Cloning and sequencing of the *Candida albicans* homologue of *SRB1/PSA1/VIG9*, the essential gene encoding GDP-mannose pyrophosphorylase in *Saccharomyces cerevisiae*

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Two genomic fragments have been isolated from *Candida albicans* which strongly hybridize to *SRB1/PSA1/VIG9*, an essential gene which encodes GDP-mannose pyrophosphorylase in *Saccharomyces cerevisiae*. A common 2.5 kb *Xba*I–*Pst*II fragment has been identified, which Southern analysis suggests is most likely unique in the *C. albicans* genome. The fragment contains an ORF, which is 82% identical and 90% homologous to the *Srb1p/Psa1p/Vig9p* from *S. cerevisiae*, contains one additional amino acid at position 254 and is able to functionally complement the major phenotypic characteristics of *S. cerevisiae* *srb1* null and conditional mutations. The authors therefore conclude that they have cloned and sequenced from *C. albicans* the *bona fide* homologue of *SRB1/PSA1/VIG9*, named hereafter *CaSRB1*. Northern analysis data indicate that the gene is expressed in *C. albicans* under conditions of growth in the yeast and hyphal form and suggest that its expression might be regulated.

Keywords: *SRB1/PSA1/VIG9*, glycosylation, GDP-mannose, *Candida albicans*, cell wall

INTRODUCTION

Yeast cells, unlike mammalian cells, are surrounded by a thick cell wall, which is a particularly attractive target for antifungal drugs (Debono & Gordee, 1994). The yeast cell wall consists of three major components – glucans, mannans and chitin – and the structural features and interactions of these components are relatively well understood (Klis, 1994). In recent years two MAP kinase signalling pathways (*PKC1* and *HOG1*) and the Ras/cAMP dependent pathway have been shown to regulate cell wall integrity in *Saccharomyces cerevisiae* (Cid *et al.*, 1995; G. Tomlin, S. G. Oliver & L. I. Stateva, unpublished results).

The cell wall performs a number of functions, the most important of which are cell shape determination, regulation of permeability, protection against damaging

extracellular agents and, in pathogenic species like *Candida albicans*, expression of virulence properties. The cell wall is a highly dynamic organelle which can undergo modifications to its composition and structural features in response to both developmental and environmental signals. Significant variations have been reported for mannoproteins in particular. In *S. cerevisiae*, differences in mannoprotein synthesis during development of the bud and that of mature cells result in differences in permeability (de Nobel *et al.*, 1991). Mannoprotein composition and structure are also very different at the shmoo projection, which develops in response to pheromones (Lipke *et al.*, 1976; Herrero *et al.*, 1985). In *C. albicans*, cell wall mannoproteins specific only for the hyphal form have been described (Elorza *et al.*, 1985; Casanova *et al.*, 1989). For these reasons the mannoproteins, their synthesis and regulation, are a good target for the development of selective antifungal drugs. Mannans are synthesized by an elaborate mechanism of post-translational modifications carried out during passage through the secretory pathway (Lehle & Tanner, 1995; Herscovics & Orlean, 1993) and all of these (*N*-, *O*-linked glycosylation and

Abbreviations: MAP kinase, mitogen-activated protein kinase; YAC, yeast artificial chromosome.

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GPI-anchor synthesis) require a common substrate – GDP-mannose. Its synthesis is, therefore, of key importance in the biogenesis of a functional cell envelope in yeast.

The gene which encodes the GDP-mannose pyrophosphorylase in *S. cerevisiae* is an essential, cell-cycle-regulated gene. It has been independently isolated as a multicopy suppressor of both *alg1-PSA1* (Albright & Robbins, 1990; Benton *et al.*, 1996) and *dpm1* (Orlean *et al.*, 1988; J. Schultz & G. Sprague, unpublished data in the SGD database), as the *bona fide* *SRB1* (G. Tomlin, S. G. Oliver & L. I. Stateva, unpublished results) and *VIG9* (Hashimoto *et al.*, 1997). The existing mutant alleles of the *SRB1* gene produce highly pleiotropic cell wall integrity defects: dependence for growth upon the presence of osmotic stabilizers in the medium, lysis upon osmotic shock (Venkov *et al.*, 1974) and high competence for DNA transformation, including that with large YACs (Heale *et al.*, 1994). Repression of *SRB1/PSA1* expression reveals additional phenotypes suggesting that the gene is also required for bud-site selection and cytokinesis (Zhang, 1997). A number of structural and compositional differences in the major cell wall components glucan and mannan have also been reported (Maerkisch *et al.*, 1983; Blagoeva *et al.*, 1991). Mutations in *SRB1* (*srb1-1*) are synthetically lethal with *pkc1*, but cannot be rescued by overexpression of either *PKC1*, or any of the downstream components of the *PKC1*-regulated MAP kinase signal transduction pathway (Cid *et al.*, 1995). However, overexpression of *PDE2* (the gene encoding the high-affinity cAMP phosphodiesterase: Sass *et al.*, 1986) restores the wild-type phenotype of *srb1-1* mutants (G. Tomlin, S. G. Oliver & L. I. Stateva, unpublished results).

In the present paper the cloning and molecular characterization of the *SRB1/PSA1* homologue from *C. albicans* is reported. Southern analysis shows that it is unique in the *C. albicans* genome. Sequence determination indicates that it encodes a highly conserved protein, which is 82% identical and 90% homologous to its *S. cerevisiae* counterpart. The potential role of this gene in the dimorphic switch of *C. albicans* is discussed on the basis of the results of the Northern analysis of its expression under conditions of hyphal induction by serum or changes in pH.

METHODS

Strains and media. Strains of *Saccharomyces cerevisiae*, *Candida albicans* and *Escherichia coli* used in this study are listed in Table 1. Media to support the growth of *S. cerevisiae* and *C. albicans* (rich YEPD and minimal SD, containing YNB, glucose and appropriate supplements) were prepared according to Sherman *et al.* (1986). All types of media for the propagation of *srb1-1* fragile mutants were supplemented with 10% (w/v) sorbitol. Minimal medium for pH hyphal induction in *C. albicans* was prepared essentially as described by Lee *et al.* (1975). Bacterial strains were grown in LB and LB with ampicillin, as described by Sambrook *et al.* (1989).

Yeast library and plasmids. The *C. albicans* genomic library was kindly provided by Dr S. Scherer, University of

Minnesota, USA. It was constructed using the fosmid pFOS1 (Kim *et al.*, 1992) and fragments of *Sau3A*I partially digested genomic DNA from *C. albicans* 1161. The 1920 clones obtained were transferred to Hybond-N⁺ membranes (Amersham Pharmacia Biotech). These membranes were used for hybridization with a labelled *S. cerevisiae* *SRB1/PSA1* 0.8 kb *EcoRI*–*Bgl*II DNA fragment, isolated from plasmid *SRB1-9b* (kindly provided by G. Tomlin). All plasmids used in this study are listed in Table 1.

DNA preparation and manipulations. Yeast genomic DNA was isolated from spheroplasts (Davis *et al.*, 1980). Bacterial plasmid DNA, on both a small (1 ml) and a large (50–100 ml) scale, was isolated using the alkaline-lysis method of Birnboim & Doly (1979), as described by Sambrook *et al.* (1989). DNA was purified either by CsCl gradient centrifugation (Sambrook *et al.*, 1989) or by a Qiagen QUA filter plasmid Midi kit. All DNA fragments used for subcloning were gel-purified by the method of Heery *et al.* (1990). Enzymic reactions with restriction enzymes (Boehringer), T₄ ligase (Boehringer) and *Taq* DNA polymerase (Amersham Pharmacia Biotech) were all performed according to the recommendations of the manufacturers. For Southern analysis, the procedure recommended by Amersham Pharmacia Biotech was followed. All DNA probes used for hybridization were labelled with the Rediprime kit and Redivue [α -³²P]dCTP (10 mCi ml⁻¹, 1 Ci = 3.7 × 10¹⁰ Bq) from Amersham Pharmacia Biotech.

Hyphal induction, RNA extraction and Northern blotting.

Hyphal induction by serum and pH was achieved essentially by the methods of Bailey *et al.* (1996) and Sundstrom *et al.* (1990), respectively. The control alternative experiments, where the strains were growing only in the yeast form, were performed in both rich (YEPD) and minimal (Lee's medium, pH 4.5) medium at 25 °C after an initial 2 h starvation in water for synchronization of growth. The cell cycle phase of the cultures was determined by counting the budded and unbudded cells in the course of the experiments. Samples were withdrawn at various times (0.5, 1, 2, 3, 4 and 6 h) after hyphal induction (either method) and during the control experiments, and used for RNA extraction. Total RNA, isolated as described by Brown (1994), was denatured in formaldehyde/formamide, and after electrophoresis under denaturing conditions, transferred to Hybond-N membranes (Amersham Pharmacia Biotech). The membranes were treated and hybridized to labelled DNA probes, as recommended by the manufacturers. After hybridization, they were exposed to a Bio-Rad imaging screen, developed with the Bio-Rad phosphorimager (GS-363) and analysed with Molecular Analyser software. As an alternative probe for loading and transfer of the total RNA, the gene *TEF3* (Swoboda *et al.*, 1994) was used. However, *CaSRB1* mRNA levels were quantified relative to the rRNAs, loading approximately equal amounts and measuring the ethidium bromide staining intensity in each lane. The levels of expression at given time points were determined relative to that at 30 min after the start of the growth under all conditions.

DNA sequence analysis. The method employed for sequencing was essentially as described by James *et al.* (1995). Nucleotide sequence analyses and homology searches were performed using both the BLAST search program (<http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast>) and the CLUSTAL W (1.60) Multiple Sequence Alignment program (<http://www.genome.ad.jp/SIT/SIT.html>).

Genetic methods. The concomitant loss of plasmid markers and the mitotic stability of transformed strains were tested by growing the cultures to the stationary phase in rich (YEPD)

Table 1. Strains and plasmids used in this study

Strain	Relevant genotype or details	Source/reference
<i>S. cerevisiae</i>		
7SLU	<i>MATα srb1-1 leu2 ura3</i>	Stateva <i>et al.</i> (1991)
AH22	<i>MATα leu2-3, leu2-112 his4-519 can1</i>	UMIST collection
20hPS1-2d MET3-PSA1	<i>MATα leu2 ura3 trp1 ade1 his2 psa1::TRP1 pURA3/MET3-PSA1</i>	Zhang (1997)
<i>C. albicans</i>		
SC5314	Wild-type	Gillum <i>et al.</i> (1984)
CAI4	Δ <i>ura3::imm434</i> / Δ <i>ura3::imm434</i>	Fonzi & Irwin (1993)
<i>E. coli</i>		
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proABlacI</i> ^q ZAM15 Tn10(Tet ^R)]	Bullock <i>et al.</i> (1987)
Plasmids		
pUC19	<i>E. coli</i> vector	Yanisch-Perron <i>et al.</i> (1985)
pRS415	CEN-ARS-LEU2	Sikorski & Hieter (1989)
SRB1-4	YCp50 based, SRB1-2 with the <i>Clal</i> fragment removed	G. Tomlin, Dept of Biomolecular Sciences, UMIST
SRB1-9b	<i>Clal</i> – <i>Bam</i> HI fragment of SRB1 in pRS316	G. Tomlin, Dept of Biomolecular Sciences, UMIST
pA301	<i>Eco</i> RI– <i>Bam</i> HI fragment of <i>S. cerevisiae</i> ACT1	J. Rosamond, University of Manchester, UK

medium containing 10% sorbitol at 30 °C and plating out aliquots for single colonies on plates of the same medium. After incubation at 30 °C the colonies were replica-plated to selective media. Sorbitol dependence of the *S. cerevisiae* strain 7SLU (Table 1) and transformants, containing *C. albicans* SRB1/PSA1 putative homologues, was tested by comparing the growth of single colonies on minimal media with and without 10% sorbitol, in the presence of the appropriate nutritional supplements.

Cultures of strains containing the pMET3-regulated SRB1/PSA1 cassette were grown in selective SD medium at 30 °C with vigorous shaking until the early exponential phase (OD₆₀₀ 0.05), at which point they were split into two halves. To one half of each culture, methionine was added to a final concentration of 2 mM; to the other half, an equivalent volume of distilled water was added. The two cultures were then incubated further under the same conditions as before, until stationary phase.

Lysis test. The standard procedure, described by Stateva *et al.* (1991), was followed. All tests were performed after growth of the strains in selective minimal media to ensure maximum stability of the transforming plasmids.

Transformation. *E. coli* XL-1 Blue was transformed either by the calcium chloride method or by the DMSO method, as described by Sambrook *et al.* (1989). Yeast strains were transformed either by a modification of the lithium procedure of Ito *et al.* (1983), as described by Stateva *et al.* (1991) or by the DMSO enhanced procedure developed by Hill *et al.* (1991).

PCR. The SRB1 forward (5'-AGCTCAGAGGTCACTATACG-3') and reverse (5'-CGGGATCCAATACTACAGACAT-

TGATAGCC-3') primers were used to amplify the *S. cerevisiae* SRB1/PSA1. In 50 µl of PCR reaction, 5 ng DNA template (SRB1-4) was mixed with 200 ng of each primer, 1.5 mM MgCl₂, 200 µM dNTPs, 0.5 unit *Taq* polymerase (Boeringer) and PCR buffer. The PCR amplification was run for 30 cycles (94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min 15 s).

RESULTS

The SRB1/PSA1/VIG9 putative homologue from *C. albicans* is unique

In *S. cerevisiae* the gene SRB1/PSA1 is essential for growth. Speculating that SRB1/PSA1 might be a highly conserved gene among yeast species, it was decided to attempt the isolation of a *C. albicans* homologue by DNA sequence hybridization with an ordered fosmid library, using the internal 0.8 kb *Eco*RI–*Bgl*II fragment from the *S. cerevisiae* SRB1/PSA1 gene as a probe. After two consecutive rounds of screening, two strongly hybridizing clones (designated A and B) were selected for further analysis. They were shown to have inserts of around 45 kb (data not shown) with a common 6 kb *Xba*I fragment. At this initial stage of characterization two polymorphisms were observed in the restriction patterns of the two recombinant clones. Clone A contained an *Eco*RI site, absent from clone B, whilst clone B had a *Pst*I site, not found in clone A. Subcloning experiments defined a 2.5 kb *Xba*I–*Pst*I fragment as the smallest uniquely hybridizing region of the *C. albicans*

Fig. 1. Southern analysis. (a) Restriction map of the *C. albicans* probes. (b) Total genomic DNAs of *S. cerevisiae* AH22 (lane 1) and *C. albicans* SC5314 and CA14 (lanes 2 and 3, respectively) were digested with *Xba*I, *Xba*I/*Pst*I and *Hind*III and hybridized with *S. cerevisiae* *SRB1* probe (I), and with the 2.5 kb *Xba*I-*Pst*I fragments from clones A and B (II and III, respectively).

DNA inserts and this was used in subsequent experiments.

Southern analysis was used to check whether the putative *SRB1*/*PSA1* homologue from clones A and B defined unique sequences in the *Candida* genome. Total genomic DNA was isolated from two *C. albicans* strains, CA14 and SC5314, and digested with two enzymes without cleavage sites in the *SRB1*/*PSA1* gene of *S. cerevisiae* (*Xba*I and *Hind*III) and one enzyme with an internal cleavage site (*Pst*I). Genomic DNA from *S. cerevisiae* AH22 was similarly treated. The 2.5 kb *Xba*I-*Pst*I fragments from *C. albicans* clones A and B, and a PCR-amplified fragment of the *S. cerevisiae* *SRB1* gene, were used as probes. The results of the Southern analysis with the three different hybridization probes, together with the restriction map of the *Xba*I-*Pst*I fragment from *C. albicans* generated on the basis of the preliminary cloning experiments, are presented in Fig. 1. In all three cases, the signals from the homologous probing experiments were stronger [with *S. cerevisiae* DNA in Fig. 1(bI), and with *C. albicans* DNAs in Fig. 1(bII and bIII)], than those from the heterologous

probing. The hybridization pattern of the *S. cerevisiae* DNA, seen best in Fig. 1(bI), confirmed the presence of all the expected fragments. The patterns of the two strains of *C. albicans*, which were identical to each other, were significantly different from that of the *S. cerevisiae* strain: note the different *Pst*I/*Xba*I and *Hind*III patterns. However, they verified the restriction map (Fig. 1a) of the cloned *C. albicans* fragment, which has unique sites for *Xba*I and *Pst*I, and two sites for *Hind*III. There are one or two very faint additional bands which must contain regions of limited similarity; however, given the intensity of the main bands, it was concluded that, like the *SRB1*/*PSA1* gene in *S. cerevisiae*, its putative homologue from *C. albicans* is most likely also unique.

Functional complementation of *srb1-1*-associated phenotypes with the *C. albicans* *SRB1*/*PSA1* homologue

Mutations of *SRB1* are highly pleiotropic in their phenotypic effects, with the most characteristic features of *srb1-1* being dependence on osmotic stabilizers for

Fig. 2. Functional analysis test. The *srb1-1* mutant 7SLU was transformed with centromeric plasmids (for description of constructs see Methods) containing *CaSRB1* either from clone A (TRA A1 and TRA A2) or clone B (TRA B1 and TRA B2). For comparison the mutant 7SLU, as well as a transformant carrying the *S. cerevisiae* *SRB1/PSA1* gene (7SLU *ScSRB1*), are also included on the same plate, which contained minimal medium without sorbitol.

growth and lysis upon osmotic shock. These phenotypes were chosen for the functional complementation test. The *S. cerevisiae* *srb1-1* mutant strain (7SLU) was transformed with pRS415 only (Sikorski & Hieter, 1989), and with recombinant plasmids carrying either the clone A or the clone B 2.5 kb *XbaI*–*PstI* fragment. One hundred transformants for each recombinant plasmid were tested and, unlike the *srb1-1* mutant 7SLU, and the strain containing only pRS415, they were all able to grow without the addition of 10% sorbitol to the medium (examples are shown in Fig. 2). A mitotic stability test was performed on three transformants of each type. It confirmed that their newly acquired feature co-segregated in all cases with the plasmid-borne nutritional marker *LEU2*.

The ability of the putative *C. albicans* *SRB1* homologues to complement the lysis phenotype of the *srb1-1* mutant was also tested. The standard lysis test, as described in Methods, was performed with two different transformants of each type and the original mutant. All strains were grown in minimal selective media for maximum stability of the transforming plasmids. The results indicated clearly that the putative *C. albicans* *SRB1* homologues relieve the osmotic shock sensitivity of *S. cerevisiae* *srb1-1* mutants (data not shown).

To rule out the possibility of allele-specific complementation, the same recombinant plasmids were also transformed into the *srb1* null mutant, 20hPS1-2d, modified by Zhang (1997); see Table 1 for genotype. The strain contains a wild-type copy of the *SRB1/PSA1* gene under the control of the tightly regulated *MET3* promoter (Mountain *et al.*, 1991). As a result, the strain grows as wild-type in the absence of methionine, but the cells quickly stop growing and lose viability upon addition of 2 mM methionine to the medium (Zhang, 1997). Transformants of this strain carrying the putative *C. albicans* *SRB1/PSA1* homologues were able to grow normally in methionine-supplemented medium and reached cell densities comparable to those of the control cultures without methionine (Table 2).

On the basis of these results, it was concluded that the two *C. albicans* clones A and B contain a common 2.5 kb *XbaI*–*PstI* fragment, which not only uniquely hybridizes to the *S. cerevisiae* *SRB1* gene and to *C. albicans* genomic DNA, but is also able to functionally comp-

Table 2. Complementation of the lethality of *srb1* null mutants by *C. albicans* *SRB1* homologues

The results are means of three independent experiments $\pm$ SD.

Strain	OD ₆₀₀	
	Minus methionine	Plus methionine
Control*	1.2 $\pm$ 0.05	0.3 $\pm$ 0.03
TRA A1†	1.2 $\pm$ 0.03	1.2 $\pm$ 0.06
TRA A2†	1.18 $\pm$ 0.07	1.28 $\pm$ 0.01
TRA A3†	1.22 $\pm$ 0.03	1.11 $\pm$ 0.05
TRA B1‡	1.02 $\pm$ 0.04	1.22 $\pm$ 0.05
TRA B2‡	1.18 $\pm$ 0.01	1.01 $\pm$ 0.08
TRA B3‡	1.22 $\pm$ 0.02	1.05 $\pm$ 0.04

* Strain 20hPS1-2d *MET3-PSA1* (see Table 1 for relevant genotype) was used as a control.

† TRA A1, 2 and 3 are three independent transformants of 20hPS1-2d *MET3-PSA1* obtained with the *C. albicans* *SRB1/PSA1* homologue isolated from clone A.

‡ TRA B1, 2 and 3 are three independent transformants of 20hPS1-2d *MET3-PSA1* obtained with the *C. albicans* *SRB1/PSA1* homologue isolated from clone B.

lement the major phenotypic characteristics of two different *srb1* mutant alleles.

Whilst working with the *S. cerevisiae* 7SLU *srb1-1* transformants, carrying the putative *SRB1* *C. albicans* homologues, an additional phenotype was observed. The transformants carrying the recombinant plasmids, but not the cloning vector, lost viability very rapidly in stationary phase, irrespective of the type of medium (rich or minimal), or the temperature (4, 22 or 30 °C). In all cases tested, the percentage of viable cells decreased very rapidly and was almost zero after only 3 d (data not shown).

DNA sequence and predicted ORF of the *SRB1/PSA1* homologue from *C. albicans* (*CaSRB1*)

The sequence of the *SRB1/PSA1* homologues represented by both clones A and B was determined, as described in Methods. A single ORF, encoding a putative

	10	20	30	40	50	
Ca.Ap	MKGLILVGGYGTRLRPLTLTLPKPLVEFGNRP	MILHQIEALAAAGVTDIVLAVNYRPEVM	60			
Ca.Bp	MKGLILVGGYGTRLRPLTLTLPKPLVEFGNRP	MILHQIEALAAAGVTDIVLAVNYRPEVM				
Sc.Srb1p	MKGLILVGGYGTRLRPLTLTVPKPLVEFGNRP	MILHQIEALANAGVTDIVLAVNYRPEVM				
	70	80	90	100	110	
Ca.Ap	VSTLKKYE	EEYGV	SITFSV	EEPLGTAGPLKLAEEVLKDDSPFFVLNSDVICDYPFKEL	120	
Ca.Bp	VSTLKKYE	EEYGV	SITFSV	EEPLGTAGPLKLAEEVLKDDSPFFVLNSDVICDYPFKEL		
Sc.Srb1p	VE	TLKKYE	KEYGV	NITFSV	ETEPLGTAGPLKLAEDVLKDDNSPFFVLNSDVICDYPFKEL	
	130	140	150	160	170	
Ca.Ap	ADFHKAHGA	AAGTIVATKVDEPSKYGVIVH	DRDTPNLIDRFVEKPV	EFVGNRINAGLYILN	180	
Ca.Bp	ADFHKAHGA	AAGTIVATKVDEPSKYGVIVH	DRDTPNLIDRFVEKPV	EFVGNRINAGLYILN		
Sc.Srb1p	ADFHKAHGG	KG	TIVATKVDEPSKYGVIVH	DIATPNLIDRFVEKPK	EFVGNRINAGLYILN	
	190	200	210	220	230	
Ca.Ap	PSVIDLIEMRPTSIEKDP	FPILVEQKQLYSFDLEGYWM	DVGQPKDFLSGTCLYLTSLSKK	240		
Ca.Bp	PSVIDLIEMRPTSIEKDP	FPILVEQKQLYSFDLEGYWM	DVGQPKDFLSGTCLYLTSLSKK			
Sc.Srb1p	PEVIDLIEMKPTSIEKET	FPILVEEKQLYSFDLEGFW	MDVGQPKDFLSGTCLYLTSLSKK			
	250	260	270	280	290	
Ca.Ap	HPEKLC	KEKYV	HGGNVLIDPTAKIHPS	SALIGPNVTIGPNV	VVGEGAR	ICQ
Ca.Bp	HPEKLC	KEKYV	HGGNVLIDPTAKIHPS	SALIGPNVTIGPNV	VVGEGAR	ICQ
Sc.Srb1p	QPKK	LATGANIVG	NALIDPTAKISS	TAKIGPDV	VIGPNVTIGDGV	RTIRSVVLCNSTIK
	310	320	330	340	350	
Ca.Ap	DHAWVKSTIVGWN	SRIGKWART	EGVTVLGD	DDVQVKNEIYVNGAKVLP	PKHSISSNVEKESI	360
Ca.Bp	DHAWVKSTIVGWN	SRIGKWART	EGVTVLGD	DDLQVKNEIYVNGAKVLP	PKHSISSNVEKESI	
Sc.Srb1p	NHSLVKSTIVGWN	STVGQWCR	LEGVTVLGD	DEVKDEIYINGGK	VLPKHSISSDNVPKEAI	
Ca.Ap	IM	362				
Ca.Bp	IM					
Sc.Srb1p	IM					
	**					

Fig. 3. Comparison of the deduced amino acid sequences of the two putative *C. albicans* SRB1/PSA1 (Ca.Ap and Ca.Bp) and *S. cerevisiae* SRB1/PSA1 (Sc.Srb1p). Amino acids given in bold correspond to differences, asterisks to identities and dots to similarities in the deduced *C. albicans* and *S. cerevisiae* protein sequences. The amino acids in boxes (positions 254 and 288) correspond to an insertion in the *Candida* protein in comparison to the *S. cerevisiae* counterpart, and to the only difference between the two *C. albicans* protein sequences, respectively.

product of 362 amino acids, was identified. The first ATG of this ORF is 77 bp downstream from a TATAA box. The 3' region of the gene contains two in-frame stop codons 21 nucleotides downstream of the final codon and a consensus site for transcription termination (TATG) 137 nucleotides downstream of the final codon. No polyadenylation consensus sequences could be identified within the sequenced regions.

The predicted protein sequence of this new gene (called *CaSRB1*) has an estimated molecular mass of about 40 kDa (39961 Da for clone A; 39989 Da for clone B). A comparison between the protein sequences of the two *Candida* clones, together with that of *S. cerevisiae* Srb1p, is given in Fig. 3. The following points are noteworthy. First, there is only one significant difference between the two *C. albicans* clones: at position 288, the encoded amino acid is Gln in clone A and Arg in clone B. Second, there is a very high percentage of identity (82%) and homology (90%) between the *C. albicans* and *S. cerevisiae* sequences. Third, the amino acid at position 254 in the *C. albicans* sequence is not present in the corresponding position of the *S. cerevisiae* protein.

Fig. 4 shows the hydropathy profile of the predicted *CaSRB1* protein. The profile suggests that, like the *S.*

Fig. 4. Hydropathy profile of the predicted *C. albicans* homologue of SRB1/PSA1 from clone A. The Position scale runs from 0 to 400.

cerevisiae counterpart (Hashimoto *et al.*, 1997), the protein from *C. albicans* is soluble and does not have hydrophobic stretches long enough to be considered potential transmembrane domains or signal sequences for secretion.

Fig. 5. Northern analysis of total mRNA from *C. albicans* SC5314 after induction of hyphae by serum (a) and pH (e), and during growth in the yeast form in rich medium (c). Panels (a), (c) and (e) show, from top to bottom: ethidium bromide staining pattern, hybridization results with probe A, hybridization results with probe B and (a and e only) hybridization with *TEF3* as a probe (T). Times of induction and growth and conditions of controls are shown above the tracks. Panels (b), (d) and (f) show levels of expression after quantification of the Northern results, as described in Methods. Levels at all time points are expressed relative to that taken 30 min after start of experiments.

Northern analysis

The expression of *CaSRB1* was investigated during yeast-to-hyphal transition and also under conditions of yeast-only growth. The dimorphic switch was induced by either addition of serum or pH change, and both combined with a higher growth temperature (37 °C). At a pH of 4.5 and a temperature of 25 °C in minimal medium, and also in rich medium at the same temperature, *C. albicans* grows purely as a yeast. When the pH and the temperature are increased to 6.5 and 37 °C respectively, or when 10% serum is added to the rich

medium at 37 °C, the cells undergo a morphogenic switch, forming first germ tubes, then hyphae. Under the conditions of our experiments, the first germ tubes were clearly visible 30 min after induction (either method) and the hyphal transition was completed after 4 h (data not shown). The pattern of expression of the 'control' probe in our experiments, the gene *TEF3* (Swoboda *et al.*, 1994), was consistent with published results (Swoboda *et al.*, 1994). Since neither actin (Paranjape & Datta, 1991), nor indeed *TEF3* or any other *Candida* gene (Swoboda *et al.*, 1994), could be used as a classic internal loading mRNA control in this experiment,

CaSRB1 mRNA levels were quantified relative to the major rRNA species (see Methods).

Total RNA isolated from *C. albicans* growing in either yeast or hyphal form, as well as during the transition between the two forms, showed one distinct band when probed with the *CaSRB1* DNA (Fig. 5). This result clearly shows that *CaSRB1* is an expressed gene. RNA quantification, however [Fig. 5(b) and (f) for serum and pH induction, and (d) for the yeast-only growth in rich medium], suggests that the gene might be also regulated. The different induction conditions give slightly different results, although both show a high level of expression early after induction and a considerable reduction after hyphal transition (four- to fivefold, 6 h after induction). In the experiments where the transcription levels of *CaSRB1* were determined after growth in the yeast form in rich medium (Fig. 5c, d), and in minimal medium (data not shown), the pattern of expression was different from that observed under conditions of hyphal induction: there was no reduction in expression levels after 6 h. Taken together, these experiments demonstrate that similar to the situation in *S. cerevisiae* (Benton *et al.*, 1996), in *C. albicans* this gene might be also regulated, with levels of expression particularly high during periods of intense cell wall synthesis as in germ tube formation seen during the morphological switch, and bud initiation during growth in the yeast form.

DISCUSSION

We have cloned and sequenced the functional *C. albicans* homologue of *SRB1/PSA1/VIG9*. This gene is unique and essential for growth in *S. cerevisiae*. The data presented here (Fig. 1) suggest that it is most likely unique in the *C. albicans* genome, although at present it is not known whether the gene is also essential for growth in this species.

Two positive clones were identified out of a fosmid library which, according to our data, appear to define the two copies of this gene in the *C. albicans* diploid genome. They have almost identical nucleotide and thus amino acid sequences (Fig. 3). The only significant difference is an amino acid polymorphism at position 288 (Gln in clone A, Arg in clone B). Significantly, however, both copies of the *Candida* gene are able to complement, in *S. cerevisiae*, the characteristic phenotypes conferred by two different *srb1* mutant alleles, including that of the null allele (Fig. 2, Table 2, and lysis data not shown), and have similar patterns of expression under conditions of hyphae induction by pH and serum (Fig. 5). Given that *SRB1/PSA1* is an essential gene for growth in *S. cerevisiae*, the ability of the *C. albicans* homologues to give functional complementation of its null allele is strong evidence that *CaSRB1* indeed encodes GDP-mannose pyrophosphorylase in *C. albicans*.

The proteins encoded by *SRB1/PSA1/VIG9* in *S. cerevisiae* and its functional homologue in *C. albicans* *CaSRB1* appear to be highly conserved. This is shown by the high percentages of identity and homology between

their amino acid sequences (Fig. 3), as well as their almost identical hydropathy profiles (Fig. 4, and Hashimoto *et al.*, 1997). The protein from *C. albicans* contains an additional amino acid (glycine, position 254), not present in its *S. cerevisiae* counterpart, although we have no data on its functional or structural significance. We can only speculate that it might be partially responsible for the low survival of the *S. cerevisiae* 7SLU strains transformed with *CaSRB1*. All the amino acids affected in existing *SRB1* mutant alleles (G. Tomlin, S. G. Oliver & L. I. Stateva, unpublished results) are, however, conserved in the *C. albicans* homologues, giving indirect proof of their functional importance. Interestingly, there appears to be little conservation of the glycosylation capacity of the proteins. Whilst a computer search identified five potential sites for glycosylation in *SRB1/PSA1/VIG9* in *S. cerevisiae*, the same program recognized only one site in *CaSRB1*. However, our functional complementation data indicate that the glycosylation pattern, if different *in vivo*, does not affect the function of the protein in a heterologous system.

Outside the sequence of the ORF there is little similarity between the genes from *S. cerevisiae* and *C. albicans*. The putative regulatory sites are, however, located in a similar position relative to the first and the final codons of the ORF, and the *C. albicans* sequences do appear to be sufficient for function in *S. cerevisiae*, as evidenced by the complementation of different *SRB1* mutant phenotypes. Attempts at complementation of a *C. albicans* *CaSRB1* mutant with the *S. cerevisiae* gene will show whether the opposite is also true, although to date heterologous gene expression in *Candida* has met with little success.

Taken together, all our data suggest strongly that *CaSRB1* is the *C. albicans* gene encoding GDP-mannose pyrophosphorylase and we are currently developing a biochemical analysis to prove this unambiguously.

The gene encoding GDP-mannose pyrophosphorylase in *S. cerevisiae* may be considered as a morphogene, given the plethora of cell-wall-related phenotypes associated with the *srb1-1* mutation (G. Tomlin, S. G. Oliver & L. I. Stateva, unpublished results). There are other microbial mutants with altered morphology which also have defects in genes determining metabolic functions [e.g. glucose-6-phosphate dehydrogenase and phosphoglucomutase in *Neurospora crassa* (Brody & Tatum 1966, 1967); NADP-linked glutamate dehydrogenase and phosphoglucose isomerase in *S. cerevisiae* (Wilkinson *et al.*, 1996; Dickinson & Williams, 1987)]. One conclusion from these data is that many genes make an indirect contribution to morphogenesis acting via a series of pleiotropic effects. In the case of *SRB1/PSA1/VIG9*, these pleiotropic effects are determined by the demand for GDP-mannose, either directly or via the dolichol pathway linking a number of basic cellular processes which contribute to the biogenesis of a functional cell envelope [N- and O-linked glycosylation of proteins (Kukuruzinska *et al.*, 1987; Abeijon &

Hirschberg, 1992; Herscovics & Orlean, 1993); mannosylation of lipids in the Golgi (Lester & Dickson, 1993) and the synthesis of GPI anchors attached to the C-terminus of some proteins (Orlean, 1990)]. This high demand probably accounts for the high levels of expression of the gene in both *S. cerevisiae* and *C. albicans* (our Northern data; Benton *et al.*, 1996; Zhang, 1997). In *S. cerevisiae*, the expression levels of *SRB1/PSA1* vary and are tightly regulated with the cell cycle, with a peak of expression in G1 at START (Benton *et al.*, 1996). Our data indicate that in *C. albicans* as well, the levels of expression of *CaSRB1* are not constant. There are clear changes in mRNA levels following the switch to hyphal growth. Our data also suggest that there might be some periodicity in the rate of mRNA synthesis during growth in the yeast form. It will be interesting to discover how this regulation is achieved. Our future studies on *CaSRB1* mutant strains and/or strains expressing the gene under tightly controlled promoters might provide some answers.

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REFERENCES

- Abeijon, C. & Hirschberg, C. B. (1992). Topography of glycosylation reactions in the endoplasmic reticulum. *Trends Biochem Sci* **17**, 32–36.
- Albright, C. F. & Robbins, P. W. (1990). The sequence and transcript heterogeneity of the yeast gene *ALG1*, an essential mannosyltransferase involved in N-glycosylation. *J Biol Chem* **265**, 7042–7049.
- Bailey, D. A., Feldman, P. J. F., Bovey, M., Gow, N. A. R. & Brown, A. J. P. (1996). The *Candida albicans* *HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J Bacteriol* **178**, 5353–5360.
- Benton, B. K., Plump, S. D., Roos, J., Lennarz, W. & Cross, F. R. (1996). Overexpression of *Saccharomyces cerevisiae* G1 cyclins restores the viability of *alg1* N-glycosylation mutants. *Curr Genet* **29**, 106–113.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.
- Blagoeva, J., Stoyev, G. & Venkov, P. (1991). Glucan structure in a fragile mutant of *Saccharomyces cerevisiae*. *Yeast* **7**, 455–461.
- Brody, S. & Tatum, E. L. (1966). The primary effect of a morphological mutation in *Neurospora crassa*. *Proc Natl Acad Sci USA* **56**, 1290–1297.
- Brody, S. & Tatum, E. L. (1967). Phosphoglucomutase mutants and morphological changes in *Neurospora crassa*. *Proc Natl Acad Sci USA* **58**, 423–430.
- Brown, A. J. P. (1994). RNA extraction and mRNA quantitation in *Candida albicans*. In *Molecular Biology of Pathogenic Fungi: a Laboratory Manual*, pp. 127–134. Edited by B. Maresca & G. S. Kobayashi. New York: Telos Press.
- Bullock, W. O., Fernandez, J. M. & Short, J. M. (1987). XL1-Blue – a high-efficiency plasmid transforming *recA* *Escherichia coli* strain with β -galactosidase selection. *Biotechniques* **5**, 376–378.
- Casanova, M., Gil, M., Cardenoso, L., Martinez, J. P. & Sentandreu, R. (1989). Identification of wall specific antigens synthesised during germ tube formation by *Candida albicans*. *Infect Immun* **57**, 262–271.
- Cid, V. J., Duran, A., delRay, F., Snyder, M. P., Nombela, C. & Sanchez, M. (1995). Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. *Microbiol Rev* **59**, 345–386.
- Debono, M. & Gordee, R. S. (1994). Antibiotics that inhibit fungal cell wall development. *Annu Rev Microbiol* **48**, 471–497.
- Davis, R., Thomas, M., Cameron, J., John, T. S., Sherer, S. & Padget, R. (1980). Rapid DNA isolation for enzymatic and hybridisation analysis. *Methods Enzymol* **65**, 404–411.
- Dickinson, J. R. & Williams, A. S. (1987). The *cdc30* mutation in *Saccharomyces cerevisiae* results in a temperature sensitive isoenzyme of phosphoglucose isomerase. *J Gen Microbiol* **133**, 135–140.
- Elorza, M. V., Murgai, A. & Sentandreu, R. (1985). Dimorphism in *Candida albicans*: contribution of mannoproteins to the architecture of yeast and mycelial cell wall. *J Gen Microbiol* **131**, 2209–2216.
- Fonzi, W. A. & Irwin, M. Y. (1993). Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**, 717–728.
- Gillum, A. M., Tsay, E. Y. H. & Kirsch, D. R. (1984). Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* *ura3* and *E. coli* *pyrF* mutations. *Mol Gen Genet* **198**, 179–182.
- Hashimoto, H., Sakakibara, A., Yamasaki, M. & Yoda, K. (1997). *Saccharomyces cerevisiae* *VIG9* encodes GDP-mannose pyrophosphorylase, which is essential for protein glycosylation. *J Biol Chem* **272**, 16308–16314.
- Heale, S. M., Stateva, L. I. & Oliver, S. G. (1994). Introduction of YACs into intact yeast cells by a procedure which shows low levels of recombination and co-transformation. *Nucleic Acids Res* **22**, 5011–5015.
- Heery, D. M., Cannon, F. & Powel, R. (1990). A simple method for subcloning DNA fragments from gel slices. *Trends Genet* **6**, 173.
- Herrero, E., Valentin, E. & Sentandreu, R. (1985). Effect of alpha-factor on individual wall mannoproteins from *Saccharomyces cerevisiae* a-cells. *FEMS Microbiol Lett* **27**, 293–297.
- Herscovics, A. & Orlean, P. (1993). Glycoprotein biosynthesis in yeast. *FASEB J* **7**, 540–550.
- Hill, J., Ian, K. A., Donald, G. & Griffiths, D. E. (1991). DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res* **19**, 5791.
- Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**, 163–168.
- James, C. M., Indge, K. J. & Oliver, S. G. (1995). DNA sequence analysis of a 35 kb segment from *Saccharomyces cerevisiae* chromosome VII reveals 19 open reading frames including *RAD54*, *ACE1/CUP2*, *PMR1*, *RCK1*, *AMS1* and *CAL1/CDC43*. *Yeast* **11**, 1413–1419.
- Kim, U., Shizuya, H., deJong, P. J., Birren, B. & Simon, M. (1992).

Stable propagation of cosmid sized human DNA inserts in an F factor based vector. *Nucleic Acids Res* **20**, 1083–1085.

Klis, F. M. (1994). Cell wall assembly in yeast. *Yeast* **10**, 851–869.

Kukuruzinska, M. A., Bergh, M. L. E. & Jackson, B. J. (1987). Protein glycosylation in yeast. *Annu Rev Biochem* **56**, 915–944.

Lee, K. L., Buckley, H. R. & Campbell, C. C. (1975). An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia* **13**, 148–153.

Lehle, L. & Tanner, W. (1995). Protein glycosylation in yeast. In *Glycoproteins*, vol. 29a, pp. 475–509. Edited by J. Montreuil, H. Schachter & J. F. G. Vliegthart. Amsterdam: Elsevier.

Lester, R. L. & Dickson, R. C. (1993). Sphingolipids with inositolphosphate-containing head groups. *Adv Lipid Res* **26**, 253–274.

Lipke, P. N., Taylor, A. & Ballou, C. (1976). Morphogenic effects of alpha-factor on *Saccharomyces cerevisiae* a cells. *J Bacteriol* **12**, 610–618.

Maerkisch, U., Reuter, G., Stateva, L. I. & Venkov, P. (1983). Mannan structure analysis of the fragile *Saccharomyces cerevisiae* mutant VY1160. *Int J Biochem* **15**, 1373–1377.

Mountain, H. A., Bystroem, A. S., Larsen, J. T. & Korch, C. (1991). Four major transcriptional responses in the methionine/threonine biosynthetic pathway of *Saccharomyces cerevisiae*. *Yeast* **7**, 781–803.

de Nobel, J. G., Klis, F., Ram, A., Van Unen, H. J., Priem, J., Munnik, T. & Van den Ende, H. (1991). Cyclic variations in the permeability of the cell wall of *Saccharomyces cerevisiae*. *Yeast* **7**, 589–598.

Orlean, P. (1990). Dolichol phosphate mannose synthase is required *in vivo* for glycosyl phosphatidylinositol membrane anchoring, O-mannosylation, and N-glycosylation of proteins in *Saccharomyces cerevisiae*. *Mol Cell Biol* **10**, 5796–5805.

Orlean, P., Albright, C. & Robbins, P. W. (1988). Cloning and sequencing of the yeast gene for dolichol phosphate mannose synthase, an essential protein. *J Biol Chem* **263**, 17499–17507.

Paranjape, V. & Datta, A. (1991). Overexpression of the actin gene is associated with the morphogenesis of *Candida albicans*. *Biochem Biophys Res Commun* **179**, 423–427.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sass, P., Field, J., Nikawa, J., Toda, T. & Wigler, M. (1986). Cloning and characterisation of the high-affinity cAMP phosphodiesterase of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **83**, 9303–9307.

Sherman, F., Fink, G. R. & Hicks, J. B. (1986). *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sikorski, R. S. & Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27.

Stateva, L. I., Oliver, S. G., Trueman, L. J. & Venkov, P. V. (1991). Cloning and characterisation of a gene which determines osmotic stability in *Saccharomyces cerevisiae*. *Mol Cell Biol* **11**, 4235–4243.

Sundstrom, P., Smith, D. & Sypherd, P. S. (1990). Sequence analysis and expression of the two genes for elongation factor 1 α from the dimorphic yeast *Candida albicans*. *J Bacteriol* **172**, 2036–2045.

Swoboda, R. K., Bertram, G., Colthrust, D. R., Tuite, M. F., Gow, N. A. R., Gooday, G. W. & Brown, A. J. (1994). Regulation of the gene encoding translation elongation factor 3 during growth and morphogenesis in *Candida albicans*. *Microbiology* **140**, 2611–2616.

Venkov, P., Hadjiolov, A., Battaner, E. & Schlessinger, D. (1974). *Saccharomyces cerevisiae* sorbitol dependent fragile mutants. *Biochem Biophys Res Commun* **56**, 559–604.

Wilkinson, B. M., James, C. & Walmsley, R. M. (1996). Partial deletion of the *Saccharomyces cerevisiae* GDH3 gene results in novel starvation phenotypes. *Microbiology* **142**, 1667–1673.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Zhang, N. (1997). *Genetically-controlled cell lysis in the yeast Saccharomyces cerevisiae*. PhD thesis, UMIST, Manchester, UK.

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