defensin family, also show a selectively reduced activity against this fungus (6, 9, 11). The cellular basis for the apparent insensitivity of *C. glabrata* to various antifungal agents as different as azoles and cationic antifungal proteins is as yet unknown. In the current study we evaluated the role of petite mutation, fermentation, and the activity of the efflux pumps CgCDR1 and CgCDR2 in the resistance of *C. glabrata* to histatin 5.

C. albicans (ATCC 10231) and *C. glabrata* (ATCC 90030) were cultured in 1:10 diluted Sabouraud dextrose broth (SDB; Difco, Becton Dickinson, Franklin Lakes, NJ) (9) and containing either 0, 56, 112, or 225 $\mu\text{g/ml}$ of histatin 5. Assessment of the optical density at 620 nm (OD_{620}) after 48 h of incubation at 30°C showed that *C. glabrata* was able to grow even in the presence of the highest concentration of histatin 5, while the growth of *C. albicans* was inhibited by all three histatin concentrations (Fig. 1).

Since *C. glabrata* resistance to fluconazole in some cases is explained by induction or selection of petite mutants (3, 4) and petite mutation in *C. albicans* is known to abolish sensitivity to histatin 5 (7), the respiratory competence of *C. glabrata* cells grown in the presence of histatin 5 was evaluated. Two experimental approaches were followed. First, 10- μl aliquots of cells grown for 48 h in the presence of the indicated three concentrations of histatin 5 were plated on glucose- or glycerol-limited agar (7), the latter strictly supporting cellular respiration. Second, since the *C. glabrata* petite phenotype may be a transient cellular characteristic caused by mitochondria that switch between states of respiratory competence and incompetence (12), we also measured directly the respiration of *C. glabrata* cells in the histatin 5-containing cell cultures. Plated *C. albicans* cell suspensions containing histatin 5 did not grow on agar (Fig. 2). In contrast, the histatin 5-grown *C. glabrata* cells grew well on glucose- and glycerol-limited agar, the latter indicating that the presence of histatin 5 during growth in diluted SDB broth had not induced “petite” characteristics. In addition, respiratory measurements using a biological oxygen monitor (Yellow Springs Instruments, Yellow Springs, Ohio) (7) of cells grown in the presence of 56, 112, or 225 $\mu\text{g/ml}$ of histatin 5 indicated that the oxygen consumption of these cells in their respective supernatants was certainly not lower than that from

FIG. 1. Assessment of growth (OD_{620}) after 48 h of incubation of *C. albicans* and *C. glabrata* in diluted SDB containing three concentrations of histatin 5. Represented are the average values and standard errors of two independent experiments, each performed in duplicate.

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FIG. 2. Determination of the viability and respiratory competence of aliquots of histatin-grown *C. albicans* and *C. glabrata* cell suspensions plated on glycerol or glucose-limited agar (7).

cells that had been cultured in the absence of histatin 5 (10.6, 10.6, 10.2, and 9.0 nmol O₂/min/OD₆₂₀, respectively).

In previous studies it was demonstrated that cellular respiration is important in *C. albicans* sensitivity to histatin 5 (8). It was hypothesized that *C. glabrata* might escape histatin 5 activity by utilizing fermentative pathways, since theoretically dextrose (glucose) can either be fermented or assimilated. Furthermore, *C. glabrata*, but not *C. albicans*, is a Crabtree-positive fungus (22), and its respiration may be negatively affected by certain levels of glucose (15). To exclude the possibility of

substrate fermentation by *C. glabrata*, growth inhibition assays were also conducted in broth containing only glycerol as the carbon source. *C. glabrata* was insensitive in either glycerol- or glucose-limited broth, exhibiting 50% inhibitory concentrations (IC₅₀s) higher than 225 µg/ml (Fig. 3). This result shows that the resistance of this fungus to histatin 5 is not explained by mitochondrial dysfunction or lack of respiration. In contrast, *C. albicans* was sensitive to histatin 5 in glycerol- and glucose-containing broths, exhibiting IC₅₀s of 13.8 ± 4.8 and 21.2 ± 2.5 µg/ml, respectively. The sensitivity of *C. albicans* in

FIG. 3. Fungistatic activity of serially diluted histatin 5 toward *C. albicans* and three *C. glabrata* strains in diluted glycerol-limited broth (A), containing (per liter) 1 g peptone, 0.5 g yeast extract, 0.5 g glucose, and 0.5 g glycerol, and in glucose-limited broth (B), containing (per liter) 1 g peptone, 0.5 g yeast extract, 0.5 g glucose, and 0.5 g glycerol. Broth microdilution experiments were conducted as described previously (9). Graphs represent the averages of an experiment performed in duplicate and are representative of four independent experiments each performed in duplicate.

TABLE 1. Comparison of fluconazole and histatin 5 sensitivity of CgCDR1 and CgCDR2 mutants of *C. glabrata*^a

Species	Strain ^c	Property(ies)	IC ₅₀ (μg/ml)				LC ₅₀ (μg/ml) with 5 mM PPB ^d and histatin 5 at 1.5 h
			RPMI ^b Fluconazole		Diluted SDB ^c for 48 h		
			24 h	48 h	Fluconazole	Histatin 5	
<i>C. glabrata</i>	DSY562	Clinical isolate	5.0 ± 0.0	7.0 ± 1.4	26 ± 3.1	>225	>225
	DSY565	Clinical isolate	>128	>128	>128	>225	>225
	DSY1029	<i>ura3</i>	ND ^e	ND	>128	>225	71 ± 12
	DSY1041	<i>Δcgcdr1::hisG URA3 hisG</i>	8.0 ± 2.8	10 ± 0.0	8.0 ± 1.3	>225	>225
	DSY1612	<i>Δcgcdr2::hisG URA3 hisG</i>	>128	>128	>128	>225	>225
	DSY1613	<i>Δcgcdr2::hisG Δcgcdr1::hisG URA3 hisG</i>	2.3 ± 0.4	3.5 ± 0.7	2.8 ± 0.4	>225	>225
	DSY562-HFAR	High-frequency acquired resistance	>128	>128	>128	>225	>225
	DSY1041-HFAR	High-frequency acquired resistance	16 ± 6.0	46 ± 5.7	44 ± 5.1	>225	>225
	90030	ATCC ^f	7.0 ± 2.6	10 ± 1.5	104 ± 32	>225	>225
<i>C. albicans</i>	10231	ATCC	0.5 ± 0.0	0.6 ± 0.1	8.3 ± 2.9	9.5 ± 0.5	12 ± 2.0

^a Values represent the means and standard deviations of two experiments each performed in duplicate. LC₅₀, 50% lethal concentration.

^b Method M27-A2, NCCLS (13).

^c The growth inhibition assay was conducted as described by Helmerhorst et al. (9).

^d PPB, potassium phosphate buffer, pH 7.0. The killing assay was conducted as described by Helmerhorst et al. (9).

^e ND, not determined.

^f ATCC, American Type Culture Collection.

glucose broth is consistent with the fact that respiration of *C. albicans* is not suppressed by glucose (10).

Previous studies have shown that the site-directed mutation in *C. glabrata* of CgCDR1, CgCDR2, or both affects sensitivity to fluconazole, in particular when both genes were disrupted simultaneously (20). Using the standardized antifungal susceptibility protocol M27-A2 of the National Committee for Clinical Laboratory Standards (NCCLS) (14), we confirmed published results (20) that strains in which either CgCDR1 or both CgCDR genes were abolished showed a marked increased sensitivity to fluconazole (Table 1). Since the intracellular accumulation of histatins is required for their activity toward *C. albicans* (8, 13, 25), it was hypothesized that *C. glabrata* resistance could be related to the fact that histatin 5 fails to accumulate intracellularly as a result of the activity of efflux pumps. While it is unknown whether histatin 5 could be transported by the CgCDR1 and CgCDR2 pumps that are so effective in exporting fluconazole, it was assessed whether the mutants lacking one or both of these genes would show an increased sensitivity to histatin 5. Growth inhibition assays with histatin 5 were conducted in diluted SDB (9), since salts in RPMI medium interfere with histatin activity (9). As indicated in Table 1, the efficacy of fluconazole in RPMI medium and in diluted SDB was in most cases comparable, allowing the comparison of *C. glabrata* sensitivities to fluconazole and histatin 5 in diluted SDB. The results indicated that while the various *C. glabrata* mutants showed differential sensitivities to fluconazole, all *C. glabrata* strains were resistant to histatin 5 growth inhibition (IC₅₀ > 225 μg/ml). Notably, the observed histatin 5 resistance was relative to the histatin 5 sensitivity of *C. albicans*, which displayed IC₅₀ values of 9.5 ± 0.5 μg/ml. In killing assays conducted in 5 mM potassium phosphate buffer, pH 7.0 (9), similar results of *C. glabrata* resistance were obtained, except that the *ura3* *C. glabrata* strain (DSY 1029) was somewhat sensitive (Table 1). These results combined indicated that *C. glabrata* resistance to histatin 5 is independent of mutations in CgCDR1 or CgCDR2.

The data obtained here and in our previous study (9) reveal a seemingly fundamental and widespread resistance of *C. glabrata* to histatin 5. While the resistance of *C. glabrata* to fluconazole is known to occur in cells exhibiting petite mutations and/or overexpressing specific efflux pumps, the resistance to histatin 5 is not related to either of these two mechanisms. The absence of a correlation between the responses to fluconazole and histatin 5 of the various *C. glabrata* mutants evaluated suggests that the cellular characteristics providing resistance against azoles and cationic antifungal proteins in this fungus are not identical.

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