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The bZip Transcription Factor Cap1p Is Involved in Multidrug Resistance and Oxidative Stress Response in *Candida albicans*

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***Candida albicans* is an opportunistic pathogenic yeast which frequently develops resistance to the antifungal agent fluconazole (FCZ) in patients undergoing long-term therapy. FCZ-resistant strains often display a reduced intracellular FCZ accumulation which correlates with the overexpression of the ATP-binding cassette transporters *CDR1* and *CDR2* or the major facilitator (MF) *MDR1*. We have recently cloned a *C. albicans* gene, named *CAP1*, which codes for a bZip transcription factor of the AP-1 family homologous to the Yap1 protein involved in multidrug resistance and response to oxidative stress in *Saccharomyces cerevisiae*. *CAP1* was found to confer FCZ resistance in *S. cerevisiae* by transcriptionally activating *FLR1*, a gene coding for an MF homologous to the *C. albicans MDR1* gene product (A.-M. Alarco, I. Balan, D. Talibi, N. Mainville, and M. Raymond, J. Biol. Chem. 272:19304–19313, 1997). To study the role of *CAP1* in *C. albicans*, we constructed a CAI4-derived mutant strain carrying a homozygous deletion of the *CAP1* gene (CJD21). We found that deletion of *CAP1* did not affect the susceptibility of CJD21 cells to FCZ, cerulenin, brefeldin A, and diamide but caused hypersensitivity to cadmium, 4-nitroquinoline *N*-oxide, 1,10-phenanthroline, and hydrogen peroxide, an effect which was reverted by reintroduction of the *CAP1* gene in these cells. Introduction of a hyperactive truncated allele of *CAP1* (*CAP1-TR*) in CJD21 resulted in resistance of the cells to all of the above compounds except hydrogen peroxide. The hyperresistant phenotype displayed by the CJD21 *CAP1-TR* transformants was found to correlate with the overexpression of a number of potential *CAP1* transcriptional targets such as *MDR1*, *CaYCF1*, *CaGLR1*, and *CaTRR1*. Taken together, our results demonstrate that *CAP1* is involved in multidrug resistance and oxidative stress response in *C. albicans*. Finally, disruption of *CAP1* in strain FR2, selected in vitro for FCZ resistance and constitutively overexpressing *MDR1*, did not suppress but rather increased the levels of *MDR1* expression, demonstrating that *CAP1* acts as a negative transcriptional regulator of the *MDR1* gene in FR2 and is not responsible for *MDR1* overexpression in this strain.**

The opportunistic yeast *Candida albicans* is the leading etiologic agent of candidiasis, an infection affecting severely immunocompromised individuals. The last decade has seen an increase in the number of cases in which the immune system is chronically compromised, most notably with the onset of the AIDS pandemic, leading to a substantial increase in the incidence of *Candida* infections. Candidiasis is usually treated with azole antifungal agents such as ketoconazole, itraconazole, and fluconazole (FCZ), with FCZ currently the most widely used due to its high level of bioavailability and low toxicity (38). However, a growing number of studies report the occurrence of clinical failure during FCZ treatment, which correlates with increased levels of in vitro FCZ resistance in *C. albicans* clinical isolates (14, 30, 38).

In *C. albicans* and other *Candida* species, azole resistance has been associated with the alteration or overexpression of the azole cellular target 14α-lanosterol demethylase, encoded by the *ERG11*/*CYP51A1* gene (41, 53, 58). In addition, a number of studies have also shown that many resistant strains display lower intracellular accumulation of FCZ involving the participation of energy-dependent transporter-mediated drug efflux mechanisms in these strains (2, 10, 22, 36, 44). Furthermore, in some *C. albicans* FCZ-resistant clinical and experimental isolates, azole resistance has been correlated with the overexpression of the *CDR1* and *CDR2* genes, encoding ATP-binding cassette (ABC) transporters, or overexpression of the *MDR1* gene, encoding a major facilitator (MF) (43, 44, 58). However, the molecular mechanisms controlling the transcriptional activation of these transporter-encoding genes have not yet been elucidated.

Our studies of the molecular determinants of azole resistance led us to isolate a *C. albicans* gene which confers FCZ resistance when overexpressed in *Saccharomyces cerevisiae* (1). This gene encodes a protein highly homologous to the *S. cerevisiae* bZip transcription factor Yap1p and was named *CAP1*, for *C. albicans* AP-1. Yap1p has been associated with resistance to a variety of toxicants as well as with tolerance to oxidative stress induced by compounds such as H_2O_2 and diamide (21, 23, 26, 46, 49, 55, 56, 59). Yap1p is the prototype of a growing family of transcription factors which also includes *S. cerevisiae* Yap2p (7) as well as six other *S. cerevisiae* members identified through sequence similarity searches (15), in addition to *C. albicans* Cap1p (1), *Schizosaccharomyces pombe* Pap1p (51), *Kluyveromyces lactis* Klyap1p (5), and *Aspergillus nidulans* meaBp (37). Although related to the yeast transcription factor Gcn4 and to the mammalian AP-1 proteins Jun and Fos, Yap1p-like transcriptions factors are distinctive due to atypical residues present in their basic DNA binding domains (15). Interestingly, Yap1p, Yap2p, Cap1p, Pap1p, and Klyap1p constitute a subgroup within the YAP family of transcription factors since these proteins possess a highly conserved C-terminal region termed the cysteine-rich domain (CRD). This domain contains three invariably conserved cysteine residues

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TABLE 1. *C. albicans* strains used in this study

Strain	Parental strain	Relevant genotype	Reference
CAI4	SC5314	*CAP1/CAP1*	17
CJD10	CAI4	*CAP1/cap1*Δ::*hisG-URA3-hisG*	This study
CJD11	CJD10	*CAP1/cap1*Δ::*hisG*	This study
CJD20	CJD11	*cap1*Δ::*hisG-URA3-hisG/cap1*Δ::*hisG*	This study
CJD21	CJD20	*cap1*Δ::*hisG/cap1*Δ::*hisG*	This study
SGY243	A-81	*CAP1/CAP1*	24
FR2	SGY243	*CAP1/CAP1*	2
FJD10	FR2	*CAP1/cap1*Δ::*hisG-URA3-hisG*	This study
FJD11	FJD10	*CAP1/cap1*Δ::*hisG*	This study
FJD20	FJD11	*cap1*Δ::*hisG-URA3-hisG/cap1*Δ::*hisG*	This study
FJD21	FJD20	*cap1*Δ::*hisG/cap1*Δ::*hisG*	This study

and has been shown, both in Yap1p and in Pap1p, to regulate the nuclear localization of these proteins in response to oxidative stress (27, 52).

Yap1p mediates its pleiotropic phenotype through the transcriptional activation of a number of downstream target genes, including *YCF1*, which encodes an ABC transporter essential for cadmium resistance (56); *ATR1*, encoding an MF involved in 4-nitroquinoline *N*-oxide (4-NQO) and 3-amino-1,2,4-triazole resistance (11); *TRX2*, which encodes a thioredoxin conferring tolerance to oxidative stress (26); *TRR1*, encoding a thioredoxin reductase (32); *GSH1*, whose product is a γ-glutamylcysteine synthetase involved in glutathione biosynthesis (60); and *GLR1*, encoding a glutathione reductase (20). In addition, we have shown that *YAP1* mediates FCZ resistance through the transcriptional activation of *FLR1*, which codes for a multidrug transporter of the MF superfamily highly homologous to the *C. albicans MDR1* gene product (1). Cap1p and Yap1p are functional homologues, since *CAP1* overexpression in *S. cerevisiae* also leads to FCZ resistance through transcriptional activation of *FLR1* and expression of *CAP1* in a *yap1* mutant strain partially restores the ability of the cells to grow in the presence of toxic concentrations of cadmium or H_2O_2 (1). The high level of structural and functional similarities between Cap1p and Yap1p suggested that Cap1p could be involved in multidrug resistance (MDR) and oxidative stress response (OSR) in *C. albicans*. Furthermore, the *CAP1/YAP1*-dependent *FLR1*-mediated FCZ resistance phenotype in *S. cerevisiae* suggested that Cap1p could also mediate FCZ resistance in *C. albicans* through the transcriptional activation of *MDR1*. This paper reports the results of our investigations of Cap1p function in *C. albicans*.

MATERIALS AND METHODS

Yeast strains and culture conditions. Yeast strains used in this study are listed in Table 1. The cells were routinely grown in YPD or uracil-deficient SD -Ura medium at 30°C (47).

Deletion of *CAP1*. Plasmid pBC-CAP1 was generated by inserting a 3.2-kb *Kpn*I-*Hin*dIII *CAP1* fragment excised from plasmid YEp352-CAP1 (1) into plasmid pBC (Stratagene, PDI Bioscience, Aurora, Ontario, Canada) cut with *Kpn*I and *Hin*dIII. The *CAP1* open reading frame (ORF) in pBC-CAP1 was removed by replacing a 1.35-kb *Cla*I-*Bsa*BI *CAP1* fragment with a 4-kb *Bam*HI-*Bgl*II *hisG-URA3-hisG* cassette excised from plasmid pMB7 (17), generating plasmid pBC/*cap1*Δ::*hisG-URA3-hisG*. A linear 5.8-kb *cap1*Δ::*hisG-URA3-hisG* fragment was released from pBC/*cap1*Δ::*hisG-URA3-hisG* by digestion with *Sal*I and used to transform *C. albicans* CAI4 and FR2 to Ura^+ prototrophy. Transformations were performed by the lithium acetate method (18). Counterselection of the *URA3* gene was carried out on plates containing 5-fluoroorotic acid (5-FOA; 1 mg/ml; Toronto Research Chemicals Inc., North York, Ontario, Canada) as described by Boeke (6) except that uracil was replaced with uridine (25 μg/ml).

Genomic DNA isolation and Southern blot analyses. *C. albicans* genomic DNA was prepared essentially as described for *S. cerevisiae* (39) except that Zymolyase-100T (ICN Biomedicals, Costa Mesa, Calif.) was added to a final concentration of 0.4 mg/ml. Genomic DNAs (2 μg) were digested to completion with *Bgl*II, electrophoresed in triplicate on 1% agarose gels, and transferred to a nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, Ill.). Prehybridization was performed for 2 h at 65°C in 6× SSC (0.9 M NaCl, 0.9 M sodium citrate)–5× Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll, 1% polyvinylpyrrolidone)–0.5% sodium dodecyl sulfate (SDS) in the presence of 20 μg of denatured salmon sperm DNA per ml of prehybridization solution. Hybridization was carried out for 16 h at 65°C with 10^6 cpm of radiolabeled probe per ml of hybridization solution, using a 3.2-kb *Kpn*I/*Hin*dIII fragment containing the entire *CAP1* gene, a 341-bp *Acc*I DNA fragment (positions +276 to +617 relative to the start codon) internal to the *CAP1* ORF (1), or a 900-bp *Bam*HI/*Bgl*II *hisG* fragment isolated from plasmid pMB7 (17). The blots were washed at high stringency (0.1× SSC–0.1% SDS at 65°C) and exposed at −80°C with two intensifying screens.

Plasmid construction. Plasmids YEp352-CAP1 and YEp352-CAP1TR (1) were digested with *Pst*I and *Sma*I to generate a 3.2-kb *CAP1* and a 3-kb *CAP1-TR* (truncated form) fragment, respectively. Both DNA fragments were blunt ended and cloned into plasmid pMK22 (29) (obtained from R. Rachubinski, University of Alberta) linearized with *Sca*I, generating plasmids PMK-CAP1 and PMK-CAP1TR, respectively. Plasmids PMK22, PMK-CAP1, and PMK-CAP1TR were transformed in CAI4 and CJD21 cells by the lithium acetate method (18). Single Ura^+ colonies were picked and restreaked on SD −Ura medium. Individual clones for CJD21/PMK-CAP1 and CJD21/PMK-CAP1TR transformants were selected based on wild-type levels of *CAP1* expression, as determined by Northern blot analysis (data not shown).

Drug resistance assays. Stock solutions of FCZ (10 mg/ml) and cadmium sulfate (100 mM) were prepared in water. Stock solutions of 4-NQO (5 mM), and diamide (1 M) were prepared in dimethyl sulfoxide. Stock solutions of cerulenin (1 mg/ml), brefeldin A (1 mg/ml), and 1,10-phenanthroline (10 mg/ml) were prepared in ethanol. Dilutions of 30% H_2O_2 were prepared in water. FCZ was obtained from Pfizer Canada Inc. (Arnprior, Ontario, Canada), cadmium sulfate was obtained from MAT Laboratory (Beauport, Québec, Canada), and the other compounds were obtained from Sigma (Mississauga, Ontario, Canada). Drug resistance was determined by spot assay. PMK22 transformants (CAI4/PMK, CJD21/PMK, CJD21/PMK-CAP1, and CJD21/PMK-CAP1TR) were grown overnight on SD −Ura plates. Cells were then suspended in YPD to an A_{600} of 0.1. Three microliters of fivefold serial dilutions of each yeast culture were spotted onto YPD plates supplemented or not with a specific antifungal compound. Qualitative growth differences among the yeast transformants were recorded following incubation of the plates at 30°C for 2 days. Growth was not affected by the presence of ethanol (1%) or dimethyl sulfoxide (0.15%).

RNA preparation and Northern blotting. Total RNA from cells grown in YPD or in SD −Ura medium (PMK22 transformants) was prepared by using the glass beads extraction protocol (9). RNA samples (20 μg) were electrophoresed on a 7.5% formaldehyde–1% agarose gel and transferred by capillarity to a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Detection of specific RNAs was performed by hybridization with ^{32}P-labeled DNA probes as previously described (1). The *CAP1* probe was a 614-bp *Xba*I-*Hin*cII DNA fragment (positions +47 to +661 relative to the translation initiation codon [1]). An *MDR1* DNA fragment was generated by PCR with primers 5′-ACTCTTGCTGATGATACA and 5′-TATATGGATGTACGACCA and overlaps region +135 to +559 of the *MDR1* gene (positions are relative to the translation initiation codon [16]). This PCR fragment was subsequently gel purified and cloned blunt into the pCRII vector (TA cloning kit; Invitrogen). The *MDR1* DNA fragment used to generate this probe was then obtained following *Eco*RI digestion of the PCR-*MDR1* plasmid. The *C. albicans CaYCF1*, *CaGLR1*, and *CaTRR1* probes were obtained by PCR using oligonucleotides derived from sequences available from the *C. albicans* information pages on the Alces server (http://alces.med.umn.edu/Candida.html). A 220-bp *CaYCF1* fragment was amplified with primers 5′-TATCAATATTGATGGTATAG and 5′-CCTCGGTAG TCTCCCTC; a 256-bp *CaGLR1* fragment was generated with primers 5′-TCC ATCAGTGATTTTCTC and 5′-GCAACACCAAAACCTTG; and a 286-bp *CaTRR1* fragment was obtained with primers 5′-TCTGAAGTATCATGGAAC and 5′-CAGCAGAAGTGGTGGCTT. All DNA fragments were gel purified before random labeling with [α-^{32}P]ATP (40). The membranes were also hybridized with an *ACT1* probe (kindly provided by B. Magee, University of Minnesota), confirming that the RNA samples had been equally loaded and transferred to the membranes.

Preparation of antisera. A glutathione *S*-transferase (*GST*)-*CAP1* in-frame gene fusion was constructed by inserting a 341-bp *Acc*I *CAP1* fragment blunt ended with T4 DNA polymerase and inserted into the *Sma*I site of vector pGEX-4T-1 (Pharmacia), generating plasmid pGEX-CAP350. The resulting fusion protein contains 114 amino acids of the Cap1p protein (amino acid positions 93 to 207 [1]). *Escherichia coli* DH5α cells transformed with pGEX-CAP350 were treated with isopropyl-β-D-thiogalactoside (0.1 mM) for 4 h at 30°C to induce the expression of the fusion protein. The GST-Cap1p fusion protein was soluble and was thus purified from a crude bacterial lysate by affinity chromatography on immobilized glutathione (48). The purified fusion protein was used to raise polyclonal antibodies in two New Zealand White rabbits, yielding two anti-Cap1p-350 antisera of high titers (C1 and C2). The C1 antiserum was used without any further purification for the detection of Cap1p in our experiments.

Protein preparation and Western blot analysis. Total protein extracts were prepared from strains CAI4, CJD21, CJD21/PMK, CJD21/PMK-CAP1, and

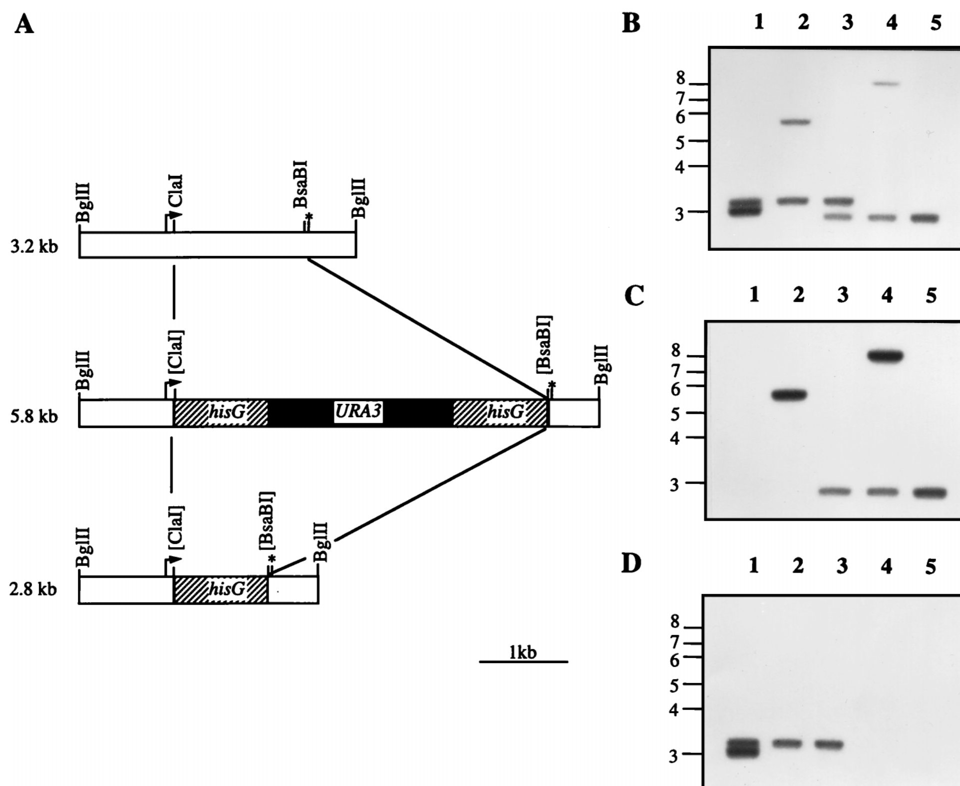

FIG. 1. Chromosomal deletion of *CAP1* in CAI4. (A) Schematic representation of the disruption strategy. The *CAP1* locus is contained within a 3.2-kb *Bgl*II fragment (top). The start (arrow) and stop (asterisk) codons of the *CAP1* ORF are indicated. The disruption cassette (middle) was generated by replacing a 1.35-kb *Cla*I-*Bsa*BI *CAP1* fragment by the 4-kb *hisG-URA3-hisG* cassette. After counterselection on 5-FOA, recombination between the two *hisG* direct repeats should generate a 2.8-kb *Bgl*II fragment (bottom). Southern blot analysis was used to characterize the different steps of the disruption (B to D). Genomic DNA was extracted from strains CAI4 *CAP1*/*CAP1* (lanes 1), CJD10 *CAP1*/*cap1*Δ::*hisG-URA3-hisG* (lanes 2), CJD11 *CAP1*/*cap1*Δ::*hisG* (lanes 3), CJD20 *cap1*Δ::*hisG-URA3-hisG*/*cap1*Δ::*hisG* (lanes 4), and CJD21 *cap1*Δ::*hisG*/*cap1*Δ::*hisG* (lanes 5). DNA samples (2 μg) were digested in triplicate with *Bgl*II, separated by electrophoresis on agarose gels, and transferred to nylon membranes. The blots were then probed with either the 3.2-kb *Bgl*II fragment comprising the entire wild-type *CAP1* gene (B), a 0.9-kb *Bam*HI-*Bgl*II *hisG* fragment (C), or a 0.6-kb *Xba*I-*Hinc*II *CAP1* internal fragment deleted in the *cap1*Δ::*hisG-URA3-hisG* allele (D). Positions of molecular size markers (in kilobases) are indicated on the left. Membranes were exposed for 6 h at −80°C with two intensifying screens.

CJD21/PMK-CAP1TR as described previously (3). Protein concentration was determined by the Bradford method (8), using bovine serum albumin as the standard. Total protein extracts (50 μg) were suspended in Laemmli sample buffer, boiled for 10 min, and separated by electrophoresis on an SDS–12% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and analyzed with the anti-Cap1p-350 polyclonal antibody at a dilution of 1:5,000, using a chemiluminescence detection system under conditions recommended by the manufacturer (Boehringer Mannheim).

RESULTS

Chromosomal deletion of the *CAP1* gene in *C. albicans* CAI4. To investigate the biological function of Cap1p in *C. albicans*, both copies of the *CAP1* gene were deleted in the FCZ-sensitive *C. albicans* strain CAI4, using the Ura-blaster strategy (17). A linear 5.8-kb *cap1*Δ*::hisG-URA3-hisG* deletion fragment was constructed as described in Materials and Methods (Fig. 1A) and used to transform CAI4. In CAI4, the *CAP1* locus is polymorphic: the two *CAP1* alleles are located on a 3.0- and a 3.2-kb *Bgl*II genomic fragment (Fig. 1B, lane 1). Deletion of the first *CAP1* allele occurred in strain CJD10 at the 3.0-kb *Bgl*II *CAP1* locus, as seen from the disappearance of the 3.0-kb *Bgl*II fragment and the appearance of a new 5.8-kb *Bgl*II fragment hybridizing with the 3.2-kb *CAP1* probe (Fig. 1B, lane 2). After counterselection on 5-FOA, a 2.8-kb *Bgl*II fragment resulting from the recombination between the two *hisG* direct repeats was detected in strain CJD11 (Fig. 1B, lane 3). This strain was used for a second round of transformation with the *cap1*Δ fragment. Deletion of the remaining 3.2-kb *Bgl*II *CAP1* allele was confirmed in strain CJD20 by the appearance of a new fragment of about 9 kb which hybridizes with the *CAP1* probe (the size of this fragment suggests that two *cap1*Δ fragments were inserted during the recombination process) (Fig. 1B, lane 4). Proper loop-out of the *URA3* marker in strain CJD21 was confirmed by the appearance of a second 2.8-kb fragment hybridizing with the *CAP1* probe (Fig. 1B, lane 5). Southern blotting analysis was also performed with a *hisG* and a *CAP1* internal probe, confirming the correct genotypes of the different strains (Fig. 1C and D). CJD21 cells are Ura$^-$ and thus suitable for transformation with a *URA3*-based vector for the expression of *CAP1* in a null mutant background.

Involvement of *CAP1* in *C. albicans* MDR and OSR. Our previous findings that *CAP1* mediates FCZ resistance when overexpressed in *S. cerevisiae* led us to investigate the role of *CAP1* in *C. albicans* FCZ resistance. First, we compared the levels of FCZ susceptibility of the CAI4 and CJD21 strains, using a spot assay. This experiment showed that deleting *CAP1* does not change the ability of the cells to grow on FCZ, as both strains displayed an FCZ MIC of 1 μg/ml in this assay (data not shown). We then analyzed the FCZ resistance profile of CJD21 cells transformed with plasmid pMK22 carrying either the full-length *CAP1* gene or a hyperactive allele of *CAP1* (*CAP1-TR*). This allele, identified previously during our isolation of the *CAP1* gene (1), codes for a truncated Cap1p protein

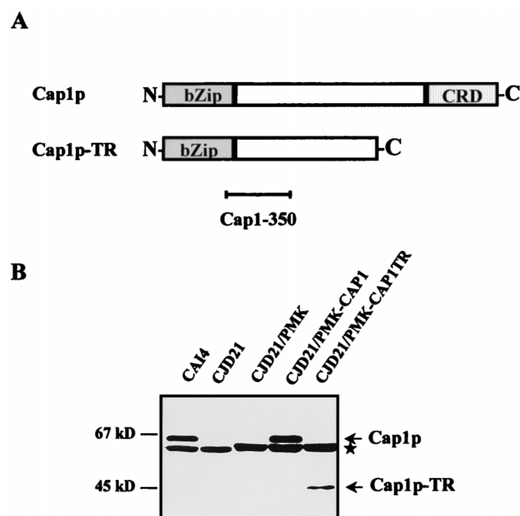

FIG. 2. Expression of the Cap1p and Cap1p-TR proteins in *C. albicans*. (A) Schematic representation of the full-length (Cap1p) and the truncated (Cap1p-TR) proteins. Positions of the bZip domain (grey) and the CRD (stippled) are indicated. The region used to generate a GST-Cap1p fusion protein (Cap1-350) is indicated. This fusion protein was used to raise an anti-Cap1p-350 polyclonal antibody. (B) Western blot analysis of Cap1p and Cap1p-TR expression. Total proteins were extracted from CAI4, CJD21, CJD21/PMK, CJD21/PMK-CAP1, and CJD21/PMK-CAP1TR cells. Protein samples (50 μg) were separated by electrophoreses on an SDS–12% polyacrylamide gel, transferred to a nitrocellulose membrane, and analyzed with the anti-Cap1p-350 polyclonal antibody. Arrows on the right indicate positions of the full-length (Cap1p) and truncated (Cap1p-TR) proteins. The position of a nonspecific cross-reacting protein is also indicated (★). Positions of molecular size standards are shown on the left.

which lacks 165 amino acids at its C terminus but is otherwise identical to the full-length protein and which is hyperactive in conferring FCZ resistance in *S. cerevisiae* (Fig. 2A) (1). Total protein extracts from CJD21 cells transformed with plasmids PMK22, PMK-CAP1, and PMK-CAP1TR were analyzed by Western blotting using an anti-Cap1p polyclonal antibody (Fig. 2B). This antibody was raised against a region of the Cap1p protein (Cap1-350) located downstream of the bZip domain and thus recognizes both the full-length and the truncated forms of Cap1 (Fig. 2A). This experiment showed that the anti-Cap1p antibody detects a 65-kDa protein in CAI4 which is absent in the CJD21 cells, thus demonstrating that this protein is Cap1p and confirming the complete deletion of the *CAP1* gene in strain CJD21. We noted a difference between the apparent molecular mass of 65 kDa for the Cap1p protein deduced from its mobility relative to the migration of the molecular mass markers and its predicted molecular weight of 55,000 based on the deduced amino acid sequence of Cap1p (1). The reasons for this difference are not known, but a similar discrepancy has been reported for the Yap1p protein (33, 50). The CJD21/PMK22-CAP1 transformants produce a 65-kDa protein similar in size and abundance to that detected in CAI4, thus confirming the proper expression of Cap1p from the plasmid-borne copy of the *CAP1* gene (Fig. 2B; compare CJD21/PMK-CAP1 with CAI4). In the CJD21/PMK-CAP1TR transformants, the anti-Cap1p antibody detects a protein with an apparent molecular mass of 45 kDa, which is in close agreement with the calculated molecular weight of 38,000 for the truncated protein encoded by the *CAP1-TR* allele (1). The truncated Cap1p appears to be less abundant than the full-length protein, most likely due to a decreased stability as previously reported for C-terminal deletion versions of Yap1p (57). Analysis of the FCZ resistance profile of the CJD21 transformants confirmed that the deletion of *CAP1* does not affect the level of FCZ susceptibility of the CJD21 cells and that reintroduction of *CAP1* in CJD21 has, as anticipated, no effect on their FCZ resistance profile when analyzed on various concentrations of FCZ (Fig. 3A). However, expression of the truncated Cap1p did confer FCZ resistance, as judged from the ability of the CJD21/PMK-CAP1TR cells to grow on FCZ at concentrations of up to 2 μg/ml, compared to cells expressing the full-length Cap1p protein, whose growth is completely abrogated at this FCZ concentration (Fig. 3A). These results are consistent with our previous finding that the truncated Cap1p protein elicits high levels of FCZ resistance in *S. cerevisiae* (1) and also establish the involvement of *CAP1* in *C. albicans* FCZ resistance.

We have shown that in *S. cerevisiae*, overexpression of *CAP1* mediates resistance not only to FCZ but also to cycloheximide and 4-NQO (1). These results, together with the fact that *S. cerevisiae YAP1* mediates resistance to a wide variety of antifungal agents (21, 23, 45, 56) and prooxidants (26, 46), prompted us to characterize the levels of susceptibility of our different transformants to a series of compounds displaying antifungal activity. As seen for FCZ, deletion of the *CAP1* gene did not affect the ability of the CJD21/PMK transformants to grow on cerulenin and brefeldin A when their growth was compared to that of the CAI4/PMK transformants, both transformants displaying similar cerulenin and brefeldin A MICs (1 and 6 μg/ml, respectively [data not shown]). Reintroduction of the full-length *CAP1* gene had no effect on cell growth in the presence of these drugs, whereas expression of the truncated Cap1p protein in strain CJD21 resulted in increased resistance of the cells to these two compounds (Fig. 3A). On the other hand, we found that the deletion of *CAP1* led to hypersensitivity of the cells to cadmium, 4-NQO, and 1,10-phenanthroline (Fig. 3A; compare CAI4/PMK and CJD21/PMK) which was compensated for by the reintroduction of *CAP1* in strain CJD21. Finally, expression of the truncated form of Cap1p in CJD21 led to a hyperresistance of the cells to these three compounds (Fig. 3A). Taken together, these results indicate that *CAP1* is a determinant of MDR in *C. albicans*.

YAP1 has been shown to be involved in OSR in *S. cerevisiae* by regulating the expression of a number of effector genes (20, 26, 32, 60). In addition, we have shown that *CAP1* complements a *yap1* deletion mutant for its ability to grow on H_2O_2 (1). Therefore, we were interested in determining if *CAP1* also participates in OSR in *C. albicans*. To this end, CAI4 and CJD21 transformants were grown on plates containing different concentrations of either diamide, a thiol oxidant (25), or H_2O_2, an oxidizing agent whose effects include lipid peroxidation, protein oxidation, and DNA damage (12). We found that deletion of *CAP1* did not affect the susceptibility of the cells to diamide, since both CAI4/PMK and CJD21/PMK cells display a diamide MIC of 1 mM (data not shown). However, the truncated Cap1p was found to confer resistance to this compound, as CJD21/PMK-CAP1TR transformants were able to grow at diamide concentrations inhibitory for the growth of the CAI4/PMK and CJD21/PMK-CAP1 transformants (Fig. 3B). On the other hand, the deletion of *CAP1* resulted in hypersensitivity of the cells to H_2O_2, as the growth of CJD21/PMK cells was completely abrogated on 20 mM H_2O_2, whereas growth of CAI4/PMK cells was unaffected at this H_2O_2 concentration (Fig. 3B). These results indicate that *CAP1* is essential for H_2O_2 tolerance in *C. albicans*. Reintroduction in the CJD21 cells of either the full-length or the truncated *CAP1* gene restored normal levels of tolerance to H_2O_2 at a concentration of 20 mM (Fig. 3B). However, only the full-length *CAP1* gene was able to restore wild-type growth in CJD21 at 50

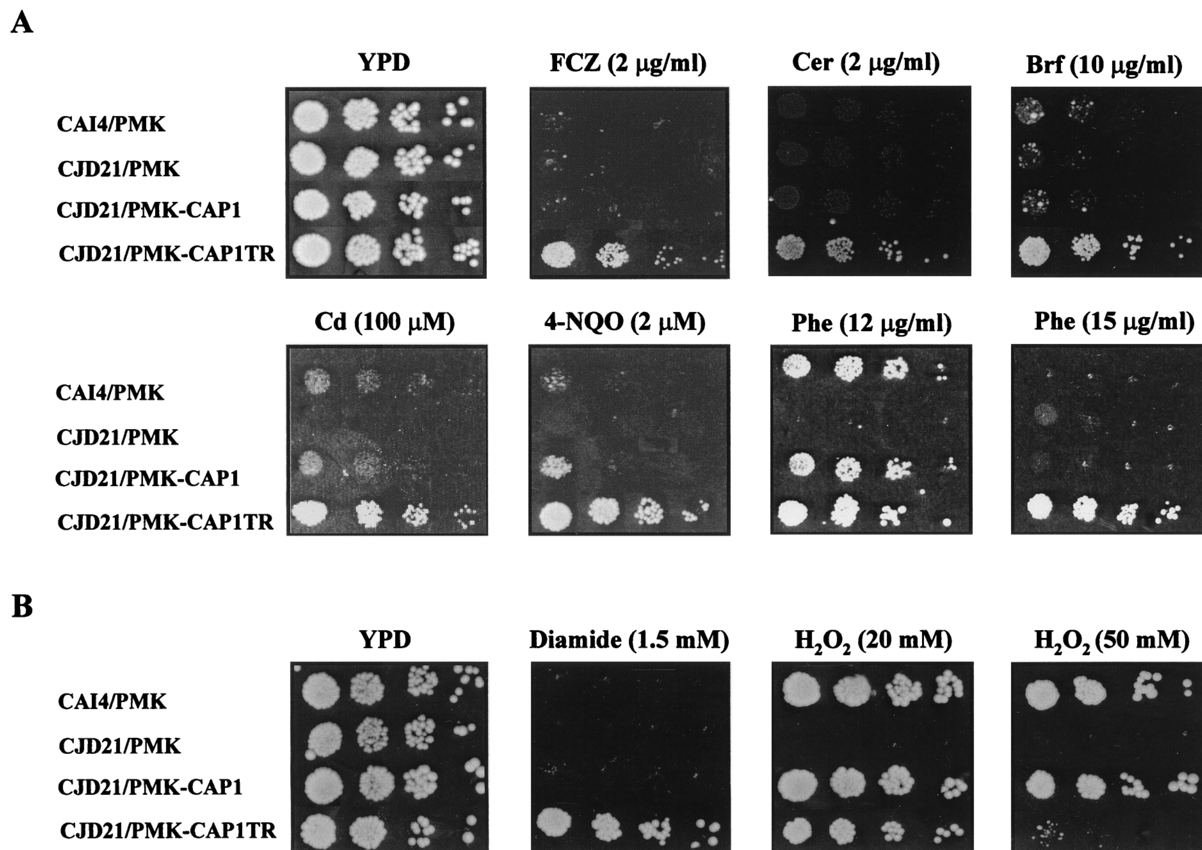

FIG. 3. Involvement of *CAP1* in *C. albicans* MDR and OSR. Strains CAI4/PMK, CJD21/PMK, CJD21/PMK-CAP1, and CJD21/PMK-CAP1TR were analyzed by spot assay (as described in Materials and Methods) for the ability to grow on YPD plates containing different antifungal (A) and prooxidant (B) agents. Growth differences were monitored after 2 days at 30°C. A representative plate of control growth on YPD medium is shown for each set of experiments (YPD). Abbreviations for substances are FCZ (fluconazole), Cer (cerulenin), Brf (brefeldin A), Cd (cadmium), 4-NQO (4-nitroquinoline *N*-oxide), and Phe (1,10-phenanthroline).

mM H_2O_2. Taken together, these results clearly establish that *CAP1*, in addition to MDR, is involved in OSR in *C. albicans*. Furthermore, the different behavior of the CJD21/PMK-CAP1TR transformants when exposed to diamide or H_2O_2 suggests that complex regulatory mechanisms control the activity of Cap1p in response to these two oxidants.

Identification of potential *CAP1* transcriptional targets. Yap1p has been shown to transcriptionally activate a number of downstream target genes, including *FLR1*, *YCF1*, *ATR1*, *TRX2*, *TRR1*, and *GLR1* (1, 11, 20, 26, 32, 56). We were thus interested in investigating if Cap1p regulates similar targets in *C. albicans* and whether the *CAP1*-mediated phenotypes observed in *C. albidans* correlate with the overexpression of such targets. To this end, total RNAs prepared from CAI4 and CJD21 cells as well as from the CJD21/PMK, CJD21/PMK-CAP1, and CJD21/PMK-CAP1TR transformants were analyzed by Northern blotting, a technique which assess steady-state levels of RNA expression, using probes for the *MDR1*, *CaYCF1*, *CaGLR1*, and *CaTRR1* genes. First, hybridization with a *CAP1* probe identified a major 2.2-kb transcript in the wild-type CAI4 strain (Fig. 4, top panel). This transcript is absent in the CJD21 and CJD21/PMK cells (Fig. 4, top panel), as expected from the complete deletion of *CAP1* in this strain. Restoration of *CAP1* expression in the CJD21/PMK-CAP1 cells was confirmed by the detection of the *CAP1*-specific transcript (Fig. 4, top panel). The introduction of the truncated *CAP1* gene in CJD21 cells led to the appearance in the CJD21/PMK-CAP1TR cells of a shorter *CAP1* transcript of approximately 1.7 kb which codes for the truncated Cap1p protein observed in Fig. 2B (Fig. 4, top panel, lane CJD21/PMK-CAP1TR). The high level of *CAP1-TR* mRNA detected in the CJD21/PMK-CAP1TR cells by Northern blotting indicates that the lower amount of truncated Cap1p as compared to full-length Cap1p observed in the Western blot (Fig. 2B) is not due to a lower level of expression of the truncated *CAP1* allele mRNA. This experiment showed that deletion of the *CAP1* gene has no effect on the basal transcription of any of the genes analyzed (Fig. 4; compare lanes CAI4 and CJD21). Analysis of the expression of the *MDR1* gene showed that the *MDR1* transcript is undetectable in all strains but is massively overexpressed in the CJD21/PMK-CAP1TR transformants (Fig. 4, *MDR1* panel). This result suggests that the FCZ-resistant phenotype of the CJD21/PMK-CAP1TR transformants may be due to the overexpression of the *MDR1* gene and demonstrates that *CAP1* modulates *MDR1* expression. Since *MDR1* expression in *S. cerevisiae* has also been shown to confer resistance to cerulenin, brefeldin A, 4-NQO, and 1,10-phenanthroline (43), it is likely that *MDR1* overexpression in CJD21/PMK-CAP1TR transformants is also responsible for the resistance of the cells to these compounds. As seen for *MDR1*, expression of the truncated Cap1p protein resulted in strong overexpression of the *CaYCF1*, *CaGLR1*, and *CaTRR1* genes. Given that the sequence homology between the *C. albicans* genes *CaYCF1*, *CaGLR1*, and *CaTRR1* genes and their *S. cerevisiae* counterparts translates into functional homology, increased expression of these genes upon expression of the Cap1p-TR protein could

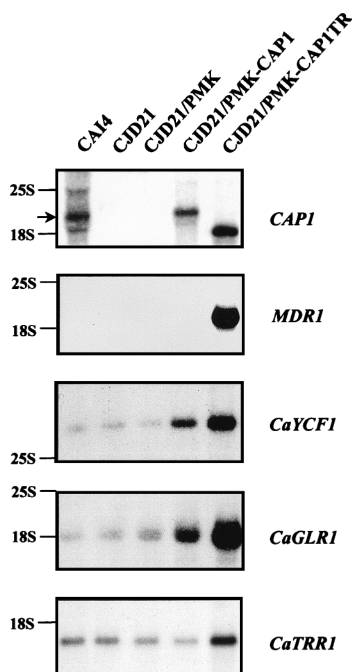

FIG. 4. Northern blot analysis of *CAP1* transcriptional targets in *C. albicans*. Total RNA was extracted from strains CAI4, CJD21, CJD21/PMK, CJD21/PMK-CAP1, and CJD21/PMK-CAP1TR. RNA samples (20 μg) were separated by electrophoresis on a 1% agarose gel, transferred onto nylon membranes, and probed with a *CAP1*, *MDR1*, *CaYCF1*, *CaGLR1*, or *CaTRR1* probe. Positions of rRNAs are indicated on the left; the position of the major *CAP1* transcript is indicated by an arrow (top panel). The membranes were exposed for either 24 h (*CAP1*), 3 days (*CaYCF1*, *CaGLR1*, and *CaTRR1*), or 12 days (*MDR1*) at −80°C with two intensifying screens.

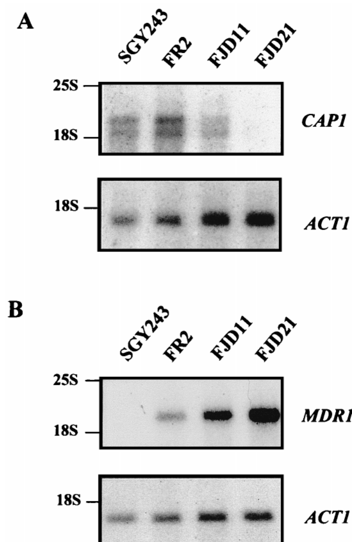

FIG. 5. Northern blot analysis of *CAP1* and *MDR1* in FR2 *cap1* disruptants. Total RNA was extracted from strains SGY243, FR2, FJD11 (*CAP1*/*cap1*Δ::*hisG*), and FJD21 (*cap1*Δ::*hisG*/*cap1*Δ::*hisG*). RNA samples (10 μg) were separated by electrophoresis in duplicate on a 1% agarose gel and transferred onto nylon membranes. The membranes were probed with a *CAP1* (A) or *MDR1* (B) probe. Both blots were subsequently hybridized with an *ACT1* probe as a control for RNA loading and transfer. Membranes were exposed for 10 days (*MDR1* and *CAP1*) or 24 h (*ACT1*) at −80°C with two intensifying screens. Positions of rRNAs are indicated on the left.

explain the cadmium and diamide resistance phenotypes of the CJD21/PMK-CAP1TR cells (Fig. 3). We also found that reintroduction of the full-length *CAP1* gene in CJD21 resulted in a small increase in *CaYCF1* and *CaGLR1* expression detected in the CJD21/PMK-CAP1 cells (Fig. 4, *CaYCF1* and *CaGLR1* panels). However, this increase did not translate into increased resistance to cadmium or diamide, respectively (Fig. 3). Finally, hybridization with a *CDR1* probe showed that *CDR1* expression is not modulated by Cap1p in these different strains (data not shown). Taken together, our results suggest that *CAP1* acts as a molecular determinant of MDR and OSR in *C. albicans* through the transcriptional control of a number of specific downstream target genes.

Chromosomal deletion of the *CAP1* gene in the *C. albicans* FCZ-resistant FR2 strain. A number of *C. albicans* FCZ-resistant clinical isolates have been shown to overexpress the *MDR1* gene (44, 58). In addition, the FCZ-resistant strain FR2, which was derived in vitro from the FCZ-susceptible strain SGY243 (24) through serial passages in medium containing increasing concentrations of FCZ, also overexpresses the *MDR1* gene (2). FCZ resistance in these strains is a stable phenotype (2, 44), suggesting that it could be caused by genetic alterations including dominant activating mutations in a *trans*-acting factor controlling *MDR1* expression. Such dominant hyperactive mutations have been identified in the transcription factors Pdr1p and Pdr3p and have been shown to elicit MDR in *S. cerevisiae* by causing the overexpression of a number of drug efflux transporters of the ABC family (reviewed in reference 4). It was thus of interest to evaluate the potential contribution of *CAP1* in the constitutive *MDR1* overexpression displayed by these FCZ-resistant isolates. Unlike clinical isolates, FR2 is auxotrophic for uracil and thus amenable to gene disruption with the Ura-blaster strategy (17). We thus deleted both alleles of the *CAP1* gene in strain FR2 and determined the consequence of this deletion on the levels of *MDR1* expression. Disruption of both *CAP1* alleles was performed as described for CAI4 except that the *CAP1* alleles are both located on a 3.2-kb *Bgl*II fragment in FR2 (data not shown). To confirm that the *CAP1* gene was completely deleted, we performed a Northern blot analysis (Fig. 5A) using total RNA extracted from the FCZ-sensitive SGY243 strain, its FCZ-resistant derivative FR2, the disruption intermediate FJD11 (*CAP1*/*cap1*Δ::*hisG*), and the homozygous disruptant FJD21 (*cap1*Δ::*hisG*/*cap1*Δ::*hisG*). Using an internal *CAP1* fragment as a probe, two *CAP1* RNA transcripts were detected in strains SGY243 and FR2 (Fig. 5A), showing that there are no quantitative or qualitative differences in *CAP1* RNA expression between the FCZ-sensitive strain SGY243 and the FCZ-resistant strain FR2. Disruption of one of the *CAP1* alleles resulted in a weaker signal with the *CAP1* probe in strain FJD11 (Fig. 5A). This signal was completely absent in the double disruptant FJD21, thus confirming the complete deletion of *CAP1* in FR2 (Fig. 5A). Northern blot analysis of the same strains with the *MDR1* probe confirmed that the FR2 strain overexpresses *MDR1* compared to its parental strain SGY243 (Fig. 5B) (2). If *CAP1* was involved in *MDR1* overexpression in FR2, it would be expected that deletion of *CAP1* would result in the downregulation of *MDR1* expression in the resulting *cap1*Δ/*cap1*Δ mutant strain. However, we found that the disruption of *CAP1* correlates with increased levels of *MDR1* expression: the heterozygous FJD11 *CAP1*/*cap1* cells have higher levels of *MDR1* RNA than their parental FR2 strain, and *MDR1* expression is further increased in the FJD21 *cap1*/*cap1* homozygous strain (Fig. 5B). These results demonstrate that *CAP1* acts as a negative transcriptional regulator of the *MDR1* gene

in FR2 and is not the determinant of the stable *MDR1* overexpression in this strain.

DISCUSSION

To unravel the biological function of Cap1p in *C. albicans*, we deleted the *CAP1* gene in strain CAI4 and analyzed the consequence of this deletion on (i) the level of expression of Cap1p transcriptional targets and (ii) the susceptibility of the cells to a variety of toxic compounds. On the one hand, we found that deletion of *CAP1* has no effect on the basal transcription of the genes analyzed, although these genes were found to be Cap1p transcriptional targets, as shown by their upregulation in the *CAP1-TR* transformants (Fig. 4). This observation is consistent with what has been found in *S. cerevisiae* for Yap1p targets such as *YCF1*, *GSH1*, and *TRX2*, whose expression is not reduced in a *yap1* deletion strain under noninducing conditions (50, 56). Rather, Yap1p has been shown to be required for stress-induced transcriptional activation of its targets (50), a situation mimicked by elevated gene dosage (56) or by expression of Yap1p derivatives carrying an altered CRD (see below). On the other hand, we found that deletion of *CAP1* in CAI4 elicits hypersensitivity to 4-NQO, 1,10-phenanthroline, cadmium, and H_2O_2, which could be reverted by reintroduction of a plasmid-borne copy of the *CAP1* gene in the *cap1* deletion strain (Fig. 3). These results clearly establish that Cap1p is a key determinant of cellular tolerance to different drugs and prooxidants in *C. albicans*. The hypersensitivity of the CJD21/PMK cells to 4-NQO and 1,10-phenanthroline could result from a defective upregulation of *MDR1* in the *cap1* mutant since overexpression of *MDR1* in *S. cerevisiae* has been shown to mediate resistance to 4-NQO and 1,10-phenanthroline (43) and since deletion of the *MDR1* gene in *C. albicans* results in hypersensitivity of the cells to 4-NQO (19, 42). Similarly, the hypersensitivity of CJD21/PMK cells to cadmium (Fig. 3A) could reflect the absence of *CaYCF1* induction upon *cap1* deletion. Alternatively, other *CAP1* transcriptional targets may be responsible for the cadmium hypersensitivity of CJD21/PMK cells, such as the recently characterized *CIP2* gene (35). Expression of this gene is massively induced upon exposure of cells to cadmium or diamide. Moreover, *CIP2* possesses a putative Yap1 response element in its promoter region (35), suggesting that cadmium and diamide induction of the *CIP2* gene expression could be *CAP1* dependent. Finally, the H_2O_2-hypersensitive phenotype of CJD21/PMK cells (Fig. 3B) could be due to a reduced induction of *CaGLR1* and/or *CaTRX2* transcripts in the absence of Cap1p, since induction of these genes by H_2O_2 in *S. cerevisiae* has been shown to be dramatically reduced in a *yap1* mutant (20, 50); alternatively, it could be due to the involvement of other, yet unidentified *CAP1* target genes involved in the OSR.

The essential role of *CAP1* in cellular tolerance to the reactive oxygen species (ROS) H_2O_2 is of particular interest since it suggests that Cap1p may be involved in *C. albicans* response to the ROS produced by the host immune system. The production of ROS such as H_2O_2, hydroxyl radicals, and superoxide anions by human phagocytic neutrophils is a major line of defense against fungal infections (34) since it has been shown to correlate directly with fungicidal activity (13), while antioxidants impair killing of *C. albicans* (54). These findings emphasize the need for *C. albicans* to adapt to the presence of ROS in order to counteract the host immune response. *CAP1* could thus be part of *C. albicans* defense mechanisms against ROS produced by the host immune system, through the transcriptional regulation of target genes coding for proteins with antioxidant activities. Experiments are currently under way to address the role of *CAP1* in tolerance to ROS and virulence of *C. albicans*.

We have previously shown that expression of the truncated *CAP1* gene in *S. cerevisiae* confers high levels of drug resistance compared to its full-length counterpart (1), thus behaving as a gain-of-function allele. A similar behavior was observed in *C. albicans*, since CJD21 cells expressing *CAP1-TR* are resistant to FCZ, cerulenin, brefeldin A, cadmium, 1,10-phenanthroline, and diamide compared to cells expressing the full-length *CAP1* (Fig. 3). CJD21 cells expressing *CAP1-TR* do not display increased resistance to drugs such as ketoconazole and fluphenazine, two substrates of the Cdr1p transporter, demonstrating that the resistance phenotype conferred by the truncated *CAP1* allele is specific for certain drugs and, by extension, for *CAP1* transcriptional targets (data not shown). Expression of the truncated *CAP1* allele in CAI4 was also found to cause drug resistance, demonstrating that this hyperactive allele of *CAP1* is dominant (data not shown). The hyperactivity of the *CAP1* truncated allele most likely reflects the absence of the highly conserved CRD, the effect of such a truncation being well characterized in the case of Yap1p and Pap1p (7, 51, 57). Upon imposition of an oxidative stress, Yap1p and Pap1p relocalize from the cytoplasm to the nucleus by a regulated protein export mechanism which involves the Crm1p/Xpo1p nuclear export factor (27, 28, 52). It has been shown that Crm1p interacts with the CRD and that this interaction is redox regulated via the conserved cysteine residues present in the CRD (28, 52). Thus, Yap1p and Pap1p are exported out of the nucleus in the absence of stress but remain in the nucleus upon imposition of an oxidative stress, due to the disruption of the Crm1p-CRD interactions. Interestingly, expression of the truncated Cap1p leads to resistance to diamide but not to H_2O_2 (Fig. 3B). This difference in behavior toward these two oxidative stress-inducing agents is reminiscent of what has been reported for Yap1p:*YAP1* mutants carrying mutations in the CRD are able to grow in the presence of diamide but not of H_2O_2, probably due to their inability to activate transcription of the *TRX2* gene (27, 55). It thus seems that the region deleted in the Cap1-TR protein, although not essential for the ability of Cap1p to activate its transcriptional targets involved in resistance to FCZ, cerulenin, brefeldin A, cadmium, 1,10-phenanthroline, and diamide, is essential for Cap1p to activate its target(s) conferring resistance to H_2O_2. We found that resistance of the CJD21/pMK-CAP1TR transformants was accompanied by the overexpression of *MDR1*, *CaYCF1*, *CaGLR1*, and *CaTRR1* (Fig. 4), demonstrating that these genes are transcriptional targets of *CAP1* and suggesting that they may be involved in the resistant phenotype conferred by the expression of Cap1p-TR in CJD21.

Our previous data suggested that the overexpression of *MDR1* in FR2 and in a number of FCZ-resistant clinical isolates could be due to a gain-of-function mutation in a transcriptional regulator of *MDR1* such as *CAP1*. However, we observed that the deletion of *CAP1* in the FR2 strain did not abolish *MDR1* expression (Fig. 5), showing that Cap1p is not responsible for the constitutive overexpression of *MDR1* in FR2. This finding suggests that FR2 probably contains a mutation in another *MDR1* transcriptional regulator or in the *MDR1* promoter itself which would be responsible for the stable *MDR1* overexpression observed in this strain. Moreover, we found that the deletion of *CAP1* in FR2 resulted in *MDR1* upregulation (Fig. 5), indicating that Cap1p behaves as a negative transcriptional regulator of *MDR1* in this strain. This finding was unexpected, in light of our previous demonstration that Cap1p (and Yap1p) behaves as a positive transcriptional regulator of *FLR1* in *S. cerevisiae* (1) and that deletion of *CAP1*

does not result in *MDR1* upregulation in *C. albicans* CAI4 (Fig. 4). It is possible that in FR2, the genetic alteration leading to *MDR1* overexpression also modifies the activity of Cap1p, which, in this mutated context, would function as a transcriptional repressor rather than as an activator. Nevertheless, our data showing that Cap1p is not responsible for the constitutive overexpression of *MDR1* in FR2 suggests, by extension, that *CAP1* is probably not responsible for the constitutive overexpression of *MDR1* observed in a number of FCZ-resistant clinical isolates. Identifying the molecular mechanisms leading to *MDR1* upregulation is becoming particularly important given recent data showing that overexpression of *MDR1* constitutes a very early and widespread event in the emergence of FCZ resistance in *C. albicans* and related species (31, 58). The molecular dissection of the *cis*- and *trans*-acting regulatory elements controlling the expression of *MDR1* will help answer these questions.

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