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Characterisation of human steroid hormone transport mediated by Cdr1p, a multidrug transporter of *Candida albicans*, belonging to the ATP binding cassette super family

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Abstract

Cdr1p, a multidrug transporter from a pathogenic yeast *Candida albicans*, confers resistance to several unrelated drugs including anti-*Candida* drugs. We demonstrate that Cdr1p can specifically transport human steroid hormones namely β-estradiol and corticosterone. *Saccharomyces cerevisiae* transformant S-12, harbouring the *CDR1* gene, accumulated about 3-fold less [^{3}H]β-estradiol and about 2-fold less [^{3}H]corticosterone than the non-transformed strain. When *CDR1* was expressed in AD strain (AD-CDR1) which had seven ATP binding cassette (ABC) superfamily of putative transporter genes disrupted, the net accumulation of these hormones as compared to S-12 was significantly lower. Efflux of β-estradiol and corticosterone was inhibited by a 100-fold higher (200 nM) concentration of β-estradiol, corticosterone, ergosterol or dexamethasone, but progesterone which could not be transported by Cdr1p did not affect the efflux and thus accumulation. Interestingly, some of the drugs viz. cycloheximide, chloramphenicol, fluconazole and *o*-phenanthroline, to which *CDR1* confers resistance, could also prevent efflux and enhance accumulation to some extent. In conclusion, we show that human steroid hormones could be the substrates for Cdr1p and the energy dependent transport mediated by it is specific for estradiol and corticosterone.

Keywords: Multidrug resistance; *CDR1*; ABC transporter; *C. albicans*; Steroid transport

1. Introduction

Multidrug resistance (MDR), where cells exhibit simultaneous resistance to multiple chemically unrelated chemotherapeutic agents, has been one of the principal causes of failure of chemotherapy [1–3]. The elucidation of the mechanism by which cells develop resistance to toxic effects of potent chemotherapeutic agents has revealed a great deal about the process of drug uptake, metabolism and extrusion. Overexpression of a 170 kDa glycoprotein (P-glycoprotein) was shown to be responsible for this phenomenon in mammals including humans [1–3]. Such MDR proteins are widely distributed throughout the evolutionary scale [4–6]. Yeast is no exception, in fact more than two dozen loci responsible for pleiotropic drug resistance (PDR) have been identified mostly in *Saccharomyces cerevisiae* and a few in *Schizosaccharomyces pombe* [7,8]. The class of trans-

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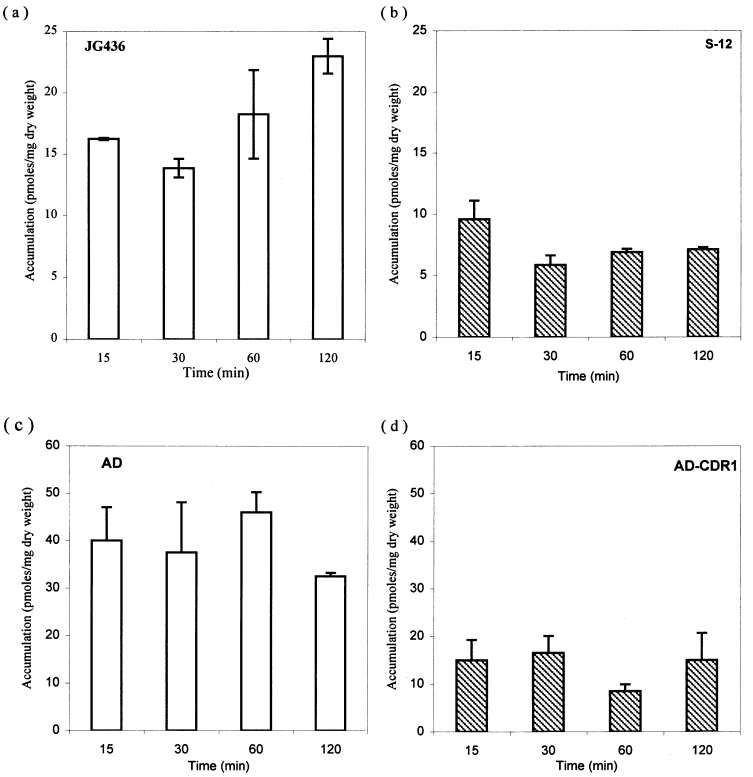

Fig. 1. Time course of β-estradiol transport. a: β-estradiol transport in host strain JG436, a *PDR5* disruptant. Cells (yeast transformants) from mid-log phase were centrifuged and washed twice at 500×*g* and resuspended in fresh YNB medium as 10% cell suspension. 1.5 ml of cell suspension was incubated for 5 min in shaking water bath at 150 rpm at 30°C. The reaction was started by the addition of 2 nM of [^{3}H]β-estradiol (91 Ci/mmol) or 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 which were always less than 10%, were deducted from the final values. b: β-estradiol transport in S12. c: β-estradiol transport in strain AD1234568 (AD). d: β-estradiol transport in transformant of AD (AD-CDR1). The transport was carried out as described in a. The bar in each figure shows standard deviation and values are the mean of three independent experiments.

porters in yeasts not only includes ATP binding cassette (ABC) family of transporters and multidrug resistance-associated protein family (MRP) of transporters but also a family of membrane facilitators (MFS), which are basically symporters [9]. In addition, regulatory networks of PDR, genes backed by transcription regulators, are also very well characterised [7,9].

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* [6]. In recent years, the incidence of *C. albicans* cells acquiring resistance to azoles and polyenes has increased considerably [6,10,11]. In this regard, the cloning and sequencing of a multidrug transporter, *CDR1* (Candida drug resistance) in *C. albicans*, which is a homologue of *S. cerevisiae* multidrug efflux pump *PDR5* [7,9], was an important step towards an understanding of the mechanism of drug resistance [12]. A few more homologues of *CDR1* have been identified recently in *C. albicans* [13,14] and few genes specific to fluconazole and benomyl resistance have also been characterised [6,8,15,16]. 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 [17,18] and MDR2, which is a specific phosphatidylcholine translocator, between

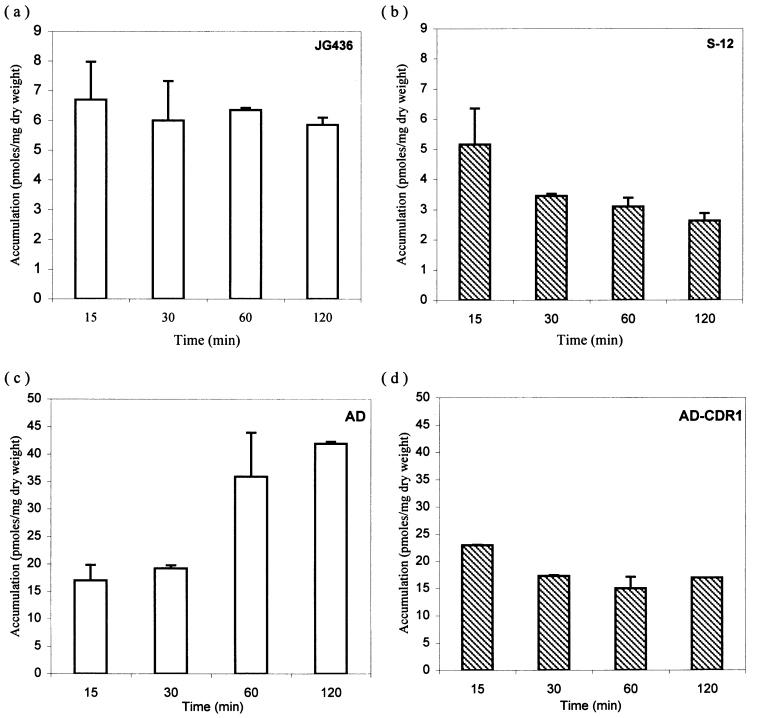

Fig. 2. Kinetics of corticosterone transport in host and transformed yeast, harbouring the *CDR1* gene. a: Corticosterone transport in host strain JG436. b: Corticosterone transport in S-12. c: Corticosterone transport in AD1234568 (AD). d: Corticosterone transport in transformant of AD (AD-CDR1). The transport of corticosterone was carried out as described in Fig. 1a. The bar in each figure shows standard deviation and values are the mean of three independent experiments.

lipid monolayers [19], none of the characterised 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* [7,9]. *STE6* codes for an ABC protein and is known to export mating factor in yeast [20]. 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 [21,22].

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* promoter [9,12]. In addition, *CDR1* promoter has regulatory elements which are not found in *PDR5* promoter. In view of above facts and considering the sites of infection of *C. albicans* in humans, it was most appropriate to ascertain if this pathogenic yeast has also adapted to transport steroids. This communication, for the first time, describes in detail the specificity of an energy dependent steroid transport, mediated by Cdr1p, in a background where seven ABC transporters are deleted. We also demonstrate that drugs, which are effluxed out by Cdr1p, competitively inhibit steroid accumulation.

2. Materials and methods

2.1. Strains and growth media

S. cerevisiae JG436 (*Mat a*, *PDR5*::Tn5, *leu2*, *met5*, *ura*3-52, *mak71, KRB1*) [12] was a kind gift from Dr. J. Golin, Catholic University of America, Washington, DC, USA. *S. cerevisiae* AD 1234568

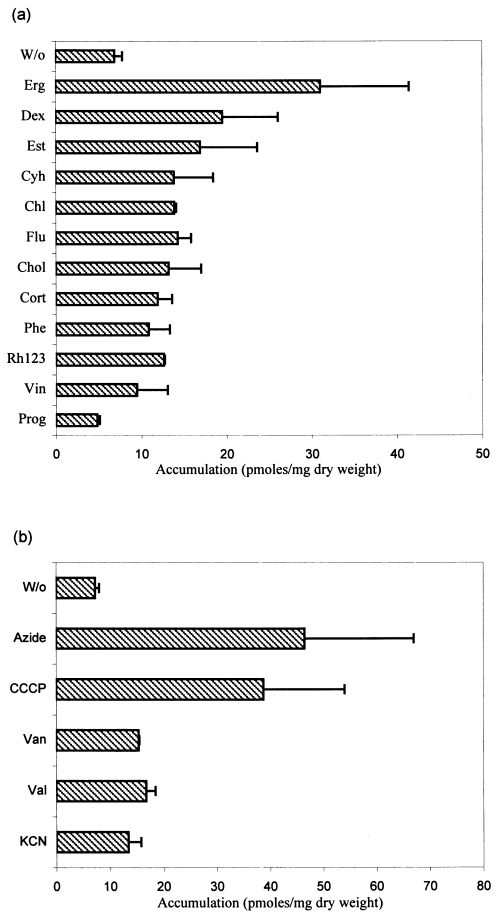

Fig. 3. Specificity of β-estradiol efflux mediated by CDR1. a: Competition of β-estradiol transport in AD-CDR1 cells, by steroids and drugs. Transport was carried out in the presence of 100-fold (200 nM) cold steroids or drugs. W/o, without competitor; Erg, ergosterol; Dex, dexamethasone; Est, β-estradiol; Cyh, cycloheximide; Chl, chloramphenicol; Flu, fluconazole; Chol, cholesterol; Cort, corticosterone; Phe, phenanthroline; Rh123, rhodamine123; Vin, vinblastine; and Prog, progesterone. b: 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-chlorophenylhydrazone (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 the legend of Fig. 1a. The bar in each figure shows standard deviation and values are the mean of three independent experiments.

(Mat α, *pdr1-3*, *his*, *ura3*, *pdr5*Δ, *snq2*Δ, *pdr10*Δ, *pdr11*Δ, *pdr15*Δ, *yor1*Δ, *ycf1*Δ), was a kind gift from A. Goffeau and A. Decottignies, Université Catholique de Louvain, Belgium. Both the strains were transformed with the *CDR1* gene by a method described earlier [12] and the resulting transformants harbouring *CDR1* were designated S-12 and AD-CDR1.

2.2. Transport of [^{3}H]β-estradiol and corticosterone

The accumulation of steroid hormone [^{3}H]β-estradiol and [^{3}H]corticosterone was determined by the modified method described elsewhere [21]. The results presented show net accumulation of steroids at the indicated time and not the efflux.

3. Results and discussion

3.1. Transport of β-estradiol and corticosterone is mediated by Cdr1p

S. cerevisiae strain JG436, hypersensitive to cycloheximide due to disrupted *PDR5*, was used as a host to express *CDR1* of *C. albicans*. The resulting strain S-12, hyper-resistant to cycloheximide and other drugs [12], was used to check 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 [21]. The strain JG436 showed steady accumulation of [^{3}H]β-estradiol (Fig. 1a). However, due to continuous efflux of these substrates mediated by Cdr1p, strain S-12 showed much less accumulation (at 60 min JG436 had 19 pmoles/mg dry weight of [^{3}H]β-estradiol while it was 7.0 pmoles/mg dry weight in S-12) (Fig. 1a and b). Similarly, [^{3}H]corticosterone was rapidly effluxed out from S-12 cells (at 60 min JG436 had 6.5 pmoles of [^{3}H]corticosterone, while it was 3 pmoles per mg dry weight in S-12 cells) (Fig. 2a and b).

Although JG436 does not have a functional Pdr5, it is known to harbour several other putative efflux pumps, which might still mask the function of Cdr1p [23]. We therefore used a *S. cerevisiae* strain AD1234568 (called as AD hereafter in this paper) constructed by A. Decottignies, in A. Goffeau's group, where in seven ABC transporter genes, e.g. *PDR5*, *PDR10*, *PDR11*, *PDR15*, *SNQ2* (*Saccharomyces* nitroquinoline oxide resistance gene), *YCF1*

(yeast cadmium factor gene) and *YOR1* (yeast oligomycin resistance gene) were disrupted. So all the following transport experiments have been done with strain AD harbouring *CDR1* called as AD-CDR1. Interestingly, when transport of [^{3}H]β-estradiol and [^{3}H]corticosterone was studied in AD-CDR1, the results were more dramatic. As can be seen from Fig. 1c and d, the AD strain showed higher accumulation of [^{3}H]β-estradiol (50 pmoles/mg dry weight), the uptake could never reach above 20 pmoles/mg dry weight in AD-CDR1 and at 60 min it was less than 10 pmoles/mg dry weight. Similar was the case with [^{3}H]corticosterone, where the accumulation was much less in AD-CDR1 (Fig. 2c and d). It is important to point out here that none of these transformants was able to show the efflux of [^{3}H]progesterone, suggesting that progesterone may not be a substrate of Cdr1p (data not shown). Although both S-12 and AD-CDR1 showed reduced accumulation as compared to their host lacking *CDR1* gene, the difference in accumulation levels was very significant in AD-CDR1. The deletion of seven ABC type transporters in AD-CDR1 ensured unmasked functioning of Cdr1p. A range of [^{3}H]estradiol concentrations (0.1 to 10 nM) was used to determine the K_m of estradiol accumulation. Total accumulation of estradiol was measured at each concentration up to 1 h. The resulting accumulation was plotted (Linweaver-Burk plot), to obtain K_m values, using Sigma plot (Jandel Scientific),. However, the K_m values, which were in the range of 0.5–1.0 nM (data not shown), do not directly reflect the affinity of estradiol.

3.2. β-estradiol and corticosterone transport mediated by Cdr1p is specific

Cdr1p is known to efflux many unrelated hydrophobic xenobiotics and as a result it is able to confer resistance to a variety of drugs [12]. The transport of steroid hormones by Cdr1p provided an opportunity to check in vivo specificity of this pump. AD-CDR1 cells were exposed to a 100-fold higher concentration of other steroid hormones (200 nM) before assaying for [^{3}H]β-estradiol accumulation. An excess concentration (100-fold) of β-estradiol, corticosterone, dexamethasone, cholesterol and ergosterol prevented efflux of labeled substrate and led to increased accumulation (Fig. 3a). Progesterone, on the other hand, had no effect on the transport of either of the steroids. Several drugs, e.g. cycloheximide, *o*-phenanthroline, chloramphenicol, fluconazole and rhodamine 123, which are expected to be effluxed by Cdr1p were also able to affect the exit of both the hormones (Fig. 3a). The transport of corticosterone also showed similar specificities (data not shown).

3.3. Transport 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 (Krishnamurthy et al., unpublished results). 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 (data not shown). 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), potassium cyanide (KCN) and valinomycin (ionophore) also prevented the exit of β-estradiol (Fig. 3b), albeit to a lesser extent. Transport of corticosterone showed similar results indicating that it is also energy dependent (data not shown).

In conclusion, our 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 [24]. The interaction of some of the azoles such as ketoconazole, with the corticosteroid receptor and binding protein has been suggested [25]. Moreover, the presence of a 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* (Krishnamurthy et al., submitted for publication).

The steroid efflux system mediated by Cdr1p, could also be a part of the total sterol homeostasis of *Candida* cells. Studies are underway in our laboratory to confirm the above hypothesis.

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