The DNA-binding domain of CaNdt80p is required to activate *CDR1* involved in drug resistance in *Candida albicans*

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CaNdt80p, the *Candida albicans* homologue of the *Saccharomyces cerevisiae* transcription factor ScNdt80p, has been identified as a positive regulator of *CDR1*, which encodes an efflux pump involved in drug resistance in *C. albicans*. To investigate the involvement of the putative DNA-binding domain of CaNdt80p in drug resistance, chimeras of CaNdt80p and ScNdt80p were constructed. Interestingly, the DNA-binding domain of ScNdt80p could functionally complement that of CaNdt80p to activate *CDR1p-lacZ* in *S. cerevisiae*. Consistently, CaNdt80p containing a mutation in the DNA-binding domain failed to activate *CDR1p-lacZ* in *S. cerevisiae*. Furthermore, a copy of *CaNDT80* with the same mutation also failed to complement the drug-sensitive phenotype caused by a null mutation in *C. albicans*. Thus, the DNA-binding domain of CaNdt80p is critical for its function in drug resistance in *C. albicans*.

Received 28 March 2006

Accepted 13 July 2006

INTRODUCTION

Candida albicans is the most frequently isolated fungal pathogen in humans, and has caused morbidity in seriously debilitated and immunocompromised hosts (Edwards, 1990). The prevalence of nosocomial candidaemia increased 27-fold from 1981 to 1993 at a hospital in Taiwan (Chen *et al.*, 1997; Hung *et al.*, 1996). In the USA, yeast infections also rank as the fourth-most-common cause of nosocomial bloodstream infection (Beck-Sague & Jarvis, 1993; Pfaller *et al.*, 1998). Coinciding with the increased usage of antifungal drugs, incidences of drug resistance have also multiplied (Pfaller *et al.*, 2000; Vanden Bossche *et al.*, 1994; Yang & Lo, 2001).

Overexpression of efflux pumps, such as ATP-binding cassette (ABC) transporters, has been shown to be the major mechanism for drug resistance in clinical isolates (Karababa *et al.*, 2004; Marger & Saier, 1993; Michaelis & Berkower, 1995). Mutations on *CDR1* in *C. albicans* result in an increased susceptibility to azole drugs (Sanglard *et al.*, 1996), which is consistent with the observation that overexpression of *CDR1* contributes to the drug resistance in clinical isolates (Lopez-Ribot *et al.*, 1998). Recently, two transcription factors, CaNdt80p and Tac1p, have been identified as

positive regulators of *CDR1*, and mutations of either gene result in an increased drug susceptibility (Chen *et al.*, 2004; Coste *et al.*, 2004).

CaNdt80p is a *C. albicans* homologue of the *Saccharomyces cerevisiae* ScNdt80p (Chen *et al.*, 2004). Nevertheless, CaNdt80p is reported to be involved in drug resistance in *C. albicans* (Chen *et al.*, 2004), while ScNdt80p is a meiosis-specific transcription factor in *S. cerevisiae* (Abi-Said *et al.*, 1997; Chu *et al.*, 1998; Chu & Herskowitz, 1998). CaNdt80p is 672 amino acids (aa) in length and ScNdt80p 592 aa (Fig. 1a). The sequence from amino acids 221 to 592 of CaNdt80p and that from amino acids 1 to 330 of ScNdt80p share 35% identity and 53% similarity (Fig. 1b). Interestingly, there is no similarity between the N terminal of CaNdt80p and the C terminal of ScNdt80p (Fig. 1a) (Chen *et al.*, 2004). The amino acid residues from 1 to 330 of ScNdt80p have been shown to be important for the DNA-binding activity. The residues responsible for the DNA-binding capability of ScNdt80p have been reported. Among them, the 177Arg residue is the most important one for ScNdt80p to bind DNA. When this 177Arg residue is mutated, the DNA-binding capability decreases 27-fold (Montano *et al.*, 2002). Whether the putative DNA-binding domain of CaNdt80p also plays an important role in its function(s) has not been reported.

Abbreviation: β -Gal, β -galactosidase.

Fig. 1. Comparison of CaNdt80p in *C. albicans* and ScNdt80p in *S. cerevisiae*. (a) Overall comparison between CaNdt80p and ScNdt80p. For CaNdt80p, the promoter (pro) is in the solid black box, the activation domain (AD) is in the black diagonally hatched box, and the DNA-binding domain (BD) is in the cross-hatched box. For ScNdt80p, the promoter is in the horizontally lined box, the DNA-binding domain (BD) is in the open box, and the activation domain (AD) is in the grey diagonally hatched box. (b) Protein sequences of the DNA-binding domains of CaNdt80p and ScNdt80p. Similar residues of the two sequences are shaded. The mutated residue in CaNdt80p is indicated by an arrow, and the numbers represent the positions of amino acid residues in CaNdt80p and ScNdt80p.

In this study, we have constructed chimeras of CaNdt80p and ScNdt80p, and have found that the DNA-binding domain of ScNdt80p could functionally complement that of CaNdt80p to activate *CDR1p-lacZ*. Furthermore, complementation of a *Cand80/Cand80* mutant with a copy of *Cand80* carrying a mutation in the DNA-binding domain failed to restore drug resistance, in contrast to the wild-type *CaNDT80* gene. In conclusion, the DNA-binding domain of CaNdt80p is critical for activating the *CDR1* promoter and participating in the regulation of drug-resistance activity in *C. albicans*.

METHODS

Strains and media. *S. cerevisiae* strains, *C. albicans* strains, plasmids and primers used in this study are listed in Tables 1, 2, 3 and 4, respectively. Yeast peptone dextrose (YPD; 1% yeast extract, 2% peptone, 2% glucose), and synthetic dextrose (SD; 0.67% yeast nitrogen base without amino acids, 2% glucose) were prepared as described in Sherman *et al.* (2002).

Domain swapping. The schematic diagram in Fig. 2 shows the various chimeras used in this study. The sequence containing the

promoter and the 5' portion of *CaNDT80* (from 578 bp upstream to 666 bp downstream of the translation initiation site; denoted as -578 to 666 bp) was amplified from LOB45 (Chen *et al.*, 2004) by PCR with primers HJL382 and HJL383, HJL384 or HJL436, which introduced a *SacI* site at the 5' end and a *XmaI* or *XhoI* site at the 3' end. The DNA fragment containing the DNA-binding domain of ScNdt80p (from +4 to +992 bp) was amplified from LOB46 (Chen *et al.*, 2004) with the primers HJL385 and HJL387 to introduce a *XmaI* site at the 5' end and a *ClaI* site at the 3' end. The entire ORF of *ScNDT80* (from +4 bp to +2517 bp) was amplified by primers HJL385 and HJL386 to introduce a *XmaI* site at the 5' end and a *ClaI* site at the 3' end. The PCR products were cloned into PCRII-TOPO (Invitrogen, K65001) and sequenced. The inserted DNA fragments were then cloned into the high-copy-number vector pRS426 at appropriate restriction sites to construct plasmids LOB114 to LOB117. Different chimeras (Fig. 2) were used to transform *S. cerevisiae* strain SLO1 (Chen *et al.*, 2004) containing the *CDR1p-lacZ* reporter to construct SLO75, -77, -79 and -81. The LOB46 plasmid containing *ScNDT80* was also used to transform the *S. cerevisiae* strain SLO1 (Chen *et al.*, 2004), to construct SLO71.

In vitro assay of β -galactosidase activity. The overnight *S. cerevisiae* cell cultures were diluted into 20 ml SD plus adenine and histidine (SD/Ad/His) medium in a 250 ml flask to OD₆₀₀ 0.2. The cells were harvested by centrifugation at 3000 r.p.m. for 10 min at 4 °C (Sorvall RT7) after being grown at 30 °C for 4 h. Each cell

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
10560-2B	<i>MATa his3::hisG ura3-52 leu2::hisG</i>	Chen <i>et al.</i> (2004)
SLO1	<i>MATa his3::hisG ura3-52 leu2::hisG ade3::CDR1p-lacZ-LEU2</i>	Chen <i>et al.</i> (2004)
SLO2	<i>MATa his3::hisG ura3-52 leu2::hisG ade3::CDR1p-lacZ-LEU2</i> (pRS426)	Chen <i>et al.</i> (2004)
SLO5	<i>MATa his3::hisG ura3-52 leu2::hisG ade3::CDR1p-lacZ-LEU2</i> (LOB45)	Chen <i>et al.</i> (2004)
SLO71	<i>MATa his3::hisG ura3-52 leu2::hisG ade3::CDR1p-lacZ-LEU2</i> (LOB46)	This study
SLO75	<i>MATa his3::hisG ura3-52 leu2::hisG ade3::CDR1p-lacZ-LEU2</i> (LOB114)	This study
SLO77	<i>MATa his3::hisG ura3-52 leu2::hisG ade3::CDR1p-lacZ-LEU2</i> (LOB115)	This study
SLO79	<i>MATa his3::hisG ura3-52 leu2::hisG ade3::CDR1p-lacZ-LEU2</i> (LOB116)	This study
SLO81	<i>MATa his3::hisG ura3-52 leu2::hisG ade3::CDR1p-lacZ-LEU2</i> (LOB117)	This study
SLO85	<i>MATa his3::hisG ura3-52 leu2::hisG ade3::CDR1p-lacZ-LEU2</i> (LOB119)	This study

Table 2. *C. albicans* strains used in this study

Strain	Genotype	Source
SC5314		Gillum <i>et al.</i> (1984)
YLO132	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG</i> <i>arg4::hisG/arg4::hisG Candt80::GFP-ARG4/Candt80::URA3-dpl200</i>	Chen <i>et al.</i> (2004)
YLO133	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG</i> <i>arg4::hisG/arg4::hisG Candt80::GFP-ARG4/Candt80::URA3-dpl200</i> <i>ENO1/eno1::ENO1-tetR-ScHAP4-3xHA-HIS1</i>	Chen <i>et al.</i> (2004)
YLO137	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG</i> <i>arg4::hisG/arg4::hisG Candt80::GFP-ARG4/Candt80::URA3-dpl200::CaNDT80-HIS1</i>	Chen <i>et al.</i> (2004)
YLO263	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG</i> <i>arg4::hisG/arg4::hisG Candt80::GFP-ARG4/Candt80::URA3-dpl200::Candt80^{R432A}-HIS1</i>	This study

pellet was resuspended in 250 µl breaking buffer (1 mM DTT, 20%, v/v, glycerol, 0.1 M Tris/HCl, pH 8.0) with 12.5 µl 40 mM PMSF, and placed on ice. Acid-washed glass beads were added to just below the liquid level, and the suspension was vortexed at maximum speed for 60 s, followed by an additional incubation for 60 s at 4 °C. The vortexing procedure was repeated four times, and then an extra 300 µl breaking buffer was added. The cell extract was centrifuged at 3000 r.p.m. for 5 min at 4 °C. The supernatant was transferred to a fresh ice-cold Eppendorf tube for β-galactosidase (β-Gal) assay and Bradford quantitative protein assay.

Yeast extract (10 µl) was added to a 12 × 75 mm disposable glass tube containing 990 µl cold Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄) with β-mercaptoethanol (2.7 ml l⁻¹). A total of 200 µl ONPG (Sigma, N1127; 4 mg ml⁻¹ in Z buffer) as enzyme substrate was added to start the reaction at *t* = 0. The reaction was stopped by adding 500 µl 1 M Na₂CO₃. Bio-Rad Protein Assay Dye (Catalogue no. 500-0006) was used to determine the protein concentration of the cell extract. Based on the Bradford dye-binding procedure, the protein concentration of the supernatant was determined by comparison with a standard curve of A₅₉₅ versus

Table 3. Plasmids used in this study

Plasmid	Description	Source
pRS426	—	Christianson <i>et al.</i> (1992)
LOB45	pRS426- <i>CaNDT80</i>	Chen <i>et al.</i> (2004)
LOB46	pRS426- <i>ScNDT80</i>	Chen <i>et al.</i> (2004)
LOB49	pGEM- <i>HIS1-CaNDT80</i>	Chen <i>et al.</i> (2004)
LOB114	pRS426- <i>CaNDT80p-CaNDT80</i> (AD)- <i>ScNDT80</i> (BD)	This study
LOB115	pRS426- <i>CaNDT80p-CaNDT80</i> (AD)	This study
LOB116	pRS426- <i>CaNDT80p-ScNDT80</i> (ORF)	This study
LOB117	pRS426- <i>CaNDT80p-CaNDT80</i> (AD)- <i>ScNDT80</i> (ORF)	This study
LOB119	pRS426- <i>Candt80</i> ^{R432A}	This study
LOB122	pGEM- <i>HIS1-Candt80</i> ^{R432A}	This study

Table 4. Primers used in this study

Name	Sequence	Location
HJL237	TCCCCACCACCAATCTACC	<i>CaNDT80</i> : +1311 to +1330*
HJL238	GTTTTGCTTACTGTGGAGGAG	<i>CaNDT80</i> : +1786 to +1766
HJL293	AGTCATTGAACGAATTCTTGGCTG	<i>CaEFB1</i> : +38 to +61
HJL294	TTCTTCAACAGCAGCTTGTAAGTC	<i>CaEFB1</i> : +591 to +568
HJL382	CTTCCGCGGTTGTGGCGATTTCACTTTC	<i>CaNDT80</i> : -578 to -559
HJL383	TTTTCCCGGGCATCATTCTGACAAGAGTTT	<i>CaNDT80</i> : -14 to +6
HJL384	ATTACCCGGGAGCCAAAGGTTGTGGTGTT	<i>CaNDT80</i> : +666 to +648
HJL385	TAAACCCGGGAATGAAATGGAACACAGAT	<i>ScNDT80</i> : +4 to +24
HJL386	GGATCTCGAGTGTCAGCCAAATGCATAC	<i>ScNDT80</i> : +2517 to +2498
HJL387	CCATCGATTATTAGTTAGAAGGTGATCTTC	<i>ScNDT80</i> : +992 to +974
HJL436	GGATCTCGAGTTAAGCCAAAGGTTGTGGTGTT	<i>CaNDT80</i> : +666 to +648
HJL562	GATCCAGCACACTGCTAAAGCAGATAAAGGTCCACAGTTCC	<i>CaNDT80</i> : +1275 to +1315
HJL567	GGAAGTGTGGACCTTTATCTGCTTTAGCAGTGTGCTGGATC	<i>CaNDT80</i> : +1315 to +1275
HJL800	CCTAACGTATCGAGTGAAG	<i>CaNDT80</i> : +46 to +64
HJL801	CATTGTAGTGCACATCTG	<i>CaNDT80</i> : +337 to +319
HJL802	CCAGAGATAGGCTCACG	<i>ScNDT80</i> : +1558 to +1574
HJL803	CCAGATGGTAGAGTACCG	<i>ScNDT80</i> : +1832 to +1815
HJL804	CATCCACCGATTCTCCAAG	<i>ScEFB1</i> : +5 to +24
HJL805	GTCTTCGTCTTCTTCAATGC	<i>ScEFB1</i> : +579 to +460

*The A of the ATG translation initiation site is +1.

protein concentration, obtained by the serial twofold dilution of 500 µg ml⁻¹ BSA down to 62.5 µg ml⁻¹. β-Gal activity units were calculated (moles ONPG cleaved per minute per milligram protein) by the following equation:

$$(A_{420} \times 378) / (\text{time} \times \text{volume of extract} \times \text{protein concentration})$$

where time is in minutes, volume of extract in millilitres, and protein concentration in milligrams per millilitre.

RT-PCR. We used two-step RT-PCR to detect the level of mRNA in different strains. Total RNA was isolated by the acid hot phenol method (Schmitt *et al.*, 1990), followed by RQ1 RNase-free DNase (Promega, M610A) treatment to digest any contaminating DNA. First, 1 µg total RNA from each strain was used as template with oligo-dT as primer to synthesize the first-strand cDNA with StrataScript Reverse Transcriptase (Stratagene, 600085). The product of each reaction was diluted in sterile double-distilled H₂O, then used as the template to carry out the PCR amplification. Each reaction contained 50 µl PCR mixture including 0.25 µM each primer, 0.2 mM dNTPs, 1× buffer (10 mM Tris/HCl, pH 9.0, 1.5 mM MgCl₂, 50 mM KCl) and 1 U *Taq* polymerase (Amersham, 27-0799-06). The reaction conditions were as follows: 95 °C for 30 s, 57 °C for 30 s and 72 °C for 80 s for 35 cycles. The primers HJL237 and HJL238 were used to detect the *CaNDT80* mRNA in different *C. albicans* strains, and the primers HJL800 and HJL801 were used for *CaNDT80* in different chimeras in *S. cerevisiae* strains. The primers HJL802 and HJL803 were used for the detection of *ScNDT80*. The primers HJL293 and HJL294, the sequences of which were deduced from the intron and exon of the *C. albicans* *CaEFB1* gene, were used to distinguish the PCR products resulting from either the genomic DNA (928 bp DNA fragment) or the spliced RNA (564 bp DNA fragment) (Schaller *et al.*, 2003). For *S. cerevisiae*, the primers HJL804 and HJL805 were used for the detection of *ScEFB1*.

Quantitative analysis of mRNA level by real-time PCR. The cells were harvested at OD₆₀₀ 0.7–0.9 after being grown in 20 ml SD

liquid medium at 30 °C. Total RNA was isolated by the acid hot phenol method (Schmitt *et al.*, 1990), followed by RQ1 RNase-free DNase (Promega, M610A) treatment to digest any contaminating DNA. A real-time PCR was performed in a Rotor-Gene 3000 instrument (Corbett Research) with a TITANIUM *Taq* PCR kit (BD Clontech, 639210) and SYBR Green I Nucleic Acid stain (Cambrex, 50513) to determine the level of mRNA. The sample set-up was carried out automatically by a CAS-1200 instrument (Corbett Research). The real-time PCR was performed according to the instructions of the manufacturer, and the expression of *CaTEF3* in each strain was used as the loading control. The relative quantification was based on two standard curves for comparisons, and the results were given as a ratio (Kofron *et al.*, 1999). The level of *CaNDT80* mRNA isolated from the cells containing the wild-type *CaNDT80* gene was assigned the value 1, and the relative level of mRNA isolated from different strains was normalized accordingly.

Site-directed mutagenesis. The Arg432 residue of *CaNdt80p* was altered by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene, 200519). The primers HJL562 and HJL567 were used to mutate the *CaNdt80p* 432Arg to Ala on LOB45 and LOB49, to construct LOB119 and LOB122, respectively. Then, LOB119 was used to transform the *S. cerevisiae* strain SLO1 (Chen *et al.*, 2004) containing the *CDR1p-lacZ* reporter, to generate SLO85. LOB122 was linearized at the *SpeI* site located in the promoter of *CaNDT80* before being used to transform the *Candt80/Candt80* homozygous mutant YLO132 (Chen *et al.*, 2004) to generate the YLO263 (*Candt80/Candt80::Candt80^{R432A}*) strain. All constructs were assessed by PCR and sequencing.

Antifungal susceptibility tests. The Etest (Pfaffler *et al.*, 2003; Tapia *et al.*, 2003) was used to determine susceptibility to antifungal agents for *C. albicans*. Homogenized isolated colonies grown on SD medium overnight were transferred in 0.85% NaCl to achieve a density of 5 × 10⁶ cells ml⁻¹. A sterile swab was dipped into inoculum suspension and then used to swab the entire agar surface of an SD plate evenly. Fluconazole Etest strips (AB BIODISK) were applied to

Plasmid	Strain	Insert	Relative β -Gal activity (fold)
pRS426	SLO2	None	1.0

LOB45	SLO5		5.4 $\pm$ 1.9
LOB46	SLO71		1.1 $\pm$ 0.21
LOB116	SLO79		1.5 $\pm$ 0.18
LOB115	SLO77		1.2 $\pm$ 0.07
LOB114	SLO75		3.1 $\pm$ 0.85
LOB117	SLO81		3.4 $\pm$ 0.92
LOB119	SLO85		1.4 $\pm$ 0.15

Fig. 2. Different chimeras having different effects on the expression of the *CDR1p-lacZ* reporter in *S. cerevisiae*. For CaNdt80p, the promoter (pro) is in the solid black box, the activation domain (AD) is in the black hatched box, and the DNA-binding domain (BD) is in the cross-hatched box. For ScNdt80p, the promoter is in the horizontally lined box, the DNA-binding domain (BD) is in the open box, and the activation domain (AD) is in the grey hatched box. The β -Gal activity of the strain (SLO2) containing vector alone (pRS426) was defined as 1, and the β -Gal activity of cells containing different chimeras was normalized accordingly. The mutated residue is marked by an asterisk.

the plate when the excess moisture had been absorbed completely. The agar dilution method was also used to determine the susceptibility to antifungal agents of *C. albicans*. First, cells were diluted to OD₆₀₀ 2 ($\sim 2 \times 10^7$ cells ml⁻¹) and were spotted at ~ 0.5 μ l per spot with a replica device (Oxoid) onto plates containing different drugs, along with 10-fold serial dilutions. Fluconazole and miconazole, in 0.1% DMSO, were prepared to final concentrations of 5 and 0.3 μ g ml⁻¹, respectively. Cells were grown on SD media containing 0.1% DMSO in the absence or presence of drugs for 2 days at 30 °C.

RESULTS AND DISCUSSION

ScNdt80p failed to activate *CDR1p-lacZ* in *S. cerevisiae*

CaNdt80p was annotated as the *C. albicans* homologue of the *S. cerevisiae* transcription factor ScNdt80p due to the high degree of identity between the C terminal of CaNdt80p and the N terminal of ScNdt80p, a known DNA-binding domain (Fig. 1). Interestingly, there is no similarity between the N terminal of CaNdt80p and the C terminal of ScNdt80p (Chen *et al.*, 2004). Thus, to investigate the closeness of functionality between these two proteins, we wished to determine whether ScNdt80p, like CaNdt80p, can activate *CDR1p-lacZ* in *S. cerevisiae*. The β -Gal activity of *CDR1p-lacZ* was determined

by liquid assay, and the results are summarized in Fig. 2. *CaNDT80* in a high-copy-number vector (pRS426) increased the expression of *CDR1p-lacZ* approximately fivefold in *S. cerevisiae* SLO5, consistent with our previous report (Chen *et al.*, 2004). On the other hand, *ScNDT80* in the same vector did not affect the expression of *CDR1p-lacZ* in *S. cerevisiae* SLO71. Since *ScNDT80* is tightly regulated and expressed only during meiosis (Pak & Segall, 2002), we constructed a chimera in which *ScNDT80* was under the control of the *CaNDT80* promoter (LOB116) to further determine the effect of ScNdt80p in activating the expression of *CDR1p-lacZ*. The expression of *CDR1p-lacZ* was not induced by ScNdt80p in *S. cerevisiae* SLO79 (Fig. 2), even though the *ScNDT80* gene under the control of the *CaNDT80* promoter was expressed (Fig. 3a, lane 12). The fact that ScNdt80p failed to activate *CDR1p-lacZ* may be due to the difference in activation domain between ScNdt80p and CaNdt80p.

The DNA-binding domain of ScNdt80p can functionally complement that of CaNdt80p in *S. cerevisiae*

Since there is high degree identity between the C terminal of CaNdt80p and the DNA-binding domain of ScNdt80p, it is

Fig. 3. Expression of different chimeras in *S. cerevisiae* and both *CaNDT80* and *Candt80*^{R432A} alleles in *C. albicans*. (a) RT-PCR was performed with primers specific for *ScEFB1*, *ScNDT80* and *CaNDT80*. Total RNA was isolated from different *S. cerevisiae* strains. The template for lane 1 was genomic DNA from the parental *S. cerevisiae* strain (10560-2B). The remaining templates were cDNAs from different *S. cerevisiae* strains: lane 2, SLO2; lane 3, SLO5; lane 4, SLO71; lane 5, SLO79; lane 6, SLO77; lane 7, SLO75; lane 8, SLO81; lane 9, SLO85; lane 10, water as a negative control; lane 11, SLO5; and lane 12, SLO79. Lanes 2–10 were from reactions containing primers for amplifying *CaNDT80*, and lanes 11–12 were for *ScNDT80*. (b) RT-PCR was performed with primers specific for *CaEFB1* and *CaNDT80*, separately. The total RNAs were isolated from different *C. albicans* strains. The template for lane 1 was genomic DNA from the wild-type *C. albicans* strain (SC5314), and the remaining templates were cDNAs from different strains: lane 2, *Candt80/Candt80::CaNDT80* (YLO137); lane 3, *Candt80/Candt80* (YLO133); lane 4, *Candt80/Candt80::Candt80*^{R432A} (YLO263); lane 5, water as negative control. The sizes of the RT-PCR products are shown on the right.

possible that the DNA-binding domain of ScNdt80p can functionally complement the C-terminal putative DNA-binding domain of CaNdt80p. To assess this possibility, we constructed a chimera containing the promoter and the N terminal of CaNdt80p fused with the DNA-binding domain of ScNdt80p (LOB114). The results of the β -Gal liquid assay are summarized in Fig. 2. This chimera in SLO75 induced the expression of *CDR1p-lacZ* approximately threefold. Consistently, the chimera containing the promoter and the N terminal of CaNdt80p fused with the full-length ORF of ScNdt80p in SLO81 also induced the expression of *CDR1p-lacZ* approximately threefold. In contrast, overexpression of the N terminal of CaNdt80p alone in SLO77 did not have any effect on the expression of *CDR1p-lacZ*. These data suggest that CaNdt80p lacking the putative DNA-binding domain failed to activate *CDR1p-lacZ* in *S. cerevisiae*. Furthermore, the DNA-binding domain of ScNdt80p could complement the function possessed by the putative DNA-binding domain of CaNdt80p. Therefore, this putative domain shares at least one function with the DNA-binding domain of ScNdt80p.

CaNdt80p with a mutation of the DNA-binding domain failed to activate *CDR1p-lacZ* in *S. cerevisiae*

The DNA-binding capability of ScNdt80p decreased approximately 19-, 27- and 14-fold when the DNA-binding domain contained a mutation at residues Arg111, Arg177 and Arg254, respectively. Several homologues of ScNdt80p from higher eukaryotes, including proteins from *Neurospora crassa*, *Dictyostelium discoideum*, *Caenorhabditis elegans*, *Drosophila melanogaster* and humans, have been reported (Montano *et al.*, 2002). Interestingly, Arg177 and Arg254 are absolutely conserved, and Arg111 is highly conserved (Arg or Lys), in all these proteins (Montano *et al.*, 2002). Since the DNA-binding domain of CaNdt80p is important for activating *CDR1p-lacZ* in *S. cerevisiae*, CaNdt80p with mutations of the DNA-binding domain could fail to activate *CDR1p-lacZ*. To assess this possibility, we mutated the Arg432 amino acid of CaNdt80p, corresponding to ScNdt80p residue Arg177, known to be the most important residue for the DNA-binding activity of ScNdt80p (Fig. 1b)

(Montano *et al.*, 2002). We found that overexpression of *Candt80*^{R432A} in the SLO85 strain failed to activate *CDR1p-lacZ* in *S. cerevisiae* (Fig. 2). In addition, this is also consistent with the concept that this domain of CaNdt80p is important for the activity associated with DNA binding.

Construction of the *Candt80/Candt80*^{R432A} *C. albicans* strain

Though we showed that the DNA-binding domain of CaNdt80p is important for activating *CDR1p-lacZ* in *S. cerevisiae*, we were still not sure if the DNA-binding domain of CaNdt80p indeed plays a role in drug resistance in *C. albicans*. To test this hypothesis, we reintroduced the *Candt80*^{R432A} mutated allele into the locus of *CaNDT80* in the *Candt80/Candt80* mutant to determine if this mutated allele, like the wild-type *CaNDT80* gene, could restore the drug susceptibility phenotype to the *Candt80/Candt80* mutant. After assessing the constructed genome with PCR and sequencing, the expression of this mutated allele in the reintroduced *Candt80/Candt80::Candt80*^{R432A} strain was also determined by RT-PCR (Fig. 3b), along with the *Candt80/Candt80::CaNDT80* strain. First, there was no genomic DNA contamination in the preparations of total RNA, since none of the 928 bp intron-containing *CaEFB1*

DNA fragment was amplified from cDNA samples (Fig. 3b, lanes 2–4). The wild-type *CaNDT80* (Fig. 3b, lane 2) and *Candt80*^{R432A} (Fig. 3b, lane 4) alleles were expressed. We also determined the level of both the *CaNDT80* and the *Candt80*^{R432A} alleles by real-time PCR, and the results indicated that both alleles were expressed at the same level.

The *Candt80*^{R432A} allele with a mutation of the DNA-binding domain failed to complement *Candt80/Candt80* mutant cells

Consequently, to further elucidate the function of the DNA-binding domain of CaNdt80p in *C. albicans*, we analysed the drug susceptibility of different strains by the Etest assay and by the agar dilution method. The Etest results are shown in Fig. 4(a). The MIC to fluconazole of the *Candt80/Candt80* cells was 0.5 mg l⁻¹. The MICs to fluconazole of the *Candt80/Candt80* mutant cells containing a copy of the wild-type *CaNDT80* gene (*Candt80/Candt80::CaNDT80*) increased to 1.5 mg l⁻¹. Thus, the wild-type *CaNDT80* gene conferred drug resistance on the *Candt80/Candt80* mutant cells. In contrast, the *Candt80/Candt80::Candt80*^{R432A} cells were as sensitive to the drug as the *Candt80/Candt80* mutant cells. The results of the agar dilution method are shown in Fig. 4(b). All cells tested grew in the absence of drug. The

Fig. 4. The *Candt80*^{R432A} allele failed to complement *Candt80/Candt80* mutant cells. Susceptibility to azole drugs was determined by both the Etest assay (a) and the agar dilution method (b). Fluconazole strips (FL, 0.016–256 µg ml⁻¹) were used for the Etest. For agar dilution, cells were grown on media in the absence of drug, or in the presence of 5 µg fluconazole ml⁻¹ or 0.3 µg miconazole ml⁻¹. The results of both assays for four tested strains, *CaNDT80/CaNDT80* (SC5314), *Candt80/Candt80::CaNDT80* (YLO137), *Candt80/Candt80* (YLO133), and *Candt80/Candt80::Candt80*^{R432A} (YLO263) were photographed after 2 days growth at 30 °C.

Candt80/Candt80 mutant cells with the wild-type *CaNDT80* allele were more susceptible to the drug than the wild-type cells. This may be due to the dosage effect described previously (Chen *et al.*, 2004; Kohler & Fink, 1996). The *Candt80/Candt80::CaNDT80* cells with the wild-type *CaNDT80* allele were more resistant to drugs than the *Candt80/Candt80* mutant cells, consistent with the Etest results and our previous report that CaNdt80p is involved in drug resistance (Chen *et al.*, 2004). It was noteworthy that the *Candt80/Candt80::Candt80^{R432A}* cells with the mutated *Candt80^{R432A}* allele were as sensitive to drugs as the *Candt80/Candt80* mutant cells. The results of both the Etest assay and the agar dilution method suggested that, unlike the wild-type *CaNDT80* allele, *Candt80^{R432A}* failed to complement the lack of drug resistance of the *Candt80/Candt80* cells.

We have shown that the fact that ScNdt80p failed to activate *CDR1p-lacZ* in *S. cerevisiae* may have been due to the differences in the activation domains between ScNdt80p and CaNdt80p. Nevertheless, we have been able to show that the DNA-binding domain of ScNdt80p could complement that of CaNdt80p to activate *CDR1p-lacZ*. Furthermore, CaNdt80p containing a mutation of the DNA-binding domain failed to complement the drug sensitivity caused by a null mutation in *C. albicans*. Thus, the DNA-binding domain of CaNdt80p is critical for the drug resistance of *C. albicans*, and it may have the same DNA-binding site as that of ScNdt80p.

ACKNOWLEDGEMENTS

This work was in part supported by grants 94-2320-B-400-001 and 94-2320-B-009-001 from the Taiwan National Science Council, and grant CL-094-PP-05 from the Taiwan National Health Research Institutes.

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