Phosphorylation of *Candida glabrata* ATP-binding Cassette Transporter Cdr1p Regulates Drug Efflux Activity and ATPase Stability*§

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Fungal ATP-binding cassette transporter regulation was investigated using *Candida glabrata* Cdr1p and Pdh1p expressed in *Saccharomyces cerevisiae*. Resephosphorylation of Pdh1p and Cdr1p was protein kinase A inhibitor-sensitive but responded differentially to Tpk isoforms, stressors, and glucose concentration. Cdr1p Ser307, which borders the nucleotide binding domain 1 ABC signature motif, and Ser484, near the membrane, were dephosphorylated on glucose depletion and independently rephosphorylated during glucose exposure or under stress. The S484A enzyme retained half the wild type ATPase activity without affecting azole resistance, but the S307A enzyme was unstable to plasma membrane isolation. Studies of pump function suggested conformational interaction between Ser484 and Ser307. An S307A/S484A double mutant, which failed to efflux the Cdr1p substrate rhodamine 6G, had a fluconazole susceptibility 4-fold greater than the Cdr1p expressing strain, twice that of the S307A mutant, but 64-fold less than the control null strain. Stable intragenic suppressors indicative of homodimer nucleotide binding domain 1-nucleotide binding domain 1 interactions partially restored rhodamine 6G pumping and increased fluconazole and rhodamine 6G resistance in the S307A/S484A mutant. Nucleotide binding domain 1 of Cdr1p is a sensor of important physiological stimuli.

Infections caused by *Candida* sp. are most frequently seen in immunocompromised individuals, including AIDS and leukemia patients. *Candida albicans* remains the leading cause of candidiasis, but the incidence of drug-resistant non-*albicans* *Candida* infections has become an increasingly significant clinical problem. *Candida glabrata* is among the most common of these pathogens, (1), with many clinical isolates showing a 16- to 64-fold higher minimum inhibitory concentration (MIC) of fluconazole (FLC) than *C. albicans* (2). Azole drugs such as FLC and itraconazole, which target lanosterol 14α-demethylase and block the synthesis of ergosterol, are well tolerated and widely used in the treatment of fungal disease. They are, however, fungistatic substrates of pleiotropic drug resistance (PDR) family ATP-binding cassette (ABC) transporters, and resistant fungi that overexpress these pumps are frequently isolated in the clinic (3). The *C. glabrata* PDR family ABC transporters Cdr1p and Pdh1p, which efflux azole agents and structurally unrelated compounds, are among the primary causes of the intrinsic resistance of *C. glabrata* toazole drugs (4–8). The two pumps have >70% amino acid sequence identity and transport a similar spectrum of substrates, but Cdr1p had greater drug efflux activity for most substrates. Structural information on fungal single subunit ABC transporters is rudimentary, and the molecular and regulatory features that determine their enzyme activity and substrate specificity are poorly understood. Such information is required for the rational design of pump inhibitors and antifungal drugs that are not pump substrates.

There are few reports of the effects of post-translational modification on the activity of fungal ABC transporters. Serine 420, a casein kinase-dependent phosphorylation site adjacent to the Cdr1p ABC signature motif, is the only experimentally proven fungal ABC transporter phosphorylation site. We have shown that the ATPase activity of Cdr1p and the drug efflux activity of Pdh1p are regulated by phosphorylation (7). Cdr1p cannot be phosphorylated at the position equivalent to Ser420, whereas Pdh1p phosphorylation was regulated by protein kinase A (PKA) at one or more sites not homologous to Pdr5p Ser420. More than one type of phosphorylation therefore occurs in fungal PDR family pumps.

This report describes the differential regulation of *C. glabrata* Cdr1p and Pdh1p expressed in *S. cerevisiae*. Immunological, physiological, and biochemical methods were applied to site-directed mutants in putative phosphorylation sites and to kinase-deletion mutants. PKA catalytic subunit isoforms differentially affected pump phosphorylation, and the effects of the phosphorylation of two putative novel sites in Cdr1p were determined. A phosphorylation site adjacent to the Cdr1p ABC signature motif

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1 The abbreviations used are: MIC, minimum inhibitory concentration; FLC, fluconazole; PDR, pleiotropic drug resistance; ABC, ATP-binding cassette; PKA, protein kinase A; NBD, nucleotide binding domain; CSM, complete synthetic medium; Rh6G, rhodamine 6G; p-PKAs, phospho-(Ser/Thr) PKA substrate; p-Akts, phospho-Akt substrate; p-Thr, phospho-Threonine; BIM, bisindolylmaleimide I.
Phosphorylation of C. glabrata Cdr1p

in nucleotide binding domain 1 (NBD1) affected the extent of multidrug efflux and the in vitro stability of ATPase activity while mutation of another cytoplasmic site nearer the membrane diminished transport at low glucose concentrations. Mutation in both sites eliminated the pumping activity of Cdr1p, whereas intragenic suppressors obtained by exposing the double mutant to FLC partially restored pump function. The Cdr1p NBD1 is a functional sensor of cell physiology and stress that may regulate multidrug efflux and the transcriptional regulator and thus constitutively heterologously expresses the Pdr1p implicitly related plasma membrane ATPase Pma1p (Fig. 1, white arrowheads) by the three antibodies provided an internal control for both the CDR1-AD and PDR1-AD preparations.

**RESULTS**

**Antibodies Recognizing Cdr1p or Pdh1p Phosphorylations**—We have previously described the S. cerevisiae strains CDR1-AD and PDR1-AD, which contain a mutant Pdr1p transcriptional regulator and thus constitutively heterologously hyperexpress Cdr1p and Pdh1p, respectively, from the PDR5 promoter (7, 10). Cdr1p and Pdh1p were hyperexpressed at comparable levels (~10% of plasma membrane protein) and were readily distinguished as heterologous plasma membrane proteins on Coomassie Blue-stained SDS-polyacrylamide gels due to the deletion of seven similar-sized, endogenous pump proteins. Each strain also showed the expected high level resistance to azole drugs (7). The pumps heterologously expressed in S. cerevisiae were therefore correctly folded and properly folded and correctly targeted to the plasma membrane, and immediately rephosphorylated when 2% glucose was added (7). The glucose concentration dependence of each phosphorylation was therefore determined (Fig. 2A). Early stationary phase YPD cultures were glucose-starved for 3.5 h and then treated for 10 min with 10 μM, 1 mM, or 100 mM glucose.

**Glucose-sensitive Phosphorylation of Pdh1p and Cdr1p—**We previously found that the Cdr1p and Pdh1p pumps were extensively dephosphorylated after a few hours of glucose starvation and immediately rephosphorylated when 2% glucose was added (7). The glucose concentration dependence of each rephosphorylation was therefore determined (Fig. 2A). Early stationary phase YPD cultures were glucose-starved for 3.5 h and then treated for 10 min with 10 μM, 1 mM, or 100 mM glucose.

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**Experimental Procedures**

**Yeast Strains and Growth Media—**Growth and selection media, and the methods used to prepare and mutate S. cerevisiae strains expressing Pdh1p and Cdr1p, are described in the Supplemental Data section.

**Drug Susceptibility Assays—**Agar diffusion assays on YPD (Qbiogene, Irvine, CA) agar plates, microplate MIC assays in HEPES and MES-buffered CSM (Qbiogene, Inc.) were performed, and the drugs and chemical compounds used were obtained as previously described (7).

**Analysis of Pump Protein Phosphorylation—**Glucose-starved yeast were obtained by incubation in CSM minus glucose for 3.5 h, and crude plasma membrane fractions were prepared as previously described (7) using GTED-20 buffer (10 mM Tris-HCl, pH 7.0, 0.5 mM EDTA, and 20% (v/v) glycerol) instead of the previous homogenization buffer. The membrane fractions were analyzed by SDS-PAGE and immunoblotting as previously described (7) using 1/2000 dilutions of anti-phospho (serine/threonine) protein kinase A substrate antibody, anti-phospho- (serine/threonine) Akt substrate antibody, and anti-phospho threonine antibodies together with anti-rabbit IgG conjugated with horseshadish peroxidase secondary antibody (Amersham Biosciences).

**ATPase Assay—**Purified plasma membrane fractions were prepared from cells grown in YPD to early stationary phase (A600nm = 7.0–9.0) and oligomycin-sensitive ATPase activities of samples were measured as previously described (7).

**Fluorometric Assay of Rhodamine 6G Efflux—**Log phase (A600nm = 1.5) cells grown in CSM-URA (Qbiogene, Inc., Irvine, CA) medium were stored overnight on ice. The cells were harvested by centrifugation, washed twice with distilled water, and then incubated in HEPES buffer (50 mM HEPES-NaOH, pH 7.0) containing 5 mM 2-deoxyglucose at 30 °C for 30 min to deplete intracellular energy levels. The cells were preloaded with 15 μM Rh6G for 30 min, washed twice, and resuspended in HEPES buffer at A600nm = 15 (1.5 × 10^6 cells ml^-1). Cell samples (50 μl) were incubated at 30 °C for 5 min and 50 μl of glucose at twice the final concentration added to start the reaction. After 8 min the cells in 80-μl samples were removed by passage through a Multi-well Filter plate (Acro Prep, Pall Corp.) placed on a Multiscreen resist vacuum manifold (BMG Labtechnologies GmbH, Offenburg, Germany) underneath. The Rh6G content of the eluate, combined with two 80-μl washes with ice-cold HEPES buffer, was quantitated using a POLARstar OPTIMA (BMG LaTechtechnologies) fluorometer (excitation and emission wavelengths of 485 and 520 nm, respectively) with Fluostar OPTIMA fluorometer (BMG Labtechnologies) to show relative protein expression levels. The 170-kDa Cdr1p and Pdh1p were indicated with a black arrowhead, and the control 100-kDa plasma membrane H^-ATPase (Pma1p) band is indicated with a white arrowhead. Representative data from several experiments are shown.

**Fluorometric Assay of Rhodamine 6G Efflux—**Log phase (A600nm = 1.5) cells grown in CSM-URA (Qbiogene, Inc., Irvine, CA) medium were stored overnight on ice. The cells were harvested by centrifugation, washed twice with distilled water, and then incubated in HEPES buffer (50 mM HEPES-NaOH, pH 7.0) containing 5 mM 2-deoxyglucose at 30 °C for 30 min to deplete intracellular energy levels. The cells were preloaded with 15 μM Rh6G for 30 min, washed twice, and resuspended in HEPES buffer at A600nm = 15 (1.5 × 10^6 cells ml^-1). Cell samples (50 μl) were incubated at 30 °C for 5 min and 50 μl of glucose at twice the final concentration added to start the reaction. After 8 min the cells in 80-μl samples were removed by passage through a Multi-well Filter plate (Acro Prep, Pall Corp.) placed on a Multiscreen resist vacuum manifold (BMG Labtechnologies GmbH, Offenburg, Germany) underneath. The Rh6G content of the eluate, combined with two 80-μl washes with ice-cold HEPES buffer, was quantitated using a POLARstar OPTIMA (BMG LaTechtechnologies) fluorometer (excitation and emission wavelengths of 485 and 520 nm, respectively) with Fluostar OPTIMA software and a standard curve of Rh6G in the HEPES buffer.
Pdh1p remained partially phosphorylated after glucose starvation, but all three antibodies detected glucose-dependent phosphorylation at a glucose concentration (10 mM) that failed to induce Pma1p phosphorylation. The different phosphorylation patterns detected with each antibody indicated that Pdh1p was also phosphorylated at multiple sites. The >100-fold differential in the glucose-dependence of Cdr1p and Pdh1p phosphorylation suggested that the two pumps were modified by different kinases/phosphatases or had sites with different susceptibilities to phosphorylation.

Pdh1p phosphorylation at p-PKAs sites is inhibited by PKA inhibitors (7). A panel of PKA catalytic subunit (Tpk) single and double deletion mutants was used to identify the TPK gene product that regulated Pdh1p phosphorylation in PDH1-AD. The three genes (TPK1, -2, and -3) encoding these PKA catalytic subunit isoforms can be deleted without lethality, either singly or up to two at a time (11). In YPD culture, identical Pdh1p phosphorylation signals were observed, even in the double deletion mutants PDH1-TPK1Δ2, -TPK1Δ3, and -TPK2Δ3Δ (Fig. 2B). In contrast, phosphorylation at the PKA and Akt sites was not induced by 10 μM glucose in PDH1-TPK1Δ3Δ, and PKA site phosphorylation was dramatically decreased in PDH1-TPK1Δ3Δ (Fig. 2B). Thus Tpk3p was the main contributor to glucose-dependent phosphorylation of Pdh1p PKA and Akts sites at low glucose concentrations, and only Tpk2p could augment this process.

Stress Sensitivity of Cdr1p and Pdh1p Phosphorylation—The glucose-dependent differences in Cdr1p and Pdh1p phosphorylation patterns suggested that the two pumps might be phosphorylated in discrete physiological contexts. This hypothesis was initially tested for Cdr1p phosphorylation in glucose-starved CDR1-AD by adding 1 mM glucose together with a stressor for 10 min (Fig. 3A). The 1 mM glucose supplied sufficient ATP as kinase substrate, because the Pma1p band was strongly phosphorylated even in the absence of Akt site phosphorylation of Cdr1p (Figs. 2A and 3A). The pump substrate FLC (45 μg/ml) did not affect phosphorylation (data not shown), but oxidative stress (2 mM H₂O₂), osmotic stress (500 mM NaCl), and heat shock (42 °C) for 10 min induced p-Akt site phosphorylation, albeit to a lesser extent than 100 mM glucose (Fig. 3A). Stress-dependent phosphorylation was not detected with the p-Thr antibody. Pdh1p in PDH1-AD was highly phosphorylated in 1 mM glucose, and stress treatments caused no additional phosphorylation. In contrast, stress with 2 mM H₂O₂ alone decreased Pdh1p phosphorylation and gave no rephosphorylation of Cdr1p. However, Cdr1p phosphorylation was not affected when the yeast in YPD culture were stressed (H₂O₂, NaCl, or 42 °C) for 10 min (data not shown). Under these conditions, the effects of multiple kinases and phosphatases may have been complex and/or compensatory.

Rephosphorylation of Cdr1p Akt sites was inhibited by the PKA inhibitors H-89 and amide 14–22 but not by the H-89 homologue H-8, which has a Ki for PKA 30-fold higher than H-89 (12), or the protein kinase C inhibitor bisindolylmaleimide I (BIM) (Fig. 3B). A panel of TPK single or double deletion mutants constructed in CDR1-AD was tested for the effects of NaCl- and glucose-dependent rephosphorylation of Cdr1p (Fig. 3C). Apart from CDR1-TPK1ΔΔ and CDR1-TPK2ΔΔ, which reduced Cdr1p phosphorylation by about 50% during both treatments, p-Akts site re-phosphorylation was unaffected. Thus Tpk2p and to a lesser extent Tpk1p and Tpk3p may play a role with other kinases susceptible to PKA inhibitors in the phosphorylation of Cdr1p Akts sites.

Identification of Putative Phosphorylation Sites in Cdr1p—Modest phosphorylation signals suggested the presence of few p-Akts sites in Cdr1p. The p-Akts antibody recognizes phosphorylated Ser or Thr in the -5(K/R)XX(S/T) motif and cross-reacts with the phosphorylated -3(K/R)XX(S/T) motif (manufacturer’s information). The full size, single subunit, ABC transporter Cdr1p comprises two nucleotide binding domains (NBD1 and NBD2) that each contain the Walker A, Walker B, and ABC signature motifs, alternating with two pairs of six transmembrane segments, as illustrated in Fig. 4A. We constructed the yeast strains CDR1-M1 to CDR1-M9, which expressed equivalent amounts of Cdr1p (Fig. 4B), and each contained a point mutation (S/T → A) at each one of the nine putative p-Akts recognition sites. Of these sites, only M2, M4, and M7 may be recognized by the phospho-PKA substrates antibody (phosphorylated Ser or Thr in RXX(S/T)). Unlike the essentially normal phosphorylation of Cdr1p p-Akts sites in the CDR1-M3-CDR1-M9 mutants in 12-h YPD (early
stationary phase) cultures, Cdr1p phosphorylation was eliminated in CDR1-M1 and >90% inhibited in CDR1-M2 (Fig. 4B). Rephosphorylation of Cdr1p-M1 and Cdr1p-M2 was not detected in response to stressors plus 1 mM glucose (Fig. 4, C and D), and 100 mM glucose gave rephosphorylation to about 50% of the control level in each mutant (Fig. 4D). These results suggested that Ser307 and Ser484 are the dominant Akt sites in Cdr1p. This hypothesis was confirmed by finding, in the S307A/S484A double mutant strain CDR1-M1,2, that 100 mM glucose gave no Cdr1p p-Akts site rephosphorylation (Fig. 4E). Thus, the phosphorylation of M1 and M2 sites responded comparably to 12-h YPD culture (low glucose) and stressors, whereas glucose-induced rephosphorylation of the two sites occurred independently. Interestingly, the S307A mutation of the M1 site mutation reduced Thr phosphorylation by one-third in 12-h YPD cultures (Fig. 4B), and the S509A mutation at the membrane-associated M3 site similarly affected Akts site rephosphorylation of Cdr1p in 100 mM glucose (Fig. 4E).

**Rhodamine 6G Efflux from Pdh1p, Cdr1p, and Point Mutant Yeast**—The effect of the M1 and M2 mutations on energy-dependent drug efflux by Cdr1p was quantitated by comparably pre-loading CDR1-AD, CDR1-M1, CDR1-M2, CDR1-M1,2, and AD1–8u cells with the pump substrate rhodamine 6G (Rh6G) after 2-deoxyglucose treatment and then stimulating efflux by

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**Fig. 3. Stress- and kinase-dependent phosphorylation of Cdr1p.** A, glucose-starved pSK-AD, CDR1-AD, or PDH1-AD cells were stressed or treated with 100 mM glucose for 10 min as indicated in the top panel. Stress experiments that included 1 mM glucose are indicated. Phosphorylation patterns and expression levels of pump proteins were determined as in the previous figures. B, glucose-starved CDR1-AD (−glucose) cells were treated with protein kinase inhibitors H-89, H-8, 14–22 amide (14–22), bisindolylmaleimide I (BIM), or a solvent control (1% Me_2SO plus 1% water). After 10 min, 500 mM NaCl plus 1 mM glucose, or 100 mM glucose was added, the cells cultured for 10 min, and crude membrane fractions prepared for analysis. C, single and double TPK gene deletion mutants were constructed from CDR1-AD as described for PDH1-AD (Fig. 2). Glucose-starved CDR1-AD and the derivative TPK deletion mutants were treated with 500 mM NaCl, and 1 mM glucose, or 100 mM glucose for 10 min. The phosphorylation patterns and protein expression levels of the crude membrane fractions were analyzed.
adding glucose (Fig. 5). Fluorometric measurements showed that the CDR1-M2 strain (half-maximal rate of Rh6G pumping, 2.5 mM glucose; maximal pumping rate, 5–10 mM glucose) pumped Rh6G at rates up to 80% of the CDR1-AD strain (half-maximal rate of Rh6G pumping, 1 mM glucose; maximal rate, 5–10 mM glucose). In contrast, the CDR1-M1 strain required at least 5 mM glucose for a significant rate of Rh6G efflux and reached a maximal rate at 20 mM glucose, which was only 30% that of the CDR1-AD strain. The CDR1-M1.2 strain showed no glucose-dependent Rh6G efflux, even at 100 mM glucose. All strains that effluxed Rh6G showed a 30% decrease in the pumping rate at glucose concentrations between 20 and 100 mM glucose.

The pumping activities of Cdr1p and its point mutants were confirmed by flow cytometric measurement of cellular Rh6G content, which monitors combined dye uptake and efflux (see Supplemental Data, Fig. S1). These data also showed that strain CDR1-M1.2 did not efflux Rh6G even in the presence of 100 mM glucose and that phosphorylation of the Cdr1p-M1 site may be required for Rh6G efflux at low glucose concentrations.

**Lability of ATPase Activity in CDR1-M1—** The Cdr1p drug efflux pumps were functional in CDR1-M1, CDR1-M2, and CDR1-M8 cells and therefore expected to retain significant ATPase activity on cell fractionation. Plasma membrane fractions from these strains contained equivalent amounts of Cdr1p proteins, CDR1-M8 had a normal oligomycin-sensitive ATPase activity with a broad pH profile, but CDR1-M2 showed a decrease of ~50% in the ATPase activity compared with CDR1-AD (Fig. 6).

**Phosphorylation sites of Cdr1p detected by the phospho-(Ser/Thr) Akt substrate antibody.** A, the nine putative phospho-Akt substrate antibody recognition sites (p-Akts sites) T[(K/R)X(S/T) or (K/R)(K/R)(S/T); or (K/R)//(K/R)X(S/T)] of Cdr1p are shown (M1–M9), with Ser in black and Thr in gray. The conserved motifs of the nucleotide binding cassette, Walker A, B, and ABC signature, and twelve trans-membrane domains (TMD) are depicted with gray bars, whereas regions external to the plasma membrane (PM) are shown in black. CDR1-M1 through CDR1-M9 yeast strains, whose Ser or Thr residues were replaced by Ala at each M1–M9 site, were constructed. B, the phosphorylation patterns and expression levels of Cdr1p in crude membrane fractions from CDR1-AD and the point mutated derivatives CDR1-M1 through CDR1-M9 grown to early stationary phase in YPD medium were measured as described in previous figures. The ratios of intensities relative to CDR1-AD (1.0), given at the bottom of each panel, were measured using Scion Image. C, CDR1-AD and its derivative mutant strains were glucose-starved for 3.5 h and treated with 1 mM glucose and 500 mM NaCl for 10 min. The phosphorylation status of the Akt sites in each mutant Cdr1p was analyzed as above. D, glucose-starved CDR1-AD, -M1, and -M2 yeasts were treated with stressors and 1 mM glucose, or 100 mM glucose for 10 min as indicated in the top panel. The patterns of Cdr1p phosphorylation were analyzed as above. The relative band intensities are shown for each stress condition. E, CDR1-AD, the point mutants CDR1-M1 through CDR1-M9, and CDR1-M1,2 were glucose-starved for 3.5 h and then treated with 100 mM glucose for 10 min. The phosphorylation of Cdr1p was analyzed as above. In B–E, Cdr1p and the equivalent bands from the point mutant derivatives are indicated with the black arrowhead, whereas the Pma1p band is indicated with the white arrowhead. Representative data, from multiple experiments using several independently isolated clones of each strain, are shown.
residue and aligns with Ser\textsuperscript{307} of NBD1. The membranes from CDR1-M1, however, lacked detectable oligomycin-sensitive ATPase activity. The M1 site may therefore be critical for the \textit{in vitro} ATPase activity. Experiments that included ATP during membrane isolation allowed the recovery of small amounts (10\%) of vanadate and oligomycin-sensitive ATPase activity compared with the control strain (see Supplemental Data Table SIII). A statistically significant proportion of this activity (\(p < 0.01\)) can be attributed to S307A Cdr1p that survived membrane isolation due to the protective ATP.

**Drug Susceptibilities of CDR1-AD and PDH1-AD Derivative Yeasts**—The MIC\textsubscript{50} values for antifungal agents were determined for PDH1-AD and CDR1-AD derivative strains to assess whether the phosphorylation of Cdr1p and Pdh1p affected drug efflux activity (Table I). As expected, the Pdh1p- and Cdr1p-expressing strains were more resistant toazole agents than the control pSK-AD strain but were equally susceptible to flucytosine extended the data on antifungal susceptibility re- 

Among the CDR1-AD derivatives, only CDR1-M1 and CDR1-M2 were within two dilutions of the values for pSK-AD. The efflux activity (Table I) can be explained by partially functional mutant Cdr1p operating at glucose concentrations \(>5\) \(\mu\)M. The lack of Rh6G pumping by CDR1-M1,2 (Fig. 5), however, seemed inconsistent with its relatively high MIC for FLC and other azoles (Table I), although this was less pronounced in disk diffusion assays. CDR1-M1,2 was also more resistant to FLC than either PDH1-AD or the AD1–8u\textsuperscript{−} host strain (Table I). Even though identical FLC and Rh6G susceptibilities were observed with three separately isolated clones for each construct under study, we excluded the possibility that secondary mutations in the genetic background of the strains might affect susceptibilities to azoles. 

The relatively high FLC MIC value for the CDR1-M1 strain (Table I) can be explained by partially functional mutant Cdr1p (to ensure the incorporation of the \textit{CDR1} gene) showed FLC and Rh6G susceptibilities identical to those for the strains providing the transforming DNA (Table II). The drug resistance of each donor strain was thus conferred by the expression of Cdr1p and not an extragenic determinant. The unexpectedly high FLC and Rh6G liquid MIC\textsubscript{50} values for CDR1-M1,2 were therefore due to Cdr1-M1,2p overexpression. It was possible, however, that suppressor mutations were selected during growth in liquid MIC assays.

Progeny that survived FLC exposure during MIC determinations in buffered FLC-containing CSM-URA medium, using either glucose or the non-fermentable substrate glycerol as energy source, were isolated. Sequencing of several independent isolates showed that the S307A and S484A mutations were maintained in these progeny independent of the energy source. In addition, three independently isolated intragenic suppressor mutations were obtained that did not change the Ser\textsuperscript{307} and Ser\textsuperscript{184} background: a ΔA349 mutation after growth on glycerol and D32H and V353L mutations after growth on glucose. In contrast to the inactive parental CDR1-M1,2 strain, all three suppressor strains showed glucose-dependent Rh6G pumping comparable to the CDR1-M1 mutant. An Rh6G efflux experiment conducted with the representative suppressor strain CDR1-M1,2 48a is shown in Fig. 5. The Cg\textit{CDR1-URA3} cassette was obtained by PCR of genomic DNA from each of the three suppressor strains and used to transform strain AD1–8u\textsuperscript{−}. The Ura\textsuperscript{+} transformants that also grew on 5 \(\mu\)g/ml FLC showed FLC and Rh6G resistance comparable to the CDR1-M1 strain (Table II). The suppression mutation phenotypes therefore result from intragenic modification of Cdr1p in CDR1-M1,2. The selection of the suppressor mutants occurred at a low frequency (suppressor strains were detectable in <10\% of MIC determinations). This frequency was not high enough to compromise the MICs forazole drugs and other xenobiotic substrates of CDR1-M1,2.

**DISCUSSION**

Phosphorylation of ABC Transporters—Phosphorylation mediated by PKA and PKC affects the function of numerous human ABC transporters, including ABCA1, MDR1, and CFTR (13–15). We previously demonstrated that PKA-dependent phosphorylation of \textit{C. glabrata} Pdh1p was important for drug efflux and Cdr1p ATPase specific activity was glucose-dependent and possibly regulated by phosphorylation (7). \textit{C. glabrata} Cdr1p and Pdh1p have about 70\% amino acid sequence identity with \textit{S. cerevisiae} Pdr5p and thus belong to the \textit{PDR} family, the
largest ABC transporter family in *S. cerevisiae* (16, 17). The *S. cerevisiae* drug efflux transporters Pdr5p, Snq2p, and Yor1p are all phosphorylated, and casein kinase I phosphorylation of Ser420 in Pdr5p is important for enzyme turnover (9), but the equivalent site is absent in Cdr1p. Phosphorylation of Thr613 and Ser623 in the D-box linking the two homologous halves of Ste6p has been implicated in enzyme turnover (18). The Cdr1p-Akts antibody recognition sites at Ser307 and Ser484 are therefore distinct from the sites affecting Pdr5p and Ste6p turnover. The Ser307 and Ser484 sites appear physiologically important. They affect the glucose dependence of pump activity, and glucose-dependent Rh6G efflux was eliminated by the S307A/S484A double mutation. The expression of S307A, S484A, and the S307A/S484A double mutant Cdr1ps at levels comparable with the wild type protein implies that misfolding does not target the mutant proteins for early degradation. The PKA-dependent phosphorylation of human ABCA1 (13) provides a precedent for phosphorylation at Ser307.

### Kinases Responsible For the Phosphorylation of Pdh1p and of Cdr1p at M1 and M2 Sites—

The glucose-dependent rephosphorylation of Pdh1p in starved cells was blocked by PKA inhibitors (7) and was primarily affected by Tpk3p and to a lesser extent by Tpk2p. Antibody recognition of phosphorylated PKA sites implies that these phosphorylations were direct. Of the nine putative phosphorylation sites tested in glucose-starved Cdr1p, only Ser307 and Ser484 strongly affected the rephosphorylation pattern. Unlike Pdh1p phosphorylation, neither site was recognized by the p-PKA substrates antibody after stress or at glucose concentrations 100-fold higher than those required for Pdh1p p-Akts site rephosphorylation. The blockage

### Table I

| Strain          | MIC<sub>80</sub><sup>a</sup> Fluconazole (µg/ml) | Miconazole (µg/ml) | Ketoconazole (µg/ml) | Fluocytosine (µg/ml) | Amphotericin B (µg/ml) |
|-----------------|-----------------------------------------------|--------------------|----------------------|----------------------|------------------------|
| pSK-AD          | 0.5                                          | 0.016              | 0.063                | 0.5                  | 0.25                   |
| CDR1-AD         | 128                                          | 2                  | 4                    | 1                    | 0.5                    |
| CDR1-M1         | 64                                           | 2                  | 4                    | 1                    | 0.5                    |
| CDR1-M2         | 128                                          | 2                  | 4                    | 1                    | 0.5                    |
| CDR1-M3         | 128                                          | 2                  | 4                    | 1                    | 0.5                    |
| CDR1-M4         | 128                                          | 2                  | 4                    | 1                    | 0.5                    |
| CDR1-M5         | 128                                          | 2                  | 4                    | 1                    | 0.5                    |
| CDR1-M6         | 128                                          | 2                  | 4                    | 1                    | 0.5                    |
| CDR1-M7         | 128                                          | 2                  | 4                    | 1                    | 0.5                    |
| CDR1-M8         | 128                                          | 2                  | 4                    | 1                    | 0.5                    |
| CDR1-M9         | 128                                          | 2                  | 4                    | 1                    | 0.5                    |
| CDR1-M1,2       | 32                                           | 1                  | 2                    | 1                    | 0.5                    |
| CDR1-TPK1Δ      | 128                                          | 2                  | 4                    | 2                    | 0.5                    |
| CDR1-TPK2Δ      | 256                                          | 4                  | 8                    | 2                    | 0.5                    |
| CDR1-TPK3Δ      | 128                                          | 2                  | 4                    | 2                    | 0.5                    |
| CDR1-TPK1Δ2Δ    | 256                                          | 4                  | 4                    | 2                    | 0.5                    |
| CDR1-TPK1Δ3Δ    | 128                                          | 2                  | 4                    | 1                    | 0.25                   |
| CDR1-TPK2Δ3Δ    | 256                                          | 4                  | 8                    | 1                    | 0.25                   |
| CDR1-SCH9Δ      | 256                                          | 4                  | 8                    | 0.25                 | 0.25                   |
| CDR1-HOG1Δ      | 256                                          | 4                  | 8                    | 2                    | 1                      |
| PDH1-AD         | 16                                           | 0.5                | 1                    | 1                    | 0.5                    |
| PDH1-TPK1Δ      | 16                                           | 0.5                | 1                    | 1                    | 0.5                    |
| PDH1-TPR2Δ      | 16                                           | 0.5                | 1                    | 1                    | 0.5                    |
| PDH1-TPK3Δ      | 16                                           | 0.5                | 1                    | 1                    | 1                      |
| PDH1-TPK1Δ2Δ    | 16                                           | 1                  | 2                    | 0.5                  | 0.5                    |
| PDH1-TPK1Δ3Δ    | 16                                           | 0.5                | 1                    | 2                    | 1                      |
| PDH1-TPK2Δ3Δ    | 16                                           | 0.5                | 1                    | 1                    | 0.5                    |

* MIC<sub>80</sub> values (µg/ml) were the lowest concentration of drug that inhibited the growth yield by at least 80% compared with the growth yield for a non-drug control.
of Cdr1p rephosphorylation by the PKA inhibitors H89 and 14-22 and the 50% reduction in Cdr1p phosphorylation in CDR1-TPK1ΔΔ and CDR1-TPK2ΔΔ only, but not CDR1-TPK2a, suggested a role for Tpk2p in phosphorylation at either Ser307 or Ser484 that may be complemented by Tpk1p plus Tpk3p. Alternatively, other kinases affected by PKA inhibitors could directly or indirectly cause the Ser307 and Ser484 rephosphorylation signals.

The M1 site in Cdr1p (Fig. 7A) contributes to putative yeast PKA (PKVs), and CKII (SIAE) sites (see www.cbs.dtu.dk/da-H9004) contributes to putative yeast PKA, Akt, and CaMII kinase sites. Although not close homologues of Akt, deletion of SCH9, one of the four yeast kinases categorized in the Akt family (19), gave slightly higher levels of multidrug drug resistance and an increased, rather than decreased, Akt site phosphorylation that slightly higher levels of multidrug drug resistance and an increased, rather than decreased, Akt site phosphorylation that might occur widely in fungi, including fungal pathogens like C. albicans. Ser307 at the Cdr1p M1 site is five amino acids C-terminal to the NBD1 ABC signature “LSGGQ” motif, which is not perfectly conserved in fungi. The sequence around Ser307 is highly conserved in the PDR family transporters, SNQ2, and known PDR homologues in other pathogenic fungi, including C. albicans, Aspergillus fumigatus, and Filobasidiella neoformans (Fig. 7A). In addition, an M1 Ser or Thr is found in four S. cerevisiae non-PDR family ABC transporters.

The LSGGQ consensus motifs are normally highly conserved between NBDs of ABC transporters. The NBD1 ABC signature motif VSGGE of fungal PDR family proteins corresponds with a LNVEQ consensus in the NBD2 of these proteins (Fig. 7B). A similar sequence to the M1 region is found in mammalian (hABCG1, hABCG2, and hABCG8) and insect (White and Brown) ABCG proteins (Fig. 7C) (17). ABC transporters with sequences similar to that around the M1 site of human MDR1, a multidrug pump functional homologue of Cdr1p, are widely distributed from Protista to mammals, and include the yeast mitochondrial peptide transporter MDL1 (Fig. 7C). Most of the human ABCA and ABCC (CFTR/MRP) family transporters have Ser or Thr at M1 equivalent sites. Alanine is the main amino acid in the M1 and M8 sites of MDR1 type multidrug transporters, and all ABCC (MDR1) family transporters have alanine at the M1 site, consistent with the substantial pump function of the CDR1-M1 strain. PKA-dependent phosphorylation was recently reported at sites equivalent to M1 in both NBDs of the full-size human ABCA1 (13). Phosphorylation of the NBD2 site was important for phospholipid efflux, but the effect of phosphorylation on ATPase activity was not reported.

The multiple defects caused by S307A mutation in NBD1, together with the phosphorylation of M1 but not the aligned M8 site in Cdr1p, indicate that the two NBDs in Cdr1p are functionally non-equivalent. Furthermore, the Rh6G efflux activity, drug resistance, p-Akts site phosphorylation, and plasma membrane nucleoside triphosphatase activity of CDR1-M8 and the CDR1-AD strain were identical. Thus the M8 site in NBD2 is not critical for Cdr1p function. The C193A mutation, in a catalytically active GST fusion of the N-terminal cytoplasmic domain of C. albicans Cdr1p, causes a loss of 95% of enzyme activity (25). We recently found that the equivalent C189A mutation in the NBD1 Walker A motif of Cdr1p gave 60% of normal ATPase activity and susceptibilities to FLC and

### Table II

| Strain       | MIC<sub>FLC</sub> (µm) | MIC<sub>Rh6G</sub> (µm) |
|--------------|------------------------|-------------------------|
| CDR1-AD      | 192                    | 200                     |
| CDR1-M1      | 128                    | 50                      |
| CDR1-M2      | 256                    | 200                     |
| CDR1-M1,2    | 48/32                  | 12.5                    |
| CDR1-M1,2 glucose-grown revertant B5 (V335L) | 128 | 25 |
| CDR1-M1,2 glucose-grown revertant 48a (D32H) | 96 | 25 |
| CDR1-M1,2 glyceroL-grown revertant16a (ΔA349) | 48 | 25 |
| CDR1-AD recloned in AD1–8u | 256 | 200 |
| CDR1-M1 recloned in AD1–8u | 96 | 50 |
| CDR1-M2 recloned in AD1–8u | 192 | 200 |
| CDR1-M1,2 recloned in AD1–8u<sup>-</sup> | 48 | 12.5 |
| CDR1-M1,2 B5 recloned in AD1–8u<sup>-</sup> | 128 | 25 |
| CDR1-M1,2 48a recloned in AD1–8u<sup>-</sup> | 96 | 50 |
| CDR1-M1,2 16a recloned in AD1–8u<sup>-</sup> | 64 | 25 |

<sup>a</sup> MIC<sub>FLC</sub> as defined in Table I.
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Rh6G intermediate between CDR1-M1 and CDR1-M2. In contrast, a K989A mutant in the aligned residue in the Walker A motif of NBD2 showed no ATPase activity and had PLC and Rh6G susceptibilities comparable with strain AD1–8u. These results confirm the functional non-equivalence of the two NBDs of Cdr1p, show that NBD2 is required for basal pump activity, and suggest that NBD1 regulates enzyme activity.

If NBD1-TM6-NBD2-TM6 transporters like Cdr1p were functional monomers, drug pumping should involve the binding of ATP molecules using the Walker A and B motifs from one NBD and the ABC signature motif from the other NBD (26). The residue homologous to Ser307 in the human TAP1 NBD would give the enzyme activation seen at glucose concentrations below 20 mM and significant enzyme deactivation at glucose concentrations above 20 mM by regulating the frequency and/or turnover of productive contacts between like NBDs.

**Functional Role of M2 Phosphorylation**—The primary sequence around the M2 site, which is C-terminal of NBD1 and closer to the first transmembrane domain, is strongly conserved in Cdr1p, Pdh1p, and Pdr homologues and differs from Snq2p, which has basic residues at both the −3 and −2 positions (Fig. 7D). The S484A mutation blocked M1 and M2 site phosphorylation in a 12-h YPD culture, decreased plasma membrane ATPase activity by 50%, but only modestly affected transport activity at glucose concentrations <10 mM. Partial suppression of the effects of the S307A mutation on Rh6G pumping by Ser484 and the non-additive drug resistance properties of the S307A/S484A double mutation suggest conformational interactions between these two sites. Lack of suitable structural data for homology modeling precludes further interpretation.

**Drug Resistance of Mutant Strains**—Although CDR1-M1 and CDR1-M2 showed major reductions in Rh6G efflux, both strains were 2- to 5-fold more susceptible than CDR1-AD to a panel of drugs, which includes clinical antimicrobials used for drug susceptibility assays should support sufficient pump function to detoxify the M1 mutant at glucose concentrations >5 mM. Because ATPase activity is required for ABC transporter pump function, the CDR1-M1 enzyme must be sufficiently stable for phosphorylation/conformational change.
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at Ser\textsuperscript{484} to support partial pump function, even though only ~5% of the activity of the enzyme survives plasma membrane isolation in the presence of protective concentrations of ATP. The CDR1-M1,2 strain, at glucose concentrations that activate the S307A or S484A pumps, failed to efflux Rh6G. The identical FLC and Rh6G resistances, obtained for independent CDR1-M1,2 isolates and upon recloning of this CDR1-URA3 construct in the FLC- and Rh6G-hypersensitive AD1–8u strain, show that overexpression of an apparently non-functional Cdr1p gives significant drug resistance. Although the mechanism responsible has yet to be determined, enhanced ergosterol-rich lipid raft formation, an undetected enzyme-stabilizing modification, or residual activity of the hyperexpressed Cdr1p may be important.

Drug resistance assays selected a low frequency of intragenic suppressors (e.g. D32H, V353L, and A349) that assembled productive S307A/S484A Cdr1p with enhanced Rh6G pumping and diminished susceptibility to FLC and Rh6G. We speculate that the A349 or V353L mutations may modify the structure of the H-loop in NBD1. The Cdr1p H-loop lacks the “invariant” H thought to contribute to NBD interactions as a result of ATP binding. The Blastp algorithm aligns Ala\textsuperscript{349} with this amino acid, and its deletion may alter the configuration of neighboring amino acids. The D32H mutation produces an His\textsubscript{2} motif just three residues C-terminal to another His residue. This might introduce a structure-deforming divalent metal ion binding pocket or facilitate complementary interaction between homodimeric N termini. The effects of these suppressor mutations on enzyme function and their possible location at homodimer inter-subunit boundaries are consistent with NBD1 mediating multiple responses to growth and stress by modulating the efficiency of productive inter-subunit interactions. A complex pattern of phosphorylation events, primarily involving the Ser\textsuperscript{307} site, the compensatory Ser\textsuperscript{484} site, and possibly involving other sites, regulate the overall ATPase and pumping activity of homodimeric Cdr1p. A similar, but differentially glucose-sensitive mechanism, probably regulates Pdh1p.

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