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Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: transcriptional activation by heat shock, drugs and human steroid hormones

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Abstract

We have examined the expression of *CDR1* (*Candida* drug resistance gene) in different stress conditions. There was a significant but transient enhancement of *CDR1* expression associated with elevated temperatures. Most noteworthy transcriptional activation was observed with miconazole and vinblastine. Interestingly, β-estradiol and progesterone were also able to enhance *CDR1* expression. Elevated levels of *CDR1* and *CDR2* (a homologue of *CDR1*) mRNA were found in some azole-resistant clinical isolates of *C. albicans*. *CaMDR1* (benomyl-resistant) expression, however, did not differ among all the resistant isolates. Our results confirm the existence of multiple mechanisms of azole resistance in *C. albicans*. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: ABC protein; Multidrug resistance; Expression; Stress induced; *Candida albicans*

1. Introduction

The dimorphic pathogenic yeast *Candida albicans* is naturally more resistant to several drugs, e.g. cycloheximide, benomyl and methotrexate, than *Saccharomyces cerevisiae* [1,2]. In addition, the incidence of *C. albicans* cells acquiring resistance to azoles and polyenes has increased considerably in recent years, which has posed serious problems for its successful chemotherapy [3]. Azole resistance in *C. albicans* can arise by several mechanisms, viz., mutations in the target enzyme, $P450_{DM}$ [4] or lesions in other ergosterol biosynthetic enzymes (e.g. $\Delta^{5,6}$ sterol desaturase) [5], which compensates for the inhibition of $P450_{DM}$ by azoles. In addition, energy-dependent drug transporters of the multidrug resistance (MDR) type have been implicated in azole resistance [2,4]. Three *C. albicans* proteins, encoded by the *CDR1*, *CDR2* and *CaMDR1* (benomyl-resistant) genes, have been shown to play a role in fluconazole resistance [4,6–8].

In an attempt to delineate the functional role of *CDR1*, we have investigated factors which influence

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CDR1 expression. We demonstrate that *CDR1* expression is induced by environmental stimuli, e.g. temperature, drugs and human steroid hormones. The expression also appears to be growth stage-specific. The expression pattern of *CDR1*, *CDR2* and *CaMDR1* in azole-resistant clinical isolates of *C. albicans* confirms the existence of multiple mechanisms for azole resistance in *C. albicans*.

2. Materials and methods

2.1. Yeast isolates and culture conditions

C. albicans ATCC 10261 and all other clinical isolates used in this study were routinely grown and maintained in YEPD medium (yeast extract 1%, bactopeptone 2%, glucose 2%) at 30°C. Clinical isolates were a kind gift from Tanya Parkinson and Chris Hitchcock, Pfizer Ltd. Fluconazole was kindly provided by Pfizer Ltd., Sandwich, Kent, UK. Itraconazole and ketoconazole were kind gifts from the Janssen Research Foundation, Beerse, Belgium. All the chemicals including steroids and other drugs were from Sigma, and were of analytical grade.

2.2. Isolation of total RNA and Northern analysis

Total RNA isolation from *C. albicans* cells was done as described earlier [9]. For Northern analysis the standard protocol was used [10]. Northern transfer was performed overnight as given in the standard laboratory protocol [10] using Hybond™ nylon membrane (Amersham). RNA was fixed by UV cross-linking (Stratagene) as per the manufacturer's instructions. A 4.2-kb *Bam*HI fragment of *CDR1* or *CDR2* (PCR probe) or *CaMDR1* (1.8-kb *Eco*RV fragment) as described by Sanglard et al. [7], labelled with [α-^{32}P]dCTP using the random primer kit from Life Technologies (USA), was used as molecular probe for Northern analysis [6].

3. Results

3.1. CDR1 expression is temporally regulated

In order to ascertain the physiological role of *CDR1* as well as its role in drug resistance, *CDR1* mRNA levels were checked during the growth phase (Fig. 1a). The level of expression of *CDR1* was higher at 4 h and 6 h of growth (lanes 1 and 2, Fig. 1b),

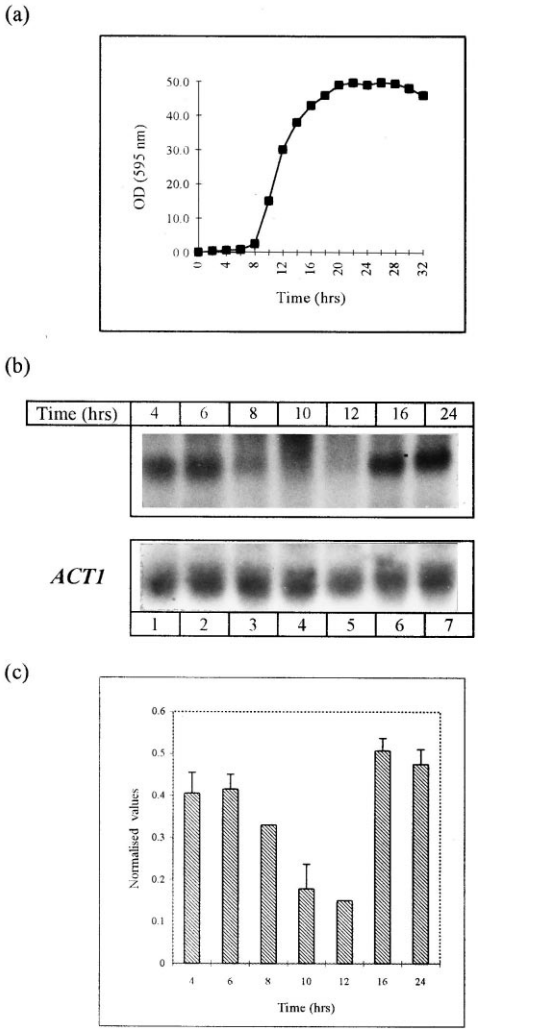

Fig. 1. Northern blots of total RNA. a: Growth curve of ATCC 10261 used in this study. b: During the growth phase of *C. albicans* (ATCC 10261). The cells were harvested at indicated times (lanes 1–7) and total RNA was extracted as described in Section 2. 40 μg of RNA was loaded for electrophoresis. After transfer to Hybond N$^+$ nylon membrane (Amersham), the blot was sequentially probed with [α-^{32}P]dCTP-labelled DNA fragments (*CDR1* and *ACT1*). The blot was hybridised and stripped as described in Section 2. d: Densitometric scan of Northern blot of b. The bars show the extent of variation between three sets of experiments. No bars are seen at the 8-h and 12-h points because there was very little variation.

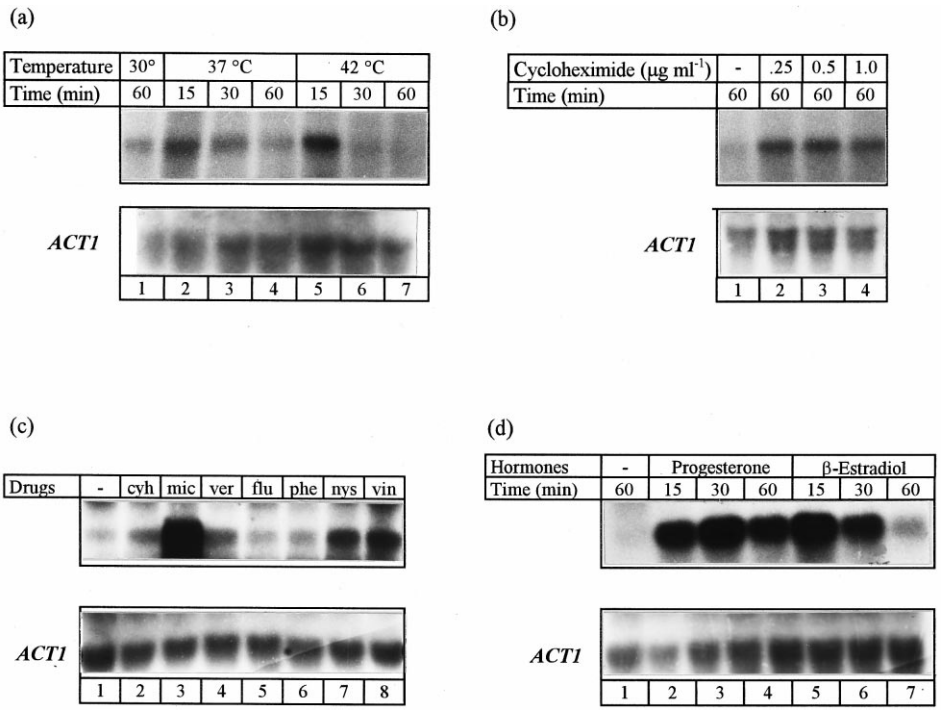

Fig. 2. Northern blots of total RNA under different stresses. a: Expression of *CDR1* under elevated temperatures. *C. albicans* strain ATCC 10261 was grown to the mid-exponential phase (10 h) in YEPD at 30°C and then shifted to two different temperatures (37°C and 42°C); the cultures were allowed to grow for 15, 30 and 60 min (lanes 2–7) before harvesting for the isolation of total RNA. The total RNA isolated from cells grown at 30°C for the same time period was taken as control (lane 1). b: Cycloheximide-induced expression of *CDR1*. *C. albicans* strain ATCC 10261 was grown to mid-exponential phase (10 h) in YEPD at 30°C as described in Section 2. Then different concentrations of cycloheximide (0.25, 0.5 μg and 1 μg ml^{-1}) were added (lanes 2–4, respectively) to the media and the cells were allowed to grow to the indicated time at 30°C. Cells grown without the drug for the same period of time were taken as control (lane 1). c: Expression of *CDR1* in the presence of different drugs. Cycloheximide (cyh) 1 μg ml^{-1}, miconazole (mic) 100 μg ml^{-1}, verapamil (ver) 10 μM, fluconazole (flu) 50 μg ml^{-1}, *o*-phenanthroline (phe) 20 μg ml^{-1}, nystatin (nys) 1 μg ml^{-1} and vinblastine (vin) 1 μM were added directly to the media at mid-exponential phase (10 h) and the cells were allowed to grow for another 60 min before processing for RNA isolation (lanes 2–8). Cells grown for the same time without the addition of drugs were taken as control (lane 1). d: β-Estradiol- and progesterone-induced expression of *CDR1*. *C. albicans* strain ATCC 10261 was grown to mid-exponential phase (10 h) in YEPD at 30°C as described. Then β-estradiol (1 mM) and progesterone (1 mM) were added to the media and the cells were allowed to grow to the indicated time at 30°C (lanes 2–4, progesterone; lanes 5–7, β-estradiol). Cells grown without hormones for the same period of time were taken as control (lane 1).

declined during mid-exponential phase (lanes 4 and 5, Fig. 1b) and again increased at late exponential and stationary phase (lanes 6 and 7, Fig. 1b). Normalised densitometric scans in Fig. 1b showing statistical variations also confirm that *CDR1* transcription is temporally regulated (Fig. 1c). It must be pointed out that the both 8-h and 10-h points (Fig. 1c) do not depict any bars since there were no significant variations observed at these hours of growth.

3.2. Heat shock and unrelated drugs induce expression of *CDR1*

The sequencing of *CDR1* has earlier revealed the existence of two putative heat shock elements (HSE) at −192 and −323 bp upstream of the translation start site [6]. In order to study if heat stress could induce expression of *CDR1*, *C. albicans* cells were grown at 30°C up to 10 h, were transferred to 37°C or 42°C and were harvested after 15, 30 and

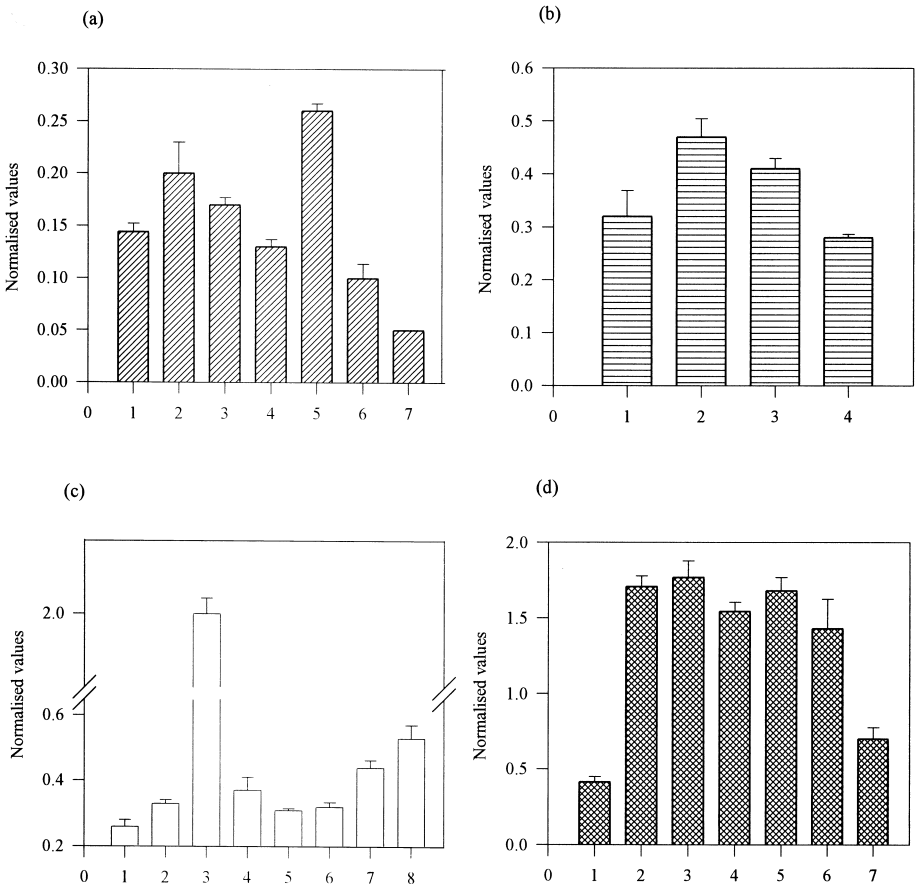

Fig. 3. Relative mRNA levels of *CDR1* calculated from Fig. 2a–d. Quantitations were performed after scanning the autoradiograms in a densitometer (PDI, USA). Each signal was quantitated and normalised with the signal obtained from the *ACT1* probe, which served as an internal control for loading of total RNA. Arbitrary values are plotted after normalisation. Fig. 2 shows a typical set of Northern blots while Fig. 3 represents the average of densitometric scan values of three Northern blots with error bars showing deviation from mean values.

60 min of incubation (lanes 2–7, Fig. 2a). As a control, an aliquot of cells was incubated at 30°C for the same period of time. There was a significant increase in expression of *CDR1* after 15 min of exposure of cells to 37°C or 42°C, after which transcript levels decreased gradually (Figs. 2a and 3a).

CDR1 was previously shown to confer resistance to several drugs and metabolic inhibitors [6]. We therefore examined whether some of these compounds could induce transcription of *CDR1*. Cycloheximide caused an increase in *CDR1* mRNA levels (Figs. 2b and 3b), which was time-dependent and of longer duration than the temperature-induced expression. Maximal induction was obtained using 0.25 μg ml^{-1} cycloheximide and addition of higher concentrations did not result in any further increase in mRNA levels (lanes 2–4, Figs. 2b and 3b).

A range of drugs was tested for their effects on *CDR1* transcription, including antifungal agents (miconazole, fluconazole and nystatin), a transcription inhibitor (*o*-phenanthroline) and anticancer drugs (verapamil and vinblastine). All of the drugs tested caused an increase in *CDR1* mRNA levels after 60 min exposure, with miconazole having the greatest effect (lane 3, Figs. 2c and 3c).

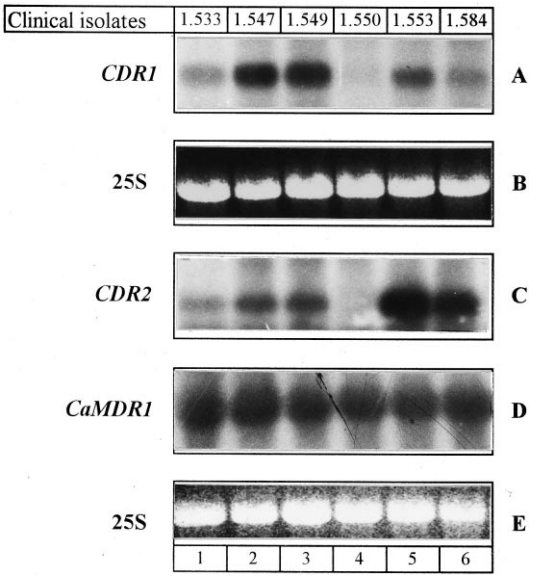

Fig. 4. Expression of *CDR1*, *CDR2* and *CaMDR1* in azole-resistant clinical isolates of *C. albicans*. Northern blot analysis of total RNA from different clinical isolates of *C. albicans* was carried out. Cells were harvested at 10 h of growth and total RNA was isolated as described in Section 2. Lanes 1–6 represent different azole-resistant clinical isolates. Panel B shows the gel load (25S rRNA) of panel A while panel E shows the gel load of panels C and D.

3.3. *CDR1* expression is induced by β-estradiol and progesterone

In this study, we have for the first time demonstrated that human steroid hormones can transcriptionally activate *CDR1* promoter. The *CDR1* transcript levels were monitored in the presence of β-estradiol and progesterone (Fig. 2d). The steroid-induced activation was very rapid which was evident within 15 min of exposure of cells to the hormones (Fig. 2d). β-Estradiol and progesterone were both able to enhance *CDR1* transcript levels to about 4.5-fold as compared to the control (Figs. 2d and 3d) within 15–30 min of exposure.

3.4. Some of the resistant clinical isolates of *C. albicans* have overexpressed *CDR1* and *CDR2*

We have examined *CDR1* mRNA levels in fluconazole-resistant clinical isolates (MICs 50–100 μg ml^{-1}). It is evident from Fig. 4 that isolates Y01.547 and Y01.549 (lanes 2 and 3) particularly showed high expression of *CDR1* as compared to other strains. Interestingly, isolates Y01.553 and Y01.584, which did not show high levels of *CDR1* transcript, exhibited enhanced levels of expression of *CDR2*. On the other hand, *CaMDR1* expression did not vary significantly in these isolates. It must be mentioned that a low level of expression of all three genes, viz. *CDR1*, *CDR2* and *CaMDR1*, was observed in four azole-sensitive isolates of *C. albicans* (data not shown).

4. Discussion

The human multidrug resistance *MDR1* gene promoter has been shown to contain heat shock consensus elements and is induced in response to elevated temperatures and to other chemical stress-inducing agents [11]. *PDR5* (*YDR1*/*STS1*) and *SNQ2*, the *S. cerevisiae* homologues of *CDR1*, have been shown to be stimulated by stress [12,13]. Since the *CDR1* gene contains two heat shock (stress) elements at positions −192 and −323 bp upstream of the translation start site [6], we analysed *CDR1* expression under different stress conditions, including high temperature and drug treatment, and showed that *CDR1* is also a stress-inducible gene. *CDR1* mRNA levels also increased on entry into stationary phase, in common with many other genes which are known to be stress-induced [14]. Several unrelated drugs were also able to induce *CDR1* transcription. The overexpression induced by miconazole, nystatin and vinblastine was most noteworthy.

If one considers the fact that an increase in sex hormone levels leads to a rise in the incidence of *Candida* infections, our present results are significant. It is tempting to speculate that the hormonal environment of the host may affect drug resistance of *C. albicans*. In this regard it is pertinent to mention a recent report where it has been shown that in *S. cerevisiae*, the human steroid hormone β-estradiol is transported through Pdr5p and Snq2p and the disruption of their genes resulted in the accumulation of β-estradiol [15,16]. Our earlier results also indicate that β-estradiol and corticosterone are substrates of Cdr1p [17]. The presence of corticosteroid and estrogen binding proteins in *C. albicans* and other species

of *Candida* has also been established [18]. In the light of these results, our study suggests a possibility of a steroid-receptor cascade linked to multidrug resistance of *C. albicans*. The fact that human steroid responsive element (SRE) is present in the promoter region of *CDR1* (Prasad et al., personal communication) supports our argument. Whether the transient increase in *CDR1* expression, induced by drugs and steroid hormones in this study, has any clinical relevance or only represents a general stress response to xenobiotic exposure in growth cultures remains to be seen.

We have shown that some of the azole-resistant isolates of *C. albicans* show high levels of expression of *CDR1* and of *CDR2*, particularly in those isolates where *CDR1* expression was low (Fig. 4). The expression of *CaMDR1* did not vary significantly in these isolates. In conclusion, we have demonstrated in this study that *CDR1* expression is growth phase-specific and is inducible by short exposure of cells to elevated temperatures, unrelated drugs and human steroid hormones. The difference in the levels of *CDR1*, *CDR2* and *CaMDR1* expression in the azole-resistant isolates suggests multiple mechanisms of resistance. Functional analyses of other multidrug resistance genes (Prasad et al., unpublished results) would certainly clarify the mechanisms of drug resistance in *C. albicans*.

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