Fluconazole Transport into *Candida albicans* Secretory Vesicles by the Membrane Proteins Cdr1p, Cdr2p, and Mdr1p

Luiz R. Basso, Jr., Charles E. Gast, Yuxin Mao, and Brian Wong*

Division of Infectious Diseases, Department of Medicine, Oregon Health & Science University, Portland, Oregon 97239

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A major cause of azole resistance in *Candida albicans* is overexpression of *CDR1*, *CDR2*, and/or *MDR1*, which encode plasma membrane efflux pumps. To analyze the catalytic properties of these pumps, we used *ACT1*- and *GAL1*-regulated expression plasmids to overexpress *CDR1*, *CDR2*, or *MDR1* in a *C. albicans* *cdr1 cdr2 mdr1*-null mutant. When the genes of interest were expressed, the resulting transformants were more resistant to multiple azole antifungals, and accumulated less [3H]fluconazole intracellularly, than empty-vector controls. Next, we used a *GAL1*-regulated dominant negative *sec4* allele to cause cytoplasmic accumulation of post-Golgi secretory vesicles (PGVs), and we found that PGVs isolated from *CDR1*-, *CDR2*-, or *MDR1*-overexpressing cells accumulated much more [3H]fluconazole than did PGVs from empty-vector controls. The *K*ₐₚₛ (expressed in micromolar concentrations) and *V*ₘₐₓₛ (expressed in picomoles per milligram of protein per minute), respectively, for [3H]fluconazole transport were 0.8 and 0.91 for Cdr1p, 4.3 and 0.52 for Cdr2p, and 3.5 and 0.59 for Mdr1p. [3H]fluconazole transport by Cdr1p and Cdr2p required ATP and was unaffected by carbonyl cyanide 3-chlorophenylhydrazone (CCCP), whereas [3H]fluconazole transport by Mdr1p did not require ATP and was inhibited by CCCP. [3H]fluconazole uptake by all 3 pumps was inhibited by all other azoles tested, with 50% inhibitory concentrations (*IC₅₀*s; expressed as proportions of the [3H]fluconazole concentration) of 0.2 to 5.6 for Cdr1p, 0.3 to 3.1 for Cdr2p, and 0.3 to 3.1 for Mdr1p. The methods used in this study may also be useful for studying other plasma membrane transporters in *C. albicans* and other medically important fungi.

*Candida albicans* is a major cause of serious infections in immunocompromised patients. Azole antifungals are widely used to treat *C. albicans* infections, but resistance to this class of drugs has been reported frequently (40). The ability of *C. albicans* to pump azoles out of the cell is an important drug resistance mechanism, and several groups have shown that the most important azole efflux pumps in the plasma membrane of *C. albicans* are the ATP-binding cassette (ABC) transporters Cdr1p and Cdr2p and the major facilitator superfamily (MFS) transporter Mdr1p (40). A great deal is now known about transcriptional regulation of the genes encoding these pumps (i.e., *CDR1*, *CDR2*, and *MDR1*), but less is known about these pumps’ catalytic properties (3–8, 10, 12, 13, 27, 28, 30, 47, 56–58). One reason for this is that the inaccessibility of the cytoplasmic face of the plasma membrane precludes direct examination of these transporters’ abilities to pump azoles out of intact cells. To circumvent this problem, several groups have studied the ability of *C. albicans* or of *Saccharomyces cerevisiae* cells expressing the *C. albicans* genes of interest to pump fluorescent marker compounds out of the cell. These studies have provided important insights into the energetics and kinetics of these pumps, but the fluorescent compounds used in most of these studies are unrelated structurally or functionally to theazole antifungals (14, 19, 23, 36, 38, 50, 52). Moreover, the fact that *C. albicans* translates the codon CTG as leucine rather than serine complicates the interpretation of results obtained by heterologous expression of CTG-containing *C. albicans* genes in *S. cerevisiae* or other convenient hosts (17, 21, 39, 45, 48, 50).

In the 1990s, a new method was developed for studying plasma membrane efflux pumps (32, 41). The general strategy was to overexpress the transporter of interest in a temperature-sensitive *S. cerevisiae sec6-4* mutant and to use post-Golgi secretory vesicles (PGVs) isolated from spheroplasts to study the transporter’s catalytic properties. Since the membranes of PGVs and whole cells are oriented in opposite directions, transporters that pump substrates out of whole cells pump the same substrates into the lumens of PGVs. Thus, isolated PGVs are especially useful for studying the properties of plasma membrane efflux pumps, and this approach has been used to characterize the transport properties of multiple membrane transporters from yeast, fungi, and mammalian cells (9, 22, 32, 41, 42, 44). Of particular relevance to the present study is the work of Cannon et al. (2), who expressed *C. albicans* *CDR1* in *S. cerevisiae sec6-4* mutants and showed that PGVs isolated from these cells transported [3H]fluconazole into to their lumens in a time-dependent manner. This study established the feasibility of using PGVs to study azole transport by a *C. albicans* membrane transporter, but this approach has not been used to study the catalytic properties of *C. albicans* Cdr1p in detail or to study azole transport by other *C. albicans* efflux pumps.

In an earlier study from this laboratory, Mao et al. (29) showed that *GAL1*-regulated overexpression of a dominant negative allele [sec4(S28N)] of the essential post-Golgi secretion pathway gene *SEC4* in *C. albicans* inhibited the growth and secretion of soluble aspartyl proteases from cells and caused PGVs to accumulate in the cytoplasm. These results suggested that it should be possible to generate PGVs with
C. albicans in the plasma membranes of intact C. albicans cells, (ii) developed a method for isolating functional PGVs from C. albicans, and (iii) used the resulting PGVs to study the catalytic properties of Cdr1p, Cdr2p, and Mdr1p.

MATERIALS AND METHODS

Strains and media. Candida albicans SC5314 was from W. Fonzi (Georgetown University, and C. albicans DSY1050 (Δcdr1::hisGΔcdr2::hisG Δcdr1::hisG URA3::hisG Δcdr1::hisG) was from D. Sanglard (University of Lausanne, Lausanne, Switzerland). C. albicans DSY1050F is a ura3 derivative of C. albicans DSY1050; it was obtained by (i) selecting for growth of C. albicans DSY1050 on 5-fluoroorotic acid (FOA) and (ii) testing stable uridine auxotrophs for growth in minimal medium lacking uridine when they were transformed with plasmids encoding C. albicans URA3.

C. albicans was grown in YP medium (1% yeast extract, 2% peptone) or in minimal YNB medium (0.67% yeast nitrogen base without amino acids) containing 2% glucose, 2% galactose, or 2% raffinose. C. albicans transformed with plasmids conferring hygromycin B resistance were grown either in YP medium with 600 μg hygromycin B per ml and either 2% glucose, 2% galactose, or 2% raffinose or in YNB medium buffered to pH 7.0 with 0.15 M HEPES-NaOH plus 600 μg hygromycin B per ml and either 2% glucose, 2% galactose, or 2% raffinose. C. albicans was grown in YP medium plus galactose, after which the cells were diluted to an optical density at 600 nm (OD600) of 5 in RPMI 1640 medium containing either glucose or galactose, 80 μg/ml uridine, 0.165 M morpholinepropanesulfonic acid (MOPS) (pH 7.0), and graded concentrations of fluconazole, voriconazole, posaconazole, miconazole, itraconazole, clotrimazole, or caspofungin. The presence or absence of visible growth was scored after incubation at 35°C for 48 h.

[1H]Fluconazole accumulation by C. albicans cells. Intracellular [1H]fluconazole was quantified as described by Sanglard et al. (46), with modifications. The frozen cells were resuspended in ATC plus hygromycin B, and the cells were washed in 10 mM Tris-HCl (pH 7.5)–5 mM NaCl, collected by centrifugation, and stored at −80°C.

Isolation and properties of post-Golgi secretory vesicles. C. albicans DSY1050F transformed with pACT1-CDR1, pACT1-CDR2, pACT1-MDR1, or pACT1 was transformed again with pS28N, and the resulting transformants were expanded in YNB-glucose plus hygromycin B. To induce PGV accumulation, cells were washed in YNB-galactose and were then grown for 7 h in YNB-galactose plus hygromycin B at 30°C. Cell growth was stopped by the addition of NaN3, collected by centrifugation, and stored at −80°C. Post-Golgi secretory vesicles were isolated as described by Ruetz and Gрос (42), with modifications. The frozen cells were resuspended in 100 mM Tris-SO4 (pH 9.4) at 25°C, collected by centrifugation, and converted to spheroplasts with Zymolase 20T (25 mg/g [wet weight] of cells, 1 h, 30°C) in SM buffer (1.4 M sorbitol, 20 mM HEPES–KOH [pH 7.0] supplemented with 10 mM NaCl2, 2 mM MgCl2, and 40 mM β-mercaptoethanol). The spheroplasts were washed twice in SM buffer with 10 mM NaCl2, and then incubated on ice in SM buffer (5 mg of cells) supplemented with 1 mM CaCl2, 5 mM MnSO4, and concanavalin A (1.5 mg/g of cells) for 15 min. The spheroplasts were collected by centrifugation and washed twice with cold SM buffer, after which they were incubated in hypotonic lysis buffer (0.6 M sorbitol, 20 mM HEPES–KOH [pH 7.0], 2 mM EDTA, protease inhibitor cocktail for use with fungal and yeast extracts [diluted 1:100; Sigma]) for 10 min on ice. The spheroplasts were further disrupted by Dounce homogenization (30 strokes), and the lysate was centrifuged (10,000 × g, 10 min, 4°C). The supernatant was centrifuged to remove additional cell debris (13,000 × g, 10 min, 4°C). The supernatant was then centrifuged (100,000 × g, 45 min, 2°C) to pellet the PGVs, which were resuspended in glucose or nitrate vesicle buffer (30 mM sucrose, 10 mM Tris–HEPES [pH 7.5], and either 100 mM potassium glucose or 100 mM potassium nitrate) with 5 mM EGTA. The resuspended PGV samples were centrifuged (100,000 × g, 45 min, 2°C). The final pellet was resuspended in glucose or nitrate vesicle buffer. Total protein concentrations in PGV samples were determined with the Bradford reagent using bovine serum albumin as a reference. PGV samples were adjusted to 0.5 mg of total protein/ml in glucose or nitrate buffer. [1H]Fluconazole uptake was initiated by adding PGV samples to prewarmed (37°C) glucose or nitrate buffer supplemented with ATP (2.5 mM), creatine phosphate (10 mM), creatine phosphokinase (3 μM/ml), and [1H]fluconazole (0.05 μM). [1H]Fluconazole transport was interrupted by the addition of an ice-cold stop solution (200 mM sucrose, 10 mM Tris–HCl [pH 7.5], 25 μM fluconazole), and the PGVs were collected as described previously. After two
TABLE 2. Effects of CDR1, CDR2, or MDR1 overexpression on antifungal MICs for DSY1050F cells

| Antifungal | MIC (μg/ml) for DSY1050F cells transformed with the following plasmid: |
|------------|-------------------------------------------------|
|            | None | pACT | pACT1-CDR1 | pACT1-CDR2 | pACT1-MDR1 |
| Fluconazole | 0.032 | 0.032 | 0.25 | 0.187 | 0.125 |
| Voriconazole | 0.016 | 0.016 | 0.187 | 0.032 | 0.062 |
| Posaconazole | 0.032 | 0.032 | 0.187 | 0.062 | 0.125 |
| Miconazole | 0.004 | 0.004 | 0.032 | 0.016 | 0.008 |
| Itraconazole | 0.125 | 0.125 | 0.50 | 0.187 | 0.250 |
| Clotrimazole | 0.008 | 0.008 | 0.062 | 0.016 | 0.032 |
| Caspofungin A | 0.125 | 0.125 | 0.125 | 0.125 | 0.125 |

RESULTS

Effects of expressing CDR1, CDR2, and MDR1 in C. albicans.

The cdr1 cdr2 mdr1-null mutant C. albicans DSY1050F had a much lower fluconazole MIC than did its wild-type parent, C. albicans SC5314. When C. albicans DSY1050F was transformed with pACT1-CDR1, pACT1-CDR2, or pACT1-MDR1, the fluconazole MICs increased at least 4-fold, and similar increases were observed in the MICs of several other antifungal azoles. In contrast, transformation of C. albicans DSY1050F with pACT1 alone had no effect on susceptibility to anyazole antifungal, and transformation of C. albicans DSY1050F with pACT1-CDR1, pACT1-CDR2, or pACT1-MDR1 had no effect on susceptibility to the echinocandin antifungal caspofungin (Table 2).

Whether these changes in the fluconazole MIC were associated with differences in intracellular fluconazole levels was assessed by quantifying intracellular [3H]fluconazole levels at intervals after the cells of interest were incubated in 0.05 μM [3H]fluconazole. We found that (i) C. albicans DSY1050F cells transformed with pACT1 accumulated substantially more intracellular [3H]fluconazole than did C. albicans SC5314 cells and (ii) C. albicans DSY1050F cells transformed with pACT1-CDR1, pACT1-CDR2, or pACT1-MDR1 accumulated less intracellular [3H]fluconazole than did pACT1-transformed controls and more intracellular [3H]fluconazole than did C. albicans SC5314 (Fig. 1A). Furthermore, immunoreactive proteins of the sizes expected for Flag-tagged Cdr1p, Cdr2p, and Mdr1p, respectively, were found in whole-cell lysates of the pACT1-CDR1, pACT1-CDR2, and pACT-MDR1 transformants probed with anti-Flag antibodies but not in Western blots of lysates of pACT1-transformed controls (empty vector) (100 μg total protein per lane).

FIG. 1. Effect of ACT1-regulated overexpression of CDR1, CDR2, or MDR1. (A) Intracellular [3H]fluconazole levels after incubation in 0.05 μM [3H]fluconazole for the times shown were highest in C. albicans DSY1050F cells transformed with pACT1 alone (empty vector), lower in C. albicans DSY1050F cells transformed with pACT1-CDR1 (CDR1), pACT1-CDR2 (CDR2), or pACT1-MDR1 (MDR1), and lowest in wild-type C. albicans SC5314. Data are means ± SD for 3 experiments. (B) Immunoreactive proteins of the sizes expected for Flag-tagged Cdr1p, Cdr2p, and Mdr1p, respectively, were demonstrated in Western blots of lysates of the pACT1-CDR1, pACT1-CDR2, and pACT-MDR1 transformants probed with anti-Flag antibodies.
CDR2, and pACT1-MDR1 transformants but not in lysates of pACT1-transformed controls (Fig. 1B).

Since overexpression of CDR1, CDR2, and MDR1 could increase fluconazole MICs and decrease intracellular [3H]fluconazole levels by decreasing fluconazole uptake and/or by increasing fluconazole efflux, we also examined the effects of expressing CDR1, CDR2, and MDR1 under the control of the regulatable C. albicans GAL1 promoter. The fluconazole MICs were substantially higher when C. albicans transformed with pGAL1-CDR1, pGAL1-CDR2, or pGAL1-MDR1 (but not pGAL1 alone) was incubated in inducing medium (galactose) than when these transformants were incubated in repressing medium (glucose) (Fig. 2A). Next, the pGAL1-CDR1, pGAL1-CDR2, and pGAL1-MDR1 transformants were incubated in YNB medium with the noninducing and nonrepressing sugar raffinose (2%) and 0.05 μM [3H]fluconazole until steady-state intracellular [3H]fluconazole levels were achieved, and intracellular [3H]fluconazole was quantified after either 2% galactose or 2% raffinose was added to the cell suspensions. Intracellular [3H]fluconazole levels fell substantially by 2 h and then returned to baseline levels by 6 h after 2% galactose was added to the pGAL1-CDR1, pGAL1-CDR2-, or pGAL1-MDR1 transformants, but not after 2% galactose was added to controls transformed with pGAL1 (Fig. 2B). In contrast, intracellular [3H]fluconazole levels did not change when 2% raffinose was added to pGAL1-CDR1, pGAL1-CDR2, pGAL1-MDR1, or pGAL1 transformants (data not shown). To determine if intracellular [3H]fluconazole increased to baseline levels by 6 h because of galactose depletion, we added 2% galactose to suspensions of pGAL1-CDR1, pGAL1-CDR2-, or pGAL1-MDR1 transformants at 0, 1, 2, 3, and 4 h. Intracellular [3H]fluconazole levels in these transformants again fell sharply by 1 to 2 h and remained low through 6 h (data not shown). Lastly, Flag-tagged forms of Cdr1p, Cdr2p, and Mdr1p, respectively, were demonstrated by Western blotting of whole-cell lysates of pGAL1-CDR1, pGAL1-CDR2, and pGAL-MDR1 transformants 2 to 4 h after these cells were exposed to galactose, but not by Western blotting of raffinose-exposed or pGAL1-transformed controls (Fig. 2C).

Isolation and properties of post-Golgi secretory vesicles. The results summarized above constituted strong direct evidence that Cdr1p, Cdr2p, and Mdr1p pumped fluconazole out of C. albicans cells, but the inaccessibility of the cytoplasmic face of the plasma membrane precluded detailed characterization of these transporters’ catalytic properties. Since the membranes of PGVs and those of whole cells are oriented in opposite directions, and since PGVs isolated from temperature-sensitive S. cerevisiae sec4-4 mutants have been used to study multiple eukaryotic plasma membrane efflux pumps (9, 22, 42, 43), we reasoned that it should be possible to use PGVs from pACT1-CDR1-, pACT1-CDR2-, or pACT1-MDR1-transformed C. albicans DSY1050F cells to characterize Cdr1p, Cdr2p, or Mdr1p, respectively. In earlier studies, GAL1-regulated overexpression of a dominant negative allele of the post-Golgi secretion pathway gene SEC4 in wild-type C. albicans caused PGVs to accumulate in the cytoplasm (29) and interfered with the targeting of fluorescently labeled Cdr1p to the plasma membrane (25). Therefore, we introduced the GAL1-regulated plasmid (pS28N) that Mao et al. (29) and Lee et al. (26) had used to overexpress the dominant negative sec(4)(S28N) allele into C. albicans DSY1050F cells that had previously been transformed with either pACT1-CDR1, pACT1-CDR2, pACT1-MDR1, or pACT1 alone. When the resulting transformants were shifted from glucose (repressing) to galactose (inducing) medium, PGVs accumulated intracellularly (Fig. 3A), and subcellular fractions prepared by

FIG. 2. Effect of GAL1-regulated overexpression of CDR1, CDR2, or MDR1. (A) Fluconazole MICs were higher when C. albicans DSY1050F cells transformed with pGAL1-CDR1 (CDR1), pGAL1-CDR2 (CDR2), or pGAL1-MDR1 (MDR1) were incubated in galactose than when they were incubated in glucose, whereas the fluconazole MICs of pGAL1-transformed controls (empty vector) were the same in galactose and glucose. (B) Intracellular [3H]fluconazole levels fell when 2% galactose was added to pGAL1-CDR1-, pGAL1-CDR2-, or pGAL1-MDR1-transformed C. albicans DSY1050F cells that had been incubated in YNB medium with 2% raffinose and 0.05 μM [3H]fluconazole until steady-state intracellular [3H]fluconazole levels were attained (16 h), but not when 2% galactose was added to pGAL1-transformed controls. Data are means ± SD for 3 experiments. (C) Immunoreactive proteins of the sizes expected for Flag-tagged Cdr1p, Cdr2p, and Mdr1p, respectively, were demonstrated in Western blots for controls exposed to galactose or raffinose. (100 μg total protein per lane).
differential centrifugation of lysed spheroplasts of galactose-incubated pS28N transformants contained many more intact PGVs than did the corresponding fractions from glucose-incubated controls (Fig. 3B). Furthermore, abundant amounts of Flag-tagged Cdr1p, Cdr2p, and Mdr1p, respectively, were demonstrated by Western blotting of PGV-containing fractions from galactose-incubated pACT1-CDR1-, pACT1-CDR2-, or pACT1-MDR1-transformed cells, but not by Western blotting of the corresponding fractions from pACT1-transformed controls (Fig. 3C).

$[^3H]$fluconazole transport by *C. albicans* PGVs. Having established that intact PGVs containing Cdr1p, Cdr2p, or Mdr1p can be isolated from *C. albicans* transformants, we next tested the PGV fractions for their abilities to accumulate $[^3H]$fluconazole. PGVs isolated from CDR1-, CDR2-, or MDR1-overexpressing *C. albicans* cells accumulated substantially more $[^3H]$fluconazole than did PGVs from pACT1-transformed controls (Fig. 4A). $[^3H]$fluconazole uptake by Cdr1p-, Cdr2p-, and Mdr1p-containing PGVs conformed to Michaelis-Menten kinetics (Fig. 4B), and the $K_m$ and $V_{max}$ values, respectively, were 0.80 ± 0.20 μM and 0.91 ± 0.15 pmol/mg protein/min for Cdr1p, 4.3 ± 1.0 μM and 0.52 ± 0.10 pmol/mg protein/min for Cdr2p, and 3.5 ± 1.2 μM and 0.59 ± 0.06 pmol/mg protein/min for Mdr1p (means ± standard deviations [SD] for 3 experiments).

The level of $[^3H]$fluconazole accumulation by PGVs from CDR1- or CDR2-overexpressing *C. albicans* was markedly lower in the absence of ATP than in its presence, and accumulation was inhibited by the ATPase inhibitor orthovanadate and by the nonhydrolyzable ATP analog 5'-adenyl-β-γ-imidodiphosphate (AMP-PNP). In contrast, the absence of ATP and the presence of orthovanadate or AMP-PNP had little effect on $[^3H]$fluconazole accumulation by PGVs from MDR1-overexpressing transformants (Fig. 5). Whether $[^3H]$fluconazole transport required a membrane potential and/or proton motive force was assessed by measuring $[^3H]$fluconazole accumulation by PGVs in buffers containing the permeable anion nitrate or the nonpermeable anion gluconate and also in the presence or absence of the proton ionophore carboxyl cyanide 3-chlorophenylhydrazone (CCCP). The level of $[^3H]$fluconazole accumulation by PGVs from MDR1-overexpressing transformants was markedly lower in gluconate-containing buffer than in nitrate-containing buffer, and accumulation was also inhibited by CCCP. In contrast, the levels of $[^3H]$fluconazole accumulation by PGVs from CDR1- or CDR2-overexpressing transformants were similar in gluconate- and nitrate-containing buffers and were only slightly affected by CCCP (Fig. 5). Lastly, verapamil (a general modulator of P glycoproteins) markedly inhibited $[^3H]$fluconazole transport by PGVs containing Cdr1p, Cdr2p, or Mdr1p (Fig. 5).

Effects of alternative compounds on $[^3H]$fluconazole transport. Since the results summarized above established that Cdr1p-, Cdr2p-, and Mdr1p-mediated $[^3H]$fluconazole transport could be quantified using isolated *C. albicans* PGVs, we next examined the abilities of 22 unlabeled compounds to inhibit Cdr1p-, Cdr2p-, and Mdr1p-mediated $[^3H]$fluconazole uptake by PGVs. These potential inhibitors included several antifungal azoles, compounds that have been reported to interact withazole transport in whole-cell assays, known substrates of other eukaryotic plasma membrane efflux pumps, and selected related compounds. All of the antifungal azoles we tested substantially inhibited $[^3H]$fluconazole uptake by PGVs isolated from CDR1-, CDR2-, and MDR1-overexpressing cells (Fig. 6), with IC$_{50}$s ranging from 0.2 to 0.3 for voriconazole to 2.0 to 5.6 for clotrimazole (Table 3). Among the other compounds we examined, inhibition of $[^3H]$fluconazole transport with IC$_{50}$s of <10 was observed only for β-estradiol, cycloheximide, and 5-fluorouracil with Cdr1p and for methotrexate with Mdr1p. No compound other than azoles inhibited $[^3H]$fluconazole transport by Cdr2p with an IC$_{50}$ of <10 (Fig. 6; Table 3).

**DISCUSSION**

The goals of this study were to determine directly if Cdr1p, Cdr2p, and Mdr1p transport fluconazole out of *C. albicans* cells, to develop a method for studying the catalytic properties of *C. albicans* plasma membrane transport proteins, and to use this method to characterize Cdr1p, Cdr2p, and Mdr1p. The principal new findings were that (i) ACT1- or GAL1-regulated overexpression of CDR1, CDR2, or MDR1 increased the fluconazole MIC and decreased intracellular $[^3H]$fluconazole levels in the *C. albicans* cdr1 cdr2 mdr1-null mutant DSY1050F; (ii) PGVs isolated from *C. albicans* cells overexpressing both the dominant negative sec4(S28N) allele and either CDR1, CDR2, or MDR1 actively transported $[^3H]$fluconazole into their lumens and thus could be used to study the transport properties of Cdr1p, Cdr2p, and Mdr1p; and (iii) studies of CDR1-, CDR2-, and MDR1-overexpressing whole *C. albicans* cells and of PGVs isolated from these cells indicate that Cdr1p, Cdr2p, and Mdr1p transport multiple antifungal azoles across the plasma membrane.

Although a large body of evidence shows that overexpression of CDR1, CDR2, and MDR1 is a major cause of fluconazole resistance in *C. albicans*, direct genetic and biochemical evidence that Cdr1p, Cdr2p, and Mdr1p pump fluconazole out of *C. albicans* cells is limited. For example, it has been shown that intracellular $[^3H]$fluconazole levels decreased when CDR1 (20, 33) and MDR1 (37) were overexpressed in *S. cerevisiae* and also that fluconazole MICs increased when CDR1, CDR2 (36, 53), and MDR1 (31) were overexpressed in *C. albicans*. The levels to which CDR1, CDR2, and MDR1 were expressed in *C. albicans* in the present study did not approach the expression levels that others achieved in *S. cerevisiae* or *C. albicans*; this may have been due to our use of eposomal rather than integrating vectors and our use of the ACT1 or GAL1 promoters instead of fluconazole-inducible promoters. Nevertheless, we were able to show that ACT1- and GAL1-regulated overexpression of CDR1, CDR2, or MDR1 caused both increased fluconazole MICs and decreased intracellular $[^3H]$fluconazole concentrations. To our knowledge, this is the first direct demonstration that Cdr1p, Cdr2p, and Mdr1p pump fluconazole out of *C. albicans* cells. One reason that it was possible to show that ACT1- or GAL1-regulated expression of CDR1, CDR2, or MDR1 increased fluconazole MICs and decreased intracellular $[^3H]$fluconazole levels was that the *C. albicans* cdr1 cdr2 mdr1-null mutant used in our experiments had a much lower fluconazole MIC and much higher intracellular $[^3H]$fluconazole levels than did its wild-type parent.

When the methods of Mao et al. (29) and Ruetz and Gros
FIG. 3. Isolation of *C. albicans* post-Golgi vesicles (PGVs). (A) Transmission electron microscopy showed that membrane-bound PGVs accumulated in the cytoplasm when pACT1- and pS28N-transformed *C. albicans* DSY1050F cells were incubated for 7 h in 2% galactose, but not when they were incubated in 2% glucose. (B) In addition, there were many more intact PGVs in 100,000 × g pellets prepared from lysed spheroplasts of pACT1- and pS28N-transformed *C. albicans* DSY1050F cells incubated in galactose than in glucose-incubated controls. (C) Lastly, immunoreactive proteins of the sizes expected for Cdr1p, Cdr2p, and Mdr1p, respectively, were demonstrated by probing Western blots of the 100,000 × g pellets from *C. albicans* DSY1050F cells transformed with pS28N and either pACT1-CDR1 (CDR1), pACT1-CDR2 (CDR2), or pACT1-MDR1 (MDR1) with anti-Flag monoclonal antibodies, but not by probing Western blots of the 100,000 × g pellets prepared from pACT1-transformed controls (empty vector) (10 μg total protein per lane).
were adapted to isolate PGVs from C. albicans cells, transmission electron microscopy showed that (i) PGVs accumulated intracellularly when the GAL1-regulated dominant negative sec4(S28N) allele was overexpressed and (ii) subcellular fractions prepared from these cells were enriched for intact PGVs. Moreover, Western blotting with anti-Flag antibodies showed that PGVs from C. albicans cells contained abundant amounts of each recombinant protein of interest, whereas PGVs from empty-vector controls did not. Most importantly, PGVs from C. albicans cells accumulated substantially more [3H]fluconazole than did PGVs from empty-vector controls. We concluded from these results that PGVs from C. albicans specifically transported [3H]fluconazole across their membranes and thus could be used to examine directly these pumps’ catalytic constants, energy requirements, and inhibitor profiles.

Membrane fractions from C. albicans transformants have previously been used to determine the respective apparent K_m and V_max values for the ATPase activities of Cdr1p, Cdr2p, and Mdr1p (23), but so far as we are aware, none of these pumps’ K_m and V_max values for azoles had been determined prior to the present study. We also found that [3H]fluconazole transport by Cdr1p and Cdr2p (but not by Mdr1p) required ATP and was inhibited by ATP inhibitors and also that [3H]fluconazole transport by Mdr1p (but not by Cdr1p or Cdr2p) required a transmembrane proton gradient and was blocked by CCCP. These results were not surprising, because (i) Cdr1p and Cdr2p are members of the ABC transporter superfamily, (ii) Mdr1p is a member of...
the MFS transporter family (40), and (iii) Cdr1p- and Cdr2p-mediated transport across the plasma membranes of whole \textit{S. cerevisiae} cells was known to be ATP dependent (11, 21, 49). However, we found that orthovanadate had only a slight inhibitory effect on $[^3H]$fluconazole uptake by PGVs from \textit{MDR1}-overexpressing \textit{C. albicans} cells, whereas orthovanadate increased intracellular $[^3H]$fluconazole levels in \textit{MDR1}-overexpressing whole \textit{S. cerevisiae} cells in an earlier study (20). Two possible explanations for these differing results are that (i) other vanadate-sensitive transporters may also pump $[^3H]$fluconazole out of whole \textit{S. cerevisiae} cells or (ii) a step required to process and/or transport Mdr1p to the plasma membrane of \textit{S. cerevisiae} may be inhibited by vanadate.

One striking finding was that $[^3H]$fluconazole transport into PGVs by Cdr1p, Cdr2p, and Mdr1p was markedly inhibited by all of theazole antifungals tested. These results suggest that all three pumps of interest transport multiple azoles through the plasma membrane, and this conclusion was supported by the observation that \textit{C. albicans} DSY1050F cells expressing \textit{CDR1}, \textit{CDR2}, or \textit{MDR1} had higher MICs for all azoles tested than empty-vector controls. Our results were consistent with those of previous studies showing that overexpression of \textit{CDR1} or \textit{CDR2} in \textit{S. cerevisiae} increased the MICs of multiple antifungal azoles (14, 15, 17, 18, 23, 33, 35, 36, 45, 50, 52). However, previous studies indicated that Mdr1p is more selective than Cdr1p or Cdr2p (16, 20, 23, 37). For example, Lamping et al. (23) showed that the fluconazole MICs for \textit{MDR1}-overexpressing \textit{S. cerevisiae} were markedly higher than those for controls, whereas the itraconazole MICs for the same transformants were not. We found that itraconazole markedly inhibited $[^3H]$fluconazole uptake by Mdr1p-containing PGVs but also that \textit{MDR1} overexpression in whole \textit{C. albicans} cells resulted in only a 2-fold increase in the itraconazole MIC. Since compounds can inhibit the transport of a labeled substrate by ABC transporters without themselves being transport substrates, and since overexpression of \textit{MDR1} increased the MIC of itraconazole less than the MICs of other azoles, our results do not necessarily imply that itraconazole is transported by Mdr1p. The apparent differences in Mdr1p-mediated itraconazole transport between our study and earlier studies of \textit{S. cerevisiae}
TABLE 3. IC₅₀s for recombinant Cdr1p, Cdr2p, and Mdr1p

| Substrate      | Cdr1p    | Cdr2p    | Mdr1p    |
|----------------|----------|----------|----------|
| Fluconazole    | 1.0 ± 0.2| 1.0 ± 0.2| 1.0 ± 0.7|
| Voriconazole   | 0.2 ± 0.0| 0.2 ± 0.1| 0.3 ± 0.2|
| Posaconazole   | 0.8 ± 0.3| 0.6 ± 0.3| 0.7 ± 0.4|
| Miconazole     | 0.7 ± 0.3| 0.9 ± 0.4| 3.1 ± 1.1|
| Itraconazole   | 4.4 ± 1.0| 2.4 ± 0.6| 0.8 ± 0.4|
| Clotrimazole   | 5.6 ± 1.3| 3.1 ± 1.0| 2.0 ± 0.7|
| β-Estradiol    | 1.9 ± 0.3| 21 ± 7.5 | 22 ± 10  |
| 5-Fluorouracil | 9.6 ± 2.3| 10 ± 4.9 | 15 ± 9.0 |
| Pregestosterone| 21 ± 10  | 32 ± 12  | ≥50      |
| Cereulin       | 33 ± 11  | 30 ± 9.1 | 17 ± 6.2 |
| Cycloheximide  | 7.6 ± 2.8| 27 ± 9.6 | ≥50      |
| Carnosine      | 12 ± 4.5 | ≥50      | ≥50      |
| Am. imidazole  | 13 ± 3.6 | ≥50      | ≥50      |
| Dichlorobenzene| 16 ± 3.0 | ≥50      | ≥50      |
| Daunorubicin   | 23 ± 4.9 | ≥50      | ≥50      |
| Corticosterone | 14 ± 3.8| 17 ± 6.8 | ≥50      |
| Caspofungin    | ≥50      | ≥50      | ≥50      |
| Cyt. arabinose | ≥50      | ≥50      | 20 ± 4.2 |
| Methotrexate   | ≥50      | ≥50      | 6.7 ± 2.2|
| Triazole       | ≥50      | ≥50      | 16 ± 3.0 |
| Colchicine     | ≥50      | ≥50      | 11 ± 4.2 |
| Brefeldin A    | ≥50      | ≥50      | 11 ± 3.7 |

aData: Am. imidazole, 1-3-aminopropyl-imidazole; cyt. arabinose, cytosine β-D-arabinofuranoside.

bExpressed as the ratio of the concentration of each compound to the concentration of fluconazole. Data are means ± SD for 3 experiments.

may have been due to the different promoters used to drive MDR1 expression, the use of plasmids to express MDR1 in our study versus integrating vectors in earlier studies, and/or the presence of two CUG codons in the MDR1 ORF. For all of these reasons, definitive conclusions about the ability or inability of Mdr1p to transport raftaconazole through the plasma membrane will require studies of labeled raftaconazole, which was not available for these studies.

Two other compounds also influenced [³H]fluconazole uptake by the efflux pumps of interest. First, [β]-estradiol markedly inhibited [³H]fluconazole transport by Cdr1p (IC₅₀ 1.9) but not by Cdr2p or Mdr1p. These findings support the observation by Krishnamurthy et al. (21) that overexpression of CDR1 in S. cerevisiae decreased intracellular [³H]estradiol levels. Since C. albicans occupies at least one niche with high estrogen levels (i.e., the vaginal mucosa), and since estrogens have profound effects on the growth, morphology, and gene expression of C. albicans (1, 5, 21, 24, 59), one would expect C. albicans to have mechanisms for regulating intracellular estrogen levels. Since Cdr1p may play a role in this process, we plan in future studies (i) to test the abilities of PGVs from CDR1-overexpressing C. albicans to accumulate β-[³H]estradiol and (ii) to compare the effects of estrogens on C. albicans null mutants and wild-type controls. Second, methotrexate inhibited [³H]fluconazole uptake by Mdr1p (IC₅₀ 6.7) but not that by Cdr1p or Cdr2p. Earlier studies showed that overexpression of MDR1 in S. cerevisiae reduced intracellular levels of [³H]methotrexate and also that exposure of these cells to unlabeled fluconazole slightly inhibited intracellular [³H]methotrexate accumulation (20); our results support a role for Mdr1p in pumping methotrexate out of C. albicans cells.

We also found that several compounds that others have reported as potential substrates for Cdr1p, Cdr2p, or Mdr1p did not inhibit [³H]fluconazole transport by these transporters. For example, overexpression of CDR1 or CDR2 in S. cerevisiae increased brefeldin A MICs (36, 45), but brefeldin A did not reduce [³H]fluconazole uptake by PGVs from CDR1- or CDR2-overexpressing C. albicans. Similarly, overexpression of MDR1 in S. cerevisiae increased cycloheximide MICs (20, 38), but cycloheximide did not inhibit [³H]fluconazole transport by PGVs isolated from MDR1-overexpressing C. albicans. Lastly, earlier studies have reported conflicting results for the effects of CDR2 overexpression on resistance to the antifungal echinocandin caspofungin. Schuetz-Muehlbauer et al. (48) reported that overexpression of CDR2 in S. cerevisiae increased caspofungin MICs measured on solid media but not those in liquid media. In contrast, Niimi et al. (35) found that overexpression of CDR2 in S. cerevisiae did not significantly increase caspofungin MICs, and Silver et al. (51) found no significant differences between the caspofungin MICs of C. albicans clinical strains that did or did not overexpress CDR2. In our studies, caspofungin did not inhibit the uptake of [³H]fluconazole by PGVs isolated from CDR1-, CDR2-, or MDR1-overexpressing C. albicans, and overexpression of CDR1, CDR2, or MDR1 in C. albicans DSY11050F had no effect on caspofungin MICs. Since membrane transporters can have different binding sites for different transport substrates (50), the observations that brefeldin A, cycloheximide, and caspofungin did not inhibit [³H]fluconazole uptake by PGVs isolated from CDR1-, CDR2-, or MDR1-overexpressing C. albicans cells do not rule out the possibility that these compounds are transported out of the cell by the transporters of interest. Definitive conclusions that Cdr1p, Cdr2p, or Mdr1p can or cannot pump brefeldin A, cycloheximide, or caspofungin out of the cell would require studies of the radiolabeled compounds of interest, which were not available for this study.

In summary, we have shown that ACT1- and GAL1-regulated overexpression of C. albicans CDR1, CDR2 and MDR1 in a C. albicans cdr1 cdr2 mdr1-null mutant resulted in increased fluconazole MICs and decreased intracellular [³H]fluconazole concentrations, thereby providing strong direct evidence that Cdr1p, Cdr2p, and Mdr1p transport fluconazole out of C. albicans cells. We also developed a new method for isolating C. albicans PGVs with abundant amounts of catalytically active Cdr1p, Cdr2p, or Mdr1p in their membranes, and we used these PGVs to analyze the energy requirements, kinetic constants, and inhibitor profiles of [³H]fluconazole transport by recombinant Cdr1p, Cdr2p, and Mdr1p. In addition to providing new information about the catalytic properties of three important C. albicans drug efflux pumps, the methods and approaches used in this study should be useful for studying other plasma membrane transporters from C. albicans and other medically important fungi.

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