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Proteomic Analysis of Mrr1p- and Tac1p-Associated Differential Protein Expression in Azole-Resistant Clinical Isolates of *Candida albicans*

Christopher F. Hoehamer^{1,2}, Edwin D. Cummings³, George M. Hilliard³, Joachim Morschhäuser⁴, and P. David Rogers^{1,2,3,*}

¹Departments of Clinical Pharmacy, Pharmaceutical Sciences, and Pediatrics, Colleges of Pharmacy and Medicine, University of Tennessee Health Sciences Center, Memphis, Tennessee 38163

²Children's Foundation Research Center of Memphis, Le Bonheur Children's Medical Center, Memphis, Tennessee 38103

³Department of Molecular Sciences, University of Tennessee Health Sciences Center, Memphis, Tennessee 38163

⁴Institut für Molekulare Infektionsbiologie, Universität Würzburg, D-97070, Würzburg, Germany

Abstract

Azole resistance in *Candida albicans* is frequently caused by the overexpression of multi-drug efflux pump genes *MDR1*, *CDR1*, and *CDR2* due to gain-of-function mutations in the zinc cluster transcription factors Mrr1p and Tac1p. In this study, we performed a comparative proteomic analysis to identify proteins whose expression level is influenced by these transcription factors. Both 2-DE and PMF were used to examine the expression profiles of six pairs of matched *C. albicans* isolates carrying gain-of-function mutations in either *MRR1* or *TAC1* resulting in the overexpression of either *MDR1* or *CDR1* and *CDR2*. Using this approach, 17 differentially expressed proteins were identified in the *MDR1*-overexpressing isolates, while 14 were identified in the isolates that overexpress *CDR1* and *CDR2*. Furthermore, we found that the expression of many of these proteins was increased in a wild-type strain of *C. albicans* after the introduction of a gain-of-function allele of *MRR1* or *TAC1*. Moreover, disruption of *MRR1* and *TAC1* in isolates carrying gain-of-function mutations resulted in decreased expression of these proteins, confirming their regulation by Mrr1p or Tac1p. Several proteins involved in heat shock and carbohydrate metabolism were differentially expressed in all clinical isolate sets, but these proteins were not dependent upon either Tac1p or Mrr1p.

*Correspondence: Professor P. David Rogers, Pharm. D., Ph.D., FCCP., Children's Foundation Research Center at Le Bonheur Children's Medical Center, Room 304 West Patient Tower, 50 North Dunlap Street, Memphis, Tennessee, 38103. Phone: (901) 287-5387. Fax: (901) 448-1741. drogers@utmem.edu.

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We have no financial or commercial conflicts of interest to declare.

Keywords

Azole resistance; *Candida albicans*; drug efflux pumps; fluconazole

1. Introduction

The opportunistic human fungal pathogen *C. albicans* is present on mucosal surfaces of the gastrointestinal and urogenital tracts in many healthy individuals with no clinical symptoms; however, this fungus is also a major cause of both superficial and systemic infections, including oropharyngeal candidiasis (OPC), the most frequently observed infection among patients with acquired immune deficiency syndrome (AIDS) [1, 2]. Treatment of these infections with the antifungal agent fluconazole is effective; however, prolonged exposure to this drug can lead to the emergence of azole-resistant strains of *C. albicans* and ultimately therapeutic failure [3–5].

The azole antifungals specifically inhibit the biosynthesis of ergosterol, the major sterol within the fungal membrane, by irreversibly binding to the heme group in the active site of the *ERG11* gene product and azole target, 14 α -lanosterol demethylase [6]. To date several mechanisms of azole resistance have been characterized in *C. albicans* [7–13]. First, point mutations in *ERG11* can cause reduced affinity of Erg11p to azoles [8, 13]. Additionally, overexpression of *ERG11* results in the increased production of 14 α -lanosterol demethylase, requiring more azole for inhibition of its activity [14]. Moreover, azole resistance is also caused in part by insufficient levels of effective antifungal concentrations within the cell due to the constitutive overexpression of multi-drug efflux pump genes, such as the major facilitator superfamily gene, *MDR1*, as well as the ATP-binding cassette (ABC) transporter genes *CDR1* and *CDR2* [9–12, 14]. The up-regulation of *MDR1* confers an azole-resistant phenotype specific for fluconazole, whereas overexpression of *CDR1* and *CDR2* causes azole resistance by mediating efflux of a variety of structurally diverse azoles out of the cell [7]. Furthermore, it has been shown that resistant strains of *C. albicans* isolated from patients with OPC possess a combination of distinct azole resistance mechanisms that can function synergistically, resulting in high-levels of fluconazole resistance [15].

Recent studies have implicated the zinc cluster transcription factors Mrr1p and Tac1p in the regulation of antifungal resistance genes *MDR1*, *CDR1*, and *CDR2* in clinical isolates of *C. albicans*, respectively [16, 17]. These transcription factors both have zinc binuclear cluster Zn(2)-Cys(6) DNA-binding motifs and each presumably binds sequence specific response elements in the promoters of their target genes for transcriptional activation. Recent studies have shown that gain-of-function mutations in these transcription factors cause constitutive, hyperactive transcription of their target genes and confer multi-drug resistance [17, 18], but the specific mechanisms by which these mutations constitutively activate the function of these transcription factors is unknown. Moreover, while gene expression and location profiling studies have identified sets of genes that are targeted by these transcriptional regulators, their influence on protein expression has yet to be examined [17, 19].

In an effort to identify proteins that are coregulated with the multi-drug efflux pump genes *MDR1*, *CDR1*, and *CDR2* upon activation of their respective transcriptional regulators, we

examined six pairs of matched clinical isolates of *C. albicans* originally obtained from AIDS patients diagnosed with OPC who failed azole therapy. Previous studies have demonstrated that these azole-resistant isolates overexpress either *MDR1* (isolates F5, G5, 6692) or *CDR1* and *CDR2* (isolates Gu5, C56, 5674) [11, 13, 20–22]. As overexpression of azole resistance genes in these resistant isolates is due to gain-of-function mutations in their respective transcription factors, we measured changes in protein expression between the azole-susceptible and azole-resistant isolates in each of these matched isolate pairs using a comparative proteomic approach to reveal the differentially expressed proteins co-regulated with either Mdr1p or Cdr1p and Cdr2p. In order to determine which differentially expressed proteins were influenced specifically by activation of Mrr1p or Tac1p, we examined the protein expression profiles of an *MRR1* disruption mutant derived from clinical isolate F5 and a *TAC1* disruption mutant derived from clinical isolate 5674. We also examined the protein expression profiles of two additional mutant strains, each containing a gain-of-function allele of either *MRR1* or *TAC1* introduced into a wild-type background. This investigation expands on previous proteomic studies in *C. albicans* and identifies proteins that are specifically co-regulated with known azole resistance genes and those that are influenced by Mrr1p or Tac1p. Our findings also provide unique insight into the correlation between gene and protein expression profiling as all of the isolate pairs examined here have previously been subjected to microarray analysis [17, 19]. It is possible that some of these differentially expressed proteins contribute directly to azole antifungal resistance.

2. Materials and Methods

2.1 Organisms and culture conditions

All strains used in this study are detailed in Table 1. The clinical *C. albicans* matched isolate pairs used in this study were originally obtained from AIDS patients diagnosed with OPC who failed azole therapy. Briefly, the matched isolate pairs (susceptible/resistant) used in this study overexpress *MDR1* (F1/F5, G1/G5, and 5833/6692) or *CDR1/CDR2* (Gu2/Gu5, C43/C56, and 5457/5674). Strains SC_zcf36MK3A and SZY91 are derivatives of strain SC5314 in which the endogenous *MRR1* and *TAC1* alleles, respectively, were disrupted and gain-of-function alleles were introduced [17, 19]. *MRR1* and *TAC1* disruption mutants F5MRR1M4B and SZY31 were derived from clinical isolates F5 and 5674, respectively [17, 19]. In three independent experiments, *C. albicans* isolates were cultured overnight at 30°C with agitation in 10 mL of Yeast Peptone Dextrose (YPD, Sigma, St. Louis, MO) broth consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose. For each isolate, cells were diluted into 500 mL of YPD broth to an OD₆₀₀ of 0.2 and subsequently grown for 4.5 hours at 30°C to early logarithmic phase in a shaking incubator rotating at 250 rpm.

2.2 Preparation of protein extracts

Early logarithmic *C. albicans* cells were collected by centrifugation at 3000 × *g* for 10 minutes. The cell pellets were washed three times with ultrapure water and broken with 0.5 mm diameter glass beads using a Mini-Bead Beater 8 (BioSpec Products, Inc., Bartlesville, OK) in cold 50 mM Tris buffer (pH 7.4) containing 1 mM EDTA and Complete protease inhibitor cocktail (Roche, Penzberg, Germany). Briefly, equal volumes of glass beads were

added to cell pellets in 2 mL conical bottom tubes and beaten for six 20-second bursts. Between bursts, the samples were incubated on ice for one minute. After disruption, the samples were centrifuged at $20,000 \times g$ for 15 minutes at 4°C to remove the glass beads, unbroken cells, and particulate debris from the homogenate. The supernatant was collected, and the protein content was quantitated using the bicinchoninic acid (BCA) method using BSA as a standard [23]. The protein samples were stored at -80°C for further studies.

2.3 2-DE

For 2-DE, samples containing 300 μg of total protein were processed using the 2-D cleanup kit according to the manufacturer's protocol (Bio-Rad Laboratories, Inc, Hercules, CA). The resulting acetone-washed protein pellets were subsequently added to 300 μL of rehydration buffer consisting of 7 M urea, 2 M thiourea, 50 mM DTT, 4% (w/v) CHAPS, 0.6% (v/v) Bio-Lyte 3–10 ampholytes, and 0.002% (w/v) bromophenol blue. The samples were then subjected to IEF on a PROTEAN IEF Cell (Bio-Rad) over a pI range from four to seven using 17 cm ReadyStrip IPG strips (Bio-Rad) at 20°C . The IPG strips were subjected to passive rehydration for 12 hours and run at 250V for the first 15 minutes, followed by a linear increase in voltage to a maximum of 10,000V, where they ran for an additional five hours. After IEF, the IPG strips were reduced in equilibration solution consisting of 1.5 M Tris-HCl (pH 8.8), 7 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, and 1% (w/v) DTT for 10 minutes followed by an alkylation step in the same equilibration buffer containing 1.5% (w/v) iodoacetamide instead of the DTT for 10 minutes. Proteins were subsequently separated in the second dimension based on their relative molecular mass using homogenous 12% acrylamide gels (20cm \times 20cm 1mm) containing 2.6% *N, N'*-methylene-bis-acrylamide and run at a constant 200V for six to seven hours at 15°C in a PROTEAN[®] plus Dodeca[™] Cell (Bio-Rad). After electrophoresis, the gels were fixed, washed, and stained with Sypro[®] Ruby.

2.4 Gel imaging and analysis software

2-D gels were imaged on an FX imager (BioRad) at the medium Sypro[®] Ruby intensity setting at a resolution of 300 dpi. Spot comparison of 2-D gel images was performed using PDQuest software version 7.1 (BioRad) to measure the differences in spot intensity. Protein spots were compared between gel images using an automated matching program, and any mismatched spots were corrected using a manual matching feature in the software. After background removal, the individual spot volume for each resolved protein spot was normalized against the total spot volume and area in the gel. Protein spots that were consistently differentially expressed in mean normalized spot volume by at least 1.5 fold (50%) in three independent experiments were selected for excision and subsequent identification. Statistical analysis was performed using a Student's *t*-test.

2.5 Protein trypsinization and extraction

Protein spots were excised from gels and transferred to 1.5 mL microfuge tubes pre-washed three times with 50% methanol and 10% glacial acetic acid. Proteins were then destained twice in 200 μL of 50% acetonitrile at 23°C and vortexed for 30 minutes. The samples were subsequently placed in a 96-well Millipore PTFE 0.45 μm filter plate with the wells

pretreated three times with 20% methanol and 0.1% TFA. Trypsinization of the proteins was carried out by adding 200 μL of neat acetonitrile to the wells. The solution was removed, and the samples dried under vacuum for one minute. Another 200 μL of acetonitrile was applied to the samples and removed by vacuum. The washed proteins were subsequently digested in a 100 μL aliquot of fresh digestion buffer containing 12.5 ng/ μL ultrapure sequencing grade trypsin (Promega, Madison, WI), 25 mM ammonium bicarbonate, and 10% acetonitrile and incubated at 37°C overnight. The tryptic polypeptides were extracted in a 25 μL aliquot of fresh extraction solution containing 50% acetonitrile and 5% TFA.

2.6 Protein identification and database search

Protein identification was carried out according to the methods described by Cummings *et al.* 2007 [24]. Briefly, MS spectra were recorded on an Ultraflex matrix assisted laser desorption ionization reflecting time-of-flight (MALDI-ToF/ToF) mass spectrometer (Bruker Daltonics, Bremen, Germany). The autolytic trypsin fragment peaks of 842.509 m/z and 2211.104 m/z were used for internal calibration of peptide mass spectra with mass resolution between 2500 and 8000 m/z . External calibration of this instrument was carried out using a 2:1 mixture of des-R-bradykinin and ACTH (Sigma). Mass spectra parameter settings were 20 kV for the extraction voltage and 11.8 kV for the reflector voltage. Both FlexControl version 2.0 and FlexAnalysis version 2.0 software were used for recording the mass spectra data and sample analysis, respectively. For each sample, the S/N was calculated using the SNAP algorithm using the default settings within the FlexAnalysis software. For each protein identified, MALDI-TOF mass fingerprint data were blasted against a custom database (<http://genolist.pasteur.fr/CandidaDB/>) containing known *C. albicans* ORF DNA sequences using PROWL software. For each protein identified, there was a Z-score value calculated from the software that measured the significance of the identification. A Z-score of 1.65 ranked the mass data search to a 95% level of confidence of nonrandom matches to the specific ORF.

3. Results

A description of the *C. albicans* isolates used in this study is provided in Table 1. In three independent experiments, the combination of 2-DE and PMF revealed the identities of 31 differentially expressed proteins that were found to be coregulated with the overexpression of either *MDR1* or *CDR1* and *CDR2* by at least 1.5-fold (Tables 2 and 3). The overexpression of proteins corresponding to these efflux pump genes was not detected by this approach as we analyzed only the soluble proteins. We report here the proteins that were found to be differentially expressed in at least two of the three pairs of matched isolate sets that overexpress either *MDR1* or *CDR1* and *CDR2*. A summary of all differentially expressed proteins in each of these matched sets can be found in the supplementary data section tables S1–S3 for proteins coregulated with Mdr1p and tables S4–S6 for those coregulated with Cdr1p and Cdr2p.

3.1 Identification of differentially expressed proteins modulated by Mrr1p activating mutations

A total of 17 differentially protein spots were identified in the *MDR1*-overexpressing azole-resistant isolates F5, G5, and 6692 when compared to their azole-susceptible counterparts F1, G1, and 5833, respectively. Among them, 16 proteins were found to be up-regulated in the azole-resistant isolates, while only one protein (Gnd1p) was shown to be down-regulated. The proteins that were consistently up-regulated in all *MDR1*-overexpressing resistant isolates were Ipf5987p, Adh4p, Oye32p, Ipf17186p, Gpx1p, Tdh3p, Grp2p, Eno1p, Ifd1p, Ifd4p, and Ifd5p. Selected differentially expressed proteins are visualized in Figure 1A, which shows detailed regions of 2-D gels displaying Mrr1p-associated changes in expression of selected soluble proteins from the azole-susceptible isolate F1 carrying wild-type alleles of *MRR1*, azole-resistant isolate F5 harboring gain-of-function mutations in both alleles of *MRR1*, and the homozygous *mrr1* strain F5MRR1M4B, a derivative of isolate F5. The changes in the expression of these proteins depicted in this figure are also representative of the alterations we observed in azole-resistant isolates G5 and 6692. The putative biological function, molecular properties, and relative fold changes of these proteins are summarized in Table 2. The protein expression profiles of mutant strain SC zcf36MK3A, containing a gain-of-function allele of *MRR1* from isolate F5 showed similar patterns of differential protein expression to those observed in azole-resistant isolates F5, G5, and 6692 when compared to its wild-type strain SC5314 (Figure 1B). The proteins that were differentially expressed as a result of the *MRR1* gain-of-function allele were Ipf5987p, Adh4p, Ipf17186p, Gpx1p, Grp2p, Ifd1p, Ifd4p, Ifd5p, and Ifd6p. Two proteins, Grp2p and Ipf17186p, were found to be represented by multiple spots in all three *MDR1*-overexpressing resistant clinical isolates as well as in strain SC zcf36MK3A (Figure 1B). Disruption of *MRR1* in fluconazole-resistant isolate F5 (F5MRR1M4B) returned the expression of all 10 of these proteins to the same levels as observed in fluconazole susceptible isolate F1 (Figure 1A). These data confirm that these 10 proteins are indeed regulated by Mrr1p and suggest that at least Eno1p and Tdh3p, while increased in expression in resistant clinical isolates F5, G5, and 6692, are not regulated by Mrr1p.

3.2 Identification of differentially expressed proteins modulated by Tac1p activating mutations

In three independent experiments, 14 differentially expressed proteins were identified in the *CDR1*- and *CDR2*-overexpressing azole-resistant isolates Gu5, C56, and 5674 compared to the azole-susceptible isolates Gu2, C43, and 5457, respectively (Table 3). The expression of 10 proteins was found to be up-regulated in the azole-resistant strains while the expression of four proteins was shown to be down-regulated (Table 3). Selected differentially expressed proteins are visualized in Figure 2A, which shows detailed regions of 2-D gels displaying Tac1p-associated changes in expression of selected soluble proteins from the azole-susceptible isolate 5457 carrying wild-type alleles of *TAC1*, azole-resistant isolate 5674 with gain-of-function mutations in both alleles of *TAC1*, and the homozygous *tac1* strain SZY31, which is a derivative of isolate 5674. The alterations in expression of these proteins shown in this figure are also representative of those changes observed in azole-resistant isolates Gu5 and C56. The proteins determined to be differentially expressed in all three

azole-resistant isolates were Enolp, Tdh3p, Gpx1p, Ipf4065p, Ipf15297p, Pdc11p, Snz1p and Ssb1p. Interestingly, the differential expression of a heat shock protein 70 (Ssb1p) was found to be down-regulated in one spot and up-regulated in another adjacent spot on the gel (data not shown). This horizontal shift in position of Ssb1p was observed in the protein expression profiles of all *CDR*-overexpressing azole-resistant isolates. Similarly, alcohol dehydrogenase (Adh1p) also displayed a relative horizontal shift in position, but this shift was only observed in the 2-D expression profile of SZY91 when compared to that of SC5314 (Figure 2B). The protein expression profiles of SZY91, containing a gain-of-function allele of *TAC1*, revealed similar patterns of differential protein expression to those observed in azole-resistant isolates Gu5, C56, and 5674. The differentially expressed proteins identified after the introduction of a *TAC1* allele were Adh1p, Ipf4065p, Ipf15297p, Gpx1p, and Snz1p (Figure 2B). Disruption of *TAC1* in fluconazole-resistant isolate 5674 returned the expression of all five of these proteins to the same levels as observed in fluconazole susceptible isolate 5457 (Figure 2A). This confirms that these five proteins are indeed regulated by Tac1p, and demonstrates that Enolp, Tdh3p, Pdc11p, and Ssb1p, while all increased in expression in resistant clinical isolates Gu5, C56, and 5674, are not regulated by Tac1p.

4. Discussion

The proteomic methods used in this study have proven to be an effective approach to analyze the alterations in protein expression between azole-susceptible and –resistant clinical isolates of *C. albicans* [21, 25]. It was recently reported that specific mutations in the transcription factor genes *MRR1* and *TAC1* result in constitutive overexpression of *MDR1*, *CDR1*, and *CDR2* as well as other Mrr1p and Tac1p target genes and multi-drug resistance [17]. Sequencing of the *MRR1* alleles in the matched *C. albicans* azole-resistant isolates used in the present study revealed single nucleotide substitutions, resulting in a proline to serine (P683S) exchange in isolate F5 and a glycine to valine (G997V) substitution in isolate G5 [17]. When these mutated *MRR1* alleles were introduced into a drug-susceptible strain of *C. albicans*, the resulting mutant exhibited constitutive *MDR1* overexpression and multi-drug resistance to fluconazole, diamide, brefeldin A, and cerulenin. Likewise, studies of matched clinical isolates identified multiple mutations in Tac1p, including the amino acid change from asparagine to aspartic acid at position 977 (N977D) that induced increased *CDR1* and *CDR2* expression and associated drug resistance [18]. Introduction of that mutated *TAC1* allele into a *tac1/tac1* background, followed by loss of heterozygosity, conferred *CDR1* and *CDR2* overexpression and drug resistance.

In the present study, we examined six matched clinical isolate sets and mutants disrupted for *MRR1* or *TAC1* carrying gain-of-function mutations in these genes in an effort to identify proteins whose expression is influenced by these transcription factors. With the exception of the proteins associated with carbohydrate metabolism, our findings confirm that each of these transcription factors regulates a different set of unique proteins associated with specific mechanisms of azole resistance.

The azole-resistant clinical isolates F5, G5, and 6692 belong to a collection of well-characterized strains of *C. albicans* that have been shown to overexpress *MDR1*. Several

proteins identified in this study were previously observed to be differentially expressed in association with *MDR1* overexpression, including, Ifd1p, Ifd4p, Ifd5p, Ifd6p, and Ipf5987p [21]. Proteins encoded by the *IFD* gene family are putative aldo-keto reductases and are classified as homologues of the putative aryl-alcohol dehydrogenases in the *S. cerevisiae* family of proteins, *YPL088W*. Ifd4p is also known as Csh1p and has been shown in *C. albicans* to function in cell-surface hydrophobicity and to mediate the binding to host target proteins [26]. Another aldo-keto reductase found co-regulated with Mdr1p was Ipf5987p, which is a homologue of the *YPR127W* gene product in *S. cerevisiae* and is also regulated by Yrr1p and Yrm1p [27]. It has previously been demonstrated that neither overexpression nor disruption of this gene affected drug resistance or oxidative stress resistance in *C. albicans* clinical isolates G5 and F5 [21]. Among the differentially expressed proteins identified in the isolates that overexpress *MDR1*, the aldo-keto reductases were the most abundantly overrepresented. It is tempting to speculate that their overexpression may contribute to azole resistance through detoxification of reactive substrates produced from azole stress.

Another protein found to be consistently differentially expressed in the *MDR1*-overexpressing isolates was Grp2p, which was shown to be up-regulated in two different spots in azole-resistant isolates F5, G5, and 6692 (Figure 1A). This protein was also previously found to be differentially expressed in *C. albicans* clinical isolate 12–99, an azole-resistant isolate that overexpresses *MDR1*, *CDR1*, *CDR2*, and *ERG11* [25]. Grp2p putatively functions as a stress-induced reductase and is homologous to the NADPH-dependent methylglyoxal reductase or Gre2p in *S. cerevisiae*. It is believed that Gre2p functions in the detoxification of methylglyoxal-derivatives, and its expression is induced by a variety of cellular stress responses, including those in response to osmotic and oxidative stress, heat shock, heavy metals [28, 29]. The involvement of Gre2p in ergosterol metabolism has also been reported [30]. Mutant strains lacking *GRE2* displayed both impaired growth and a striking induction of ergosterol biosynthesis enzymes, including, Erg10p, Erg19p, and Erg6p after membrane stress. Furthermore, these *gre2* mutants also exhibited impaired tolerance to ergosterol biosynthesis inhibitors.

Another protein up-regulated with *MDR1* is Ipf17186p, a protein that belongs to the ThiJ/PfpI protein family [31]. Its ortholog in *S. cerevisiae* is Hsp31p, and expression of *HSP31* is regulated by the transcription factor Yap1p during oxidative stress [32]. Additionally, it has been shown that a mutant lacking this gene is hypersensitive to ROS generators, including hydrogen peroxide, diamide, *tert*-butyl hydroperoxide, and menadione, which suggests that Hsp31p also functions in protecting the cell against oxidative stressors [32]. Similarly, Oye32p is a putative NAD(P)H oxidoreductase, whose expression is also induced during oxidative stress [33, 34]. Mutants lacking this gene display increased levels of ROS compared to the wild-type strain; therefore, this enzyme may be involved in intracellular redox homeostasis in *C. albicans* [35].

It was previously shown that while disruption of *MDR1* in two fluconazole resistant isolates (including isolate F5) diminished fluconazole resistance, disruption of *MRR1* in these isolates had an even stronger effect than inactivation of *MDR1*, suggesting that *MRR1* controls expression of factors in addition to *MDR1* that contribute to azole resistance in strains F5 and G5. In an effort to determine which differentially expressed proteins

identified in this study were influenced by Mrr1p, we examined the protein expression profiles of a wild-type strain of *C. albicans* containing the gain-of-function *MRR1* allele from isolate F5. We observed changes in the expression levels of many proteins previously identified in the clinical isolates (Table 2). We also examined a *MRR1* disruption mutant derived from isolate F5 and found increased expression of these proteins to be dependent upon Mrr1p (Table 2 and Figure 1A). It is possible that at least one of these proteins under the influence of Mrr1p directly contributes to azole resistance in these clinical isolates.

The azole-resistant clinical isolates Gu5, C56, and 5674 are among a variety of well-known clinical isolates previously shown to overexpress the ATP-binding cassette transporter genes *CDR1* and *CDR2*. We identified 14 proteins that were differentially expressed in these azole-resistant isolates. Those proteins identified whose genes have previously been shown to be differentially expressed in association with *CDR1* and *CDR2*, include, Ipf4065p, Gpx1p, and Snz1p [19, 36]. Gpx1p or glutathione peroxidase is an enzyme whose up-regulation was observed in all azole-resistant isolates. This enzyme is involved in the glutathione redox cycle and generally functions in the cell to reduce harmful oxidants using glutathione as a source of reducing power. Studies have implicated Gpx1p to function in quinone resistance in tumor cells [37, 38], and in human drug-resistant cancer cells, overexpression of this enzyme is commonly coregulated with increased activities of the ABC transporter P glycoprotein [39].

Another protein found to be differentially expressed in these azole-resistant isolates was a member of the heat shock protein 70 family, Ssb1p. This protein was found to be down-regulated in one spot and up-regulated in another adjacent spot, displaying a slight horizontal shift in location in the gel. This shift may be due to a change in overall net charge of the protein, possibly as a result of a phosphorylation event. Ssb1p is a cytosolic ATPase that functions as a ribosome-associated molecular chaperone that is involved in the folding of nascent polypeptide chains. These proteins are stress-induced and are considered the major class of Hsp70 chaperones. It has been shown that the Hsp70 protein Ssz1p (formerly Pdr13p) in *S. cerevisiae* functions to increase the activity of the pleiotropic drug resistance (PDR) transcription factor Pdr1p, resulting in increased expression of the ABC transporter genes *YOR1* and *PDR5* with associated drug resistance to cyclohexamide and oligomycin [40]. More recently, it has been shown that another cytosolic Hsp70p chaperone Ssa1p in *S. cerevisiae* is a negative regulator of the PDR transcription factor Pdr3p [41]. The overexpression of Ssa1p repressed expression of *PDR5* and attenuated drug resistance to cyclohexamide. It is tempting to speculate that the Hsp70 protein Ssb1p plays a similar role in regulating the transcription factor Tac1p and influencing azole resistance.

As gain-of-function mutations in Tac1p result in increased transcription of its target genes and associated drug resistance, our aim was to identify differentially expressed proteins whose expression is influenced by Tac1p. We studied the protein expression profiles of a wild-type strain of *C. albicans* containing a *TAC1* allele that possesses a gain-of-function mutation (N972D) from azole-resistant isolate 5674. We detected changes in the expression levels of proteins identified in isolates Gu5, C56, and 5674, including, Adh1p, Ipf4065p, Ipf15297p, Gpx1p, and Snz1p (Table 3). We also examined a *TAC1* deletion mutant derived

from isolate 5674, and found increased expression of these proteins to be dependent upon Tac1p.

Interestingly the up-regulation of enzymes involved in carbohydrate metabolism such as enolase (Eno1p), glyceraldehyde-3-phosphate dehydrogenase (Tdh3p), and pyruvate decarboxylase (Pdc11p) was observed in the azole-resistant isolates studied. The up-regulation of Gap1p and 6-phosphogluconate dehydrogenase (Gnd1p) was reported in a clinical azole-resistant isolate of *C. albicans* [25], and another study revealed a variety of up-regulated glycolytic enzymes, including Gap1p (Tdh3p) and Pdc11p, in a laboratory-derived fluconazole-resistant strain of *C. albicans* [42]. Perhaps, glycolysis is up-regulated to produce ATP and reducing equivalents such as NADH and NADPH to complement the azole resistance proteins, particularly the ABC transporters *CDR1* and *CDR2*, ATP-dependent chaperones, and the aldo-keto reductases. However, it is important to note that while introduction of gain-of-function mutant alleles of *MRR1* or *TAC1* imparts fluconazole resistance, this did not result in increased expression of these metabolic enzymes. Likewise, disruption of *MRR1* in isolate F5 or *TAC1* in isolate 5674 did not influence the expression of these proteins. Each isolate pair used in this study consists of a pre-treatment isolate and a post-treatment failure isolate. Each treatment failure isolate shares two things in common relative to its respective matched pre-treatment isolate: the development of azole resistance and an increased duration of exposure to the host. It is therefore possible that up-regulation of these proteins reflects adaptation to the host environment.

Our data also provide a unique opportunity to closely examine the overlap between gene and protein expression as all of the isolates studied here have previously been examined by microarray analysis [17, 19, 36]. Overall, there was a significant degree of overlap when comparing our DNA microarray data to the data obtained from our proteomic analysis in these matched isolates, especially for the *MDR1*-overexpressing isolates F5, G5, and 6692 (Tables 2 and 3). The proteins that were found to be differentially expressed in these isolates whose genes were previously shown to be differentially expressed by DNA microarray analysis, include, Gpx1p, Grp2p, Ifd1p, Ifd4p, Ifd5p, Ifd6p, Ino1p, Ipf4065p, Ipf5987p, Oye32p, Adh4p, Ipf15297p, Ipf17186p, and Snz1p. The correlation between gene and protein expression in this study confirms that a majority of these differentially expressed proteins identified are regulated at the transcriptional level. Furthermore, this integrated approach of studying mRNA and protein expression in combination provides a more global understanding of the regulation of targets of Tac1p and Mrr1p.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABC	ATP binding cassette transporter
AIDS	Acquired immune deficiency syndrome
OPC	oropharyngeal candidiasis

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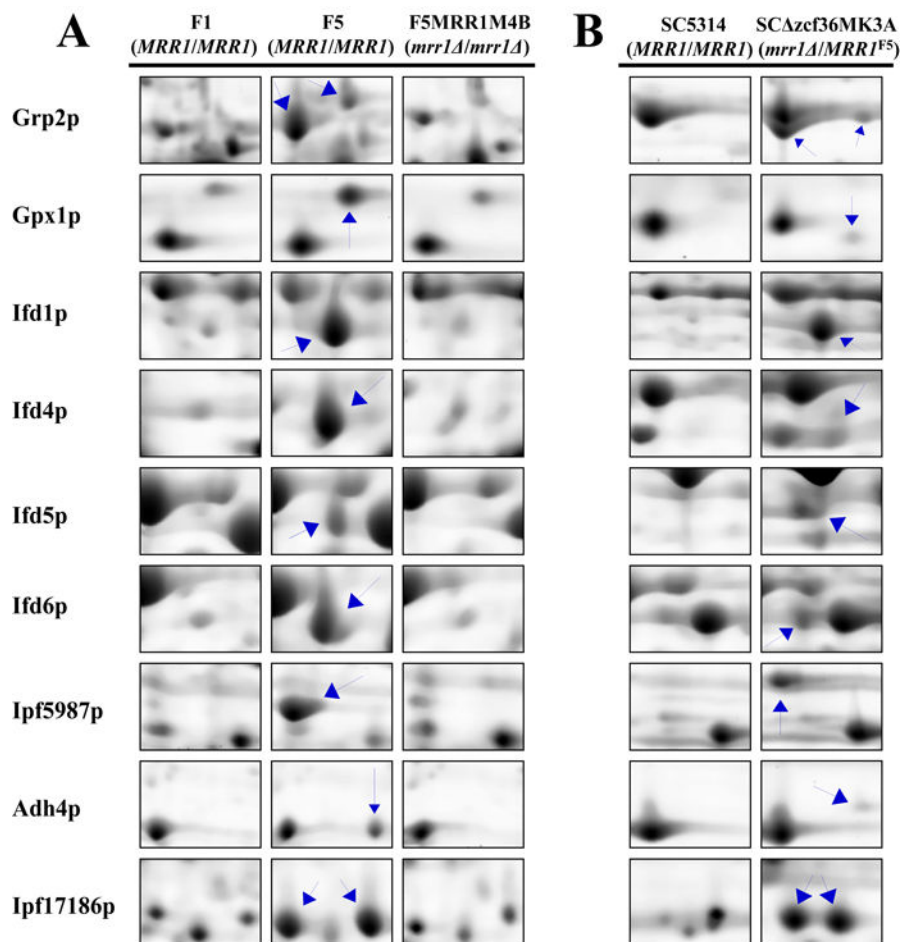


Figure 1. Detailed regions of SYPRO® Ruby –stained 2-D gels displaying *MRR1*-associated changes in protein expression of selected soluble proteins extracted from (A) azole-susceptible isolate F1 with wild-type alleles of *MRR1*, azole-resistant isolate F5 carrying gain-of-function mutations in both alleles of *MRR1*, F5-derivative mutant strain F5MRR1M4B lacking both alleles of *MRR1*, (B) wild-type strain SC5314 with endogenous *MRR1* alleles, and mutant strain SC Δzcf36MK3A with an introduced gain-of-function *MRR1* allele from azole-resistant isolate F5.

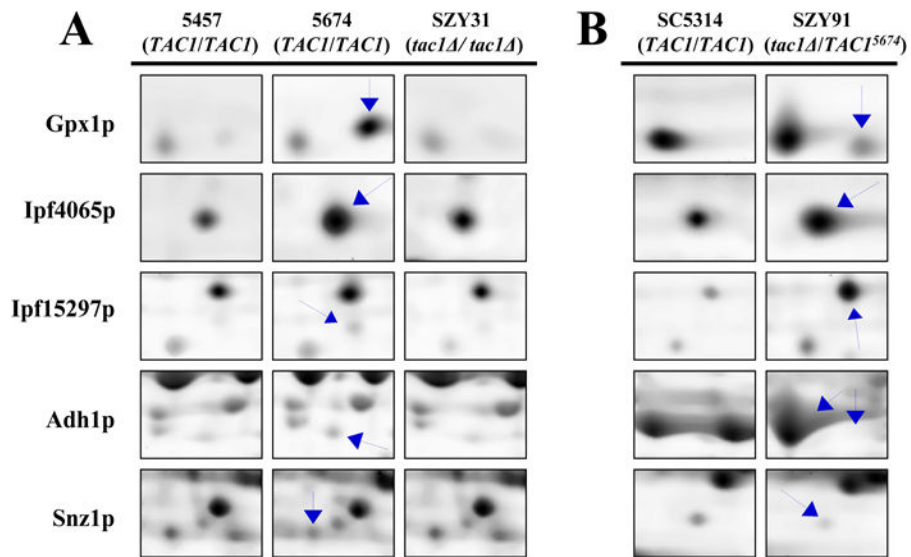


Figure 2. Detailed regions of SYPRO® Ruby –stained 2-D gels displaying *TAC1*-associated changes in protein expression of selected soluble proteins extracted from (A) azole-susceptible isolate 5457 with wild-type alleles of *TAC1*, azole-resistant isolate 5674 carrying gain-of-function mutations in both alleles of *TAC1*, 5674-derivative mutant strain SZY31 lacking both alleles of *TAC1*, (B) wild-type strain SC5314 with endogenous *TAC1* alleles, and mutant strain SZY91 with an introduced gain-of-function allele of *TAC1* from azole-resistant isolate 5674.

Table 1

Description of *C. albicans* clinical isolates and mutant strains used in this study.

C. albicans strain	Relevant Genotype or Description	Description of Mutations			Reference	Reference source
		Protein	Mutation	Reference		
F1	Clinical isolate from patient F, fluconazole-susceptible	-	-	-	-	[10]
F5	Clinical isolate from patient F, fluconazole-resistant	Mrr1p	P683S	[17]	-	[10]
G1	Clinical isolate from patient G, fluconazole-susceptible	-	-	-	-	[10]
G5	Clinical isolate from patient G, fluconazole-resistant	Mrr1p	G997V	[17]	-	[10]
5833	Clinical isolate, fluconazole-susceptible	-	-	-	-	[20]
6692	Clinical isolate, fluconazole-resistant	Mrr1p	T360L_K335N	[22]	-	[20]
Gu2	Clinical isolate, fluconazole-susceptible	-	-	-	-	[11]
Gu5	Clinical isolate, fluconazole-resistant	Tac1p	L979E	[Unpub. data]	-	[11]
C43	Clinical isolate, fluconazole-susceptible	-	-	-	-	[12]
C56	Clinical isolate, fluconazole-resistant	Tac1p	N977D	[18]	-	[12]
5457	Clinical isolate, fluconazole-susceptible	-	-	-	-	[20]
5674	Clinical isolate, fluconazole-resistant	Tac1p	N972D	[44]	-	[20]
SC5314	Wild-type <i>C. albicans</i> model strain	-	-	-	-	[43]
SC zcf36MK3A	<i>mrr1</i> :: <i>FRT/MRR1(F5)-caSAT1</i>	Mrr1p	P683S	[17]	-	[17]
SZY91	<i>tac1</i> :: <i>FRT/TAC1-2(5674)-MPA^R-FLP</i>	Tac1p	N972D	[44]	-	[44]
FSMRR1M4B	<i>mrr1</i> :: <i>FRT/mrr1</i> :: <i>FRT</i>	-	-	-	-	[17]
SZY31	<i>tac1</i> :: <i>FRT/tac1</i> :: <i>FRT</i>	-	-	-	-	[44]

A summary of the differentially expressed proteins co-regulated with Mdr1p identified by 2-DE and MALDI-TOF MS

Table 2

Protein	Systematic ^a Name	Function	pI	Mr (kDa)	Z-score ^b		Protein coverage (%)		Matched peptides		Fold change		t-test p-value						
					F5	G5	F5	G5	F5	G5	F5/F1	G5/G1	F5	G5	F5	G5			
Eno1p	orf19.395	Enolase (2-Phosphoglycerate dehydratase)	5.5	47.2	2.4	2.4	2.4	2.4	21	61	53	7	20	16	2.1 ± 0.5	2.2 ± 0.7	2.4 ± 0.7	0.04	0.00
Tdh3p	orf19.6814	Glyceraldehyde-3-phosphate dehydrogenase ^f	6.6	35.8	2.4	2.4	1.8	4.5	25	38	38	9	9	10	2.0 ± 1.9	1.9 ± 0.3	1.8 ± 0.4	0.21	0.05
Gpx1p	orf19.86	Glutathione peroxidase ^c	6.6	18.1	2.4	2.4	2.4	35	41	39	7	8	8	8	3.7 ± 1.0	3.8 ± 0.3	3.1 ± 0.6	0.00	0.00
Grp2p	orf19.4309	Reductase ^{c, d, f}	6.0	37.6	2.4	2.4	2.4	31	22	18	9	9	5	5	4.1 ± 0.9	4.4 ± 1.1	11.6 ± 0.8	0.00	0.01
Grp2p	orf19.4309	Reductase ^{c, d, f}	6.0	37.6	2.3	2.4	2.4	28	35	55	8	13	22	22	5.5 ± 2.3	5.3 ± 0.9	13.4 ± 3.9	0.07	0.02
Ifd1p	orf19.1048	Putative aryl-alcohol dehydrogenase ^{c, d, e}	5.6	39.1	2.4	1.8	2.2	42	28	21	15	8	9	9	10 ± 6.2	3.9 ± 0.4	6.4 ± 1.3	0.11	0.00
Ifd4p	orf19.4477	Putative aryl-alcohol dehydrogenase ^{c, d, e}	6.0	38.3	2.4	2.4	2.3	37	46	31	15	19	8	10.1 ± 4.7	27.6 ± 16.2	2.1 ± 1.1	0.08	0.19	
Ifd5p	orf19.1048	Putative aryl-alcohol dehydrogenase ^{c, d, e}	5.4	39.2	2.2	2.4	2.4	31	33	33	9	12	8	4.4 ± 1.9	13.9 ± 6.2	6.9 ± 1.1	0.11	0.20	
Ipf5987p	orf19.7306	Aldo-keto reductase ^{c, e}	5.5	39.0	2.3	2.4	2.4	43	60	44	9	17	11	4.7 ± 0.8	201 ± 46	3.2 ± 0.3	0.02	0.00	
Oyc32p	orf19.3131	Putative NADPH-dependent flavin oxidoreductase ^c	5.9	47.5	2.4	1.8	1.3	30	21	16	10	6	4	7.3 ± 2.5	5.4 ± 3.2	1.7 ± 0.3	0.00	0.04	
Adh4p	orf19.271	2,4-dienoyl-coenzyme A reductase ^c	7.0	28.1	2.4	2.4	2.4	28	22	25	8	6	7	25.2 ± 5.3	4.8 ± 0.4	21.8 ± 7.5	0.02	0.00	
Ipf17186p	orf19.251	Heat shock protein 31 of DJ-1/Pfp1 family ^c	4.7	25.8	2.3	2.4	2.4	20	23	48	5	7	9	6.1 ± 0.6	4.5 ± 0.5	5.2 ± 0.6	0.00	0.00	
Ipf17186p	orf19.251	Heat shock protein 31 of DJ-1/Pfp1 family ^c	4.7	25.8	2.1	2.3	2.4	25	35	36	9	7	7	7.9 ± 0.2	10.8 ± 2.2	7.7 ± 1.2	0.04	0.00	
Adh1p	orf19.3997	Alcohol dehydrogenase ^f	8.6	46.1	N/A	2.4	2.3	N/A	39	42	N/A	14	13	ND	2 ± 0.6	1.7 ± 0.1	N/A	0.08	0.01
Pdc11p	orf19.2877	Pyruvate decarboxylase	5.4	62.4	N/A	2.4	2.3	N/A	36	17	N/A	15	7	ND	1.7 ± 0.7	2.5 ± 1.7	N/A	0.19	0.16
Ifd6p	orf19.4476	Putative aryl-alcohol dehydrogenase ^{c, d, e}	5.9	39.1	2.2	1.2	N/A	26	20	N/A	8	5	N/A	5.1 ± 4.9	6.0 ± 0.4	ND	0.15	0.00	
Gnd1p	orf19.5024	6-Phosphogluconate dehydrogenase ^d	6.1	56.9	2.4	N/A	2.4	18	N/A	33	8	8	N/A	18	0.4 ± 0.1	ND	0.4 ± 0.1	0.00	N/A

^aSystematic names are according to <http://www.candidagenome.org>.

^bZ-score values of 1.20 and 1.65 rank the mass data search to a 90% and 95% level of confidence of nonrandom matches to the specific open reading frame, respectively.

^cGenes that were found to be differentially expressed by microarray analysis [17].

^dProteins previously shown to be differentially expressed in clinical isolates of *C. albicans* by Hooshdaran *et al.* 2004 [25].

^eProteins differentially expressed in clinical isolates of *C. albicans* by Kusch *et al.* 2004 [21].

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^f Proteins found differentially expressed between *C. albicans* strain SC5314^S and SC5314^R by Yan *et al.*, 2007 [42].

Proteins in bold were observed to be differentially expressed in *C. albicans* mutant SC_zcf36MK3A.

ND-Not detected

N/A-Not applicable

Table 3

A summary of the differentially expressed proteins co-regulated with Cdr1p and Cdr2p identified by 2-DE and MALDI-TOF MS.

Protein	Systematic ^a Name	Function	pI	Mr (kDa)	Z-score ^b	Protein coverage (%)			Matched peptides			Fold change		t-test p-value					
						Gu5	C56	5674	Gu5	C56	5674	Gu5/Gu2	C56/C43	5674/5457	Gu5	C56	5674		
Eno1p	orf19.395	Enolase (2-Phosphoglycerate dehydratase)	5.5	47.2	2.4	2.0	2.3	49	21	40	18	7	15	2.0 ± 0.3	1.6 ± 0.6	2.2 ± 0.4	0.05	0.11	0.00
Tdh3p	orf19.6814	Glyceraldehyde-3-phosphate dehydrogenase ^{d, e}	6.6	35.8	2.4	2.3	2.1	29	18	46	8	6	10	1.7 ± 0.3	2.1 ± 1.1	2 ± 0.5	0.00	0.08	0.03
Gpx1p	orf19.87	Glutathione peroxidase ^c	6.6	18.1	2.4	2.0	2.4	37	23	26	7	5	6	4.2 ± 1.3	12 ± 8	8.5 ± 1.5	0.00	0.05	0.01
Ip4065p	orf19.1862	Unknown function ^c	5.2	13.1	2.3	1.7	2.2	56	47	50	6	4	4	2.0 ± 0.3	3.9 ± 1.5	2.2 ± 0.2	0.01	0.01	0.01
Ip15297p	orf19.3053	Unknown function ^c	4.3	20.3	1.8	1.9	2.3	25	39	59	5	5	8	1.8 ± 0.2	6.1 ± 2.5	1.6 ± 0.1	0.00	0.01	0.01
Pdc11p	orf19.2877	Pyruvate decarboxylase	5.4	62.4	2.4	2.4	2.4	31	34	35	14	14	11	2.2 ± 0.8	1.6 ± 0.2	2.3 ± 0.6	0.00	0.04	0.00
Ssb1p	orf19.6367	Heat shock protein 70	5.3	66.4	2.4	2.3	2.4	29	39	40	15	14	24	1.7 ± 0.4	2.2 ± 0.1	1.6 ± 0.3	0.00	0.03	0.06
Adh1p	orf19.271	Alcohol dehydrogenase ^e	8.6	46.1	2.3	2.4	2.4	20	50	53	7	16	20	2.0 ± 0.4	510 ± 456	1.5 ± 0	0.01	0.01	0.00
Atp2p	orf19.5653	F1F0 ATPase complex, beta subunit	4.9	55.7	2.4	2.4	N/A	51	52	N/A	18	16	N/A	1.8 ± 0.4	1.8 ± 0.5	ND	0.05	0.00	ND
Ino1p	orf19.7585	Myoinositol-1-phosphate synthase ^c	5.3	57.8	2.3	N/A	24	28	N/A	25	9	N/A	11	2.7 ± 0.5	ND	2.6 ± 0.9	0.01	ND	0.02
Ssb1p	orf19.6367	Heat shock protein 70	5.3	66.4	2.4	1.7	1.6	56	19	14	28	7	8	0.5 ± 0.3	0.5 ± 0	0.5 ± 0	0.08	0.03	0.00
Snz1p	orf19.2947	Stationary phase protein ^c	5.8	31.8	2.3	2.4	1.7	24	24	15	7	7	5	0.5 ± 0.1	0.4 ± 0.2	0.5 ± 0	0.00	0.02	0.00
Ado1p	orf19.5591	Adenosine kinase	5.0	38.2	N/A	2.4	2.3	N/A	43	35	N/A	11	10	ND	0.4 ± 0.1	0.5 ± 0.1	ND	0.04	0.01
Apt1p	orf19.1448	Adenine phosphoribosyltransferase	5.2	20.9	2.1	N/A	2.3	49	N/A	52	6	N/A	7	0.2 ± 0.3	ND	0.5 ± 0.1	0.02	ND	0.02

^aSystematic names are according to <http://www.candidagenome.org>.

^bZ-score values of 1.65 or greater ranked the mass data search to a 95% level of confidence of nonrandom matches to the specific open reading frame.

^cGenes that were found to be differentially expressed in microarray analysis [19].

^dProteins found to be differentially expressed in clinical isolates of *C. albicans* by Hooshdaran *et al.* 2004 [25].

^eProteins previously found to be differentially expressed in *C. albicans* strain SC5314 by Yan *et al.* 2007 [42].

Proteins in bold were observed to be differentially expressed in *C. albicans* mutant SZY91.

ND-Not detected

N/A-Not applicable