

## The ATP Binding Cassette Transporters Pdr5 and Snq2 of *Saccharomyces cerevisiae* Can Mediate Transport of Steroids *in Vivo*\*

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Multiple or pleiotropic drug resistance in the yeast *Saccharomyces cerevisiae* can arise from overexpression of the Pdr5 and Snq2 ATP binding cassette multidrug transporters. Expression of Pdr5 and Snq2 is regulated by the two transcription factors Pdr1 and Pdr3, as multidrug-resistant *pdr1* and *pdr3* gain-of-function mutants overexpress both drug efflux pumps. One such *pdr1* mutant allele was previously cloned in a genetic screen by its ability to suppress the squelching toxicity mediated by an estradiol-inducible chimeric VP16-human estrogen receptor (VEO) expressed in yeast (Gilbert, D. M., Heery, D. M., Losson, R., Chambon, P., and Lemoine, Y. (1993) *Mol. Cell. Biol.* 13, 462–472).

In this study, we demonstrate that relief of estradiol toxicity in yeast cells expressing VEO requires functional *PDR5* and *SNQ2* genes, since a  $\Delta pdr5 \Delta snq2$  double deletion leads to an increased estradiol toxicity. Furthermore, using *URA3* as an estradiol-inducible reporter gene, we show that Pdr5 and Snq2, when overexpressed from high-copy plasmids, can reduce the intracellular concentration of estradiol. In contrast, a  $\Delta pdr5 \Delta snq2$  double deletion mutant accumulates almost 30-fold more intracellular estradiol than the isogenic wild type. Indirect immunofluorescence showed that a *pdr1-3* mutant massively overexpresses Pdr5 at the plasma membrane, suggesting that estradiol efflux from the cells occurs across the plasma membrane. Our data demonstrate that Pdr5 and Snq2 can transport steroid substrates *in vivo* and suggest that steroids and/or related membrane lipids could represent physiological substrates for certain yeast ABC transporters, which are otherwise involved in the development of pleiotropic drug resistance.

Pleiotropic drug resistance (PDR)<sup>1</sup> in yeast is a well documented phenomenon that appears quite similar to P-glycoprotein (Pgp or Mdr1) and MRP-mediated multidrug resistance in

mammalian cells (1, 2). For example, two homologous yeast ABC transporter genes, *PDR5* (3, 4) and *SNQ2* (5), were recently shown to represent functional and structural homologues of mammalian Mdr1 and MRP (6), since their overexpression in yeast is associated with PDR development. Elevated levels of Pdr5 and Snq2 lead to resistance against a variety of structurally unrelated cytotoxic compounds including mycotoxins (3), cycloheximide (7), 4-nitroquinoline *N*-oxide and sulfomethuron methyl (5). However, each transporter mediates resistance to only a distinct subset of drugs, and there is very little overlap in the substrate specificity of Pdr5 and Snq2 (3, 8).

Transcription of both *PDR5* and *SNQ2* is controlled by the transcription regulatory proteins Pdr1 and Pdr3 (4, 9, 10). Loss-of-function mutations such as  $\Delta pdr1$  and  $\Delta pdr3$  deletions result in a dramatic decrease of Pdr5 and Snq2 expression (11) and cause a marked drug hypersensitivity. In turn, several gain-of-function alleles of *pdr1* and *pdr3* have been isolated by their ability to confer a pleiotropic drug resistance phenotype (12, 13). In such drug-resistant *pdr* mutants, expression of the drug efflux pumps Pdr5 and Snq2 is dramatically increased (11), and it has been shown that *pdr1*-mediated cycloheximide resistance requires the presence of functional *PDR5* (14).

Notably, another *pdr1* mutant allele was previously cloned as a suppressor of the estradiol-induced squelching phenomenon caused by a VP16-estrogen receptor fusion protein (VEO) expressed in yeast (15). Induction by estradiol of such a chimeric protein, which has the hormone-binding and the DNA-binding domain of the human estrogen receptor fused to the acidic activation domain of the viral protein VP16, leads to a toxic transcriptional transactivation known as transcriptional squelching and causes lethality. The squelching phenomenon caused by an activator is thought to be due to its competition for a functional interaction with other limiting yet unknown transcription factors (15). The attempt to isolate such limiting factors from a yeast genomic library surprisingly resulted in the repeated cloning of a *pdr1* gain-of-function allele (15). One possible mechanism for the Pdr1-mediated suppression of VEO toxicity would be that Pdr1 itself represents the limiting transcriptional intermediary factor interacting with VEO (15). However, the authors proposed that overexpression of estradiol-specific efflux pumps in the isolated *pdr1* mutant would be a more likely explanation for the apparent squelching suppression (15).

In this report, we show by using two different experimental strategies that gene dosage variation of *PDR5* and *SNQ2*, both of which were shown to be targets for the transcription factors Pdr1 and Pdr3 (11), can efficiently modulate the intracellular estradiol concentration. Our results demonstrate that suppression of VEO squelching by a *pdr1* gain-of-function allele is the consequence of the overexpression of estradiol-exporting efflux

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<sup>1</sup> The abbreviations used are: PDR, pleiotropic drug resistance; MDR, multidrug resistance; ABC, ATP binding cassette; E2, estradiol; ERE, estrogen-response element; 5-FOA, 5-fluoroorotic acid; hER, human estrogen receptor; VEO, chimeric receptor containing the DNA binding domain and hormone binding domain of the hER and the acidic activation domain of VP16; kb, kilobase(s).

TABLE I  
Genotypes of strains used in this study

Strain	Genotype	Parental strain	Ref.
PL3	<i>MAT<math>\alpha</math> ura3-<math>\Delta</math>1 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 trp1::3ERE-URA3</i>		20
YPH500	<i>MAT<math>\alpha</math> ura3-52 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 trp1-<math>\Delta</math>63 lys2-801<sup>amb</sup> ade2-101<sup>oc</sup></i>		17
YKKB-13	<i>MAT<math>\alpha</math> ura3-52 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 trp1-<math>\Delta</math>63 lys2-801<sup>amb</sup> ade2-101<sup>oc</sup> <math>\Delta</math>pdr5::TRP1</i>	YPH500	3
YYM3	<i>MAT<math>\alpha</math> ura3-52 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 trp1-<math>\Delta</math>63 lys2-801<sup>amb</sup> ade2-101<sup>oc</sup> <math>\Delta</math>pdr5::TRP1 <math>\Delta</math>snq2::hisG</i>	YKKB-13	11
YYM5	<i>MAT<math>\alpha</math> ura3-52 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 trp1-<math>\Delta</math>63 lys2-801<sup>amb</sup> ade2-101<sup>oc</sup> <math>\Delta</math>snq2::hisG</i>	YPH500	This study
YALA-B1	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his3-11,115 trp1-1 PDR1</i>		11
YALA-G4	<i>MAT<math>\alpha</math> ura3-52 leu2-3,112 his3-11,115 trp1-1 pdr1-3</i>		11
YYM6	<i>MAT<math>\alpha</math> ura3-<math>\Delta</math>1 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 trp1::3ERE-URA3 <math>\Delta</math>snq2::hisG</i>	PL3	This study
YYM7	<i>MAT<math>\alpha</math> ura3-<math>\Delta</math>1 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 trp1::3ERE-URA3 <math>\Delta</math>pdr5::hisG</i>	PL3	This study
YYM8	<i>MAT<math>\alpha</math> ura3-<math>\Delta</math>1 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 trp1::3ERE-URA3 <math>\Delta</math>pdr5::hisG <math>\Delta</math>snq2::hisG</i>	YYM7	This study

pumps such as Pdr5 and Snq2. Furthermore, our results suggest that steroid derivatives or related membrane lipids could represent physiological substrates for the yeast ABC transporters Pdr5 and Snq2.

#### EXPERIMENTAL PROCEDURES

**Media, Culture Conditions, and Spot Tests**—Rich medium (YPD) and synthetic medium (SD), supplemented with auxotrophic components, as well as medium containing 5-FOA (5-fluoroorotic acid) were prepared essentially as described elsewhere (16). Yeast strains to be tested for growth on agar plates containing estradiol were grown to exponential growth phase. Identical volumes of a cell suspension ( $A_{600} = 0.025$ ), as well as a 1:10 dilution of the culture, were spotted onto the plates. Estradiol was prepared as a 2 mM stock dissolved in absolute ethanol or freshly in dimethyl sulfoxide.

**Yeast Strains and Transformations**—All *S. cerevisiae* strains including the parental wild type strain YPH500 (17) used in this study are listed in Table I. Strains harboring  $\Delta$ snq2::hisG and  $\Delta$ pdr5::hisG deletions were constructed in a one-step gene replacement procedure (18) by transforming the corresponding parental strains with the *SacI-SalI*  $\Delta$ snq2::hisG-URA3-hisG and *SalI-XbaI*  $\Delta$ pdr5::hisG-URA3-hisG fragments obtained from pYM25 and pYM31, respectively. Transformants were grown on minimal plates containing uracil and 5-FOA to select for the desired  $\Delta$ snq2::hisG and  $\Delta$ pdr5::hisG deletion strains. Correct integration of the deletion constructs and proper looping-out was confirmed by PCR analysis of genomic DNA isolated from several transformants (19).

Construction of strain PL3, in which transcription of the *URA3* reporter gene can be induced by estradiol via three estrogen-responsive elements in the promoter (*3ERE-URA3*), has been described in detail elsewhere (20). Transformations of yeast strains were carried out essentially as described previously (21), except for transformations with the VEO (VP16-estrogen receptor fusion) expressing plasmid pDGY3, which were performed using the spheroplast method (16). Several pDGY3 transformants were tested for reversion to estradiol resistance on appropriate selective plates containing 100  $\mu$ M estradiol. Assays for growth phenotypes on estradiol plates were carried out only with fresh transformants with a reversion frequency of less than  $10^{-6}$ .

**Plasmid Constructions**—The plasmids pDGY3 (2 $\mu$ , *dLEU2*, VP16-estrogen receptor fusion) (15), YE

90 (2 $\mu$ , *HIS3*), and YE

90HEGO (2 $\mu$ , *HIS3*, human estrogen receptor) (20) were described in the references cited. Plasmid pYM40 was constructed by inserting *PDR5* as a 5.8-kb *SphI-AvrII* fragment of pYSTS1 (3) into the *SphI-XbaI* sites of YEplac181 (2 $\mu$ , *LEU2*). The *SNQ2* gene was cloned as a 6.8-kb *XhoI-SphI* fragment obtained from pEH22 (22) into the corresponding sites of YEplac181, resulting in plasmid pYM36.

The  $\Delta$ pdr5::hisG-URA3-hisG deletion plasmid was constructed as follows. A 4-kb *EcoRI-BamHI* fragment containing the *hisG-URA3-hisG* element of plasmid pYM24 (23) was inserted in the *EcoRI-BamHI* sites of pSP73 (Promega), to yield plasmid pYM28. An internal 2.5-kb *BglIII* sequence of *PDR5* in pBluescript was then replaced by the 4-kb *BamHI-BglIII* *hisG-URA3-hisG* fragment obtained from pYM28 to generate plasmid pYM31. The construction of the  $\Delta$ snq2::hisG-URA3-hisG disruption plasmid pYM25 has been described elsewhere (11).

**Immunoblotting of Yeast Extracts and Indirect Immunofluorescence Analysis**—The preparation of yeast cell extracts for immunoblotting was carried out exactly as described previously (23). Total extracts equivalent to 0.5  $A_{600}$  of cells were electrophoresed through a 6% SDS-polyacrylamide gel (24) and transferred to nitrocellulose membranes by standard methods (25). Proteins on immunoblots were visualized using the ECL chemoluminescence detection system (26).

Polyclonal antibodies recognizing Pdr5 (23) and Snq2 (11) were de-

scribed in the references cited. Antibodies against the plasma membrane ATPase Pma1 were generously provided by Ramon Serrano and used as described elsewhere (23). For indirect immunofluorescence analysis and subcellular localization of Pdr5, affinity-purified (27) polyclonal anti-Pdr5 antiserum was employed using published experimental conditions (23).

**In Vivo Estradiol Accumulation Assay**—[<sup>3</sup>H]Estradiol (specific activity 151 Ci/mmol; Amersham) was added to a final concentration of 2 nM to 2-ml cultures of logarithmically growing yeast cells (1.0  $A_{600}$ ). [<sup>3</sup>H]Estradiol uptake into cells was allowed for 1 h at 30 °C with gentle shaking. Cells were washed 4 times with cold PBS, 2% glucose by brief centrifugation steps and finally resuspended in 50  $\mu$ l of PBS. Intracellular amounts of [<sup>3</sup>H]estradiol were quantified by liquid scintillation counting of whole yeast cells using a Packard 1600 TR Tri-Carb counter.

#### RESULTS

**Disruption of PDR5 and SNQ2 Increases VEO-mediated Estradiol Toxicity**—Searching a yeast genomic library for genes that could provide relief of the toxicity mediated by VEO, a hER-VP16 fusion protein, resulted in the repeated cloning of a *pdr1* allele (15). Pdr1, a transcriptional regulator that can mediate PDR in yeast, has been shown to regulate the expression of several drug pumps, including the ABC transporters Pdr5 and Snq2 (9, 11). The *pdr1* allele cloned by Gilbert *et al.* (15) was also shown to confer increased resistance to cycloheximide, a known Pdr5 substrate, implying that overexpression of Pdr5 in the *pdr1* mutant might actually diminish VEO toxicity. Therefore, we wanted to test if VEO toxicity on media containing estradiol is increased in strains that do not express functional Pdr5 or Snq2 pumps. In these strains, the intracellular availability of estradiol should be increased, and, consequently, VEO toxicity should be elevated.

The pDGY3 plasmid encoding the VEO fusion protein was transformed into isogenic yeast strains in which *PDR5*, *SNQ2*, or both genes were deleted. Several fresh transformants were assayed for their growth phenotype on estradiol-containing medium. As shown in Fig. 1, growth of the  $\Delta$ pdr5  $\Delta$ snq2 double disruptant was severely reduced on plates containing 10–25 nM estradiol, whereas isogenic strains with single deletions of either *PDR5* or *SNQ2* did not differ in growth compared to the wild type strain. Increasing the estradiol concentration to 50–100 nM led to a complete cell growth arrest by VEO in all strains examined (data not shown). These results demonstrate that the intracellular estradiol concentration was elevated in the absence of functional *PDR5* and *SNQ2*, which led to an increased activation of the toxic VEO fusion protein. Furthermore, these data suggest that the relief of VEO toxicity by the *pdr1* allele cloned by Gilbert *et al.* (15) is most likely due to the overexpression of estradiol-specific efflux pumps such as Pdr5 and Snq2, preventing the accumulation of estradiol and the induction of VEO toxicity.

**Overexpression of Pdr5 and Snq2 Decreases the Intracellular Availability of Estradiol**—As shown above, the absence of functional *PDR5* and *SNQ2* genes leads to an enhanced estradiol-mediated VEO toxicity. To show that overexpression of both Pdr5 and Snq2 can reduce intracellular estradiol concentra-

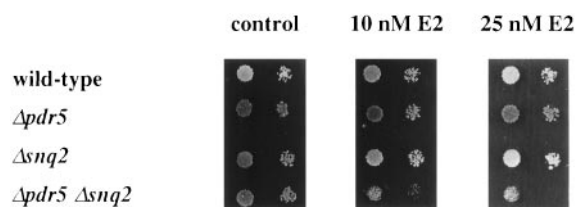


FIG. 1. **Increased estradiol toxicity mediated by VEO in a  $\Delta pdr5 \Delta snq2$  disruptant.** Exponentially grown cultures of YPH500 (wild type), YKKB-13 ( $\Delta pdr5$ ), YYM5 ( $\Delta snq2$ ), and YYM3 ( $\Delta pdr5 \Delta snq2$ ) expressing the VEO fusion protein were tested for growth on selective plates in the absence (control) or presence (10 nM and 25 nM) of the indicated estradiol concentrations.

tions and thereby suppress transcriptional squelching, we used a yeast strain whose growth is essentially estradiol-dependent on a medium lacking uracil (20). In this strain, *URA3* serves as a reporter gene for the presence of intracellular estradiol, since transcriptional activation of *URA3* is induced by the human estrogen receptor (hER) in an estradiol-dependent manner via three estrogen-responsive elements (*3ERE*) placed upstream of the *URA3* gene. This reporter strain expressing the full-length hER is completely estradiol-dependent for growth on a medium lacking uracil (20). In contrast, its growth is inhibited by 5-FOA, since the *URA3* gene product converts 5-FOA to a toxic metabolite.

To test whether or not increased *PDR5* and *SNQ2* gene dosage can modulate intracellular estradiol concentrations, we transformed the reporter strain containing *3ERE-URA3* and hER with high-copy *PDR5* and *SNQ2* plasmids, along with the appropriate control plasmids. Serial dilutions of several independent transformants were assayed for growth in the presence or absence of estradiol on selective plates either lacking uracil or containing uracil plus 5-FOA. As shown in Fig. 2A, strains harboring a control plasmid (lanes marked – in Fig. 2) were unable to grow on plates lacking uracil, whereas strains expressing the hER were able to grow on plates lacking uracil only in the presence of estradiol; in the absence of estradiol, these strains also failed to grow (data not shown). Strains expressing hER plus *PDR5* or *SNQ2* from a high-copy plasmid were unable to grow on media lacking uracil when the estradiol concentration was low (1 nM) compared to the strain containing the empty 2 $\mu$  vector. At elevated estradiol concentrations, all strains expressing the hER containing empty 2 $\mu$  vector, 2 $\mu$  *PDR5*, or 2 $\mu$  *SNQ2* were able to grow on medium lacking uracil (data not shown). Taken together, these results suggest that intracellular estradiol is effluxed from the cells by the plasma membrane pumps Pdr5 and Snq2.

Analysis of the strains for growth on medium supplemented with uracil and containing 5-FOA, both with and without estradiol, gave similar results (Fig. 2B). In the presence of estradiol, the strain expressing the hER did not grow on 5-FOA, since transcriptional activation of the *URA3* gene results in a Ura3-mediated production of a metabolite toxic to yeast cells. Overexpression of *SNQ2* appeared to relieve this toxic effect (Fig. 2B), while the *PDR5* overexpressing strain failed to grow under the same conditions. However, *PDR5* overexpression seemed to affect growth on 5-FOA regardless of the presence of estradiol, because growth of the *PDR5* overexpressing strain was slightly inhibited on the control plate lacking estradiol.

To compare the expression levels of Pdr5 and Snq2 in different strains, we performed immunoblot analysis with the set of strains analyzed in Fig. 2 (Fig. 3). Immunoblotting revealed that Pdr5 expression was only slightly increased in the strain harboring the 2 $\mu$  *PDR5* plasmid when compared to the strain with the control vector. In contrast, the 2 $\mu$  *SNQ2* plasmid led to a dramatic Snq2 overexpression when compared to wild type

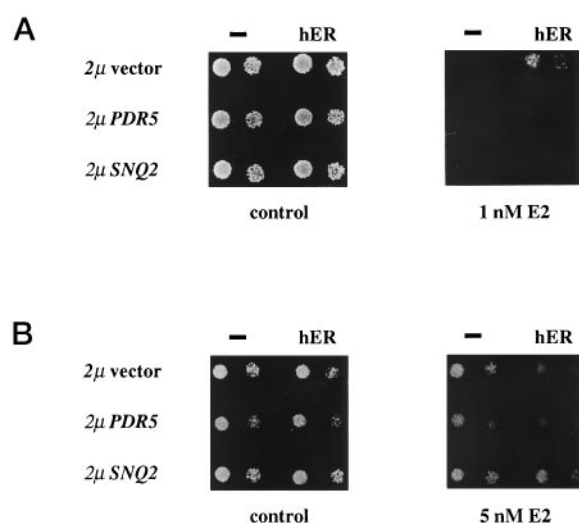


FIG. 2. **Overexpression of Pdr5 and Snq2 inhibit estradiol-mediated *URA3* induction.** The reporter strain PL3 (*3ERE-URA3*) carrying YEp90 (–) or YEp90HEGO (*hER*) was transformed with the empty vector YEplac181 (2 $\mu$  vector), the *PDR5* overexpressing plasmid pYM40 (2 $\mu$  *PDR5*) or the *SNQ2* overexpressing plasmid pYM36 (2 $\mu$  *SNQ2*). Transformants were analyzed for their estradiol-dependent growth phenotype by spot tests. A, growth phenotypes on plates selecting for plasmid maintenance (control) and on a plate lacking uracil containing estradiol (1 nM E2). B, growth phenotypes on selective 5-FOA plates in the absence (control) or in the presence of estradiol (5 nM E2).

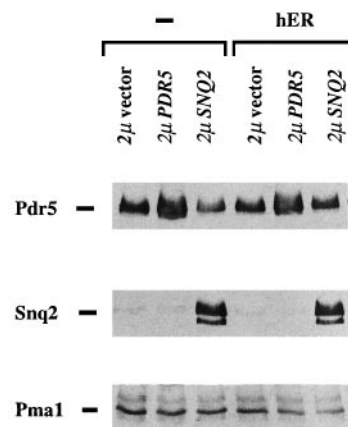
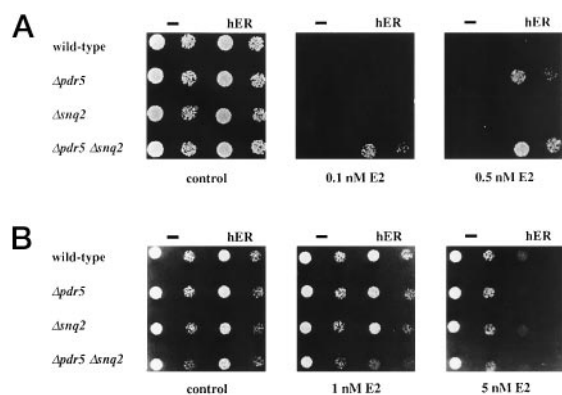


FIG. 3. **Protein levels of Pdr5 and Snq2 in the PL3 reporter strain.** Total cell extracts were prepared from strain PL3 transformed with both YEp90 (–) or YEp90HEGO (*hER*) and with the empty vector YEplac181 (2 $\mu$  vector), the *PDR5* overexpressing plasmid pYM40 (2 $\mu$  *PDR5*), or the *SNQ2* overexpressing plasmid pYM36 (2 $\mu$  *SNQ2*). The extracts were analyzed by immunoblotting using polyclonal antibodies against Pdr5 and Snq2. Equivalent protein loading in each lane was verified by immunoblotting with polyclonal anti-Pma1 antibodies (*Pma1*).

levels. Immunological detection of the plasma membrane ATPase Pma1 served as a control for equal protein loadings in each lane. Thus, the expression levels of Pdr5 and Snq2 were not comparable in these strains. Consequently, the results do not allow for an accurate assessment concerning the individual contribution of Pdr5 and Snq2 to modulating intracellular estradiol levels.

**Major Role of Pdr5 in Modulating the Intracellular Estradiol Concentration**—To determine the individual contributions of *PDR5* and *SNQ2* to the modulation of intracellular estradiol concentrations, we constructed *3ERE-URA3* reporter strains deleted for *PDR5*, *SNQ2*, or both ABC transporter genes. The isogenic set of PL3-derived strains were then transformed with YEp90 vector alone and YEp90HEGO (*hER*) and analyzed for

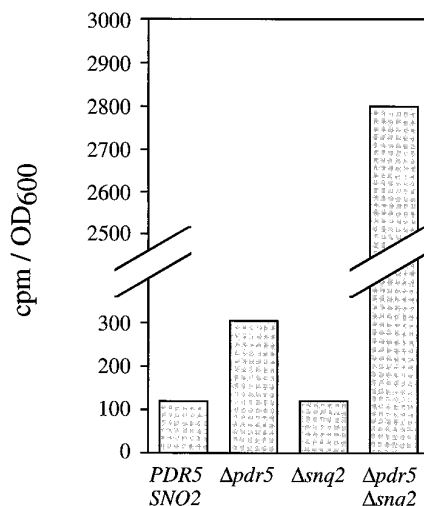


**FIG. 4. Predominant role of Pdr5 in the modulation of intracellular estradiol.** Isogenic *3ERE-URA3* reporter strains PL3 (wild-type), YYM7 ( $\Delta pdr5$ ), YYM6 ( $\Delta snq2$ ), and YYM8 ( $\Delta pdr5 \Delta snq2$ ) were transformed with YE<sub>p</sub>90 (–) or YE<sub>p</sub>90HEGO (*hER*) and analyzed for an estradiol-dependent growth phenotype. *A*, growth phenotypes on plates selecting for plasmid maintenance (*control*), and on plates lacking uracil with the indicated concentrations of estradiol (0.1 nM E2) and (0.5 nM E2). *B*, growth phenotypes on selective 5-FOA plates in the absence (*control*) or in the presence of the indicated estradiol concentrations (1 nM E2) and (5 nM E2).

their estradiol-dependent growth phenotype on a medium lacking uracil (Fig. 4A) and on a medium containing 5-FOA (Fig. 4B). As shown in Fig. 4A, a  $\Delta pdr5 \Delta snq2$  double deletion strain expressing the *hER* was able to grow on medium containing very low levels of estradiol (0.1 nM), reflecting the increased intracellular concentration of estradiol. Disruption of *PDR5* apparently had a major effect on the accumulation of estradiol, since a  $\Delta pdr5$  single disruptant strain was able to grow at 0.5 nM estradiol, whereas the  $\Delta snq2$  single disruptant failed to grow at this concentration. Increasing the estradiol concentration led to growth of all strains, and we were unable to find conditions that allowed for growth of the  $\Delta snq2$  single disruptant but not the wild type strain (data not shown). Nevertheless, the marked difference in the estradiol-dependent growth phenotypes of the single disruptants *versus* the  $\Delta pdr5 \Delta snq2$  double deletion strain indicate a synergistic action of Pdr5 and Snq2 in influencing the intracellular estradiol concentrations. In fact, this is further supported by results obtained from analyzing the estradiol-dependent growth characteristics on 5-FOA-containing medium (Fig. 4B). The  $\Delta pdr5 \Delta snq2$  double-disrupted strain showed a severe growth deficiency at 1 nM estradiol, whereas both single disruptants could grow under the same conditions (Fig. 4B). Although these results show that Pdr5 seems to play a major role in effluxing estradiol from cells, it seems obvious that both ABC transporters appear to contribute to this process.

**Synergistic Contribution of Pdr5 and Snq2 to Modulating Intracellular Estradiol Levels**—As shown above, deletion of both membrane pumps led to elevated intracellular estradiol, which also resulted in an increased estradiol-dependent modulation of both VEO and the *URA3* reporter gene expression. These results suggest that Pdr5 and Snq2 may act as estradiol exporting pumps. To further investigate this idea, we directly quantified the estradiol accumulation by an *in vivo* estradiol-uptake assay using [<sup>3</sup>H]estradiol.

As shown in Fig. 5, [<sup>3</sup>H]estradiol accumulation is increased about 3-fold in a  $\Delta pdr5$  deletion strain, whereas a  $\Delta snq2$  deletion does not significantly change the estradiol accumulation when compared to wild type levels. However, deletion of *SNQ2* in a  $\Delta pdr5$  background dramatically potentiates the estradiol accumulation observed in a  $\Delta pdr5$  single-deletion strain, leading to almost 10-fold and 30-fold elevated intracellular [<sup>3</sup>H]estradiol in the  $\Delta pdr5 \Delta snq2$  double disruptant over the  $\Delta pdr5$



**FIG. 5. [<sup>3</sup>H]Estradiol uptake and accumulation in a  $\Delta pdr5 \Delta snq2$  disruptant.** Exponentially growing cultures of YPH500 (*PDR5 SNQ2*), YKKB-13 ( $\Delta pdr5$ ), YYM5 ( $\Delta snq2$ ), and YYM3 ( $\Delta pdr5 \Delta snq2$ ) were incubated with [<sup>3</sup>H]estradiol for 1 h. Intracellular amounts of [<sup>3</sup>H]estradiol were quantified by liquid scintillation counting.

and  $\Delta snq2$  single mutants, respectively (Fig. 5). In agreement with the results obtained from assaying the growth phenotypes of the *URA3* reporter strains (Fig. 4A), these data show that Pdr5 can modulate the intracellular estradiol levels, presumably by mediating cellular efflux of the steroid. Moreover, these results indicate that Snq2, although it does not appear to have a major influence on estradiol export on its own, contributes to intracellular estradiol modulation in a synergistic manner only in concert with Pdr5.

**Pdr5 Is Localized to the Plasma Membrane in *pdr1-3* Mutants**—We have previously demonstrated that Pdr5 is localized to the plasma membrane in wild type yeast cells (23). In addition, we have shown that *pdr1-3* gain-of-function mutants overexpress Pdr5 and Snq2 by about 10-fold over wild type levels (11). However, it has been reported that overexpression of certain yeast proteins can result in mislocalization (28). To test whether the normal Pdr5 localization to the plasma membrane is affected in *pdr1-3* mutants, we performed indirect immunofluorescence on both a wild type and a *pdr1-3* mutant strain, which, like the *pdr1* mutant allele cloned by Gilbert *et al.* (15), exhibits increased cycloheximide resistance (11, 14).

As shown in Fig. 6, both the wild type and the *pdr1-3* strain display a ring-like fluorescence signal typical for plasma membrane localization. However, consistent with a dramatic overexpression of Pdr5, the signal is much more intense in the *pdr1-3* mutant strain. These results demonstrate that Pdr5 is not only overexpressed in *pdr1-3* mutants, but also that it is correctly localized and targeted to the plasma membrane as observed in the wild type situation. These data suggest that the mechanism of intracellular steroid depletion is likely to be Pdr5-mediated efflux across the plasma membrane to the extracellular space.

## DISCUSSION

In this report we show that Pdr5 and Snq2, two yeast ABC transporters associated with high-copy-mediated pleiotropic drug resistance are able to modulate intracellular estradiol levels. We took advantage of two different experimental systems allowing for monitoring intracellular estradiol concentrations *in vivo*. First, a human estrogen receptor-VP16 chimeric fusion protein that leads to cell growth arrest upon induction by estradiol (15); second, a *3ERE-URA3* reporter strain whose growth on a medium lacking uracil is entirely estradiol-de-

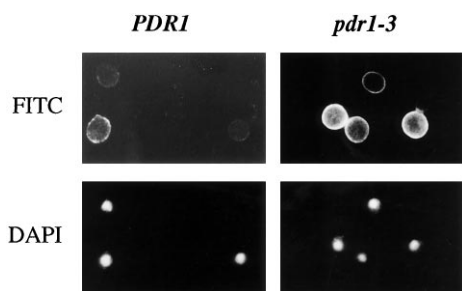


FIG. 6. **Pdr5 is overexpressed in the plasma membrane of a *pdr1-3* mutant.** Indirect immunofluorescence of Pdr5 in wild type YALA-B1 (*PDR1*) and mutant YALA-G4 (*pdr1-3*) cells. Cells were fixed, spheroplasted, and incubated with affinity-purified anti-Pdr5 antibodies. Pdr5 was visualized with fluorescein isothiocyanate-conjugated secondary goat anti-rabbit IgG (*FITC*). Nuclear DNA was visualized by staining with 4,6-diamino-2-phenylindole hydrochloride (*DAPI*).

pendent (20). In both reporter systems, we demonstrated that deletions of *PDR5* and *SNQ2* potentiate the action of estradiol, whereas overexpression of these genes inhibited the action of estradiol in the *3ERE-URA3* reporter strain. The simplest explanation for these results is that Pdr5 and Snq2 can act as estradiol-specific efflux pumps thereby counteracting the intracellular estradiol accumulation.

Another possible explanation for our results is that Pdr5 overexpression causes its mislocalization or accelerated endocytic delivery to the vacuole where Pdr5 is normally degraded (23). This could lead to an enhanced transport of estradiol to the vacuole, but not the extracellular space. Thus, estradiol resistance would be the consequence of its accumulation in the vacuole rather than to cellular efflux. However, the present work demonstrates that Pdr5, even when drastically overexpressed like in *pdr1* gain-of-function mutants (11), is localized at the cell surface as observed for the wild type situation. Therefore, our data indicate that the suppression of inducible VEO toxicity by the *pdr1* allele could be attributable to increased estradiol efflux via the plasma membrane mediated by Pdr5 and by the related Snq2 membrane ABC pump. In agreement with that, we have shown that a  $\Delta pdr5 \Delta snq2$  strain accumulates about 30-fold more intracellular estradiol than a wild type strain due to the lack of efflux pumps at the cell surface. Although we consider this possibility highly unlikely, we cannot entirely exclude that depletion of intracellular steroids is the consequence of changes in membrane lipid composition and/or permeability caused by gene dosage variation of *SNQ2* and *PDR5* rather than an active transport process across the plasma membrane. First, a reduced membrane permeability would likely result in an impaired steroid uptake in the  $\Delta pdr5 \Delta snq2$  mutant, which is the opposite of what we observed. Second, in the case of an increased membrane permeability, one would not expect the drastically enhanced estradiol accumulation as observed in the  $\Delta pdr5 \Delta snq2$  mutant.

Interestingly, a genetic screen for mutants enhancing the responsiveness to dexamethasone of the rat glucocorticoid receptor expressed in yeast recently resulted in the isolation of a *pdr5* (*lem1*) loss-of-function mutant (29). This report identified the *PDR5* gene as a modulator for the intracellular levels of glucocorticoids such as dexamethasone. Surprisingly though, the authors reported that the *pdr5* mutation had no significant effect on the intracellular accumulation of estradiol. These results appear in contradiction to our observations. However, it could be that the *pdr5* mutant isolated by Kralli *et al.* (29) only affects certain Pdr5 substrates such as dexamethasone and corticosterone, whereas recognition and transport of other steroid substrates including estradiol is not affected by this mutation. Hence, we suggest that the transport of different sub-

strates, including drugs and steroids, is brought about by a dedicated substrate specificity that involves several binding sites or binding zones within the folded Pdr5 transporter in the plasma membrane. Notably, a G185V mutation in the human P-glycoprotein also leads to a dramatic switch in substrate specificity causing preferential recognition of colchicine (30, 31), whereas wild type Mdr1 mediates transport of a variety of different cytotoxic drugs (1), including bioactive peptides such as the yeast  $\alpha$ -factor peptide mating pheromone (32, 33).

In summary, our studies and those carried out by others (29), reveal a new class of substrates, namely steroids and glucocorticoids, for the yeast ABC pumps Pdr5 and Snq2, and confirm earlier suggestions that both transporters have additional functions to their proposed role in cellular detoxification (3). This is exciting, since it uncovers yet another common functional feature between Pdr5, Snq2, and mammalian Mdr1 and MRP, whose overexpression render tumors and cultured cells resistant to a variety of cytotoxic drugs (1). Interestingly, dexamethasone-induced apoptosis in mouse lymphoma cells is linked to overexpression of the mouse *mdr1* gene (34). Moreover, human Mdr1, like Pdr5, can also transport steroids, glucocorticoids, and mineralocorticoids (30, 31, 35) both *in vitro* and *in vivo*, and it was suggested that the physiological function of P-glycoprotein in the human adrenal cortex might involve the transport and/or targeted secretion of steroid hormones or other membrane lipids (31). In fact, by analogy to *PDR5*, it was proposed earlier that Pgp or similar ABC transporters in mammalian cells could be controlling glucocorticoid potency and steroid hormone action by cell- and/or substrate-specific efflux of receptor ligands (36). Indeed, a yet unidentified ATP-dependent export system for steroids such as cortisone and dexamethasone has been reported in mammalian L929 cells (37). Like yeast Pdr5 (36), the steroid export system in L929 cells can be inhibited by the immunosuppressive agent FK506. Based on this finding, the authors suggest that similar mechanisms may account for the glucocorticoid efflux both in yeast and in L929 cells (36).

Although a classical steroid hormone signaling pathway as it exists in mammalian cells has not been described in yeast, steroid derivatives must have an important role in yeast. Actually, the most important sterol in yeast, ergosterol, represents the main sterol compound of the plasma membrane (38). Thus, it is tempting to speculate that the yeast Pdr5 and Snq2 ABC transporters or newly identified yeast homologues, which now include Pdr10, Pdr11, Pdr12, and Pdr15 (39, 40), may somehow play a role in modulating the cell surface sterol composition, thereby regulating the permeability and rigidity of the plasma membrane. Several lines of evidence seem to support this idea. First, very recent findings show that progesterone, a steroid which is normally not a substrate for human Mdr1 although it can tightly bind to the transporter (31), can inhibit cholesterol biosynthesis in cultured cells (41). A model was proposed according to which cholesterol biosynthesis is impaired in progesterone-treated cultured cells, since Mdr1-mediated transport of cholesterol precursors like lanosterol from the plasma membrane to the endoplasmic reticulum, the site of cholesterol biosynthesis, is severely reduced due to the specific Mdr1 inhibition by progesterone (42). Second, another close homologue of human Mdr1, Mdr2 (43), is also believed to mediate the transmembrane transport and/or flipping of membrane lipids including phosphatidylcholine and cholesterol or even cholesterol metabolites such as bile acids (44). Homozygous *mdr2* knock-out mice develop a severe liver disease due to the lack of lipid transport into the bile via the canalicular membrane (45). There is also evidence that the mouse *mdr2* analogue, when functionally expressed in yeast secretory ves-

icles, may act as a phosphatidylcholine-specific flippase *in vitro* (46).

Data presented in this work as well as work from other laboratories (29) have demonstrated that the yeast ABC transporters Pdr5 and Snq2 can mediate the transmembrane transport of steroids and glucocorticoids *in vivo*. Ongoing studies in our laboratory attempt to test the idea of a specific involvement of yeast ABC drug efflux pumps in maintaining a certain plasma membrane ergosterol composition. The availability of yeast strains deleted for steroid-specific efflux pumps such as Pdr5 and Snq2 also provides us with tools to molecularly identify by functional cloning in yeast their mammalian counterparts, some of which could act as steroid efflux pumps *in vivo*, thereby playing an important role in regulating both steroid hormone action and response in mammalian cells.

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