Endosomal Trafficking Defects Can Induce Calcium-Dependent Azole Tolerance in Candida albicans

Arturo Luna-Tapia, Hélène Tournu, Tracy L. Peters, Glen E. Palmer

Department of Clinical Pharmacy, Division of Clinical and Experimental Therapeutics, College of Pharmacy, University of Tennessee Health Sciences Center, Memphis, Tennessee, USA

The azole antifungals arrest fungal growth through inhibition of ergosterol biosynthesis. We recently reported that a Candida albicans vps21Δ/Δ mutant, deficient in membrane trafficking through the late endosome/prevacuolar compartment (PVC), continues to grow in the presence of the azoles despite the depletion of cellular ergosterol. Here, we report that the vps21Δ/Δ mutant exhibits less plasma membrane damage upon azole treatment than the wild type, as measured by the release of a cytoplasmic cellular Ca2+. And, in the presence of fluconazole, enhanced expression of a calcineurin-responsive RTA2-GFP reporter. Furthermore, the azole tolerance phenotype of the vps21Δ/Δ mutant is dependent upon both extracellular calcium levels and calcineurin activity. These findings underscore the importance of endosomal trafficking in determining the cellular consequences of azole treatment and indicate that this may occur through modulation of calcium- and calcineurin-dependent responses.

Several important antifungal drugs inhibit the synthesis of ergosterol, a lipid that modulates the thickness, fluidity, and permeability of fungal cell membranes (1). These drugs include the azoles, which inhibit lanosterol demethylase (Erg11p). Both the depletion of cellular ergosterol and the accumulation of intermediate sterol species are thought to cause plasma membrane dysfunction and, ultimately, growth arrest following azole treatment (2). The emergence of azole-resistant fungal isolates is a problem of increasing medical significance (3). Several specific mechanisms that contribute to the development of azole resistance in the prevalent human fungal pathogen Candida albicans have been described. These mechanisms include increased expression of the target enzyme, point mutations that alter the target enzyme’s affinity for the azoles (4, 5), and increased expression of efflux pumps, such as Cdr1p and Mdr1p, that export the azoles out of the fungal cell (6–8). However, the azole resistance of many fungal isolates is not fully accounted for by these well-characterized mechanisms. We recently reported that membrane trafficking through the late endosome/prevacuolar compartment (PVC) has a significant impact upon C. albicans azole tolerance (9). Specifically, we found that a deletion of VPS21, which encodes a Rab GTPase required for PVC trafficking, substantially enhanced C. albicans growth in the presence of the azoles. The azole tolerance of the vps21Δ/Δ mutant does not depend upon established mechanisms of azole resistance such as the activity of the well-characterized drug efflux pumps Mdr1p and Cdr1p, or increased target protein expression, but occurs despite the depletion of cellular ergosterol (9). This phenotype resembles an exaggerated form of “trailing growth,” a phenomenon that is observed using standard CLSI antifungal susceptibility testing protocols (10), in which a subset of C. albicans isolates appear to be azole susceptible at early time points but display significant growth at later time points (10). The objective of this study was to define the mechanism(s) underlying the azole tolerance of the C. albicans vps21Δ/Δ mutant.

MATERIALS AND METHODS

Growth conditions. C. albicans was routinely grown at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) supplemented with uridine (50 μg/ml) when necessary. Transformant selection was carried out on minimal YNB medium (6.75 g/liter yeast nitrogen base without amino acids, 2% dextrose, 2% Bacto agar) supplemented with the appropriate auxotrophic requirements, as described previously for Saccharomyces cerevisiae (11), or 50 μg/ml uridine.

Plasmid construction. Plasmid pLUX (12) was kindly provided by William Fonzi (Georgetown University). Plasmids pLUXVPS21 (13), pKE1 (14), and pKE1-NLUC (15) were described previously. All oligonucleotides used in this study are listed in Table S1 in the supplemental material. The VPH1 open reading frame (ORF) with 3′- and 3′-untranslated region (UTR) sequences was amplified from SC5314 genomic DNA (gDNA) with HiFi Platinum Taq (Invitrogen) and primer set VPH1AMPF and VPH1AMPR-KpnI and cloned between the KpnI and SalI sites of pKE1 in place of the ACT1prF-KpnI and ACT1prR-SalI and cloned between the ScaI and KpnI restriction sites of plLUX to produce plasmid pLUXVPH1.

For the construction of reporter plasmid pRTA2prGFPpy, 1,000 bp of the RTA2 promoter was amplified from SC5314 gDNA with primer pair RTA2prF-KpnI and RTA2prR-Sall and cloned between the KpnI and Sall sites of pKE1 in place of the ACT1 promoter. The GFPpy (green fluorescent protein) coding sequence was then amplified using primers GFPAMPF-Sall and GFPAMPR-MluI and cloned downstream of the RTA2 promoter between the Sall and MluI sites.

C. albicans strains. All strains used in this study are listed in Table S2 in the supplemental material. The vps21Δ/Δ, aps3Δ/Δ, and vps21Δ/Δ.
aps2ΔΔ mutants were constructed in previous studies (13, 14, 16, 17). Control strain YPH6284 (18) was kindly provided by Judith Berman (Tel Aviv University). Transformation of C. albicans with DNA constructs was performed by using the lithium acetate method (19). Gene deletion strains were constructed by a PCR-based approach described previously by Wilson et al. (20), using ura3ΔΔ his1ΔΔ arg4ΔΔ strain BWP17 (kindly provided by Aaron Mitchell, Carnegie Mellon University). Strain CA4 was kindly provided by William Fonzi (Georgetown University).

VPH1 deletion cassettes were amplified by PCR with primers VPH1DISF and VPH1DISR, using pRS-ARG4ΔSpel, pGEM-HIS1, or pDDB57 (containing a recyclable URA3-dpl200 marker) (20, 21) as the template. Each VPH1 allele was sequentially deleted by using HIS1 and ARG4 markers to generate vph1ΔΔ ura3ΔΔ vector fully restores pLUXVPH1 after linearization with NheI. The correct integration of the VPH1ΔΔ allele was detected by PCR with the following primers sets: ARG4INTF2 plus VPH1DETR2 and URA3INTF2 plus VPH1DETR2 and URA3INTR2 plus VPH1DETR2. The correct integration of the pLUX vector fully restores URA3 and adjacent IRO1 loci, and this was confirmed by PCR using primer pairs LUXINTDETF and LUXINTDETR.

A previously described vps21ΔΔ ura3ΔΔ arg4ΔΔ strain (13) was used to construct the vps21ΔΔΔ vph1ΔΔ mut double mutant. The first allele of VPH1 was deleted with the URA3-dpl200 selection marker, and the second allele was deleted with the ARG4 marker. Replacement of the first VPH1 allele with the URA3-dpl200 selection marker was confirmed by using primer pairs URA3INTF2 plus PHHTDETR2 and URA3INTR2 plus PHHTDETR2. The resulting vps21ΔΔΔ vph1ΔΔΔ double mutant was then selected on YNB agar plates supplemented with uridine and 5-fluoroorotic acid (5-FOA) (22) to select for ura3ΔΔ segments that had excised the URA3 marker gene. The correct excision of the URA3 marker from the vph1ΔΔΔ vps21ΔΔΔ strain was confirmed by using the flanking primer pair VPH1AMPF and VPH1AMPR-Kpnl. Prototrophic and isogenic mutant and reconstituted control strains were made by transforming the strains (see Fig. S1 in the supplemental material), and the

RESULTS

The C. albicans vps21ΔΔΔ mutant is less susceptible than wild type to azole-induced membrane damage. We previously reported that the vps21ΔΔΔ mutant exhibits enhanced growth in the presence of the azole antifungals versus wild-type (WT) controls, despite ergosterol depletion (9). We therefore examined plasma membrane integrity following azole treatment using a luciferase-based assay (15) to determine if the vps21ΔΔΔ mutant exhibited signs of membrane damage. A cytoplasmic version of nanoluciferase (NLuc; Promega Corp.) (23) was expressed in mutant and control strains (see Fig. S1 in the supplemental material), and the amount of NLuc released into the culture supernatant was com-
pared following treatment with fluconazole. Fluconazole treatment at 35°C caused significant Nluc release from the WT strain, indicating a loss of plasma membrane integrity (Fig. 1A). However, at this temperature, the vps21Δ/Δ mutant released significantly less Nluc upon fluconazole treatment than the WT, indicating less membrane damage. In contrast, at 30°C, the mutant released levels of Nluc that were similar to, or even slightly higher than, those released by the WT (Fig. 1B), indicating that fluconazole induces similar levels of membrane permeabilization in either strain at the lower temperature. This is consistent with our previously reported observation that the continued growth of the vps21Δ/Δ mutant in the presence of the azoles (as measured by the OD600) occurs at 35°C but not at 30°C (9). To further confirm the temperature dependence of the azole tolerance of the vps21Δ/Δ mutant, we compared its growth with and without fluconazole using two additional measures, dry weight and CFU (Fig. 1C and D). By both measures, at 35°C, the vps21Δ/Δ mutant was more tolerant of fluconazole than the WT, while at 30°C, the growth of both strains was severely inhibited. As such, it appears that at 35°C, the azole antifungals cause less membrane damage to the vps21Δ/Δ mutant than to the WT, and this likely accounts for its continued growth.

Azole tolerance of the C. albicans vps21Δ/Δ mutant does not depend upon Vph1p function. A recent study reported that inhibition of the V-ATPase proton pump that is responsible for the acidification of the vacuole may in part underlie the antifungal activity of the azoles (1). In S. cerevisiae, a vps21Δ/Δ mutant is deficient in the delivery of the V-ATPase to the fungal vacuole and thus vacuolar acidification, as evidenced by reduced quinacrine accumulation (26). In contrast, we observed that the C. albicans vps21Δ/Δ mutant accumulates more quinacrine than the control strains, consistent with increased vacuolar acidification (see Fig. S2 in the supplemental material). To determine if the azole tolerance of the vps21Δ/Δ mutant depends upon V-ATPase activity, we deleted both alleles of VPH1, a gene encoding a vacuole-specific subunit of the V-ATPase (27). However, the loss of VPH1 did not affect the susceptibility of the wild-type control strain to fluconazole or the trailing growth of the vps21Δ/Δ mutant (Fig. 2). These data demonstrate that the azole tolerance of the vps21Δ/Δ mutant does not depend upon V-ATPase activity.

The vps21Δ/Δ mutant has abnormal intracellular calcium levels. Ca2+-based signaling has been shown to induce adaptive responses that promote C. albicans survival following azole treatment (28). The fungal vacuole is the major storage site for intra-

FIG 1 The C. albicans vps21Δ/Δ mutant exhibits less membrane damage following azole treatment. (A and B) The cytoplasmic Nluc expression vector pKE1-Nluc was introduced into the vps21Δ/Δ mutant and wild-type control (CAI4) strains. Each strain was then grown in 96-well plates in YNB medium with various concentrations of fluconazole or DMSO solvent (minus-drug control). After 24 h of incubation at 35°C (A) or 30°C (B), the culture supernatant was removed from each well, and Nluc activity was assayed by using a luminescent substrate. Nluc activity measured in relative light units was normalized for fungal growth (OD600) and expressed as a percentage of the value for the minus-drug controls. The means and standard deviations of data from three biological replicates are shown for each drug concentration. (C and D) vps21Δ/Δ mutant and WT strains of C. albicans were introduced into YNB at 2 × 10⁶ cells/ml in the presence of 0.5% DMSO or 5 μg/ml of fluconazole (FLU) and grown at 30°C or 35°C. After 24 h, biomass was measured as dry weight (C), and cell viability was determined as CFU (D). Dry weight and CFU are expressed as percentages of the values for the DMSO control for each strain. The means ± standard deviations of data from two biological replicates are shown for panels C and D. The means for each group were compared by using a two-tailed t test. *, P < 0.05.
cellular Ca\(^{2+}\) (29). Therefore, to determine if the vacuolar trafficking defects of the vps21\(\Delta\) \(\Delta\) mutant impact cellular Ca\(^{2+}\) homeostasis, we compared the levels of intracellular free calcium using the calcium-responsive dye Fura 2-AM (30). The wild-type strain treated with the antiarrhythmic drug amiodarone (AMD), which causes cytoplasmic Ca\(^{2+}\) surges in S. cerevisiae (31), was used as a positive control and induced a dose-dependent increase in the F\(_{340}/F_{380}\) ratio (see Fig. S3 in the supplemental material). Interestingly, the F\(_{340}/F_{380}\) ratio of Fura 2-AM-stained vps21\(\Delta\) \(\Delta\) mutant cells was also significantly higher than that of WT control cells, indicating that the mutant has abnormally high intracellular Ca\(^{2+}\) levels (Fig. 3).

The azole tolerance of the vps21\(\Delta\) \(\Delta\) mutant is dependent upon extracellular calcium availability. We next examined if the azole tolerance phenotype of the vps21\(\Delta\) \(\Delta\) mutant was affected by the availability of extracellular Ca\(^{2+}\). The addition of 1 mM CaCl\(_2\) to the medium had little effect upon the growth of either the vps21\(\Delta\) \(\Delta\) mutant or control strains in the presence of fluconazole (Fig. 4D and E). Dihydropyridines were previously shown to induce azole tolerance in C. albicans (28). Upon azole treatment at 35°C, suggesting that it sustains less membrane damage. We also found that the vps21\(\Delta\) \(\Delta\) mutant releases less cytoplasmic content than do WT strains following fluconazole treatment at 35°C, which facilitates the influx of external Ca\(^{2+}\) into the cytoplasm.

The azole tolerance of the vps21\(\Delta\) \(\Delta\) mutant is dependent upon calcineurin activity. Calcineurin-dependent activation of the calcineurin serine/threonine protein phosphatase was previously shown to induce azole tolerance in C. albicans (28). Upon azole exposure, increased calcineurin activity enhances the transcription of a number of specific genes involved in plasma membrane maintenance and the stress response, including RTA2 (33, 34). Since the vps21\(\Delta\) \(\Delta\) mutant has abnormal levels of intracellular Ca\(^{2+}\), we investigated the possibility that calcineurin-dependent responses contribute to its azole tolerance. To compare calcineurin-responsive gene expression levels in mutant and control strains, we used a reporter construct consisting of the GFP coding sequence (35) under the control of the calcineurin-responsive RTA2 promoter (34, 36). As expected, treatment with fluconazole enhanced GFP expression in the control strain (Fig. 5A). Furthermore, treatment with the calcineurin inhibitor cyclopiazonic suppressed fluconazole-induced expression of GFP (see Fig. S4 in the supplemental material), establishing the responsiveness of this reporter construct to calcineurin activity. Interestingly, treatment of the vps21\(\Delta\) \(\Delta\) mutant with fluconazole induced a significantly larger response from the RTA2pr-GFP reporter than that observed for the WT control strain (Fig. 5A). Finally, inhibition of calcineurin activity using either cyclopiazonic (Fig. 5B to D) or tacrolimus (FK506) (see Fig. S5 in the supplemental material) was sufficient to abolish the growth of the vps21\(\Delta\) \(\Delta\) mutant in the presence of fluconazole. Thus, the ability of the vps21\(\Delta\) \(\Delta\) mutant to grow in the presence of the azole antifungals depends upon calcineurin activity.

DISCUSSION

In a previous study, we established that the C. albicans vps21\(\Delta\) \(\Delta\) vacuolar trafficking mutant, deficient in transport through the PVC, is able to grow in the presence of fluconazole at 35°C despite ergosterol depletion (9). In contrast, a C. albicans vps15\(\Delta\) \(\Delta\) mutant, deficient in retrograde trafficking from the PVC back to the Golgi apparatus (37), as well as two mutants unable to form multivesicular bodies within the PVC (38) are sensitive to fluconazole. Collectively, these data indicate that membrane trafficking through the PVC is an important determinant of the cellular consequences of azole treatment. Here, we report that the vps21\(\Delta\) \(\Delta\) mutant releases less cytoplasmic content than do WT strains following fluconazole treatment at 35°C, suggesting that it sustains less membrane damage. We also found that the vps21\(\Delta\) \(\Delta\) mutant has abnormally high levels of intracellular Ca\(^{2+}\) compared to those of the WT under routine culture conditions. Finally, the mutant’s continued growth in the presence of the azoles depends upon the availability of extracellular Ca\(^{2+}\) and is abrogated by inhibitors of the calcineurin phosphatase.
The fungal vacuole is the major intracellular storage site of calcium. Accordingly, the transient cytoplasmic Ca$^{2+}$/H$^{+}$ fluxes that activate calcineurin are regulated by Yvc1p, a channel that releases vacuolar calcium (31), and the Pmc1p and Vcx1p pumps that sequester cytoplasmic calcium into the vacuole (39, 40). As for many integral membrane proteins, Yvc1p, Pmc1p, and Vcx1p are likely delivered from the Golgi apparatus to the vacuole via the PVC (41). Thus, inefficient localization of these calcium transporters to the fungal vacuole in the vps21Δ/Δ mutant may underlie the elevated cytoplasmic calcium levels. Abnormal Ca$^{2+}$ homeostasis could potentially increase the basal level of calcineurin activity and/or affect the magnitude or duration of calcineurin signaling in the mutant, in turn enhancing azole tolerance. This is supported by the RTA2 reporter data, which indicate that the vps21Δ/Δ mutant exhibits enhanced expression of calcineurin-responsive genes in the presence of fluconazole. However, in preliminary studies, we have found that the deletion of either the PMC1 or VCX1 gene alone is not sufficient to impact the susceptibility of C. albicans to fluconazole or induce trailing growth under standard CLSI assay conditions (see Fig. S6 in the supplemental material). Nonetheless, it is clear from our experiments that chemical inhibition of calcineurin is sufficient to abrogate the growth of the vps21Δ/Δ mutant in the presence of fluconazole. Interestingly, Liu et al. (42) showed that dihydropyridine treatment suppresses the fluconazole-induced expression of the two calcineurin subunits, sensitizing azole-resistant C. albicans isolates to fluconazole. Thus, the

FIG 4 Azole tolerance of the C. albicans vps21Δ/Δ mutant depends upon extracellular calcium. (A) The susceptibility of the C. albicans vps21Δ/Δ mutant to fluconazole was compared to those of the wild-type (YJB6284) and isogenic reconstituted strains in medium supplemented with 1 mM CaCl$_2$ by using standard CLSI broth microdilution protocols. Growth was measured as the OD$_{600}$ and expressed as a percentage of the values for the minus-drug (DMSO) control wells. (B and C) Comparison of the growth of the C. albicans vps21Δ/Δ mutant in the presence of various concentrations of the Ca$^{2+}$ chelator EGTA without (B) and with (C) 1 μg/ml of fluconazole. For each strain, growth is expressed as a percentage of the values for the minus-fluconazole, minus-EGTA control. (D and E) Comparison of the growth of the vps21Δ/Δ mutant, wild-type (YJB6284), and reconstituted strains in dose-response experiments with the Ca$^{2+}$ channel blocker nisoldipine (NIS) in the absence (D) or presence (E) of 1 μg/ml fluconazole. The growth of each strain is expressed as a percentage of the growth in the minus-drug (DMSO) control wells. The means ± standard deviations of data from two biological replicates are shown in each panel. The means for each group were compared by using a two-tailed $t$ test. *, $P < 0.05$. 

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resensitization of the \textit{vps21}\textsuperscript{Δ/Δ} mutant by nisoldipine is also consistent with the mutant’s azole tolerance depending upon enhanced calcineurin-dependent responses.

The azole tolerance of the \textit{vps21}\textsuperscript{Δ/Δ} mutant contrasts with data for a previously reported \textit{vps15}\textsuperscript{Δ/Δ} mutant defective in retrograde trafficking from the PVC back to the Golgi apparatus. Despite also exhibiting elevated expression levels of calcineurin-dependent genes, this mutant is highly sensitive to fluconazole (37). The reasons for this are unclear; however, it is apparent that the specific PVC trafficking step affected is a crucial determinant of the physiological impact of the azoles on the fungal cell. In support of this notion, we found that blocking of the AP-3-mediated Golgi apparatus-to-vacuole trafficking route through the deletion of \textit{APS3} (17) is sufficient to eliminate the trailing growth of the \textit{vps21}\textsuperscript{Δ/Δ} mutant (see Fig. S7 in the supplemental material), indicating that its azole tolerance depends upon this pathway. However, the loss of the AP-3-mediated pathway alone has no impact on the azole susceptibility of \textit{C. albicans} (9). Collectively, these findings indicate that complex interactions occur between the PVC membrane-trafficking and ergosterol biosynthetic pathways of \textit{C. albicans}.

Studies performed to date have suggested that the trailing-
growth phenotype is usually associated with in vivo susceptibility to azole therapy (43, 44). However, it is unclear how, or if, the azole-tolerant phenotype of the $rps21A/\Delta$ mutant relates to that of trailing clinical isolates or if these clinical isolates have abnormal endosomal trafficking. A first step in establishing the potential clinical relevance of our observations will be to examine the azole susceptibility of the $rps21A/\Delta$ mutant in vivo using an appropriate mouse model of infection.

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