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

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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 [^{3}H]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 [^{3}H]fluconazole than did PGVs from empty-vector controls. The K_ms (expressed in micromolar concentrations) and V_{max}s (expressed in picomoles per milligram of protein per minute), respectively, for [^{3}H]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. [^{3}H]fluconazole transport by Cdr1p and Cdr2p required ATP and was unaffected by carbonyl cyanide 3-chlorophenylhydrazone (CCCP), whereas [^{3}H]fluconazole transport by Mdr1p did not require ATP and was inhibited by CCCP. [^{3}H]fluconazole uptake by all 3 pumps was inhibited by all other azoles tested, with 50% inhibitory concentrations (IC_{50}s; expressed as proportions of the [^{3}H]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 the azole 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 [^{3}H]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

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TABLE 1. Oligonucleotide sequences used to construct the pYM71 plasmid and to clone *CDR1*, *CDR2*, and *MDR1* from *C. albicans*

Name	Sequence (5′ → 3′)
GAL1pt-5	GGTGGTGGTCTAGAGGGAAGATCTGATATTGACGAAG
GAL1pt-3	GGTGGTGGTTAATTAAGGTATAACTCTTTCTTATAAAAATCGG
CDR1-5	CGGACTTTAATTAAATGTCAGATTCTAAGATGTCGTCGCAAG
CDR1-3	GCCTGACCGCGGTTATTTATCATCATCATCTTTATAATCAATATCATGATCTTTATAATCACCATCAT GATCTTTATAATCTTTCTTATTTTTTTTCTCTCTGTTACCCTTTGG
CDR2-5	CGGACTTTAATTAAATGAGTACTGCAAACACGTCTTTGTC
CDR2-3	GCCTGACCGCGGTTATTTATCATCATCATCTTTATAATCAATATCATGATCTTTATAATCACCATCAT GATCTTTATAATCTTTTTTCATCTTCTTTTCTCTATTACCTTTTGG
MDR1-5	CGGACTTTAATTAAATGCATTACAGATTTTTGAGAGATAGTTTTG
MDR1-3	GCCTGACCGCGGTTATTTATCATCATCATCTTTATAATCAATATCATGATCTTTATAATCACCATCAT GATCTTTATAATCATTAGCATACTTAGATCTTGATCTCAACTT

Cdr1p, Cdr2p, or Mdr1p in their membranes by overexpressing the *C. albicans sec4*(*S28N*) allele in *C. albicans* cells that also overexpress *CDR1*, *CDR2*, or *MDR1* and to use these vesicles to study the catalytic properties of Cdr1p, Cdr2p, and Mdr1p. Therefore, in the present study, we (i) examined the abilities of recombinant Cdr1p, Cdr2p, and Mdr1p to transport fluconazole across 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 (Δ*cdr1*::*hisG*/Δ*cdr1*::*hisG* Δ*cdr2*::*hisG*/Δ*cdr2*::*hisG* Δ*mdr1*::*hisG-URA3-hisG*/Δ*mdr1*::*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-fluorootic 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 1,000 μg hygromycin B per ml and either 2% glucose, 2% galactose, or 2% raffinose. Plasmids were amplified in *Escherichia coli* DH5α in Luria-Bertani medium with 100 μg ampicillin per ml.

Plasmids and transformation methods. PCR with oligonucleotides CDR1-5 and CDR1-3, CDR2-5 and CDR2-3, and MDR1-5 and MDR1-3 (Table 1), respectively, was used to amplify the *CDR1*, *CDR2*, and *MDR1* open reading frames (ORFs) from *C. albicans* SC5314 genomic DNA and to fuse a 3× Flag epitope tag to each recombinant protein's C terminus. The resulting PCR products were ligated into the PacI and SacII restriction sites in the multicopy plasmid pYM70, which contains *C. albicans ARS2*, a synthetic hygromycin B resistance marker, and the *C. albicans ACT1* promoter (GenBank accession number GU937092). Plasmid pYM71 is identical to pYM70 except that the *ACT1* promoter was replaced by the *C. albicans GAL1* promoter, which was amplified from *C. albicans* SC5314 genomic DNA by PCR with oligonucleotides GAL1pt-5 and GAL1pt-3 (Table 1). The accuracy of all plasmid constructions was verified by DNA sequencing. Plasmid pS28N is a multicopy plasmid that contains *C. albicans URA3* and the dominant negative *C. albicans sec4*(*S28N*) allele under the control of the *C. albicans GAL1* promoter (29).

C. albicans DSY1050F was transformed by the lithium acetate method (54), and the resulting transformants were selected and expanded on appropriate media.

Antifungal susceptibility testing. The NCCLS (now CLSI) M27-A broth microdilution method (34) was used to test *C. albicans* strains for antifungal susceptibility. The strains of interest were grown on YP medium plus glucose or on YP medium plus galactose, after which the cells were diluted to an optical density at 600 nm (OD_{600}) of 5×10^{-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.

[^{3}H]fluconazole accumulation by *C. albicans* cells. Intracellular [^{3}H]fluconazole was quantified as described by Sanglard et al. (46), with modifications. pACT1-CDR1-, pACT1-CDR2-, pACT1-MDR1-, or pACT1-transformed *C. albicans* cells were incubated in YP-glucose plus hygromycin B, and pGAL1-CDR1-, pGAL-CDR2-, pGAL1-MDR1-, or pGAL1-transformed *C. albicans* cells were incubated in YP-raffinose plus hygromycin B. The cells were harvested by centrifugation, washed in YNB-glucose or YNB-raffinose, and resuspended to an OD_{600} of 30 in the same medium containing [^{3}H]fluconazole (final concentration, 0.05 μM; specific activity, 20 Ci/mmol; Amersham Biosciences). The cells were then shaken at 30°C; aliquots were removed at intervals and added to cold stop solution (the same medium plus 25 μM unlabeled fluconazole); the cells were collected on 0.45-μm-pore-size nitrocellulose filters; the filters were washed twice with cold stop solution; and [^{3}H]fluconazole was quantified by liquid scintillation counting. Each result was expressed as the mean of duplicate measurements from three independent experiments.

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 NaN_3 (10 mM), and the cells were washed in 10 mM Tris-HCl (pH 7.5)–5 mM NaN_3, collected by centrifugation, and stored at −80°C.

Post-Golgi secretory vesicles were isolated as described by Ruetz and Gros (42), with modifications. The frozen cells were resuspended in 100 mM Tris-SO_4 (pH 9.4) at 25°C, collected by centrifugation, and converted to spheroplasts with Zymolyase 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 NaN_3, 2 mM EDTA, and 40 mM β-mercaptoethanol. The spheroplasts were washed twice in SM buffer with 10 mM NaN_3 and were then incubated on ice in SM buffer (5 ml/g of cells) supplemented with 1 mM $CaCl_2$, 5 mM $MnSO_4$, 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 gluconate or nitrate vesicle buffer (50 mM sucrose, 10 mM Tris-HEPES [pH 7.5], and either 100 mM potassium gluconate 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 gluconate 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 gluconate or nitrate buffer. [^{3}H]fluconazole uptake was initiated by adding PGV samples to prewarmed (37°C) gluconate or nitrate buffer supplemented with ATP (2.5 mM), creatine phosphate (10 mM), creatine phosphokinase (3 μg/ml), and [^{3}H]fluconazole (0.05 μM). [^{3}H]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

additional washes with stop solution, the radioactivity level was determined by liquid scintillation counting.

To minimize the nonspecific effects of other transporters, organelles, and/or membranes, [^{3}H]fluconazole uptake by PGVs from empty-vector controls was subtracted from uptake by PGVs containing Cdr1p, Cdr2p, or Mdr1p. The kinetic constants K_m and V_{max} were calculated by the Lineweaver-Burk method, using PGVs that were exposed to graded concentrations of [^{3}H]fluconazole for 10 s. The abilities of other compounds to inhibit [^{3}H]fluconazole uptake were assessed by adding PGVs to a reaction mixture containing the compound of interest at a concentration 50-fold higher than that of [^{3}H]fluconazole. PGVs were collected at 10 s and 30 s (data not shown) and were processed as described above. Any compound that did not inhibit [^{3}H]fluconazole uptake (mean uptake, ≥85% of control values; P, ≥0.05 versus control) was considered to have a 50% inhibitory concentration (IC_{50}) of ≥50. The IC_{50}s of all other compounds were determined by retesting over a range of concentrations (0.125-fold to 64-fold relative to the concentration of [^{3}H]fluconazole in the medium [0.05 μM]), using an equation for a sigmoid plot {Y = min + (max − min)/[1 + 10exp(X − logIC_{50})], where X is log(inhibitor), Y is pmol/mg total protein, min is the minimum [^{3}H]fluconazole pmol/mg total protein, and max is the maximum [^{3}H]fluconazole pmol/mg total protein}. The IC_{50} of each compound is the amount required to inhibit fluconazole uptake by 50%, expressed as the ratio of the concentration of that compound to the concentration of [^{3}H]fluconazole.

Electron microscopy. Whole *C. albicans* cells and PGV samples were examined by transmission electron microscopy as described by Walworth and Novick (55).

Western blotting. The presence of the recombinant proteins of interest in whole *C. albicans* cells or in isolated PGVs was assessed by Western blotting (16). Lysates of whole cells or isolated PGVs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and probed with a mouse monoclonal anti-Flag primary antibody (Sigma) and a goat anti-mouse secondary antibody. The bands of interest were detected with ECL Western blotting reagents (GE Healthcare).

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 any azole 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 [^{3}H]fluconazole levels at intervals after the cells of interest were incubated in 0.05 μM [^{3}H]fluconazole. We found that (i) *C. albicans* DSY1050F cells transformed with pACT1 accumulated substantially more intracellular [^{3}H]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 [^{3}H]fluconazole than did pACT1-transformed controls and more intracellular [^{3}H]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-

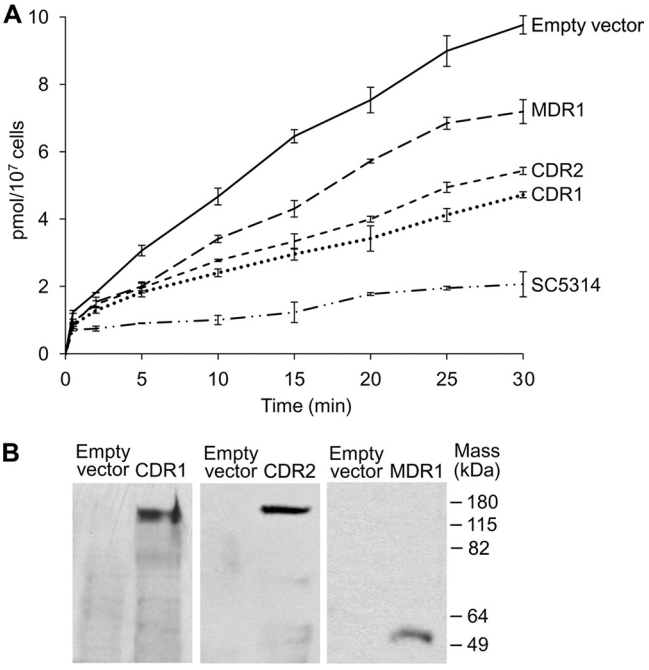

FIG. 1. Effect of *ACT1*-regulated overexpression of *CDR1*, *CDR2*, or *MDR1*. (A) Intracellular [^{3}H]fluconazole levels after incubation in 0.05 μM [^{3}H]fluconazole for the times shown were highest in *C. albicans* DSY1050F cells transformed with pACT1 alone (empty vector), lower in *C. albicans* 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 but not in Western blots of lysates of pACT1-transformed controls (empty vector) (100 μg total protein per lane).

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 [^{3}H]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 [^{3}H]fluconazole until steady-state intracellular [^{3}H]fluconazole levels were achieved, and intracellular [^{3}H]fluconazole was quantified after either 2% galactose or 2% raffinose was added to the cell suspensions. Intracellular [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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 pGAL-*CDR1*, pGAL-*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).

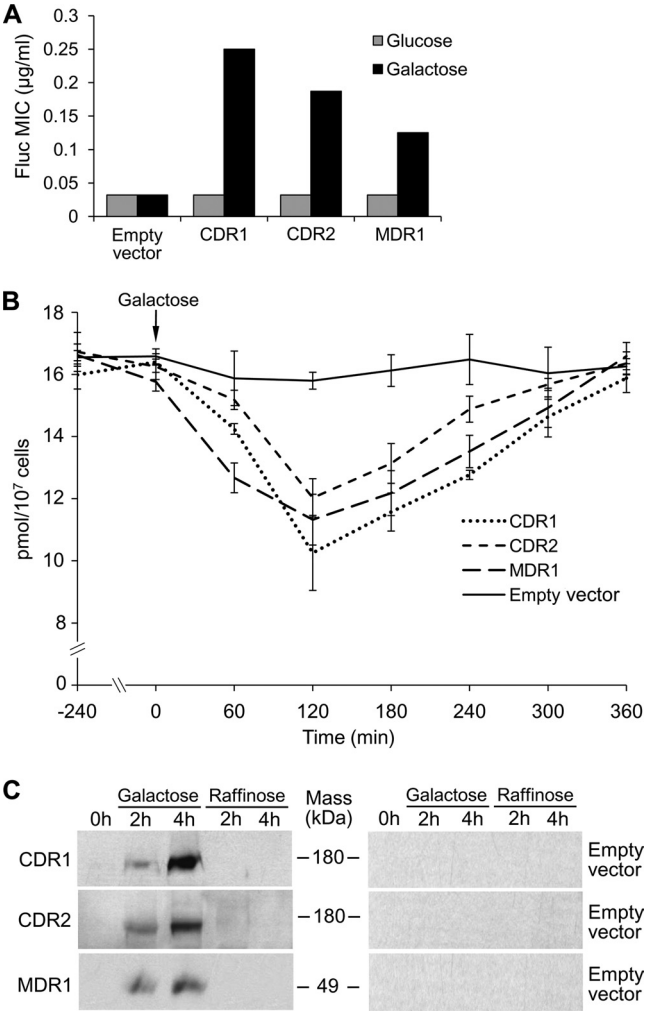

FIG. 2. Effect of *GAL1*-regulated overexpression of *CDR1*, *CDR2*, or *MDR1*. (A) Fluconazole MICs were higher when *C. albicans* DSY1050F cells transformed with pGAL1-*CDR1* (CDR1), pGAL1-*CDR2* (CDR2), or pGAL1-*MDR1* (MDR1) were incubated in galactose than when they were incubated in glucose, whereas the fluconazole MICs of pGAL-transformed controls (empty vector) were the same in galactose and glucose. (B) Intracellular [^{3}H]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 [^{3}H]fluconazole until steady-state intracellular [^{3}H]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 probed with anti-Flag monoclonal antibodies 2 h and 4 h after the pGAL1-*CDR1*, pGAL1-*CDR2*, and pGAL1-*MDR1* transformants were exposed to galactose, but not in Western blots for controls exposed to raffinose or for pGAL1-transformed controls exposed to galactose or raffinose (100 μg total protein per lane).

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 sec6-4* 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 *sec4*(*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

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).

[^{3}H]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 [^{3}H]fluconazole. PGVs isolated from *CDR1*-, *CDR2*-, or *MDR1*-overexpressing *C. albicans* cells accumulated substantially more [^{3}H]fluconazole than did PGVs from pACT1-transformed controls (Fig. 4A). [^{3}H]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 [^{3}H]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′-adenylyl-β-γ-imidodiphosphate (AMP-PNP). In contrast, the absence of ATP and the presence of orthovanadate or AMP-PMP had little effect on [^{3}H]fluconazole accumulation by PGVs from *MDR1*-overexpressing transformants (Fig. 5). Whether [^{3}H]fluconazole transport required a membrane potential and/or proton motive force was assessed by measuring [^{3}H]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 carbonyl cyanide 3-chlorophenylhydrazone (CCCP). The level of [^{3}H]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 [^{3}H]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 [^{3}H]fluconazole transport by PGVs containing Cdr1p, Cdr2p, or Mdr1p (Fig. 5).

Effects of alternative compounds on [^{3}H]fluconazole transport. Since the results summarized above established that Cdr1p-, Cdr2p-, and Mdr1p-mediated [^{3}H]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 [^{3}H]fluconazole uptake by PGVs. These potential inhibitors included several antifungal azoles, compounds that have been reported to interact with azole 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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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 episomal rather than integrating vectors and our use of the *ACT1* and *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 [^{3}H]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 [^{3}H]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 [^{3}H]fluconazole levels than did its wild-type parent.

When the methods of Mao et al. (29) and Ruetz and Gros

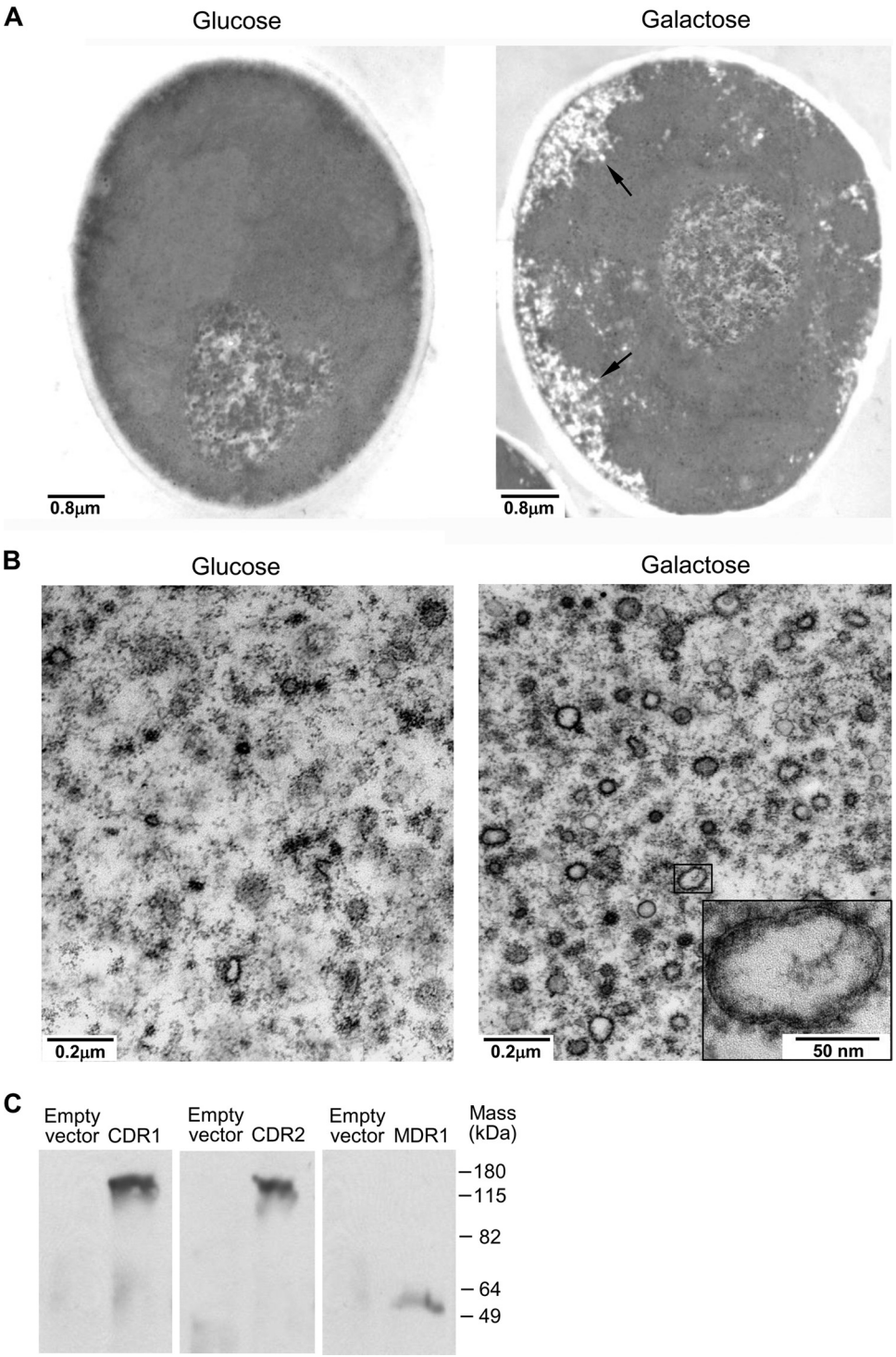

FIG. 3. Isolation of *C. albicans* post-Golgi vesicles (PGVs). (A) Transmission electron microscopy showed that membrane-bound PGVs accumulated in the cytoplasm when pACT1- and pS28N-transformed *C. albicans* DSY1050F cells were incubated for 7 h in 2% galactose, but not when they were incubated in 2% 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).

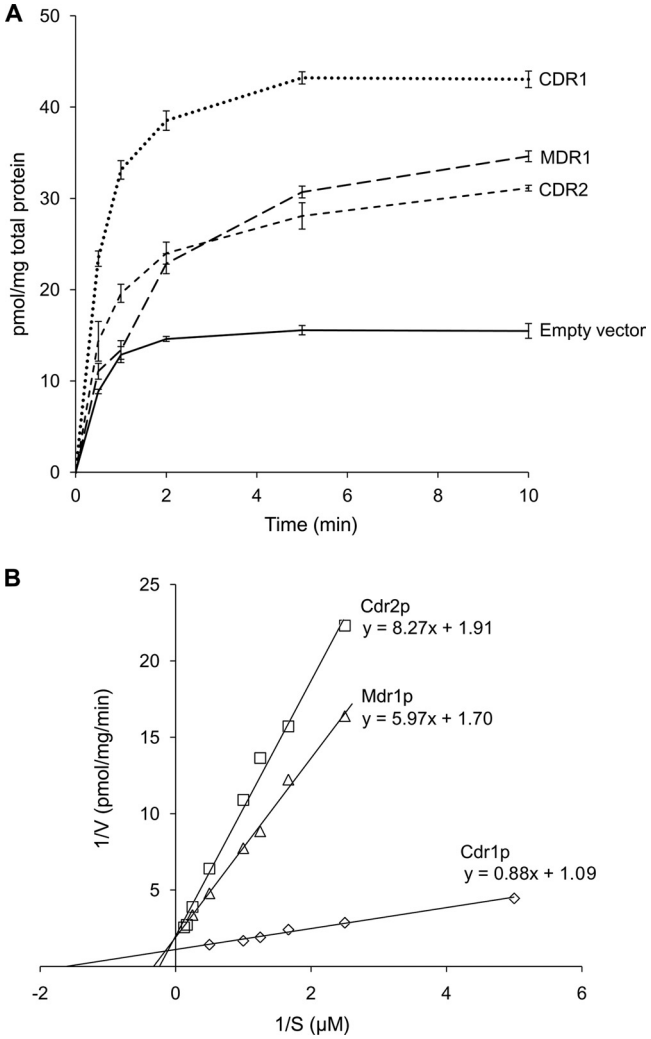

FIG. 4. [^{3}H]fluconazole accumulation by PGVs. (A) PGVs isolated from *C. albicans* DSY1050F cells transformed with pACT1-*CDR1* (CDR1), pACT1-*CDR2* (CDR2), or pACT1-*MDR1* (MDR1) accumulated substantially more [^{3}H]fluconazole after incubation in 0.05 μM [^{3}H]fluconazole for the times shown than did PGVs from pACT1-transformed controls (empty vector). Data are means ± SD for 3 experiments. (B) Lineweaver-Burk plots of initial rates of [^{3}H]fluconazole uptake by PGVs containing Cdr1p, Cdr2p, or Mdr1p.

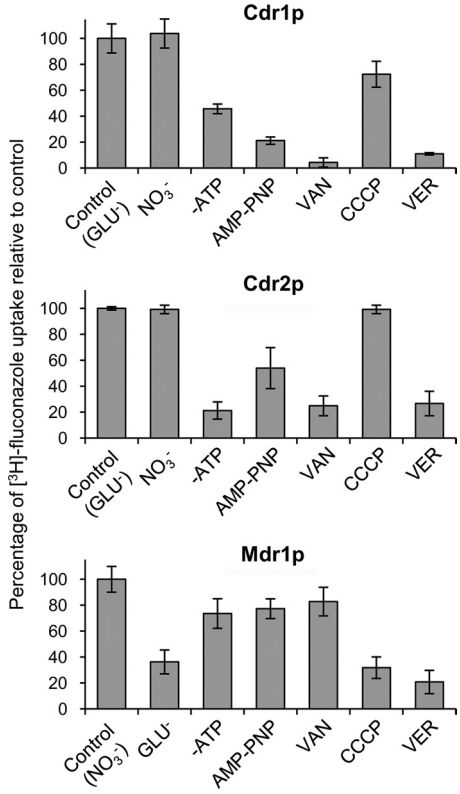

FIG. 5. Energy dependence of [^{3}H]fluconazole transport. PGVs isolated from *C. albicans* DSY1050F transformed with pACT1-*CDR1* (Cdr1p) or pACT1-*CDR2* (Cdr2p) accumulated much more [^{3}H]fluconazole after 10 s in gluconate buffer with ATP (GLU^-) than in gluconate buffer without ATP (−ATP) or in gluconate buffer with ATP plus the nonhydrolyzable ATP analog AMP-PNP, the ATP inhibitor sodium orthovanadate (VAN), or the P-glycoprotein inhibitor verapamil (VER). In contrast, the proton ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) had little effect on [^{3}H]fluconazole transport by Cdr1p or Cdr2p. PGVs isolated from *C. albicans* DSY1050F cells transformed with pACT1-*MDR1* (Mdr1p) accumulated much more [^{3}H]fluconazole after 10 s in nitrate buffer (NO_{3-}) than in gluconate buffer, and they accumulated much less [^{3}H]fluconazole in nitrate buffer plus CCCP or in the presence of verapamil. In contrast, the absence of ATP or the presence of AMP-PNP or VAN had little effect on [^{3}H]fluconazole transport by Mdr1p. Data are means ± SD for 3 experiments.

(42) were adapted to isolate PGVs from *CDR1*-, *CDR2*-, and *MDR1*-overexpressing *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 *CDR1*-, *CDR2*-, and *MDR1*-overexpressing *C. albicans* contained abundant amounts of each recombinant protein of interest, whereas PGVs from empty-vector controls did not. Most importantly, PGVs from *CDR1*-, *CDR2*-, and *MDR1*-overexpressing *C. albicans* cells accumulated substantially more [^{3}H]fluconazole than did PGVs from empty-vector controls. We concluded from these results that PGVs from *CDR1*-, *CDR2*-, and *MDR1*-overexpressing *C. albicans* specifically transported [^{3}H]fluconazole across their membranes and thus could be used to examine directly these pumps' catalytic constants, energy requirements, and inhibitor profiles.

Membrane fractions from *CDR1*-, *CDR2*-, and *MDR1*-overexpressing *S. cerevisiae* 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 [^{3}H]fluconazole transport by Cdr1p and Cdr2p (but not by Mdr1p) required ATP and was inhibited by ATP inhibitors and also that [^{3}H]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

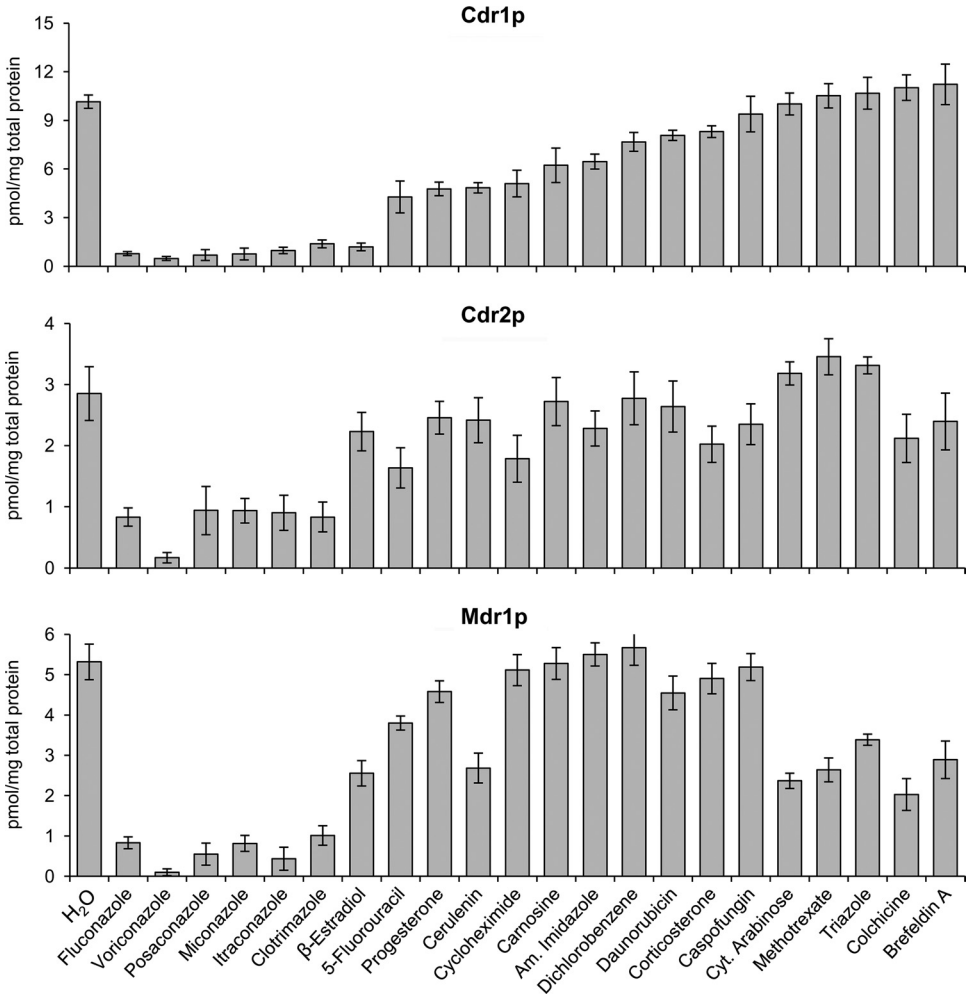

FIG. 6. Inhibitors of [^{3}H]fluconazole transport by Cdr1p, Cdr2p, and Mdr1p. [^{3}H]fluconazole uptake by PGVs isolated from *C. albicans* DSY1050F cells transformed with pACT1-*CDR1* (Cdr1p), pACT1-*CDR2* (Cdr2p), or pACT1-*MDR1* (Mdr1p) was quantified after incubation for 10 s in the presence of 50-fold molar excesses of the compounds listed. Data are means ± SD for 3 experiments. Am. Imidazole, 1-3-aminopropylimidazole; Cyt. Arabinose, cytosine β-D-arabinofuranoside.

the MFS transporter family (40), and (iii) Cdr1p- and Cdr2p-mediated transport across the plasma membranes of whole *S. cerevisiae* cells was known to be ATP dependent (11, 21, 49). However, we found that orthovanadate had only a slight inhibitory effect on [^{3}H]fluconazole uptake by PGVs from *MDR1*-overexpressing *C. albicans* cells, whereas orthovanadate increased intracellular [^{3}H]fluconazole levels in *MDR1*-overexpressing whole *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 [^{3}H]fluconazole out of whole *S. cerevisiae* cells or (ii) a step required to process and/or transport Mdr1p to the plasma membrane of *S. cerevisiae* may be inhibited by vanadate.

One striking finding was that [^{3}H]fluconazole transport into PGVs by Cdr1p, Cdr2p, and Mdr1p was markedly inhibited by all of the azole 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 *C. albicans* DSY1050F cells expressing *CDR1*, *CDR2*, or *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 *CDR1* or *CDR2* in *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 *MDR1*-overexpressing *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 [^{3}H]fluconazole uptake by Mdr1p-containing PGVs but also that *MDR1* overexpression in whole *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 *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 *S. cerevisiae*

TABLE 3. IC_{50}s for recombinant Cdr1p, Cdr2p, and Mdr1p

Substrate[a]	IC_{50}[b] for:		
	Cdr1p	Cdr2p	Mdr1p
Fluconazole	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.7
Voriconazole	0.2 ± 0.0	0.3 ± 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
Progesterone	21 ± 10	32 ± 12	≥50
Cerulenin	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

[a] Am. imidazole, 1-3-aminopropyl-imidazole; cyt. arabinose, cytosine β-D-arabinofuranoside.

[b] Expressed 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 itraconazole through the plasma membrane will require studies of labeled itraconazole, which was not available for these studies.

Two other compounds also influenced [^{3}H]fluconazole uptake by the efflux pumps of interest. First, β-estradiol markedly inhibited [^{3}H]fluconazole transport by Cdr1p (IC_{50}, 1.9) but not that by Cdr2p or Mdr1p. These findings support the observation by Krishnamurthy et al. (21) that overexpression of *CDR1* in *S. cerevisiae* decreased intracellular β-[^{3}H]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-[^{3}H]estradiol and (ii) to compare the effects of estrogens on *C. albicans* null mutants and wild-type controls. Second, methotrexate inhibited [^{3}H]fluconazole uptake by Mdr1p (IC_{50}, 6.7) but not that by Cdr1p or Cdr2p. Earlier studies showed that overexpression of *MDR1* in *S. cerevisiae* reduced intracellular levels of [^{3}H]methotrexate and also that exposure of these cells to unlabeled fluconazole slightly inhibited intracellular [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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. Schuetzer-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 [^{3}H]fluconazole by PGVs isolated from *CDR1*-, *CDR2*-, or *MDR1*-overexpressing *C. albicans*, and overexpression of *CDR1*, *CDR2*, or *MDR1* in *C. albicans* DSY1050F 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 [^{3}H]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 [^{3}H]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 [^{3}H]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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