Identification and study of a Candida albicans protein homologous to Saccharomyces cerevisiae Ssr1p, an internal cell-wall protein

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After screening of a Candida albicans genome database, the product of an ORF (IPF 3054) that has 62% homology with Saccharomyces cerevisiae Ssr1p, an internal cell-wall protein, was identified and named CaSsr1p. The deduced amino acid sequence shows that CaSsr1p contains an N-terminal hydrophobic signal peptide, is rich in Ser and Thr amino acids and has a potential glycosylphosphatidylinositol-attachment signal. CaSsr1p is released following degradation of isolated cell walls by zymolase (mainly a 1,3-β-glucanase) and therefore seems to be covalently linked to the β-glucan of the cell walls. Both disruption and overexpression of the CaSSR1 gene caused an increased sensitivity to calcofluor white, Congo red and zymolase digestion. These results suggest that CaSsr1p has a structural role associated with the cell-wall β-glucan.

INTRODUCTION

Candida albicans is an opportunistic pathogenic fungus in humans which can cause either septicemic or mucosal infections (Odds, 1988, 1994). The number of fungal infections caused by C. albicans has increased dramatically in the last few decades, due to the rise in the number of immunocompromised patients and their life expectancies (Fox, 1993). C. albicans is a dimorphic organism capable of reproducing by budding (yeast cells) or by producing germ tubes (mycelial cells) depending upon environmental factors (Odds, 1988); this morphological transition has been associated with pathogenicity (Calderone & Braun, 1991; Odds, 1988; Sentandreu et al., 1993). Because the cell wall of C. albicans is the fungal structure responsible for the initial interaction with the host and for the characteristic shape of each growth form, several studies have focused on its biosynthesis and function (Gozalbo et al., 1994; Sentandreu et al., 1993; Valentin et al., 2000).

The cell wall of C. albicans is a complex biochemical entity composed mainly of three components, namely, glucans (1,3-β- and 1,6-β-glucan), mannoproteins and chitin (Fleet, 1991; Valentin et al., 2000). The β-glucans are the main components, accounting for 50–60% by weight of the cell wall in C. albicans and other fungi. Chitin, a linear polymer of 1,4-β-linked N-acetylg glucosamine units, is a relatively minor (1–10%) but important constituent. In most fungi, β-glucans and chitin polymers account for the rigidity of the cell wall as well as its morphology (Sentandreu et al., 1994). Mannoproteins represent 30–40% of the total cell wall and determine the surface properties, enabling C. albicans cells to interact and adhere to host tissues (Chaffin et al., 1998).

Cell-wall mannoproteins can be divided into three groups according to the methods used for their extraction. One group can be solubilized by detergents, such as SDS, or chaotropic agents, such as urea, and is formed by mannoproteins loosely associated with other components of the cell wall (Elorza et al., 1985; Valentin et al., 1984). The second group can be extracted by reducing agents, DTT or β-mercaptoethanol (Casanova et al., 1989; Orlean et al., 1986). The third group can be released from the cell wall only following enzymic degradation of β-glucans and chitin with β-glucanases or chitinase (Kapteyn et al., 1999; Marcilla et al., 1991; Montijn et al., 1994; Valentin et al., 1984; Van Rijnsum et al., 1991). Only the mannoproteins in this latter group are covalently bound to β-glucan and chitin. Two types of glucanase-extractable proteins have been reported: glycosylphosphatidylinositol (GPI)-dependent cell-wall proteins and proteins of the Pir family (proteins with internal repeats). The proteins of the first type have, as common characteristics, a high Ser/Thr content and a putative GPI-attachment site (Klis, 1994; van der Vaart et al., 1995). Proteins of the Pir family can also be extracted from the cell wall by a mild NaOH treatment (Kapteyn et al., 1999; Mrsa et al., 1997). The presence of a GPI anchor has been

Abbreviations: CR, Congo red; CW, calcofluor white; GPI, glycosylphosphatidylinositol; IPF, individual protein file.

A table showing putative GPI-proteins in Candida albicans can be found in Microbiology Online.
demonstrated in cell-wall proteins of different fungal species (Frieman et al., 2002; Moukadiri et al., 1997; Staab et al., 1999; Wojciechowicz et al., 1993). The presence of the GPI anchor seems to play an important role in the biology of C. albicans, as mutants are affected in morphogenesis, virulence and cell-wall composition (Richard et al., 2002). The total number of glucanase-extractable mannoproteins identified in C. albicans so far is small, but it is likely that this number will increase in the very near future as a result of homology studies following BLAST searches in the genome database of this fungus.

In the present study, we have taken a sequence-dependent approach to identify cell-wall ORFs by screening the genome database of C. albicans for cell-wall proteins by an in silico analysis. From all the ORFs identified as potential cell-wall proteins, we selected IPF 3054 for further study. This ORF has a putative GPI-anchor sequence, is rich in Ser/Thr and has a high homology to the Saccharomyces cerevisiae Ssr1p cell-wall protein, which was cloned and studied by our group (Moukadiri et al., 1997).

METHODS

Database analysis. ORFs of the C. albicans genome database (http://genolist.pasteur.fr/CandidaDB/) were used for sequence analysis. The PSORT II program, developed by Nakai & Horton (1999; http://psort.nibb.ac.jp), was used to predict the subcellular localization of proteins derived from the ORFs. The ORFs with greater than 33% probability for extracellular localization – including cell wall – were taken as candidates for cell-wall proteins. These ORFs were analysed for (i) the presence of a signal peptide at the N terminus using the SIGNALP V2.0.2 program (Nielsen et al., 1997; http://www.cbs.dtu.dk/services/SignalP-2.0/), (ii) the presence of putative N-glycosylation sites (N-X-S/T) using the PROSITE program (Gattiker et al., 2002; Sigrist et al., 2002; http://us.expasy.org/tools/scanprosite/), (iii) the presence of a putative GPI-attachment site (Hamada et al., 1998) in the C terminus of the protein and (iv) their Ser/Thr amino acid content.

Strains, growth conditions and transformations. The C. albicans and Escherichia coli strains used in this study are listed in Table 1. C. albicans cells were grown routinely in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) (Sherman, 1991) or SD medium (0·7% yeast nitrogen base without amino acids, 2% glucose) supplemented with the appropriate nutrients in amounts specified by Sherman (1991). YPD and SD media were solidified with 2% agar. For germ tube induction, cells were cultured in modified Lee’s medium as described previously (Elorza et al., 1988).

E. coli DH5α was used for plasmid propagation. It was grown routinely in LB medium (0·5% yeast extract, 1% tryptone, 0·5% NaCl) supplemented with 100 μg ampicillin ml−1 (LBA) when required. E. coli was transformed as described by Hanahan (1983).

Nucleic acid manipulations and analysis. Genomic DNA from C. albicans was prepared using the method described by Fujimura & Sakuma (1993); total RNA isolation was done as described previously (Ramón et al., 1996). Plasmid purification was performed using the FlexiPrep Kit commercial system (Amersham Biosciences). Standard DNA manipulation techniques were carried out using standard protocols (Sambrook et al., 1989). DNA probes (amplicons IAE12 and IAE34) for Southern blot and Northern blot analysis were labelled by random primed incorporation of digoxigenin-labelled 2′-deoxyuridine 5′-triphosphate (DIG-labelled DNA) using the DIG DNA Labelling Kit (Roche) according to the manufacturer’s instructions. Southern blot hybridization was performed as described by Ramón et al. (1996); Northern blot hybridization was done as described previously (Montero et al., 1998). DNA and RNA concentrations were determined by measuring absorbance (A260) in a GeneQuant II RNA–DNA calculator spectrophotometer (Amersham Biosciences).

Plasmid construction for disruption of the CaSSR1 gene. The CaSSR1 gene was disrupted by replacing part of the ORF (from amino acid 29 to 128) with a hisG::URA3::hisG cassette (Fonzi & Irwin, 1993). The disruption cassette construction was achieved by using a two-step PCR amplification procedure with genomic DNA as template. In the first step, an amplicon of 607 bp was obtained from the genomic DNA using the sense primer IAE1 (5′-AGAAAGCTTCAGAATAGGAGCGGACGACCC-3′) and the antisense primer IAE2 (5′-AGCTTTGACAACTAAAACACCCGG-3′) containing engineered HindIII and PstI restriction sites (underlined), respectively. The amplicon obtained (IAE12) was digested with HindIII and PstI, then subcloned into p5921 (Fonzi & Irwin, 1993) containing the hisG::URA3::hisG cassette; the resulting plasmid was named pIAE1-2 and contained the 5′ region of the gene (positions −521 to +86 with respect to the start codon). In the second step, an amplicon of 444 bp containing the last 321 bp of the ORF plus the first 123 bp of the 3′ downstream non-coding region was obtained by using the sense primer IAE3 (5′-TAAA- GGATCGCAATCTAGTTCAGCCAGGG-3′) and the antisense primer IAE4 (5′-CATTGGAGCTCTATCTAAGAATACCAACC-3′) containing engineered restriction sites BamHI and SacI, respectively.

Table 1. Strains used in this study

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<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
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<td>Wild-type</td>
<td>Guillum et al. (1984)</td>
</tr>
<tr>
<td>CAI4</td>
<td>Δura3::imm434/Δura3::imm434</td>
<td>Fonzi &amp; Irwin (1993)</td>
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<tr>
<td>30541-1</td>
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<td>This work</td>
</tr>
<tr>
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<td>This work</td>
</tr>
<tr>
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<td>This work</td>
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<tr>
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<td>This work</td>
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<td>30543</td>
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<td>This work</td>
</tr>
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</tr>
<tr>
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<td>F 80 lacM15 recA1 endA1 gyrA96 thi-1 (r− m−) supE44 relA1 deoRΔ(lacZYA–argF)U169</td>
<td>Hanahan (1983)</td>
</tr>
</tbody>
</table>
 Identification of a *C. albicans* cell-wall protein

***RESULTS***

**In silico screening for potential cell-wall proteins**

The cell-wall proteins associated with glucan have some common characteristics: (i) a signal sequence for secretion at the N terminus, (ii) a Ser/Thr-rich sequence which could provide sites for glycosylation and (iii) a C-terminal GPI-attachment site and a stretch of hydrophobic amino acid residues. On the basis of these characteristics, a *C. albicans* database was analysed *in silico*. More than 100 ORFs were identified as putative cell-wall proteins. A similar analysis has been performed by P. de Groot & F. Klis (University of Amsterdam) (http://www.pasteur.fr/recherche/unites/Galar_Fungail/data.html). Comparison of both analyses shows small differences. In our analysis, cell-wall proteins CaAls12, CaRbt1, CaSpr1, CaYck3 and IPFs (individual protein files) 331, 12201 and 15911 are not present as they lack a signal peptide; however, IPFs 564, 652, 2053 and 19968 were not included in the analysis performed by P. de Groot & F. Klis. The online version of this article (at http://mic.sgmjournals.org) contains a supplementary table showing putative GPI-proteins in *C. albicans*.

From the ORFs analysed, we selected one (IPF 3054 = CaSSR1) that presents a potential GPI region and whose product has high homology (62 %) with *S. cerevisiae* Ssr1p, an internal cell-wall protein (Moukadiri et al., 1997) (Fig. 1A).

To study whether CaSSR1 had a morphology-dependent expression, a Northern blot analysis of total RNA from yeast and mycelial cells of *C. albicans* CA14 was performed using the DIG-labelled DNA from ampiclons IAE12 and IAE34 as a probe. One transcript of 0·8 kb (Fig. 2) from both yeast and mycelium was observed. It therefore indicates that CaSSR1 has no morphological differential expression.

**Isolation of the CaSSR1 null mutant.** Disruption of CaSSR1 was achieved as described by Fonzi & Irwin (1993). CA4 cells were transformed to Ura+ prototrophy with 10 μg of an HindIII–SacI fragment from pL4. Transformed cells were selected as Ura+ in SD minimal medium lacking uridine and checked for integration of the cassette at the CaSSR1 locus by Southern blot analysis. One of the heterozygous disruptants recovered (designated *C. albicans* 30541-1) was used to select spontaneous Ura+ derivatives in SD minimal medium containing 5-fluoro-orotic acid (Boeke et al., 1984). These clones were analysed by Southern blot hybridization to identify those that had undergone intrachromosomal recombination between hisG repeats. One of these Ura+ derivatives (termed *C. albicans* 30542-1) was used for replacement of the second CaSSR1 allele in a similar way using the HindIII–SacI fragment from pL4. Transformed cells were selected as Ura+ and integration into the correct allele was verified by Southern blot analysis. One of the Ura+ transformants (designated *C. albicans* 30542-1) was used for 5-fluoro-orotic acid selection to Ura+ auxotrophy. Ura- segregants were screened by Southern blot analysis using digestion with BglII to identify those carrying both disrupted CaSSR1 alleles. One of these CaSSR1 null mutants was designated *C. albicans* 30542-2.

**Cell-wall purification.** Purified cell walls were obtained as described previously for *S. cerevisiae* (Pastor et al., 1984; Valentín et al., 1984) except that intact cells were broken with glass beads (1·5 g (mg dry cells)−1) by shaking in a vortex mixer at room temperature for eight periods of 1 min each with intermediate periods of 1 min on ice. Using this method, breakage of the whole cell population was obtained, as monitored under the phase-contrast microscope. The purification procedure was continued by repeated washing (1200 g, 5 min) of the cell-wall pellet in cold PMSF (1 mM). The pellet was collected and operationally defined as the cell wall.

**Solubilization of cell-wall proteins.** Conditions for solubilization of cell-wall proteins with SDS or zymolyase 20T have been described (Pastor et al., 1984; Valentín et al., 1984); growth media and β-mercaptoethanol extracts were obtained as described previously (Casanova et al., 1989; Elorza et al., 1988).

**SDS-PAGE and Western blot analysis.** Proteins were separated basically as described by Laemmli (1970) on SDS-10% (w/v) polyacrylamide gels. For Western blot analysis, proteins were electro-pheroretically transferred from SDS-PAGE gels on to nitrocellulose filters (Hybond-C Extra; Amersham Biosciences) according to the method of Towbin et al. (1979). Filters were probed with rabbit antibodies against *C. albicans* yeast cells (PAbL) at a dilution of 1:1000, followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad). Antiserum binding was visualized by using the ECL (enhanced chemiluminescence) fluorescent labelling kit (Roche) following the manufacturer’s instructions. Luminescence was recorded by exposing the filter to a radio-autographic X-Omat film (Kodak).

**Subcloning of the CaSSR1 ORF into pADH.** The CaSSR1 ORF was obtained by PCR using the sense primer IAE5 (5′-CACAAGATCTTGGTCTTTTTTTTTTAAG-3′) containing an engineered BglII (underlined) site and the antisense primer IA6 (5′-CACAATCGGTTCTCAAAAAACGACC-3′) containing an engineered XhoI (underlined) site. A DNA polymerase with 3′→5′ proofreading activity (EcoTaq-Plus; Ecogen) was used to improve fidelity. We obtained an amplicon of 705 bp, from the ATG start codon to the TAA stop codon, which was subcloned into the commercial vector pGEM-T Easy (Promega). The amplicon was rescued by digestion with BglII/XhoI and ligated to BglII/XhoI-digested pADH (Bertram et al., 1996) to give plasmid pADH-CaSSR1, which was used to transform the null mutant. The resulting transformed strain (*C. albicans* 30543) was tested for CaSSR1 overexpression.

**Phenotypic analysis of mutants and the overexpressing strain.** Calcofluor white (CW) and Congo red (CR) sensitivities were tested by streaking cells on to plates containing different concentrations of CW or CR following the method described by van der Vaart et al. (1995). Aliquots (3 μl) of serial 1/10 dilutions of cells that had been grown overnight and adjusted to an OD600 value of 1 were deposited on to the surface of YPD or SD plates containing different concentrations of CW (0–200 μg ml−1) or CR (0–25 μg ml−1); these samples were then grown at 28°C and monitored for 3 days. Sensitivity to zymolyase was also tested following the method described by van der Vaart et al. (1995). Exponentially growing cells were adjusted to an OD600 value of 0·5 (~4 × 106 cells ml−1) in 10 mM Tris/HCl, pH 7·5, containing 50 μg zymolyase 20T ml−1 and the decreases in the optical density were monitored over a 90 min period.

(underlined). The amplicon obtained (IAE34) was ligated into the BamH I and Sac I sites of pIAE1-2 to create plasmid pL4 in which 297 bp (33 %) of the coding region were deleted.

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Structural analysis of the amino acid sequence encoded by CaSSR1

CaSSR1 encodes a putative polypeptide of 234 aa with a calculated molecular mass of 22,553 Da and a pI of 4.59. Analysis of the predicted amino acid sequence revealed an N-terminal region with characteristics of a signal peptide (von Heijne, 1986) and a predicted cleavage site at positions 22–23 (...TLA–AP...) (Fig. 1A, B). Hydropathy analysis (Kyte & Doolittle, 1982) of the deduced amino acid sequence showed that the hydrophobic signal sequence is followed by a neutral region representing the mature protein (Fig. 1C). Assuming the cleavage site is at position 22, the mature protein has 212 aa residues with a calculated molecular mass of 20,248 Da. Eighty-one of the 212 aa residues are Ser or Thr (38 %), localized in the central part of the protein, indicating that CaSsr1p could be a highly O-glycosylated protein. The protein is also rich in Ala, Glu, Gly, Leu, Lys and Val residues. Another structural characteristic of CaSsr1p is the high proportion of Cys residues in the first 60 N-terminal amino acid residues (Fig. 1B). The Cys residues should be able to participate in disulphide bond formation within CaSsr1p, contributing to the structure of the protein, or might participate in the formation of disulphide bonds with other Cys groups of other proteins in the cell wall. Amino acid repeats SSSA, SSSAA, SSE and STTA are found. The sequence also shows a putative GPI-attachment site. According to Nuoffer et al. (1993), the possible GPI-attachment site in CaSsr1p would be represented by G(215) (7 aa residues after the hydrophobic tail) (Fig. 1). The polar (amino acids 211–213) and hydrophobic (amino acids 222–234) regions described as necessary for GPI attachment by Caras et al. (1987, 1989) are

![Alignment of the CaSsr1p amino acid sequence with that of ScSsr1p from S. cerevisiae.](image1)

**Fig. 1.** (A) Alignment of the CaSsr1p amino acid sequence with that of ScSsr1p from *S. cerevisiae*. The alignment was performed with the CLUSTAL programs and shaded using BOXSHADE 3.31 software. Residues that are identical are shaded black, whereas conserved residues are shaded grey. Dashes represent gaps to maximize alignment. ⬤, Putative GPI-attachment site. (B) Structural features of CaSsr1p. The hydrophobic N- and C-terminal domains (grey and black bars, respectively), the Ser/Thr-rich domain (hatched bar) and the Cys residues (●) are indicated. (C) Hydropathic plot from the deduced amino acid sequence of CaSsr1. Values above and below the horizontal line indicate hydrophobic and hydrophilic regions, respectively.

![Hydropathic plot from the deduced amino acid sequence of CaSsr1.](image2)
also present in the sequence, as shown in the Kyte–Doolittle hydrophilicity plot (Fig. 1C). All described features are common to other cell-wall proteins found in other fungal species (Lipke et al., 1989; Lu et al., 1994; Moukadiri et al., 1997; Roy et al., 1991; Teunissen et al., 1993; van der Vaart et al., 1995). The difference between the predicted size of CaSsr1p (22.5 kDa) and that deduced from the mobility in SDS-PAGE (70 kDa) could be accounted for by O-glycosylation and GPI modification (Fig. 6).

**Construction of a CaSSR1 null mutant**

To investigate the function of the CaSsr1p protein, construction of null mutants by targeted gene disruption and analysis of the resulting phenotype was carried out. Disruption of the CaSSR1 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 297 bp of the CaSSR1 ORF. A linear HindIII–SacI fragment including the cassette flanked by CaSSR1 sequences was used to transform *C. albicans* CAI4 to Ura⁺.

Twelve of the resulting Ura⁺ transformants were analysed and 10 of them contained the desired insert at the CaSSR1 locus (data not shown). Southern blot analysis of a representative isolate, *C. albicans* 30541-1, after digestion with BglII, revealed that the cassette had integrated in the allele contained in a 3.08 kb BglII fragment originating a 7.08 kb fragment (Fig. 3); this is consistent with the replacement of one allele of CaSSR1 with the transforming DNA. The 3.08 kb BglII fragment corresponds to the other allele which was still present in the Ura⁺ transformants. Ura⁻ segregants of *C. albicans* 30541-1 were selected on medium containing 5-fluoro-orotic acid (Boeke et al., 1984) and examined by Southern blot analysis. Nine of the 12 independent segregants examined had undergone intrachromosomal recombination between the hisG repeats, resulting in the excision of the URA3 marker and one copy of hisG, whereas three of them had experienced an interchromosomal recombination event, reverting to the parental genotype (data not shown). Southern blot analysis of a representative intrachromosomal recombinant, *C. albicans* 30541-2, is shown in Fig. 3. The 7.08 kb BglII fragment seen in *C. albicans* 30541-1 was absent, and a new band of 3.8 kb was present; this size corresponds to the correct intrachromosomal event.

The homozygous Cassr1 null mutant was generated after transformation of *C. albicans* 30541-2 with the same

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**Fig. 2.** Northern blot analysis of total *C. albicans* RNA isolated from cells growing as yeast (Y) or mycelium (M). Total RNA was electrophoresed through an agarose/formaldehyde gel and transferred to a nylon membrane. The blotted membrane was hybridized with DIG-labelled DNA probe (amplicons IAE12 and IAE34). The corresponding ethidium bromide-stained gel is shown on the right and the position of rRNA (18S and 28S) is indicated by arrowheads. The arrowhead on the left marks the 0.8 kb transcript.

**Fig. 3.** Disruption of the *C. albicans* CaSSR1 locus. Southern blot analysis of genomic DNAs from CAI4, 30541-1, 30541-2, 30542-1 and 30542-2 (see Table 1 for genotype details) digested with BglII and hybridized with a DIG-labelled DNA probe (amplicons IAE12 and IAE34). The wild-type and disrupted alleles are shown on the left.
Genomic DNA was isolated from transformants and screened by Southern blot analysis of a representative Ura\(^+\) isolate that exhibited a hybridization pattern consistent with targeting of the previously undisrupted allele, \textit{C. albicans} 30542-1, as shown in Fig. 3. The parental 3·08 kb \textit{BglII} fragment was absent, a 7·08 kb fragment appearing instead, indicating a correct integration. \textit{C. albicans} 30542-1 was plated on 5-fluoro-orotic-acid-containing medium to select Ura\(^-\) segregants. Five Ura\(^-\) segregant isolates were screened by Southern blot analysis and each exhibited a single 3·8 kb \textit{BglII} fragment that hybridized with the probe. Northern blot analysis demonstrated that no mRNA was present in RNA samples from the null mutant \textit{C. albicans} 30542-2 (data not shown).

**Characterization of CaSSR1 mutants**

Proof that \textit{CaSSR1} encodes a cell-wall protein was obtained after the disruption of the two alleles of \textit{CaSSR1} and comparison of the zymolyase extracts from parental and null mutant strains cell walls by Western blot analysis with PAbL rabbit antibodies. As shown in Fig. 6, the antibody recognizes a 70 kDa species in the parental strain but not in the null mutant. Therefore, it was concluded that \textit{CaSSR1} encodes a cell-wall protein that is bound to \(\beta\)-glucan. No differences were observed in the protein patterns of the \(\beta\)-mercaptoethanol extract, SDS extract and spent medium (data not shown).

Phenotypic analyses of the mutants in comparison with their parental strain were performed. The specific growth rates of the yeast form of the parental strain \textit{C. albicans} CAI4 and the Ura\(^-\) strains 30541-2 and 30542-2 were similar and no differences in morphology were observed, as in \textit{S. cerevisiae ssr1} (Moukadiri et al., 1997). No differences in the kinetics of germ tube formation were observed between the mutants and the parental strain. Changes in the cell wall were studied by testing the sensitivities of the mutants to CW, CR and zymolyase, as described by van der Vaart et al. (1995) for \textit{S. cerevisiae}. Sensitivities to CW and CR did increase with respect to parental strain (Fig. 4) and the sensitivity to zymolyase was also increased in the null mutant (Fig. 5). These results suggest that the lack of

![Fig. 4](image)

**Fig. 4.** Sensitivities to CW (A) and CR (B) of the parental, heterozygous (\(\text{Cas}_{1}\Delta/\text{CaSSR1}\)) 30541-2 and homozygous (\(\text{Cas}_{1}\Delta/\text{Cas}_{1}\Delta\)) 30542-2 strains. Cells were grown in YPD medium and a 1/10 dilution series of each strain was inoculated on to SD medium containing the indicated amounts (in \(\mu\)g ml\(^{-1}\)) of CW or CR.

![Fig. 5](image)

**Fig. 5.** Sensitivities to zymolyase of the parental strain (○), null mutant (△) and overexpressing strain (■). Exponentially growing cells were incubated in 50 \(\mu\)g zymolyase 20T ml\(^{-1}\) and the decreases in the optical density were monitored.
Overexpression of CaSsr1p leads to a defective cell wall, as happens in S. cerevisiae ssr1 (Moukadiri et al., 1997).

Study of the CaSSR1-overexpressing strain

Overexpression of CaSSR1 was achieved by subcloning an amplicon containing CaSSR1 in a pADH episomal vector (Bertram et al., 1996). Overexpression was confirmed by Western blot analysis of zymolyase extracts (Fig. 6). Neither changes in morphology nor growth rates of cells were observed; however, the behaviour of the overexpressing strain was very similar to the null mutant. Overexpression of CaSSR1 affected the sensitivity of these cells to zymolyase, they being more sensitive than the parental strain, in a similar way to the null mutant (Fig. 5). These results could indicate that any deviation from the optimal amounts of CaSsr1p in the cell wall makes these cells slightly more sensitive to drugs that interfere with the assembly of cell-wall polymers.

DISCUSSION

Following a sequence-dependent approach to identify cell-wall ORFs by screening the genome database of C. albicans, we found the prospective protein code for one ORF (CaSSR1) that has 62 % homology with Ssr1p of S. cerevisiae, an internal cell-wall protein, which is β-glucanase-extractable (originally named 1cwp; Moukadiri et al., 1997). Analytical determinations demonstrated that CaSSR1 was expressed in C. albicans growing with yeast or mycelial morphology.

The results obtained revealed that CaSSR1 encodes a cell-wall protein: (i) its disruption leads to the absence of a 70 kDa protein band in the material released from isolated cell walls by zymolyase (Fig. 6); (ii) overexpression of this gene leads to an increase in the amount of the 70 kDa protein band (Fig. 6); and (iii) the deduced amino acid sequence presents the typical characteristics of a cell-wall protein, i.e. presence of a cleavable signal peptide, richness in Ser and Thr amino acids susceptible to O-glycosylation, as well as the probability of being modified by GPI addition.

The theoretical molecular mass of mature CaSsr1p is 20-2 kDa but in SDS-PAGE the protein appears with an apparent mobility of 70 kDa. This experimental mobility could be due to O-glycosylations and/or GPI-addition modifications. This anomalous electrophoretic behaviour has also been reported for other Ser- and Thr-rich cell-wall proteins (Lu et al., 1994; Moukadiri et al., 1997; van der Vaart et al., 1995). In S. cerevisiae, Ssr1p (Icw) has an apparent mass of 140 kDa in SDS-PAGE, whereas in C. albicans its size is 70 kDa. This difference in size may be due to the fact that ScSsr1p has an N-glycosylation site (Moukadiri et al., 1997), whereas this is lacking in CaSsr1p. As a consequence, ScSsr1p appears as a poly-disperse band after extraction by zymolyase, whilst CaSsr1p does not. This polydispersity could be conferred by the size of the different mannan chains and by the presence of glucan side chains attached to the mannoprotein moiety of certain β-glucanase-extractable cell-wall proteins (Montijn et al., 1994; van der Vaart et al., 1995). CaSsr1p shares some common features with ScSsr1p, such as an abundance of Ser and Thr residues susceptible to O-glycosylation and a putative GPI-attachment site, which could play a role in the covalent linkage to the glucan network.

To obtain some information about the possible function of CaSsr1p, we analysed the phenotype of Cassr1Δ mutants and the overexpression of CaSsr1p. Both the null mutant and the overexpressing strain behaved similarly to the parental strain with respect to growth rates, morphology and kinetics of germ tube formation, indicating that CaSsr1p is not absolutely necessary for viability in cells growing under the experimental conditions and for the dimorphic transition. CW and CR are compounds that interfere with the assembly of polymers in the cell wall (Elorza et al., 1983; Kopecka & Gabriel, 1992) and they have been used for detecting S. cerevisiae cell-wall mutants (Ram et al., 1994; van der Vaart et al., 1995); when both the Cassr1Δ null mutant and an overexpressing strain were grown in media containing either CW or CR, an increased sensitivity was observed when compared to the parental strain. This result suggests that in a normal cell-wall structure CaSsr1p must be present in an adequate concentration with respect to the other cell-wall components, and that any deviation from this concentration causes a change in the architecture of that structure as in S. cerevisiae (Moukadiri et al., 1997).

Sensitivity to zymolyase has also been used to find cell-wall defects (van der Vaart et al., 1995). In the present work, depletion of CaSsr1p caused an increase in sensitivity to zymolyase, as happens in S. cerevisiae (Moukadiri et al., 1997); however, overabundance of Ssr1p in S. cerevisiae does not induce changes in sensitivity when compared with the parental strain, whereas in C. albicans the overexpression of CaSSR1 renders the cells as sensitive to zymolyase as the null
mutant. These results suggest that this increased sensitivity should be related to changes in the structure of the glucan network rather than a decrease in the thickness of the outer layer of the cell wall, as suggested by van der Vaart et al. (1995) for Cwp2p in *S. cerevisiae*.

We do not know whether CaSsr1p forms disulphide bridges with other cell-wall proteins, but if that is the case, the protein is also bound to β-glucan because it is not released by reducing agents (i.e. β-mercaptoethanol).

Further studies with modified versions of CaSsr1p should provide insights into the organization and structure of the *C. albicans* cell wall.

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**REFERENCES**


Montero, M., Marcilla, A., Sentandreu, R. & Valentín, E. (1998). A *Candida albicans* 37 kDa polypeptide with homology to the laminin...
receptor is a component of the translational machinery. Microbiology 144, 839–847.


