Identification and Characterization of Cor33p, a Novel Protein Implicated in Tolerance towards Oxidative Stress in Candida albicans

K. Sohn, M. Roehm, C. Urban, N. Saunders, D. Rothenstein, F. Lottspeich, K. Schröppel, H. Brunner, and S. Rupp

Fraunhofer, IGB, 70569 Stuttgart, Germany; MPI for Infection Biology, 10117 Berlin, Germany; MPI for Biochemistry, 82152 Martinsried, Germany; and Institut für Klinische Mikrobiologie, Immunologie und Hygiene, Universität Erlangen, 91054 Erlangen, Germany

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We applied two-dimensional gel electrophoresis to identify downstream effectors of CPH1 and EFG1 under hypha-inducing conditions in Candida albicans. Among the proteins that were expressed in wild-type cells but were strongly downregulated in a cph1Δ/efg1Δ double mutant in α-minimal essential medium at 37°C, we could identify not-yet-characterized proteins, including Cor33-1p and Cor33-2p. The two proteins are almost identical (97% identity) and represent products of allelic isoforms of the same gene. Cor33p is highly similar to Ciplp from Candida sp. but lacks any significant homology to proteins from Saccharomyces cerevisiae. Strikingly, both proteins share homology with phenylcoumaran benzylic ether reductases and isoflavone reductases from plants. For other hypha-inducing media, like yeast-peptone-dextrose (YPD) plus serum at 37°C, we could not detect any transcription for COR33 in wild-type cells, indicating that Cor33p is not hypha specific. In contrast, we found a strong induction for COR33 when cells were treated with 5 mM hydrogen peroxide. However, under oxidative conditions, transcription of COR33 was not dependent on EFG1, indicating that other regulatory factors are involved. In fact, upregulation depends on CAP1 at least, as transcript levels were clearly reduced in a Δcap1 mutant strain under oxidative conditions. Unlike in wild-type cells, transcription of COR33 in a tsa1Δ mutant can be induced by treatment with 0.1 mM hydrogen peroxide. This suggests a functional link between COR33 and thiol-specific antioxidant-like proteins that are important in the oxidative-stress response in yeasts. Concordantly, cor33Δ deletion mutants show retarded growth on YPD plates supplemented with hydrogen peroxide, indicating that COR33 in general is implicated in conferring tolerance toward oxidative stress on Candida albicans.

The increasing occurrence of severe fungal infections represents a serious problem in the treatment of patients with impaired immune systems. For most of these opportunistic systemic infections, Candida albicans is the predominant causative organism. Much effort has therefore been put into studies in order to understand how this fungus mediates pathogenesis in humans. An important step toward this understanding was the discovery that a mitogen-activated protein kinase activation function, especially for hypha-specific proteins like HWP1, is also important for gene transcription even under nonfilamentous conditions (23, 27).}

* Corresponding author. Mailing address: Fraunhofer, Inst. f. Grenzflächen- und Bioverfahrenstechnik, Nobeschtr. 12, 70569 Stuttgart, Germany. Phone: 49-711-9704045. Fax: 49-711-9704200. E-mail: rup@igb.fhg.de.

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same protein, which shares significant homology with Cip1p from *Candida* sp., as well as with plant oxidoreductases. Transcription of *COR33* is most strongly upregulated under treatment with hydrogen peroxide, indicating a functional role of these proteins during oxidative-stress response. Deletion mutants of *COR33* revealed retarded growth on solid media containing hydrogen peroxide, suggesting that these proteins might be implicated in conferring tolerance toward oxidative stress on *C. albicans*.

**MATERIALS AND METHODS**

**Strains and media.** Strains of *C. albicans* and transforming plasmids that were used in this study are summarized in Table 1. Wild-type and mutant strains of *C. albicans* were grown overnight in yeast-peptone-dextrose (YPD) at 30°C. As indicated YPD, YPD plus 10% fetal calf serum, or α-MEM plus 2% glucose was inoculated with overnight cultures of *C. albicans*. The cultures were incubated at 30°C (YPD) or 37°C (YPD plus 10% serum) and subsequent centrifugation at 13,000 g for 5 min. DNA was resolubilized in Tris-EDTA (TE) buffer, and RNA was degraded by incubation with RNase A for 5 min at 37°C. DNA was precipitated as described above and finally solubilized in TE buffer.

For the isolation of total RNA, cells were harvested by centrifugation at 1,700 × g for 5 min. The pellets were washed once in H₂O, and then 1 volume of TES solution (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS), as well as 1 volume of acidic phenol (saturated solution), was added. The sample was vortexed and incubated for 1 h in a water bath at 65°C. The RNA in the supernatant was extracted using phenol and chloroform and was subsequently precipitated by addition of 0.1 volume of 3 M sodium acetate (pH 5.3) and 2.5 volumes of 100% EtOH (−20°C). The pellets were washed twice with 70% EtOH (−20°C) and were finally solubilized in TE buffer for Northern blot analysis.

**Northern and Southern blot analyses.** For Northern blot analysis, about 15 μg total RNA was gel electrophoresed and blotted onto nylon membranes according to standard protocols (25). The following primers were used to PCR amplify a specific probe for *COR33* (5′-CGG GGA TCC ATG TGT TCA ATT ACT-3′) and 5′-GGA TGC TTT AAC TTC GTT C-3′), for *EFG1* (5′-CGG GGA TCC ATG TGT TCA ATT ACT-3′) and 5′-CGG GGA TCC ATG TGT TCA ATT ACT-3′), for *ACT1* (5′-CGG GGA TCC ATG TGT TCA ATT ACT-3′) and 5′-GGA TGC TTT AAC TTC GTT C-3′), and for *ACT1* (5′-CGG GGA TCC ATG TGT TCA ATT ACT-3′) and 5′-GGA TGC TTT AAC TTC GTT C-3′). PCR products were purified and subsequently labeled with [α-32P]dCTP in a Klenow reaction using random primers. Hybridization was performed at 65°C for 8 h with subsequent washing steps in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS and 0.1× SSC plus 1% SDS.

Southern blot analysis was performed according to standard protocols (25) using 10 μg of EcoRIV-digested genomic DNA. The blots were probed with a DNA fragment that was PCR amplified using primers *COR33* ORF (5′-ATT TGG TTC TGT TCC AGG AGC-3′) and *COR33b* FR2_SacII (5′-TCC CGG GGA TGG TTC GTA CGT AG-3′). After hybridization, Southern and Northern blots were exposed to phosphor screens (Molecular Dynamics) for 24 h. The screens were scanned using a Storm PhosphorImager (Molecular Dynamics).

**Two-dimensional gel electrophoresis.** Proteins were separated by two-dimensional gel electrophoresis according to their isoelectric points and molecular weights using the IPGphor system (Amersham Biosciences). Briefly, protein extracts were solubilized in lysis buffer (9.8 M urea, 2% ampholines, pH 7 to 9, 4% NP-40, and 100 mM dithiothreitol) and applied to IPG strips, pH 4 to 7/11 cm (Amersham Biosciences), for isoelectric focusing. After rehydration (50 V for 12 h), the proteins were focused in consecutive steps at 300 V for 1 h, at 600 V for 1 h, at 900 V for 1 h, and finally at 2,500 V for 10 h. The strips were then equilibrated for 15 min in 10 ml equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromphenol blue) supplemented with 100 mg dithiothreitol. Subsequently, the strips were equilibrated

**TABLE 1. Strains and plasmids**

| Strain or plasmid | Genotype or properties | Source |
|-------------------|------------------------|--------|
| SC5314            | Wild type              |        |
| CA14              | ura3::mm34/ura3::mm344 |        |
| JKC19             | ura3::mm34/ura3::mm344 |        |
| HLC52             | ura3::mm34/ura3::mm344 |        |
| HLC54             | ura3::mm34/ura3::mm344 |        |
| RSC11             | ura3::mm34/ura3::mm344 |        |
| RSC12             | ura3::mm34/ura3::mm344 |        |
| RSC2              | ura3::mm34/ura3::mm344 |        |
| RSC3              | ura3::mm34/ura3::mm344 |        |
| RSC66             | ura3::mm34/ura3::mm344 |        |
| CJ12D1            | ura3::mm34/ura3::mm344 |        |
| pCaEXP            | Reintegration of a wild-type copy of URA3 into the RP10 locus | 4 |
| pSFU1             | URA3-FLP cassette      | 22     |
| pSCOR33           | pSFU1 deletion cassette with COR33 flanking regions | This study |
| pCaCOR33          | Reintegration of a wild-type copy of COR33 and URA3 into the RP10 locus | This study |
| pMK22             | Control plasmid restoring URA prototrophy |        |
| pMK22-CAP1        | Integration of a wild-type CAP1 allele into strain CJ12D1 | 1 |
| pMK22-CAP1-TR     | Integration of a truncated CAP1 allele into strain CJ12D1 | 1 |
in 10 ml of equilibration buffer containing 250 μg iodacetamide. Then, the strips were placed on top of 12% SDS-acrylamide gels and run in SDS electrophoresis buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS) at constant voltage (120 V). After electrophoresis, the protein spots were visualized either by using Coomassie brilliant blue (R250; Serva) or by ammoniacal silver staining (http://www.expasy.org/ch2d/protocols).

Identification of proteins. To identify the proteins, approximately 1 mg protein from cellular extracts was separated by preparative two-dimensional gel electrophoresis. The gels were stained using Coomassie brilliant blue, and the spots corresponding to Cor33-1p and Cor33-2p were cut out. Proteins were in-gel digested using trypsin, and the resulting peptides were eluted from the gel matrix using acetonitrile. The peptide mixture was separated by CN reverse-phase chromatography, and the amino acid sequences of the individual peptides were determined by Edman degradation. Identification of the open reading frames was carried out by tblastn homology searches using the C. albicans genome database (http://www-sequence.stanford.edu/group/candida/index.html).

Plasmid construction. Generation of the COR33 deletion constructs was accomplished by ligation of the up- and downstream flanking regions FR1 and FR2 of COR33 into plasmid pSFU1 (22). For this purpose, oligonucleotides COR33b_F1_ApaI (5'-GCT GCC GCA TGA TGT GGC TGT ATC-3') and COR33b_F1_Xhol (5'-CCG CTC GGA GAT TGA TTG ATG AAG AAG-3'), as well as COR33b.FR2_NotI (5'-ATA AGA ATG CGG CCG CTG TTC CAA GTG GTA TTT TC-3') and COR33b.FR2_SacII (5'-TCC CCG CGG GGA TGT TTC AAT TTC GTT C-3') were used to PCR amplify the 5' upstream flanking region, FR1, from nucleotide positions 489 to 47 before the start codon and the 3' downstream flanking region, FR2, from nucleotide positions 22 to 536 after the stop codon, respectively. Flanking region FR1 was ligated into ApaI/Xhol-digested pSFU1 to generate pSFU3. Subsequently, FR2 was cloned into NotI/SacII-digested pSCOR3, resulting in plasmid pSCOR3. The correct insertion of both fragments was confirmed by PCR and restriction analysis. Afterward, the FR1-URA3 flipper-FR2 cassette from plasmid pSCOR3 was excised by ApaI/SacII digestion and subsequently used for transformation of strain CAI4.

For the reintroduction of a functional copy of COR33 into the cor3Δ deletion strain, a genomic fragment containing COR33, including approximately 1,400 bp of the upstream regulatory sequence and 500 bp downstream of the open reading frame, was PCR amplified using primers COR33a-rek_forR (5'-CCG GCC TCG AAA AAA GGG GAC ATG TTG TTG-3') and COR33a-rek_revN (5'-CCG GGA TGT GTA GAT TGA TTT TC-3') and COR33b- rek_revN (5'-CCG GCC TCG AAA AAA GGG GAC ATG TTG TTG-3'). The resulting PCR fragment was ligated into plasmid pCaExp digested with BamHI to yield plasmid pCaCOR33, which was used for the transformation of RS22.

Construction of COR33 deletion and revertant strains. COR33 was deleted in the uridine-auxotrophic C. albicans strain CAI4 by FLP-mediated site-specific recombination as described previously (22). The FLP cassette containing both flanking regions of COR33 was excised from plasmid pSCOR3 by digestion using ApaI/SacII. Strain CAI4 was transformed with the COR33-FLP cassette using the lithium acetate method (3). Deletion of cor3Δ mutant strains was accomplished by integration of the Stu-linearized plasmids pCaExp and pCaCOR33 into the RP10 locus. Deletion of the open reading frame of COR33, as well as reconstitution, was confirmed by PCR and Southern blot analysis.

Plate assays. Overnight cultures of C. albicans wild-type cells, as well as mutant strains RS33 and RS66, were grown in YPD at 30°C. Fresh YPD was inoculated with the overnight cultures and grown to an optical density at 600 nm of 1. Series of 10-fold dilutions were prepared in YPD, and approximately 103, 104, 105, and 106 cells were spotted onto YPD plates supplemented with either 5 mM hydrogen peroxide or 0.5 mM cadmium chloride. Cells were also applied to YPD plates without any supplement as a control. The plates were documented after incubation at 30°C for 2 days using a flat-bed scanner.

RESULTS

Identification of Cor33-1p and Cor33-2p. Previous studies have demonstrated that the two transcription factors CPH1 and EFG1 are critical for filamentation and virulence in C. albicans, indicating that proteins regulated by CPH1 and EFG1 are implicated in pathogenesis. In order to identify downstream factors controlled by these two transcription factors, we prepared protein extracts from wild-type and cph1Δ efg1Δ mutant strains of C. albicans that were grown in α-MEM at 37°C for 8 h. Under these conditions, wild-type cells of C. albicans generated hyphae, in contrast to the cph1Δ efg1Δ double mutant, which grew exclusively in the yeast form (27). The proteins from these crude extracts were separated by two-dimensional gel electrophoresis, and the corresponding protein patterns were compared with each other. Overall, more than 500 different spots could be detected by two-dimensional gel electrophoresis, of which 10 spots showed a significant difference in expression patterns between wild-type cells and the cph1Δ efg1Δ mutant. Two prominent spots were detectable in extracts from wild-type cells but were strongly downregulated or even missing in the cph1Δ efg1Δ double mutant (Fig. 1A and B), indicating that the expression of the corresponding proteins is dependent on CPH1 or EFG1. As estimated from 2D gels, the apparent molecular masses for both proteins are approximately 33 kDa. For identification, both spots were cut out from preparative 2D gels, and peptides were derived by tryptic in-gel digests of the corresponding proteins. The resulting peptides were separated by reverse-phase high-performance liquid chromatography, and their sequences were determined by Edman degradation. At least five different peptide sequences for each protein were derived by microsequencing (Fig. 2). Subsequently, assembly 19 of the C. albicans genome database was searched for homologous sequences using these peptide sequences. Two open reading frames (ORFs) (ORF19.113 and ORF19.7761) exactly matching the peptide sequences were found in the database (Fig. 2). The molecular weights and isoelectric points of the amino acid sequences deduced from these open reading frames were in good agreement with the apparent migration behavior of the corresponding spots in the 2D gels. Computer analysis revealed no signal sequences for both proteins, indicating that the proteins are most likely cytosolic. Strikingly, the amino acid sequences of Orf19.113p and Orf19.7761p are highly homologous, sharing 97% identity over the entire amino acid sequence. This suggests that the two open reading frames represent polymorphic alleles of the same gene. In fact, both open reading frames are annotated as allelic genes of the same genomic locus in the C. albicans genome database (http://www-sequence.stanford.edu/group/candida/index.html). Further database searches re-
revealed strong similarities of ORF19.113 and ORF19.7761 to CIP1 from Candida sp. strain HN95 (55% identity and 71% homology at the protein level; accession number Y13973), a protein that can be induced by cadmium stress, but so far with no known physiological function (14). Interestingly, both open reading frames also share significant homology with oxidoreductases from plants, especially with phenylcoumaran benzylic ether reductases and isoflavonoreductases from various species (25% identity and 46% homology to phenylcoumaran benzylic ether reductases from Tsuga heterophylla at the protein level; accession number AF242495). A putative consensus sequence for NAD(P)H-binding sites (GXXGXXG) is conserved at their N termini (Fig. 2), indicating that the corresponding proteins in fact might function as oxidoreductases. Additionally, an acyl-coenzyme A dehydrogenase signature was predicted (Fig. 2), also strongly suggesting a possible function as an oxidoreductase in C. albicans. Therefore, we have named ORF19.113 and ORF19.7761 COR33-1 and COR33-2 (for candida oxidoreductase with an apparent molecular mass of 33 kDa), respectively. Remarkably, Cor33-1p and Cor33-2p do not show any significant homologies to proteins from Saccharomyces cerevisiae.

**Transcriptional regulation of COR33.** 2D gel electrophoresis showed that Cor33-1p and Cor33-2p were expressed in wild-type cells but were strongly downregulated in a cph1Δ/Δ double mutant under the hypha-inducing conditions of α-MEM at 37°C. We next addressed the question of whether these proteins were accordingly regulated on the transcriptional level and if both transcription factors, CPH1 and EFG1, were critical for their transcription. We isolated total RNAs from wild-type, cph1Δ, efg1Δ, and cph1Δ/efg1Δ mutant strains grown in α-MEM at 37°C for 8 h to test the transcript level of COR33 by Northern blot analysis. Wild-type cells displayed a high transcript level for COR33 that was significantly reduced in the cph1Δ/efg1Δ double mutant (Fig. 3A, lanes 1 and 4). This transcriptional regulation was very similar to the corresponding protein expression pattern found by 2D gel electrophoresis (Fig. 1A and B), indicating that regulation of COR33-1p and Cor33-2p was accomplished on the transcriptional level. In contrast to the cph1Δ mutant, in which downregulation of COR33 transcription does not exceed 50% of wild-type levels (Fig. 3A, lanes 1 and 2, and data not shown), the transcription of COR33 was almost entirely downregulated in the efg1Δ single mutant, suggesting that EFG1 in particular was essential for its transcription (Fig. 3A, lanes 1 and 3). Interestingly, almost no transcription has been detected for COR33 in wild-type or mutant strains cultured in YPD at 30°C or YPD plus sodium chloride to the culture medium (14). In order to test whether transcription of COR33 from C. albicans was also induced by cadmium, RNA was isolated from YPD cultures supplemented with 0.5 mM cadmium chloride. Transcription of COR33 was upregulated 8 h after induction, but the transcript level was
lower than the levels following induction using α-MEM at 37°C (Fig. 3B, lanes 1 to 3).

We have assumed a function for the product of COR33 in C. albicans as an oxidoreductase from the significant degree of homology to various oxidoreductases from plants, as well as from putative NAD(P)H binding sites at their N termini. In this context, we tested whether cells induced COR33 when they were exposed to oxidative stress. For this purpose, we determined the transcript levels of COR33 from cells treated with 5 mM hydrogen peroxide for 1 h. Strikingly, COR33 transcription was dramatically induced under peroxide stress, even more strongly than in cells that were grown for 8 h in α-MEM at 37°C (Fig. 3B, lanes 2 and 4). To analyze upregulation of COR33 during oxidative stress in a time-dependent manner, we performed a time course experiment following transcription of COR33 at different time points after induction of C. albicans wild-type cells with 5 mM hydrogen peroxide in YPD at 30°C. The cells were harvested for the isolation of total RNA after 0 (lane 1), 30 (lane 2), 60 (lane 3), 120 (lane 4), or 240 min (lane 5). The blots were tested using probes specific for COR33 or ACT1.

FIG. 3. Transcriptional regulation of COR33. (A) Northern blot analysis using total RNAs of wild-type cells (lanes 1, 5, and 9), cph1Δ (lanes 2, 6, and 10), efg1Δ (lanes 3, 7, and 11), and cph1Δ/efg1Δ (lanes 4, 8, and 12) mutant strains cultured in α-MEM at 37°C (lanes 1 to 4), in YPD at 30°C (lanes 5 to 8), or in YPD plus 10% serum at 37°C (lanes 9 to 12). The blots were tested using probes specific for COR33 or ACT1. (B) Northern blot analysis using total RNAs from wild-type cells cultured in YPD at 30°C for 8 h (lane 1), in α-MEM at 37°C for 8 h (lane 2), in YPD plus 0.5 mM cadmium chloride at 30°C for 8 h (lane 3), or in YPD plus 5 mM hydrogen peroxide at 30°C for 1 hour (lane 4). The blots were tested using probes specific for COR33 or ACT1. (C) Time course experiment following transcription of COR33 and EFG1 at different time points after induction of C. albicans wild-type cells with 5 mM hydrogen peroxide in YPD at 30°C. The cells were harvested for the isolation of total RNA after 0 (lane 1), 30 (lane 2), 60 (lane 3), 120 (lane 4), or 240 min (lane 5). The blots were tested using probes specific for COR33 or ACT1. (D) Northern blot analysis of wild-type (wt) cells (lane 1) and an efg1Δ mutant (lane 2) cultured in YPD plus 5 mM hydrogen peroxide for 1 hour at 30°C. The blots were tested using probes specific for COR33 or ACT1. (E) Northern blot analysis of wild-type cells (lane 1) and a cap1Δ mutant CJD21-PMK (lane 2); a corresponding cap1Δ/CAP1 revertant strain, CJD21-CAP1 (lane 3); and a cap1Δ/CAP1-TR mutant strain expressing a hyperactive form of CAP1, CJD21-CAP1-TR (lane 4), cultured in YPD plus 5 mM hydrogen peroxide for 1 hour at 30°C. The blots were tested using probes specific for COR33 or ACT1.
all after 4 hours (Fig. 3C, lanes 4 and 5), demonstrating that up- and downregulation of COR33 are very rapid under oxidative-stress conditions. In contrast, the transcript levels of EFG1 did not alter in such a particular manner in the same experiment. Despite a small decrease 30 min after addition of hydrogen peroxide, the EFG1 levels remained constant during this time course (Fig. 3C), showing that EFG1 and COR33 transcription did not correlate under these conditions. In fact, COR33 transcription during oxidative stress was not dependent on EFG1, as transcription of COR33 was also upregulated in an efg1Δ mutant in a manner similar to that of wild-type cells (Fig. 3D, lanes 1 and 2). This is in contrast to EFG1-dependent regulation when cells were cultured in α-MEM, suggesting that for the transcriptional regulation of COR33, diverse pathways and transcription factors are involved under different conditions.

Cap1p is a central regulator for adaptation to oxidative stress in C. albicans (1, 35). This protein is homologous to the bZIP protein Yap1p (12) that is involved in multidrug resistance and response to oxidative stress in S. cerevisiae (16). In order to test whether transcription of COR33 under oxidative conditions is regulated by Cap1p in C. albicans, we analyzed transcript levels of COR33 in a cap1Δ mutant strain that was challenged by the addition of hydrogen peroxide (Fig. 3E). Actually, no transcript was detected in the cap1Δ deletion strain (Fig. 3E, lane 2), indicating that CAP1 is involved in the regulation of COR33 under these conditions. However, transcription of COR33 was restored to wild-type levels in a cap1Δ/ CAP1 reconstituted strain (Fig. 3E, lane 3). Furthermore, COR33 transcription was strongly upregulated under these conditions in a mutant strain expressing a truncated form of Cap1p that is hyperactive (1), clearly demonstrating that activation of COR33 transcription is dependent on CAP1 (Fig. 3E, lane 4).

Among the proteins that are upregulated in a COR33-like fashion when cells are treated with hydrogen peroxide, we also found Tsa1p, which is known to be required for oxidative-stress response in S. cerevisiae (34). Tsa1p belongs to a family of thiol-specific antioxidant-like proteins that confer tolerance against oxidative, as well as reductive, stress, not only in S. cerevisiae, but also in C. albicans (32). In order to determine whether transcription of COR33 is somehow linked to TSA1 transcription, we analyzed the relative transcript levels for COR33 in a tsalΔ deletion mutant versus wild-type cells under mild oxidative conditions by applying 0.1 mM hydrogen peroxide for 40 min. Under these conditions, COR33 transcription was not yet induced in wild-type cells (Fig. 4, lane 1), but transcription in the tsalΔ deletion mutant was noticeably elevated (Fig. 4, lane 3). This upregulation clearly depended on the absence of TSA1, as well as on induction using hydrogen peroxide. Reintegration of a functional copy into the tsalΔ deletion strain largely restored the repression of COR33 transcription, suggesting a functional relationship or a parallel mechanism between COR33 and TSA1.

Phenotypic characterization of cor33Δ deletion strains. In order to functionally characterize Cor33p, we deleted the corresponding genes using the FLP recombinase approach (22). A DNA cassette containing the selectable marker URA3, an FLP recombinase under a SAP2 promoter and two FRT sites, was used to replace COR33-1 and COR33-2 by homologous recombination (Fig. 5). Subsequently, induction of FLP recombinase expression resulted in the excision of the whole cassette via the FRT sites. A restriction site for EcoRV was present approximately 200 base pairs downstream of the COR33-1 stop codon that was missing in COR33-2. This restriction polymorphism allowed discrimination between COR33-1, revealing two fragments 5.0 and 1.7 kb in size, and COR33-2, showing only one fragment of 6.8 kb in Southern blot analysis (Fig. 5B, lane 1). Transformation of the deletion cassette into C. albicans resulted in the complete disappearance of the band at 6.8 kb specific for COR33-2 (Fig. 5B, lane 2), whereas the two fragments of 5.0 and 1.7 kb corresponding to COR33-1 were still detectable. Along with the high degree of identity between COR33-1 and COR33-2, these data are in agreement with both genes representing polymorphic allelic genes (see also assembly 19 of the Candida albicans genome database). Consequently, we constructed a cor33-1Δ/cor33-2Δ deletion mutant by sequential disruption of both alleles. All deletion mutants were confirmed by Southern blot analysis (Fig. 5B), as well as Northern blot analysis (data not shown). Revertant strains of the corresponding deletion mutants were constructed by reintegrating COR33-1 under the control of its endogenous promoter into the RP10 locus using the pCaExp plasmid (4).

Compared to wild-type strains, cor33Δ deletion mutants did not show any obvious growth defects under many laboratory conditions. Similarly, we found no defect in filamentation for the deletion mutant of COR33 under hypha-inducing conditions, like YPD plus serum or α-MEM at 37°C. Given that COR33 was strongly upregulated when cells were treated with hydrogen peroxide (Fig. 3B and C), we expected a specific role
for \( \text{COR33} \) in the response to oxidative stress. In order to test whether \( \text{COR33} \) was implicated in the protection of \( \text{C. albicans} \) against oxidative stress, we plated wild-type cells and a \( \text{COR33} \)/H9004 deletion mutant, as well as the corresponding \( \text{COR33} \) revertant, in serial dilutions on YPD plates supplemented with 5 mM hydrogen peroxide (Fig. 6A). In contrast to wild-type cells, the \( \text{cor33} \)/H9004 deletion mutant revealed significant growth retardation on H\(_2\)O\(_2\) plates compared to YPD plates (Fig. 6A, lane 2). However, a corresponding revertant strain, in which a functional copy of \( \text{COR33} \) was reintegrated into the \( \text{RP10} \) locus, grew significantly better on H\(_2\)O\(_2\) plates, although its growth rate did not completely reach the wild-type level, which might be due to a dose effect (Fig. 6A, lanes 1 and 3). Evidently, \( \text{COR33} \) plays a role in conferring tolerance toward oxidative-stress conditions, although a similar phenotype could not be observed in liquid media containing hydrogen peroxide (data not shown). As the transcription of \( \text{COR33} \) was also induced when cadmium was added to the culture medium, we also tested whether \( \text{COR33} \) mediates tolerance against this toxic heavy metal. However, we have not found any difference between the \( \text{cor33} \Delta \) deletion strain and wild-type cells either in liquid media or on solid plates supplemented with cadmium (Fig. 6B).
We also performed virulence studies in order to analyze the contribution of \textit{COR33} to mediating pathogenesis. Applying the systemic mouse model, we did not find any difference in the survival rates of mice injected with wild type cells or \textit{cor33}/\textit{H9004} mutant strains in the tail vein (data not shown), indicating that \textit{COR33} is not critical to establish a systemic infection in this animal model.

**DISCUSSION**

Applying a proteomics approach using two-dimensional gel electrophoresis, we were able to identify \textit{Cor33}-1p and \textit{Cor33}-2p from wild-type extracts of \textit{Candida albicans}. The two proteins share 97\% identity, representing allelic polymorphic isoforms of the same protein in \textit{C. albicans}, and are therefore simply termed \textit{Cor33}p. Initially, we found \textit{COR33} upregulated in an \textit{EFG1}-dependent manner under the hypha-inducing conditions of \textit{a/MEM} at 37°C, but \textit{COR33} does not represent a hypha-specific factor, nor is \textit{EFG1} critical for its activation under other conditions. The most potent induction for \textit{COR33} transcription is oxidative-stress conditions using hydrogen peroxide, indicating that the cultivation of \textit{C. albicans} in \textit{a/MEM} may represent a mild oxidative milieu, resulting in moderate transcription of \textit{COR33}. Even though transcription of \textit{COR33} is dependent on \textit{EFG1} in \textit{a/MEM}, no effect at all has been found on the upregulation of \textit{COR33} in an \textit{efg1\Delta} mutant strain that was induced using hydrogen peroxide, suggesting that additional regulatory factors are required under strong oxidative conditions. In fact, transcription of \textit{COR33} under such conditions was dependent on \textit{CAP1}, which represents an important regulator of the oxidative-stress response in \textit{C. albicans} (1). In silico analysis of the $\text{−1 to −1487 bp}$ region upstream of the \textit{COR33} start codon using MatInspector (http://www.genomatix.de/) revealed, in addition to five E boxes comprising the consensus site \text{CANNTG} for DNA binding of \textit{EFG1} (19), three \text{TTACTAA} sites representing binding domains for \textit{Yap1p} from \textit{S. cerevisiae} (8). As the \textit{Yap1p} homolog in \textit{C. albicans}, \textit{Cap1p}, is also able to bind to \textit{Yap1p} consensus sites (35), these DNA motifs might represent possible consensus sites for DNA binding of \textit{Cap1p} in the upstream regulatory sequence of \textit{COR33}.

No significant homologs of \textit{Cor33}p are present in \textit{Saccharomyces cerevisiae}, showing that the protein is not conserved among all yeasts. The most significant homology is to \textit{Cip1p} from \textit{Candida} sp. strain HN95. Even though the exact classification for \textit{Candida} sp. strain HN95 is not yet clear, \textit{Cor33}p, based on homology, might represent a corresponding ortholog in strain SC5314 of \textit{C. albicans}. \textit{Cip1p} was identified using differential-display reverse transcription-PCR as being strongly upregulated in \textit{Candida} sp. strain HN95 cells exposed to cadmium stress compared to untreated cells (14). Cadmium is a
toxic heavy metal that has a detrimental effect on the growth and metabolism of microorganisms, plants, and animals. More recently, it has been shown that cadmium exerts mutagenic effects by inhibiting an essential DNA repair mechanism. Low micromolar concentrations of Cd^{2+} specifically inactivate DNA mismatch repair, leading to hypermutability in yeasts (15). Induction of CIP1 transcription is specific for cadmium and has not been observed when cells were treated with copper, mercury, or lead (14). It has therefore been proposed that CIP1 belongs to a class of detoxification-related genes that are crucial to establish a specific cellular response to meet cadmium stress. Similar to CIP1, COR33 is also induced when cells are treated with cadmium chloride. However, induction of COR33 by cadmium is relatively low compared to the transcript levels observed in C. albicans treated with hydrogen peroxide. This upregulation of transcription for COR33 is very rapid, as the maximum transcription level is reached 60 min after induction. Obviously, hydrogen peroxide represents a much more potent inducer for COR33 transcription than cadmium does. Recently, our finding that COR33 is strongly induced when cells are treated with hydrogen peroxide was also shown by global transcriptional profiling using C. albicans genome-wide DNA microarrays (6). When C. albicans was challenged with hydrogen peroxide, COR33 was already upregulated 10 min after induction. Moreover, in addition to GST3 (glutathione S-transferase) and CTA1 (catalase), COR33 is the most strongly upregulated gene under this type of oxidative-stress condition among all genes and open reading frames present in the genome of C. albicans (6). This implies that COR33, similar to glutathione S-transferase and catalase, plays an important role in conferring tolerance toward oxidative stress in general and particularly in metabolizing hydrogen peroxide. In fact, a cor33Δ deletion mutant exhibits susceptibility and growth retardation when cultured on plates supplemented with hydrogen peroxide, indicating that Cor33p is somehow implicated in the oxidative-stress response. However, no growth phenotype has been observed when mutant cells were grown on YPD plates containing cadmium chloride, suggesting that Cor33p might have no crucial function in intracellular detoxification during cadmium stress and that induction of COR33 transcription in cells treated with cadmium chloride might represent only an indirect adaptation, rather than a specific transcriptional response, under these conditions.

In addition to Cor33p, Tsalp is among the proteins that are induced when C. albicans is exposed to oxidative stress using hydrogen peroxide (32). Tsalp shares significant homology with thioredoxin-dependent peroxidases and belongs to the Tsa/AhpC family. In C. albicans, Tsalp is an abundant protein that localizes to the nucleus and the cytoplasm in the yeast form, as well as to the cell surface in hyphae (31). Tsalp is a thiol-specific antioxidant-like protein that, similar to Cor33p, confers tolerance toward oxidative stress on C. albicans (32). Remarkably, we found that in a tsalΔ deletion mutant, induction of COR33 transcription is accomplished under significantly milder oxidative conditions than in wild-type cells. This indicates that Tsalp and Cor33p might function in parallel pathways, in which Tsalp could represent a component of the general and constitutive machinery that provides protection against oxidative stress. In contrast, Cor33p is induced only when the oxidative level has reached a critical value or Tsalp function is somehow impaired. Alternatively, Tsalp could serve as a protectant for Cor33p under oxidative conditions. Thus, if Tsalp function fails, C. albicans upregulates COR33 transcription to compensate for oxidized and nonfunctional Cor33p.

Noticeably, in addition to the strong homology of Cor33p to Cip1p, this protein also shares significant homology with plant phenylcoumaran benzylic ether reductases. Recently, a large number of phenylcoumaran benzylic ether reductases have been identified by screening for plant genes that are induced by fungal elicitors or during defensive responses (24). At least 19 homologs have been reported in both angiosperms and gymnosperms, including Arabidopsis thaliana (2), Zea mays (24), and T. heterophylla (10), for example. Phenylcoumaran benzylic ether reductases are associated with the phenylpropanoid biosynthesis pathways in lignifying cells (33). They catalyze the reduction of the benzylic ether moieties of both dehydrodiconiferyl alcohol and dihydrodehydrodiconiferyl alcohol using NADPH as a cofactor. In this context, it is also worth mentioning that heterologous expression of phenylcoumaran benzylic ether reductases also confers tolerance toward oxidizing agents on yeast. In a tolerance screening toward the thiol-oxidizing drug diamide, the phenylcoumaran benzylic ether reductase homolog in A. thaliana was found to reduce the susceptibility of a Δcyp1 mutant to oxidative stress in S. cerevisiae (2). However, the mechanism through which this tolerance is accomplished at the molecular level is not yet clear. It is therefore difficult to speculate on the molecular function that Cor33p might exert to confer tolerance toward oxidative stress on C. albicans. In fact, the role of Cor33p in oxidative-stress response in C. albicans does not seem to be critical for virulence in a systemic mouse model, which is similar to Tsalp, which also causes no virulence phenotype (32). However, the functional interaction between Tsalp and Cor33p, as well as the identification of Cor33p substrates or downstream effectors, might help to get a better understanding about the functions of both proteins and how oxidative-stress response is specifically handled in C. albicans.

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