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The *Candida albicans CDR3* Gene Codes for an Opaque-Phase ABC Transporter

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We report the cloning and functional analysis of a third member of the *CDR* gene family in *Candida albicans*, named *CDR3*. This gene codes for an ABC (ATP-binding cassette) transporter of 1,501 amino acids highly homologous to Cdr1p and Cdr2p (56 and 55% amino acid sequence identity, respectively), two transporters involved in fluconazole resistance in *C. albicans*. The predicted structure of Cdr3p is typical of the *PDR*/*CDR* family, with two similar halves, each comprising an N-terminal hydrophilic domain with consensus sequences for ATP binding and a C-terminal hydrophobic domain with six predicted transmembrane segments. Northern analysis showed that *CDR3* expression is regulated in a cell-type-specific manner, with low levels of *CDR3* mRNA in CAI4 yeast and hyphal cells, high levels in WO-1 opaque cells, and undetectable levels in WO-1 white cells. Disruption of both alleles of *CDR3* in CAI4 resulted in no obvious changes in cell morphology, growth rate, or susceptibility to fluconazole. Overexpression of Cdr3p in *C. albicans* did not result in increased cellular resistance to fluconazole, cycloheximide, and 4-nitroquinoline-*N*-oxide, which are known substrates for different transporters of the *PDR*/*CDR* family. These results indicate that despite a high degree of sequence conservation with *C. albicans* Cdr1p and Cdr2p, Cdr3p does not appear to be involved in drug resistance, at least to the compounds tested which include the clinically relevant antifungal agent fluconazole. Rather, the high level of Cdr3p expression in WO-1 opaque cells suggests an opaque-phase-associated biological function which remains to be identified.

The selective transport of molecules across membranes is a fundamental cellular process. Over the past decade, several studies have revealed the existence of a superfamily of polytopic membrane proteins implicated in the active transport of various molecules (e.g., proteins, peptides, sugars, ions, and antibiotics) (20). The characteristic feature of these proteins is the presence of highly conserved consensus sequences for ATP binding and hydrolysis. They have been named ABC (ATP-binding cassette) transporters (20). Some members of the ABC superfamily have been extensively studied because of their medical importance. These include the cystic fibrosis transmembrane regulator (CFTR) protein, a chloride channel defective in patients with cystic fibrosis (37), the P-glycoproteins (Pgp), and the multidrug resistance-associated protein (Mrp), whose overexpression causes resistance to several different chemotherapeutic drugs, a phenotype known as multidrug resistance (MDR) (12, 16). Transporters of the ABC superfamily are evolutionarily conserved proteins that have been identified in a wide variety of cell types, including bacteria, protozoans, fungi, plants, nematodes, insects, and mammalian cells (20). They can be localized in the plasma membrane, as are Pgp and CFTR, or in the membranes of intracellular compartments (endoplasmic reticulum, vacuole, peroxisome, or mitochondria) (20, 48). The normal cellular functions and substrates for a large number of ABC transporters are still unknown.

In the yeast *Saccharomyces cerevisiae*, the first ABC transporter to be identified was Ste6p, a close homolog of the mammalian Pgp (26, 28). Ste6p transports **a**-factor, a peptide pheromone that is required for mating (26, 28) and, unlike Pgp, is unable to confer drug resistance (29). Functional cloning strategies in *S. cerevisiae* have identified a number of ABC transporters associated with drug or heavy-metal resistance: Pdr5p, whose overexpression leads to resistance to various growth inhibitors such as cycloheximide, chloramphenicol, staurosporine, and sporidesmin (6, 8, 22); Snq2p, a close homolog of Pdr5p which confers resistance to 4-nitroquinoline-*N*-oxide (4-NQO), sulfomethuron methyl, and phenanthroline (42); Ycf1p, a vacuolar glutathione-cadmium conjugate pump closely related to mammalian Mrp and CFTR (47); and Yor1p, a Ycf1p homolog conferring resistance to oligomycin and reveromycin (13, 24). In addition, several additional open reading frames (ORFs) predicted to code for ABC transporters have been identified with the yeast genome sequencing project, but their potential role in MDR has yet to be determined (14, 29, 48).

Candida albicans, the etiologic agent of candidiasis, is an important fungal pathogen of humans. It is a naturally occurring diploid yeast with no known sexual stage and has therefore been classified as an imperfect fungus (Deuteromycetes). *C. albicans* can cause severe infections in immunocompromised individuals. Azole derivatives such as fluconazole (FCZ) are often used for clinical treatment, but resistance to this class of drugs is becoming increasingly common in patients undergoing long-term or prophylactic treatment (36). A number of studies investigating the mechanisms of FCZ resistance in *C. albicans* and other *Candida* species have previously shown that resistant strains fail to accumulate FCZ due to an increased drug efflux, suggesting the participation of transporter-mediated drug resistance mechanisms in these strains (33, 41). Two *C. albicans* genes of the ABC family, *CDR1* and *CDR2*, have been isolated by functional complementation of an *S. cerevisiae pdr5* null mutant strain, screening for cycloheximide and FCZ resis-

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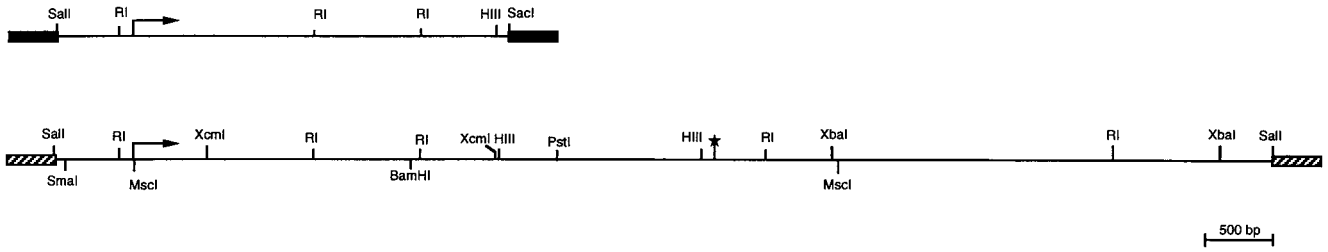

FIG. 1. Maps of genomic DNA clones overlapping the *CDR3* gene. (Top) restriction map of a 3.2-kb *Sal*I-*Sac*I WO-1 genomic DNA fragment derived from clone H6CDR and cloned in vector pUC19 (black boxes). The arrow indicates the position of the initiator methionine at the beginning of an incomplete ORF (34). (Bottom) restriction map of a 9-kb *Sal*I fosmid fragment in vector YEp352-*Sal*I (hatched boxes) overlapping the complete *CDR3* gene (arrow, initiator methionine; ★, stop codon; HIII, *Hin*dIII; RI, *Eco*RI).

tance, respectively (34, 40). These genes code for proteins displaying extensive sequence homology with each other and with *S. cerevisiae* Pdr5p and Snq2p (34, 40). These proteins are typically formed by two similar halves, each with an N-terminal hydrophilic domain that contains the ATP-binding motifs followed by a C-terminal hydrophobic domain with six predicted transmembrane (TM) segments (6, 34, 40, 42). Together with the *Schizosaccharomyces pombe* brefeldin A resistance gene *BFR1* (32) and other *S. cerevisiae* ORFs of unknown function, they form a subgroup within the ABC superfamily of transporters referred to as the *PDR* subgroup, or cluster I (14, 29, 48). Overexpression of *CDR1* or *CDR2* in *S. cerevisiae* results in cellular cross-resistance to a wide range of toxic compounds, including a number of azole derivatives and different metabolic inhibitors (34, 40). *C. albicans* strains carrying a *cdr1*Δ or a *cdr1*Δ *cdr2*Δ mutation display increased sensitivity to these different drugs as well as an increased intracellular accumulation of [^{3}H]FCZ, demonstrating that Cdr1p and Cdr2p function as drug efflux pumps in these cells (39, 40). Interestingly, *CDR1* and *CDR2* have been found to be overexpressed in *C. albicans* FCZ-resistant isolates from AIDS patients with oropharyngeal candidiasis, indicating that both genes are involved in the emergence of clinical FCZ resistance (40, 41).

In this paper, we report the isolation of a new member of the *C. albicans PDR/CDR* gene family, named *CDR3*. This gene codes for a protein which displays a high degree of structural conservation with Cdr1p and Cdr2p, including similarities in length, amino acid sequence, and predicted membrane topology. Analysis of the drug resistance phenotype of *C. albicans* cells overexpressing *CDR3* indicates that Cdr3p is not able to confer resistance to a number of toxic compounds which are known substrates for different transporters of the *PDR/CDR* family, including FCZ. Rather, our finding that *CDR3* is highly expressed in WO-1 opaque cells suggests an opaque-phase-associated biological function for this transporter.

MATERIALS AND METHODS

Yeast strains and culture conditions. *C. albicans* CAI4 (17), WO-1 (45), and LSPQ 5902 (an FCZ-resistant clinical isolate kindly provided by G. St-Germain, Laboratoire de Santé Publique du Québec, Sainte-Anne de Bellevue, Québec, Canada) were used in this study. Yeast strains were grown in YPD or Ura-deficient SD medium at 30°C (43). Differentiation of WO-1 white to opaque cells was carried out as described by Anderson and Soll (3). Hypha formation in CAI4 yeast cells was induced as described by Lee et al. (27).

Isolation of the *CDR3* gene. The following oligonucleotide primers, corresponding to a conserved region overlapping the C-terminal ATP-binding domains of *S. cerevisiae PDR5* (6), *PDR10* (GenBank Z49821), *PDR15* (GenBank U32274), and *C. albicans CDR1* (34), were synthesized: 5′-ATGTTGATGGT(A)TGGGTT(C)AAA(G)CCA(T)GG and 5′-GTTC(C)AAC(G)ATT(C)TAAACCTTCACCAG. PCR was performed on genomic DNA from *C. albicans* WO-1 in a typical reaction containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 50 μM deoxynucleoside triphosphates, 100 pmol of each oligonucleotide primer, 0.2 μg of genomic DNA template, and 1 U of *Taq* DNA polymerase (Pharmacia) in a final volume of 50 μl. The program consisted of one cycle of 4 min at 94°C, 2 min at 40°C, and 1 min at 72°C, followed by 29 cycles of 1 min at 94°C, 2 min at 40°C, and 1 min at 72°C, with a final elongation step of 11 min at 72°C. The PCR products were gel purified, cloned into the pCRII vector (TA cloning kit; Invitrogen), and analyzed by DNA sequencing. One of the resulting inserts (clone H6) was used to screen a YEp352-based *C. albicans* WO-1 genomic DNA library (a gift from C. Boone, Queen's University, Kingston, Ontario, Canada), yielding a positive clone containing an incomplete ORF (H6CDR) as determined by DNA sequencing. A *C. albicans* 1006 fosmid library was probed with a 0.7-kb *Eco*RI-*Hin*dIII fragment derived from the 3′ end of H6CDR, which yielded five positive fosmids (5F3, 7G3, 11D8, 13C10, and 16D6; a gift from B. Magee, University of Minnesota, St. Paul). A 9-kb *Sal*I fragment positive for this probe and shared by the five fosmids was excised from fosmid 11D8 and subcloned into vectors YEp352 (21) and pGEM-5Zf (Promega, Madison, Wis.) at the *Sal*I site, generating plasmids YEp352/CDR3 and pGEM5/CDR3, respectively.

Sequencing of a 5.3-kb region overlapping the entire *CDR3* gene was performed in plasmid YEp352/CDR3 on both DNA strands with custom-synthesized oligonucleotides, using the automated sequencing facilities of the Sheldon Biotechnology Center (McGill University, Montréal, Québec, Canada). Sequence analyses were performed with the Wisconsin package, version 9.0 (Genetics Computer Group, Madison, Wis.).

RNA preparation and Northern blotting. Total RNA was extracted by the hot phenol extraction method (Fig. 3) (25) or the glass bead extraction protocol (Fig. 5A) (11). RNA samples (20 μg) were electrophoresed on a 7.5% formaldehyde–1% agarose gel and transferred by capillarity to a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Specific RNAs were detected under conditions of high stringency as described previously (1). The *CDR3* probe was generated by PCR with primers 5′-CCAAGACATCACAAGCAG and 5′-GCAACACCTAATTTACCT and overlaps a region from positions +4 to +344 of the *CDR3* gene (positions are relative to the translation initiation codon [Fig. 2]). The *CDR1* probe, which overlaps a region from +1 to +339 of the *CDR1* coding domain, was generated by PCR with primers 5′-ATGTCAGATTCTAAGATG and 5′-CAGGATCTGATTCAAACA (34). The Op4 probe was synthesized by PCR with primers 5′-ATGAAGTTTTCACAAGCC and 5′-GGACAATAGCACCTTGAA and overlaps bases +1 to +349 of the Op4 cDNA sequence (30). A *C. albicans ACT1* probe (provided by B. Magee) was also used as an internal control to monitor RNA loading and transfer.

Deletion of *CDR3*. A *cdr3* deletion plasmid (pGEM5/*cdr3*Δ::*hisG-URA3-hisG*) was generated by replacing, in pGEM5/CDR3, a 3.7-kb *Xcm*I-*Hin*dIII internal fragment overlapping most of the *CDR3* coding region with a 4-kb *Sal*I-*Bgl*II fragment from plasmid pMB-7 encompassing the *hisG-URA3-hisG* deletion cassette (17). A linear 9.3-kb *cdr3*Δ::*hisG-URA3-hisG* fragment was obtained from pGEM5/*cdr3*Δ::*hisG-URA3-hisG* following digestion with *Sal*I and used to disrupt *CDR3* in strain CAI4, essentially as described previously (17).

***CDR3* overexpression.** To overexpress *CDR3* in *C. albicans*, the coding region of the gene was cloned into vector YPB1-ADHpt under the control of the *C. albicans ADH1* promoter (kindly provided by A. Brown, University of Aberdeen, Aberdeen, United Kingdom) (4). This construction was performed in three steps. First, an *Xba*I site was introduced 35 bp upstream of the *CDR3* initiation codon by PCR using primers 5′-GCTCTAGAACTAAGAGGGCATTCAC (forward; the *Xba*I site is underlined) and 5′-CGGTAATAGAAGGAAGATG (reverse) as well as *C. albicans* 1006 genomic DNA as the template. The reverse primer corresponds to positions +1592 to +1610 in *CDR3*, downstream of the first *Eco*RI site located at position +1282 in the coding region of the gene (Fig. 1). This 1.6-kb PCR fragment was gel purified and digested with *Xba*I and *Eco*RI, and the resulting 1.3-kb fragment was cloned into pUC19 cut with *Xba*I and *Eco*RI, to yield plasmid pUC/CDR3[1.3-kb XbaI-RI]. Second, a 5.5-kb *Msc*I fragment encompassing *CDR3* from positions +4 bp to +5.5 kb (Fig. 1) was excised from YEp352/CDR3 and cloned into pUC/CDR3[1.3-kb XbaI-RI] cut with *Msc*I (at position +4), thus reconstituting the original coding region in plasmid pUC/CDR3-MscI. Third, a 5.5-kb *Xba*I fragment was isolated from

plasmid pUC/CDR3-MscI (positions −35 to +5.5 kb), blunt ended, and cloned into the blunt-ended *Bgl*II site of the YPB-ADHpt vector, downstream of the *C. albicans ADH1* promoter (4), to yield plasmid YPB-ADHpt/CDR3. This construct was used to transform CIB4 cells, along with vector YPB-ADHpt as a negative control, as described for *S. cerevisiae* (7).

Preparation of antisera. A *GST-CDR3* in-frame gene fusion was constructed by inserting a 605-bp *Msc*I-*Xcm*I *CDR3* fragment blunt ended with T4 DNA polymerase into the *Sma*I site of vector pGEX-4T-3 (Pharmacia), to generate plasmid pGEX-CDR3. This fusion protein contains 201 amino acids from the NH_2 terminus of Cdr3p (amino acid positions 2 to 203). *Escherichia coli* DH5α cells transformed with pGEX-CDR3 were treated with isopropyl-β-D-thiogalactoside (0.1 mM) for 4 h at 37°C to induce the expression of the fusion protein. The Gst-Cdr3 fusion protein, mostly insoluble, was purified from inclusion bodies by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by electroelution and used to raise polyclonal antisera in two New Zealand White rabbits. Two anti-Cdr3p (R3A and R3B) antisera of high titers were obtained and used without any further purification. The anti-Cdr1p polyclonal antiserum R1B was raised against a Gst-Cdr1 fusion protein containing 112 amino acids from the NH_2 terminus of Cdr1p (2).

Protein preparation and Western blot analysis. Total protein extracts were prepared from the CIB4[YPB-ADHpt] and CIB4[YPB-ADHpt/CDR3] transformants and from strain LSPQ 5902 as follows. Twenty OD_{600} (optical density at 600 nm) units of saturated cell cultures grown in Ura-deficient SD were harvested by centrifugation and resuspended to a final concentration of 1.5 g (wet weight) per ml in TE (50 mM Tris [pH 7.5], 1.5 mM EDTA) in the presence of protease inhibitors (phenylmethylsulfonyl fluoride at 1 mM; leupeptin, pepstatin A, and aprotinin, each at 5 μg/ml). Then 0.5 volume of acid-washed glass beads (425 to 600 μm; Sigma) was added to the cell suspension, and cells were lysed by vigorous vortexing five times in 1-min bursts, interrupted by at least 1 min of cooling on ice. Glass beads and unbroken cells were removed by centrifugation at 1,000 rpm for 5 min in a microcentrifuge; supernatants were collected and stored at −80°C. Total membranes were prepared as described previously (35). Protein concentration was determined according to Bradford (9), using bovine serum albumin as the standard. Proteins from total extracts (25 μg) or crude membranes (50 μg) were suspended in Laemmli sample buffer, heated at 55°C for 10 min, and separated by SDS-PAGE on a 7.5% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and analyzed with the anti-Cdr1p (R1B) or anti-Cdr3p (R3B) polyclonal antibody at a dilution of 1:2,000, using a chemiluminescence detection system under conditions recommended by the manufacturer (Boehringer Mannheim).

Drug resistance assays. Stock solutions of FCZ (a gift from Pfizer Canada Inc.) and CYH (Sigma) were prepared in water at a concentration of 10 mg/ml. 4-NQO (Sigma) was dissolved in dimethyl sulfoxide at a concentration of 5 mM. Drug resistance was determined by microtiter plate assays as previously described (1).

Nucleotide sequence accession number. The nucleotide sequence of the *CDR3* gene has been deposited in the GenBank database under accession no. U89714.

RESULTS

Isolation of the *C. albicans CDR3* gene. A DNA sequence alignment of highly conserved regions overlapping the C-terminal ATP-binding domain of four members of the yeast *PDR*/*CDR* gene family was used to design two degenerate oligonucleotides. These oligonucleotides were used as primers in a PCR with *C. albicans* WO-1 genomic DNA as the template, under conditions of low stringency. A major PCR product of the expected size (approximately 375 bp) was gel purified and cloned into the pCRII vector. Among nine clones sequenced, two identical inserts (H6 and H9) were found to display 76% nucleotide sequence identity with *CDR1*. One of these two inserts (H6) was used to screen a YEp352-based *C. albicans* WO-1 genomic DNA library by colony hybridization, yielding one positive clone (H6CDR). Sequence analysis of a 3.2-kb *Sal*I-*Sac*I subfragment derived from H6CDR revealed the presence of an incomplete ORF (Fig. 1). A full-length clone was obtained from *C. albicans* 1006 fosmid clones hybridizing with a ^{32}P-labeled 0.7-kb *Eco*RI-*Hin*dIII fragment derived from the 3′ end of H6CDR. Five positive fosmids (5F3, 7G3, 11D8, 13C10, and 16D6) were analyzed and found to share a 9-kb *Sal*I fragment hybridizing with the probe. DNA sequencing confirmed that this 9-kb *Sal*I fragment encompassed the full-length ORF (Fig. 1). This ORF was highly homologous to, but different from, the previously reported *CDR1* and *CDR2* genes (34, 40) and was thus named *CDR3*. *CDR3* maps to chromosome 4 of the *C. albicans* genome, whereas both *CDR1* and *CDR2* map to chromosome 3 (data available at http://alces.med.umn.edu/bin/genelist?genes).

Sequence analysis of the *CDR3* gene and of its deduced polypeptide. A 5,316-bp sequence from the 9-kb *Sal*I fragment has been determined and is shown in Fig. 2. The 5′-flanking region contains a number of noncanonical TATA boxes (−100 TTATTTAAT, −214 TTATAATT, and −242 TATATT) in the vicinity of the most upstream in-frame ATG codon (19). This codon appears to be the authentic translational start site, since the surrounding sequence (−5 AAAAAATG) is in good agreement with the consensus sequence for translation initiation in yeast (−5 AA/YAA/TAATG) (15). A consensus sequence for transcription termination in yeast ($TAGN_{22}TATG$ $TN_{38}TTT$) is found in the 3′-flanking region (52).

CDR3 codes for a predicted protein of 1,501 amino acids (Fig. 2), with a calculated molecular mass of 170.3 kDa and an estimated isoelectric point of 7.74. Cdr3p has a predicted structure characteristic of the Pdrp/Cdrp family and is formed by two similar halves, each with an N-terminal hydrophilic domain followed by a C-terminal hydrophobic domain (14, 29, 48) (Fig. 2). The TMpred algorithm predicts six high-score TM segments for each of the two hydrophobic domains of Cdr3p (23) (Fig. 2). Both hydrophilic domains include an ATP-binding cassette which comprises Walker A, Walker B (50), and ABC signature (PROSITE entry PS00211) (5) motifs which display sequence features distinctive of the Pdrp/Cdrp subfamily of ABC transporters (14, 29, 48) (Fig. 2). In its N-terminal hydrophilic domain, Cdr3p contains a degenerate Walker A motif where the highly conserved lysine residue is replaced by a cysteine (178 GRPGAGCS), as previously reported for other members of the Pdrp/Cdrp family (6, 14, 29, 34, 40, 42). A perfect match with a consensus ABC signature is found downstream (294 ISGGERKRLSIAEVT), followed by a Pdrp/Cdrp-specific Walker B motif (318 **D**NST**R**GL**D**) that contains two aspartic acid residues highly conserved among ABC proteins (shown in boldface) as well as other residues conserved within proteins of the Pdrp/Cdrp family (underlined) (14). The C-terminal hydrophilic domain of Cdr3p contains a typical Walker A motif (876 GASGAGKT). A degenerate ABC signature can be identified where the usual serine or alanine residue at position 2 and the invariable glycine at position 4 of the signature are replaced by an asparagine and a glutamic acid residue, respectively (982 LNVEQRKRLTIAVEL). The absence of a consensus ABC signature in the C-terminal ATP-binding domain is also typical of members of the Pdrp/Cdrp family (6, 14, 34, 40, 42). Finally, a Walker B motif is present where the first aspartate is typically preceded by four hydrophobic residues (1003 LVFL**D**EPTSGL**D**).

BLAST and FASTA homology searches of protein sequence databases with the full-length Cdr3p sequence show that the highest homology was with Cdr1p (34) and Cdr2p (40). Pairwise sequence comparisons indicate that Cdr3p displays 56% sequence identity and 74% similarity with Cdr1p and 55% sequence identity and 75% similarity with Cdr2p, while Cdr1p and Cdr2p are 84% identical and 92% similar (40). These results indicate that Cdr1p and Cdr2p are more closely related to each other than to Cdr3p, probably reflecting a shorter evolutionary distance between Cdr1p and Cdr2p. The highest degree of sequence homology among the three proteins is found in the segments overlapping the two predicted intracellular domains that contain the ATP-binding sequences. The homology decreases in the membrane-associated domains, with the area of greatest divergence found in the N-terminal segment of the proteins (data not shown).

The BLAST and FASTA homology searches also revealed a

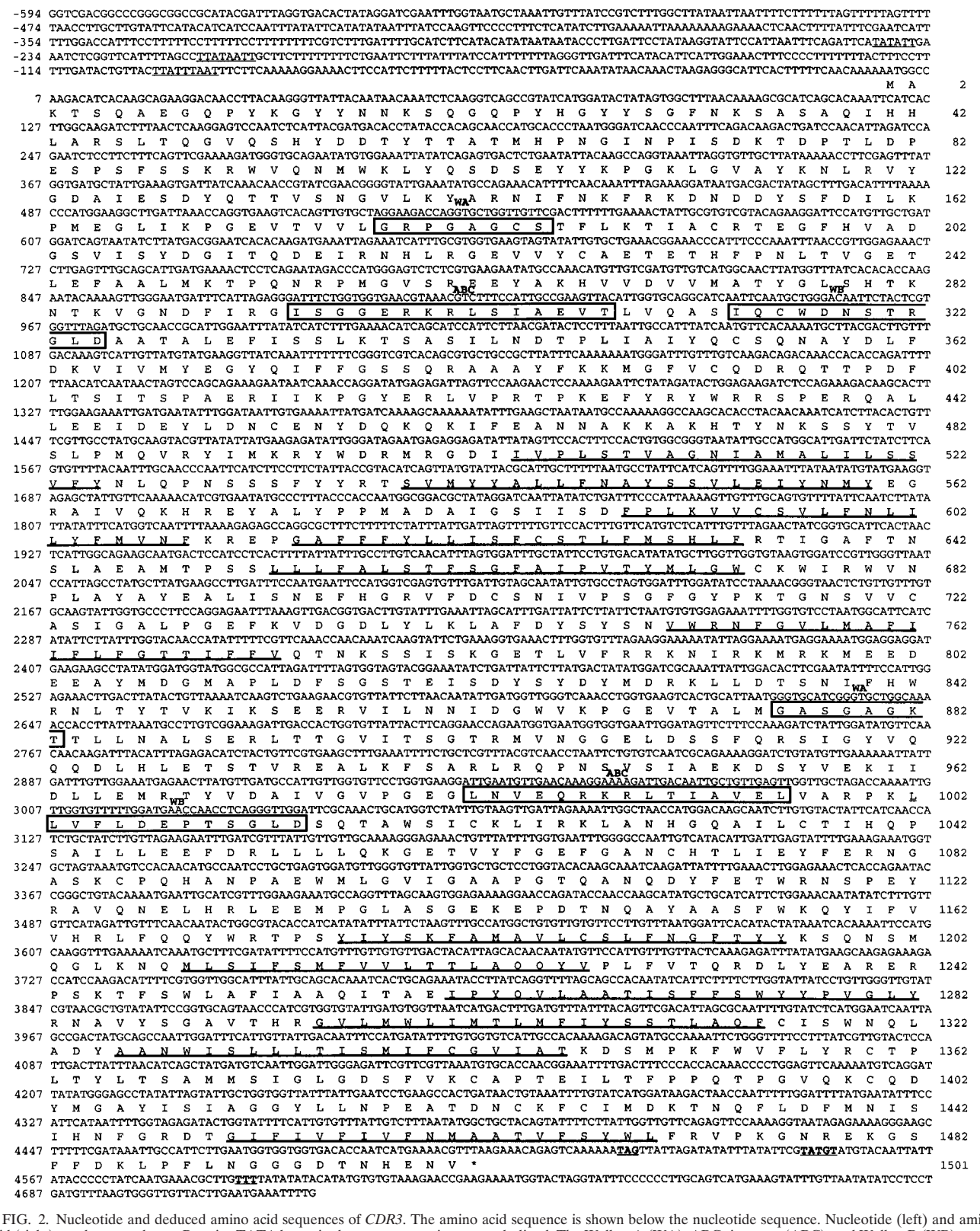

FIG. 2. Nucleotide and deduced amino acid sequences of *CDR3*. The amino acid sequence is shown below the nucleotide sequence. Nucleotide (left) and amino acid (right) numbers are shown. Putative TATA boxes in the promoter region are underlined. The Walker A (WA), ABC signature (ABC), and Walker B (WB) motifs of the ATP-binding cassettes are indicated (boxed). The 12 predicted membrane-spanning domains are underlined. The consensus sequence for transcription termination in the 3′-flanking region is shown in boldface and underlined.

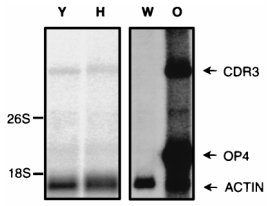

FIG. 3. Northern blot analysis of *CDR3* cell-type-specific expression. Total RNA was extracted from CAI4 yeast (lane Y), CAI4 hyphal (lane H), WO-1 yeast white (lane W), and WO-1 yeast opaque (lane O) cells. RNA samples (20 μg) were separated by electrophoresis on an agarose gel and transferred to a nylon membrane. The membrane was hybridized simultaneously with the *CDR3* and actin probes (left) or with the *CDR3*, opaque-phase-specific Op4 (30), and actin probes (right). The positions of the 26S and 18S rRNAs are indicated on the left. The specific *CDR3*, Op4, and actin RNA transcripts are shown on the right.

high level of homology between Cdr3p and *S. cerevisiae* Pdr5p (52% identity and 70% similarity; GenBank L19922), Pdr10p (51% identity and 69% similarity; GenBank Z49821), and Pdr15p (51% identity and 69% similarity; GenBank U32274). The homology between Cdr3p and two other members of the Pdrp family in *S. cerevisiae*, Snq2p (39% identity and 60% similarity; GenBank X66732) and Pdr11p (30% identity and 55% similarity; GenBank P40550), is lower than with the other Pdrs. The high degree of sequence similarity between Cdr3p and drug resistance-associated members of the Pdrp/Cdrp family, such as *C. albicans* Cdr1p and Cdr2p and *S. cerevisiae* Pdr5p and Snq2p, suggested that Cdr3p could function as a drug transporter.

Analysis of the cell-type-specific expression of *CDR3*. *C. albicans* displays a high degree of phenotypic plasticity. It is a dimorphic organism, capable of growing as either yeast or hyphae, depending on the environmental conditions (46). A number of *C. albicans* strains can also undergo a variety of high-frequency phenotypic transitions. Among these switchings, the white-opaque transition in the strain WO-1 has been thoroughly documented, with white and opaque cells differing in many respects, including size, shape, sugar assimilation, and gene expression (reviewed in reference 46). It was thus of interest to determine if the expression of *CDR3* was modulated in these different cell types. To this end, strains CAI4 (17) and WO-1 (45) were differentiated by using well-established protocols. CAI4 yeast and hyphae cells were obtained by culturing the cells in Lee's liquid medium at 25 and 37°C, respectively (27), whereas opaque cells were obtained after plating the WO-1 strain at 25°C on Lee's modified medium containing phloxine B (3). Total RNA prepared from CAI4 yeast and hyphae cells as well as from WO-1 white (yeast) and WO-1 opaque (yeast) was analyzed with a *CDR3* gene-specific probe (Fig. 3). This probe is derived from the 5′ region of the *CDR3* gene, where the DNA sequence homology with *CDR1* and *CDR2* is very low, and does not cross-hybridize with the other *CDR* genes under conditions of high stringency (data not shown). This analysis showed that *CDR3* is expressed at low levels in CAI4 yeast and hyphal cells (Fig. 3, lanes Y and H), at undetectable levels in WO-1 white cells (Fig. 3, lane W), and at high levels in WO-1 opaque cells (Fig. 3, lane O). These results demonstrate that *CDR3* expression is regulated in a cell-type-specific manner and suggest an important biological function for this ABC transporter in WO-1 opaque cells.

Chromosomal deletion of *CDR3*. To investigate the biological function of Cdr3p in *C. albicans*, both copies of the *CDR3* gene were deleted in strain CAI4, using the "ura-blaster" strategy (17). To this end, a linear 9.3-kb *Sal*I *cdr3*Δ::*hisG-URA3-hisG* fragment was constructed as described in Materials and Methods (Fig. 4A) and used to transform CAI4. A Ura$^+$ transformant, CIB1, displayed a hybridization pattern characteristic of the deletion of the first allele, with the appearance of a new 3.0-kb *Eco*RI fragment hybridizing with the wild-type probe (Fig. 4B, lane 2). CIB1 was plated on 5-fluoro-orotic acid, and Ura$^-$ revertants were checked by Southern blotting for loss of the *URA3* gene. Strain CIB2, which displayed a new 2.3-kb *Eco*RI fragment resulting from the homologous recombination between the two *hisG* repeats (Fig. 4B, lane 3), was used for a second round of transformation with the *cdr3* deletion fragment. Strain CIB3 had the correct genotype, namely, the disappearance of the remaining 1.5-kb *Eco*RI wild-type fragment and the appearance of a new 3.0-kb *Eco*RI fragment with the wild-type probe (Fig. 4B, lane 4). Strain CIB3 was plated on 5-fluoro-orotic acid, yielding the homozygous strain CIB4 *cdr3*Δ::*hisG*/*cdr3*Δ::*hisG* with a doublet at 2.3 kb (Fig. 4B, lane 5). Southern blotting analysis was also performed with a *hisG* probe, confirming the correct genotypes of the different strains (Fig. 4C). The successful disruption of the two *CDR3* alleles in the diploid strain CAI4 demonstrates that *CDR3* is not essential for viability under the conditions tested. In addition, no obvious changes in cell morphology, growth rate, or FCZ susceptibility were associated with the *cdr3* deletion (data not shown). As CIB4 cells generated from the final step of the disruption are auxotrophic for uridine, they were thus suitable for transformation with a *URA3*-based vector for the overexpression of *CDR3* in a null mutant background.

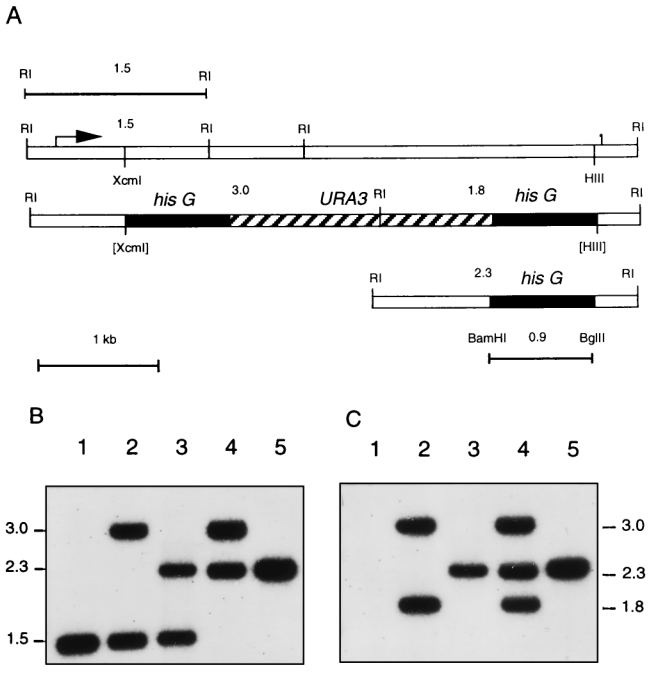

FIG. 4. Deletion of *CDR3* in strain CAI4. (A) Schematic representation of the strategy used for the *CDR3* deletion. *Eco*RI (RI) restriction map of the wild-type *CDR3* (top), the *cdr3*Δ::*hisG-URA3-hisG* (middle), and the *cdr3*Δ::*hisG* (bottom) alleles. A 1.5-kb *Eco*RI fragment derived from the wild-type allele (above the restriction map) as well as a 0.9-kb *Bam*HI/*Bgl*II *hisG* fragment (below the restriction map) were used as probes to monitor the different steps of the deletion. (B and C) Southern blot analysis of the different *cdr3*Δ strains. The genomic DNA samples digested with *Eco*RI were from strains CAI4 (lanes 1), CIB1 (lanes 2), CIB2 (lanes 3), CIB3 (lanes 4), and CIB4 (lanes 5). The blots were probed with the 1.5-kb *Eco*RI wild-type *CDR3* (B) or the 0.9-kb *Bam*HI/*Bgl*II *hisG* (C) fragment. Sizes are indicated in kilobases.

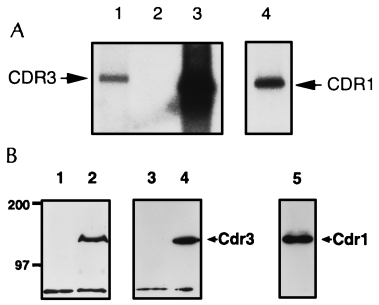

FIG. 5. Analyses of *CDR3*-overexpressing cells. (A) Northern blot. Total RNA was extracted from WO-1 opaque cells (lane 1), from the CIB4 (*cdr3*Δ::*hisG*/*cdr3*Δ::*hisG*) strain transformed with plasmid YPB-ADHpt (lane 2) or plasmid YPB-ADHpt/CDR3 (lane 3), and from the FCZ-resistant strain LSPQ 5902 (lane 4). RNA samples (20 μg) were separated by electrophoresis on an agarose gel and transferred to a nylon membrane. The membrane was cut and hybridized with a gene-specific *CDR3* (lanes 1 to 3) or *CDR1* (lane 4) probe. Positions of the specific *CDR3* and *CDR1* RNA transcripts are indicated. (B) Western blot. Membrane proteins (50 μg) from WO-1 white (lane 1) and WO-1 opaque (lane 2) cells as well as total protein extracts (25 μg) from the CIB4[YPB-ADHpt] (lane 3), CIB4[YPB-ADHpt/CDR3] (lane 4), and FCZ-resistant LSPQ 5902 (lane 5) strains were separated by electrophoresis on an SDS–7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was cut and probed with an anti-Cdr3p (lanes 1 to 4) or an anti-Cdr1p (lane 5) antibody. Molecular mass standards shown on the left are myosin (200 kDa) and phosphorylase *b* (97 kDa). Cdr3p and Cdr1p are indicated by arrows.

Overexpression of *CDR3*. A *C. albicans CDR3* overexpression plasmid was constructed by inserting the coding region of the *CDR3* gene under the control of the strong *ADH1* promoter into vector YPB-ADHpt (4), yielding plasmid YPB-ADHpt/CDR3. Strain CIB4 was transformed with this plasmid or with YPB-ADHpt alone, as a control. Total RNA was prepared from these transformants and separated by electrophoresis on an agarose gel along with RNA from WO-1 opaque cells and from *C. albicans* LSPQ 5902. This strain overexpresses both *CDR1* and *CDR2* but not *CDR3* and displays high levels of cross-resistance to the azole derivatives FCZ, ketoconazole, and itraconazole, as well as to a number of different toxic compounds (data not shown). The gel was transferred to a nylon membrane and hybridized with a *CDR3* (Fig. 5A, lanes 1 to 3) or with a *CDR1* (Fig. 5A, lane 4) gene-specific probe. These probes are derived from the 5′ region of the *CDR3* and *CDR1* genes, where the DNA sequence homology is very low, and do not exhibit cross-hybridization under conditions of high stringency (data not shown). This experiment confirmed that the *CDR3* transcripts were indeed absent from CIB4 cells homozygous for the *cdr3* disruption (Fig. 5A, lane 2). The CIB4[YPB-ADHpt/CDR3] transformant was found to express very high levels of *CDR3* RNA (Fig. 5A, lane 3), exceeding the endogenous levels of *CDR3* RNA in opaque cells (Fig. 5A, lane 1) as well as the levels of *CDR1* RNA in the FCZ-resistant clinical isolate LSPQ 5902 (Fig. 5A, lane 4). Probing these Northern blots with the constitutively transcribed *ACT1* gene confirmed that all lanes contained equivalent amounts of RNA (data not shown).

A Western blot analysis was also performed to analyze Cdr3p in these strains. Total cellular extracts were prepared from the CIB4[YPB-ADHpt] and CIB4[YPB-ADHpt/CDR3] transformants as well as from strain LSPQ 5902. In addition, total membranes were prepared from WO-1 white and opaque cells. Proteins were separated by SDS-PAGE and analyzed with an anti-Cdr3p (Fig. 5B, lanes 1 to 4) or an anti-Cdr1p (Fig. 5B, lane 5) polyclonal antibody. These antibodies, which were raised against the NH_2-terminal region of Cdr3p and Cdr1p where the amino acid sequence homology between the two proteins is low, do not display cross-reactivity (data not shown). The anti-Cdr3p antibody specifically recognized a band of the expected molecular mass (approximately 170 kDa) (Fig. 5B, lanes 2 and 4). This band is present in the CIB4[YPB-ADHpt/CDR3] transformant (Fig. 5B, lane 4) and is absent from CIB4 cells carrying the empty vector (Fig. 5B, lane 3), demonstrating that this protein is indeed Cdr3p. This result indicates that the high levels of *CDR3* mRNA detected in the CIB4[YPB-ADHpt/CDR3] transformant (Fig. 5A, lane 3) translate into high levels of Cdr3p expression (Fig. 5B, lane 4). As expected, Cdr3p was present in WO-1 opaque cells (Fig. 5B, lane 2) but completely absent from WO-1 white cells (Fig. 5B, lane 1), confirming that (i) Cdr3p expression in the WO-1 strain is opaque specific and that (ii) Cdr3p is a membrane-associated protein. Finally, strain LSPQ 5902 expressed Cdr1p (Fig. 5B, lane 5) at levels roughly similar to those of Cdr3p in the CIB4[YPB-ADHpt/CDR3] transformant (Fig. 5B, lane 4). Therefore, the CIB4[YPB-ADHpt/CDR3] transformant was appropriate to determine if overexpression of Cdr3p can cause drug resistance.

Drug susceptibility analysis of *CDR3*-overexpressing cells. CIB4[YPB-ADHpt/CDR3] and CIB4[YPB-ADHpt] transformants were compared for their levels of susceptibility to drugs with antifungal properties, using a microtiter plate growth inhibition assay. The MDR strain LSPQ 5902 was also included in these experiments as a positive control. For this purpose, the cells were grown for 24 h in medium containing increasing concentrations of FCZ (Fig. 6A) or cycloheximide (Fig. 6B), which are known substrates of Cdr1p and Cdr2p (34, 40, 41), and of 4-NQO (Fig. 6C), a known substrate of the *S. cerevisiae* Snq2p transporter (42). These experiments demonstrated that CIB4 cells overexpressing *CDR3* were as susceptible to the three compounds tested as CIB4 cells in which *CDR3* is not expressed, unlike drug-resistant LSPQ 5902 cells, which were capable of growth at much higher concentrations of the three drugs (Fig. 6). These results indicate that despite a high degree of sequence conservation with Cdr1p and Cdr2p, Cdr3p does not appear to be involved in drug resistance, at least to the compounds tested which include the clinically relevant antifungal agent FCZ.

DISCUSSION

Using degenerate oligodeoxyribonucleotides overlapping the conserved ATP-binding domain from different transporters of the yeast *PDR*/*CDR* family, we have identified a new *CDR* gene in *C. albicans* that we have named *CDR3*. This gene codes for a protein highly homologous to *C. albicans* Cdr1p and Cdr2p and *S. cerevisiae* Pdr5p, three transporters involved in resistance to FCZ (6, 34, 40). The strong sequence similarity between Cdr3p and these transporters suggested that Cdr3p could also confer FCZ resistance. However, three separate pieces of evidence indicate that this does not seem to be the case. First, disruption of the two *CDR3* alleles in CAI4 did not result in an increased susceptibility of the cells to FCZ (data not shown). Second, overexpression of Cdr3p in *C. albicans* did not render the cells more resistant to FCZ (Fig. 6A). Third, the expression of *CDR3* was undetectable in a panel of FCZ-resistant *C. albicans* clinical isolates, unlike that of *CDR1* and *CDR2*, whose levels were found to correlate with the level of FCZ resistance in these strains (data not shown). In addition, we found that overexpression of Cdr3p did not render the cells more resistant to cycloheximide (Fig. 6B) and 4-NQO (Fig. 6C) as well as to cerulenin, brefeldin A, and phenanthroline (data not shown), which are all known substrates of different

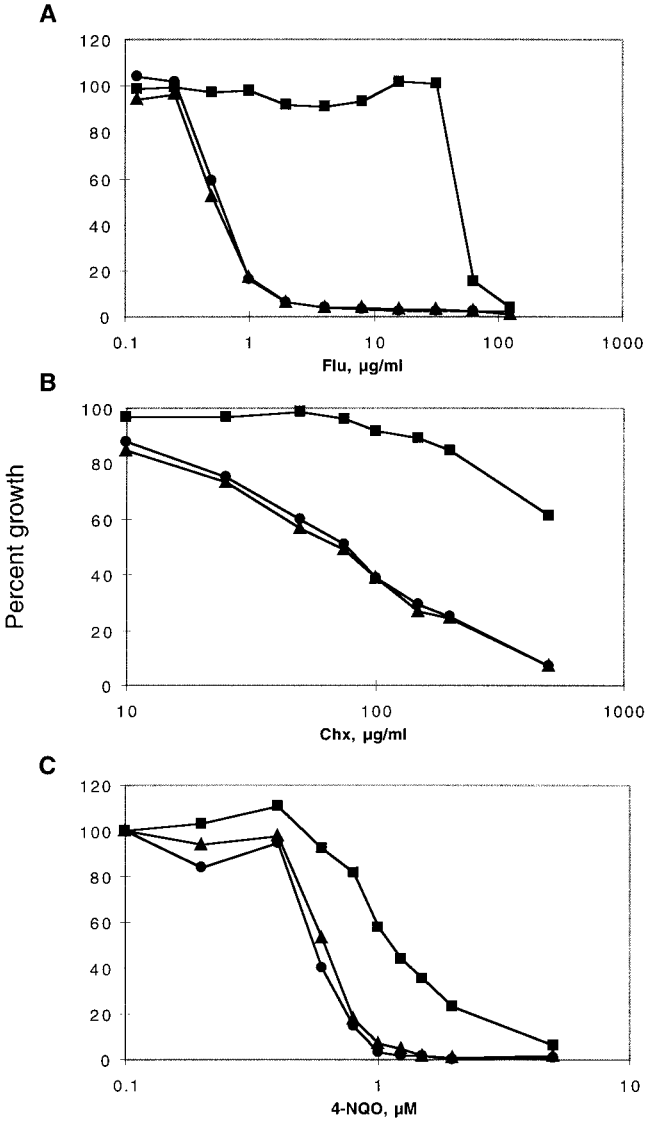

FIG. 6. Drug susceptibility phenotype of *CDR3*-overexpressing cells. The CIB4 strain transformed with plasmid YPB-ADHpt (circles) or plasmid YPB-ADHpt/CDR3 (triangles) and the *CDR1*-overexpressing FCZ-resistant strain LSPQ 5902 (squares) were tested by microtiter plate assay for the ability to grow in the presence of increasing concentrations of FCZ (Flu; A), cycloheximide (Chx; B), or 4-NQO (C). Cell growth is presented as the percentage of growth in drug-containing medium relative to drug-free medium. Values represent the average of two independent experiments performed in duplicate.

Pdrp/Cdrp transporters. Taken together, these data suggest that Cdr3p is not involved in drug resistance, although it is possible that it can confer resistance to other toxic compounds that were not tested in the course of our experiments.

The inability of Cdr3p to confer FCZ resistance compared to Cdr1p and Cdr2p can be due to a different subcellular localization. The availability of isoform-specific anti-Cdrp antibodies will allow us to test this hypothesis (2). Alternatively, differences in primary structure resulting in different substrate specificities may underlie the observed inability of Cdr3p to confer drug resistance. Consistent with this hypothesis is the observation that Cdr1p and Cdr2p display overlapping but distinct substrate specificities upon overexpression in *S. cerevisiae*: both Cdr1p and Cdr2p can confer resistance to drugs such as FCZ, cycloheximide, cerulenin, and brefeldin A, whereas only Cdr1p can confer resistance to sulfomethuron methyl and only Cdr2p can confer resistance to crystal violet (40). Taken together, the structural similarities and functional differences among Cdr1p, Cdr2p, and Cdr3p suggest that the three Cdr proteins may participate in similar transport functions of distinct substrates. This situation is reminiscent of the mouse *mdr* gene family, which codes for the three highly homologous Pgp isoforms: Mdr1, Mdr2, and Mdr3 (18). Both Mdr1 and Mdr3 can confer drug resistance when overexpressed in mammalian cells, while Mdr2 cannot (18). It has been recently shown that Mdr2 and Mdr3 can function as phospholipid translocases, demonstrating that Mdr2 can indeed function as a transporter (38, 49). The inability of Mdr2 to confer drug resistance appears to be linked to a reduced drug-binding capacity of the protein (10). Furthermore, a functional analysis of chimeric genes obtained by exchanging homologous domains between the Mdr1 and Mdr2 proteins has shown that the functional differences detected between Mdr1 and Mdr2 in terms of the ability to confer drug resistance reside within the predicted TM domains (18). By using a similar strategy, it will be possible to take advantage of the distinct functional properties between Cdr3p and Cdr1p/Cdr2p to identify, in chimeric molecules, structural domains required for drug recognition and transport.

Determination of the complete sequence of the *S. cerevisiae* genome has revealed the presence of 30 proteins belonging to the ABC superfamily (14, 48). Based on sequence comparison analyses, these proteins have been grouped into different subfamilies, one of which being referred to as the *PDR* subgroup, or cluster I (14, 48). Proteins from the *PDR* subgroup display distinctive structural features, including an NH_2-ABC-TM-ABC-TM-COOH organization and the presence of degenerate sequences for ATP binding (14, 48). Thus, genes coding for proteins of the *PDR* subfamily probably share a common evolutionary origin and may also be functionally related. In addition to the previously identified *PDR5* and *SNQ2* genes (6, 42), this subgroup contains seven new ORFs predicted to code for proteins homologous to Pdr5p and Snq2p (14, 48). The biological functions of these proteins as well as their potential roles in MDR remain to be determined. The presence of several *PDR5* homologs in the *S. cerevisiae* genome suggests that, besides *CDR1* and *CDR2*, other members of the *CDR* gene family may exist in *C. albicans*. Our identification of the *CDR3* gene indeed confirms this hypothesis. Furthermore, recent work from other laboratories and from the *C. albicans* sequencing project has identified partial sequences for at least two additional genes predicted to code for proteins belonging to the *CDR* subfamily: *CDR4* and *CDR99*, which are most homologous to *S. cerevisiae PDR10* and *PDR15*, respectively (data available at http://alces.med.umn.edu/bin/genelist?genes). It will be interesting to see if these transporters are involved in MDR and what biological function they carry out in *C. albicans.*

C. albicans can spontaneously switch at high frequency between a number of different phenotypes in an inheritable fashion, and it has been suggested that this characteristic may reflect the ability of this pathogenic yeast to rapidly adapt to different environmental conditions (44, 45). In particular, the white-to-opaque switch in strain WO-1, which involves a transition between a white hemispherical to a flat grey opaque colony morphology, has been extensively investigated (reviewed in reference 46). This switch has been shown to also affect a variety of cellular properties, including cell size and shape, adhesiveness, antigenicity, lipid content, and susceptibility to antifungal compounds (46). While white cells are very

similar to other *C. albicans* strains, opaque cells are larger and more elongated, and they contain a large vacuole and pimples on their cell wall (46). By differential hybridization of an opaque cDNA library, two opaque-phase-specific genes have been identified: *SAP1*/*PEP1*, coding for a secreted aspartyl proteinase (31, 51), and *OP4*, coding for a protein of unknown function (30). *SAP3*, another member of the *SAP* gene family in *C. albicans*, was subsequently cloned and found to be expressed specifically in opaque cells (51). Our finding that *CDR3* is expressed in opaque cells and not in white cells of the WO-1 strain identifies yet another opaque-phase-specific gene and suggests an opaque-phase-associated function and substrate for the Cdr3p transporter. Deletion of the *CDR3* gene in WO-1 cells and analysis of the resulting phenotype in the opaque-phase transition should prove useful in trying to elucidate this function.

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