Evidence for a Molecular Diode-based Mechanism in a Multispecific ATP-binding cassette (ABC) Exporter

SER-1368 AS A GATEKEEPING RESIDUE IN THE YEAST MULTIDRUG TRANSPORTER Pdr5

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Background: Once an ABC multidrug transporter efflux pump expels a substrate, its reentry must be prevented.

Results: Ser-1368 in Pdr5 prevents drug reflux during transport against a concentration gradient.

Conclusion: Pdr5 may function as a molecular diode.

Significance: The behavior of an S1368A mutant suggests an important mechanism of drug exclusion.

ATP-binding cassette multidrug efflux pumps transport a wide range of substrates. Current models suggest that a drug binds relatively tightly to a transport site in the transmembrane domains when the protein is in the closed inward facing conformation. Upon binding of ATP, the transporter can switch to an outward facing (drug off or drug releasing) structure of lower affinity. ATP hydrolysis is critically important for remodeling the drug-binding site to facilitate drug release and to reset the transporter for a new transport cycle. We characterized the novel phenotype of an S1368A mutant that lies in the putative drug-binding pocket of the yeast multidrug transporter Pdr5. This substitution created broad, severe drug hypersensitivity, although drug binding, ATP hydrolysis, and intradomain signaling were indistinguishable from the wild-type control. Several different rhodamine 6G efflux and accumulation assays yielded evidence consistent with the possibility that Ser-1368 prevents reentry of the excluded drug.

ATP-binding cassette (ABC)² multidrug transporters use the energy from ATP binding and hydrolysis to efflux a broad array of structurally and mechanistically dissimilar compounds. Overexpression of these proteins is a major clinical problem in the treatment of cancers and pathogens (1–3).

Understanding the transport cycle of these complex, polytopic proteins is a major challenge, but it is generally agreed that several conformational changes take place during one turn of the catalytic cycle. The atomic structure of several ABC transporters, notably Sav1866 (4), the Escherichia coli maltose transporter (5–7), and the Caenorhabditis elegans P-gp homologue (8), as well as kinetic studies with P-gp (9), lend credence to the idea that a high affinity drug-binding conformation switches upon ATP binding or hydrolysis to a drug-releasing structure of lower affinity.

When a drug molecule is released from one of multiple drug-binding sites, it leaves the drug-binding pocket. Implicit, but rarely mentioned in this discussion, is the assumption that when a drug is expelled from the cell, it is prevented from returning to the interior via the transporter’s binding pocket, which is now facing the extracellular milieu. Several ways to exclude a drug can be posited from studies of multidrug transporters, such as Pdr5 and P-gp, and these are by no means mutually exclusive. By one mechanism, the lowered affinity of the drug-binding site in the drug-releasing conformation reduces the probability of rebinding and subsequent reflux. In the case of P-gp, studies with the photoaffinity substrate iodooarylazidoprazosn (IAAP) indicate a 30× reduction in binding affinity following ATP hydrolysis (9). That might succeed to some degree, but it is also possible that the external concentration of a xenobiotic drug is often high enough to allow rebinding and some reentry.

A second exclusion mechanism is suggested from the observation that with both P-gp and Pdr5, high concentrations of a subset of drug substrates cause allosteric (trans) inhibition of the ATPase activity (10–14). Gupta et al. (15) proposed that a failure of drug release hinders the conformational freedom of the transporter which is locked in an outward facing conformation. As a consequence, nucleotide exchange is impaired, resulting in reduced ATPase activity. In the case of both P-gp and Pdr5, however, several potent and clinically important substrates do not inhibit the ATPase activity at even very high concentrations, or inhibition is incomplete (10–14).

One might think that drugs that do not cause trans inhibition of ATPase activity could slip back through the transporter. Therefore, another alternative proposed by Gupta et al. (15) is that ABC efflux pumps are molecular diodes or unidirectional gates that are remodeled after drug release and ATP hydrolysis so that back transport is eliminated even if some drug is bound to a transport site.
**Pdr5-mediated Drug Release**

We characterized the phenotype of an S1368A mutation in transmembrane helix 11 (TMH11) of Pdr5. An atomic model of Pdr5 (16) places Ser-1368 deep in the proposed drug-binding pocket of Pdr5. This residue has low lipophilicity and high sequence entropy and is thought to face into the open, drug-binding pocket. The S1368A mutant strain exhibits profound, broad drug hypersensitivity to agents that we showed are not transported from the same drug-binding site. In fact, the behavior of the mutant in drug-binding assays is indistinguishable from an isogenic wild-type (WT) control. The S1368A mutant appears to be biochemically competent. Its localization in purified plasma membrane (PM) vesicles, ATPase activity, and signaling capability are indistinguishable from WT. Because of its unusual behavior in several kinds of transport and reflux assays with rhodamine 6G (R6G), we posit that S1368A prevents the reflux of drugs during transport.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmid**—All of the yeast strains we used were isogenic and derived from R-1, a strain lacking all PM ABC transporters. This strain offers numerous other advantages for genetic and biochemical analyses, which are described in detail elsewhere (17, 18). We cultured the strains at 30 °C. We used the pSS607-integrating plasmid (12) for site-directed mutagenesis, as described previously (17). This plasmid has a WT PDR5 gene under the transcriptional control of its own upstream region as well as a URA3-selectable marker. We used strains containing two copies of either WT or a mutant PDR5 gene to make purified PM vesicles for ATPase and IAAP photoaffinity-labeling experiments. The rationale for using and the method for generating these strains are described elsewhere (17). We used single-copy strains for all other experiments, including most of the whole-cell R6G transport studies. Unless otherwise noted, we grew our strains in yeast extract, peptone, dextrose (YPD) medium at 30 °C.

**Chemicals and Media**—We purchased all chemicals from Sigma-Aldrich, except for 5-fluororotic acid and G-418, which we obtained from Research Products International (Mt. Prospect, IL), and cliambazole, cerulenin, cyproconazole, tebuconazole, and imazalil, which we obtained from LKT Laboratories (St. Paul, MN). All chemicals were dissolved in DMSO except for 5-fluororotic acid and G-418, which we purchased from PerkinElmer Life Sciences (Waltham, MA) and [3H]R6G (1.5 Ci/mol) from Moravek Biochemicals (Brea, CA).

**Measurement of Relative Resistance of Strains**—We determined the relative resistance of strains to clotrimazole (clo), cliambazole, imazalil, cyproconazole, tebuconazole, and cerulenin. We placed 990 μl of YPD broth in sterile glass tubes. We added 5 μl of drug stock solution (5 mM) to the first tube. We introduced the desired concentration of drug (usually 1.56–25.0 μM) as a 2-fold dilution into tubes and added 0.5 × 10^5 cells (typically 2–5 μl). After culturing for 24 h, we measured absorbance at 600 nm (A_600). We used this value to calculate the percentage of inhibition (%I) as follows: %I = 1 – (Ac/Ao) × 100, where Ac is the A_600 of the culture at a given concentration of drugs, and Ao is the A_600 of the positive control (19). We then determined the IC_{50} as the concentration of drugs resulting in 50% inhibition. We used isogenic WT and Δpdr5 strains as controls for comparing the susceptibility of the mutants to the drugs. We performed statistical analyses of the curves with Prism GraphPad software (San Diego, CA). We determined the cell density after 24-h rather than 48-h growth. Therefore, our IC_{50} values were somewhat different from those reported previously (14, 18, 20).

**Site-directed Mutagenesis**—We introduced mutations into pSS607 with a QuickChange Lightning site-directed mutagenesis kit (Invitrogen). We designed mutant primers with a genomics program provided by Agilent Technologies (Santa Clara, CA). The mutant plasmids were introduced into XL-Gold E. coli by transformation, as described in the QuikChange instruction manual. We extracted plasmid DNA from the transformants with an IBI miniprep kit (MIDSCI, St. Louis, MO) and had it sequenced commercially to confirm the presence of the mutation in the plasmid (Retrogen). We introduced the mutant plasmid DNA into R-1 with a Sigma-Aldrich yeast transformation kit. Genetic testing confirmed that the construct was correctly inserted (17).

**DNA Extraction and PCR Recovery of DNA**—We extracted chromosomal DNA with Qiagen Puregene Yeast/Bacteria kit B (Qiagen, Valencia, CA), and we amplified PDR5 by PCR. Consistently good amplification requires relatively pure DNA (260/280, ~2.0; 260/230, ~2.2) at a concentration of 60–120 ng. We performed 40 rounds of amplification as described previously (18). We sent the PCR product of ~4.5 kbp to SeqWright (Houston, TX) for purification and sequencing. The entire coding region was sequenced to confirm that only the desired alteration was present.

**Preparation of Purified PM Vesicles**—We prepared purified PM vesicles as described recently (14). We determined the protein concentration of PM vesicle protein with a bicinchoninic acid kit (Peribo, Rockland, IL).

**Gel Electrophoresis of PM Vesicle Proteins**—We solubilized samples containing 10 μg of PM vesicle protein in SDS-PAGE for 30 min at 37 °C. We separated the proteins on NuPAGE 7% Tris acetate gels (125–150 V for ~80 min; Invitrogen).

**Western Blots of Pdr5 in PM Vesicles**—We conducted Western blotting with 10 μg of PM vesicle protein as described previously (14). We performed the transfer from the gel to the nitrocellulose membrane (400 mA, 60 min) with an X Cell II apparatus (Invitrogen). We purchased all of the antibodies from Santa Cruz Biotechnology, Inc. (Dallas, TX). We diluted the polyclonal goat anti-Pdr5 and anti-Pma1 antibodies 1:1000 and 1:500 (yC18 and yN20, respectively). We blocked the polyclonal goat anti-Pdr5 and anti-Pma1 antibodies from Santa Cruz Biotechnology, Inc. (Dallas, TX). We diluted the polyclonal goat anti-Pdr5 and anti-Pma1 antibodies 1:1000 and 1:500 (yC18 and yN20, respectively). We blocked the nitrocellulose membranes for 30 min with 5% nonfat milk in PBS containing 1% Tween 20. Following this, we incubated the filters in primary antibody overnight at 4 °C. We washed them three times for 15 min in PBS, 1% Tween 20 before adding a 1:5000 dilution of secondary antibody (donkey, anti-goat IgG horseradish peroxidase; SC2033) and incubating them at room temperature for 2 h. We developed blots with a Novex ECL horseradish peroxidase chemiluminescent substrate reagent kit (Invitrogen).

**Assay of ATPase Activity**—We measured Pdr5-specific ATPase activity as described previously (12) for 8 min at 35 °C.
with 12 μg of purified PM vesicle protein derived from cells bearing two copies of either WT or mutant PDR5 genes (17). We verified all ATPase results by carrying out assays with at least two independent PM vesicle preparations/strain. We used 3 mM ATP to assay allosteric inhibition of the ATPase activity by clo, as described previously (12). Kinetic analyses were performed with GraphPad Prism software (San Diego, CA).

Cross-linking of [125I]IAAP to Pdr5—Purified membranes, prepared from double-copy-bearing yeast cells (30 μg of protein/125 μl) were incubated with clo for 5 min in ATPase buffer, followed by incubation with [125I]IAAP (3.5 nM) at room temperature for 5 min under subdued light. We photo-cross-linked the samples for 10 min with 365-nm UV light in an ice water bath, followed by electrophoresis on 7% NuPAGE gels (Invitrogen). We quantified the gels as described previously (15). We also exposed the dried gels to X-ray film to create an autoradiogram of the samples.

R6G Transport in Whole Cells—Initially, we measured R6G transport with whole cells grown at 30 °C in SD + ura, his medium as described previously (14, 17). For the time course experiments, we loaded 1–2 × 10^6 cells suspended in 100 μM of 0.05 mM Hepes buffer (pH 7.0) minus glucose for 90 min in the presence of 10 μM R6G. Following loading, we pelleted cells in a microcentrifuge tube and removed the supernatant before resuspending them in 300 μl of 0.05 mM Hepes, 1 mM glucose. Later we reduced the buffer molarity to 0.02 mM Hepes to eliminate the sporadic toxicity that we observed with the higher concentration. We incubated the tubes at 30 °C for various times. We terminated transport reactions by placing the tubes in an ice water bath. We determined cell fluorescence with a FACSort (BD Biosciences) with an excitation wavelength of 529 nm and an emission wavelength of 535 nm. We analyzed the data with a CellQuest program. We expressed retained fluorescence in arbitrary units.

We also performed R6G transport experiments in which the cells were not preloaded with this drug and thus had to work against a concentration gradient. To do this, we inoculated 5 ml of YPD growth medium with 0.5 × 10^7 cells/ml in the presence of R6G and incubated these cultures at 30 °C in a shaking water bath for 20 h. We also used spectroscopy (600 nm) to monitor the retained R6G and incubated these cultures at 30 °C in a shaking water bath for 20 h. We also used spectroscopy (600 nm) to monitor the retained R6G and incubated these cultures at 30 °C in a shaking water bath for 20 h. We also used spectroscopy (600 nm) to monitor the retained R6G.

Statistical Analyses—Statistical analyses were performed with GraphPad Prism software. Error bars in the figures or a “±” designation indicates S.E.

**RESULTS**

An S1368A Substitution Results in Severe, Broad Drug Hypersensitivity—We constructed an S1368A substitution as part of a systematic study designed to identify drug-binding residues. Previous work by Egner et al. (21) established that mutations in the residues of TMH11 (then predicted to be TMH10) affect the substrate specificity of Pdr5. These include Ser-1360 and Thr-1364. These results suggested that at least some of these residues form part of a drug-specific binding site. A recent atomic model of Pdr5 is consistent with this possibility (16). We used that model to select and make alanine substitutions in Phe-1363, Thr-1364, Ser-1366, Ser-1368, Phe-1494 (TMH12), and Phe-1495. We tested these mutations for their sensitivity to six established Pdr5 substrates. Some of the mutants had mild, drug-specific phenotypes not found in the WT. The T1364A substitution, for example, was 2.5 times more sensitive to climbazole than the WT, but both strains had the same IC_50 for clo and similar transport kinetics for R6G.4 In contrast, the S1368A mutant was profoundly drug-hypersensitive to all six Pdr5 substrates. These data are found in Fig. 1, where the relative resistance to these antifungal agents is compared with an isogenic WT control. These drugs vary in structure and degree of hydrophobicity, with a logP range of 1.2 (cerulenin) to 5.4 (clo). Of these, only clo is a significant allosteric (trans) inhibitor of Pdr5-ATPase activity (14). In each instance, the substitution was 5–6 times more sensitive than the WT.

Ser-1368 is not an especially well conserved residue. In an alignment with 16 other members of the Pdr subfamily, the equivalent position is often (12/16) occupied by leucine or isoleucine. Three members, however, have a serine in this position; one has a threonine, and one has an asparagine residue. In the atomic model of Pdr5 (16), Ser-1368 lies deep in the proposed drug-binding pocket. To determine whether S1368A caused misfolding of Pdr5 and therefore reduced PM localization, we prepared purified PM vesicles and performed immunoblotting with a Pma1-loading control. These data (Fig. 2A) demonstrate that this substitution does not reduce the level of Pdr5 in purified PM vesicles from that of the WT.

To determine whether the phenotype we observed was attributable to the mutation we constructed, we transformed the strain with a plasmid (pSS607) bearing a WT PDR5 gene. We confirmed that the transformant we used had a mutant and a WT allele by plating cells on 5-fluoroorotic acid medium (17, 18) and qualitatively testing the resulting segregants for their relative clo resistance by replica plating. We recovered both WT and S1368A mutant alleles. We then tested the transformant for its resistance to clo relative to an isogenic WT strain

4 J. Mehla, unpublished data.
The resulting killing curves were similar (Fig. 2B), suggesting that the expression of the PDR5 gene in the plasmid completely restored resistance. Therefore, we concluded that the profound drug sensitivity exhibited by the S1368A mutant is attributable solely to the alanine substitution in Pdr5.

ATPase Activity and Allosteric Inhibition of Enzyme Activity Are Unaltered in S1368A—We recently analyzed several loss-of-function mutations in the Pdr5 transmission (signal) interface that exhibit a characteristic phenotype. Most show at least some reduction in ATPase activity (14, 17, 18, 20). V656A (intracellular loop-2) and S558Y (TMH2), which exhibited severe, broad drug hypersensitivity much like S1368A, also had a Pdr5-specific ATPase that was more resistant to allosteric inhibition by clo. S1368A, however, had an ATPase activity that was indistinguishable from WT. In Fig. 3A and B, we show Henri-Michaelis-Menten plots from two independent PM vesicle preparations from each strain. The $V_{\text{max}}$ of the WT and S1368A enzymes in the first preparation were ~415 and 459 nmol of $P_i$/min/mg of protein, respectively. In the second preparation, they were 349.2 (WT) and 347.7 (S1368A) nmol/min/mg.

We evaluated the allosteric (trans) inhibition by clo and R6G of these ATPases (Fig. 3C). With respect to clo, the IC$_{50}$ for both enzymes was ~2.2 $\mu$M, a value also much like those observed previously with WT vesicles (14). The inhibition was nearly complete, with only about 10% of the ATPase activity remaining when vesicles from either strain were treated with 20 $\mu$M clo. The WT and S1368A ATPase activities also showed nearly identical behavior when R6G was used as an allosteric inhibitor (Fig. 3D). In light of these data, it appears unlikely that intradomains signaling is affected by the S1368A mutation.

The Effect of the S1368A Substitution Extends beyond a Single Drug-binding Site—The S1368A substitution failed to show any drug specificity, implying that it was not a drug-binding site mutant. It was possible, however, that all six substrates that we tested bound and were transported from the same region of the drug-binding pocket. To find out, we tested three of these drugs for their ability to inhibit R6G efflux in a concentration-dependent manner using WT cells. None of these particular substrates inhibited Pdr5 ATPase to any significant degree (14). Our results are presented in Fig. 4A.

Imazalil and tebuconazole showed complete, concentration-dependent inhibition of R6G efflux. When a 100 $\mu$M concentration of either drug is included in the transport assay, the cells retain a level of fluorescence that approaches that of the
untreated Δpdr5 control (about 800 arbitrary units). This indicated that the binding sites or transport pathway of these drugs overlap with R6G. However, although S1368A was profoundly sensitive to cerulenin (Fig. 1), this drug failed at any concentration to inhibit R6G transport. In a previous report, Downes et al. (14) demonstrated that imazalil and cerulenin are substrates of comparable strength with respect to Pdr5 transport capability (for a discussion of how substrate strength is evaluated, see Golin et al. (22)). Therefore, the S1368A strain was sensitive to cerulenin (Fig. 1). This result strongly suggested that the S1368A mutant exerted its effect beyond a single, drug-specific binding residue and served an alternative role.

Further support for the conclusion that Ser-1368 does not have a major drug-binding role comes from photoaffinity labeling experiments. Hanson et al. (23) demonstrated that photoaffinity labeling with IAAP is inhibited by R6G and clo in a concentration-dependent manner. We evaluated the capability of clo to inhibit IAAP labeling in the S1368A mutant PM vesicles. In the presence of clo, the IC₅₀ was 5.0 M in WT and 4.9 M in S1368A mutant vesicles. Therefore, although S1368A was profoundly clo-sensitive, a large scale distortion of the clo-binding region by this mutation is very unlikely.

R6G Transport in Cells—Although the S1368A mutant exhibited a WT level of ATPase activity, this mutant exhibited profound drug hypersensitivity to all of the tested compounds. In this regard, it was phenotypically similar to recently studied signaling mutants, such as S558Y (17), V656A (14), and D1042N (20). For instance, in a recent study, the D1042N mutant was 10 and 4.3 times more sensitive than the WT to clo and cyproconazole, respectively (20). Relative to the WT control, S1368A was ~5 and 6 times more sensitive to clo in two sets of experiments (Figs. 1 and 2). This mutant was also 5–6 times more sensitive to cyproconazole than the WT strain. Thus, D1042N and S1368A exhibited similar drug hypersensitivity. The S558Y, V656A, and D1042N mutants also showed very poor R6G efflux from whole cells. D1042N exhibited an efflux rate that was >10 times slower than the WT control (20). Therefore, we expected a similar result with S1368A. We compared the transport capability of the S1368A mutant with that of an isogenic control WT strain (Fig. 5A). Under these experimental conditions, transport takes place along an R6G concentration gradient. The difference between the mutant and WT was surprisingly modest. The R6G fluorescence half-life was ~2.7 min in the WT and 6.1 min in the mutant. S1368A mutant transport was therefore only ~2.3 times slower than in the WT. In Fig. 5B, we show a representative histogram plot of our data. The surprisingly strong transport behavior of the S1368A mutant was also concentration-independent. The data in Fig.
As a control, we loaded the Δpdr5 strain with 10 μM R6G. Following loading, the observed cell-associated R6G fluorescence was 1561 arbitrary units. After incubation for 30 min under conditions allowing efflux, this strain still retained 1294 arbitrary units (83% of the preloaded fluorescence). Therefore, the loss of fluorescence in WT and S1368A cells was due almost entirely to Pdr5-mediated R6G transport.

The ability of competing substrates to reduce R6G efflux was determined by measuring the concentration-dependent inhibition of R6G efflux was determined by measuring the cell-associated R6G fluorescence after 30 min of efflux. Shown are the R6G inhibition curves generated by imazalil (black line, WT; orange, red, dashed line, S1368A). The WT curves are the same ones found in Fig. 4A and are shown here for comparison. In these experiments, n = 2 for tebuconazole and imazalil, and n = 3 for 3,9-diacytcarbazole.

FIGURE 6. S1368A shows a much greater R6G transport deficiency when the external concentration is high regardless of time and buffer conditions. A, cell-associated R6G accumulation was measured after long term growth (20 h at 30 °C) in YPD (nutrient) medium containing varying concentrations of R6G (10−100 μM), as described under “Experimental Procedures.” Shown in each experiment are WT (black lines) and S1368A (red lines). B, the ratio of cell-associated fluorescence in S1368A/WT is shown for each concentration of R6G. C, the effect of R6G on the growth of WT and S1368A in YPD medium was determined (black lines, WT; red lines, S1368A; n = 3). D, efflux at various time points in YPD. Assays were performed as described under “Experimental Procedures.” Wild-type (black line) and mutant (red line) cells were suspended in YPD broth containing 20 μM R6G and incubated at 30 °C. The ratio of retained fluorescence in S1368A to WT is shown (n = 2).

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The ability of competing substrates to reduce R6G efflux was also largely unchanged in S1368A. The data in Fig. 5D show that inhibition of R6G by imazalil and tebuconazole yielded inhibition curves in the S1368A mutant strain that were similar to the isogenic WT control. Interestingly, there was a small difference between the strains when 3,9-diacytcarbazole was used as a competitive inhibitor. The S1368A mutant required about twice the concentration (200 μM) as the WT to achieve nearly full inhibition. The difference was too small to explain the difference in measures of drug hypersensitivity. These results provided additional evidence that the S1368A mutant does not affect drug binding to a large degree.

Because the initial rate of transport exhibited by the S1368A mutant was only mildly affected, but the drug-sensitive phenotypes were quite strong, we decided to test R6G efflux under conditions that were similar to the ones used to assay drug toxicity. We set up a series of parallel YPD cultures made with small inocula and varying concentrations of R6G. After a 20-h incubation, we analyzed the retained fluorescence under conditions allowing efflux, this strain still retained 1294 arbitrary units (83% of the preloaded fluorescence). Therefore, the loss of fluorescence in WT and S1368A cells was due almost entirely to Pdr5-mediated R6G transport.

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cience in S1368A versus the WT (Fig. 6B). At these relatively high concentrations of R6G, the mean ratios range from 21 to 30, but the values are not significantly different from each other because their S.E. values heavily overlap. The IC50 for S1368A was about 4 times lower than for WT (Fig. 6C). The Δpdr5 strain grew only at the 10 μM concentration of R6G (at about 25% of the WT). However, enough cells were available to determine the cell-associated fluorescence. The value of 1129 arbitrary units was similar to those observed with the Δpdr5 control used in the experiments described above in Fig. 5. The striking differences between the experiments shown in Figs. 5 and 6 implied that the S1368A was impaired to a much greater extent when the transport took place against the R6G concentration gradient.

Alternatively, because the experiments also differed in assay time and conditions (buffer versus YPD), these parameters might have caused the great disparity between the experiments found in Figs. 5 and 6. To determine which factor was the major explanation for the differences in our results, we performed a series of shorter time course experiments. In the first, we suspended 10⁶ cells in logarithmic growth phase in YPD broth with 20 μM R6G and sampled the cultures for growth and fluorescence at various intervals (Fig. 6D). Within the first 5 min of incubation in the presence of R6G, ~5 times more fluorescence appeared in S1368A cells than in the WT. At 90 min, the difference was 12 times.

In the second series of experiments, we performed a parallel set (90 min) of transport assays in either YPD or Hepes-glucose buffer. Fig. 6E shows the results for 20 μM R6G. The results with Hepes buffer were similar to those with YPD. In each case, the S1368A mutant cells exhibited ~13 times more fluorescence than the WT. Thus, the difference between the experiments in Figs. 5 and 6 was not due to differing effects of YPD broth and Hepes-glucose buffer.

Therefore, S1368A cells exhibited a severe transport deficiency when efflux occurred against a concentration gradient but only a mild one when the extracellular R6G was removed prior to efflux to observe transport along the resulting concentration gradient.

**Transport of [³H]R6G against a Concentration Gradient of Unlabeled R6G**—Our transport studies suggested that S1368A is particularly deficient when transport takes place against a concentration gradient, as in the experiments found in Fig. 6. To test this observation further, we loaded Δpdr5, WT, and S1368A cells with 20 μM R6G in the absence of glucose. Under these conditions, the three strains typically accumulated ~1600 arbitrary units. Following loading, we collected cells by centrifugation, removed the supernatant, and resuspended the cells in either YPD or Hepes minus glucose, each containing 20 μM unlabeled R6G, for 15 min. We determined the amount of retained [³H]R6G as described under “Experimental Procedures” (Fig. 7). In the absence of glucose, the strains showed approximately the same amount of label exchange (Fig. 7A). A one-way ANOVA test indicated that the differences between the three strains were not significant (p = 0.112).

When transport took place in YPD against a concentration gradient established by preloading the cells with unlabeled R6G (Fig. 7B), the WT strain, as expected, accumulated only a very
small amount of [3H]R6G. The mean value for nine independently grown WT cultures was 0.15 ± 0.03 pmol/10^7 cells, or only ~4.2% of the amount observed with passive exchange (3.50 ± 0.46 pmol/10^7 cells). In contrast, the nine independently grown S1368A cultures accumulated ~3 times more [3H]R6G than the WT control (0.40 ± 0.04 pmol/10^7 cells). A D’Agostino-Pearson omnibus test indicated that the data were consistent with a normal distribution. We therefore used an unpaired t test to compare the WT and S1368A mutant data. Our results were significant, with a p value of <0.001. Com pared with the WT strain, the ∆pdr5 strain accumulated about 12 times the amount of [3H]R6G (1.47 ± 0.40 pmol/10^7 cells), a value about half that observed as a result of passive exchange in Hepes minus glucose buffer.

In one experiment, we measured reflux after 15 and 90 min (Fig. 7C). The level of cell-associated [3H]R6G in the WT was small, and there was no significant change over this time interval (0.16 ± 0.01 pmol/10^7 cells at 15 min, 0.21 ± 0.09 pmol/10^7 cells at 90 min). In contrast, we observed a significant increase in S1368A mutant cell-associated [3H]R6G (0.45 ± 0.09 pmol/10^7 cells at 15 min, 1.17 ± 0.27 pmol/10^7 cells at 90 min). At 90 min, the ∆pdr5 strain had accumulated 3.49 ± 0.94 pmol/10^7 cells.

**DISCUSSION**

The S1368A mutation presents a novel phenotype never before seen in any ABC transporter. S1368A exhibited profound, broad multidrug hypersensitivity, and yet its ATPase activity was indistinguishable from WT, as was the allosteric inhibition (trans inhibition) of this enzyme by clo. Previously reported mutations in Pdr5 with broad hypersensitivity, such as K911A (Walker A), E1036Q, and G312A, exhibited either a strong reduction in steady-state ATPase activity (13, 20) or a loss of communication between the transmembrane domains and NBDs causing a large reduction in allosteric inhibition (14, 17). This includes the S1360F mutant, which is in the same TMH as S1368A (21, 24). The most surprising feature of this mutant, however, was its behavior in a series of R6G transport assays. The S1368A mutant was only modestly deficient when it was engaged in transport along an R6G concentration gradient. Therefore, the process of drug selection and the actual conformational changes required for transport appear largely unperturbed by this mutation. Mutants with hypersensitivity profiles very similar to that of S1368A, such as D1042N and V656A, that are involved in intradomain signaling or mutants that have little or no ATPase activity, such as E1036Q (Walker B) and G312A (Signature motif), have a profound R6G transport deficiency that mirrors their behavior in toxicity tests. For instance, when the D1042N mutant (which has broad drug hypersensitivity similar to S1368A) was tested for R6G efflux, the fluorescence half-life was >30 min compared with about 3 min for the WT (20). In contrast, the half-life of the R6G fluorescence in S1368A (Fig. 5A) was only 6.1 min.

However, the S1368A mutant transporter was greatly impaired when it had to work against a concentration gradient as indicated by two types of experiments. In the first, we incubated cells with a known concentration of R6G in glucose (YPD) medium and quantified the accumulation. In the second, we measured the uptake of radioactive R6G into cells during active extrusion of nonradioactive R6G. Under both conditions, S1368A accumulated ~5 times more R6G than the wild-type cells in a 15-min period (Figs. 6 and 7). As the transport time increased, so did the difference between these strains (Figs. 6D and 7C).

The most straightforward explanation for these observations is that Ser-1368 is not required for drug binding and transport per se but is essential for efficient net transport of xenobiotic compounds from the drug-binding pocket. The most appealing model that accords with our data is that this residue helps create a molecular diode or one-way gate in Pdr5 (15). According to this model, multidrug efflux pumps evolved a mechanism to avoid undesired reuptake of a drug that has just been transported. For instance, these transporters might do this through the collapse of the drug-binding regions prior to resetting of the transmembrane domains to their inward facing or high drug affinity conformation. The S1368A mutation, however, creates a leaky diode or gate. Fig. 8 illustrates the differences in the gating of the S1368A and WT strains. Nucleotide exchange results in the conformational switch from one that is inward facing and drug binding (Fig. 8, i) to one that is outward facing and drug-releasing (ii). ATP hydrolysis (iii) remodels the outward facing binding sites so that little or no drug can be bound when the transporter switches back (iv) to the inward facing conformation to begin a new transport cycle. The WT strain (Fig. 8A) has no difficulty functioning against a concentration gradient. In contrast, as a result of the S1368A substitution, Pdr5 does not remodel properly following ATP hydrolysis. When the external concentration of transported drug is relatively low (Fig. 8B), reflux is minimized. The phenotype is more severe when the extracellular concentration is high (Fig. 8C). Under these conditions, a significant amount of drug remains associated with the transporter when it switches back to the inward facing conformation. Thus, the drug leaks back into the cell. The decrease in net transport rate results over time in the accumulation of drugs in the cytoplasm or intracellular organelles. The S1368A mutant with its leaky gate therefore accumulates a severalfold higher concentration of cytotoxic drugs and exhibits broad, profound hypersensitivity.

An alternative explanation to the molecular diode model is its exact opposite; the S1368A substitution creates occlusion or trapping of drugs in the pump and slows their exit out of the cell. According to this explanation, however, S1368A should have exhibited a severely impaired transport phenotype when the cells were preloaded to a high concentration with R6G (the non-steady-state experiments found in Fig. 5), yet that was not the case. Furthermore, one would expect that a locked (clogged) conformation would affect the conformational freedom of the transporter and alter the behavior of its ATPase activity.

Both the reflux model and the less appealing occlusion model predict a concentration-dependent effect. Unfortunately, the relatively low specific activity of the commercially available [3H]R6G (and, therefore, a lack of sufficient sensitivity) precludes a direct test of concentration dependence.

The binding pocket of Pdr5 is a busy place with multiple drug-binding regions (23, 25) and intradomain signaling residues (24). The work in this report demonstrates that these
alone are insufficient for the successful removal of xenobiotic compounds from the cell.

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