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Induced Expression of the *Candida albicans* Multidrug Resistance Gene *CDR1* in Response to Fluconazole and Other Antifungals

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The *Candida albicans CDR1* gene encodes a member of the ABC-type family of multidrug transporters which has been shown to be involved in azole resistance. Using an in-frame gene fusion between the *CDR1* open reading frame and the green fluorescent protein allele *yEGFP3*, an optimized derivative for its use in *C. albicans*, we show here how the *CDR1-yEGFP3* gene expression is induced in response to azoles as well as to other structurally unrelated drugs like cycloheximide. Moderate increases were observed for calcofluor, canavanine, 5′-fluorcytosine, cilofungin and caffeine, while no induction was found for the antifungals benomyl and amphotericin B or hydrogen peroxide at subinhibitory concentrations. The use of confocal microscopy enabled us to localize the Cdr1p fusion protein at the cell periphery, thus suggesting a cytoplasmic membrane localization. These results suggest deregulation of *CDR1* gene as a putative mechanism for the generation of azole resistance in this clinically important pathogenic fungus.

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KEY WORDS — *Candida albicans*; multidrug resistance; Fluconazole; antifungal drugs

INTRODUCTION

The increase in the incidence of fungal infections (Fox, 1993; Shaberg *et al*., 1991) requires new and better strategies to fight against fungal diseases. Systemic fungal infections represent a major therapeutic problem due to the limited number of available and highly selective drugs. Amphotericin B, the 'gold standard' in the treatment of systemic fungal infections, presents serious side-effects and non-appropriate pharmacokinetics (now partially overcome with the development of novel liposomal formulations), which severely limit its usefulness (Bennett, 1996). The development of novel orally administrated azoles (ketoconazole, fluconazole and itraconazole) was a major improvement in antifungal therapy. Among these compounds, fluconazole soon became one of the drugs of choice for the treatment of deep fungal diseases in patients with HIV infection and AIDS due to its high bioavailability (>90%) and selectivity. However, shortly after its introduction in 1988, resistance to fluconazole appeared as a major mechanism limiting its usefulness in AIDS patients, who frequently relapsed after prolonged therapy (Ng and Denning, 1993). Resistance directly correlated with the dose of fluconazole and/or length of treatment, with frequent cross-resistance to other azoles (Rex *et al*., 1995).

Of the three basic general mechanisms involved in antimicrobial resistance (modification of the target enzyme, reduced accumulation of the drug and drug covalent modification), only the first two have been described for azoles (Odds, 1993; vanden Bossche *et al*., 1994; Hitchcock, 1993). A first mechanism described in *Candida* spp. involves an alteration of the target fungal enzyme, the cytochrome P-450-dependent lanosterol 14α-demethylase, either rendering it less susceptible to

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azole inhibition (vanden Bossche *et al.*, 1990; vanden Bossche, 1997; White, 1997) or causing its overproduction (vanden Bossche *et al.*, 1992). A second major mechanism involves a reduced intracellular accumulation of the drug, which has been suggested to be mediated through changes in membrane lipid composition that alter permeability (Hitchcock *et al.*, 1986, 1987a,b) or, more recently, through an active efflux system involving drug pumps.

While a pleiotropic multidrug resistance network has been identified in the non-pathogenic yeast *Saccharomyces cerevisiae* (reviewed in Balzi and Goffeau, 1991, 1994, 1995), there are only a few genes described in *Candida albicans* with a defined role in drug resistance. The first one, *MDR1* (formerly *BEN*^{r}), was cloned on the basis of conferring resistance to benomyl and methotrexate in *S. cerevisiae* (Fling *et al.*, 1991) but it was later shown to be a multidrug resistance gene in *S. cerevisiae* (Ben-Yaacov *et al.*, 1994) belonging to the MFS (Major Facilitators Superfamily) family of drug transporters (Marger and Saier, 1993). *MDR1* is homologous to a *C. maltosa* cycloheximide resistance gene (Sasnauskas *et al.*, 1992) and *Schizosaccharomyces pombe* amiloride resistance gene *car1* (Jia *et al.*, 1993). Gene disruption experiments demonstrated its role in virulence (Becker *et al.*, 1995) as well as its involvement in resistance to different drugs in *C. albicans* (Goldway *et al.*, 1995; Sanglard *et al.*, 1996). A second gene, *CDR1*, was cloned by complementation of a *S. cerevisiae pdr5* mutant (Prasad *et al.*, 1995) and shown to encode a member of the ABC (ATP-Binding Cassette) family of multidrug transporters on the basis of its primary sequence and the multidrug resistance phenotype that it conferred when overexpressed in *S. cerevisiae* (Prasad *et al.*, 1995). The role of *CDR1* in multidrug resistance in *C. albicans* is reinforced by recent observations which indicate that some—but not all—clinical isolates from drug-treated AIDS patients have increased *CDR1* mRNA transcripts (Sanglard *et al.*, 1995). In addition *cdr1* mutants have been shown to be more sensitive to some antifungals and other metabolic inhibitors (Sanglard *et al.*, 1996). Another gene, *CDR2*, which is highly homologous to *CDR1*, has also been isolated recently and shown to play a role in fluconazole resistance (Sanglard *et al.*, 1997).

In this work, we have made use of a GFP derivative optimized for its use in *C. albicans* as gene reporter of gene expression and protein localization (Cormack *et al.*, 1997) to demonstrate how *CDR1-yEGFP3* expression is induced by fluconazole and other compounds and to suggest a specific cytoplasmic membrane localization of its gene product.

MATERIALS AND METHODS

Strains and growth conditions

The *C. albicans* strain RM1000 (*ura3 ::imm434/ura3 ::imm434 his1 ::hisG his1 ::hisG*) used throughout this work is a derivative of strain CAI-4 (*ura3 ::imm434/ura3 ::imm434*; Fonzi and Irwin, 1993) in which the *C. albicans HIS1* gene was deleted (Negredo *et al.*, 1997). Strain CNC50 (*ura3 ::imm434/ura3 ::imm434 his1 ::hisG his1 ::hisG CDR1-yEGFP3-URA3*) is a derivative of strain RM1000 in which the construction *CDR1-yEGFP3* has been integrated in the genome at the *CDR1 locus* (see below).

Yeast cells were grown in minimal medium (20 g/l glucose, 0·7 g/l yeast nitrogen base without amino acids) supplemented with a mixture containing all amino acids except uracil at 37°C (*C. albicans* strains) or 30°C (*S. cerevisiae* strains) with agitation. The *Escherichia coli* strain used for plasmid amplification was DH5αF′ (*supE44D lacU169* (φ80 *lacZ M15*) *hsdR17 recA1* end*A1 gyrA96 thi-1 relA1* F′; Hanahan, 1988).

Genetic constructions and molecular biology techniques

Molecular biology techniques were carried out using standard protocols (Sambrook *et al.*, 1989, 1993).

Construction of the *CDR1-yEGFP3* gene fusion was carried out in a three-step process. First, a 5·93 kbp *Sa*/I-*Af*/III from plasmid pN21 (Prasad *et al.*, 1995) comprizing the entire *CDR1* gene was ligated with the 2·31 kbp *Sa*/I-*Af*/III fragment from pUC19 to yield pUC-CDR1. Second, the green fluorescent protein gene allele *yEGFP3* (Cormack *et al.*, 1996, 1997) was introduced as a *Hin*dIII-*Pst*I fragment in the *C. albicans* autoreplicative vector pRM10 digested with *Hin*dIII-*Pst*I, thus yielding pGFP10. Finally, a blunt-ended *Sa*/I-*Nco*I fragment from pUC-CDR1 (obtained through DNA polymerase I (Klenow enzyme) treatment), which comprized most of the *CDR1* gene (see Figure 1), was inserted at the unique *Hin*dIII site (also similarly blunt ended) of plasmid pGFP10 to yield pCDR1-GFP. Homologous

recombination at the *CDR1 locus* was forced through digestion of pCDR1-GFP with *Hin*dIII, and checked by PCR using the oligonucleotides O-CDR1 (5′-GGAGTAGCAAGTGTGTCAAG AACC-3′, complementary to *CDR1* gene) and O-GFP3 (5′-ACCAAAATTGGGACAACACCA GTG-3′, complementary to *GFP3* allele gene). Therefore, the integration resulted in a duplication of the *CDR1* gene in one chromosome, with the *CDR1-yEGFP3* fusion separated from *CDR1* by vector sequences.

Genetic transformation

Transformation of *C. albicans* was achieved using the standard *S. cerevisiae* protoplast transformation (Beggs, 1978) with some minor modifications (Navarro-Garcia *et al*., 1995; Herreros *et al*., 1992). Transformants were recovered directly on selective (uracil deficient) minimal plates. Three independent transformants were initially analysed for induction by fluconazole (which behave similarly), but only one transformant, named CNC50, was selected for subsequent extensive analyses.

Quantification of induction of CDR1-yEGFP3 *expression and localization of this fusion protein*

CDR1-yEGFP3 induction in response to different compounds (Figure 3) was quantified by flow cytometry. Basically, 10^4 exponentially growing cells of *C. albicans* were inoculated in wells containing 200 µl of minimal medium supplemented with the required antifungal at different concentrations. Plates were incubated for 20 h at 37°C with agitation in a iEMS (Integrated EiA Management System; LabSystems). Cells were spun down in a microfuge and the supernatant was removed. This incubation time was chosen from time-course experiments in order to standardize the measures and to avoid a significant cell death if using more prolonged incubation times. The pellet was washed twice with PBS, resuspended in the same buffer and propidium iodide (0·005% (v/v)) was added to each sample (if applicable) to quantify cell death (de la Fuente *et al*., 1992). Cells were immediately analysed using a FACScan flow cytometer (Becton-Dickinson, San José, CA). 10^6 cells were analysed per sample, exciting at 488 nm and recording the emission at 520 nm. The mean fluorescence of the population was taken as a measure of *CDR1-yEGFP3* expression. The Minimal Inhibitory Concentration (MIC) was determined as described (National Committee for Clinical Laboratory Standards, 1992) (80% inhibition for azoles). For technical reasons, experiments involving fluconazole in Figure 2 were carried out similarly, but test tubes of 2 ml were used instead of 96-microtiter plates. The same samples were analysed by confocal microscopy for subcellular localization of the *CDR1* gene fusion using the same excitation and emission wavelengths.

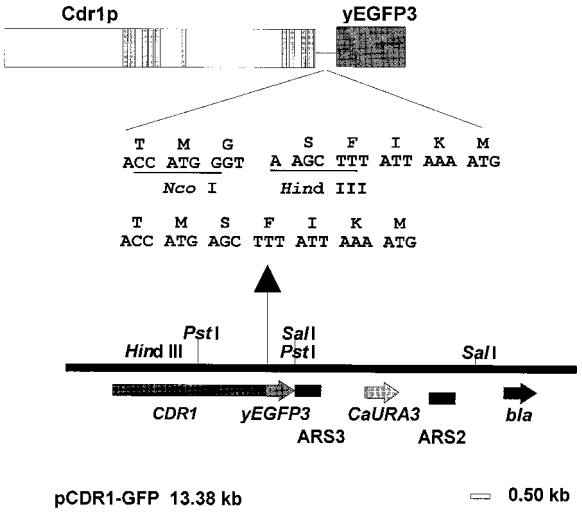


Figure 1. Schematic representation of the *CDR1-yEGFP3* genetic construction and the *C. albicans* pCDR1-GFP vector obtained to force homologous recombination in *C. albicans*.

RESULTS

Involvement of fluconazole in CDR1-yEGFP3 *regulation*

In order to analyse the role of *CDR1* in *C. albicans* multidrug resistance, we constructed an in-frame gene fusion between *CDR1* and *yEGFP3*, an allele of the green fluorescent protein (Chalfie *et al*., 1994) in which both the peculiar genetic code of some *Candida* spp. (Kawaguchi *et al*., 1989; Yokogawa *et al*., 1992; Santos and Tuite, 1995) as well as the codon usage of *C. albicans* have been taken into account. Protein fusion was done between the amino acid methionine at the end of the ten spanning domain (M1332) of Cdr1p and the entire yEGFP3p, including the N-terminal amino acids SFIK just before the ORF of *yEGFP3* (Figure 1).

We first subcloned the *CDR1-yEGFP3* gene into the autoreplicative *E. coli–S. cerevisiae–C. albicans*

shuttle vector pRM10. When *C. albicans* RM1000 cells were transformed with plasmid pCDR1-GFP, only 10–20% of the transformants showed increased levels of fluorescence over control cells (that is, transformed with vector pGFP10). In addition, a significant heterogeneity of fluorescence was detected in the population of these positive transformants. To circumvent this problem, we integrated the above-mentioned construction in the genome forcing recombination at the *CDR1 locus*, therefore obtaining CNC50 strain. CNC50 strain displayed a similar MIC to all the antifungals tested.

Cultures of CNC50 were inoculated in minimal media with different fluconazole concentrations, and 20 h later fluorescence was measured precisely by flow cytometry. A sharp increase in cell fluorescence was observed at concentrations above the MIC (1·25 μg/ml under these conditions) which was also evident at half the MIC (Figure 2a). Induction reached a maximum at the highest concentration tested, 10 μg/ml, where an eight- to nine-fold increase was observed in *CDR1-yEGFP3* induction, although significant cell death (about 10% as determined using propidium iodide staining (de la Fuente *et al.*, 1992)) was observed under these conditions. Prolonged incubation times did not result in increased levels of fluorescence (data not shown). This induction was reversible, as deduced from experiments in which fluconazole-treated cultures were allowed to resume growth in medium devoid of antibiotic and disappeared in 2 h (after a 2 h lag period) under these conditions (Figure 2b). Time-course experiments revealed that the rate of induction (but not the induction itself) was largely independent of fluconazole concentration (data not shown). We conclude from these results that *CDR1-yEGFP3* gene expression is transcriptionally regulated in response to fluconazole.

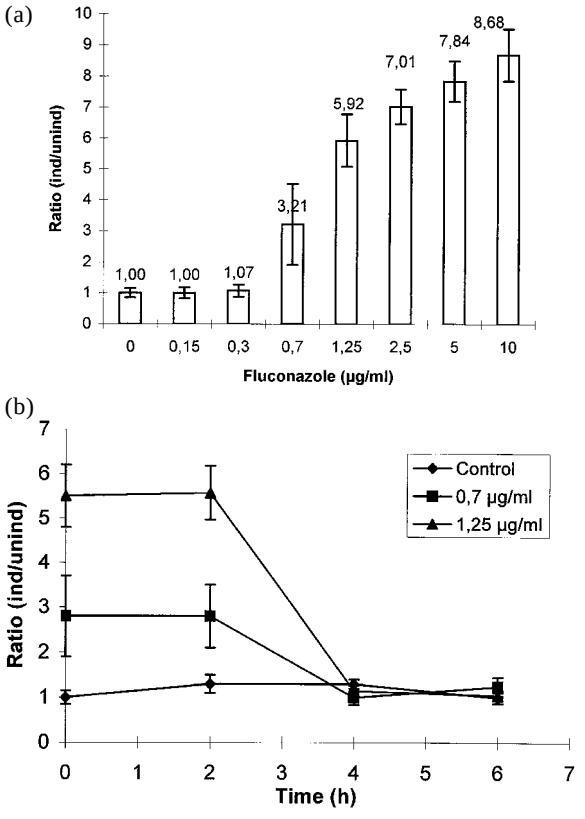


Figure 2. (a) Regulation of *CDR1-yEGFP3* expression by fluconazole. Flow cytometric quantification of the fluorescence in CNC50 *C. albicans* strain in response to fluconazole. The mean fluorescence intensity of CNC50 strain (normalized to the mean fluorescence of the cells grown in the absence of fluconazole) is plotted against the fluconazole concentrations used in the culture medium. The values given are therefore the ratio of induced/uninduced. Under the conditions used in the assay, the MIC to fluconazole was 1·25 μg/ml. Data are the mean of three independent experiments, the vertical bar indicating the standard deviation. (b) Reversibility of the fluconazole-induced expression of *CDR1-yEGFP3* fusion. The mean fluorescence of cultures of *C. albicans* CNC50 strain growing in the absence of fluconazole (given as the ratio of induced/uninduced, see (a), is plotted versus the time (in h) grown in the absence of fluconazole. These cultures were initially grown at the fluconazole concentrations given in the figure (0·7 and 1·25 μg/ml). As a parallel control, a culture was grown without fluconazole. Data are the mean of two independent experiments, the vertical bar indicating the standard deviation.

Regulation of CDR1-yEGFP3 *by multiple drugs*

The putative role of *CDR1* in multidrug resistance prompted us to analyse the role of other antifungals and metabolic inhibitors in *CDR1-yEGFP3* induction. The effect of these compounds was quantified in a similar way to fluconazole and classified according to the induction ratio (as determined between the expression level in fully induced cultures and the uninduced controls). The highest subinhibitory concentration was used for fungicidal antifungals. All azoles (miconazole, econazole, ketoconazole and fluconazole) as well as cycloheximide were found to induce *CDR1-yEGFP3* expression (six- to seven-fold induction ratio, with fluconazole being a potent inductor (eight- to nine-fold; Figure 3). Calcofluor, canavanine, 5′-fluorcytosine, cilofungin and caffeine were found to induce *CDR1-yEGFP3* expression

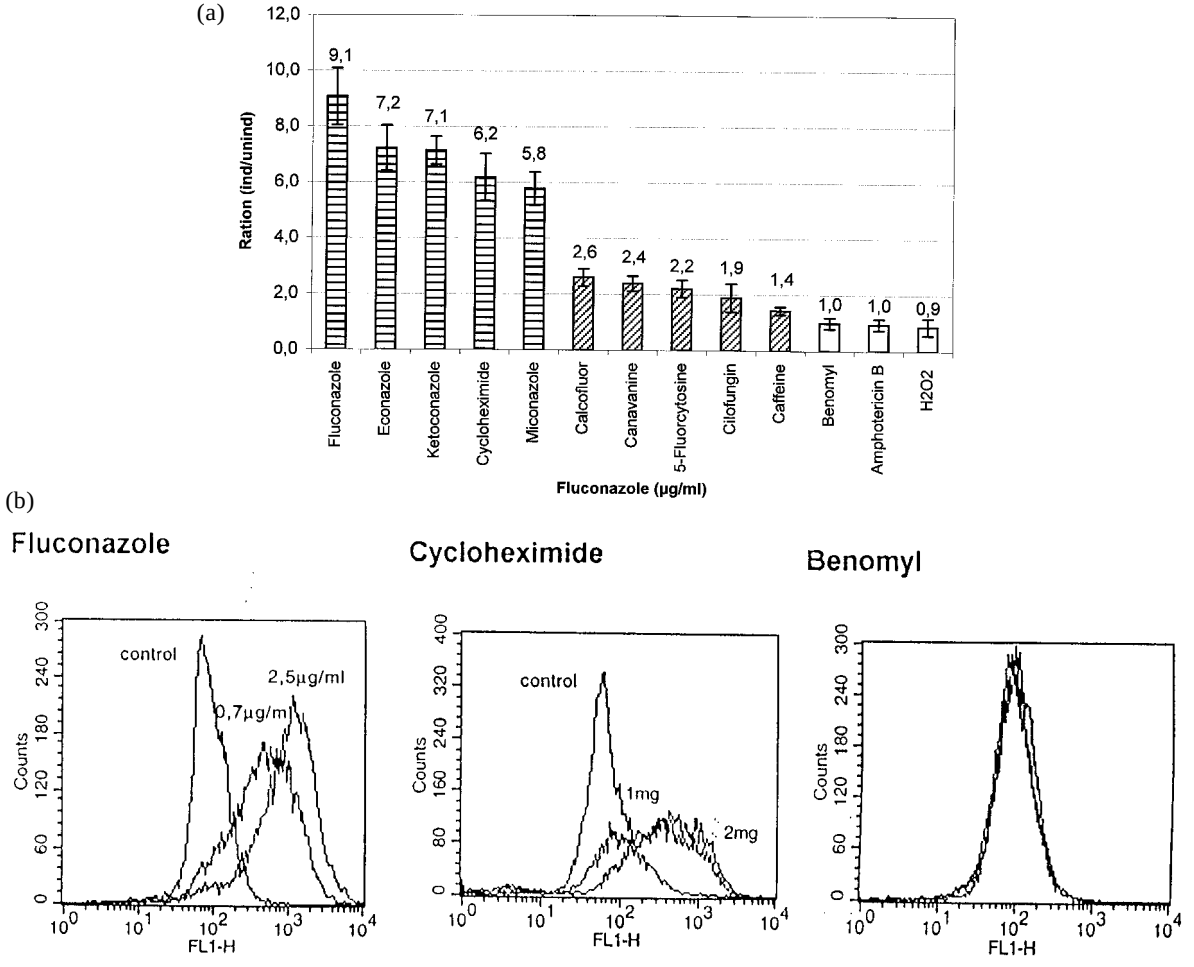
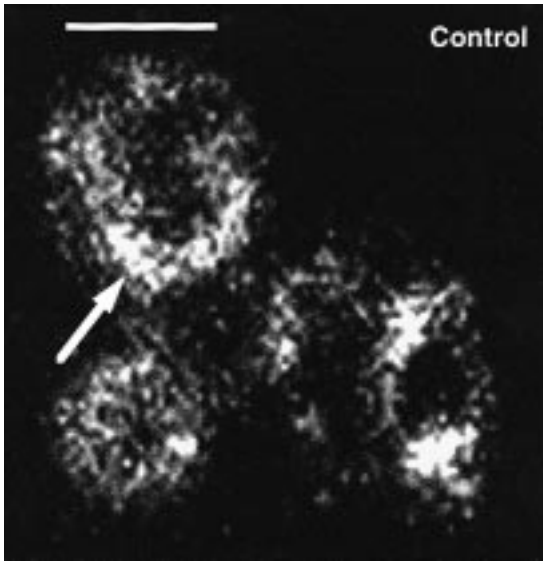


Figure 3. (a) Induction of *CDR1-yEGFP3* expression by other antifungals. Flow cytometric analysis of CNC50 *C. albicans* grown in medium with different drugs. The induction ratio (see Figure 2a) was obtained from the mean fluorescence of fully induced cultures and the uninduced control. The concentration of drugs used for maximal induction were estimated from experiments similar to the one described with fluconazole (Figure 2a) avoiding significant cell lysis, and are the following: fluconazole (10 µg/ml), econazole (2·5 µg/ml), ketoconazole (5 µg/ml), cycloheximide (2 mg/ml), miconazole (5 µg/ml), calcofluor (0·5 mg/ml), canavanine (5 µg/ml), 5-fluorcytosine (2·5 µg/ml), cilofungin (1·25 µg/ml), caffeine (20 mM), benomyl (40 µg/ml), amphotericin B (0·3 µg/ml), hydrogen peroxide (6·25 mM). Data are the mean of three independent experiments, the vertical bar indicating the standard deviation. (b) Fluorescence profiles determined by flow cytometry of cells treated with different antifungals. The intensity of fluorescence (FL1-H; *x*-axis) is plotted against the number of cells (counts; *y*-axis) for cultures of CNC50 strain treated with fluconazole, cycloheximide or benomyl at the concentrations indicated in the figure. A representative histogram is shown.

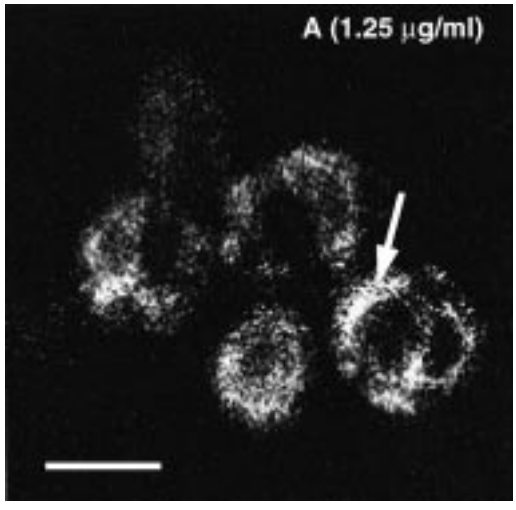
very moderately while benomyl (Figure 3b), amphotericin B or hydrogen peroxide did not induce expression at all. The pattern of distribution of fluorescence was, however, different among these compounds, with cycloheximide generating broader distributions of the fluorescence pattern in the population (Figure 3b) compared to fluconazole. These results suggest that *CDR1* expression is regulated in response to many structurally unrelated compounds.

Localization of CDR1

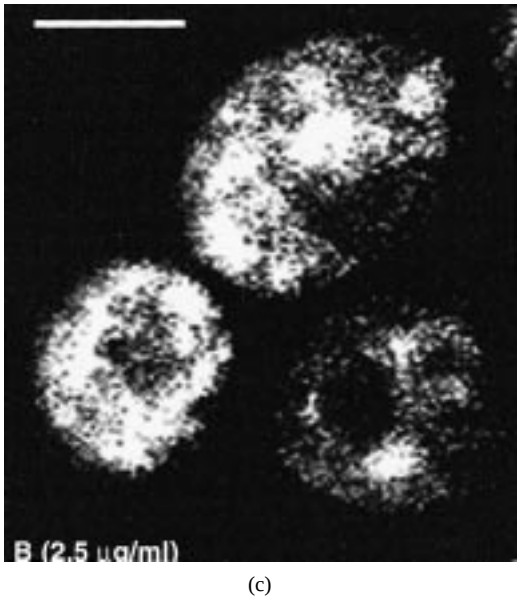
The localization of the *CDR1-yEGFP3* protein fusion was investigated using confocal microscopy (Figure 4). When CNC50 cells were grown under non-inducing conditions (that is, in the absence of fluconazole), fluorescence localized at the cell periphery as a clear signal which became more evident at the poles and the bud scars of the cell (Figure 4). No fluorescence was observed in control



(a)



(b)



(c)

Figure 4. Localization of Cdr1-yEGFP3p protein fusion *in vivo* in *C. albicans* using confocal microscopy. Cells of CNC50 strain were grown in minimal medium supplemented with different fluconazole concentrations (control, without fluconazole; A, 1·25 μg/ml; B, 2·5 μg/ml) and prepared for confocal microscopy as described in Materials and Methods. The arrow indicates an example of peripheral fluorescence. No fluorescence was observed in the control strain RM1000 (thus devoid of Cdr1-yEGFP3p; data not shown).

strains transformed with plasmids lacking the GFP construction (data not shown). Similar results were observed for cultures treated at concentrations of fluconazole equal or below the MIC (1·25 μg/ml) (Figure 4A) or even for cells from *C. albicans* strain RM1000 transformed with the autoreplicative plasmid pCDR1-GFP (data not shown). These results suggest that the protein fusion is localized to the cell membrane and suggest such localization for Cdr1p. Interestingly, when a similar analysis was carried out on cells grown in minimal medium supplemented with 2·5 μg/ml fluconazole (induced state), a more diffuse fluorescence signal within the cytoplasm was observed in addition to the signal at the cell periphery (Figure 4B), suggesting delocalization of the protein fusion in these conditions.

DISCUSSION

Although drug efflux mechanisms are well known in bacterial systems (Levy, 1992), their involvement in microbial resistance in pathogenic fungi is less documented. *CDR1*, a member of the ABC-family of transporters (Higgins *et al*., 1992; Higgins, 1995), has been shown to play a role in azole resistance in *C. albicans* as deduced from the high level expression found in some clinical isolates recovered from patients receiving long-term antifungal therapy (Sanglard *et al*., 1995, 1996). In this work we have addressed the localization and regulation of Cdr1p, making use of a recently described GFP able to be used in *C. albicans* (Cormack *et al*., 1997). GFP was chosen because of its utility both as a transcriptional gene reporter and as a fusion tag to monitor protein localization (Cubitt *et al*., 1995). Integration of the protein fusion in the genome was used to minimize problems derived from overexpression as well as to obtain a homogeneous population expressing the fusion protein. Although we cannot rule out the possibility of mislocalization due to the use of a protein fusion, confocal microscopy analyses revealed a peripheral localization for the protein construct, a result which is in accordance with the primary structure of the Cdr1p protein (Prasad *et al*., 1995), its proposed role as an active drug efflux pump and the situation for the close *S. cerevisiae* homologue Pdr5p (Decottignies *et al*., 1994). More diffuse patterns, however, were observed when cells were treated with fluconazole concentrations above the MIC, a result which could reflect mislocalization caused by its overproduction (four- to eight-fold). An alternative explanation would be that the delocalization could be caused by the altered physiological situation (membrane functionality modifications?) due to the antifungal. This latter explanation is supported by the fact that cells transformed with autoreplicative plasmids allowing similar increases in protein expression (Pla *et al*., 1995; copy number around 4–5) display localization patterns similar to fluconazole-free cells (data not shown). In any case, the results presented here are, to our knowledge, the first demonstration of localization of a protein gene fusion *in vivo* in *C. albicans* using this GFP allele and may represent a very useful approach to monitor protein localization and perform large-scale analysis of *C. albicans* gene function using strategies similar to those described for *S. cerevisiae* (Burns *et al*., 1994).

Another major conclusion from this work is that *CDR1* transcription is probably induced to different extents in response to different antifungals that apparently do not display structural similarities. Maximum expression was found for the azoles and cycloheximide and was effective at concentrations just below (1/2 ×) or above (2–8 ×) the MIC, ranging from two- to eight-fold induction. Induction should not be considered specific for drugs primarily interfering with membrane function since cycloheximide (a protein synthesis inhibitor) effectively induces *CDR1-yEGFP3* expression at subinhibitory concentrations. These results are in accordance with recent data which suggest, using an experimental system of generation of fluconazole-resistant mutants, that *MDR1* is more specific to fluconazole while *CDR1* mediates multidrug resistance (Albertson *et al*., 1996). Our data are also in accordance with the situation in *S. cerevisiae*, where the close homologue *PDR5* (Balzi *et al*., 1994) is not only responsible for drug resistance upon amplification (Leppert *et al*., 1990), but is also regulated by the transcription factors *PDR1* and *PDR3* (Balzi *et al*., 1994; Meyers *et al*., 1992). It would be predictable that in *C. albicans*, similar yet undefined transcription factors should exist which would be involved in *CDR1* expression and other additional genes involved in multidrug resistance (Goldway *et al*., 1995; Albertson *et al*., 1996; Sanglard *et al*., 1996, 1997). Although we have not observed major changes in *CDR1-yEGFP3* expression for cells growing at 30, 37 or 42°C (data not shown), our results do not rule out the possibility that other types of signals like heat shock or different stress signals may also regulate *CDR1* transcription.

While our work suggests that *CDR1* deregulation could be involved in clinical resistance—either as the result of mutations at the promoter region (*cis*) or in other genes acting *in trans*, it is also evident that other additional mechanisms, like structural mutations in the *CDR1* ORF, could also play a role. Therefore, the generation of fluconazole resistance could arise as a multi-step and gradual process (involving regulatory and structural mutations) which could depend on the length and intensity of the antifungal therapy. The analysis of *CDR1* regulation in clinical isolates obtained from patients receiving long-term fluconazole therapy should enable the clarification of this mechanism in clinical resistance.

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