Deletions of the Endocytic Components *VPS28* and *VPS32* in *Candida albicans* Lead to Echinocandin and Azole Hypersensitivity

Muriel Cornet,[1,2] Claude Gaillardin,[1]* and Mathias L. Richard[1]

*Microbiologie et Génétique Moléculaire, Institut National Agronomique Paris-Grignon, Institut National de la
Recherche Agronomique UMR1238, Centre National de la Recherche Scientifique UMR2585,
78850 Thiverval-Grignon, France,[1] and Microbiologie, Hôtel-Dieu, 1 Place du Parvis Notre-Dame,
75181 Paris Cedex 04, Assistance Publique-Hôpitaux de Paris, Université Paris V,
Paris Descartes, Paris, France[2]*

Vps28p and Vps32p act in both the endocytic and the pH signaling pathways in yeasts and are required for *Candida albicans* virulence. Here, we show that deletions of *VPS28* and *VPS32* increase the susceptibility of *C. albicans* to cell wall disruption agents, echinocandin and azole antifungal agents.

The incidence of invasive fungal infections is rising continuously, and *Candida albicans* is now a major cause of nosocomial infections (11, 15, 17). Although antifungal resistance among invasive *C. albicans* isolates is not common, there is concern about the development of azole resistance due to prophylactic use of fluconazole (13). Thus, in recent years, interest has focused on developing and licensing new agents, such as echinocandins or new triazoles (7, 10, 12, 23).

Vacuolar protein sorting (Vps) factors act in the endosomal sorting complexes required for transmembrane protein recycling and degradation during the last steps of endocytosis (1). Recently, some Vps factors have been documented as required for activation of the Rim pathway, which governs pH sensing in fungi (2, 3, 9, 16, 24). We previously established that Vps28p and Vps32p affect virulence in *C. albicans* through Rim-dependent and -independent pathways (3). Here, we show that, in addition to virulence, these Vps factors affect cell wall maintenance and the susceptibility of *C. albicans* to antifungal agents.

We analyzed strains with *VPS* deleted for sensitivity to two cell wall-perturbing agents: calcofluor white (CFW) and sodium dodecyl sulfate (SDS). Homozygous deletions of *VPS28* ($vps28^{-/-}$) and *VPS32* ($vps32^{-/-}$) were constructed in the BWP17 strain as previously described (3, 6). Complementation of each mutant was achieved by reintroduction of the wild-type allele at the *HIS1* locus. As controls for any Rim-mediated effect, we analyzed the $rim101^{-/-}$ null mutant (4) and introduced *RIM101SL*, a truncated constitutively active allele of *RIM101* (3, 5), into the $vps28^{-/-}$, $vps32^{-/-}$, and $rim101^{-/-}$ mutants and into the DAY286 control strain at the *HIS1* locus (Table 1).

Both the $vps28^{-/-}$ and $vps32^{-/-}$ mutants were hypersensitive to CFW and SDS, with the wild-type phenotype partially restored by complementation (Fig. 1A). This suggests a cell wall disorder compensated for by chitin accumulation and corroborates previous reports of enhanced CFW binding to the cell walls of *vps* mutants (3). Indeed, SDS hypersensitivity reflects facilitated access of detergents to the mutant membrane, whereas hypersensitivity to CFW, a known inhibitor of chitin incorporation in the wall, results from disruption of the chitin-based compensatory mechanism (18, 19). Nikkomycin Z, a chitin synthase inhibitor, was used to investigate this cell wall defect: an increased sensitivity of the $vps28^{-/-}$ mutant was observed, whereas no major effect was observed in the case of the $vps32^{-/-}$ mutant, either on plates (Fig. 1B) or in liquid assays (not shown). This suggests that the compensatory mechanisms elicited by the two *vps* mutations (e.g., chitin overproduction versus chitin deposition) may differ. In *Saccharomyces cerevisiae*, deletions of *VPS28* and *VPS32* led to CFW hyperresistance, unlike other *vps* mutations affecting the same complexes, which also suggests subtle differences in the precise compensatory mechanisms triggered by *vps* mutations (22).

Mutations in the *VPS* genes affect Rim101p activation (3).

TABLE 1. *Candida albicans* susceptibilities[a]

Strain	Geometric mean MIC (μg/ml)[b]			
	Fluconazole	Voriconazole	Caspofungin	Micafungin
ATCC 90028	0.96	0.03	0.05	0.06
DAY185 (control strain)	0.62	0.02	0.06	0.03
$vps28^{-/-}$	0.39	0.01	0.02	0.01
$vps32^{-/-}$	0.32	0.01	0.02	0.01
$vps28^{-/-}$+*VPS28*	0.62	0.03	0.03	0.04
$vps32^{-/-}$+*VPS32*	0.68	0.02	0.03	0.04
DAY286+*RIM101SL*	0.75	0.03	0.04	0.02
$vps28^{-/-}$+*RIM101SL*	0.75	0.02	0.007	0.001
$vps32^{-/-}$+*RIM101SL*	0.62	0.01	0.005	0.001
$rim101^{-/-}$	0.11	0.002	0.005	0.001
$rim101^{-/-}$+*RIM101SL*	0.42	0.01	0.03	0.03

[a] Calculated from at least 10 independent experiments. All strains derive directly from BWP17 (His⁻ Ura⁻ Arg⁻) (4) and are prototrophic. Double disruptions restored a Ura⁺ Arg⁺ phenotype. The His⁺ information was brought by the plasmids carrying *VPS28*, *VPS32*, or *RIM101SL* or by an empty vector, all integrated at *HIS1*. DAY286 is an Arg⁺ Ura⁺ derivative of DAY185.
[b] Results are MIC minus 2 (prominent growth reduction or a 50% reduction in optical density) at 24 h for CAS and MCF and at 48 h for FLC and VRC.

* Corresponding author. Mailing address: Laboratoire de Microbiologie et Génétique Moléculaire, INRA, CBAI, 78850 Thiverval-Grignon, France. Phone: 33 1 30 81 54 53. Fax: 33 1 30 81 54 57. E-mail: claude.gaillardin@grignon.inra.fr.
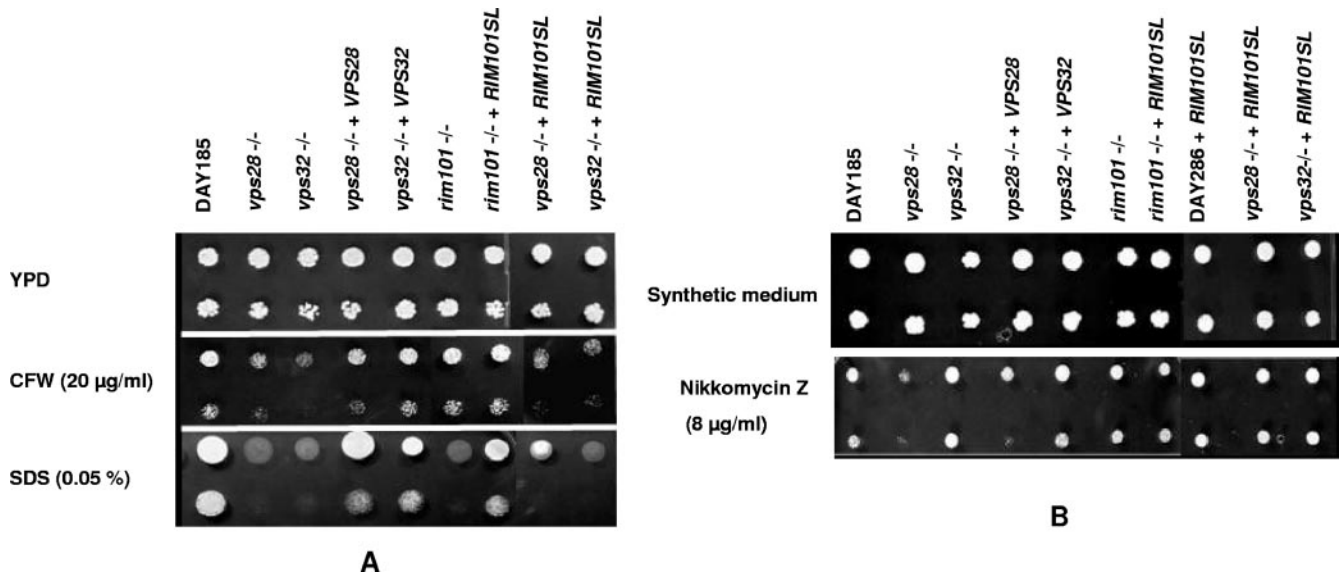
FIG. 1. Sensitivities of *C. albicans* strains to CFW, SDS, and nikkomycin Z. The strains are indicated above the lanes. All strains are prototrophic derivatives of the Arg⁻ Ura⁻ His⁻ strain BWP17 (4). DAY286 is a Ura⁺ Arg⁺ derivative of BWP17 that was transformed by a plasmid carrying *RIM101SL* and *HIS1*. (A) Droplets (3.5 μl) at two dilutions (10^5 and 10^4 cells/ml) were spotted on yeast extract-peptone-dextrose (YPD) medium and YPD medium supplemented with 20 μg/ml CFW (Sigma; fluorescent brightener 28) or 0.05% SDS. The plates were incubated at 30°C for 48 h, except for the plates with 0.05% SDS, which were incubated for 5 days. (B) Droplets (3.5 μl) at two dilutions (10^5 and 10^4 cells/ml) were spotted on synthetic complete medium (3) and synthetic medium supplemented with 8 μg/ml of nikkomycin Z (Sigma). The plates were incubated at 30°C for 48 h.
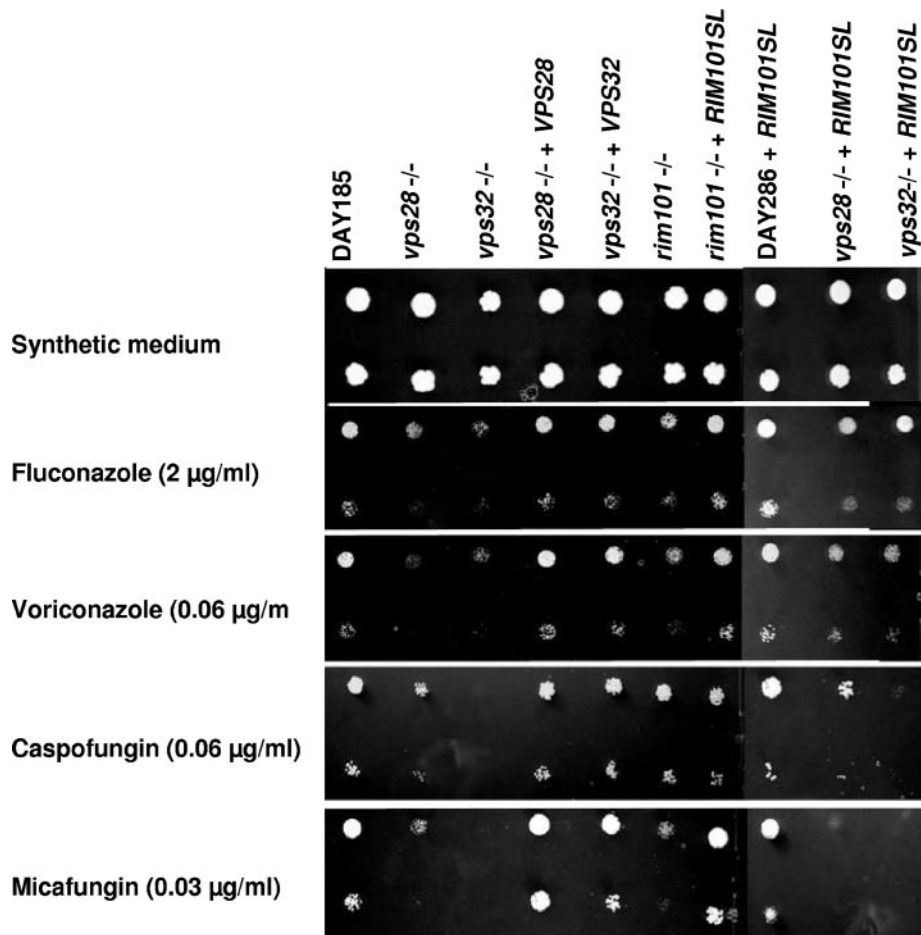

FIG. 2. Sensitivities of *C. albicans* strains to antifungal agents. The strains are indicated above the lanes. All strains are prototrophic derivatives of the Arg⁻ Ura⁻ His⁻ strain BWP17 (4). Droplets (3.5 μl) at two dilutions (10^5 and 10^4 cells/ml) were spotted on synthetic complete medium (3) and synthetic complete medium buffered at pH 7.0 with 150 mM HEPES and supplemented with antifungal agents. The plates were incubated at 37°C for 48 h.

Inactivation of *RIM101* resulted in SDS, but not CFW or nikkomycin Z, susceptibility (Fig. 1, *rim101*$^{-/-}$). SDS sensitivity elicited by *vps* mutations, however, is independent of Rim101p activation, since expression of *RIM101SL*, while suppressing the SDS sensitivity of the *rim101*$^{-/-}$ mutant, had no effect on *vps28*$^{-/-}$ and *vps32*$^{-/-}$ mutant susceptibilities (Fig. 1A). Similarly, the CFW sensitivities of *vps* mutants, which were not bypassed by *RIM101SL* and were not displayed by the *rim101*$^{-/-}$ mutant, appeared to be independent of an effect of *vps* genes on the Rim pathway. Expression of *RIM101SL*, surprisingly, increased the nikkomycin Z tolerance of the control strain (DAY286+*RIM101SL*) and of the *vps28*$^{-/-}$ mutant (Fig. 1B). This shows that, although inactivation of the Rim pathway has no effect on nikkomycin Z tolerance, constitutive activation of the pathway increases tolerance for unknown reasons.

The yeast cell wall is constructed around large, intricate glucan molecules (8). Since *VPS* genes affect cell wall integrity, we tested them for a possible effect on susceptibility to antifungal drugs, like echinocandins, which specifically target β-1,3-glucan synthesis.

The MICs of the echinocandins caspofungin (CAS) and micafungin (MCF) and of the azoles fluconazole (FLC) and voriconazole (VRC) were determined using the CLSI (formerly NCCLS) broth microdilution method (12a). We discarded experiments showing reduced growth of the strains with deletions compared to the DAY185 control (4). Both *vps28*$^{-/-}$ and *vps32*$^{-/-}$ null mutants showed MICs different from that of DAY185 (Table 1), but the results were not consistently reproducible.

Indeed, although the CLSI protocol is recommended for clinical MIC testing, agar plate assays seem to be more reliable for mutant analysis (20, 21). Agar plate assays, carried out on synthetic complete medium containing semi-inhibitory drug concentrations, confirmed that both *vps28*$^{-/-}$ and *vps32*$^{-/-}$ null mutants were more susceptible to FLC, VRC, CAS, and MCF than the isogenic DAY185 control strain (Fig. 2). Growth inhibitions in these assays were clear, thus validating MIC observations (Fig. 2 shows one example). Growth inhibition of the strains with *VPS* deleted was observed over the following concentration ranges (in μg/ml): 1 to 4 for FLC, 0.03 to 0.12 for VRC and CAS, and 0.015 to 0.06 for MCF. The slight growth defect observed for the *vps32*$^{-/-}$ mutant on the no-drug control plates was confirmed by growth rate measurements (3) but was not significant enough (2.3 h versus 1.7 h) to account for the hypersensitivity of the strain. The susceptibilities of the strains with *VPS* deleted to amphotericin B and flucytosine were not affected (data not shown). Complementation restored the susceptibility of the control strain to all drugs tested (Fig. 2 and Table 1). The *rim101*$^{-/-}$ deletion strain was also hypersensitive to FLC, VRC, CAS, and MCF, and introduction of the constitutive allele *RIM101SL* restored wild-type susceptibility (the *rim101*$^{-/-}$ strain is hypersensitive to 0.12 μg/ml of CAS on agar plates [data not shown]). *RIM101SL* also restored normal resistance levels to azoles in *VPS* mutants (Fig. 2 and Table 1), confirming direct involvement of the Rim pathway in azole sensitivity, as in *S. cerevisiae* (14). The echinocandin hypersensitivity of the *vps* null mutants appeared to be independent of the Rim pathway, since the *RIM101SL* allele had no suppressive effect on the echinocandin hypersensitivity of *vps28*$^{-/-}$ and *vps32*$^{-/-}$ mutants.

In conclusion, our data show that deletions of *VPS28* and *VPS32* increase *C. albicans* susceptibility to drugs that directly interfere with cell wall assembly at the level of chitin synthesis and/or deposition (nikkomycin Z and CFW) or at the level of β-1,3-glucan network construction (echinocandins) independently of the Rim pathway. These data suggest that *vps* mutations perturb localization and/or recycling of surface proteins involved in cell wall maintenance. They also lead to azole hypersensitivity, but the latter phenotype seems to result directly from their effect on the Rim pathway.

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