Cdr1p IS A VERSATILE TRANSPORTER
3.2 Cdr1p is a versatile transporter

3.2.1 Introduction

The studies involving MDR in yeast have attracted much attention, since some of the species are also pathogenic to humans. *Candida albicans* is a dimorphic, opportunistic human pathogen, which is naturally more resistant to several drugs than *S. cerevisiae* (Prasad, et al., 1996). In recent years, the incidence of *C. albicans* cells acquiring resistance to azoles and polyenes has increased considerably (Prasad, et al., 1996; Vanden Bossche, 1995; Hitchcock, 1993). In this regard, the cloning and sequencing of a multidrug transporter, *CDRI* (*Candida Drug Resistance*) in *C. albicans*, which is a homologue of *S. cerevisiae* multidrug efflux pump *PDR5* (Balzi and Goffeau, 1995; Balzi and Goffeau, 1994), was an important step towards understanding the mechanism of drug resistance (Prasad, et al., 1995b). A few more homologues of *CDR1* have been identified recently in *C. albicans* (Walsh, et al., 1997; Sanglard, et al., 1997) and few genes specific to fluconazole and benomyl resistance have also been characterised (Sanglard, et al., 1996; Prasad, et al., 1995; Prasad, et al., 1996; Fling, et al., 1991). The knowledge of physiological substrates and role of MDRs continue to be elusive. Except for human MDR1, which is a general phospholipid translocator and steroid transporter (Ueda, et al., 1992; Van Helvoort, et al., 1996) and MDR2, which is a specific phosphatidylcholine translocator, between lipid monolayers (Ruetz and Gros, 1994c), none of the characterized genes is so distinctly identified with its functions. Yeast MDRs are no exception and from host of genes identified as putative efflux pumps, none could be related with their physiological roles, the only exception being STE6, of *S. cerevisiae* (Balzi and Goffeau, 1995; Balzi and Goffeau, 1994). STE6 codes for an ABC protein and is known to export mating factor in yeast (Kuchler, et al., 1989). The fact that *S. cerevisiae* having disrupted *SNQ2* and *PDR5*, shows higher accumulation of estradiol, as compared to their parental strain, suggests that steroids could be physiological substrates for these ABC pumps (Kolaczkowski, et al., 1996; Mahe, et al., 1996).

Though Cdr1p is a homologue of PDR5p and SNQ2p, it has different profile of drug resistance and regulation of its activity. PDR1 and PDR3, which are well known transcription factors which regulate *PDR5* expression, do not have homologous binding domains in *CDR1*
Results and Discussion

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3.2.2.1 Cdr1p mediates efflux of β-estradiol and corticosterone

*S. cerevisiae* strain JG436, which had disrupted *PDR5* and was hypersensitive to cycloheximide, was used as a host to express *CDRI* of *C. albicans*. The resulting strain S-12-4, hyper resistant to cycloheximide and other drugs (Prasad, et al., 1995b), was used to assess whether Cdr1p could transport human steroid hormones. It is pertinent to mention here that steroids, which are small hydrophobic molecules, enter *S. cerevisiae* cells by diffusion (Mahe, et al., 1996; Kolaczkowski, et al., 1996). It is evident from Fig 25a & b and Fig 26a & b that JG436, which did not have functional *PDR5* or *CDR1*, showed steady accumulation of [³H]-β-estradiol and [³H]-corticosterone. However, due to continued efflux of these substrates, strain S-12-4 showed much less accumulation (at 60 min, JG436 had 19 pmoles/mg dry weight of [³H]-β-estradiol while it was 7.0 pmoles/mg dry weight in S-12-4). Similarly, [³H]-corticosterone was rapidly effluxed out from S-12 cells (at 60 min, JG436 had 6.5 pmoles of [³H]-corticosterone, while it was 3 pmoles per mg dry wt in S-12 cells) (Fig 25a & b and Fig 26a & b).

Although JG436 did not have a functional *PDR5*, it is known to harbor several putative efflux pumps, which might mask the functionality of Cdr1p (Balzi and Goffeau, 1994). Therefore, a *S. cerevisiae* strain AD1234568 (called as AD here after) constructed by Goffeau’s group which had seven disrupted ABC transporter genes e.g. *PDR5, PDR10,*
Fig 25. **Time course of β-estradiol transport.** (a) β-estradiol transport in host strain JG436, a PDR5 disruptant. (b) β-estradiol transport in S12-4. Cells (yeast transformants) from mid log phase were centrifuged and washed twice at 500 ×g and resuspended in fresh YNB medium as 10% cell suspension. 1.5 ml of cell suspension was incubated for 5 min in shaking water bath at 150 rpm at 30°C. The reaction was started by the addition of 2 nM of [³H]-β-estradiol (91 Ci/mmol). An aliquot of cells was withdrawn at indicated time intervals, filtered rapidly and washed thrice with 10 mM PBS, pH 7.4, containing 2% glucose, on Millipore manifold filtration assembly using 0.45 μm cellulose nitrate filter (Millipore, USA). Control experiments, without cells, were also performed and the background count on the filters, were deducted from the final values.
FIG 26. Time kinetics of corticosterone transport in host and transformed yeast, harboring CDR1 gene. (a) Corticosterone transport in host strain JG436. (b) Corticosterone transport in S-12-4. Cells (yeast transformants) from mid log phase were centrifuged and washed twice at 500 xg and resuspended in fresh YNB medium as 10% cell suspension. 1.5 ml of cell suspension was incubated for 5 min in shaking water bath at 150 rpm at 30 °C. The reaction was started by the addition of 2 nM of [³H]-corticosterone (81 Ci/mmol). An aliquot of cells was withdrawn at indicated time intervals, filtered rapidly and washed thrice with 10 mM PBS, pH 7.4, containing 2% glucose, on Millipore manifold filtration assembly using 0.45 μm cellulose nitrate filter (Millipore, USA). Control experiments, without cells, were also performed and the background count on the filters, were deducted from the final values. Bar in each figure shows standard deviation and values are the mean of three independent experiments.
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PDR11, PDR15, SNQ2, YCF1 and YOR1, was used. Use of this strain, to test the functionality of Cdr1p, ensured non-interference of many putative efflux pumps. So, all the following experiments have been thus done with strain AD harboring CDR1 called as AD-CDR1. Interestingly, when efflux of [3H]-β-estradiol and [3H]-corticosterone was studied in AD-CDR1, the results were more dramatic. As can be seen from the Fig 27a & b and 28a & b, AD strain showed high accumulation of [3H]-β-estradiol (40-50 pmoles/mg dry weight), the uptake could never reach above 20 pmoles/mg dry weight in AD-CDR1 and in 60 min it was less than 10 pmoles/mg dry weight. Similar was the case with another steroid hormone where [3H]-corticosterone accumulation was much less in AD-CDR1. Although both S-12 and AD-CDR1 showed reduced accumulation as compared to their host lacking functional CDR1, the difference in accumulation levels owing to rapid efflux was very significant in AD-CDR1. Thus, it may be possible that Cdr1p efflux pump functions much more efficiently when other putative pumps are not functional. It should be pointed out that the efflux of steroid hormone is indirectly measured as net accumulation where fluctuation in total accumulation was taken as an indication of efflux rate, since the influx is expected to be by diffusion, owing to hydrophobic nature of steroids used. A range of [3H]-estradiol concentrations (0.1 to 10 nM) was used to determine the Km of estradiol accumulation. Total accumulation of estradiol was measured at each concentration upto 1 hr. The resulting accumulation was plotted (Lineweaver-Burk plot) to obtain Km values, using Sigma plot (Jandel Scientific). However, the Km values, which were in the range of 0.5-1.0 nM, do not directly reflect the affinity of estradiol.

3.2.2.2 Progesterone is not a substrate for Cdr1p

Both the host JG436 and its transformants S-12-4 accumulated similar amount of [3H]-progesterone (140-160 pmoles/mg dry weight) independent of time, indicating the inability of Cdr1p to efflux this substrate. It would mean that progesterone may not be a substrate of Cdr1p (Fig 29a & b). That progesterone might not be the substrate was also observed in case of few other ABC transporters (Mahe, et al., 1996; Kolaczkowski, et al., 1996). Interestingly, it is shown that [3H]-progesterone binds to Mdr1p with high affinity and it is slated to be a
FIG. 27 Time kinetics of $\beta$-estradiol transport. (a) $\beta$-estradiol transport in strain AD1234568 (AD). (b) $\beta$-estradiol transport in transformant of AD (AD-CDR1). The transport was carried out as mentioned in (a). Bar in each figure shows standard deviation and values are the mean of three independent experiments. Cells (yeast transformants) from mid log phase were centrifuged and washed twice at 500 xg and resuspended in fresh YNB medium as 10% cell suspension. 1.5 ml of cell suspension was incubated for 5 min in shaking water bath at 150 rpm at 30°C. The reaction was started by the addition of 2 nM of $[^3\text{H}]$-$\beta$-estradiol (91 Ci/mmol). An aliquot of cells was withdrawn at indicated time intervals, filtered rapidly and washed thrice with 10 mM PBS, pH 7.4, containing 2% glucose, on Millipore manifold filtration assembly using 0.45 μm cellulose nitrate filter (Millipore, USA). Control experiments, without cells, were also performed and the background count on the filters, were deducted from the final values.
FIG. 28. Time kinetics of corticosterone transport in host and transformed yeast, harboring CDR1 gene. (a) Corticosterone transport in host strain AD. (b) Corticosterone transport in AD-CDR1. Cells (yeast transformants) from mid log phase were centrifuged and washed twice at 500 xg and resuspended in fresh YNB medium as 10% cell suspension. 1.5 ml of cell suspension was incubated for 5 min in shaking water bath at 150 rpm at 30 °C. The reaction was started by the addition of 2 nM of [3H]-corticosterone (81 Ci/mmol). An aliquot of cells was withdrawn at indicated time intervals, filtered rapidly and washed thrice with 10 mM PBS, pH 7.4, containing 2% glucose, on Millipore manifold filtration assembly using 0.45 μm cellulose nitrate filter (Millipore, USA). Control experiments, without cells, were also performed and the background count on the filters, were deducted from the final values. Bar in each figure shows standard deviation and values are the mean of three independent experiments.
FIG. 29. Time course of progesterone transport. (a) Progesterone transport in host strain JG436, a PDR5 disruptant. (b) Progesterone transport in S-12-4. Cells from mid log phase were centrifuged and washed twice at 500 xg and resuspended in fresh YNB medium as 10% cell suspension. 1.5 ml of cell suspension was incubated for 5 min in shaking water bath at 150 rpm at 30 °C. The reaction was started by the addition of 2 nM of [3H]-progesterone (85 Ci/mmol). An aliquot of cells was withdrawn at indicated time intervals, filtered rapidly and washed thrice with 10 mM PBS, pH 7.4, containing 2% glucose, on Millipore manifold filtration assembly using 0.45 μm cellulose nitrate filter (Millipore, USA). Control experiments, without cells, were also performed and the background count on the filters, were deducted from the final values. Bar in each figure shows standard deviation and values are the mean of three independent experiments.
modulator of Mdr1p activity (Sharom, 1997). However, it remains to be seen if progesterone could also modulate Cdr1p.

3.2.2.3 Specificity of efflux of β-estradiol and corticosterone

Cdr1p is known to efflux out many unrelated hydrophobic xenobiotics and as a result it is able to confer resistance to a variety of drugs (Prasad, et al., 1995). The transport of steroid hormones by Cdr1p provided an opportunity to check in vivo specificity of this pump. AD-CDR1 cells were exposed to 100-fold concentration of other steroid hormones (200 nM) before assaying for [³H]-β-estradiol and [³H]-corticosterone accumulation. An excess concentration of estradiol, corticosterone and ergosterol prevented efflux of labeled estradiol and corticosterone and led to increased accumulation (Fig 30 and 31). Progesterone and dexamethasone on the other hand had no effect on the efflux of either of the steroids. Several drugs e.g. cycloheximide, o-phenanthroline, chloramphenicol, fluconazole and rhodamine 123, which are expected to be effluxed out by Cdr1p were also able to affect the exit of both the hormones indicating the specificity of efflux.

3.2.2.4 Efflux of β-estradiol and corticosterone is energy dependent

Cdr1p has been shown to contain adenosine triphosphatase (ATPase) and uridine triphosphatase (UTPase) activities, which have features very distinct from plasma membrane ATPase (PM-ATPase) e.g. broad substrate specificities and pH optima. The external pH did not significantly affect the ability to transport these hormones, which correlates well with the broader pH optimum of nucleotide triphosphatase activity of Cdr1p. The efflux of β-estradiol was inhibited by sodium azide as well as by other inhibitor of energy metabolism, carbonyl cyanide m-chlorophenylhydrazone (CCCP). Other inhibitors such as sodium orthovanadate (ATPase inhibitor), pottasium cyanide (KCN) and valinomycin (ionophore) also prevented the exit of β-estradiol (Fig 32a), albiet to a lesser extent. Transport of corticosterone showed similar results indicating that it is also energy dependent (Fig. 32b).
FIG. 30. Specificity of β-estradiol efflux mediated by CDR1 shown by competition of β-estradiol transport in AD-CDR1 cells, by steroids and drugs. Transport was carried out as described in legends of Fig. 27 in the presence of 100 fold (200 nM) cold steroids or drugs. W/o, without competitor; Erg, ergosterol; Dex, dexamethasone; Estra, β-estradiol; Cyh, cycloheximide; Chl, chloramphenicol; Flu, fluconazole; Chol, cholesterol; Corti, corticosterone; Phe, phenanthroline; Rh123, rhodamine123; Vin, vinblastine and Prog, progesterone.
FIG. 31. Specificity of corticosterone efflux mediated by CDR1. Competition of corticosterone transport in AD-CDR1 cells, by steroids and drugs. Transport was carried out as described in legend of Fig 31, in the presence of 100-fold (200 nM) cold steroids or drugs. W/o, without competitor; Erg, ergosterol; Dex, dexamethasone; Estra, β-estradiol; Cyh, cycloheximide; Chl, chloramphenicol; Flu, fluconazole; Chol, cholesterol; Cort, corticosterone; Phe, phenanthroline; Vin, vinblastine and Prog, progesterone. Bar in each figure shows standard deviation and values are the mean of three independent experiments.
FIG. 32 Inhibition of β-estradiol and corticosterone efflux by different inhibitors. (a) Inhibition of β-estradiol efflux by different inhibitors. The cells were exposed to indicated concentrations of inhibitors, Sodium azide (10 mM); CCCP, Carbonyl cyanide m-chloro phenylhydrazone (100 μM); KCN, potassium cyanide (1 μg/ml); Van, Sodium orthovanadate (100 μM) and Val, valinomycin (200 nM), 10 min before the commencement of the transport and the transport was carried out as described in legend of Fig 28b. (b) Inhibition of corticosterone efflux by different inhibitors. Bar in each figure shows standard deviation and values are the mean of three independent experiments.
3.2.2.5 β-Estradiol and Rhodamine 123 efflux in azole resistant clinical isolates of C. albicans

The overexpression of CDR1 has been linked to azole resistance in C. albicans (discussed in the following section) where multiple efflux mechanism has been suggested (Krishnamurthy, et al., 1998b; White, 1997b; Sanglard, et al., 1995b). To check the efflux of [3H]-β-estradiol and Rh 123 in azole resistant clinical isolates of C. albicans, cells were grown till midlog phase and used for uptake experiments as mentioned in "Methods". When the accumulation of an indicator dye Rh 123 and [3H]-β-estradiol (reflecting the efflux) was checked in azole resistant clinical isolates of C. albicans, it was observed that isolates Y1.363, Y1.550, and Y1.552 showed more accumulation of Rh 123 and [3H]-β-estradiol than Y1.358 and Y1.549 (Fig 33a and b). The level of accumulation of these substrates did not correlate with the level of azole resistance of these isolates (TABLE XVII, mentioned in next chapter). This would mean that the steroids, which could be physiological substrates of Cdr1p, could be effluxed out independent of azole resistance of C. albicans in at least some cases. There could be still some unknown factors, which might affect the accumulation of these hormones in clinical isolates. It is tempting to speculate that one of these factors could be the level of steroid binding proteins in these clinical isolates.

In conclusion, these results demonstrate that Cdr1p, a multidrug transporter, can selectively mediate energy dependent transport of human steroid hormones with high affinity and specificity. It is possible that these hormones might be physiological substrates of Cdr1p. In this regard, it is pertinent to mention that, corticosteroid and estrogen binding proteins in C. albicans and other species of Candida have been identified and related to its pathogenicity (Feldman, 1996). The interaction of some of the azoles such as ketoconazole, with the corticosteroid receptor and binding protein has been suggested (Stover, et al., 1983). Moreover, the presence of a putative steroid response element in the promoter of CDR1 (unpublished results) and the upregulation of CDR1 transcription by β-estradiol, strongly suggest a possibility of a steroid receptor cascade, linked to multidrug resistance in C. albicans (described in following section). The steroid efflux system mediated by Cdr1p could also be a part of the total sterol homeostasis of Candida cells.
FIG. 33. Accumulation of rhodamine 123 and [3H]-β-estradiol in different azole resistant clinical isolates. Transport of rhodamine 123 and [3H]-β-estradiol was done as mentioned in "Methods". All the isolates were grown till midlog in YPD at 30 °C, harvested and transport was carried out as mentioned in Fig 17a & b. Bars represent SD of three independent experiments.
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