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Upregulation of *ERG* Genes in *Candida* Species by Azoles and Other Sterol Biosynthesis Inhibitors

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Infections due to *Candida albicans* are usually treated with azole antifungals such as fluconazole, but treatment failure is not uncommon especially in immunocompromised individuals. Relatedly, in vitro studies demonstrate that azoles are nonfungicidal, with continued growth at strain-dependent rates even at high azole concentrations. We hypothesized that upregulation of *ERG11*, which encodes the azole target enzyme lanosterol demethylase, contributes to this azole tolerance in *Candida* species. RNA analysis revealed that *ERG11* expression in *C. albicans* is maximal during logarithmic-phase growth and decreases as the cells approach stationary phase. Incubation with fluconazole, however, resulted in a two- to fivefold increase in *ERG11* RNA levels within 2 to 3 h, and this increase was followed by resumption of culture growth. *ERG11* upregulation also occurred following treatment with other azoles (itraconazole, ketoconazole, clotrimazole, and miconazole) and was not dependent on the specific medium or pH. Within 1 h of drug removal *ERG11* upregulation was reversed. Azole-dependent upregulation was not limited to *ERG11*: five of five *ERG* genes tested whose products function upstream and downstream of lanosterol demethylase in the sterol biosynthetic pathway were also upregulated. Similarly, *ERG11* upregulation occurred following treatment of *C. albicans* cultures with terbinafine and fenpropimorph, which target other enzymes in the pathway. These data suggest a common mechanism for global *ERG* upregulation, e.g., in response to ergosterol depletion. Finally, azole-dependent *ERG11* upregulation was demonstrated in three additional *Candida* species (*C. tropicalis*, *C. glabrata*, and *C. krusei*), indicating a conserved response to sterol biosynthesis inhibitors in opportunistic yeasts.

The dimorphic yeast *Candida albicans* is a common cause of vaginitis and, in immunocompromised individuals, of oropharyngeal and systemic infections. Antifungal azoles such as fluconazole (oral and intravenous) and miconazole (topical) are used for treatment or prophylaxis of most *C. albicans* infections. While azoles have little or no toxicity they generally lack fungicidal activity. Consequently, in immunocompromised individuals azoles must be administered for extended periods of time. This practice, combined with the increased use of azoles in recent years, is most likely responsible for the increased isolation of azole-resistant strains of *C. albicans* and of intrinsically resistant *Candida* species such as *C. glabrata* and *C. krusei* (2, 17, 28, 40). Even in the absence of resistance, treatment failures or recurrent infections are not uncommon, especially in immunocompromised individuals (28). These clinical limitations associated with azole use have in vitro correlates. In susceptibility assays, significant "trailing" growth occurs even at high fluconazole concentrations with many *Candida* isolates (23, 26, 30). In agar diffusion assays, trailing is visualized as background growth which may obscure the zone of inhibition (33).

Azoles inhibit the enzyme lanosterol demethylase in the sterol biosynthetic pathway. This pathway is conserved in eukaryotes, leading to cholesterol in mammals and ergosterol in fungi (Fig. 1). In *Saccharomyces cerevisiae* and *C. albicans* sterols have been shown to be important in membrane fluidity, membrane permeability, cell morphology, enzyme activity, and cell cycle progression (5, 13, 18, 19, 20). Sterol precursors are also involved in heme and glycolipid biosynthesis as well as protein prenylation (20). In addition to azoles, other classes of antifungals target ergosterol synthesis, including allylamines (e.g., terbinafine) and morpholines (e.g., fenpropimorph).

Lanosterol demethylase is encoded by the gene *ERG11* (also known as *CYP51*). Multiple mechanisms for azole resistance in *C. albicans* clinical isolates have been identified, including increased expression of multidrug transporters (encoded by *CDR1*, *CDR2*, and *MDR1*), mutations in lanosterol demethylase that reduce azole binding, and increased expression of *ERG11* (7, 22, 34, 36, 41, 42). Genetic studies with *S. cerevisiae* confirm that each of these mechanisms can operate alone to confer various degrees of azole resistance (1, 16, 36). Specifically, *GAL1* promoter-mediated overexpression of *ERG11* in *S. cerevisiae* was recently shown to confer high-level fluconazole resistance, further implicating *ERG* gene upregulation as a factor in azole resistance (14).

Regulation of the ergosterol biosynthetic pathway has been studied in some detail in *S. cerevisiae*. Changes in the activities of selected enzymes or in the levels of expression of selected *ERG* genes in response to sterol availability, genetic lesions in the pathway, or treatment with specific inhibitors have been described (3, 6, 13, 20, 24, 31, 37). More recently, microarray techniques in combination with the *S. cerevisiae* genome sequence have permitted investigation of global changes in gene expression associated with *ERG* mutations and inhibitor treatment (4, 32).

In light of its potential role in modulating azole susceptibility, we have examined the effects of azole treatment on *ERG* expression in *C. albicans*. We report that azole exposure leads to *ERG11* upregulation, which was followed by resumed culture growth. In addition, we demonstrate that (i) other *ERG* genes are upregulated by azole treatment, (ii) other inhibitors of ergosterol biosynthesis upregulate *ERG11*, and (iii) azole

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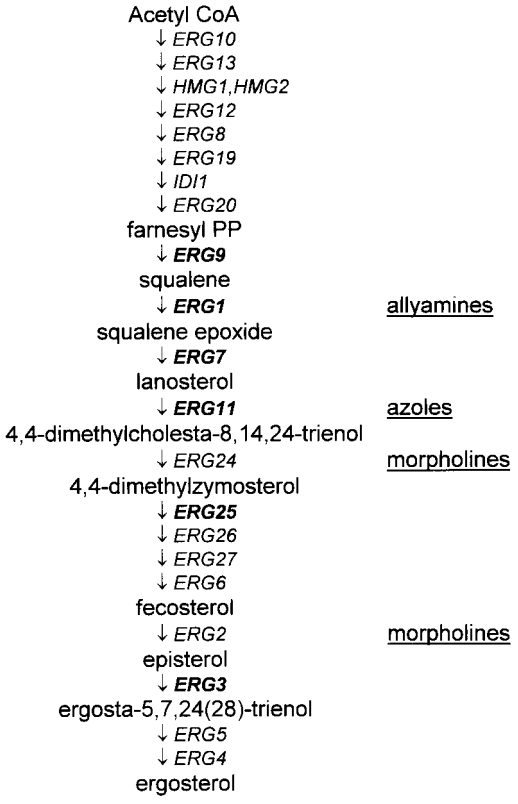

FIG. 1. Ergosterol biosynthesis pathway. Only selected substrates or products are shown. Genes (in italics) that encode enzymes in the pathway are labeled according to the convention for *S. cerevisiae*; those whose expression was monitored in this study are shown in bold. The three groups of sterol biosynthesis inhibitors examined in this study are underlined and are shown to the right of the gene encoding the targeted enzyme. CoA, coenzyme A.

treatment upregulates *ERG11* expression in other *Candida* species.

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MATERIALS AND METHODS

Strains, media, and drugs. The strains used in this study are listed in Table 1, along with their azole susceptibilities. *Candida* species were maintained on liquid or agar YPD medium (1% yeast extract, 2% peptone, 2% dextrose). Susceptibility assays were performed in RPMI 1640 medium with L-glutamate and without $NaHCO_3$ (Sigma, St. Louis, Mo.), buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS). DOB medium (0.17% yeast nitrogen base, 2% dextrose, 0.5% ammonium sulfate) was prepared as recommended by the manufacturer (Bio 101, Inc., Vista, Cal.). RNA expression studies were done in YPD or, where indicated, RPMI 1640 medium or DOB medium. The following drugs were purchased from the indicated supplier: fluconazole, Pfizer (Groton, Conn.); terbinafine, Novartis (East Hanover, N.J.); itraconazole and ketoconazole, Jannsen (Titusville, N.J.); fenpropimorph, Cresent Chemical (Hauppauge, N.Y.); and miconazole and clotrimazole, Sigma. The concentrations of the fluconazole stocks were 2 mg/ml in saline, and the stocks were stored at 4°C; the concentrations of all others were 10 mg/ml in dimethyl sulfoxide and the stocks were stored at −20°C.

Susceptibility assays. Susceptibility testing of *Candida* species was done according to the guidelines in document M27-A (26) in flat-bottom 96-well polystyrene microtiter plates. Briefly, a 100-μl volume of RPMI 1640 medium was added to a series of wells, except for the initial well, to which 195 μl was added. Drugs (5 μl) were added to the initial well at twice the maximum concentration to be tested (16 μg/ml for itraconazole and ketoconazole), and twofold serial dilutions were made by transferring 100 μl of this solution to subsequent wells. When testing for fluconazole susceptibility, only 174.4 μl of RPMI 1640 medium was added to the initial well, as 25.6 μl of the drug stock was required to achieve a final concentration of 256 μg/ml in 200 μl. The final well in each series received no azole and served as a growth control. Control series received RPMI 1640 medium alone or RPMI 1640 medium plus 0.5% DMSO depending upon the drug vehicle used. Logarithmic-phase cultures of yeast were diluted in RPMI 1640 medium, and 100 μl was added to each well to give a final density of 10^4 cells/ml. Plates were incubated at 35°C, and the absorbance at 630 nm was read at 24 and 48 h with a microplate reader (Bio-Tek Instruments, Winooski, Vt.). Dilutions (fivefold) of additional drug-free wells were prepared and served as references for readings of the MIC (80% reduction in turbidity).

RNA isolation. Logarithmic-phase cultures (3×10^7 to 5×10^7 cells/ml) were exposed to drugs at the indicated concentrations and times at 30°C (or 35°C where indicated) with shaking. Controls received an equivalent amount of DMSO or were untreated. RNA was extracted as described previously (9). Briefly, at the indicated times, 10^8 cells from each culture were transferred to microcentrifuge tubes, pelleted, and washed twice in ice-cold 50 mM sodium acetate–10 mM EDTA buffer (pH 4.5). The pellet was resuspended in 200 μl of ice-cold sodium acetate–EDTA buffer, followed by the addition of 200 μl of glass beads, 20 μl of 10% sodium dodecyl sulfate, and 200 μl of prewarmed buffer-saturated phenol. The cells were disrupted by periodic vigorous vortexing and incubation at 65°C for 5 to 10 min. The samples were cooled on ice and centrifuged, and the aqueous phase was reextracted with phenol-chloroform-isoamyl alcohol (25:24:1), followed by extraction with chloroform-isoamyl alcohol (24:1). (The reextractions were omitted when RNA was isolated for slot blot analysis.) RNA was precipitated overnight with 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate and was collected by centrifugation.

RNA hybridization analysis. For slot blot analysis, RNA was dissolved in 500 μl of H_2O and denatured by the addition of 200 μl of 37% formaldehyde and 300 μl of 20× SSPE (20× SSPE is 3.6 M sodium chloride, 0.2 M sodium phosphate, and 20 mM EDTA [pH 7.0]) with incubation at 65°C for 15 min. Either 100 μl (for the *ACT1* probe) or 250 μl (for the other probes) of denatured RNA was applied to a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, Ind.) with a Bio-Dot SF apparatus (Bio-Rad, Richmond, Calif.). The membranes were rinsed in 2× SSPE and UV cross-linked. Slot blots were hybridized overnight to *C. albicans ACT1*-, *ERG11*-, *CDR1*-, or *CDR2*-specific PCR products (see Table 2) that were random primer labeled with digoxigenin-dUTP, as recommended by the manufacturer (Boehringer Mannheim). The blots were washed twice under high-stringency conditions (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% sodium dodecyl sulfate at 68°C) and prepared for chemiluminescence detection with CSPD substrate, as recommended. After 15 min of incubation at 37°C, the blots were exposed to Kodak X-OMAT LS film for 1 to 15 min. RNA levels were quantified with a densitometer (Bio-Rad GS-670 imaging densitometer with Molecular Analyst software), with *ERG11*, *CDR1*, and *CDR2* levels normalized to the levels of the *ACT1* controls.

Reverse transcription-PCR (RT-PCR) analysis. RNA pellets were washed in 70% ethanol and resuspended in 50 μl of RNase-free water. Aliquots (1 μg) were treated with RNase-free DNase I as recommended by the manufacturer (Promega, Madison, Wis.). cDNAs were prepared with reverse transcriptase (Moloney murine leukemia virus reverse transcriptase; New England Biolabs, Beverly, Mass.), as recommended, by using the reverse primers listed in Table 2. Each reaction had a parallel reaction that lacked reverse transcriptase to confirm that the PCR product was derived from cDNA rather than genomic DNA contamination. PCR was performed with the primer sets listed in Table 2. Initially, genes were amplified by PCR for 23 cycles to determine levels of expression. Genes expressed at medium to high levels (genes for which PCR products were observed at 23 cycles; *ACT1*, *ERG11*, *ERG3*, and *CDR*) were subjected to three independent amplifications of 20, 23, and 25 cycles to ensure that amplification during the logarithmic phase was obtained. Genes expressed at low levels (little or no PCR product at 23 cycles; *ERG9*, *ERG1*, *ERG7*, and *ERG25*) were am-

TABLE 1. Strains used in this study and azole susceptibilities

Candida species	Strain	MIC (μg/ml)					
		Fluconazole		Itraconazole		Ketoconazole	
		24 h	48 h	24 h	48 h	24 h	48 h
C. albicans	ATCC 24433	0.25	1.0	0.016	0.031	0.016	0.031
	ATCC 90028	0.25	2.0	0.016	0.031	0.016	0.062
	630-15.3[a]	0.25	0.50	0.016	0.016	0.016	0.016
	707.15[a]	0.50	>256	0.031	>8.0	0.016	>8.0
C. glabrata	ATCC 66032	16	128	0.062	2.0	0.031	2.0
C. tropicalis	ATCC 750	0.50	>256	<0.031	0.12	<0.031	0.50
C. krusei	ATCC 14243	32	64	0.031	0.062	0.031	0.25

[a] Obtained from J. Rex (30).

TABLE 2. Primers used in this study

Gene	Protein	GenBank accession no.	Primer	Location[a]	Sequence (5′ to 3′)[b]
ERG9	Squalene synthase	D89610	Forward	448 to 467	AAAATGGGTAATGGTATGGC
			Reverse	895 to 914	ACTTGGGGAATGGCACAAAA
ERG1	Squalene epoxidase	U69674	Forward	583 to 605	TTGACAWTTAKTTGTGATGGTAT
			Reverse	1243 to 1265	CTTTGGAAATATTTGAAACAACC
ERG7	Squalene cyclase	L04305	Forward	1584 to 1603	TATCCATACGTGGAATGTAC
			Reverse	2133 to 2154	TGTATAWACCTAATGCCTTAAT
ERG11	Lanosterol 14α-demethylase	X13296	Forward	903 to 931	ATTGGTATTCTTATGGGTGGTCAACATAC
			Reverse	1390 to 1416	CCCAATACATCTATGTCTACCACCACC
ERG25	C-4 Methyl sterol oxidase	AF051914	Forward	235 to 257	ATTCCWTATTTTAGAAAATGGAA
			Reverse	805 to 825	GWAATCCCACCATCTAAAAGA
ERG3	Δ5,6-Desaturase	AF069752	Forward	459 to 482	CCWMTTTGAAAAACCAAATG
			Reverse	961 to 984	GAATTGACCGTAGTTGTAGTTGAA
ACT1	Actin	X16377	Forward	975 to 1001	ACCGAAGCTCCAATGAATCCAAAATCC
			Reverse	1465 to 1491	GTTTGGTCAATACCAGCAGCTTCCAAA
CgACT1	*C. glabrata* actin	AF069746	Forward	45 to 64	ATGTGTAAGGCKGGKTTTGC
			Reverse	1050 to 1069	ATCCACATTTGTTGGAAKGT
CDR	ABC transporters *CDR1* and *CDR2*	X77589 and U63812	Forward	901 to 924	GTGGTGTTTCCGGTGGTGAAAGAAA
			Reverse	1379 to 1403	CCTGGTGTTGGATCGTTCACATTCA
CDR1	ABC transporter *CDR1* specific	X77589	Forward	−184 to −163	TTTTTTTTTTTAGTTCATCATC
			Reverse	242 to 262	GGTCATTATTTATTTCTTCAT
CDR2	ABC transporter *CDR2* specific	U63812	Forward	−187 to −167	CTCTATTATGAATACTAGTAG
			Reverse	239 to 258	TTGTTCATGACTATTGTTGC
MDR1	Major facilitator superfamily transporter	X53823	Forward	451 to 476	GAGTCGTAGCTACATTGCCATTAACA
			Reverse	1014 to 1040	GGTGATTTCTAATGGTCTCCATAATGT

[a] Location refers to the nucleotide sequence within the *C. albicans* gene (except *CgACT1*), with the "A" of the start codon (ATG) representing +1.
[b] Code for mixed bases within the primer sequence: W = A + T, K = G + T, and M = A + C.

plified for 25, 28, and 30 cycles. Cycling conditions for *ACT1*, *CDR*, and *ERG11* were 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. All other genes were amplified with an annealing temperature of 52°C due to differences in primer melting temperatures. The PCR products were subjected to gel electrophoresis and ethidium bromide staining, and relative intensities were determined with a densitometer, with normalization to *ACT1* levels.

RESULTS

***ERG11* expression varies with growth phase.** In initial experiments, *C. albicans* gene expression was monitored by RNA slot blot hybridization. The probes used were specific for their targeted RNAs, as determined by preliminary Northern and Southern blotting and, in the case of the *CDR1* and *CDR2* probes, by slot blot hybridization to RNAs from strains from which those genes had been deleted (35). In subsequent experiments, gene expression was monitored by RT-PCR. While RNA hybridization is potentially more quantitative, RT-PCR facilitates the analysis of larger numbers of samples and of multiple genes. In selected experiments in which both assays were used, comparable results were obtained (compare Fig. 2A and 5).

Both slot blot (Fig. 2A) and RT-PCR (see Fig. 5) analyses revealed that the level of *ERG11* expression in *C. albicans* strain 24433 varies dramatically with the growth phase. *ERG11* RNA levels were maximal during logarithmic-phase growth (0 h; 3×10^7 to 5×10^7 cells/ml) and then decreased 3- to ≥10-fold as the cells approached the stationary phase (5 to 10 h; $\geq 1 \times 10^8$ cells/ml). The levels of *ACT1* RNA were relatively constant over this interval. In contrast, expression of the multidrug transporter gene *CDR1* increased as cells approached stationary phase (Fig. 2A), in agreement with previous studies (10, 15). *CDR2* expression was not detected in untreated logarithmic- or stationary-phase cultures (data not shown).

Azole treatment of *C. albicans* leads to *ERG11* upregulation. Treatment of *C. albicans* cultures with the triazole fluconazole at a concentration of 2 to 9 μg/ml resulted in four- to fivefold increases in *ERG11* RNA levels within 1.5 to 2 h of incubation (Fig. 2A and B). This increase was in marked contrast to the late-logarithmic-phase-associated reduction observed in the untreated controls. Lower but reproducible upregulation was also detected in cultures treated with as little as 0.5 μg of fluconazole per ml (data not shown). *ERG11* expression remained elevated for up to 18 h (Fig. 2A). Treatment with four other azoles (the triazole itraconazole and the imidazoles ketoconazole, clotrimazole, and miconazole, all at 0.5 μg/ml) had an effect comparable to that of fluconazole (Fig. 2B). Furthermore, comparable fluconazole-dependent *ERG11* upregulation was observed in three of three additional *C. albicans* strains tested, including a strain (strain 707.15) which exhibits high-level trailing (Table 1; data not shown).

Fluconazole (2 or 8 μg/ml) treatment of *C. albicans* cultures also resulted in increased *CDR1* expression. However, this was observed only after 10 to 18 h of azole treatment (Fig. 2A). *CDR2* expression was not detected (data not shown).

Correlation of *ERG11* upregulation and *C. albicans* growth in fluconazole-treated cultures. Under the conditions used, fluconazole at 9 μg/ml completely inhibited the growth of a *C. albicans* culture during the initial 3 h of treatment, whereupon growth resumed (Fig. 3). In the same experiment, flu-

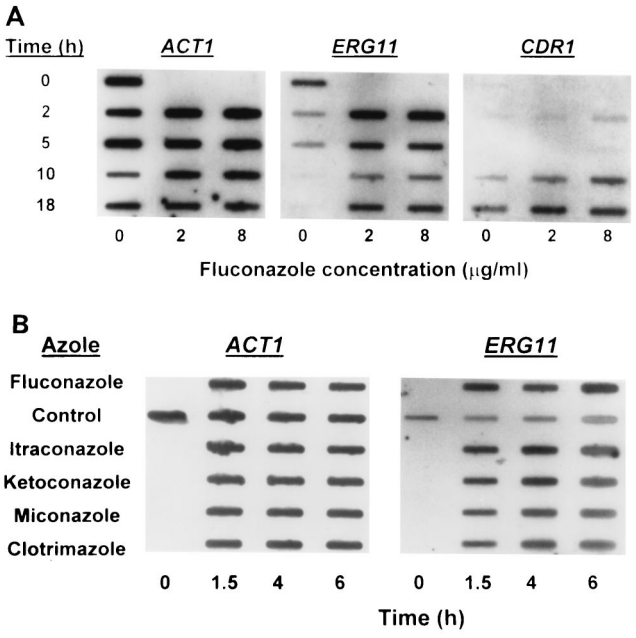

FIG. 2. Effect of azole treatment on *ERG11* expression in *C. albicans*. (A) RNA slot blot analysis of *ACT1*, *ERG11*, and *CDR1* expression in *C. albicans* 24433 following fluconazole treatment (0, 2, or 8 μg/ml) for the indicated times. Similar results were observed in four independent experiments and with a second *C. albicans* strain (strain ATCC 90028). (B) RNA slot blot analysis of *ACT1* and *ERG11* expression in *C. albicans* 24433 following treatment with the indicated azoles for the indicated times. Azole concentrations were 9 μg/ml (fluconazole) and 0.5 μg/ml (all others). The control received DMSO vehicle (final concentration, 0.25%). Similar results were observed in a second independent experiment.

conazole treatment resulted in a fivefold increase in *ERG11* RNA levels (relative to those for the control at 0 h) after 2 h. Thus, *ERG11* upregulation directly preceded and presumably contributed to the resumption of *C. albicans* growth.

The experiment described above was repeated, except that the fluconazole was removed at the 2-h time point by careful washing. The 2.6-fold increase in *ERG11* RNA levels after 2 h

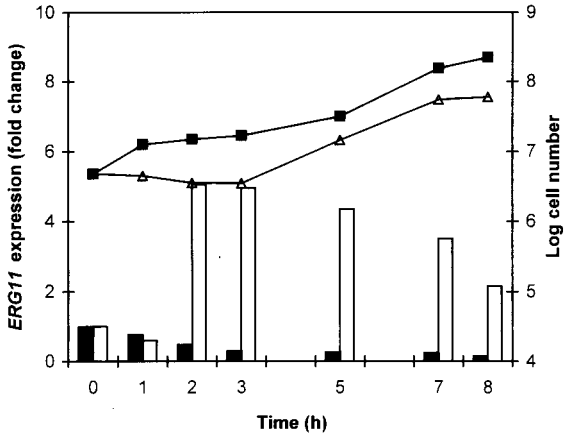

FIG. 3. Correlation of *ERG11* expression and *C. albicans* 24433 growth in control and fluconazole-treated cultures. RNA levels (determined from slot blot analysis and normalized to *ACT1* levels) are represented as fold increase or decrease relative to the level for the control at 0 h; solid bars, 0 μg of fluconazole per ml; open bars, 9 μg of fluconazole per ml. *C. albicans* growth is represented as cell number (determined in a hemocytometer) at the indicated times and treatments: no drug (■) and 9 μg of fluconazole per ml (△). The data shown represent the averages of three independent experiments.

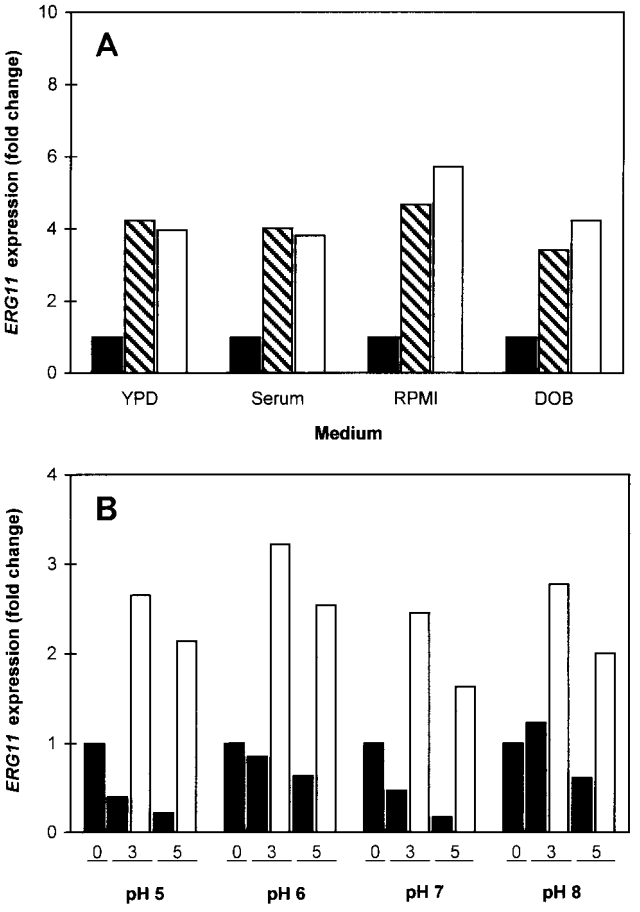

FIG. 4. Effects of medium and pH on fluconazole-dependent *ERG11* upregulation in *C. albicans* 24433. (A) Media were YPD, YPD supplemented with 20% fetal bovine serum (Serum), RPMI 1640 medium prepared according to the susceptibility testing guidelines in document M27-A (26) (RPMI), and supplemented yeast nitrogen base (DOB). Effects on *ERG11* expression were analyzed by slot blot analysis with normalization to *ACT1* RNA levels and are represented as the fold change relative to the level for the untreated controls (solid bars). Treatment was for 2 h with fluconazole at 1 (hatched bars) or 9 (open bars) μg/ml. The data shown are the averages of two independent experiments. (B) RPMI 1640 medium was adjusted to the indicated pH, and *ERG11* expression was analyzed by RT-PCR. Incubation was at 35°C instead of 30°C to conform with the guidelines in document M27-A (26). Treatment was for 0, 3, or 5 h, as indicated, with no drug (solid bars) or fluconazole at 9 μg/ml (open bars). The data represent the averages of four RT-PCRs from two independent experiments.

of fluconazole treatment was reduced to 1.1-fold after 0.5 h and to 0.5-fold (comparable to that for the untreated control) after 1 h of further incubation in drug-free medium (data not shown). Thus, fluconazole-dependent *ERG11* upregulation is reversible.

***ERG11* upregulation is independent of medium and pH.** The activity of fluconazole against *C. albicans* is affected by changes in medium and pH (23, 29, 33). Therefore, the effects of these variables on fluconazole-dependent upregulation of *ERG11* were examined. *ERG11* expression after 2 h of fluconazole treatment was compared in YPD (used in all experiments described above), YPD with 20% fetal bovine serum (which induces germ tubes), a defined RPMI 1640 medium (according to the guidelines in document M27-A [26] for susceptibility testing), and a defined minimal medium (supplemented yeast nitrogen base [DOB medium]). Comparable levels of upregulation were observed in all four media (Fig. 4A), although there were different basal levels of *ERG11* expression that

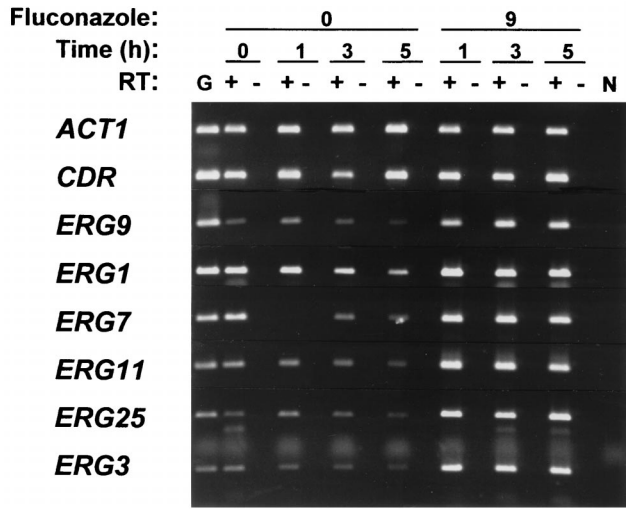

FIG. 5. Global upregulation of *C. albicans ERG* genes following fluconazole treatment. RT-PCR was used to analyze the relative expression of *ACT1*, *CDR* (*CDR1* and *CDR2*), and the indicated *ERG* genes in cultures (strain 24433) treated with fluconazole at 9 μg/ml (labeled 9) or no drug (labeled 0) for 0, 1, 3, or 5 h, as indicated. Equal volumes from cDNA reactions with (+) or without (−) reverse transcriptase (RT) were amplified in parallel to detect genomic DNA contamination. Lane G, PCR positive control containing genomic DNA; lane N, PCR negative control without added template. The data shown are representative of two independent experiments, and similar data were obtained with an additional *C. albicans* isolate (isolate 630-15.3). Due to differences in basal levels of expression, PCR cycle numbers were optimized for each gene to ensure logarithmic-phase amplification. The cycle numbers used were 23 (*ERG11* and *ACT1*), 25 (*CDR*), 28 (*ERG3*, *ERG7*, *ERG9*), and 30 (*ERG1* and *ERG25*). On the basis of previous slot blot data, *CDR* expression is primarily due to *CDR1*.

positively correlated with the medium-dependent growth rate (data not shown).

Fluconazole-induced upregulation was further examined in RPMI 1640 medium buffered to pH 5, 6, 7, and 8. Alteration of the pH did not significantly alter the ability of *C. albicans* to upregulate *ERG11* in response to azole treatment (Fig. 4B). Alteration of the pH also had no detectable effect on the expression of the multidrug transporter genes *CDR1*, *CDR2*, and *MDR1* (data not shown).

Azole treatment induces a global increase in sterol biosynthesis gene expression. The data presented above demonstrate that azole treatment upregulates expression of *ERG11*, which encodes the azole target lanosterol demethylase. The effects of azole treatment on the expression of other *ERG* genes was examined next. Three of the genes examined (*ERG9*, *ERG1*, and *ERG7*) encode enzymes that act upstream, and two (*ERG25* and *ERG3*) encode enzymes that act downstream of lanosterol demethylase in the ergosterol biosynthesis pathway (Fig. 1). As with *ERG11* (see above), the expression of these genes was maximal in logarithmic-phase cells (0 h) and decreased as the cultures approached the stationary phase (3 to 5 h) (Fig. 5). Similarly, fluconazole (9 μg/ml) treatment of these cultures resulted in increased levels of expression of all five *ERG* genes, in addition to *ERG11*. This global *ERG* upregulation was also observed in a second *C. albicans* strain (strain 630-15.3; data not shown).

Inhibitors targeting other sterol biosynthesis enzymes upregulate *ERG11*. Since fluconazole upregulated the expression of multiple *ERG* genes, it seemed likely that inhibitors acting at other steps in the sterol biosynthesis pathway might upregulate *ERG11*. The allylamine terbinafine inhibits the *ERG1* product squalene epoxidase, which acts upstream of the *ERG11* product lanosterol demethylase (Fig. 1). Nevertheless, slot blot analysis showed that treatment with terbinafine (1 and 9 μg/ml) for 3 to 5 h upregulated *ERG11* RNA two- to threefold (relative to that at 0 h) (Fig. 6). The morpholine fenpropimorph inhibits the *ERG24* and *ERG2* products, which act downstream of *ERG11* (Fig. 1). Similarly, RT-PCR revealed that treatment with fenpropimorph (1 and 9 μg/ml) upregulated *ERG11* 1.8- to 2.2-fold (Fig. 6).

Azole-dependent *ERG11* upregulation is conserved in other *Candida* species. The ability of fluconazole to upregulate *ERG11* in *C. tropicalis*, *C. glabrata*, and *C. krusei* was examined. As indicated in Table 1, these three species are either moderately (*C. glabrata*) or highly (*C. krusei*) resistant to fluconazole or exhibit high-level trailing (*C. tropicalis*; the MIC at 48 h was substantially greater than the MIC at 24 h). RT-PCR was used with a single primer pair that represented conserved *ERG11* sequences present in all *Candida* species tested. As for *C. albicans*, fluconazole (9 μg/ml) treatment for 3 to 5 h resulted in *ERG11* upregulation in *C. tropicalis* (1.7- to 2.4-fold), *C. glabrata* (1.8-fold), and *C. krusei* (2.0-fold) (Fig. 7). Itraconazole has a relatively high level of activity against several fluconazole-resistant fungi, including *C. krusei* (Table 1). *C. tropicalis*, *C. glabrata*, and *C. krusei* upregulated *ERG11* 1.8- to 2.5-fold in response to itraconazole treatment (0.1 μg/ml for 3 to 5 h) (Fig. 7).

DISCUSSION

Azoles are the most widely used group of antifungals. Fluconazole, in particular, has negligible toxicity, excellent bio-

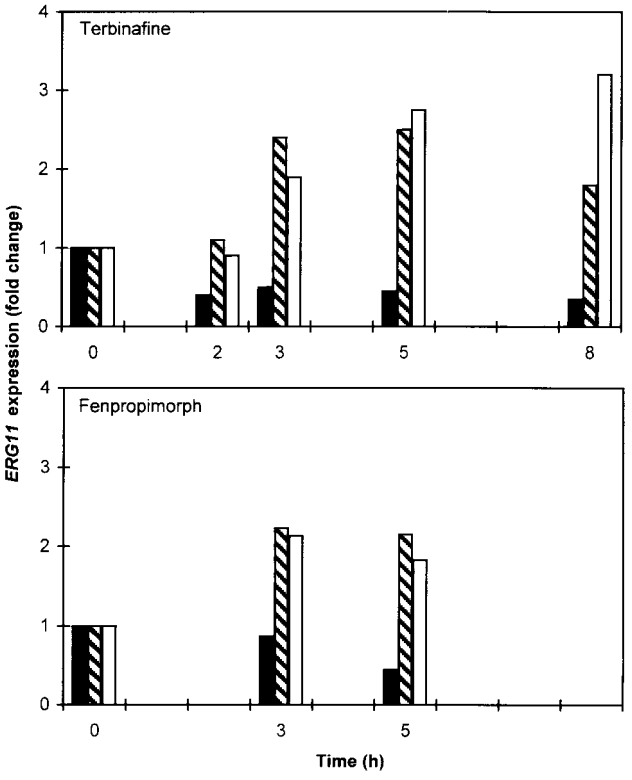

FIG. 6. Upregulation of *C. albicans ERG11* following treatment with terbinafine or fenpropimorph. *ERG11* RNA levels were examined by slot blot hybridization (terbinafine) or RT-PCR (fenpropimorph), normalized to *ACT1* RNA levels, and represented as the fold change relative to the RNA levels at 0 h. Cultures were treated with no drug (solid bars) or the indicated drug at 1 (hatched bars) and 9 (open bars) μg/ml for the indicated times.

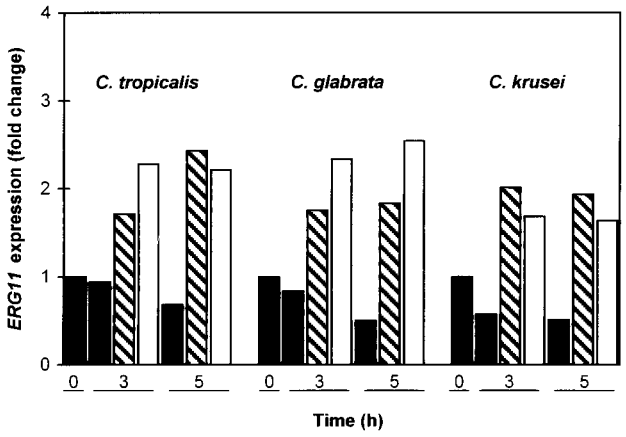

FIG. 7. Upregulation of *ERG11* in three additional *Candida* species following azole treatment. RNA levels were examined by RT-PCR after 0, 3, and 5 h of treatment, as indicated, and are represented as the fold change relative to the levels at 0 h. Cultures were treated with no drug (solid bars), fluconazole at 9 μg/ml (hatched bars), or itraconazole at 0.1 μg/ml (open bars).

availability, and a moderately high level of activity against commonly encountered yeasts such as *C. albicans*. Other azoles such as itraconazole have potent and broader-spectrum activity that extends to fluconazole-resistant *Candida* species and many molds. However, most azole treatments only suppress and do not eliminate fungal infections. In immunocompromised individuals, this leads to a requirement for long-term treatment, which in turn leads to a selection for azole-resistant fungal strains. In vitro, the nonfungicidal activity of azoles is readily apparent in the trailing observed in broth dilution assays (23, 30) and the background growth observed in agar diffusion assays such as the E-test (33). Indeed, these assays indicate that fluconazole and potentially other azoles lack even fungistatic activity toward common fungal pathogens. Understanding the cellular responses to these drugs may enhance our understanding of antifungal resistance and facilitate the development of new antifungal agents.

We hypothesized that the ability of *C. albicans* to tolerate azole treatment was at least partly due to the upregulation of the *ERG11* gene, which encodes the azole target lanosterol demethylase. This hypothesis derived from previous reports that *ERG11* was constitutively upregulated in certain fluconazole-resistant clinical isolates (7, 22, 41; A. M. Alarco, I. Balan, F. Comte, G. St-Germain, D. Falconer, T. Parkinson, C. A. Hitchcock, and M. Raymond, Abstr. ASM Conf. Candida and Candidiasis, abstr. C22, p. 54, 1999). Also, in *S. cerevisiae ERG11* overexpression was directly shown to confer fluconazole resistance (14; K. W. Henry and T. D. Edlind, unpublished data). In support of the hypothesis, our data demonstrate *C. albicans ERG11* upregulation in response to treatment with any of five different azoles. Upregulation is maximal 2 to 3 h after treatment and is most readily visualized in late-logarithmic-phase cultures in which *ERG11* expression is normally declining. The effect was observed in four different media over a pH range of 5 to 8 and was reversed following drug removal.

Fluconazole treatment of *C. albicans* upregulated not only *ERG11* encoding the azole target but also five of five other *ERG* genes tested. Consistent with this global upregulation, treatment with compounds that inhibit enzymes upstream (terbinafine) and downstream (fenpropimorph) of lanosterol demethylase also led to *ERG11* upregulation. Finally, *ERG11* upregulation in response to azole treatment was demonstrated in three other *Candida* species with various fluconazole susceptibilities: moderately resistant *C. glabrata*, highly resistant *C. krusei*, and *C. tropicalis* which exhibits substantial trailing.

Our studies were limited to the examination of *ERG11* RNA levels rather than protein levels or enzyme activity. This was unavoidable due to the lack of specific antibodies or staining procedures and the inability to reliably measure lanosterol demethylase activity in the presence of azole. However, related studies with *S. cerevisiae* demonstrate that *ERG* mRNA levels correlate with the corresponding enzyme activities (6, 20, 24).

The results that we observed with *Candida* are similar to those previously reported for *S. cerevisiae*. For example, *S. cerevisiae ERG9* expression increases following treatment with lovastatin (hydroxymethylglutaryl coenzyme A reductase inhibitor), zaragosic acid (squalene synthase inhibitor), or ketoconazole (13, 31). Genetic lesions in ergosterol biosynthesis result in both increased levels of *ERG9* expression and increased ERG9 activity (13, 24). Enzyme inhibitors and genetic lesions in ergosterol biosynthesis also cause an increase in the levels of *ERG3* mRNA (3, 37). Similarly, in *C. glabrata* deletion of *ERG3* was associated with upregulation of *ERG11* and deletion of *ERG11* was associated with *ERG3* upregulation (8). Recent studies with *S. cerevisiae* and microarrays for investigation of genome-wide expression in response to antifungal treatment or mutations in the ergosterol pathway support earlier studies and more clearly demonstrate global changes in gene expression. Specifically, Bammert and Fostel (4) and unpublished data cited by Rosamond and Allsop (32) document global *ERG* gene upregulation in response to azole, terbinafine, and amorolfine treatment or mutations in *ERG1*, *ERG11*, *ERG6*, *ERG2*, and *ERG5*. Since a complete *C. albicans* genome sequence is not yet publicly available, genome-wide microarray analysis with this organism in response to antifungal treatment has not been reported. It would no doubt further enhance our understanding of the antifungal response in this opportunistic pathogen.

Although *ERG* upregulation is likely to contribute significantly to the survival of azole-treated cells, our data do not support a specific connection between *ERG* upregulation and the trailing phenotype that is variably expressed in *Candida* isolates. Previous work has shown that lowering the pH reduces trailing growth in several *C. albicans* isolates (23; T. D. Edlind, K. W. Henry, and S. K. Katiyar, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 297, p. 549, 1999), but pH had little effect on azole-dependent upregulation of *C. albicans ERG11* (Fig. 4B). Additionally, azole-dependent upregulation of *ERG11* did not vary in magnitude or kinetics between low-trailing-level strain *C. albicans* 630.15-3 and high-trailing-level strain 707.15 (30) (Table 1 and data not shown). Thus, the basis for trailing and its variation among *Candida* isolates remains to be defined.

The molecular mechanism behind the global upregulation of *ERG* genes in response to azoles and other sterol biosynthesis inhibitors is unknown. It is clearly initiated by either the depletion of a late product (e.g., ergosterol) of the pathway or the accumulation of an early substrate or toxic sterol by-product. Current evidence argues against accumulation of a specific substrate or by-product as the initiator of global *ERG* upregulation. Specifically, several different inhibitors that act on different enzymes in the ergosterol biosynthesis pathway cause upregulation of *ERG* genes in *S. cerevisiae* (4, 6, 13, 37) or, as shown here, *Candida* species. The substrates or by-products that accumulate in these treated cells would presumably vary depending upon the inhibitor used. These and other data have led to the suggestion that in *S. cerevisiae* the levels of ergosterol

or other sterol formed late in the pathway regulate *ERG* expression (3, 27), and this may be the case in *Candida* species as well. As in yeasts, the inhibition of sterol biosynthesis in mammalian cells results in increased levels of sterol biosynthesis gene expression and enzyme activity (11, 12, 21, 25, 38, 39). Thus, while the mechanism remains unknown, it appears to be evolutionarily conserved.

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