Role of Pir1 in the construction of the *Candida albicans* cell wall

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Searches in a *Candida albicans* database (<http://genolist.pasteur.fr/CandidaDB/>) identified two Individual Protein Files (IPF 15363 and 19968) whose deduced amino acid sequences showed 42% and 45% homology with *Saccharomyces cerevisiae* Pir4. The two DNA sequences are alleles of the same gene (*CaPIR1*) but IPF 19968 has a deletion of 117 bases. IPF 19968 encodes a putative polypeptide of 364 aa, which is highly O-glycosylated and has an N-mannosylated chain, four cysteine residues and seven repeats. Both alleles are expressed under different growth conditions and during wall construction by regenerating protoplasts. The heterozygous mutant cells are elongated, form clumps of several cells and are hypersensitive to drugs that affect cell wall assembly. CaPir1 was labelled with the V5 epitope and found linked to the 1,3- β -glucan of the *C. albicans* wall and also by disulphide bridges when expressed in *S. cerevisiae*.

INTRODUCTION

The cell wall of *Candida albicans* plays essential roles in growth and development, and in interactions with mammalian cells and tissues during infection and colonization. Besides its primary protective role in shielding the cell against osmotic, chemical and biological harm, the wall of *C. albicans* is involved in many other functions, including morphogenesis, and some activities that may be termed 'social', such as morphological responses, antigenic expression, adhesion and cell–cell interaction (Calderone & Fonzi, 2001; Berman & Sudbery, 2002; Sundstrom, 2002). There are many data supporting the idea that temporal and spatial regulation of the synthesis and assembly of wall polymers are critical for the properties of the wall, which thus do not exclusively depend on their chemical composition, but also on the way that different polymers interact and assemble to give the final structure.

The cell wall is a composite of chitin fibrils immersed in an amorphous matrix made of β -glucans and mannoproteins. The three components are dispersed throughout the cell wall, although the mannoproteins are mostly concentrated on the outer surface (Sentandreu *et al.*, 2001; Klis *et al.*, 2002; Kapteyn *et al.*, 1999b).

In *C. albicans* and *Saccharomyces cerevisiae* some wall proteins are released by hot SDS and the remaining ones are solubilized in the form of supramolecular highly poly-disperse complexes, after the degradation of the structural

skeleton by β -glucanases and chitinase (Marcilla *et al.*, 1991, 1993). Three types of proteins that are released by β -glucanases and chitinase have been detected: glycosylphosphatidylinositol proteins (GPI-dependent wall proteins), Pir proteins (proteins with internal repeats) and other proteins. The proteins of the first group are linked to 1,3- β -glucan through a 1,6- β -glucan connector whereas the proteins of the second group are highly O-glycosylated, and are attached to the 1,3- β -glucan in the cell wall by unknown alkali-labile bonds, suggesting that they are retained through an O-glycosidic linkage (Mormeneo *et al.*, 1995; Mrša & Tanner, 1999; Kapteyn *et al.*, 2000; Klis *et al.*, 2001). The third type of proteins detected in the cell walls lack signal peptide and are probably exported by a putative, non-classical export pathway: enolase, aconitase, pyruvate kinase, phosphoglycerate mutase, methionine synthase and many others have been identified (Cleves *et al.*, 1996; Eroles *et al.*, 1997; Pardo *et al.*, 1999, 2000).

Pir proteins seem to be also present in *C. albicans* and other fungi, as immunological and Southern and Northern experiments suggested the presence of a protein related to the heat-shock-inducible product of the *HSP150/PIR2* gene of *S. cerevisiae* (Kandasamy *et al.*, 2000; Kapteyn *et al.*, 2000; Jaafar *et al.*, 2003).

Kapteyn *et al.* (1999a) published evidence that the amount of Hsp150/Pir2 in the walls of 1,6- β -glucan-deficient mutants increases and suggested that this phenomenon is part of a general compensatory mechanism in response to cell wall weakening caused by low levels of 1,6- β -glucan (Kapteyn *et al.*, 1999a).

Abbreviations: ConA, concanavalin A; Endo-F, endoglycosidase F; IPF, individual protein file.

This paper reports identification of a homologue of *S. cerevisiae* Pir4 by a BLAST search in a *C. albicans* genomic database (<http://genolist.pasteur.fr/CandidaDB/>). This new *C. albicans* Pir protein, CaPir1, has two alleles of different sizes. Both alleles seem to be expressed equally. After cloning, the *C. albicans* Pir protein was expressed in *S. cerevisiae* and overexpressed in *C. albicans*. The protein was labelled with V5 epitope and found to be linked by alkaline sensitive linkages to the wall.

METHODS

Micro-organisms and growth conditions. *C. albicans*, *S. cerevisiae* and *Escherichia coli* strains, and the plasmids used in this study, are listed in Tables 1 and 2, respectively. Strains were maintained on slants of YPD solid medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar). Inocula were prepared by transfer of loopfuls of cells from slants to YNB or YPD liquid medium, depending on the

experiment, and the cells were incubated by shaking overnight at the appropriate temperature. YNB-maltose medium contained 2% maltose, 0.167% YNB and 0.5% (NH₄)₂SO₄. *E. coli* DH5α was grown at 37 °C in Luria–Bertani (LB) broth supplemented with ampicillin (100 µg ml^{−1}) when appropriate. Agar was added to 1.5% for LB plates.

Preparation of wall and soluble fractions. Yeast cells were collected by centrifugation (3000 g, 10 min), washed twice with chilled distilled water, then suspended in a small volume of 1 mM PMSF and broken by vortexing with glass beads (5 mg beads per mg cells); complete cell breakage was obtained. Cell breakage was assessed by examining the preparation with a phase-contrast microscope. The cell walls were sedimented (3000 g, 10 min) from the cell-free homogenate, washed four times with chilled distilled water, then boiled for 10 min (twice) with 2% SDS in distilled water, and finally washed a further six times with chilled 1 mM PMSF in distilled water. Isolated walls were freeze-dried and stored at −20 °C until further use.

Table 1. Strains

Strain	Genotype	Source
<i>C. albicans</i>		
SC5314	Wild-type	Gillum <i>et al.</i> (1984)
CAI4	Δura3::imm434/Δura3::imm434	Fonzi & Irwin (1993)
CAI4/pADH-15	Δura3::imm434/Δura3::imm434 pADH-15 URA3	This work
CAI4/pADH-15-myc	Δura3::imm434/Δura3::imm434 pADH-15-myc URA3	This work
CAI4/pMAL-15-V5	Δura3::imm434/Δura3::imm434 pMAL-15-V5 URA3	This work
CAPIR15	IPF19968/Δipf15363::URA3	This work
CAPIR19	Δipf19968/IPF15363::URA3	This work
<i>S. cerevisiae</i>		
BMA 64-1A	MATα, ura 3-1 trp1-Δ2 leu2-3,112 his 3-11 ade 2-1 can 1-100	EUROFAN
pir4Δ	MATα ura 3-1 trp1-Δ2 leu2-3,112 his 3-11 ade 2-1 can 1-100 pir4::KanMX4	Moukadiri <i>et al.</i> (1999)
pir4Δ/pADH-15	MATα ura 3-1 trp1-Δ2 leu2-3,112 his 3-11 ade 2-1 can 1-100 pir4::KanMX4 pADH-15 URA3	This work
pir4Δ/pADH-15-myc	MATα ura 3-1 trp1-Δ2 leu2-3,112 his 3-11 ade 2-1 can 1-100 pir4::KanMX4 pADH-15-c-myc URA3	This work
<i>E. coli</i>		
DH5α	F [−] ϕ80 dlacΔM15 recA1 endA1 gyrA96 thi-1 hsdR17 (r _k [−] m _k [−]) supE44 relA1 deoR Δ(lacZYA–argF)U169	Hanahan (1985)

Table 2. Plasmids

Plasmid	Characteristics	Reference
pADH-pl	Contains the promoter of ADH1 of <i>C. albicans</i> , CaURA3, Amp ^R	Bertram <i>et al.</i> (1996)
Clp10-MAL2p	Contains the promoter of CaMAL2, CaURA3, Amp ^R	Backen <i>et al.</i> (2000)
p5921	Contains the hisG–URA3–hisG cassette, CaURA3, Amp ^R	Fonzi & Irwin (1993)
p15-1,2	p5921 containing the 15-1,2 fragment	This work
p15-c	p15-1,2 containing the 15-3,4 fragment	This work
pADH-15	pADH-pl containing the ORF of CaPIR1	This work
pADH-15-myc	pADH-pl containing the ORF of CaPIR1 fused to the c-myc epitope	This work
pMAL-15-V5	Clp10-MAL2p containing the ORF of CaPIR1 fused to the V5 epitope	This work

The cell walls (100 mg dry weight) were suspended in a solution of 0.01 M ammonium acetate, pH 6.3, containing 2% (v/v) β -mercaptoethanol, shaken for 3 h at 28 °C and then pelleted. The supernatant was concentrated by freeze-drying and dissolved in Laemmli solution (1970). The cell wall residue was then extracted again with 15 mM NaOH (Kapteyn *et al.*, 1999a).

Protein gel electrophoresis and Western blot techniques.

Proteins were separated by SDS-PAGE performed basically as described elsewhere (Laemmli, 1970) in 10% (w/v) acrylamide gels loaded with 20 μ g protein. The gels were stained with Coomassie brilliant blue or transferred onto Hybond-C nitrocellulose membranes as described by Towbin *et al.* (1979) and Burnette (1981), and immunodetected with a polyclonal antibody preparation against the material extracted by Zymolyase from *C. albicans* yeast cell walls (PAbL) (1:1000), or anti-c-myc (1:500) and anti-V5 (1:500) monoclonal antibodies (Invitrogen). Detection was carried out by the enhanced chemiluminiscent (ECL) method from Amersham Biosciences, following the manufacturer's instructions.

Mannoproteins were stained by the protocol described by Hawkes (1982) and modified by Millete & Scott (1984). To remove N-glycosylated chains of mannoproteins, samples containing 15 μ g protein were treated with endoglycosidase F (Endo-F; Roche) following the manufacturer's instructions.

Protoplast preparation and regeneration. Preparation and regeneration of *C. albicans* CAI4 protoplasts were carried out as described by Elorza *et al.* (1983). Regeneration was performed for 30 min, 1 h, 2 h, 3 h and 5 h. Regenerated protoplasts in each condition were recovered by centrifugation (10 min, 2000 g) and stored at -20 °C.

Mycelium induction. For germ tube induction, cells were cultured in modified Lee's medium as described previously (Elorza *et al.*, 1988).

Heat-shock assay. *C. albicans* CAI4 was grown overnight at 25 °C in YNB medium at 200 r.p.m. One millilitre was inoculated into a 110 ml YNB medium preheated at 25 °C and was grown at 25 °C to OD₆₀₀ 0.6. Two 50 ml samples were taken. One was added to 50 ml YNB medium preheated at 25 °C and incubated at 25 °C for 30 min. The other was added to 50 ml YNB medium preheated at 53 °C and incubated at 37 °C for 30 min. Cultures were harvested at room temperature by centrifugation (2000 g, 5 min) and stored frozen until RNA extraction.

Genetic constructions. To construct the heterozygous *PIR1* mutants, part of the gene (522 bp) was replaced with a *hisG-URA3::hisG* cassette (Fonzi & Irwin, 1993). This cassette was made by a two-step PCR amplification procedure. In the first step, an amplicon of 512 bp was obtained from genomic DNA using primers 15-1 (TTCCGAGCTCGTTAGTATTGTTACTTTGTTAG) and 15-2 (TTCCGGTACCTTTGTTATCATTTCTAGTCAATG) containing engineered *SacI* and *KpnI* restriction sites, respectively (underlined). The amplicon obtained (15-1,2), which included the nucleotides -291 to 224, was digested with *SacI* and *KpnI* and cloned into p5921 previously digested with the same enzymes, producing plasmid p15-1,2. In a second step, a fragment of 411 bp was obtained by using primers 15-3 (TTCCGTCGACTACTGCTGAAAAATGTTGCTAAAGC) and 15-4 (TTCCCTGCAGTTTAACAGTTGACAAATTC-AATG), which have *SalI* and *PstI* restriction sites, respectively (underlined). This new amplicon, 15-3,4, which included nucleotides 746 to 1185, was digested with *SalI* and *PstI* and cloned into p15-1,2 digested with the same enzymes, producing plasmid p15-c.

Disruption of both alleles was attempted as described by Fonzi & Irwin (1993). *C. albicans* CAI4 cells were transformed with 10 μ g of a *SacI*-*PstI* fragment from p15-c. Cells were selected in YNB medium lacking

uridine and integration was checked by PCR, RT-PCR and Southern blot analysis. Some of the heterozygous disruptants obtained from each allele (*PIR1*₁₅₃₆₃/*pir1*₁₉₉₆₈ Δ and *pir*₁₅₃₆₃ Δ /*PIR*₁₉₉₆₈) were plated on YNB medium containing uridine and 5-fluoro-orotic acid to select Ura⁻ revertants produced as a result of recombination of the flanking *hisG* repeats (Boeke *et al.*, 1984).

C. albicans CAI4/pADH-15 and *S. cerevisiae* *pir4* Δ /pADH-15 strains with constitutive expression of *CaPIR1* were obtained by transformation of *S. cerevisiae* *pir4* Δ mutant or *C. albicans* CAI4 with plasmid pADH-15. To construct this plasmid the *CaPIR1* coding region was PCR-amplified. Primers designed to introduce a *BglII* site at position -1 with respect to the first nucleotide of the coding region and an *EcoRV* site in the 3' end were used. The primer sequences were ADH 15 5' (TACTTGAGATCTATGAAGTACTCTACACTTGTAG) and ADH 15 3' (GGGCCCGATATCTTAATAGTTGACAAACTC); they include *BglII* and *EcoRV* sites respectively (underlined). The PCR product was digested with *BglII* and *EcoRV* and ligated with the 12.1 kb *BglII*-*EcoRV* fragment of pADH-pl (Bertram *et al.*, 1996) to generate pADH-15.

To construct pADH-15-myc the coding region of *CaPIR1* was amplified by PCR using the oligonucleotides ADH 15 5' and ADH 15-myc 3' (CCTTGATATCTAAAGATCCTCTTCTGAGATGAGTTT-TTGTTCACAGTTGACAAATTCAATGACAC) containing the *c-myc* sequence (in bold), the stop codon TAA, an *EcoRV* restriction site (underlined) and 23 bp of the 3' part of the *CaPIR1* gene sequence. The PCR product obtained using these primers produced a 1150 bp band that was eluted and digested with *BglII* and *EcoRV*, and ligated with the 12.1 kb fragment of pADH-pl digested with the same enzymes. This new construct was called pADH-15-myc.

Plasmid pMAL-15-V5 was constructed to tag the *CaPIR1* protein with a V5 epitope. The *CaPIR1* coding region was amplified with primers designed to introduce an *EcoRV* site at position -1 with respect to the first nucleotide of the coding region and an *MluI* site at the 3' end. The sequence corresponding to the V5 epitope was added at the 3' end. The primer sequences used were 15-V5 5' (TTAGGATATCATGAAGTATTCTACACTTGTTAGTATTGCTGC), which includes the *EcoRV* site (underlined), and 15-V5 3' (GACCACGCGTTTACGTTAGTAATCGAG-ACCGAGGAGAGGGTTAGGGATAGGCTTACCACAGTTGACAAA-TTCAATG), which contains a *MluI* site (underlined), the stop codon TAA and the V5 epitope sequence (in bold). The 1162 bp PCR product was digested with *EcoRV* and *MluI* and ligated with a 5.68 kb *EcoRV*/*MluI* Clp10-MAL2p fragment to generate the plasmid pMAL-15-V5. The identity of all PCR products was confirmed by cloning and sequencing.

Clp10-MAL2p (Backen *et al.*, 2000) contains the inducible *C. albicans* MAL2 promoter, the gene *CaRP10* and the *C. albicans* *URA3* as marker.

pMAL-15-V5 was digested with *NcoI* and the linear 6.9 kDa fragment was used to transform *C. albicans* CAI4. Integration was performed in the *RP10* locus. Transformants were recovered in YNB medium and grown in YNB-maltose medium to induce tagged protein expression.

Transformation of *C. albicans* and *S. cerevisiae*. *S. cerevisiae* was transformed using the lithium acetate method as described by Ito *et al.* (1983) and modified by Gietz & Sugino (1988). *C. albicans* was transformed using the protocol described by Gietz *et al.* (1992).

Phenotypic analysis of the *pir1/PIR1* heterozygous strains.

Morphological differences and alterations in growth rates were tested. Calcofluor white and Congo red sensitivity were tested by streaking cells onto YNB medium plates containing increasing concentrations of the two compounds as described Van der Vaart *et al.* (1995). Cells were grown in YNB liquid medium overnight and adjusted to OD₆₀₀ 1, serial decimal dilutions were done and drops

of 3 µl were placed onto the surface of YNB plates containing increasing concentrations of Calcofluor white (0–150 µg ml⁻¹) and Congo red (0–30 µg ml⁻¹). Plates were incubated at 28 °C and monitored after 3 days.

RT-PCR analysis. Total RNA was prepared from *C. albicans* CA14 after growth in different conditions by the method described by Langford & Gallwitz (1983).

RNA was treated with RNase-free DNase I (Amersham Biosciences) to eliminate genomic DNA contamination. The first-strand cDNA synthesis reaction was catalysed by the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen) and the primers used to amplify IPF 15363 and IPF 19968 were ADH 15 5' and ADH 15 3'. PCR generated an amplicon of 1120 bp from the first allele and another of 1003 bp for IPF 19968. In a parallel experiment and as an internal control the RT-PCR was performed using new primers that amplify *CaRPS0* (Baquero *et al.*, 2001). This gene carries an intron, and genomic DNA gave a fragment of 870 bp and cDNA one of 545 bp. The oligonucleotides used in this case were YST11B (TTTGACTTAACCTCCAGAAGACGG) and YST12B (ACCTCTTAATCTCAAGACTTCTCTAGC). cDNA was quantified in a GeneQuant II spectrophotometer (Amersham Pharmacia Biotech) and all samples contained the same quantity of first-strand cDNA (1 µg) for PCR amplification. In some experiments, several cycles of amplification were analysed and PCR products were run on 0.8% agarose gels.

RESULTS

Identification of putative Pir proteins in *C. albicans*

N-terminal sequencing of a protein extracted by β-mercaptoethanol from *S. cerevisiae* cell walls with an apparent mobility of 40 kDa in SDS-PAGE gave the sequence DVITQIGDGQVQA. This sequence was 100% identical to a peptide contained in *S. cerevisiae* ORF YJL158c (Castillo *et al.*, 2003). This ORF has been previously described by Moukadiri *et al.* (1999) and other researchers as Pir4/Ccw5p/Ccw1p/Scw8p/Cis3. In order to localize Pir4 homologues in *C. albicans*, isolated cell walls, previously extracted with hot SDS, were treated with β-mercaptoethanol and the released material fractionated by SDS-PAGE. No homologues to the 40 kDa *S. cerevisiae* protein were detected, instead a polydisperse material, higher than 70 kDa, was found (data not shown). After this result, homology searches were carried out *in silico*. To find potential Pir4 homologues in *C. albicans*, a database of this organism (<http://genolist.pasteur.fr/CandidaDB>) was analysed using a BLAST approach. Two ORFs IPF (Individual Protein File) 15363 and IPF 19968, were identified as homologues to *S. cerevisiae* Pir4. The two ORFs showed homology of 42% and 45%, respectively, with the ScPir4 over the entire amino acid sequence. The homology was higher (65%) when only the C-terminal parts were compared (Fig. 1). Both *C. albicans* IPFs had almost the same amino acid sequence; the differences were that IPF 19968 had a gap of 39 aa and a point mutation (Leu₂₈₁/Ser₂₄₂) (Fig. 1); this means that these two IPFs could be two alleles of the same gene. To investigate this, the promoter sequences of both IPFs were analysed. Comparison of the promoter sequences indicated that the two IPFs corresponded

to the same gene. By analogy with ScPir4, the *C. albicans* gene was named *CaPIR1*.

Structural analysis of the amino acid sequence encoded by *CaPIR1*

CaPIR1 encodes two putative polypeptides, of 385 aa (IPF 15363) and 364 aa (IPF 19968), respectively, with calculated molecular masses of 40.5 and 36.4 kDa and pIs of 4.85 and 4.75. Analysis of the predicted amino acid sequences revealed an N-terminal region with characteristics of a signal peptide (von Heijne, 1986) and a predicted cleavage site at positions 18–19 (...SLA-AT...). Assuming that the cleavage site is at position 18, the mature proteins consisted of 367 aa and 328 aa, respectively, with a calculated molecular mass of 38.9 kDa (IPF 15363) and 34.7 kDa (IPF 19968). Approximately 20% of the amino acid residues are Ser or Thr in both IPFs, indicating that CaPir1 could be a highly O-glycosylated protein. One potential N-glycosylation site (N-Xaa-S/T) was identified at amino acid position 233–235 (NST) in IPF 15363 and 194–196 (NST) in IPF 19968. Another structural feature of CaPir1 is the presence of four Cys residues in the C-terminal part of the protein (-C-66aa-C-16aa-C-12aa-C-COOH) (Fig. 1), which is characteristic of the ScPir protein family. ScPir proteins are also identified by the presence of amino acid repeats in their sequences; in this regard ScPir4 has one [-(S-Q-(I/V)-(S/T/G/N/H)-D-G-Q-(L/I/V)-Q-(A/I/V)-(S/T/A)-] whereas CaPir1 has nine (IPF 15363) or seven (IPF 19968) with a slightly different structure [-(A/K/Q)-Q-I-(S/T/G/N)-D-G-Q-I-Q-H-Q-T-].

Expression patterns of *CaPIR1*

The presence of different alleles of *CaPIR1* opened the possibility of finding if both alleles were expressed equally under the same environmental conditions or if they had a different expression control. The expression of *CaPIR1* was examined by RT-PCR. Fig. 2(a, b) shows the RT-PCR amplification of *CaPIR1*-specific fragments (1120 and 1003 bp) from first-strand cDNA derived from cells growing in different conditions. Different samples, containing the same quantity of first-strand cDNA, were prepared and subjected to different cycles of amplification. Fig. 2(a, b) shows that the quantity of *CaRPS0*-specific amplicon (545 bp) was approximately the same in each of the first-strand cDNA samples. The presence of one intron in the corresponding region of the *CaRPS0* genomic DNA allows differentiation between bands amplified from cDNA and any contaminating genomic DNA (870 bp) (Baquero *et al.*, 2001). The results indicated that the two alleles of *CaPIR1* were expressed in the same amount independently of the cell morphology, yeast or mycelium (Fig. 2a). In *S. cerevisiae* at least one of the Pir proteins is a heat-shock protein (Hsp150/Pir2; Kapteyn *et al.*, 1999a); to determine if CaPir1 could be a heat-shock protein and so a functional homologue of ScPir2, a semi-quantitative RT-PCR was performed from cells growing at 25 °C and then transferred to 37 °C. As shown in Fig. 2(b), no differences in expression were observed, suggesting that CaPir1 is not a heat-shock

Pir4p	mqfknvalaasvaalsatasaegytpgepwstltptgsiscgaa-eyttt	49
15363p	mkystlv---siaafistslaat-vpdehystlspakipsgastdfsgt	46
19968p	mkystlv---siaafistslaat-vpdehystlspakipsgastdfsgt	46
	*..... *... *... *... *... *... *... *... *	
Pir4p	fgiavqaitsska-----krdv-----	66
15363p	fgiqvvtvesasalstdtatstltnrnnkheatpvagitdggvghgttg	96
19968p	fgiqvvtvesasalstdtatstltnrnnkheatpvagitdggvghgttg	96
	*** *... *... *	
Pir4p	-----lsqigdgqvc-----tsaataqatdsq	89
15363p	vsalkqisdgqgvgghqnaaqpiagisdgqgghgttakatatpvqgindgg	146
19968p	vsalkqisd-----gg	107
	←-----39 aa-----→	
Pir4p	aqatttatp-----	98
15363p	lghgttvpvvaqisdgqgghgttakatatpvqgigdgqgghgttvqpvaqi	196
19968p	lghgttvpvvaqisdgqgghgttakatatpvqgigdgqgghgttvqpvaqi	157
	* *... *	
Pir4p	-----	98
15363p	sdggqghgtvkasatpvqgigdgqgghgttaaaattasavkqindggqgh	246
19968p	sdggqghgtvkasatpvqgigdgqgghgttaaaattasavkqindggqgh	207

Pir4p	--tssekissaskststnatssscatpslkds-----scknsgtle	137
15363p	gtttaenvakaqsdgqaiatgspssnstlsdddlssstipkaessannle	296
19968p	gtttaenvakaqsdgqaiatgspssnstlsdddlssstipkaessannle	257
	*..... *... *... *... *... *... *... *	
Pir4p	ltlkdgvltakgrigsivanrqfddgpppagaiaagwsitedgyla	187
15363p	mtlhdsvlkdtherwgaivanhqfddgpppagaiaagwsik-dgyla	345
19968p	mtlhdsvlkdtherwgaivanhqfddgpppagaiaagwsik-dgyla	306
	 *... *... *... *... *... *... *	
Pir4p	lgdsdvfyqclsgnfyndqnvaeqcsaihleavslvd	227
15363p	lgdsnifyqclsgdfynlydenvakqcsavklsviefvn	385
19968p	lgdsnifyqclsgdfynlydenvakqcsavklsviefvn	346
	***..... *... *... *... *... *... *... *	

Fig. 1. Alignment of the *S. cerevisiae* Pir4 sequence and the sequences deduced from IPFs 19968 and 15363. Internal repetitions are shadowed in black, the point mutation (Leu₂₈₁/Ser₂₄₂) in grey and the four conserved cysteine residues in bold. The arrow indicates the 39 aa lacking in the sequence deduced from IPF 19968.

protein. One interesting observation was made from protoplasts under cell wall regeneration conditions. *C. albicans* protoplasts were incubated in a regeneration medium, and after 30, 60, 120, 180 and 300 min, samples were taken and the *CaPIR1* expression examined by RT-PCR. The results (Fig. 2c) indicated that *CaPIR1* expression increased with time of regeneration, showing a maximum after 120 min of incubation.

Construction of *CaPIR1* mutants

To investigate the function of CaPir1 protein, construction of null mutants by targeted gene disruption was attempted. Disruption of the *CaPIR1* gene was performed by using a strategy originally developed for *S. cerevisiae* (Alani *et al.*, 1987) and modified for use in *C. albicans* (Fonzi & Irwin, 1993). This method uses a cassette consisting of the *C. albicans* *URA3* gene flanked by direct repeats of the *Salmonella typhimurium* *hisG* gene. This cassette was used to replace approximately 50 % of the ORF of the two alleles of *CaPIR1* (IPF 15363 and IPF 19968). A linear *SacI*–*PstI* fragment from plasmid p15-c, including the cassette flanked by *CaPIR1* sequences, was used to transform *C. albicans* CAI4 to Ura⁺ (see Methods).

Fourteen of the resulting Ura⁺ transformants were analysed and eleven of them contained the desired insert at the

CaPIR1 locus (data not shown). Southern blot analysis of different isolates, after digestion with *SacI*, revealed that the cassette had integrated into one of the two *CaPIR1* alleles (Fig. 3a), giving rise to a 13.8 kb fragment; this is consistent with the replacement of one allele of *CaPIR1* with the transforming DNA. The 10 kb *SacI* fragment corresponds to the other allele which was still present in the Ura⁺ transformants. To determine which of the two alleles had been disrupted, an RT-PCR assay was performed. As shown in Fig. 3(b), disruptants of the two alleles were obtained. A representative isolate of each mutant was chosen; these isolates were named *C. albicans* CAPIR15 and *C. albicans* CAPIR19, heterozygous mutants for allele IPF 15363 and IPF 19968, respectively. Ura[–] segregants were selected on medium containing 5-fluoro-orotic acid (Boeke *et al.*, 1984) and examined by Southern blot analysis. More than 60 independent segregants were examined and all of them had experienced an interchromosomal recombination event, reverting to the *C. albicans* CAI4 genotype (data not shown). By this method of disruption only heterozygous mutants in one or other of the two *CaPIR1* alleles were obtained (Fig. 3b) so we tried to obtain null mutants by the technique of Wilson *et al.* (1999). By this technique again only heterozygous mutants were obtained (data not shown). By using this technique it is not necessary to obtain any Ura[–] segregants to disrupt the second allele, and the fact that no homozygous mutants were obtained with both techniques

Fig. 2. (a, b) Expression of *CaPIR1* mRNA under different conditions measured by semiquantitative RT-PCR: (a) yeast and mycelium; (b) heat shock (25 and 37 °C). Samples containing 1 µg cDNA were amplified and several cycles of amplification were analysed (cycle numbers are shown at the top of each panel). (c) Protoplast regeneration at 28 °C (30, 60, 120, 180 and 300 min regeneration) measured by RT-PCR, using as control cDNA obtained from an exponential-phase CAI4 culture in YNB medium at 28 °C (C). Samples containing 1 µg cDNA were subjected to 30 cycles of amplification and then run on a 0.8% agarose gel. Primers that amplified a 545 bp fragment of *CaRPS0* (a constitutive gene with a intron in its sequence) were used as control. Primers that amplified the complete ORF of *CaPIR1* were used to measure *CaPIR1* expression.

could indicate that *CaPIR1* is an essential gene for cell viability.

Phenotypic analysis of the *Capir1/CaPIR1* mutants

Phenotypic analysis of the heterozygous mutants obtained in comparison with the parental strain was performed. The specific growth rates of the cells at 28 °C and 37 °C on minimal medium were slower in both heterozygous mutants than in the parental strain (data not shown). In addition, cells of *C. albicans* CAPIR15 and CAPIR19 mutant strains showed some differences in morphology when they were grown at 28 °C or 37 °C (Fig. 4a). Mutant cells appeared elongated and many of them had an abnormal shape with a tendency to form clumps of different numbers of cells (Fig. 4a).

Possible alterations in the cell wall were studied by testing the sensitivities of the two heterozygous strains, *C. albicans* CAPIR15 and CAPIR19, to Calcofluor white and Congo red, as described in Methods. Sensitivities to these compounds did increase in the two mutant strains with respect to *C. albicans* CAI4 parental cells (Fig. 4b), suggesting that both *CaPIR1* alleles, IPF 15363 and IPF 19968, contribute to maintaining the architecture of the cell wall.

Overexpression and cellular localization of *CaPIr1* in *C. albicans*

Overexpression of *CaPIR1* was achieved by subcloning an amplicon containing the IPF 15363 allele of *CaPIR1* in a pADH episomal vector (Bertram *et al.*, 1996) under the control of the *ADH1* promoter. The new plasmid was named pADH-15 and it was used to transform *C. albicans* CAI4. To

Fig. 3. (a) Southern blot analysis of genomic DNAs from *C. albicans* CAI4, CAPIR15 and CAPIR19 digested with *SacI* and hybridized with a DIG-labelled DNA probe (amplicons 15-1,2 and 15-3,4). The wild-type and disrupted alleles are shown on the left. (b) RT-PCR analysis of the transformants using ADH 155' and ADH 153' as primers. Primers that amplified a 545 bp fragment of *CaRPS0* (a constitutive gene with a intron in its sequence) were used as control. Primers that amplified the complete ORF of *CaPIR1* were used to measure *CaPIR1* expression.

Fig. 4. (a) Morphology of *C. albicans* strains CAI4 and CAPIR15 in YNB medium at 28 °C and 37 °C. (b) Sensitivities to Calcofluor white (CFW) and Congo red (CR) of the parental (1) and heterozygous strains (CAPIR15 and CAPIR19; 2 and 3 respectively). Cells were grown in YPD medium and a 1/10 dilution series of each strain was inoculated on to YNB medium containing the indicated amounts of CR and CFW.

determine in which cell wall fraction the overexpressed material was located, a Western blot analysis of β -mercaptoethanol and alkaline cell wall extracts, and also the spent medium, using PABL antibodies, was performed. An increased amount of the material released by alkaline solutions, but not in the material released by β -mercaptoethanol or present in the spent medium, was observed in the strain that overexpressed *CaPIR1* when compared with *C. albicans* CAI4 (Fig. 5a). No change in morphology or growth rate of cells was observed in the overexpressing *C. albicans* strain.

To confirm that the overexpressed material, detected in the alkaline extracts, corresponded to CaPir1, and not to other kinds of material recognized by the PABL antibody, the allele IPF 15363 was tagged with the V5 epitope. This epitope is a 14 aa peptide (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr) that has been described as being convenient for tagging *C. albicans* extracellular proteins (Spreghini *et al.*, 2003). The V5 epitope was introduced into the C-terminal end of CaPir1 as described in Methods. As shown in Fig. 5(b), the recombinant protein was bound

directly to the 1,3- β -glucan, as it could only be released from the cell wall by treatment with alkaline solutions. Small amounts of the protein were also detected in the spent medium (Fig. 5b).

The difference between the predicted size of CaPir1 (about 40 kDa in the case of the allele IPF 15363) and that deduced from the mobility in SDS-PAGE (about 180 kDa, Fig. 5b) could be accounted for by *N*- and/or *O*-glycosylation. To determine if CaPir1 was modified postrationally by *N*-glycosylation, the material released to the spent medium by *C. albicans* expressing CaPir1 tagged with V5 was treated with Endo-F. Western blot analysis with anti-V5 antibodies showed that the 180 kDa species disappeared after Endo-F treatment, and a new species of 110 kDa appeared (Fig. 5c), indicating that CaPir1 was *N*-glycosylated. There is a substantial difference between the molecular mass of CaPir1 (IPF 15363) treated with Endo-F (110 kDa) as determined by SDS-PAGE, and its predicted molecular mass (40.5 kDa) calculated from the deduced amino acid sequence. This discrepancy could be accounted for by the potential *O*-glycosylation, as 20 % of its amino acids are Ser/Thr, which is known to increase the apparent size in the Laemmli gel system.

Expression of *CaPIR1* in *S. cerevisiae*

To analyse whether CaPir1 could be incorporated into the walls of *S. cerevisiae*, cells of *S. cerevisiae pir4Δ* transformed with plasmid pADH-15 were grown and their cell walls isolated. Cell walls were treated with β -mercaptoethanol or alkaline solutions; the spent medium was also analysed. The solubilized material was analysed by Western blotting using PABL antibodies and ConA-peroxidase. A main band with an apparent molecular mass of 110 kDa was detected in β -mercaptoethanol extracts (Fig. 6a). A band with a similar mobility was also detected in the spent medium and in the material extracted by alkaline solutions from the isolated cell walls of the *S. cerevisiae CaPIR1*-expressing strain (data not shown). No changes in either morphology or growth rate of cells were observed in *S. cerevisiae pir4Δ* expressing *CaPIR1*.

As indicated above, CaPir1 is *N*-glycosylated in *C. albicans*. To determine whether the protein is also *N*-glycosylated when expressed in *S. cerevisiae*, material obtained by β -mercaptoethanol was treated with Endo-F. No new species appeared (Fig. 6b), indicating that CaPir1 is not *N*-glycosylated by the enzymic machinery of *S. cerevisiae*.

DISCUSSION

Wall biogenesis in fungal cells, and specifically in *C. albicans*, is the result of several steps that are initiated by the synthesis of the proteins at the level of the endoplasmic reticulum, followed by their glycosylation in the vesicles of the secretory pathway and release to the periplasmic space, where they interact with the nascent wall polysaccharides. The process ends with bond formation between the different components, producing the final architecture of the wall. Although

Fig. 5. (a) Western blot analysis of the materials released to the culture medium (lanes 1 and 2), or extracted from purified cell walls by β -mercaptoethanol (lanes 3 and 4) or alkali solutions (lanes 5 and 6) from strains CAI4 (lanes 1, 3 and 5) and CAI4/pADH-15 (lanes 2, 4 and 6) reacted with PAbL polyclonal antibody against the cell wall of *C. albicans* in yeast morphology. (b) Western blot analysis of the material released to the culture medium (lanes 1 and 2), or extracted from purified cell walls by β -mercaptoethanol (lanes 3 and 4) or alkali solutions (lanes 5 and 6) from strains CAI4 and CAI4/pMAL-15-V5 grown in YNB-maltose medium, probed with anti-V5 antibody. (c) Western blot analysis of the material released to the culture medium by strain CAI4/pMAL-15-V5 and developed with anti-V5 monoclonal antibody, before (–) and after (+) Endo-F treatment.

some of the proteins and protein–polysaccharide bonds are fairly well known, identification of additional wall proteins and the enzymes catalysing formation of the different bonds is needed.

S. cerevisiae Pir4 was identified from the material released from isolated walls with β -mercaptoethanol (Castillo *et al.*, 2003). New potential *C. albicans* cell wall proteins related to *S. cerevisiae* Pir4 were identified by an *in silico* search of a *C. albicans* database. Only two ORFs were found (IPF 15363 and 19968; Fig. 1). Additional BLAST searches in the

C. albicans database for other homologues of the Pir family of *S. cerevisiae* (Pir1, Pir2 and Pir3) gave negative results (Toh-e *et al.*, 1993). Due to the similarity in the homology percentages, CaPir1 can not be designated as the functional homologue of a specific Pir protein.

Three main differences have been detected in the *C. albicans* proteins in comparison to ScPir4: (i) absence of a potential Kex2 site (Lys-Arg, Arg-Arg and Pro-Arg) close to the N-terminal part of the proteins (cleavages occur at the carboxyl side of pairs of basic residues: Brenner *et al.*, 1994; Mizuno

Fig. 6. (a) Analysis by SDS-PAGE and Western blotting of the materials released by β -mercaptoethanol from purified cell walls. The *S. cerevisiae* strains used were: 1, BMA64-1A; 2, *pir4* Δ ; 3, *pir4* Δ /pADH-15. The nitrocellulose membranes were probed with PAbL and ConA-peroxidase. (b) Western blot analysis of β -mercaptoethanol extracts from purified cell walls of *Scpir4* Δ /pADH-15-myc before (–) and after (+) Endo-F treatment, reacted with anti-c-myc monoclonal antibody. The 110 kDa band is arrowed.

et al., 1989; Goller *et al.*, 1998); (ii) the *C. albicans* protein encoded by IPF 15363 has nine tandem repeats of 11 aa (QITDGQVQHQT) and IPF 19968 seven tandem repeats, whereas the ScPir4 has only one repeat (SQIGDGQVQA); and (iii) a point mutation (Leu₂₈₁/Ser₂₄₂) that could be a real point mutation. A homologue of *S. cerevisiae* Hsp150/Pir2 has been reported in *C. albicans* (Kapteyn *et al.*, 2000; Kandasamy *et al.*, 2000) but the results obtained *in silico* and by mass spectrometry (unpublished results) indicated that the product of a single *PIR* gene (*CaPIR1*) is found in *C. albicans* cell wall. Therefore it is possible that the protein previously reported is the same as the one described in this paper which we have named CaPir1.

It is interesting that both IPF 15363 and IPF 19968 are expressed under normal laboratory growth conditions, after heat shock and in both yeast and mycelial forms. The codon bias index (CBI) for IPF 15363 is 0.345, suggesting that it is a poorly expressed gene. The low expression of *CaPIR1* contrasts with the situation of the *PIR* genes in *S. cerevisiae* because *PIR1*, *PIR2* and *PIR3* are among those expressed abundantly (Toh-e *et al.*, 1993).

To define the cellular function of *PIR1*, we attempted to construct a null mutant to search for informative phenotypes. However, we were unable to obtain a null mutant even though both the Fonzi & Irwin (1993) and Wilson *et al.* (1999) techniques were used. We have no explanation for these results, but the phenotype of the heterozygous mutants, independently of the allele interrupted (specific growth rates, clump formation and hypersensitivity to cell-wall-perturbing agents such as Calcofluor white and Congo red) (Elorza *et al.*, 1983; Ram *et al.*, 1994; Mrša *et al.*, 1999), and the fact that in *S. cerevisiae* there are four *PIR* genes instead of one, indicated the possibility that the null mutant is lethal. In the case of *S. cerevisiae*, sequential disruption of *PIR* genes brings increasingly irregular shape, clumping of the cells and a pronounced destabilization in the presence of Calcofluor white and Congo red (Mrša & Tanner, 1999); therefore *C. albicans PIR1* may be an essential gene.

The haploinsufficiency phenotypes indicate that both *PIR1* alleles contribute to maintaining the correct cell wall organization in wild-type strains. This situation is in some respects similar to that in *S. cerevisiae*, as the phenotype shown by this species is progressively more apparent as the number of *PIR* genes disrupted increases (Mrša & Tanner, 1999).

CaPir1 was found as a new cell wall band when expressed in *S. cerevisiae*. This band reacted with ConA, demonstrating that it was a glycoprotein, but it could not be detected in *C. albicans* as the material released from the wall was highly polydisperse and no specific antibodies were available. A recombinant CaPir1 tagged with the V5 epitope was found linked only to the 1,3- β -glucan through an alkali-sensitive bond. MALDI-TOF analysis of the materials released by β -mercaptoethanol also failed to detect CaPir1 (unpublished). Therefore it is possible that CaPir1 is only attached to the

1,3- β -glucan of the wall. This result is surprising, as a fraction of *S. cerevisiae* Pir4 molecules and CaPir1 expressed in this species are retained in the wall by disulphide bridges and as a consequence released by reducing agents.

Nothing is known about the function of CaPir1. Toh-e *et al.* (1993) isolated in *S. cerevisiae* three highly homologous genes of the *PIR* family (*PIR1*, *PIR2* and *PIR3*), and genes homologous to *PIR* have been also found in *Kluyveromyces lactis* and *Zygosaccharomyces rouxii* but not in *Schizosaccharomyces pombe*, suggesting that the *PIR* genes play a role in budding yeast. In addition, null mutants of each gene are viable, indicating that none of them is essential, but they are required for tolerance to heat shock (*PIR2/HSP150*) (Toh-e *et al.*, 1993) and determine resistance to the plant protein osmotin (Yun *et al.*, 1997; Ibeas *et al.*, 2001). By functional genomics it has been found that ScPir4 interacts with Yjr030 and Bur2 (Ito *et al.*, 2001) and it was suggested that it might strengthen the regenerating cell wall of protoplasts (Pardo *et al.*, 1999). In this context it is important to emphasize that the *S. cerevisiae* Pir family is formed by four proteins (Pir1, Pir2, Pir3 and Pir4) whereas in *C. albicans* only one Pir homologue has been found. BLAST searches to find other members in the *C. albicans* genomic database have given negative results. Recently it has been reported that ScPir2 is more efficiently retained in the wall of *S. cerevisiae* growing at low pH and it was suggested that this is also the case for other Pir proteins (Kapteyn *et al.*, 2001) and that probably all members of this protein family are at least functionally equivalent in the cell wall.

Although the actual function of CaPir1 is unknown, it may be critical in the organization of the wall because during the initial steps of protoplast regeneration (2–3 h) the levels of expression are significantly high.

Two interesting differences have been detected between CaPir1 and ScPir4 from the structural and functional points of view: (i) ScPir4 is retained in the wall by two types of bonds (disulphide bridges and covalently bound to the 1,3- β -glucan) whereas CaPir1 seems to be only attached to the 1,3- β -glucan (the protein is not detected in the material extracted by β -mercaptoethanol by immunological or mass spectrometry techniques; unpublished observations); and (ii) four proteins are found in the *S. cerevisiae* Pir family whereas in *C. albicans* only one member of this family (CaPir1, as determined *in silico*) has been found. CaPir1 has an *N*-glycosylation sequon in its sequence (NX_{aa}S/T) at positions 233–235 and the protein moves with an apparent molecular mass of 180 kDa that is reduced to 110 kDa after treatment with Endo-F. But it seems that it is not *N*-glycosylated when expressed by *S. cerevisiae*. This result is of interest but its reason is unknown; it could be due to the different specificity of the oligosaccharyltransferase (the enzyme complex that transfers the inner core of the carbohydrate moiety) in the two organisms, or to steric hindrance as occurs with *S. cerevisiae* invertase; this enzyme contains 14 sequons but only eight or nine are glycosylated (Reddy *et al.*, 1988).

Finally, the cell wall of *S. cerevisiae* cells has been engineered using Pir1 or Pir2 to anchor proteins by fusion of the corresponding genes with *PIR1* or *PIR2* and the enzymic activities produced by fusion proteins on their surface detected (Abe *et al.*, 2004), opening the possibility to express *C. albicans* heterologous proteins by fusion of the corresponding genes with *CaPIR1*.

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