*Candida glabrata* ABC Transporters Cdr1p and Pdh1p Expressed in a *Saccharomyces cerevisiae* Strain Deficient in Membrane Transporters Show Phosphorylation-dependent Pumping Properties

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Running Title: Functional Analysis of *C. glabrata* ABC Transporters.
Summary

The expression and drug efflux activity of the ATP binding cassette transporters Cdr1p and Pdh1p are thought to have contributed to the recent increase in the number of fungal infections caused by Candida glabrata. The function of these transporters and their pumping characteristics, however, remain ill-defined. We have evaluated the function of Cdr1p and Pdh1p through their heterologous hyper-expression in a Saccharomyces cerevisiae strain deleted in seven major drug efflux transporters to minimize the background drug efflux activity. Although both Cdr1p- and Pdh1p-expressing strains CDR1-AD and PDH1-AD acquired multiple resistances to structurally unrelated compounds, CDR1-AD showed, in most cases, higher levels of resistance than PDH1-AD. CDR1-AD also showed greater rhodamine 6G efflux and resistance to pump inhibitors, even though plasma membrane fractions had comparable nucleotide triphosphatase (NTPase) activities. These results indicate that Cdr1p makes a larger contribution than Phd1p to the reduced susceptibility of C. glabrata to xenobiotics. Both pump proteins were phosphorylated in a glucose-dependent manner. While the phosphorylation of Cdr1p affected its NTPase activity, the protein kinase A-mediated phosphorylation of Pdh1p, that was necessary for drug efflux, did not. This suggests that phosphorylation of Pdh1p may be required for efficient coupling of NTPase activity with drug efflux.
**Introduction**

Drug efflux mediated by membrane pump proteins is a major resistance mechanism in both cancer cells and pathogenic microorganisms. While many of the drug efflux pumps in bacteria are antiporters which harness the pH gradient across the plasma membrane to efflux molecules (1, 2), eukaryotic organisms often use ATP-binding cassette (ABC) transporters to pump compounds out of the cell at the expense of ATP hydrolysis (3-6). A central role for drug-efflux ABC transporters in multidrug resistance (MDR) has been reported for pathogenic fungi (7-14).

Infections of immunocompromised and debilitated individuals caused by *Candida* sp. are the most frequent and problematic of fungal diseases. Triazole drugs, such as fluconazole and itraconazole, inhibit lanosterol14\(\alpha\)-demethylase and block the synthesis of ergosterol. These drugs have been used widely for the treatment of patients with *Candida* infections because of their limited toxicity to humans and their favorable pharmacokinetics. Fungal infections recalcitrant to triazole therapy occur frequently, however, due to the drug-resistance of fungal strains. *Candida albicans* acquires resistance to azole drugs by over-expressing ABC transporters, antiporter proteins, or 14\(\alpha\)-demethylase, by acquiring mutations in 14\(\alpha\)-demethylase or by changing its membrane composition (15, 16). Although *C. albicans* is normally susceptible to azoles, the incidence of acquired resistance increased significantly in the 1990s before the advent of HAART therapy for AIDS patients. Throughout the 1990s there was also an increase in the incidence of candidosis caused by non-*C. albicans* *Candida* species (17) due to selection of strains with lower azole susceptibility. *C. glabrata* was one the most common species responsible for these infections which were difficult to treat (18).

The susceptibilities of *C. glabrata* clinical isolates to azole drugs, measured as minimum growth inhibitory concentrations (MICs), are 16-64 fold higher than those for *C. albicans* (19),

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1 The abbreviations used are: ABC, ATP-binding cassette; MDR, multidrug resistance; MIC, minimum growth inhibitory concentration; NTPase, Nucleotide triphosphatase; PKA, protein kinase A; PKC, protein kinase C; SFI, supernatant fluorescence intensity; TFI, total fluorescence intensity.
implying that *C. glabrata* is naturally more resistant to azoles. ABC transporters Cdr1p (13) and Pdh1p (also referred to as Cdr2p - see discussion) are thought to be the main contributors to theazole drug-resistance of *C. glabrata* (12, 20). Cdr1p is highly expressed in azole-resistant clinical isolates and it has been shown to be involved in fluconazole efflux (13). At present there is no direct evidence that Pdh1p is involved in azole efflux, although a gradual increase in Pdh1p expression in *C. glabrata* strains has been reported during exposure to fluconazole *in vivo* (12) and *in vitro* (20). These pumps were inferred to efflux divergent xenobiotics because both pumps share about 70% amino acid sequence identity with *Saccharomyces cerevisiae* Pdr5p, an ABC efflux pump that is responsible for pleiotropic drug resistance (12, 13, 21). However, the precise function of the transporters and their pumping characteristics are ill-defined. More detailed knowledge of their drug efflux mechanisms may enable the development of improved antifungal drugs that are not pumped out from the cells, or chemosensitizers that inhibit the pumping activity of these transporters in *C. glabrata*.

There are about 30 genes in *S. cerevisiae* encoding ABC transporters, some of which are responsible for the efflux of xenobiotics (5, 22-24). *C. glabrata* is a close relative of *S. cerevisiae* (18, 25) and probably has a similar number of transporters. Thus the background activities of other endogenous pumps are likely to be problematic for the precise analysis of the efflux mechanism of individual drug efflux pumps in intact *C. glabrata* cells. We recently reported the functional expression of *C. albicans* drug efflux pump CaCdr1p in *S. cerevisiae* strain AD1-8u−, which was used to resolve the problem of endogenous background drug efflux (26). Strain AD1-8u− was deleted in seven major drug efflux transporters and has a *PDR1* gain-of-function mutation that highly activates the *PDR5* promoter (26, 27). Drug efflux pump genes inserted at the *PDR5* locus of this strain are highly expressed and the pumping activities of the transporters can be measured at both the cellular level and in the plasma membrane fraction against a diminished background of endogenous drug efflux activity.

In this study, Cdr1p and Pdh1p were hyper-expressed in *S. cerevisiae* and the chemical
specificities, drug efflux activities, and the NTPase activities of plasma membrane fractions from the resultant yeast strains were analyzed. The post-translational modification of the fungal drug efflux transporters by phosphorylation, and its differential effects on the regulation of drug efflux function, were also investigated.
Materials and Methods

Bacterial and yeast strains and growth media.

Plasmids were maintained in *Escherichia coli* XL1-Blue. *E. coli* was cultured in Luria-Bertani medium (Difco Laboratories, Detroit, Mich.), to which ampicillin was added (50 µg/ml) as required. The genes described in this study were obtained from *C. glabrata* CBS138 and *C. albicans* ATCC 10261. The *S. cerevisiae* strains used were AD1-8u’ (MATα, pdr1-3, his1, ura3, Δyor1::hisG, Δsnq2::hisG, Δpdr5::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, Δpdr3::hisG Δpdr15::hisG) (27, provided by Dr. A. Decottignies and Prof. A. Goffeau, Université Catholique de Louvain, Belgium) and its derivatives expressing *C. glabrata* ABC transporters. The yeast strains were cultured in YEPD broth (Difco) or complete synthetic medium (CSM: 790 mg complete supplement mixture [Bio 101, Vista, Calif.] and 26.7 g Dropout Base [Bio 101] per 1 L). For agar plates, 2% [wt/vol] Bacto agar (Difco) was added to the medium. CSM was buffered with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 18 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) pH 7.0 for MIC assays and pump-phosphorylation experiments. In the latter case, Yeast Nitrogen Base (1.7 g/L, Difco) and ammonium sulfate (5 g/L) were added to the medium, instead of Dropout Base, to prepare CSM without glucose (CSM-Gluc). For the selection and maintenance of Ura’ strains, complete synthetic medium without uracil (CSM-URA, Bio 101) was used.

Plasmid construction and yeast transformation.

Genomic DNA was prepared from *C. glabrata* CBS138 and *C. albicans* ATCC 10261 as described previously (28). Genes required for the construction of expression vectors were amplified by PCR with the combinations of templates and primers indicated in Table 1 using KOD (+) DNA polymerase (Toyobo, Osaka, Japan). PCR products were digested with restriction enzymes and inserted into pSK-PDR5PPUS vector plasmid (26) which had previously been digested with
restriction enzymes and treated with calf intestinal alkaline phosphatase (New England Biolabs, Beverly, Mass.) as shown in Fig. 1. Correct vector construction was confirmed by DNA sequencing with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and an ABI 373 DNA sequencer. The resultant vectors, named pSK-CDR1 and pSK-PDH1, were digested with Xho I and Not I or Kpn I and Not I, respectively, to prepare their transformation cassettes for gene transfer. These cassettes and a PCR fragment of PDH1 digested with HindIII (1-3 µg) were used to transform S. cerevisiae AD1-8u' by the lithium acetate transformation protocol (Alkali-Cation Yeast kit; Bio 101). Cdr1p- and Pdh1p-expressing strains were selected by growth on CSM-URA agar followed by growth on YEPD agar containing fluconazole (5 µg/ml). CDR1 and PDH1 genes from the resultant transformants were amplified by PCR using the primers GC-1 and GC-2 or GP-1 and GP-2 (Table 1), respectively, and the DNA sequences of the PCR fragments were obtained as described above.

**Northern blot analysis of pump mRNA expression.**

Total RNA (8 µg) extracted from S. cerevisiae cells (29) was electrophoresed in an agarose gel, blotted onto Hybond-N+ nylon membrane (Amersham), and fixed with 50 mM NaOH. The membranes were hybridized at 50°C for 16 h with digoxygenin-labeled DNA probes that had been prepared with a BcaBEST™ DIG Labeling Kit (Takara, Kusatsu, Shiga, Japan). The probes consisted of: nt 1-1331 CDR1 ORF, nt 428-1957 PDH1 ORF, and the complete S. cerevisiae PMA1 (plasma membrane H⁺-ATPase) ORF as a control. After washing and blocking the blots with the appropriate buffers (Roche Diagnostics Corporation K. K., Tokyo, Japan), the membranes were incubated with alkaline phosphatase-conjugated anti-digoxygenin Fab fragments (Roche) and CDP-star (Amersham). The chemiluminescence of CDR-star was detected with X-ray film.

**Preparation of plasma membrane proteins.**

Plasma membrane fractions of yeast cells were prepared as described previously (30) with
some modifications. *S. cerevisiae* cells were cultured in YEPD broth at 27°C from an initial 
\[ \text{OD}_{600\text{nm}} = 0.2 \] for 12 h with continuous shaking. These yeasts were transferred to CSM-Gluc or 
labeled with 20 µCi/ml \[^{32}\text{P}\] ortho-phosphate, depending on the experiment. The following 
procedures were performed on ice or at 4°C. The culture (5 ml) was centrifuged at 3,000 \( x \) \( g \) for 5 
min, and the yeast cell pellet was washed with 1 ml ice cold 2 % [wt/vol] glucose (or water in some 
cases). The cells were harvested by centrifugation at 3,000 \( x \) \( g \) for 5 min and suspended in 250 µl of 
homogenizing buffer (50 mM Tris-HCl [pH 7.5], 2 mM EDTA, and 2% [wt/vol] glucose). The 
following were added to the cell suspension: 1 mM phenylmethanesulfonyl fluoride (PMSF), 1.2 
µM antipain, 1.6 µM leupeptin, 1.1 µM pepstatin A, and 400 µl glass beads (Sigma-Aldrich, St. 
Louis, Mo.). The yeast cells were lysed using a MT-360 micro tube mixer (TOMY Seiko, Co. Ltd., 
Tokyo) at maximum vibration for 10 min. The cell extract was collected and the glass beads were 
washed with up to 1 ml of homogenizing buffer containing 1 mM PMSF. The cell extract was 
centrifuged (2,000 \( x \) \( g \) for 10 min) to remove unbroken cells and cellular debris and the supernatant 
centrifuged at 20,000 \( x \) \( g \) for 45 min. The pellet was washed with 1 ml of GTED-20 buffer (10 mM 
Tris-HCl [pH7.0], 0.5 mM EDTA, and 20 % [vol/vol] glycerol) containing 1 mM PMSF and 
centrifuged at 20,000 \( x \) \( g \) for 45 min. The pellet was resuspended in 100 µl of GTED-20 buffer and 
used as the crude membrane fraction for measuring pump protein expression and for western blots.

For the measurement of plasma membrane NTPase activity, a large-scale crude membrane fraction 
was acidified with 0.1 M acetate to pH 5.0 and incubated for 5 min. The precipitated mitochondria 
were removed by centrifugation at either 5,000 \( x \) \( g \) for 30 s for glucose-fermenting cells or 8,000 \( x \) 
\( g \) for 10 min for glucose-starved cells. The supernatants were immediately neutralized (to pH7.5) 
with 1 M Tris and centrifuged at 20,000 \( x \) \( g \) for 45 min. The precipitated plasma membrane 
fractions were resuspended in GTED-20 buffer to a protein concentration of approximately 1 mg/ml 
and used in the NTPase assay. The protein concentrations of these membrane fractions were 
determined by a BCA assay (Pierce Chemical Company, Rockford, Ill.) with bovine serum albumin 
as the standard.
Analysis of expression and phosphorylation of pump proteins.

Crude membrane samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8 % acrylamide [wt/vol]) (31) and either stained with Coomassie Brilliant Blue R-250 or electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Bedford, Mass.) at 12 V for 1 h. The membranes were blocked with phosphate buffered saline containing 5 % (wt/vol) skim milk and 0.05 % (vol/vol) tween 20 (t-PBS) at room temperature for 2 h. After washing twice with PBS for 5min, the membranes were incubated with anti-phospho protein kinase A-substrate antibody (Cell Signaling Technology, Beverly, Mass.) diluted 1:1000 in t-PBS containing 1 % (wt/vol) bovine serum albumin at 4°C for 16 h. The blot was washed with PBS three times and then incubated with secondary antibody (anti-rabbit IgG conjugated with horse radish peroxidase, Amersham) diluted 1:5000 in t-PBS containing 1 % (wt/vol) skim milk, for 1 h. The membranes were washed with t-PBS containing 5 % (wt/vol) skim milk for 15min and twice with PBS alone for 15 min. Signal from the horse radish peroxidase was detected with an enhanced chemiluminescence system (ECL; Amersham). For the identification of pump proteins, membrane fractions were incubated with 80 mM iodoacetamide for 30 min before SDS-PAGE. The putative pump-containing bands were excised from the gel and the proteins were extracted with 1 % (wt/vol) SDS buffered with 20 mM Tris-HCl, pH 8.0. After concentration by precipitation with 50 % acetone, the samples in 10 mM Tris-HCl pH 9.0 were partially digested with lysyl endopeptidase (Wako, Osaka, Japan; at an enzyme:substrate molar ratio of 1:100) at 37°C for 1 h, electrophoresed, and blotted onto PVDF membrane. N-terminal sequences of several of the digested fragments were analyzed with an Applied Biosystems model 473A Protein Sequencer through the courtesy of Prof. Watabe and Dr. Nakaya at the University of Tokyo.

Drug susceptibility disk assays and chemicals.

Drug susceptibility of yeast strains was measured by filter disk assays on YEPD plates
containing 1.5% [wt/vol] agar. Exponentially growing yeast cells were seeded at a concentration of 6 x 10^4 cells/ml in the agar. Sterile Whatman paper disks, on which drug solutions had been spotted and dried at room temperature for 1 h to remove excess solvent, were placed on the agar plates. Miconazole, ketoconazole, nystatin, amphotericin B, fluocytosine, cerulenin, cycloheximide, nigericin, monensin, rhodamine 123, rhodamine 6G, staurosporine, cytochalasin D, baflomycin A1, 4-nitroquinoline N-oxide, trifluoperazine, carbonyl cyanide m-chlorophenylhydrazone (CCCP), verapamil, tri-\(n\)-methyltin chloride, and tri-\(n\)-butyltin chloride were purchased from Sigma-Aldrich. Oligomycin, cyclosporin A, and protein kinase A inhibitor 14-22 amide were purchased from Calbiochem (San Diego, Calif.). The sources of other drugs used in this study were as follows: Fluconazole (Pfizer Ltd., Sandwich, Kent, UK), Itraconazole (Jannssen Research Foundation, Beerse, Belgium), terbinafine HCl (Novartis Pharma K.K., Tokyo, Japan), adriamycin (Kyowa Hakko, Tokyo, Japan), latrunculin A (Wako), aureobasidin A (Takara), G418 (Gibco BRL, Life Technologies, Rockville, Md.), tri-\(n\)-ethylin chloride (Strem Chemicals, Newburyport, Mass.), tri-\(n\)-propyltin chloride (Merck KgaA, Darmstadt, Germany), tri-\(n\)-pentyltin chloride (Kanto Chemicals Co., Inc., Tokyo, Japan), FK506 (Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan), H-89 and H-8 (Seikagaku Corporation, Tokyo, Japan). Theonellamide F and calyculin A were kindly provided by Prof. Fusetani (Univ. Tokyo). In some experiments, drug susceptibility of yeasts grown in YEPD was measured using a 96 well microtiter plate assay. Yeast cultured in YEPD until early stationary phase were diluted to \(OD_{600nm} = 0.016\) in YEPD in microtiter plate wells (Nunc, Roskilde, Denmark), drugs were added to the indicated final concentration and the cells were incubated at 30°C for 48 h. The growth of the cells in individual wells was measured with a microplate reader (EL 312e; Bio-Tek Instruments, Winoosk, Vt.) at 590nm.

**MIC determination.**

The MICs of antifungal agents for *S. cerevisiae* cells were determined by a microdilution test based on the macrodilution reference method of the National Committee for Clinical Laboratory
Standards (32). Cells (10 µl suspension of 2 x 10^5 cells/ml) were inoculated into 90 µl of CSM buffered with MES and HEPES in microtiter plate wells. The wells contained doubling dilutions of antifungal agents in the CSM (final concentrations were as follows: fluconazole, 1024 - 0.058 µg/ml; itraconazole, 128 - 0.004 µg/ml; ketoconazole and miconazole, 32 - 0.002 µg/ml; flucytosine and nystatin, 64 - 0.031 µg/ml; amphotericin B, 16 - 0.0078 µg/ml; tri-n-methyltin chloride, 50 - 0.098 µM; other tri-n-alkyltin chlorides, 5 - 0.0098 µM). The microtiter plates were incubated at 35˚C for 48 h and then the growth of cells in individual wells was measured with a microplate reader. The MIC80 was the lowest concentration of drug that inhibited the growth yield by at least 80 % compared to the growth for a no-drug control.

**Rhodamine 6G efflux by S. cerevisiae cells.**

The efflux of rhodamine 6G from *S. cerevisiae* cells was determined as previously reported (26, 33) with slight modification. Yeast cells (1 x 10^9 cells) from YEPD cultures in late-exponential growth phase (OD_{600nm} = 5.0-8.0) were collected by centrifugation (3,000 x g, 5 min, 20 °C) and washed twice with HEPES buffered saline (HBS) containing 50 mM HEPES-NaOH (pH 7.0) and 100 mM NaCl. After 2 h incubation in HBS at 27˚C with shaking (150 rpm) the cells were centrifuged as above and suspended in 4 ml of HBS supplemented with 5 mM 2-deoxyglucose and 10 µM rhodamine 6G. The cell suspension was incubated at 27˚C with shaking for 90 min to allow rhodamine accumulation under glucose-starvation conditions. The starved cells were washed twice in HBS and finally suspended in 7.6 ml HBS. Glucose (25 µl, 40 mM) was added to a portion of the cell suspension (475 µl) to initiate rhodamine 6G efflux. At specified intervals after the addition of glucose, the cells were removed by centrifugation, and triplicate 100 µl volumes of the supernatants were transferred to the wells of 96-well flat-bottom microtiter plates. The rhodamine 6G fluorescence of the samples was measured with a CytoFluor Series 4000 spectrofluorimeter (PerSeptive Biosystems, Inc., Framingham, Ma.). The excitation wavelength was selected using a 530/525 filter and emission was detected using a 580/550 filter.
NTPase assays.

Nucleotide triphosphatase (NTPase) activity of plasma membrane fractions was measured by adapting a previously described method (26). Purified plasma membrane samples (2.5 µg protein), prepared as described above, were incubated in a solution (final volume 30 µl) containing 6 mM nucleotide triphosphate (NTP) and 7 mM MgSO₄ in 59 mM MES-Tris buffer (pH 4.0 – 8.5). To eliminate possible contributions from nonspecific phosphatases and vacuolar or mitochondrial ATPases, 0.2 mM ammonium molybdate, 50 mM KNO₃, and 10 mM NaN₃ were included, respectively, in the assay mixtures (30). Oligomycin (20 µM) was added to the assay for the control reactions. After 30 min incubation at 30°C the reaction was stopped by the addition of 32.5 µl of a solution containing 1 % (wt/vol) SDS, 0.6 M H₂SO₄, 1.2 % (wt/vol) ammonium molybdate, and 1.6 % (wt/vol) ascorbic acid. The amount of inorganic phosphate released from NTPs was measured at 690 nm after 10 min of incubation at room temperature. KH₂PO₄ solutions (0.4 - 2 mM) were used to obtain a standard curve.
Results

Construction of vectors and pump-expressing yeast strains.

*S. cerevisiae* was chosen for the analysis of *C. glabrata* efflux proteins for several reasons. It is a tractable yeast amenable to molecular genetic manipulation. It has been well studied and much is known about the genes involved in pleiotropic drug resistance (21). *S. cerevisiae* and *C. glabrata* are closely related (25) and probably possess similar machinery for the folding and post-translational modification of proteins. We used *S. cerevisiae* strain AD1-8u⁻ and the vector pSK-PDR5PPUS (26, 27) to express Cdr1p and Pdh1p (Fig. 1). Strain AD1-8u⁻ has been deleted in seven major drug efflux pumps and, as shown by our previous studies with *C. albicans* multidrug efflux pumps (26), is suitable for the investigation of pump functions due to a diminished background of endogenous ABC transporters. AD1-8u⁻ was derived from a pdr1-3 strain with a gain-of-function mutation in the transcription factor Pdr1p, resulting in constitutive hyper-induction of the *PDR5* promoter (27). Plasmid pSK-PDR5PPUS contains a multi-cloning site (MCS) for the insertion of genes of interest adjacent to the *PDR5* promoter. The cloned gene can then be excised from the plasmid together with the *PDR5* promoter, the *URA3* gene, and *PDR5* downstream sequence as a transformation cassette that confers uracil prototrophy on AD1-8u⁻ by homologous integration at the *PDR5* locus (26). This strategy was used, with modifications, for the construction of *S. cerevisiae* strains hyper-expressing Cdr1p and Pdr1p.

Plasmid pSK-PDR5PPUS does not contain a transcriptional terminator sequence downstream of the MCS, and the reported *CDR1* DNA sequence (13) contains only 182 bp of sequence downstream of the stop codon. In order to ensure efficient transcription termination a 371 bp DNA fragment of the *C. albicans CDR2* terminator region, which functions as a transcriptional terminator in the expression of *C. albicans CDR2* using the AD1-8u⁻ and pSK-PDR5PPUS system (unpublished data), was inserted in the *SpeI* site of pSK-PDR5PPUS (Fig. 1). The full-length *C. glabrata CDR1* ORF was then inserted between the *HindIII* and *EcoRI* sites of the modified pSK-
PDR5PPUS vector, and the resultant plasmid was named pSK-CGCDR1 (Fig. 1). The CDRI cassette was excised from pSK-CGCDR1 with XhoI and NotI and used to transform AD1-8u- to uracil prototrophy. More than 20 Cdr1p-expressing AD1-8u- transformants were obtained by serial selection on CSM-URA plates followed by YEPD agar containing fluconazole (5 µg/ml). Most transformants had similar growth rates and fluconazole susceptibilities. A representative clone, denoted CDR1-AD, was selected for further analysis. The DNA sequence of CDRI integrated in the CDR1-AD genome was determined and compared with the previously reported sequence (13) and that of C. glabrata strain CBS138, from which the gene was obtained for this study. The DNA sequence of CDRI in CDR1-AD was identical to that of CBS138, and 27 nucleotides (0.6%) were different from the previously reported sequence (13). These differences were predicted to cause four amino acid changes (D207E, L380F, R388E, and P1181L [where the second amino acid is the residue in CDR1-AD]), which could be explained by strain variation.

Several attempts to prepare a pSK-PDR5PPUS vector containing the complete PDH1 ORF were unsuccessful because we could not obtain E. coli strains that retained such a plasmid. Therefore, a vector (pSK-PDH1) was constructed which contained a 530 bp 5’ portion of the ORF (PDH1-U, Fig. 1) and a separate 3’ region comprising the last 98 bp of the ORF in tandem with 857 bp of transcriptional terminator region (PDH1-L, Fig. 1). The PDH1-U/PDH1-L/URA3 transformation cassette was excised from pSK-PDH1 and used to transform AD1-8u- cells to uracil prototrophy. A representative transformant, PDH1-UL-AD, was identified and transformed with a full length PDH1 PCR fragment (Fig. 1), with selection for fluconazole resistance. More than 30 fluconazole-resistant clones were obtained and most of them demonstrated similar growth rates and susceptibilities to fluconazole. The DNA sequence of PDH1 integrated into the genome of representative strain PDH1-AD was identical to that of CBS138 and contained 19 nucleotides that were different to the sequence previously reported (12). These nucleotide differences were predicted to cause 4 amino acid changes (P165T, Q438K, I734V, D839E).
Expression of pump proteins in yeast strains CDR1-AD and PDH1-AD.

The expression of pump gene mRNA and transporter proteins in yeast strains CDR1-AD and PDH1-AD were studied by northern blot analysis and SDS-PAGE (Fig. 2). CDR1-AD and PDH1-AD expressed CDR1 or PDH1 mRNA respectively (Fig. 2 A). Heterologous pump proteins expressed in AD1-8u− strain with the aid of the hyper-induced PDR5 promoter were easily observed after SDS-PAGE when stained with Coomassie Brilliant Blue R-250 (26, 27). Protein bands of approximately 170 kDa, corresponding to the heterologous transporters, were observed in crude membrane fraction samples from CDR1-AD and PDH1-AD but not from the parental strain AD1-8u− or the null mutant pSK-AD transformed to uracil prototrophy with control transformation cassette containing URA3 but no transporter gene ORF (Fig. 2 B). CDR1 is predicted to encode a protein comprising 1499 amino acids with a molecular mass of 169.3 kDa, whereas the predicted product of PDH1 contains 1542 amino acids and a molecular mass of 175.0 kDa. The hyper-expressed proteins in CDR1-AD and PDH1-AD (Fig 2B) were of the expected molecular mass, with the protein from PDH1-AD being noticeably larger than that from CDR1-AD. Proteins in membrane fractions from CDR1-AD and PDH1-AD were separated by SDS-PAGE, and electroblotted onto PVDF membrane. The hyper-expressed proteins were excised, fragmented with lysyl endopeptidase digestion and the N-terminal sequences of fragments determined. The following peptide sequence was obtained for Cdr1p: 979KILEMEQYAD988, and the following sequences for Pdh1p: 849KNMLQDTYDE858; 1007KILEMETYAD1017. This confirmed the identity of the hyper-expressed bands to be Cdr1p and Pdh1p. Analysis with NIH image software indicated that the amounts of Coomassie Blue-stained Cdr1p and Pdh1p in the respective strains were equal.

Drug susceptibilities of CDR1-AD and PDH1-AD.

The susceptibilities of strains CDR1-AD and PDH1-AD to various structurally and functionally unrelated antifungals were examined using both filter disk and liquid MIC assays. Strain susceptibility to 26 compounds was measured with the disk assay (Fig. 3). Parental strain
AD1-8u’, and pSK-AD were highly sensitive to the compounds, with pSK-AD being slightly more sensitive to azole drugs (Fig. 3). While CDR1-AD was highly resistant to several compounds (Fig. 3: disks A-D, G-L, M-S), it was sensitive to polyene drugs (Fig. 3: E, F) and to hydrophobic and hydrophilic cyclic peptides (Fig. 3: T, U). There were no significant differences in the susceptibilities of control and pump-expressing strains to G418, trifluoperazine, and CCCP; high concentrations of these drugs were required to inhibit growth of all strains (Fig. 3: X-Z). In general, PDH1-AD was less resistant than CDR1-AD. It demonstrated resistance lower than that for CDR1-AD but higher than that for the parental null strain to several drugs (Fig. 3: G-J, M, N, P, and R). PDH1-AD was susceptible to the drugs to which CDR1-AD was also sensitive (Fig 3: E,F, T-Z).

The generally greater resistance of CDR1-AD was also observed with a microdilution liquid MIC assay. While the MIC_{80} values of azole agents for CDR1-AD and PDH1-AD were more than 128 fold and 32-64 fold higher, respectively, than those for pSK-AD, CDR1-AD was only 4 fold more resistant than pSK-AD to flucytosine and neither CDR1-AD nor PDH1-AD was more than 2 fold more resistant than the control strain to polyene drugs, as expected (Table 2, Fig. 3: E-G). We have, to date, examined the susceptibility of the strains to more than 30 compounds in the drug resistance assays. PDH1-AD showed slightly higher resistance than CDR1-AD only to amphotericin B (Fig. 3: F, Table 2) and H-89 (Fig. 9).

Series of homologous compounds with systematic structural variations can be used to elucidate pump extrusion preferences. The tri-n-alkyltin chlorides are a family of structurally related compounds that interfere with mitochondrial ATPase activity, and differ in the lengths of their hydrocarbon chains. The susceptibility of S. cerevisiae cells expressing Pdr5p to tri-n-alkyltin chlorides has been reported (34). We measured the susceptibilities of CDR1-AD, PDH1-AD, and pSK-AD to these compounds in a microdilution assay (Fig. 4 A). The control strain pSK-AD was relatively resistant to tri-n-methyltin chloride (1 carbon atom in alkyl chain), whereas it was highly susceptible to tri-n-butyltin chloride (4 carbon atoms in alkyl chain). Over-expression of Cdr1p conferred resistance to all members of the tri-n-alkyltin chloride family and increased the MIC_{80}
values between 3 and 15 fold (Fig. 4B). Pdh1 over-expression conferred more modest resistance to each of the tri-\textit{n}-alkyltin chlorides, except tri-\textit{n}-methyltin chloride to which it was as sensitive as the control (Fig 4 B). It was previously reported that the expression of Pdr5p did not change the sensitivity of \textit{S. cerevisiae} to tri-\textit{n}-methyltin and tri-\textit{n}-pentyltin chlorides (34). Thus Cdr1p and Pdh1p demonstrated different properties in \textit{S. cerevisiae} than their orthologue Pdr5p, with the degree of resistance to the tri-\textit{n}-alkyltin chlorides affected by the length of the alkyl chain. This may indicate that the hydrophobicity and/or molecular size of the compounds affect substrate recognition by the pumps.

We also examined the susceptibility of CDR1-AD, PDH1-AD to metal ions. CDR1-AD, PDH1-AD, AD1-8u and pSK-AD were equally sensitive in YPD broth to serial dilutions of LiCl, NaCl, KCl, RbCl, MgCl$_2$, CaCl$_2$, MnCl$_2$, or ZnCl$_2$ (data not shown).

**Energy-dependent Cdr1p- and Pdh1p-mediated efflux of rhodamine 6G from yeast cells.**

The energy-dependent drug efflux activities of Cdr1p and Pdh1p were examined in CDR1-AD and PDH1-AD by measuring the efflux of the fluorescent dye rhodamine 6G (Fig. 5). CDR1-AD and PDH1-AD cells were pre-loaded with rhodamine 6G under glucose-starvation conditions and then the efflux of the fluorescent dye into the assay supernatant, initiated by the addition of glucose (2 mM), was measured. CDR1-AD cells accumulated less dye than PDH1-AD cells (see Fig. 5 legend). This may be due to a higher residual Cdr1p activity than Pdh1p activity under the glucose-starvation conditions. To correctly compare the efflux activity of the pumps, the ratios of fluorescence released from the cells to total fluorescence in the cellular suspensions are shown in Fig. 5. Rhodamine 6G efflux from CDR1-AD cells showed a short lag and reached a plateau five minutes after addition of glucose with the export of at least 60\% of the dye. Efflux from PDH1-AD cells showed a longer lag, was slower and it took 10 minutes for the fluorescence of the assay supernatant to reach the maximal value, which was comparable to the proportion of rhodamine 6G pumped by CDR1-AD. In other experiments CDR1-AD cells were pre-incubated with higher
concentrations of rhodamine 6G in order to achieve the same intracellular concentration as for PDH1-AD cells. In these experiments, glucose-stimulated dye efflux was slightly faster and a greater proportion of the dye (80-90%) was exported from the cells. There was no detectable rhodamine 6G efflux by AD1-8u or pSK-AD cells, or by any strain without glucose addition (indicated as (-) glucose in Fig. 5), despite each strain accumulating the dye under glucose-starvation conditions. These results indicated that Cdr1p and Pdh1p are responsible for rhodamine 6G efflux in an energy-dependent manner, and that the efflux activity of Cdr1p was higher than that of Pdh1p.

The effects of ABC transporter inhibitors on the fluconazole-resistance of CDR1-AD and PDH1-AD.

Several compounds have been reported to specifically inhibit the drug efflux activity of ABC transporters (35-40). We examined the effect of four compounds on the fluconazole-resistance of CDR1-AD and PDH1-AD. The immunosuppressant FK506 has been shown to be an inhibitor of human Mdr1 (35, 36) and S. cerevisiae Pdr5p (37). FK506 strongly reversed the fluconazole-resistance of CDR1-AD and PDH1-AD even though it did not inhibit their growth in the absence of fluconazole in either the filter disk assay (Fig. 6 A) or a liquid microdilution assay (Fig. 6 B). FK506 was less effective against the fluconazole-resistance of CDR1-AD than that of PDH1-AD. Likewise, oligomycin sensitized PDH1-AD to fluconazole in the filter disk assay (Fig. 6 A) but only had an effect on CDR1-AD at high concentrations in the liquid assay (Fig. 6 C). It is possible that oligomycin inhibited the NTPase activities of Cdr1p and Pdh1p, as has been reported for Pdr5p (38). On the other hand, high concentrations of verapamil, an inhibitor of human Mdr1-mediated drug resistance (39), only weakly sensitized PDH1-AD but not CDR1-AD to fluconazole (Fig. 6 A). Another immunosuppressant, cyclosporin A, which also reversed Mdr1-mediated MDR in human cancer cells (40), did not sensitize either strain to fluconazole (Fig. 6 A). Verapamil and cyclosporin A were both ineffective in the liquid MIC assays (data not shown).
NTPase activity of plasma membrane fractions from CDR1-AD and PDH1-AD.

A significant characteristic of Pdr5p, and some other ABC transporters, is their ability to hydrolyze not only ATP but also other NTPs (38). Plasma membrane fractions of CDR1-AD and PDH1-AD were prepared for NTPase assays. The plasma membrane ATPase (Pma1p; 100 kDa) was prominent in the plasma membrane preparations (Fig. 7) and its ATPase activity could interfere with the measurement of Cdr1p and Pdh1p ATPase activities. We therefore measured the NTPase activities of membrane fractions both in the presence and absence of oligomycin (20 µM) which inhibits the NTPase activities of ABC transporters but not that of Pma1p (30). The differences between the NTPase activities in the presence and absence of oligomycin are presented in Fig. 7 as oligomycin-sensitive NTPase activities. The oligomycin-sensitive NTPase specific activities of both CDR1-AD and PDH1-AD plasma membranes were comparable and their optimum pHs were 7.5-8.0, as observed for membranes containing Pdr5p (38), S. cerevisiae Yor1p (27) or C. albicans Cdr1p (26). The greatest difference was observed with the UTPase activity measurements, where the maximum oligomycin-sensitive activity of CDR1-AD membranes was about 1.5-fold higher than that of PDH1-AD membranes, and the optimal pH for the UTPase activity of PDH1-AD membranes was slightly more alkaline than that of CDR1-AD membranes.

Phosphorylation of Cdr1p and Pdh1p in vivo.

Coomassie blue-staining after SDS-PAGE often showed Pdh1p as a broader band than that of Cdr1p (Fig. 2 B), suggesting differences in their post-translational modification, such as phosphorylation or glycosylation. Both Cdr1p and Pdh1p were phosphorylated when CDR1-AD and PDH1-AD were cultured in YEPD broth containing [32P] ortho-phosphate (20 µCi/ml), with Pdh1p showing slightly greater phosphorylation than Cdr1p (Fig. 8 A). The nature of the phosphorylation was examined by measuring immunoreactivity on western blots with antibodies specific for phospho-protein kinase A (PKA) substrates, phospho-protein kinase C (PKC) substrates,
and phospho-tyrosine. Neither Cdr1p nor Pdh1p reacted with either the phospho-PKC substrates or phospho-tyrosine antibodies (data not shown). Pdh1p, but not Cdr1p, however, specifically reacted with antibodies to phospho-PKA substrates (Fig. 8 B). The antibodies to phospho-PKA substrates recognize phosphorylated threonine or serine if they have arginine at the –3 position (manufacturer’s information). This observation implies, but does not show unequivocally, the involvement of PKA in the phosphorylation. We therefore determined the effect of the specific PKA inhibitor H-89, and the myristoylated protein kinase A inhibitor 14-22 amide, on the phosphorylation of Pdh1p in PDH1-AD. H-89 (300 µM) and 14-22 amide (30 µM) inhibited the phosphorylation of Pdh1p in PDH1-AD cultured in YEPD broth (Fig. 8 B, upper panel) without affecting Pdh1p expression (Fig. 8 B, lower panel). These results support our hypothesis that PKA is responsible for phosphorylation of Pdh1p. H-8 is an analogue of H-89 which has comparable Ki values for other kinases but its Ki for PKA is 30-fold higher than that of H-89 (41). H-8 did not inhibit the phosphorylation of Pdh1p when it was used at a concentration of 300 µM, and did not affect expression of Pdh1p (Fig. 8 B).

We determined whether the phosphorylation of Pdh1p was constitutive or affected by cell culture conditions. The phosphorylation of Pdh1p was decreased a few hours after sub-culturing from YEPD broth to CSM medium without glucose (CSM-Gluc medium) (Fig. 8 C). Pdh1p dephosphorylated during the glucose starvation was re-phosphorylated within 10 min after the addition of 2 % glucose (Fig. 8 C). This indicated that glucose is required for the phosphorylation of PKA substrates in Pdh1p.

The effect of the PKA inhibitors on the fluconazole-resistance of PDH1-AD was used to test whether phosphorylation affected the drug-efflux activity of Pdh1p (Fig. 9). CDR1-AD and PDH1-AD had much lower susceptibilities to H-89 than pSK-AD in YEPD agar disk assays, but both strains showed some growth inhibition when H-89 was used at a high concentration (480 nmole/disk; Fig. 9 A upper disks). CDR1-AD was slightly more sensitive to H-89 than PDH1-AD. Application of both fluconazole and H-89 to the disks increased the growth inhibition of pSK-AD
and PDH1-AD but not of CDR1-AD. One explanation of these results is that H-89 inhibited the phosphorylation of Pdh1p - which is necessary for fluconazole resistance, but did not inhibit the phosphorylation of Cdr1p. The protein kinase A inhibitor 14-22 amide did not inhibit growth in the disk assay, even for pSK-AD (data not shown). 14-22 amide did, however, inhibit growth in a liquid MIC assay at 20 µM but not 4 µM (Fig. 9 B). A synergistic effect of 14-22 amide with fluconazole was observed for pSK-AD and PDH1-AD when 14-22 amide was used at concentrations 4 µM or 20 µM. The high degree of fluconazole resistance demonstrated by CDR1-AD was not affected by 14-22 amide even when it was used at 20 µM. These results suggested that the fluconazole-efflux activity of Pdh1p requires PKA phosphorylation whereas the efflux activity of Cdr1p does not. The differential phosphorylation of these homologous pump proteins indicates that their activity is probably regulated by different mechanisms.

NTPase activities of plasma membrane fractions from glucose-starved CDR1-AD and PDH1-AD.

We next asked whether the phosphorylation-dependent change in the efflux activity of Pdh1p was associated with changes in NTPase activities. The oligomycin-sensitive ATPase and CTPase activities of PDH1-AD membrane fractions containing either the phosphorylated or the dephosphorylated form of Pdh1p were measured. Attempts to obtain membrane fractions containing dephosphorylated Pdh1p from yeast treated with PKA inhibitors were confounded by difficulties in preparing from the yeast the quantity of membranes needed for NTPase assay. As an alternative approach, membrane fractions were prepared from glucose-starved pSK-AD, CDR1-AD, and PDH1-AD cells that were then either incubated with or without 2 % glucose for 10 min (Fig. 10 A). In addition to Pdh1p phosphorylation, Pma1p phosphorylation was also induced by exposure to glucose (Fig. 10 A). Although phosphorylation of Pma1p may have increased the ATPase activity (30, 42) there was negligible Pma1p ATPase activity contributing to the oligomycin-sensitive ATPase activity of pSK-AD membrane fractions, even at the lower pH optimum of ~ 6 for this enzyme (Fig. 10 B). Furthermore the CTPase activity in membrane fractions from pSK-AD,
regardless of glucose addition, were essentially undetectable, as expected for a strain deficient in membrane drug pumps (Fig. 10 B). Contrary to our expectation, incubation of CDR1-AD, but not PDH1-AD, with glucose had a profound effect on the ATPase and CTPase activities of membranes from these strains, although the NTPase activities of the membranes from cells grown in CSM medium were lower than those from cells grown in YEPD medium (compare Fig. 7 and Fig. 10 B). The ATPase and CTPase activities of membranes from CDR1-AD cells incubated with glucose were at least 1.3-fold and about 2-fold higher, respectively, than the activities from glucose-starved cells. In contrast, the ATPase and CTPase activities of membranes were the same for PDH1-AD cells incubated with or without glucose (Fig. 10 B). We considered the possibility that Pdh1p in the glucose-starved cells had been phosphorylated, and activated, by some PKA contamination in the NTPase reaction mixture. However, the inclusion of 14-22 amide (10 µM) in the assay did not affect the NTPase activities (data not shown). Because the NTPase activities of membranes from CDR1-AD cells were increased by incubation with glucose, we were led to believe that Cdr1p, like Pdh1p, was phosphorylated in response to glucose exposure (Fig 10 C). These results indicate that while Cdr1p is phosphorylated in a non-PKA mediated fashion and that its phosphorylation correlates with increased NTPase activity, PKA-mediated phosphorylation does not affect the NTPase activity of Pdh1p.
ABC drug efflux pumps in *C. glabrata*.

Two *C. glabrata* proteins with homology to ABC transporters have been reported to be associated with azole drug resistance (12, 13, 20). One of these proteins was denoted Cdr1p due to its similarity to *C. albicans* CaCdr1p (13). The other protein was first denoted Pdh1p (12), and subsequently Cdr2p (20). Cdr1p and Pdh1p show amino acid sequence homology to *S. cerevisiae* Pdr5p (74 % and 72 % respectively), as do *C. albicans* CaCdr1p and CaCdr2p. However it may be misleading to refer to Pdh1p as Cdr2p because Pdh1p shares slightly greater amino acid similarity with *C. albicans* Cdr1p (55 %) than with *C. albicans* Cdr2p (53 %). As demonstrated in this paper, *C. glabrata* Cdr1p and Pdh1p show distinct pumping and phosphorylation properties. For these reasons, we have used the original nomenclature and referred to the two drug efflux proteins as Cdr1p and Pdh1p.

Drug resistance of *S. cerevisiae* AD1-8u− cells expressing Cdr1p or Pdh1p.

*S. cerevisiae* AD1-8u− derivatives CDR1-AD and PDH1-AD, hyper-expressing *C. glabrata* ABC transporters Cdr1p and Pdh1p respectively, were highly resistant to structurally and functionally unrelated compounds (Fig. 3-5). This is the first report that Pdh1p causes MDR in yeast although its ability to confer azole resistance on *C. glabrata* was inferred previously (12, 20). CDR1-AD and PDH1-AD showed similar specificities for xenobiotic substrates, with CDR1-AD, in general, showing greater resistance than PDH1-AD. Although Miyazaki *et al.* reported that *PDH1* was over-expressed up to four-fold in clinical *C. glabrata* isolates (12), Sanglard *et al.* suggested that *CDR1* contributed more than *PDH1* to the azole-resistance of this species. This paper confirms that Cdr1p may be the main contributor to the MDR of *C. glabrata* because it is a more effective efflux pump than Pdh1p.
expression system was used to express Cdr1p or Pdh1p at high concentration in yeast plasma membranes. Such high level functional expression is a powerful tool for amplifying pumping activities and enabled the specificities of individual pumps to be measured against a minimized background of residual pump activities. We have used the hyper-expression system previously to investigate *C. albicans* Cdr1p (26). The *S. cerevisiae* AD1-8u' transformant AD1002, which hyper-expressed CaCdr1p, had azole MIC$_{80}$ values that were intermediate between those of CDR1-AD and PDH1-AD. The specificities of the three pumps to xenobiotic substrates were also similar, with these strains showing high susceptibility to amphotericin B and low susceptibility to azole drugs. Equivalent results have been demonstrated for a *pdr1-3* mutant *S. cerevisiae* strain hyper-expressing *PDR5* (33), which, like CDR1-AD and PDH1-AD, had high resistance to rhodamine 123, rhodamine 6G, monensin, and nigericin and low resistance to CCCP. These data support the proposition that Cdr1p and Pdh1p are not merely similar to Pdr5p at a sequence level, but are functional orthologs in *C. glabrata*. We have also found differences among the pumps in the magnitudes of their efflux activities and their specificities to xenobiotic substrates. The two *C. glabrata* pumps showed a difference from the reported characteristics of Pdr5p (34) in their ability to confer on cells resistance to tripentyltin chloride. The hydrophobicity and molecular mass of tri-n-alkyltin chlorides seems an important factor in determining whether an analog of these compounds is a good substrate for the *C. glabrata* pumps.

**Reversal of azole-resistance in CDR1-AD and PDH1-AD by FK506 and other drugs.**

We examined the effect of the immunosuppressant FK506 and cyclosporin A, and the anti-arrhythmic drug verapamil on the reversal of fluconazole-resistance in CDR1-AD and PDH1-AD, and therefore as potential chemosensitisers for fluconazole-resistant *C. glabrata*. Several compounds have been reported to reverse the MDR of human tumors and of *S. cerevisiae* that is caused by drug-efflux transporters. Although FK506, cyclosporin A, and verapamil have been shown to bind to human P-glycoprotein and directly inhibit the drug-efflux activity (35, 36, 39, 43),
the molecular mechanisms of MDR reversal in human cancer cells are still unclear. FK506 efficiently reversed the fluconazole-resistance of CDR1-AD and PDH1-AD (Fig. 6). The reversal was less for CDR1-AD than for PDH1-AD. This may indicate that Cdr1p effluxes FK506 more efficiently than Pdh1p and thereby retains cellular resistance to fluconazole. FK506 has also been shown to reverse MDR in Pdr5p-expressing S. cerevisiae (37, 44, 45). There is evidence that residues S1360 and T1364 in Pdr5p are responsible for the effect of FK506 (44, 45). These residues are conserved in Cdr1p at S1348 and T1352 and in Pdh1p at S1376 and T1380, suggesting that the same mechanisms operate in the chemosensitisation of the C. glabrata pumps. In contrast, cyclosporin A did not show any effect on CDR1-AD and PDH1-AD whereas the agent showed some reversal of fluconazole-resistance in AD1002 (CaCdr1p, unpublished data). Since Candida infections often occur in immunocompromised individuals, the use of the immunosuppressant FK506 as a chemosensitiser would be inadvisable. Further investigation of the mechanism of action of FK506, however, may lead to promising clinical drugs for C. glabrata infections that demonstrate MDR. It appeared that oligomycin inhibited the NTPase activities of Cdr1p and Pdh1p, as is the case for Pdr5p (38). The inhibitory activity of oligomycin allows the selective measurement of ABC transporter ATPase activity in plasma membrane fractions containing Pma1p and other proteins (26). H-89 and 14-22 amide acted synergistically with fluconazole to inhibit growth only in PDH1-AD (Fig. 9). One explanation for this result, as discussed below, is that the PKA inhibitors prevented phosphorylation of Pdh1p, but the possibility that these agents directly inhibited the transporter cannot be excluded.

The NTPase activities of the membrane fractions containing Cdr1p and Pdh1p.

Like other ABC transporters involved in drug efflux, such as human P-glycoprotein (46), S. cerevisiae Pdr5p (38), C. albicans Cdr1p (26), both Cdr1p and Pdh1p from C. glabrata showed broad specificities for NTP hydrolysis (Fig. 7). The structural similarities of these ABC transporters include having 12 transmembrane domains, two Walker A and B ATP-binding motifs, and two ABC
signature sequences. These features are highly conserved among species. The Walker motif and ABC signature sequences are identical for Cdr1p, Pdh1p, and *S. cerevisiae* Pdr5p while the sequences show 92.5% identity to those from *C. albicans* Cdr1p and Cdr2p. This similarity in the structure of ATP binding site may be responsible for the similar NTPase activities and broad NTP specificities of the *C. glabrata* orthologues of Pdr5p. The activities were optimal at pH 7.0-8.0, although the UTPase activity was lower than the other NTPase activities for Pdh1p despite similar expression levels in the membrane fractions and possession of the same Walker motifs and ABC signature sequences. Therefore regions of Pdh1p other than the ATP-binding motif may affect UTP hydrolysis. Alternatively, differences in UTP hydrolysis may be caused by altered post-translational modification patterns between Cdr1p and Pdh1p. The NTPase activities of the phosphorylated and the dephosphorylated form of Cdr1p-containing membranes were clearly different despite similar expression of the pump in membranes fractions (Fig. 10). Pdh1p, in contrast, showed no such difference in NTPase activity. Although further studies are required to determine whether the difference in the Cdr1p NTPase activities is directly caused by phosphorylation, it is evident that some post-translational modification regulates NTPase activity, at least in Cdr1p.

**The phosphorylation of *C. glabrata* drug efflux transporters**

In this study, phosphorylation of both Cdr1p and Pdh1p was observed *in vivo* (Fig. 8). Although the phosphorylation of the pumps may occur at multiple sites, be catalysed by several kinases, and the complete complement of enzymes responsible for the phosphorylation was not identified, the following results showed that at least some of the phosphorylation of Pdh1p was due to PKA. (I) An antibody that detects phospho-serine and phospho-threonine in the PKA motif, but not in the casein kinase I motif (47), reacted with Pdh1p. (II) The phosphorylation of Pdh1p was inhibited by H-89 and 14-22 amide. These inhibitors are frequently used to study phosphorylation in mammalian cells. 14-22 amide is a peptide from the protein kinase A inhibitor (PKI) protein of
rabbit skeletal muscle. This peptide has a $K_i$ of 1.4 µM for yeast PKA, which is higher than that for mammalian PKA (36 nM) but lower than that for mammalian cGMP-dependent kinase (8.0 µM; 48, 49). A myristoylated derivative of 14-22 amide has been shown to inhibit C. albicans PKA (50). The inhibitor H-89, which is structurally different from 14-22 amide, has also been shown to inhibit C. albicans PKA with $IC_{50} = 1.0$ µM, indicating it is active against fungal PKA (50). Although the specificities of H-89 and 14-22 amide for yeast PKA may be lower than for mammalian PKA, their inhibition of Pdh1p phosphorylation was probably due to effects on PKA rather than on other kinases. (III) The addition of glucose to glucose-starved PDH1-AD rapidly induced the phosphorylation of Pdh1p. Glucose is a major activator of adenylate cyclase and the Ras pathway in yeast, and the addition of glucose to starved cells leads to a rapid increase in intracellular cAMP and activation of PKA (51, 52). Although glucose addition may induce various metabolic pathways, including the synthesis of ATP itself, PKA activation is a prominent response. Our results suggest PKA as the most plausible candidate mediator of Pdh1p phosphorylation but further genetic studies will be needed to determine the overall involvement of PKA in the glucose-dependent phosphorylation of Pdh1p.

The fluconazole-resistance of PDH1-AD was reversed by the PKA inhibitors. Although other possibilities are not completely excluded, the evidence collectively indicates that the Pdh1p inactivation induced by PKA inhibition was not mediated via the inhibition of the NTPase activity. The simplest explanation for our observations is that PKA-dependent phosphorylation of Pdh1p is required for efficient coupling between ATP hydrolysis and drug efflux. There are 14 sites (Arg-X-X-Thr; Arg-X-X-Ser) in Pdh1p which, when phosphorylated, could be the epitopes recognized by the phospho-PKA substrates antibody. The coupling hypothesis could be tested by constitutive activation/inactivation of such sites using site-directed mutagenesis.

In contrast, phosphorylation of Cdr1p could not be detected using the phospho-PKA substrates antibody and the fluconazole-resistance of CDR1-AD was not significantly reversed by PKA inhibitors. The phosphorylation of Cdr1p in glucose-starved cells was increased by the addition of
glucose as in the case of Pdh1p, but this occurred by PKA-independent mechanism. The membrane fraction containing phosphorylated Cdr1p showed significantly higher NTPase activity than that containing dephosphorylated Cdr1p. As discussed above, the NTPase activity of Cdr1p may therefore be regulated by the phosphorylation.

*C. glabrata* is classified in the *Candida* genus, but its phylogenetic position and properties are closer to *S. cerevisiae* than to other *Candida* species (18, 25). Thus, *C. glabrata* proteins are probably expressed in *S. cerevisiae* with the correct post-translational modifications and protein folding, and the phosphorylation of the pumps observed in AD strains probably occurs in *C. glabrata* cells.

The regulation of ABC transporter drug-efflux activity by transcriptional control of expression has been extensively studied, but little is known about regulation via post-translational modification of pump proteins. Human P-glycoprotein is phosphorylated in the linker region by PKA and PKC *in vitro* (53-55), but this is not essential for the drug-efflux activity of the protein (56, 57). In fungi, there are few reports on the phosphorylation of drug-efflux ABC transporters, but phosphorylation of fungal transporters seems to be more important for their activity than is the case for human P-glycoprotein. The phosphorylation of a PKA site was found to be essential for cadmium efflux by *S. cerevisiae* Ycf1p although this protein shows homology to the human MRP1 and CFTR transporters rather than MDR1 or *S. cerevisiae* Pdr5p family of proteins (58). Some phosphorylation, regulated by type 2A phosphatase Sit4p, was suggested to be important for the activity of *Kluyveromyces lactis* Pdr5p (59) and phosphorylation of *S. cerevisiae* Pdr5p, Snq2p, and Yor1p has also been reported (60). Phosphorylation by casein kinase I was required for the stability of Pdr5p in the plasma membrane. One of its casein kinase I-dependent phosphorylation sites, S420, has been identified. Residue S420 is conserved in Pdh1p (S419) but changed to an alanine in Cdr1p (A409). The sequence around S419 is incompatible with PKA-dependent phosphorylation, but it could be phosphorylated by casein kinase I. Since Cdr1p and Pdh1p are close homologues of Pdr5p, both of the *C. glabrata* pump proteins are likely to be phosphorylated by casein kinase I, possibly
differentially and possibly at multiple sites.

The efflux of rhodamine 6G initiated by the addition of glucose to glucose-starved rhodamine-loaded cells was delayed and slower from PDH1-AD than from CDR1-AD (Fig. 5). The differential kinetics of rhodamine 6G efflux could be caused, in part, by the differences in the post-translational modification of Pdh1p and Cdr1p. Pdh1p also appears to be more phosphorylated than Cdr1p in AD strains (Fig. 8 A, Fig. 10 C). Thus Pdh1p may require longer to be phosphorylated to its active, rhodamine 6G-effluxing, form. This interpretation is consistent with protein phosphorylation having a role in the coupling between ATP hydrolysis and drug efflux in Pdh1p. The differential phosphorylation of these transporters may also contribute to differences in the drug efflux specificities and activities of Cdr1p and Pdh1p.

**Conclusion**

We have functionally hyper-expressed the *C. glabrata* Cdr1p and Pdh1p ABC-transporters in an *S. cerevisiae* strain depleted in drug efflux pumps, and examined their properties against a negligible background of endogenous pump proteins. Although both of the pump-expressing strains showed similar specificities to xenobiotic substrates, CDR1-AD showed higher resistance than PDH1-AD in most cases. CDR1-AD also showed higher energy-dependent rhodamine 6G efflux but had NTPase activities comparable with PDH1-AD, suggesting Cdr1p is a more effective pump for these substrates than Pdh1p. Both of the pumps were phosphorylated *in vivo* in a glucose-dependent manner, and Pdh1p was phosphorylated by a mechanism(s) that includes PKA. If the phosphorylation inhibitors used in this study acted solely through inhibition of PKA-mediated phosphorylation as predicted, and did not directly affect the pumping activity of Pdh1p, phosphorylation appeared to affect coupling between ATP hydrolysis and the drug efflux activity of the pump. Cdr1p was phosphorylated via non-PKA kinase(s). The membrane fraction containing phosphorylated Cdr1p showed significantly higher NTPase activity than the dephosphorylated pump, which suggests the activity of Cdr1p is also regulated by phosphorylation.
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Legends to Figures

Fig. 1. Constitution of plasmids and yeast strains expressing C. glabrata drug efflux pumps.

Fig. 2. Expression of C. glabrata CDR1 and PDH1 in S. cerevisiae strain AD1-8u-. (A) Northern blot analysis. Total RNA (8 µg) from parental AD1-8u– cells, AD1-8u– cells transformed with: the transformation cassette from pSK-PDR5PPUS (pSK-AD); the transformation cassette from pSK-CGCDR1 (CDR1-AD); or the transformation cassette from pSK-PDH1 followed by transformation with the full length PDH1 ORF (PDH1-AD) were hybridized with DIG-labeled CDR1, PDH1, or S. cerevisiae PMA1 (control) probes. (B) SDS-PAGE profiles. Protein (30 µg) in the crude membrane fractions of yeast was separated in an 8 % polyacrylamide gel and stained with Coomassie Brilliant Blue R-250.

Fig. 3. Resistance of CDR1-AD and PDH1-AD to various antifungal drugs and chemicals. S. cerevisiae strains: AD1-8u–; pSK-AD; CDR1-AD; and PDH1-AD were each seeded in two YEPD agar plates (plate A-L and plate M-Z) at a concentration of 6 x 10^4 cells/ml. Filter disks containing drugs or chemical agents were applied to the plates (positioned as indicated by A-Z) which were then incubated at 25˚C for 48 h. The compound applied to each disk is indicated below the plates. A representative result of several independent experiments is shown.

Fig. 4. Susceptibilities of CDR1-AD and PDH1-AD to tri-n-alkyltin chloride compounds. CDR1-AD, PDH1-AD, and pSK-AD were incubated in CSM medium containing either tri-n-methyltin, tri-n-ethyltin, tri-n-propyltin, tri-n-butyltin, or tri-n-pentyltin chlorides at 30˚C for 48 h. The MIC_{80} values were determined from the growth inhibition curves obtained by a microdilution method. The number of carbon atoms contained in the alkyl chains of each compound is indicated on the horizontal axis. The mean ± S.E. of three determinations from a representative example of
three independent experiments is shown in (A). The ratios of MIC\textsubscript{80} values for the pump expressing-strains to the control strain are shown in (B).

**Fig. 5. Energy-dependent pump-mediated efflux of rhodamine 6G from CDR1-AD and PDH1-AD.** Yeast cells were pre-loaded with rhodamine 6G under glucose-starvation conditions. Release of Rhodamine 6G from the cells was monitored by measuring the fluorescence intensities (arbitrary units) of supernatants (SFI) or of total suspensions (TFI) at specific time intervals from the addition of 2 mM glucose or distilled water (- glucose). The results are the representative data of more than three independent experiments and were determined as follows: \(\%\) rhodamine 6G exported =\(\frac{(\text{SFI at X min}) - (\text{SFI at 0 min})}{(\text{TFI at 20 min}) - (\text{SFI at 0 min})}\times 100\) (means ± S.E., n=3). The TFIs at 20 min for AD1-8u\textsuperscript{+}, pSK-AD, CDR1-AD, and PDH1-AD were 34983 ± 151, 26970 ± 352, 8026 ± 0, and 12862 ± 150 (means ± S.E., n=3), respectively.

**Fig. 6. Reversal of the fluconazole resistance of pump-expressing yeast strains.** (A) The filter disk susceptibility assay was performed on CDR1-AD and PDH1-AD as described for Fig. 3. Stock solutions of 10 mM FK506, 10mM oligomycin, 0.2 M verapamil, or 0.2 M cyclosporin A were applied to the respective disks in volumes as indicated at the top of the panels. Fluconazole (8 µg) was simultaneously applied to the disks in the row indicated with FLC. (B, C) The effects of FK506 and oligomycin on the fluconazole-resistance of CDR1-AD and PDH1-AD were verified in YEPD liquid MIC assays. The growth of the yeast cells cultured in YEPD broth containing fluconazole and fixed concentrations of FK506 (B) or oligomycin (C) at 27°C for 48 h with constant shaking was measured (OD\textsubscript{590nm}). The results shown are means ± S.E. (n=3).

**Fig. 7. Oligomycin-sensitive *C. glabrata* Cdr1p and Pdh1p NTPase activities of plasma membrane fractions.** Membrane fractions from pSK-AD, CDR1-AD, and PDH1-AD cells cultured in YEPD broth were prepared and their SDS-PAGE protein profiles are shown on the left
side of the figure (30 µg protein/lane). The NTPase activities of the membrane fractions were measured at various pH values. The oligomycin-sensitive activities were determined as the difference in NTPase activity in the presence and absence of 20 µM oligomycin. The means ± S.E. of three determinations for each membrane fraction are presented. Similar results were obtained using two other sets of membrane fractions.

Fig. 8. Phosphorylation of *C. glabrata* Cdr1p and Pdh1p. (A) Phosphorylation of drug efflux pumps. [32P] Ortho-phosphate (100 µCi) was added to YEPD broth cultures (5 ml) of yeast cells in early stationary phase and cultures were incubated at 30°C for 2h with constant shaking. Crude membrane fractions were prepared from cells as described in Materials and Methods and 30 µg protein samples were separated by SDS-PAGE with an 8 % polyacrylamide gel. Phosphorylation of proteins in the fractions was detected by autoradiography. (B) Phosphorylation of Pdh1p at PKA site(s). Yeast cells in logarithmic growth phase (OD600nm = 0.2) were incubated in YEPD broth at 27°C for 12 h with shaking, with the treatments indicated above the top panel (14-22: protein kinase A inhibitor 14-22 amide). Crude membrane fractions were extracted and proteins separated by SDS-PAGE. Phosphorylation of PKA substrates (5 µg/lane) was detected by anti-Phospho-(Ser/Thr) PKA substrate antibody (upper panel), and the expression of each pump (30 µg protein/lane) was detected by Coomassie Brilliant Blue R-250 (CBB, lower panel). Cdr1p and Pdh1p drug pumps are indicated with arrowheads at the right hand side. (C) Glucose-dependent phosphorylation of Pdh1p. PDH1-AD cells after 12 h growth in CSM were washed with distilled water three times and transferred to CSM-Gluc followed by incubation for 3 h at 27°C with shaking. For a portion of the culture, glucose (2 %) was then added and the cells harvested after 10 min incubation at 27°C. Crude membrane fractions were analyzed as above. Arrowheads at the right hand side indicate Pdh1p.

Fig. 9. Reversal of fluconazole-resistance in PDH1-AD by inhibitors of protein kinase A. (A)
The filter disk susceptibility assay was performed as described for Fig. 3 with yeast strains pSK-AD, CDR1-AD, and PDH1-AD. H-89 (30 mM) was applied to the respective disks in volumes as indicated at the top of the panels. Fluconazole (8 µg) was simultaneously applied to the lower disks indicated with FLC at the right hand side. (B) Susceptibilities of pSK-AD, CDR1-AD, and PDH1-AD cells grown in a YEPD medium to fluconazole in the presence of fixed concentrations of protein kinase A inhibitor 14-22 amide (14-22) are shown as described for Fig. 6 B. The results shown are means ± S.E. (n=3).

Fig. 10. The effect of glucose-starvation on NTPase activity in membrane fractions of CDR1-AD and PDH1-AD. (A) SDS-PAGE protein profile and phosphorylation of PKA site(s) in proteins from membrane fractions used for ATPase and CTPase assays. pSK-AD, CDR1-AD, and PDH1-AD cells were incubated in CSM-Gluc for 3 h, and then a portion of each culture had glucose added (2 %) for 10 min (indicated with (+)). Membrane fractions were obtained from both the glucose (+) and (-) cells of the three strains and separated by SDS-PAGE (10 µg proteins/lane for CBB staining and 2 µg/lane for phospho-PKA site western blotting). Arrowheads at right hand side indicate Cdr1p or Pdh1p. (B) Oligomycin-sensitive ATPase and CTPase activities of the membrane fractions were measured as described in Fig. 7 at three pH values. The results shown are means ± S.E. (n=3) obtained with the membrane preparations shown in (A) and are representative data from experiments with three separate membrane preparations. (C) Phosphorylation of Cdr1p and Pdh1p stimulated by glucose addition. Yeast cells were incubated in CSM-Gluc containing [\(^{32}\)P] orthophosphate (20 µCi) for 3 h. Glucose (2 %) was then added to a portion of each culture for 10 min before cells were harvested. Crude membrane fractions were prepared and proteins present separated by SDS-PAGE. The phosphorylation of proteins in the membrane fractions was detected by autoradiography.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Template DNA</th>
<th>Primer: sequence (oligonucleotide name)</th>
</tr>
</thead>
</table>
| *CaCDR2* terminator (4501-4871) | Genomic DNA from *C. albicans* 10261 | Sense: 5′-GACTAGTCGGATGGGTCTTATATTCAAT (AC2-1)  
                 |                                       | Antisense: 5′-GACTAGTCTTTAACTGGGACCTG (AC2-2) |
| *CDR1* (1-4500) | Genomic DNA from *C. glabrata* CBS138 | Sense: 5′-CCCAAGCTTGATGTCTCTTGCAAGTGACAA (GC-1)  
                 |                                       | Antisense: 5′-CGGAATTCCGTTATTTCTTGCAAGTTTAC (GC-2) |
| *PDH1*-U (1-530) | Genomic DNA from *C. glabrata* CBS138 | Sense: 5′-CCCAAGCTTGATGTCTCTTGCAAGTGACAA (GC-1)  
                 |                                       | Antisense: 5′-CGGAATTCCGTTATTTCTTGCAAGTTTAC (GC-2) |
| *PDH1*-L (4532-5486) | Genomic DNA from *C. glabrata* CBS138 | Sense: 5′-GACTAGTCGGCTTTAGGATGCGGAATGTATC (GP-2)  
                 |                                       | Antisense: 5′-GACTAGTCGGCTTTAGGATGCGGAATGTATC (GP-2) |

*a* Nucleotide positions shown are from the open reading frames numbered in the 5′ to 3′ direction with the first base of the translation start codon being +1.

*b* Restriction sites for the cloning are underlined.
Fig. 1

Homologous recombination

Host strain (S. cerevisiae AD1-8u−)

S. cerevisiae CDR1-AD

PDH1 PCR fragment (1-5342)

Host strain (S. cerevisiae PDH1-UL-AD)

S. cerevisiae PDH1-AD
A. fluconazole, 10 µg (32.7 nmol)
B. miconazole, 0.2 µg (0.417 nmol)
C. itraconazole, 0.15 µg (0.213 nmol)
D. ketoconazole, 0.02 µg (37.6 pmol)
E. nystatin, 60 µg (64.8 nmol)
F. amphotericin B, 90 µg (97.4 nmol)
G. flucytosine, 20 µg (155 nmol)
H. terbinafine HCl, 20 µg (61.0 nmol)
I. cerulenin, 1 µg (4.49 nmol)
J. cycloheximide, 0.2 µg (0.711 nmol)
K. nigericin, 12.5 µg (16.7 nmol)
L. monensin, 12.5 µg (18.0 nmol)
M. rhodamine 123, 100 µg (263 nmol)
N. rhodamine 6G, 60 µg (125 nmol)
O. staurosporin, 3 µg (6.43 nmol)
P. adriamycin, 30 µg (53.2 nmol)
Q. cytochalasin D, 25.4 µg (50 nmol)
R. latrunculin A, 0.5 µg (1.19 nmol)
S. bafilomycin A1, 1.87 µg (3 nmol)
T. aureobasidin A, 45 µg (40.9 nmol)
U. theonellamide F, 30 µg (18.16 nmol)
V. calyculin A, 10.1 µg (10 nmol)
W. 4-nitroquinoline N-oxide, 1 µg (5.26 nmol)
X. G418, 250 µg (361 nmol)
Y. trifluoperazine, 1 mg (2.08 µmol)
Z. carbonyl cyanide m-chlorophenylhydrazone, (CCCP), 300 µg (1.47 µmol)
TABLE 2. Antifungal susceptibilities of *S. cerevisiae* cells expressing Cdr1p or Pdh1p

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fluconazole</th>
<th>Itraconazole</th>
<th>Ketoconazole</th>
<th>Miconazole</th>
<th>Flucytosine</th>
<th>Amphotericin B</th>
<th>Nystatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSK-AD</td>
<td>0.25</td>
<td>0.007813</td>
<td>0.015625</td>
<td>0.007813</td>
<td>0.25</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>CDR1-AD</td>
<td>128</td>
<td>&gt; 32*</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>PDH1-AD</td>
<td>8</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

The results are typical of three determinations in three independent experiments.

* Itraconazole is not soluble at concentrations >32 µg/ml.
Fig. 5

Rhodamine 6G exported (%)

Time (min)

0 5 10 15 20

AD1-8u
pSK-AD
AD1-8u (-) glucose
pSK-AD (-) glucose
CDR1-AD
PDH1-AD
CDR1-AD (-) glucose
PDH1-AD (-) glucose
Fig. 7

ATPase

GTPase

UTPase

CTPase
Fig. 9

A

30 mM H-89

pSK-AD  CDR1-AD  PDH1-AD

B

14-22

Absorbance at 590 nm

FLC (µM)

Absorbance at 590 nm

FLC (µM)

Absorbance at 590 nm

FLC (µM)
Candida glabrata ABC transporters Cdr1p and Pdh1p expressed in a Saccharomyces cerevisiae strain deficient in membrane transporters show phosphorylation-dependent pumping properties

Shun-ichi Wada, Masakazu Niimi, Kyoko Niimi, Ann R. Holmes, Brian C. Monk, Richard D. Cannon and Yoshimasa Uehara

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