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Short communication

Identification of two proteins induced by exposure of the pathogenic fungus *Candida glabrata* to fluconazole

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Abstract

Candida glabrata is an increasingly important cause of opportunistic fungal infection of humans and appears to be intrinsically resistant to the triazole antifungal fluconazole. However, the mechanisms responsible for reduced susceptibility to azole drugs are not understood. Fluconazole exposure rapidly induced expression of a 169-kDa protein band in plasma membrane fractions of *C. glabrata* cells. Mass spectrometry of trypsin-digested peptide fragments showed that the induced protein band comprised the ATP binding cassette-type drug efflux transporter CgCdr1p. CgCdr1p was also functionally overexpressed in *S. cerevisiae* and similarly identified by mass spectrometry. A 61-kDa protein band in the plasma membrane fraction from *C. glabrata* was also induced by fluconazole exposure. Mass spectrometric peptide fingerprinting identified this band as lanosterol 14α-demethylase, the enzyme in the ergosterol biosynthesis pathway targeted by fluconazole. The rapid induction of a multidrug efflux pump and/or overproduction of lanosterol 14α-demethylase are mechanisms that could make *C. glabrata* appear intrinsically resistant to fluconazole. Mass spectrometric fingerprint analysis of SDS–PAGE separated plasma membrane fractions combined with heterologous hyper-expression provides a convenient method for protein identification and functional evaluation of induced proteins, even in an organism where the genome sequence database is incomplete.

Keywords: *Candida glabrata*; Proteins; Fluconazole

1. Introduction

The incidence of life-threatening nosocomial fungal infections has increased steadily in recent decades, mainly due to a growing population of immunocompromised or immunosuppressed patients whose host defenses have been impaired by primary disease states such as AIDS, or because of bone marrow and organ transplantation treatments or cancer chemotherapy [1]. Particularly prominent are the sharp increases in *Candida* blood stream infections among hospitalised patients and the rising incidence of infections caused by non-*C. albicans Candida* species that include *C. glabrata*, *C. tropicalis* and *C. krusei* [2]. *C. glabrata* is of particular importance as an emerging fungal pathogen because it appears to be innately resistant to moderate levels of fluconazole [3] and is often recovered in clinical

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isolates from AIDS patients [4–6]. Other groups affected include cancer patients receiving head and neck irradiation, liver transplant patients and females with chronic vaginitis. The increasing frequency of both invasive and superficial *C. glabrata* infections is of concern because it has often been linked with the clinical use of fluconazole [7–9] and fluconazole exposure can induce even higher levels of resistance [10].

In several fungal species a failure to accumulate azoles has been identified as a major cause of the resistance demonstrated by clinical isolates [4]. Recent studies show that this property is mainly due to enhanced multidrug efflux resulting in decreased intracellular concentrations of the ergosterol biosynthesis inhibitors [1,5,11–14]. Efflux pumps in general belong to the major facilitator superfamily (MFS) of membrane transport proteins or to the ATP-binding cassette (ABC) superfamily. The MFS pumps use electrochemical gradients while ABC-type transporters utilize ATP as their source of energy. There is little information, however, about mechanisms of *C. glabrata* resistance to azole antifungals at the protein level. Studies of mRNA expression have thus far implicated only the pumps encoded by *CgCDR1* and *CgCDR2* (formerly denoted *PDH1*) in azole efflux [4,5]. The acquisition of the respiratory incompetent petite phenotype in *S. cerevisiae* leads to induction of enhanced multidrug efflux through transcriptional activation. A similar phenomenon in *C. glabrata*, denoted high-frequency azole resistance [10,15], has been linked to the up-regulation of both *CgCDR1* and *CgCDR2.*

Ergosterol is regarded as an essential component of the membranes of many fungal species, including *Candida albicans* and non-*C. albicans Candida* species including *C. glabrata* [16]. It affects membrane permeability, the activities of membrane-bound enzymes and has an important role in mitochondrial energy transduction. Changes in ergosterol concentrations and arrangements in membranes are therefore expected to influence the activities of many metabolic pathways. The enzyme lanosterol 14α-demethylase, an essential component of the ergosterol biosynthesis pathway, is the target of the azole and triazole drugs. In some instances resistance has been correlated with either over-expression of this enzyme [1,14,17] or its genetic modification [17,18]. In some fungi, azole resistance can be caused by alterations in the ergosterol biosynthesis pathway that result in the production of alternative sterols or by an innate absence of ergosterol. These features render cells cross-resistant to amphotericin B [19].

Multiple azole resistance mechanisms can occur in *C. albicans*, even in single isolates from a patient, complicating the analysis and making it difficult to establish the role of an individual mechanism in decreased azole susceptibility [20]. In many instances, the genetically tractable yeast *Saccharomyces cerevisiae* has provided a convenient model to assess the impact of individual resistance mechanisms. We recently functionally hyper-expressed the multidrug efflux pump Cdr1p from *C. albicans* in an *S. cerevisiae* strain that is multiply deleted of membrane transporters [21]. This approach has allowed direct biochemical and physiological studies of transporter function.

Proteome analysis in *S. cerevisiae* has been accomplished by the separation and isolation of proteins by SDS–PAGE or two-dimensional gel electrophoresis. Suitably abundant polypeptides can then be characterized and identified using mass spectrometric fingerprint analysis in comparison with a whole genome database [22]. Although the genomes of *S. cerevisiae* and *C. albicans* have been completely sequenced and at least 6000 open reading frames identified in each case, systematic application of this methodology to *C. albicans* and non-*C. albicans Candida* species is in its infancy [23]. A major obstacle to the proteomic analysis of *C. glabrata* polypeptides is that only a few of the open reading frames in its genome have been determined thus far.

In this study, the expression of *CgCDR1* and *CgERG11* was induced by exposure of *C. glabrata* cells to fluconazole. The protein products of these genes were separated by SDS–PAGE of purified plasma membranes and identified by MALDI-TOF peptide fingerprinting. One of these proteins, CgCdr1p, was shown to be fully functional and confer fluconazole resistance by over-expression in an *S. cerevisiae* strain deleted of multiple transporters. The heterologously expressed polypeptide was identified against a background of *S. cerevisiae* proteins by MALDI-TOF peptide fingerprinting.

2. Experimental

2.1. Yeast strains

Three strains of *C. glabrata*: CBS138 (Schimmelcultures, Baarn, The Netherlands); 850821 (ESR: Institute of Environmental Science and Research, New Zealand Centre for Disease Control, Wellington, New Zealand); and 850920 (ESR), and *S. cerevisiae* AD1-8u$^-$ [21] were used in this study.

2.2. Growth conditions

Yeast cells were routinely cultured to early stationary phase at 30 °C in YPD (1% yeast extract, 2% peptone, 2% glucose). For fluconazole exposure, *C. glabrata* cells were resuspended in fresh YPD at an $O.D._{600\ nm}$ of 0.1. When cells reached $O.D._{600\ nm}$ = 0.3, fluconazole was added to the culture at the indicated final concentration and incubated at 30 °C for a further 7 h. Cell density was measured using a Shimadzu UV-120-02 spectrophotometer, and cell concentration was calculated from culture optical density using a standard curve.

2.3. Isolation of plasma membranes and electrophoresis

Yeasts were grown in YPD at 30 °C until they reached the late exponential phase of growth ($O.D._{600\ nm}$=7). Cells were harvested, washed with distilled water, and resuspended in homogenising buffer (50 m*M* Tris–HCl, pH 7.5, 2 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride) and plasma membranes isolated using a modification of the method described by Monk et al. [24]. Cells were disrupted using glass beads. Cell debris and unbroken cells were removed by centrifugation at 5000 *g* at 4 °C for 10 min. A crude membrane fraction was isolated from the cell-free supernatant by centrifugation at 30 000 *g* at 4 °C for 30 min. The pellet was washed in GTE buffer (10 m*M* Tris–HCl, pH 7.0, 0.5 m*M* EDTA, 20%, v/v, glycerol) and resuspended in GTE buffer. Plasma membranes were prepared by centrifugation of the supernatant obtained after selective precipitation of mitochondrial contaminants at pH 5.2 [25]. The plasma membranes were resuspended in GTE buffer and stored at −80 °C. The protein concentration was determined by a micro-Bradford assay using a Protein Assay Kit II (Bio-Rad, Hercules, CA, USA). Protein samples (30 μg/lane) were separated by electrophoresis in 7.5% SDS–polyacrylamide gels and stained with Coomassie Brilliant Blue R-250.

2.4. In-gel digestion of protein bands

Coomassie Blue-stained protein bands were excised from the gels and were subjected to in-gel digestion by Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA). Gel slices were washed for 15 min with acetonitrile and rehydrated with 10 m*M* dithiothreitol in 100 m*M* NH_4HCO_3. Proteins were reduced at 56 °C for 1 h, and then alkylated at room temperature for 45 min using 55 m*M* iodoacetamide. After two 15-min washes with acetonitrile, gel slices were dried in a vacuum centrifuge. Gel pieces were then rehydrated in a solution of trypsin (25 μg/ml) in digestion buffer (5 m*M* $CaCl_2$ in 50 m*M* NH_4HCO_3) at 4 °C for 45 min. Excess protease was removed and digestion buffer was added to cover the gel pieces. Digestion was allowed to proceed at 37 °C for 16 h. Peptides were then extracted by incubation (20 min) in 20 m*M* NH_4HCO_3, followed by three extractions with 5% formic acid in 50% acetonitrile at room temperature (20 min). The peptide extracts were pooled and concentrated to 10 μl in a vacuum centrifuge.

2.5. Desalting of samples and mass spectrometry analysis of tryptic digests

Each sample of solubilized peptides was loaded on a ZipTip C_{18} pipette tip (Millipore, Bedford, MA, USA), previously equilibrated with 50% acetonitrile followed by 0.1% trifluoroacetic acid (TFA). Tips were washed with 0.1% TFA and peptides were eluted with 3.5 μl of 75% acetonitrile in 0.1% TFA. Each desalted sample was mixed with 1 μl of MALDI matrix (saturated solution of α-cyano-4-hydroxycinnamic acid) on the MALDI-TOF target and allowed to dry. MALDI-TOF spectra were acquired and recorded with a Voyager DE-STR mass spectrometer (Applied Biosystems, Foster City, CA,

USA) using an accelerating voltage of 20 kV in the reflector mode. Acquired data were matched against the GenPept databases using the MS-Fit programs (Baker, P.R. and Clauser, K.R., http://prospector.ucsf.edu), over an Internet connection. Searches were done over the full molecular mass and isoelectric point range. Mass accuracy was set at 0.2 Da for most analysis (external calibration was done using adrenocorticotropic hormone clip 18–39 and angiotensin I as standard peptides). Possible post-translational and chemical modifications such as oxidation of methionine, N-terminal acetylation, N-terminal pyroglutamic acid, and carbamidomethylation of cysteine, were taken into consideration for the queries.

3. Results and discussion

3.1. Overproduction of proteins by exposure to fluconazole

C. glabrata appears to be innately less sensitive to fluconazole than *C. albicans* [3]. In order to investigate the organism's response to fluconazole, we examined changes in the protein profiles of plasma membranes from *C. glabrata* cells exposed to fluconazole. Fig. 1 shows that the expression of two protein bands (169 and 61 kDa) was induced in the Coomassie Brilliant Blue R250-stained SDS–PAGE profiles of plasma membranes obtained from cells exposed to fluconazole (80 μg/ml) for 7 h. Other experiments found 7 h to be the optimal time for the induction (data not shown). The intensity of both bands decreased markedly when fluconazole concentrations were lowered from 80 to 10 μg/ml (data not shown). Expression of the protein bands was induced to differing extents in the three *C. glabrata* strains. There was a comparable induction of the expression of both the 169- and 61-kDa bands in strain CBS138. There was stronger induction of both bands in strain 850821, while there was strong induction of the 61-kDa band and only weak induction the 169-kDa band in strain 850920. Exposure of strains CBS138 and 850821 to fluconazole (80 μg/ml) reduced the growth yield in YPD by at least 50% (from $O.D._{600\ nm}$=35–40 to 15–18), indicating a modification in overall cell energy metabolism had

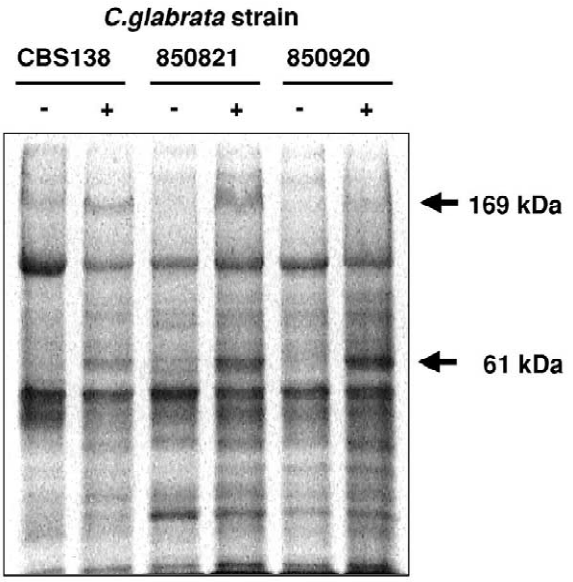

Fig. 1. Coomassie Blue-stained SDS–PAGE profile of plasma membrane fractions from *C. glabrata* CBS138, 850821 and 850920. Each strain showed induced expression of proteins with relative migrations corresponding to 169 and 61 kDa when exponential phase cells were exposed in YPD to 80 μg/ml of fluconazole (lanes marked with +) at 30 °C for 7 h.

occurred. The growth yield of strain 850920 was not altered by fluconazole exposure, but it already had a lower growth yield than the other strains in the absence of fluconazole ($O.D._{600\ nm}$=25) that could not be accounted for by the formation of respiratory incompetent petite cells. Other experiments showed that *C. glabrata* cells grown on the respiratory substrate glycerol were more sensitive to fluconazole than cells grown on glucose, while cells grown anaerobically on glucose showed enhanced fluconazole resistance. The lowering of growth yields in strains CBS138 and 850821 by exposure to fluconazole were commensurate with a block in respiratory metabolism and could not be accounted for by the formation of a significant population of petite cells (<12%).

3.2. Identification of the proteins by mass spectrometric fingerprinting

The 169- and 61-kDa bands were excised from polyacrylamide gels, and digested in-gel by trypsin.

The digested samples were subjected to MALDI-TOF mass spectrometry, and identified by comparison with sequences in the GenPept databases as described in Section 2. The MALDI-TOF data for the 169-kDa protein highly induced in the purified plasma membrane fraction from fluconazole-treated CBS138 or 850821 cells (Fig. 1) gave molecular masses for tryptic fragments that matched regions predicted for the open reading frame of *CgCDR1* (Fig. 2a, Table 1). This open reading frame encodes the 1499-amino acid protein CgCdr1p, a typical member of the ABC protein superfamily. In addition, two less intense tryptic peaks were detected in the

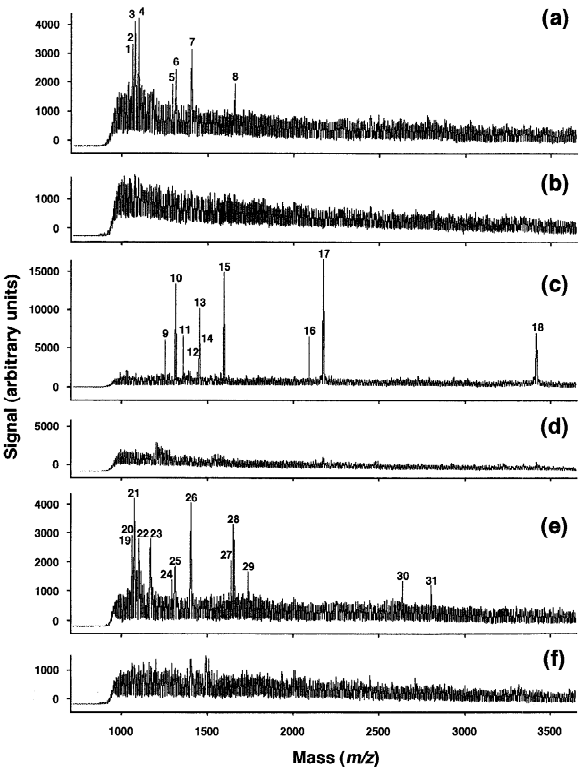

Fig. 2. Identification of SDS–PAGE separated plasma membrane proteins by mass spectrometry. Sections of polyacrylamide gels corresponding to the indicated samples were excised, in-gel digested with trypsin and eluted peptides analysed by MALDI-TOF as described in Section 2. Peaks with masses corresponding to tryptic fragments of CgCdr1p and CgErg11p are numbered. (a,c) From cells treated with fluconazole. (a,b) Gel region corresponding to 169-kDa band from *C. glabrata* strain 850821. (c,d) Gel region corresponding to 61-kDa band from *C. glabrata* strain 850821. (e,f) Gel region corresponding to 169-kDa band from *S. cerevisiae* strain CgCDR1/AD and strain AD1-8u$^-$, respectively.

169-kDa band from strain 850821 which had molecular masses that correlated with fragments predicted for CgCdr2p, a CgCdr1p homologue. These peaks, however, were also present at low levels in bands from cells not exposed to fluconazole, indicating that CgCdr2p is constitutively expressed at a low level in strain 850821. MALDI-TOF analysis of tryptic peptides from the 61-kDa protein band (Fig. 2c) from fluconazole treated *C. glabrata* 850821 cells yielded several peptide sequences that matched regions of the *CgERG11* open reading frame which encodes the 533 amino acid protein lanosterol 14α-demethylase. The masses of tryptic fragments were compared against those predicted from the known *C. glabrata* open reading frames. Only a small portion of the *C. glabrata* genome has been sequenced and so comparison was with a limited database. We therefore decided to express CgCdr1p in *S. cerevisiae* and see whether we could identify tryptic fragments in a database that was extended to include the entire *S. cerevisiae* proteome.

3.3. Validation of the protein identification method

Recently we have developed an *S. cerevisiae* host strain AD1-8u$^-$ and a vector system that allows functional expression of drug efflux pumps from other organisms [21]. The host strain is deleted in seven major membrane transporters and is hypersensitive to antifungal drugs. We have used this system to introduce the *CgCDR1* gene into the genome of *S. cerevisiae* AD1-8u$^-$, generating strain CgCDR1/AD. This strain was highly resistant to fluconazole and other azole drugs, but not to amphotericin B, and it hyper-expressed a 169-kDa protein in plasma membrane fractions. Fluconazole exposure was not required for the hyper-expression of CgCdr1p. As we have described for *CaCDR1*, a similar multidrug resistance ABC transporter from *C. albicans* [21], the *pdr1-3* mutation in the Pdr1p transcriptional regulator of the *S. cerevisiae* host was used to constitutively drive expression of *CgCDR1* under the control of the *PDR5* promoter. The tryptic digestion products obtained from the 169-kDa protein band of *S. cerevisiae* CgCDR1/AD corresponded in mass to those expected for *C. glabrata* CgCdr1p (Fig. 2a,e). No peaks corresponding to tryptic peptides from CgCdr2p or to any proteins

Table 1
Identification of peaks on chromatograms in Fig. 2

Peak	Mass (Da) measured	Database comparison[a]	Peak identification			
			Protein	Fragment position	Fragment sequence	Predicted mass (Da)
1	1040.6277	C.g.	CgCdr1	1183–1189	LFQQYWR	1040.5318
2	1048.6029	C.g.	CgCdr1	946–954	TSTVRESLR	1048.5751
3	1062.6543	C.g.	CgCdr1	1370–1376	FWIFMYR	1062.5235
4	1078.6222	C.g.	CgCdr1	1370–1376	FWIFMYR (Met-ox)	1078.5184
5	1273.7015	C.g.	CgCdr1	536–545	HNTTSTFYFR	1273.5966
6	1292.8591	C.g.	CgCdr1	327–338	GLDSATALEFIR	1292.6850
7	1383.7964	C.g.	CgCdr1	739–750	GNDYVLGDDFLR	1383.6545
8	1641.0814	C.g.	CgCdr1	790–803	QKGEILVFPQNIVR	1640.9488
9	1230.5887	C.g.	CgErg11	138–147	GVIYDCPNHR	1230.5690
10	1293.6862	C.g.	CgErg11	457–469	GVSSPYLPFGGGR	1293.6592
11	1337.7834	C.g.	CgErg11	167–177	YVPLIAEEIYK	1337.7357
12	1418.7863	C.g.	CgErg11	88–99	YGDIFSFMLLGR	1418.7142
13	1430.8237	C.g.	CgErg11	391–403	DVAIPNTSYVVPR	1430.7644
14	1434.7548	C.g.	CgErg11	88–99	YGDIFSFMLLGR (Met-ox)	1434.7091
15	1576.9050	C.g.	CgErg11	18–31	LGLSYFQALPLAQR	1576.8851
16	2077.2493	C.g.	CgErg11	118–137	LADVSAEAAYSHLTTPVFGK	2077.0606
17	2161.1816	C.g.	CgErg11	236–253	GFTPINFVFPNLPLEHYR	2161.1235
18	3413.7131	C.g.	CgErg11	404–431	DYHVLVSPGYTHLQEEFFPKPNEFNIHR	3413.6707
19	1040.6115	C.g.+S.c.	CgCdr1[b]	1183–1189	LFQQYWR	1040.5318
20	1048.6665	C.g.+S.c.	CgCdr1	946–954	TSTVRESLR	1048.5751
21	1062.6174	C.g.+S.c.	CgCdr1[b]	1370–1376	FWIFMYR	1062.5235
22	1078.6019	C.g.+S.c.	CgCdr1[b]	1370–1376	FWIFMYR (Met-ox)	1078.5184
23	1148.6591	C.g.+S.c.	CgCdr1	417–425	IINQDYINR	1148.6064
24	1273.6343	C.g.+S.c.	CgCdr1	536–545	HNTTSTFYFR	1273.5966
25	1292.7408	C.g.+S.c.	CgCdr1[b]	327–338	GLDSATALEFIR	1292.6850
26	1383.7050	C.g.+S.c.	CgCdr1	739–750	GNDYVLGDDFLR	1383.6545
27	1624.0397	C.g.+S.c.	CgCdr1	790–803	QKGEILVFPQNIVR (pyroGlu)	1623.9223
28	1640.9458	C.g.+S.c.	CgCdr1	790–803	QKGEILVFPQNIVR	1640.9488
29	1723.9428	C.g.+S.c.	CgCdr1	401–416	QTTADFLTAVTSPSER	1723.8503
30	2632.4517	C.g.+S.c.	CgCdr1	980–1003	ILEMEQYADAVVGVPGEGLNVEQR (Met-ox)	2632.2929
31	2798.3443	C.g.+S.c.	CgCdr1	507–532	QSSGVTLFMVIGNSSMAFILGSMFYK (pyroGlu)	2798.3607

[a] Databases against which fragment mass data were compared: C.g., *Candida glabrata* sequence, S.c., *Saccharomyces cerevisiae* genome database.
[b] Mass of fragment also matched fragment predicted from *S. cerevisiae* Pdr5p.

predicted from the complete genome database for *S. cerevisiae* were observed, except for four fragments (Fig. 2 and Table 1, Peaks 19, 21, 22 and 25) which had masses predicted from the homologue *S. cerevisiae* Pdr5p. The peaks could not have actually been derived from Pdr5p as the *PDR5* gene in the host strain AD1-8u$^-$ is disrupted. The appropriate control area of a gel lane containing plasma membranes from AD1-8u$^-$ did not show any peaks corresponding to those identified for CgCdr1p (Fig. 2f).

4. Conclusions

Fluconazole exposure can rapidly induce the overexpression of the 61-kDa ergosterol biosynthetic enzyme, lanosterol 14α-demethylase and the 169-kDa *C. glabrata* ABC transporter, CgCdr1p. Both classes of protein were detected in Coomassie Blue-stained SDS–PAGE bands of the plasma membranes from fluconazole-exposed *C. glabrata* cells. The induced bands were digested with trypsin and se-

quences identified by MALDI-TOF mass spectrometry and database analysis. The CgCdr1p protein band was similarly detected by functional hyperexpression of *CgCDR1* in an *S. cerevisiae* strain depleted of other major co-migrating drug transporters [21]. These experiments demonstrate that it is possible to identify an overexpressed protein in a background where thousands of open reading frames may be expressed. Previous studies of ABC transporter function in *C. glabrata* have used comparative measurements of mRNA levels, immunochemical identification, gene disruption or complementation of *S. cerevisiae* strains to elucidate roles [4,5,10,16,17]. We have similarly used Northern analysis to detect the rapid induction of the mRNAs for *CgCDR1*, *CgCDR2* and *CgERG11* in the three strains in the present study (K. Niimi, unpublished). However, it is now widely recognised that gene expression profiles evaluated at the mRNA level only have a modest correlation with the activity of their protein products [26]. This report provides the first direct identification of protein products that are induced through exposure to the triazole drug fluconazole, from essentially undetectable levels to become major constituents of the *C. glabrata* plasma membrane. MALDI-TOF peptide fingerprinting, as shown by the identification of both CgCdr1p and CgCdr2p in the present report, may also avoid the need to produce protein-specific antibodies for polypeptides that are sufficiently highly expressed compared with other co-migrating polypeptides.

Until a complete database for the *C. glabrata* genome is available, it will not be possible to exclude the miss-identification of proteins i.e., polypeptides of similar size that yield tryptic fragments with identical masses to those identified from within our limited database. For the present study, we consider this possibility unlikely because almost all peaks detected in the induced profiles could be assigned to either CgCdr1p or CgCdr2p for the 169-kDa band and to Erg11p for the 61-kDa band. It is therefore reasonable to suggest that fluconazole resistance is associated with the strong induction of one ABC pump and with an increase in the concentration of fluconazole's target. Expression of these two proteins may independently or jointly reduce the susceptibility of cells to fluconazole. We hypothesise that these mechanisms are induced sufficiently rapidly (within 7 h) to give the appearance of an innate resistance. However, these levels of resistance could be circumvented by applying suitably high concentrations of either fluconazole or a more potent azole like voriconazole [27]. But this strategy, in the longer term, would risk increased resistance through the selection of petites.

Although CgErg11p and CgCdr1p seemed to be co-induced in strains CBS138 and 850821, this did not appear to be the case for strain 850920. Comparison of the *CgERG11* promoter [16] with upstream regions of *CgCDR1* and *CgCDR2* [5,10] indicates there is no common transcriptional regulation of ergosterol metabolism and multidrug efflux, arguing against co-induction.

Pdr3p in *S. cerevisiae* responds to modifications in energy metabolism that affect mitochondrial functions, like oxidative phosphorylation, and this transcriptional regulator is autoinduced [28,29]. A similar mechanism may apply to *C. glabrata* strains CBS138 and 850821 because exposure to fluconazole significantly limited the growth yields of these strains, primarily due to an effect on oxidative metabolism. In contrast, strain 850920, in which the 61-kDa band is strongly induced but the 169 kDa is only modestly induced by fluconazole, has an intrinsically lower growth yield in the absence of fluconazole that is barely affected by the presence of 80 μg fluconazole per ml. Elevated levels of expression of *CgCDR1* and *CgCDR2* mRNA have been shown to be important for the acquisition of fluconazole resistance in *C. glabrata*, and in some strains these properties have been correlated with the formation of respiratory incompetent cells equivalent to the petite phenotype in *S. cerevisiae* [10]. Since fluconazole exposure of strains CBS138 and 850821 did not give a significant population of petite cells ($<12\%$), the induction of CgCdr1p has more in common with a retrograde signaling pathway that links the status of F_O assembly in the mitochondrial ATP synthetase with the activity of Pdr3p [29].

Upregulation of *ERG11* mRNA level has been observed in several *Candida* species (*C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei*) exposed to fluconazole or azole drugs [17]. Our observations on the rapid induction of the 61-kDa Erg11p are consistent with these previous studies. However, association of the induced protein with the plasma

membrane fraction at such high concentrations was surprising, given that this cytochrome P450 protein is normally regarded as an endoplasmic reticulum constituent. Immunolocalisation studies will be needed to discriminate between fluconazole-induced relocalisation of this protein to the plasma membrane or contamination of plasma membranes by endoplasmic reticulum that is highly enriched for Erg11p.

The direct visualisation of induced protein bands, their identification by MALDI-TOF peptide fingerprinting and assessment of their function through heterologous hyper-expression in the plasma membrane of *S. cerevisiae* provide a straight forward and information-rich approach to evaluate the consequences of multidrug efflux gene induction. This approach could be applied to the study of other inducible membrane proteins that are refractory to standard analysis.

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