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Multiple Efflux Mechanisms Are Involved in *Candida albicans* Fluconazole Resistance

GREGORY D. ALBERTSON, MASAKAZU NIIMI, RICHARD D. CANNON,* AND HOWARD F. JENKINSON

Molecular Oral Biology Laboratory, Department of Oral Biology and Oral Pathology and Centre for Gene Research, University of Otago, Dunedin, New Zealand

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Fluconazole-susceptible *Candida albicans* strains accumulated [^{3}H]fluconazole at a rate of approximately 2 pmol/min per 10^9 cells. Fluconazole accumulation was not affected by the pretreatment of cells with sodium azide or with 2-deoxyglucose. The rate of fluconazole accumulation became saturated at high fluconazole concentrations and was not affected by the addition of ketoconazole, and there was no fluconazole accumulation in cells incubated at 4°C. A fluconazole-resistant mutant of *C. albicans* SGY-243 was isolated following growth enrichment in fluconazole-containing medium. Cells of the mutant strain, designated FR2, showed a reduced rate of fluconazole accumulation compared with SGY-243 and were not resistant to other azole antifungal agents. The rates of fluconazole accumulation by *C. albicans* FR2 and the other azole-resistant strains, B59630, AD, and KB, were increased in the presence of sodium azide, suggesting that fluconazole resistance in these strains may be associated with an energy-dependent drug efflux. Fluconazole-resistant *C. albicans* strains all contained elevated amounts (2- to 17-fold) of mRNA encoding Cdr1, an ATP-binding cassette-type transporter. In addition, *C. albicans* FR2 also contained increased amounts of mRNA encoding Ben^r, a major facilitator superfamily transporter. These results suggest that fluconazole enters *C. albicans* cells by facilitated diffusion and that fluconazole resistance may involve energy-dependent drug efflux associated with increased expression of Ben^r and/or Cdr1.

Candida albicans is an opportunistic yeast that causes severe disease in immunocompromised individuals. Oropharyngeal candidiasis, for example, is one of the most common opportunistic infections of individuals undergoing immunosuppressive therapy for cancer (6) or organ transplantation and in patients with AIDS (10). The synthetic triazole fluconazole has several advantages over earlier imidazoles, such as ketoconazole, in the treatment of patients with severe candidiasis. It has a higher solubility in water, a longer plasma half-life (31 h), and a relatively low toxicity (3). Between 1988 and 1993, fluconazole was used to treat over 15 million patients, including at least 250,000 AIDS patients (18), and fluconazole treatment of patients with oropharyngeal candidosis has been adopted by many clinics (48). However, recently there has been an increased incidence of treatment failures in candidiasis patients receiving prolonged fluconazole therapy (8, 13, 27, 42), and these treatment failures have been shown to be due to reduced susceptibility of *C. albicans* to fluconazole (40). Resistance of *Candida* spp. to fluconazole has been reported more frequently than resistance to ketoconazole, and there have been few reports of resistance to the less frequently prescribed itraconazole (34). The ketoconazole-resistant *C. albicans* AD, KB, and Darlington strains have been well studied (19, 20, 23, 43, 49), and ketoconazole has been shown to enter *C. albicans* by diffusion and by an energy-dependent uptake system (7). In contrast, little is known about fluconazole uptake in *C. albicans.*

Fluconazole resistance in *C. albicans* may occur in two ways: reduced fluconazole accumulation or an altered drug target (14α-sterol demethylase, encoded by *ERG16*). There is no evidence that fluconazole is modified and inactivated by *C. albicans*. A few fluconazole-resistant *C. albicans* strains show overexpression of *ERG16* mRNA, but this is probably not the major cause of resistance in these isolates (44). Evidence suggests that a common resistance mechanism is reduced fluconazole accumulation (44, 50), either as a result of reduced cellular uptake or increased efflux. Two genes encoding proteins thought to be associated with drug efflux have been identified in *C. albicans*. The *BEN^r* gene (12) encodes a protein from the major facilitator superfamily of transporters (30) and confers resistance to benomyl and methotrexate when expressed in *Saccharomyces cerevisiae* (12). The *C. albicans CDR1* gene (38) encodes an ATP-binding cassette-type transporter (16) which complements the Δ*pdr5* mutation in *S. cerevisiae* associated with susceptibility to cycloheximide (38). A number of fluconazole-resistant *C. albicans* isolates from AIDS patients show increased amounts of Cdr1 mRNA, implicating a role for this protein in azole resistance (44). To clarify further the role of putative drug efflux proteins in *C. albicans* azole resistance, we have isolated an isogenic fluconazole-resistant mutant of *C. albicans* SGY-243. This allows the direct comparison of the expression of putative drug efflux-associated genes in the isogenic mutant with those in parental strains, as well as with those in several well-characterized azole-resistant isolates. Our results suggest that fluconazole resistance could be associated with elevated Ben^r expression while azole resistance could be associated with elevated Cdr1 expression.

MATERIALS AND METHODS

Yeast strains. The *C. albicans* strains used in this study are listed in Table 1.

Isolation of a fluconazole-resistant mutant of *C. albicans* SGY-243. A fluconazole-resistant mutant of *C. albicans* SGY-243 (*ade2*/*ade2* Δ*ura3*::*ADE2*/Δ*ura3*::*ADE2*) was isolated by serial passage through media containing fluconazole. *C. albicans* SGY-243 cells were incubated in RPMI 1640 medium (Life Technologies, Gaithersburg, Md.) containing uridine (50 μg/ml) (RPMI + Uri) at 30°C for 16 h with shaking and were diluted into fresh RPMI + Uri at a

* Corresponding author. Mailing address: Department of Oral Biology and Oral Pathology, P.O. Box 647, University of Otago, Dunedin, New Zealand. Phone: (64) 3 479 7081. Fax: (64) 3 479 0673. Electronic mail address: richard.cannon@stonebow.otago.ac.nz.

TABLE 1. *C. albicans* strains used in this study

Strain	Source	Reference
ATCC 10261	American Type Culture Collection, Rockville, Md.	
SGY-243	Squibb Institute for Medical Research, Princeton, N.J.	26
FR2	This laboratory[a]	This study
B59630	Janssen Research Foundation	33
AD	Janssen Research Foundation	43
KB	Janssen Research Foundation	43
Darlington	G. W. Pye, Pfizer Ltd.	51

[a] Isolated from SGY-243.

concentration of 4.4×10^6 cells per ml. The culture was incubated at 30°C for 4.5 h, and then fluconazole was added at a final concentration of 1 μg/ml (twice the MIC [see Table 2]). After 4.5 h of further incubation, growth of the culture reached a plateau at a cell density of 3.5×10^7 cells per ml, whereas a control culture (without fluconazole) had a cell density of 6.6×10^7 cells per ml. After additional incubation (50 h), cells at concentration of 3.3×10^6 cells per ml were subcultured from the fluconazole-containing culture into fresh RPMI + Uri with fluconazole and incubated at 30°C with shaking. After 16 h of incubation, cells from this culture were plated on minimal agar plates (yeast nitrogen base [Difco Laboratories, Detroit, Mich.] [6.7 g/liter], glucose [10 g/liter], uridine [0.05 g/liter] agar [20 g/liter]) containing fluconazole (0, 0.5, 1.0, or 2 μg/ml). One colony was picked from the plate containing 2 μg of fluconazole per ml and tested for growth on minimal agar containing higher fluconazole concentrations (4, 8, 16, or 32 μg/ml). After 24 h of incubation at 30°C, colonies had grown on all plates, although colonies were smaller on plates containing 32 μg of fluconazole per ml. A single colony was picked from the plate containing 32 μg of fluconazole per ml and was designated isolate FR2.

Determinations of MICs of antifungal agents. The MICs of antifungal agents for *C. albicans* strains were determined by a microdilution test based on the macrodilution reference method of the National Committee for Clinical Laboratory Standards (11, 14, 36).

(i) Antifungal agents. Stock solutions (10 mg/ml) of fluconazole (Pfizer Ltd., Sandwich, Kent, United Kingdom), ketoconazole (Janssen Research Foundation, Beerse, Belgium), itraconazole (Janssen Research Foundation), amphotericin B (E. R. Squibb & Sons, Princeton, N.J.), benomyl (Nippon Roche, Kamakura, Japan), cycloheximide (Sigma), and methotrexate (Nippon Roche) were prepared in either dimethyl sulfoxide (ketoconazole, itraconazole, amphotericin B, benomyl, and methotrexate), sterile water (fluconazole), or RPMI medium (cycloheximide) immediately prior to use.

(ii) Inoculum. The yeast cell inoculum was prepared from a culture grown in RPMI buffered with MOPS (morpholinepropanesulfonic acid) (165 mM, pH 6.8) at 30°C for 16 h. The RPMI medium was supplemented with uridine (50 μg/ml) for the growth of *C. albicans* SGY-243 and FR2. For determinations of the MIC of methotrexate, the folate pool of the inoculum was reduced (15) by growing the yeast in glucose-salts-biotin medium (GSB [22]) and the MIC determinations were carried out in GSB containing sulfanilamide (200 μg/ml, from 20-mg/ml acetone stock). The relationship between the optical densities of cultures and the numbers of CFU per milliliter was determined for each strain by plating cell dilutions on agar plates and counting the colonies formed after incubation of plates at 30°C for 48 h. The inoculum was added to the plates for the MIC determinations to give a final concentration of 3×10^4 CFU/ml.

(iii) MIC test. MIC assays were performed in a final volume of 100 μl in sterile plastic 96-well flat-bottom microtiter plates with covers (Greiner GmbH, Frickenhausen, Austria), with serial twofold antifungal agent dilutions in RPMI medium or in GSB for the determination of MICs of methotrexate. The final antifungal agent concentration ranges were 4 to 0.0078 μg/ml for amphotericin B, ketoconazole, and itraconazole, 128 to 0.25 μg/ml for fluconazole, 200 to 0.39 μg/ml for benomyl and methotrexate, and 1,000 to 1.95 μg/ml for cycloheximide. Uninoculated and drug-free controls were included. Inoculated microtiter plates were incubated at 30°C for 48 h on a rotary shaker (150 rpm). The plates were then agitated more vigorously to ensure homogeneous yeast cell suspensions, and the optical densities (at 492 nm) of suspensions were measured with an EAR 340 microtiter plate reader (SLT-Lab Instruments, Salzburg, Austria).

(iv) End point criterion. The optical density reading for each suspension was plotted against the antifungal agent concentration, and the MIC test end point was defined as the lowest antifungal concentration which gave >80% inhibition of growth compared with drug-free controls (36).

Biotyping of *C. albicans* strains by DNA fingerprinting. The genetic similarities of *C. albicans* strains were determined by DNA fingerprinting using a moderately repetitive *C. albicans* probe, as described by Schmid et al. (45). Genomic DNA was isolated from mid-exponential-phase yeast cells, digested with *Eco*RI, and electrophoresed on a 0.8% (wt/vol) agarose gel. The DNA fragments were vacuum blotted onto nylon membranes and hybridized with [^{32}P]dCTP-labelled Ca3 DNA probe (45) at 65°C for 16 h. After hybridization, the membranes were washed under low-stringency conditions (0.6 M NaCl, 60 mM trisodium citrate, 0.5% [wt/vol] sodium dodecyl sulfate; 60°C) and then exposed to X-ray film.

Fluconazole uptake by yeast cells. The net rate of fluconazole uptake was determined by using [^{3}H]fluconazole (specific activity, 96.92 GBq/mmol; Amersham International, Little Chalfont, United Kingdom). Cells were grown in YPD medium (yeast extract [Difco Laboratories] [10 g/liter], Bacto Peptone [Difco Laboratories] [20 g/liter], glucose [20 g/liter]) at 30°C to early log phase (optical density at 540 nm, 1.5), harvested by centrifugation ($3,000 \times g$, 5 min), and washed in 0.1 M phosphate buffer (pH 7.4) supplemented with 20 mM glucose. The cells (1 ml, 5×10^8 CFU) were incubated at 30°C for 20 min, and then [^{3}H]fluconazole was added at a final concentration of 1 μM. Samples (100 μl) were removed at various times and placed into 1 ml of ice-cold wash buffer (WB; 0.1 M phosphate buffer [pH 7.4] containing 1 mM unlabelled fluconazole), filtered on glass fiber filters (GF/C; Whatman, Kent, United Kingdom) prewetted with WB, and washed four times with 1 ml of WB. The filters were transferred to scintillation vials, scintillation cocktail (Optiphase HiSafe; Wallac Oy, Turku, Finland) was added, and the radioactivity associated with the filters was measured with a liquid scintillation counter.

Amino acid uptake by yeast cells. The rate of amino acid uptake by yeast cells was determined with a uniformly ^{14}C-labelled amino acid mixture (specific activity, 2.11 GBq/mmol; Amersham). Cells were grown and prepared as described for the fluconazole uptake experiments. The cells (1 ml; 5×10^8 CFU in 0.1 M phosphate buffer [pH 7.4] supplemented with 20 mM glucose) were incubated at 30°C for 20 min, and then uniformly ^{14}C-labelled amino acid mixture was added to a final concentration of 4.4 μM. Samples (100 μl) were removed at various times and placed into 1 ml of ice-cold 100 mM LiCl, filtered on GF/C filters (prewetted with 100 mM LiCl), and washed four times with ice-cold 100 mM LiCl (1 ml). The radioactivity associated with the filters was counted as described above.

Analysis of mRNA in *C. albicans* strains. *C. albicans* cells were grown in YPD medium at 30°C with shaking until the culture reached mid-exponential phase. Cells (1.76×10^9) were harvested by centrifugation ($4,000 \times g$, 5 min at 4°C) and washed three times with diethyl pyrocarbonate-treated water (5), and the RNA was extracted by the method of Schmitt et al. (46). Serial dilutions of RNA were vacuum blotted onto nylon membranes and then hybridized under high-stringency conditions with [^{32}P]dCTP-labelled PCR-generated *CDR1* (38), *BEN*^{r} (12), *ERG16* (*L1A1* [28]), or *ACT1* (29) probe. The probes were PCR amplified from *C. albicans* ATCC 10261 genomic DNA by using the following oligonucleotides: *CDR1*, 5′TTATGTCCAACAACAAGATGTTC3′ and 5′CTGTACATG AAAATCCAAAATCC3′; *BEN*^{r}, 5′TGAGATTCTTGGGTGGATTCTTCG3′ and 5′GGTGGCCAAATTGTCAAACAAAGG3′; *ERG16,* 5′ATGGGTGGTC AACATACTTC3′ and 5′CTTCATCAGAAGAGTTAAATC3′; and *ACT1*, 5′A CTTCTTCTCAATCTTCTGCC3′ and 5′AATGGATGGACCAGATTCGTCG 3′. The probes were partially sequenced to confirm their identities. The membranes were exposed to X-ray film, and the signals on the autoradiograms were quantified with a laser densitometer. The signals generated with the *CDR1*, *BEN*^{r}, and *ERG16* probes were expressed relative to the *ACT1* signals to correct for unequal RNA loading. The labelled probes were hybridized on Northern (RNA) blots as described previously (9).

RESULTS

Characterization of a fluconazole-resistant mutant of *C. albicans* SGY-243. *C. albicans* FR2 was isolated as a fluconazole-resistant mutant of strain SGY-243 following serial passage through media containing fluconazole (see Materials and Methods). *C. albicans* FR2, like the parental strain SGY-243, was Ura$^-$, and the patterns of the *Eco*RI genomic DNA bands that hybridized to the Ca3 moderately repetitive *C. albicans* probe (45) for these strains were identical, showing that they were isogenic. The MIC of fluconazole for FR2 grown in RPMI + Uri was 32 μg/ml (Table 2). The doubling time of FR2 cells in RPMI + Uri (1.72 h) was slightly greater than that of strain SGY-243 (1.38 h). The fluconazole resistance phenotype in FR2 was stable; cells grown for 20 generations in the absence of fluconazole retained the same resistance to fluconazole.

Cross-resistance of various *C. albicans* strains to antifungal agents. The MICs of fluconazole, itraconazole, ketoconazole, and amphotericin B for FR2 and other azole-resistant strains of *C. albicans* were determined (Table 2). The strains were designated as susceptible or resistant to each antifungal agent according to the reported ranges of the MICs of the antifungal agents for susceptible *C. albicans* strains (14, 33, 39, 47). Resistant organisms were defined as those for which the MICs of

TABLE 2. Rates of fluconazole accumulation by various *C. albicans* strains and susceptibilities to antifungal agents

Strain	Initial rate of fluconazole accumulation[a] (pmol/10^9 cells/min)	MIC (μg/ml) (resistance) of[b]:			
		Fluconazole	Itraconazole	Ketoconazole	Amphotericin B
ATCC 10261	2.53	1 (S)	0.125 (S)	0.06 (S)	0.25 (S)
SGY-243	1.95	0.5 (S)	0.004 (S)	0.08 (S)	0.015 (S)
FR2	0.29	32 (R)	0.004 (S)	0.06 (S)	0.008 (S)
B59630	0.09	64 (R)	>4 (R)	>4 (R)	0.125 (S)
AD	1.07	32 (R)	2 (S)	2 (R)	0.25 (S)
KB	0.55	32 (R)	>4 (R)	>4 (R)	0.5 (S)
Darlington	4.60	32 (R)	>4 (R)	2 (R)	2 (R)

[a] The fluconazole uptake results are the means of two separate determinations that did not vary by more than 15%.
[b] The MIC determinations were carried out at least twice. Criteria for resistance are given in the text. S, susceptible to antifungal agent; R, resistant to antifungal agent.

the following antifungal agents were as indicated: fluconazole, >10 μg/ml; itraconazole, >2 μg/ml; ketoconazole, >0.5 μg/ml; and amphotericin B, >1 μg/ml. *C. albicans* ATCC 10261 and SGY-243 were susceptible to all antifungal agents tested, and strain FR2 was resistant only to fluconazole (Table 2). The other azole-resistant *C. albicans* strains showed various levels of resistance to fluconazole and other azoles. Strain Darlington was resistant to all azoles tested and was the only strain resistant to amphotericin B.

Fluconazole accumulation by *Candida* strains. [^{3}H]fluconazole accumulation by energized *C. albicans* SGY-243 cells was linear for 10 min and approached a maximum after 30 min (Fig. 1). The rate of fluconazole accumulation by FR2 was 6.7-fold lower than that by SGY-243 and approached a maximum after 15 min. There was no fluconazole accumulation by heat-killed SGY-243 cells (Fig. 1) or by cells that were incubated at 4°C (data not shown). The initial rates of fluconazole accumulation for other *C. albicans* strains were measured (Table 2). All fluconazole-resistant *C. albicans* strains, except for Darlington, showed a reduced rate of drug accumulation compared with susceptible strains (Table 2).

Preincubation (30°C, 20 min) of *C. albicans* SGY-243 cells with sodium azide (20 mM) did not affect fluconazole accumulation significantly (Fig. 2). Under the same conditions, azide completely abolished amino acid uptake (data not shown). This suggested that fluconazole accumulation by *C. albicans* SGY-243 was not energy dependent. Replacement of glucose in the uptake assay buffer by the glycolysis inhibitor 2-deoxyglucose had no effect on the accumulation of fluconazole by *C. albicans* SGY-243 (data not shown). Azide also had no effect on the net rate of fluconazole uptake by the fluconazole-susceptible wild-type strain *C. albicans* ATCC 10261 or on the net rate of fluconazole uptake by the resistant strain Darlington (Table 3). However, azide treatment increased the net rate of fluconazole uptake by cells of all the other fluconazole-resistant *C. albicans* strains in this study, i.e., FR2 (Fig. 2), B59630, AD, and KB.

To determine whether ketoconazole interfered with fluconazole accumulation in *C. albicans*, the uptake of fluconazole by *C. albicans* SGY-243 was measured in the presence of unlabelled ketoconazole. The net uptake of fluconazole was not affected significantly by the presence of 10 μM ketoconazole (1.90 pmol/min per 10^9 cells) or 100 μM ketoconazole (1.68 pmol/min per 10^9 cells) in the uptake assay. In a separate experiment, the relationship between the rate of fluconazole

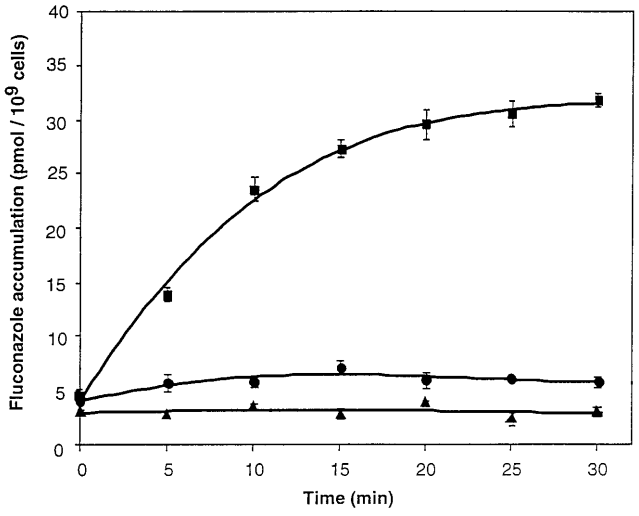

FIG. 1. Fluconazole accumulation by fluconazole-susceptible and fluconazole-resistant *C. albicans* strains. Fluconazole accumulation by *C. albicans* SGY-243 (■), *C. albicans* FR2 (●), or heat-killed (65°C, 30 min) *C. albicans* SGY-243 (▲) cells was measured as described in Materials and Methods. The results are the means ± standard deviations (error bars) of three separate determinations.

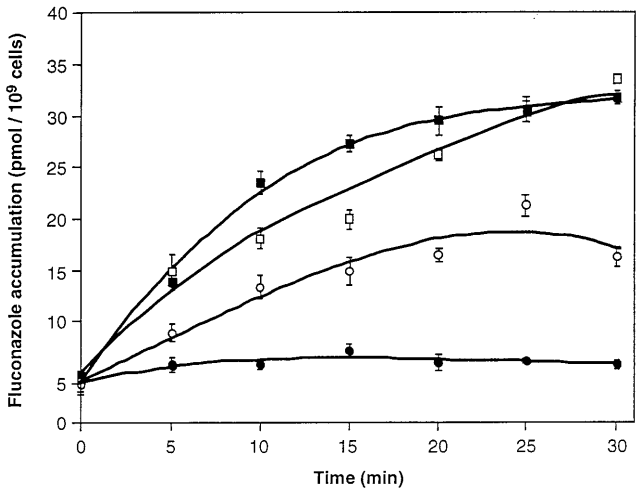

FIG. 2. Effects of azide on fluconazole accumulation by *C. albicans* SGY-243 and FR2. Fluconazole accumulation by *C. albicans* SGY-243 (■, □) or *C. albicans* FR2 (●, ○) was measured in the absence (■, ●) or presence (□, ○) of sodium azide (20 mM). The results are the means ± standard deviations (error bars) of three separate determinations.

TABLE 3. Effects of sodium azide on initial rates of fluconazole accumulation for *C. albicans* strains

Strain	Initial rate of fluconazole accumulation[a] (pmol/10^9 cells/min) in cells:		Effect of azide on rate of fluconazole accumulation (pmol/10^9 cells/min)
	Without azide	With azide[b]	
ATCC 10261	2.53	2.45	−0.08
SGY-243	1.95	1.77	−0.18
FR2	0.29	0.69	+0.40
B59630	0.09	0.40	+0.31
AD	1.07	2.34	+1.27
KB	0.55	1.90	+1.35
Darlington	4.60	4.35	−0.25

[a] Accumulation rates are the means of three determinations that did not vary by more than 15%.

[b] Cells were preincubated with 20 mM azide at 30°C for 20 min before the addition of [^{3}H]fluconazole.

accumulation by *C. albicans* SGY-243 and the fluconazole concentration was not linear, as would be expected for simple diffusion, and uptake became saturated at fluconazole concentrations of >20 μM (Fig. 3).

***CDR1* and *BEN*^{r} mRNA levels in *C. albicans* cells.** In view of the evidence that Cdr1 and/or Ben^r could be associated with drug resistance in *C. albicans* (38, 44), we compared the amounts of these mRNAs in fluconazole-resistant strains with those in fluconazole-susceptible strains. The amount of *ACT1* mRNA (encoding actin) in total RNA was also measured to standardize RNA levels and was found to be similar in all strains. In addition, all RNA preparations contained similar amounts of *PMA1* (31) mRNA, which encodes the plasma membrane proton ATPase (data not shown). The amounts of *CDR1* mRNA relative to *ACT1* mRNA in fluconazole-resistant strains were significantly higher (2- to 17-fold) than those in fluconazole-susceptible strains (Fig. 4). There was little variation in the amount of *ERG16* mRNA in each strain, and the amount of signal generated with this probe was low (Fig. 5). FR2 was the only strain to show overexpression of *BEN*^{r} mRNA. It is important to note that the relative intensities of signals generated by the different probes cannot be compared as probe length and specific activity varied. However, for each probe strain-specific variations in amounts of mRNA can be measured. Each probe hybridized with only one band on Northern blots of total RNA extracted from *C. albicans* SGY-243 or FR2 (Fig. 5 [the shadows under the *BEN*^{r} and *ERG16* signals are due to interference by rRNA in the total RNA preparation]). Growth of SGY-243 cells and, to a lesser extent, FR2 cells in the presence of sub-MIC levels of fluconazole resulted in overexpression of *ERG16* mRNA (Fig. 5), indicating that overproduction of the drug target might be a phenotypic response to intracellular fluconazole. Expression of *BEN*^{r} mRNA by FR2 was unaffected by the presence of fluconazole in the growth medium. However, there was less *CDR1* mRNA in FR2 cells grown in the presence of fluconazole, which argues against the involvement of Cdr1 in the fluconazole resistance of FR2.

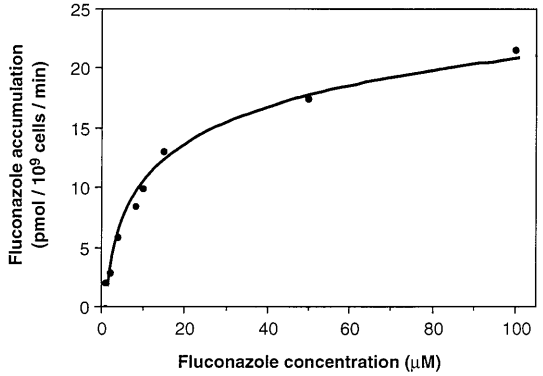

FIG. 3. Fluconazole accumulation rate as a function of fluconazole concentration for *C. albicans* SGY-243. The results are the means of two separate determinations that did not vary by more than 15%.

Susceptibilities of *C. albicans* strains to other drugs. *C. albicans BEN*^{r} expression in *S. cerevisiae* conferred resistance to benomyl, methotrexate, and cycloheximide (12, 15). Since *C. albicans* FR2 overexpressed *BEN*^{r} mRNA, we measured the susceptibilities of cells to these drugs. *C. albicans* FR2 cells were no more resistant to benomyl or methotrexate than were SGY-243 cells. The MICs of benomyl or methotrexate for all strains were greater than 100 or 200 μg/ml, respectively. The fluconazole-susceptible strains ATCC 10261 and SGY-243 were relatively susceptible to cycloheximide (MIC, 250 and 125 μg/ml, respectively). *C. albicans* FR2 and all the other azole-resistant strains were resistant to cycloheximide (MIC, >1,000 μg/ml).

DISCUSSION

Fluconazole-resistant *Candida* spp. are a significant problem for patients undergoing long-term fluconazole treatment (13, 27, 32, 41). In some cases, replacement of susceptible strains by a resistant strain has been shown to occur during fluconazole therapy (2, 37), and in other cases, a single strain has developed reduced susceptibility to fluconazole during treatment (2, 40). There is also heterogeneity in the cross-resistance pattern of fluconazole-resistant clinical isolates to other antifungal agents (13, 25, 32, 42, 49). Some fluconazole-resistant strains are cross-resistant to ketoconazole (13, 42, 49), fewer are cross-resistant to itraconazole (25, 49), and very few are resistant to amphotericin B (32). Therefore, either progressive alterations in a mechanism of resistance, such as drug permeability, gives cross-resistance to a wider variety of antifungal agents or there are several separate resistance mechanisms. The *C. albicans* strains used in this study showed a spectrum of susceptibilities to antifungal agents.

Fluconazole-susceptible *Candida albicans* cells accumulated fluconazole at rates similar to those reported by Hitchcock et al. (21) for *Candida glabrata* (1.65 pmol/min per 10^9 cells) and similar to the net uptake of triazole ICI 153066 by *C. albicans* (2.52 pmol/min per 10^9 cells). There was no accumulation of fluconazole when *C. albicans* cells were incubated on ice, and the metabolic inhibitors sodium azide and 2-deoxyglucose had a negligible effect on uptake by fluconazole-susceptible cells. Also, the rate of fluconazole accumulation became saturated at high fluconazole concentrations. These results suggest that fluconazole entered cells of susceptible strains by facilitated diffusion. Fluconazole has a structure significantly different from that of ketoconazole and displays correspondingly different pharmacokinetics. Fluconazole is a polar molecule and, compared with ketoconazole, is relatively soluble in water. The uptake of ketoconazole at concentrations higher than 0.19 μM occurs by simple diffusion, whereas at lower concentrations its accumulation appears to be an active, energy-dependent process (7). The fact that ketoconazole did not affect the accumulation of fluconazole by *C. albicans* cells is further evidence that these compounds might enter the cells by distinct mechanisms.

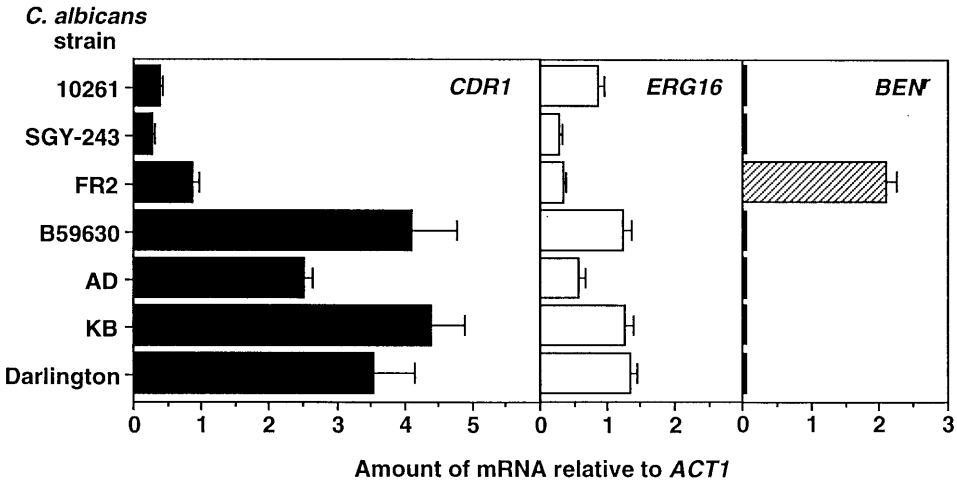

FIG. 4. Amounts of *CDR1*, *ERG16*, and *BEN*^{r} mRNAs in *C. albicans* strains relative to amounts of *ACT1* mRNA. Replicate dot blots of total RNA were hybridized with each radiolabelled probe under high-stringency conditions. The amounts of mRNA were quantified by scanning the autoradiograms of the blots with a laser densitometer. Results are the means ± standard deviations (error bars) of four determinations.

Fluconazole-resistant *C. albicans* FR2 was obtained after prolonged incubation in the presence of fluconazole, which mimicked the clinical situation. Strain FR2 was shown to have a DNA fingerprint identical to that of the parental strain SGY-243, and this genetic similarity enabled the study of factors related specifically to fluconazole resistance. Fluconazole-resistant *C. albicans* FR2, B59630, AD, and KB had much lower rates of fluconazole accumulation than did the susceptible strains. *C. albicans* AD and KB were originally isolated from patients who suffered ketoconazole treatment failure (43). These strains showed negligible accumulation of triazole ICI 153066 (43), and an alteration in membrane sterol composition (19) was proposed to be responsible for their low-level triazole accumulation and hence their azole resistance. *C. albicans* Darlington was unusual in that it accumulated fluconazole at a higher rate than did the fluconazole-susceptible strains, and this accumulation was not affected by azide. The 14α-sterol demethylase in this strain has been shown to be less sensitive to the triazole ICI 153066 (20); however, there was no correlation between the 50% inhibitory concentrations for triazole inhibition of the demethylase and the 50% inhibitory concentrations for growth inhibition. Strain Darlington is also unusual in being resistant to amphotericin B. Triazole resistance in Darlington may be the result of multiple mechanisms, including a defective $\Delta^{5,6}$ sterol desaturase (enzyme involved in sterol biosynthesis) (18, 23). *C. albicans* B59630 is a less well-characterized azole-resistant clinical isolate. It was highly resistant to fluconazole and showed the lowest rate of net accumulation. Thus, a consistent feature of these fluconazole-resistant strains was, with the exception of Darlington, a reduced ability to accumulate fluconazole.

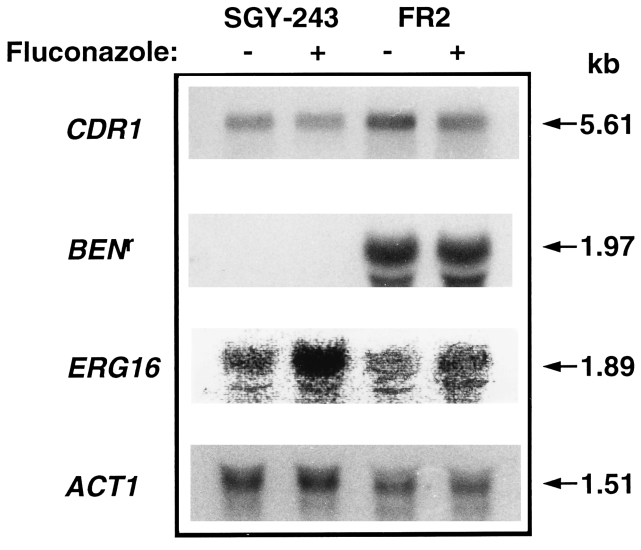

FIG. 5. *CDR1*, *BEN*^{r}, *ERG16*, and *ACT1* transcripts in *C. albicans* SGY-243 or FR2 grown in the absence (−) or presence (+) of fluconazole (0.2 times the MIC). Northern blots of total RNA (20 μg) were hybridized with [^{32}P]dCTP-labelled probes under high-stringency conditions. The signal from the *ERG16* probe was weak and has been enhanced by using the Adobe Photoshop 2.5 program.

While azide treatment had no effect on fluconazole accumulation by fluconazole-susceptible strains, in fluconazole-resistant *C. albicans* FR2, B59360, AD, and KB, the net fluconazole uptake was increased by azide treatment. This provides evidence that the resistance mechanism is energy dependent and may be accounted for by fluconazole efflux as recently proposed for drug-resistant *C. glabrata* (35).

In support of the notion that Cdr1, an ATP-binding cassette-type transporter with sequence similarity to *S. cerevisiae* Pdr5 (1), might be involved in azole resistance (44), all fluconazole-resistant *C. albicans* strains were found to contain increased amounts of *CDR1* mRNA. Furthermore, with the exception of strain Darlington, there was a correlation between the amount of *CDR1* mRNA and the azide-sensitive rate of fluconazole accumulation, taken to indicate the approximate rate of efflux. Strain FR2 was unusual in that as well as being resistant only to fluconazole, it was the only strain to show increased amounts of *BEN*^{r} mRNA as well as *CDR1* mRNA.

The *C. albicans CDR1* and *BEN*^{r} genes are currently implicated in drug resistance. Both genes were cloned originally from *C. albicans* libraries by conferring resistance to cycloheximide, chloramphenicol, and miconazole (*CDR1*) (38) or cycloheximide, benomyl, and methotrexate (*BEN*^{r}) (4, 12) on *S. cerevisiae*. However, the roles of these genes in *C. albicans* drug resistance are far from clear. Indeed, inactivation of *BEN*^{r} in

C. albicans did not lead to benomyl resistance (15), suggesting alternate mechanisms for this resistance in *C. albicans*. This is supported by the present work, which showed that isogenic mutant strain FR2 contained much more *BEN*^{r} mRNA than did strain SGY-243 but was not significantly more resistant to benomyl or methotrexate. Overexpression of *BEN*^{r} mRNA was associated with fluconazole resistance in strain FR2; however, the other fluconazole-resistant strains, which were all cross-resistant to other azoles, did not express elevated amounts of *BEN*^{r}. Taken collectively, these data suggest that azole resistance in *C. albicans* might develop as a multistep process. Resistance to fluconazole can be acquired by mutations associated with increased expression of *BEN*^{r} and/or *CDR1*, while other mutations causing increased *CDR1* expression are associated with multiazole resistance. If such mutations can be acquired at a relatively high frequency, this would permit *C. albicans* cells to survive in the presence of fluconazole or other azoles and acquire further specific drug target mutations that might confer multidrug high-level resistance (such as that exhibited by strain Darlington).

It should be noted that there is still no direct evidence that Ben^r or Cdr1 proteins bind and transport antifungal drugs. While it seems most logical to suggest that Ben^r and Cdr1 are directly involved in azole drug efflux, it is now known that membrane transporters can regulate the activities of heterologous membrane channels (17). It seems plausible then to suggest that Ben^r and Cdr1 might impinge on the activities of other (as yet undefined) systems that are directly involved in conferring multidrug resistance. Such regulatory functions might be in keeping with recent evidence from other microbial drug efflux systems in which multiple interactive mechanisms for drug resistance are suggested (24). It is important therefore that we define precisely the biochemical activities of Ben^r and Cdr1 in order to understand the molecular basis of efflux-mediated azole resistance in *C. albicans*.

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