GPI7 affects cell-wall protein anchorage in *Saccharomyces cerevisiae* and *Candida albicans*

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Glycosylphosphatidylinositol (GPI)-anchoring represents a mechanism for attaching proteins to the cell surface of all eukaryotic cells. Two localizations of GPI proteins have been observed in the yeasts *Saccharomyces cerevisiae* and *Candida albicans*: plasma membrane and cell wall. The signals and the mechanisms involved in this differential targeting are presently not well understood. Here several cell-wall-related phenotypes of a *gpi7/las21* deletion are described, where *GPI7/LAS21* encodes a GPI-anchor-modifying activity. In both organisms, the structure and composition of the cell wall was modified, with a clear increase in chitin deposition. Cell-wall-targeted proteins accumulated in the growth medium, whereas the protein content of the cell wall decreased significantly, suggesting inefficiency of the covalent linkage. The level of plasma-membrane-targeted GPI proteins was not affected. Sequence analyses revealed that gene families involved in the addition of phosphoethanolamines to the core GPI anchor are highly conserved between eukaryotes, with the exception of the *Gpi7* family which seems to be fungus-specific. These data are compatible with the notion that the phosphoethanolamine added by *Gpi7* protein to the GPI anchor is a key factor in the covalent linkage of cell-wall proteins to fungal cell-wall components.

Keywords: glycosylphosphatidylinositol, Pir proteins, Als proteins

INTRODUCTION

Numerous glycoproteins of lower or higher eukaryotes become attached to a glycosylphosphatidylinositol (GPI) anchor, many of which ultimately appear at the plasma membrane, and in some fungi in the cell wall (Tiede *et al.*, 1999). This anchor has the conserved core structure described in all eukaryotes: lipid-PO₄-myo-inositol-(6→1)-GlcNH₂-(4→1)-αMan-(6→1)-αMan-(2→1)-αMan-(6)-PO₄-(CH₂)₂-NH. In yeast, this glycan core is decorated during its assembly with a fourth mannose residue and with additional phosphoethanolamines (EtN-Ps) to form the complete precursor glycolipid (Benghezal *et al.*, 1995; Grimme *et al.*, 2001). The transfer of the complete precursor onto proteins occurs

in the lumen of the endoplasmic reticulum and is catalysed by a GPI transamidase complex (Ohishi *et al.*, 2000). GPI-anchoring is not essential at a cellular level in mammalian cells, as many GPI-deficient mutant cell lines have been established (Takeda *et al.*, 1993); however, in *Saccharomyces cerevisiae* GPI anchors are essential for growth (Leidich *et al.*, 1995). Thus, all the genes known for their involvement in the biosynthesis of GPI anchors in *S. cerevisiae* are essential, except one, *GPI7* (Benachour *et al.*, 1999). Its deletion in *S. cerevisiae* hampers GPI-anchor addition to newly synthesized proteins, resulting in cell-wall fragility and mating defects (Benachour *et al.*, 1999; Toh-e & Oguchi, 1999). In *Yarrowia lipolytica*, a dimorphic yeast, the mutant cells have a clear defect in morphogenesis and display reduced invasive growth and hypersensitivity to Calcofluor White (Richard *et al.*, 2001). Finally, in *Cgpi7* mutants of *Candida albicans*, chlamydospore formation is affected, as well as the yeast to hypha transition on solid, but not in liquid media. The cell-wall structure is also modified in the mutants, and budding patterns,

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Abbreviations: Als, agglutinin-like sequence; ConA, Concanavalin A; CWP, cell-wall protein; EtN, ethanolamine; EtN-P, phosphoethanolamine; GPI, glycosylphosphatidylinositol; Pir, protein with internal repeat.

Fig. 1. Representation of the *S. cerevisiae* GPI core structure and the main proteins involved in the addition of the three EtN groups on the different mannose groups. Man, Mannose; GlcN, glucosamine; Ins, myo-inositol.

cytokinesis and cell shape are abnormal in both liquid and solid media. *In vivo* assays revealed that these mutants have a reduced virulence, a reduced ability to colonize the gut in mice and are more sensitive to the lytic action of macrophages (Richard *et al.*, 2002).

S. cerevisiae is known to have a GPI core structure with a fourth mannose group; Smp3p, an essential protein, is described as being involved in the addition of this group (Grimme *et al.*, 2001). Various studies have described three proteins involved in the transfer of EtN-Ps onto the three first mannose groups of the GPI anchor (Benachour *et al.*, 1999; Flury *et al.*, 2000; Taron *et al.*, 2000). Mcd4p adds an EtN-P on the first mannose, Gpi7p on the second and Gpi13p/Yll031c on the third one (Fig. 1) (Flury *et al.*, 2000). Whereas both *MCD4* and *GPI13* are essential, *GPI7* is not. The lethality of the *GPI13* deletion is to be expected since its gene product is involved in the addition of the EtN-P, which links the anchor to the protein. The function of the other two EtN groups is unknown. Here we present evidence that the side chain added by Gpi7p participates in the proper anchoring of GPI-modified proteins targeted to the cell wall.

METHODS

Strains and growth conditions. The *C. albicans* strains used were SC5314 (wild-type), MLR2A42 (*ura3::imm434/ura3::imm434*, *Cagpi7::hisG/Cagpi7::hisG-CaURA3-hisG*) and MLR4A2 (*ura3::imm434/ura3::imm434*, *Cagpi7::hisG/Cagpi7::hisG-CaURA3-hisG*) (Richard *et al.*, 2002). The *S. cerevisiae* strains used were FY23 (MATa *ura3-52*, *trp1D63*, *leu2D1*) and Δ *gpi7* (MATa, *ura3-52*, *his3D200*, *LEU2*, *LYS2*, *trp1D63*) (de Groot *et al.*, 2001). *Candida* cells were grown at 37 °C, whereas *Saccharomyces* cells were grown at 30 °C in Yeast Peptone Dextrose (YPD) for pre-culture. During experiments, cells were cultured in Synthetic Complete (SC) medium, at different pH values, containing 0.17% yeast nitrogen base without amino acids (Difco), 2% glucose, 0.5% Casamino acids, 100 mM HEPES and 0.01% uracil, histidine, tryptophan or tyrosine, and 0.02% leucine when required. Two independent transformants, MLR2A42 and MLR4A2, were included in all experiments to ensure that the phenotypic traits observed were solely due to the *CaGPI7* mutation and not to unrelated mutations that may have occurred during the construction of Δ *Cagpi7* null strains. In a

previous publication, we demonstrated that the phenotypes observed were due to *CaGPI7* disruption (Richard *et al.*, 2002) by reintroducing *CaGPI7* in MLR2A42.

Quantazyme and chitinase sensitivity assays. These assays were carried out to detect possible changes in the organization of the 1,3- β -glucan and chitin layers of the cell wall. Quantazyme is a recombinant 1,3- β -glucanase (Quantum Biotechnology). The Quantazyme sensitivity assay was conducted as described by Ovalle *et al.* (1998). Briefly, cells in exponential phase (OD_{600} = 1–2) were resuspended in 40 mM β -mercaptoethanol, 50 mM Tris/HCl, pH = 7.5, to an OD_{600} of 1. After incubation for 1 h, Quantazyme dissolved in glycerol/water (1:1, v/v), was added to a final concentration of 400 U ml⁻¹. Cell lysis was determined by following the decrease in turbidity at 600 nm over time. Quantazyme sensitivity was expressed as a percentage of the initial OD_{600} . *Streptomyces griseus* chitinase (Sigma; C1525) was used in some experiments at 0.2 U ml⁻¹ for 1 h before Quantazyme addition.

SDS hypersensitivity tests. Exponential cultures were harvested and adjusted to an OD_{600} of 1; 5 μ l of tenfold serial dilutions were spotted on YPD containing increasing amount of SDS: 0.01, 0.05 and 0.1%. Plates were read after 24 h culture at 30 °C for *S. cerevisiae* and 37 °C for *C. albicans*.

Isolation of cell-wall proteins (CWPs). For these experiments, cultures were grown in SC medium at 30 °C, pH 5.5, for *S. cerevisiae* strains, and at 37 °C, pH 4.5, for *C. albicans* strains, since faint differences could only be detected at higher pH in the CWP content of the wild-type and the *gpi7* mutants of *C. albicans*. After 16 h culture in SC, cells were harvested in exponential phase (OD_{600} = 2) and washed with 10 mM Tris/HCl, pH 7.6, as described previously (Kaptein *et al.*, 1995; Montijn *et al.*, 1994). Briefly, cell walls were isolated and washed extensively with 1 M NaCl. Isolated cell walls were boiled twice in the presence of SDS, EDTA and β -mercaptoethanol to solubilize the non-covalently linked CWPs and to remove any contaminant derived from the cytosol and/or plasma membrane. SDS-extracted cell walls were freeze-dried after washing and 3 mg of the pellets were treated either with recombinant *Trichoderma harzianum* endo-1,6- β -glucanase (0.8 U g⁻¹) to release the GPI-CWPs (Kaptein *et al.*, 1996) or with 30 mM ice-cold NaOH for 17 h to release the 1,6- β -glucanase-resistant Pir-like-CWPs. Subsequently, 1,6- β -glucanase-treated cell walls were treated with ice-cold 30 mM NaOH for 17 h to release the double linked CWPs. Each enzymic digestion was stopped by adding SDS at a final concentration of 0.4% and heating for 5 min at 100 °C. Prior to heating in SDS, NaOH-treated cell walls were first neutralized by adding acetic acid.

Analysis of CWPs. CWPs were separated by electrophoresis using linear 2.6–20% polyacrylamide gels and transferred onto Immobilon-P polyvinylidene difluoride (PVDF, 0.45 μ m) membrane (Millipore) using a wet blotting apparatus. CWPs were visualized by probing the membrane with peroxidase-labelled Concanavalin A (ConA; 0.3 μ g ml⁻¹) in PBS containing 3% bovine serum albumin, 2.5 mM CaCl₂ and 2.5 mM MnCl₂ (Kaptein *et al.*, 2000). Western immunoblot analyses were performed with polyclonal antisera directed against 1,6- β -glucan, agglutinin-like sequence (Als) proteins, Cwp1p, Pir2p, Ssr1p and Gas1p. For the antisera directed against Als proteins, Cwp1p, Pir2p, Ssr1p and Gas1p, the membranes were treated with 50 mM periodic acid and 100 mM sodium acetate for 30 min before the blocking step with 5% milk

powder in PBS to enhance the specificity of the antisera. Binding of rabbit antibodies was assessed with goat anti-rabbit IgG peroxidase (Pierce) at a dilution of 1:10000 in PBS/5% milk powder. The blots were visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and Fuji Medical X-Ray film.

Determination of glucose, mannose and glucosamine content in cell walls. Isolated cell walls (about 1–1.5 mg) were hydrolysed as described previously with minor modifications (Dallies *et al.*, 1998). Briefly, cell walls were hydrolysed with 50 µl 72% sulfuric acid for 3 h at 25 °C. Then 650 µl distilled water was added to obtain a 1 M H₂SO₄ solution, followed by incubation at 100 °C for 3 h. The solution was diluted by adding 3 ml distilled water and precipitation of sulfate was carried out using 3.3 ml 40 g barium hydroxide l⁻¹. After centrifugation, the sugars were quantified using a Dionex Bio-LC system with a CarboPac PA 1 anion-exchange column. In this experiment 5 mM galactose was used as internal standard.

RESULTS

The family of genes involved in the addition of EtN-P is highly conserved between *S. cerevisiae* and *C. albicans*

To investigate whether genes involved in EtN-P addition on the GPI core structure were conserved between *S. cerevisiae* and *C. albicans*, an analysis of the sequence database of *C. albicans* was performed. This revealed that homologues of *MCD4*, *GPI7* and *GPI13*, all forming part of a major facilitator superfamily, exist in *C. albicans*. They were tentatively called *CaMCD4*, *CaGPI7* and *CaGPI13*, respectively. All predicted amino acid sequences exhibit significant similarities to each other both in sequence and in predicted structure. Percentages of identity vary between 22 and 38% among paralogues in a given organism and between 35 and 56% among *S. cerevisiae* and *C. albicans* homologues (see Fig. 2A). All predicted proteins possess between 7 and 14 predicted transmembrane domains with a large, probably luminal loop. The length and the position of the predicted loop are also conserved: its size varies between 398 and 470 aa and it begins just after the first

transmembrane domain. This analysis also identified putative motifs responsible for functional specificity. The large luminal loop, which is the most conserved region among homologues (53–67.5% identity) overlaps a predicted type I phosphodiesterase/nucleotide pyrophosphatase domain (pfam01663). Within this domain, each pair of homologues shares a very highly conserved motif of about 30 aa, which clearly permits separation of *Mcd4*-, *Gpi7*- and *Gpi13*-like proteins into three classes, the latter two being closely related and divergent from the first (Fig. 2B). Taken together, these results suggest that the number, structure and probable biochemical function of the enzymes involved in the addition of the three EtN-Ps onto the GPI core structure are highly conserved between *S. cerevisiae* and *C. albicans*.

gpi7 cells exhibit marked sensitivity to SDS

gpi7 mutants in *S. cerevisiae* and *C. albicans* have been described as being hypersensitive to Calcofluor White (Benachour *et al.*, 1999; Richard *et al.*, 2002), suggesting a cell-wall defect. To further support this hypothesis, we examined the sensitivity of the cells to SDS. SDS sensitivity suggests a loose structure for the cell wall, allowing SDS to reach the plasma membrane and to perturb its organization, leading to cell death. When *CaGpi7* mutants were grown in the presence of increasing concentrations of SDS, they were found to be hypersensitive to SDS after 24 h at 37 °C (data not shown). However, no SDS hypersensitivity was observed in the case of *S. cerevisiae gpi7* mutants (data not shown).

Composition of the mutant cell wall is modified

To better characterize the cell-wall modification in the mutants, cell-wall preparations were hydrolysed with sulfuric acid and the released sugars were separated and quantified by high performance anion-exchange chromatography. The following results represent the mean of two independent experiments and are expressed as a percentage of the respective wild-type levels (standard

Fig. 2. Sequence comparison between *S. cerevisiae* and *C. albicans* amino acid sequences. (A) Percentage identity between *MCD4*, *GPI7* and *GPI13* homologues. (B) Alignment of the highly conserved domain in the predicted amino acid sequences of all the homologues using CLUSTAL W (Thompson *et al.*, 1994). Identical amino acids are boxed.

Fig. 3. Molecular architecture of the cell wall of *S. cerevisiae* and *C. albicans*. The arrows denote the orientation of the polysaccharides from the non-reducing end to the reducing end. Mature GPI-CWPs, are believed to lose their lipid moiety once they are covalently incorporated into the cell wall. GPI-CWPs can be linked by either a 1,6- β -glucan or an alkali-sensitive bond, like Pir proteins, or by both. The model is based on data from Kollar *et al.* (1997), Kapteyn *et al.* (2000), Smits *et al.* (1999) and this study.

error on these measurements is less than 5%). The sugar composition of the *C. albicans* *Cagpi7* null mutant cell wall was 70, 102 and 412% for mannose, glucose and glucosamine, respectively, and 86, 116 and 261%, respectively, for the *S. cerevisiae* *gpi7* null mutant. The *Cagpi7* null mutants showed a fourfold increase in the chitin level, suggesting compensation of a cell-wall defect by chitin deposition, as often observed in cell-wall-defective mutants of *S. cerevisiae* (Dallies *et al.*, 1998) and *C. albicans* (Kapteyn *et al.*, 2000). Moreover, the mannose content of the cell wall decreased by 30%, indicating a defect in protein mannosylation or a decrease of mannoproteins in the mutant cell wall compared to the wild-type. Similar results were observed on cell walls of a *gpi7* mutant of *S. cerevisiae*, with a greater than twofold increase in chitin content, although the mannose level was marginally affected. Overall, disruption of *GPI7* seems to modify the cell wall of both organisms in a qualitatively similar way, resulting in a decrease of mannoproteins, which in turn is compensated by increased chitin deposition.

We reasoned that the excess of chitin in mutant cell walls might protect cells against lytic enzymes. Indeed, we found a higher resistance of the mutant cells of *C. albicans* and *S. cerevisiae* to Quantazyme, a recombinant 1,3- β -glucanase (data not shown). Furthermore, we checked whether a chitinase pre-treatment of the mutant may restore Quantazyme susceptibility. Although a 1 h treatment with chitinase alone had no effect on cell lysis under our experimental conditions, it induced a significant increase in mutant cell lysis, comparable to wild-type, during a further Quantazyme treatment in both *C. albicans* and *S. cerevisiae* (data not shown).

Fewer proteins are linked to the cell wall in the mutant cells

To investigate whether fewer mannoproteins were indeed present in the cell wall, as suggested by previous data on cell-wall composition, we analysed different fractions from hot SDS cell-wall extracts of *C. albicans*

and *S. cerevisiae* mutant and wild-type cells grown in SC medium in the yeast form. Indeed, in both *S. cerevisiae* and *C. albicans* two types of CWPs have been identified that are covalently linked to β -glucan, the GPI-dependent CWPs (GPI-CWPs) and the Pir-CWPs (Fig. 3). In *S. cerevisiae*, Pir-CWPs are cross-linked to cell-wall 1,3- β -glucan by a covalent link sensitive to mild alkali treatments (see Methods). GPI-CWPs are cross-linked by a 1,6- β -glucan to 1,3- β -glucan (and to a lesser extent to chitin), or by an alkali-sensitive bond to 1,3- β -glucan (Pir-CWPs), or by both types of bond (Kapteyn *et al.*, 1999). A recent publication demonstrated that the general organization of the *C. albicans* cell wall is very similar to that of *S. cerevisiae* (Kapteyn *et al.*, 2000). To characterize the effect of *CaGPI7* deletion on CWPs, we compared the fractions obtained after 1,6- β -glucanase digestion, mild alkali treatment, or mild alkali treatment after 1,6- β -glucanase digestion. Lectin-blotting of wild-type *C. albicans* 1,6- β -glucanase digests, using peroxidase-linked ConA, revealed a large smear and three mannosylated protein bands with apparent molecular masses of 220, 180 and 100 kDa, respectively (Fig. 4). As expected, anti-1,6- β -glucan antiserum did not recognize any of this material, indicating that the 1,6- β -glucanase did not leave any detectable 1,6- β -glucan epitope attached to the proteins (data not shown). *Cagpi7* mutants exhibited a marked reduction of ConA stained material in this test, suggesting that fewer proteins were linked to the cell wall in the mutant (Fig. 4).

Hoyer and colleagues (Hoyer *et al.*, 1995; Hoyer, 2001) have described the *ALS* gene family, encoding high-molecular-mass cell-surface proteins that are believed to be covalently linked to cell-wall glucans by a remnant part of the GPI anchor. Reprobing of the same samples with an anti-*Als* protein antiserum (Hoyer *et al.*, 1995) revealed a band at 400 kDa in the wild-type (Fig. 4), which was greatly reduced when mutant cells were probed. Analysis of SDS-extracted, alkali-treated cell walls with the same anti-*Als* protein antiserum revealed a protein band with an apparent molecular mass of ~600 kDa for both wild-type and mutant (Fig. 4),

Fig. 4. Cell walls of *C. albicans gpi7* contain less protein than wild-type cells. Western analysis of fractions after different treatments from wild-type (SC5314) and two independent *Cagpi7* null mutants (MLR2A42 and MLR4A2) using peroxidase-labelled ConA or anti-Als protein antiserum. Each lane was loaded with the equivalent of 0.3 µg dry weight of isolated SDS-extracted cell walls.

indicating that some Als proteins at least are attached by an alkali-sensitive bond. Moreover, quantitative comparison between mutant and wild-type revealed the same decrease in quantity as in the 1,6- β -glucanase digestion fraction. Finally, ConA staining of the protein fraction obtained by alkali extraction of 1,6- β -glucanase-digested cell walls showed a comparable decrease in the mutant walls as seen in the other fractions (Fig. 4). Taken together, these results indicate that the cell wall of *C. albicans* contains GPI-dependent CWPs, including at least one Als protein, that are linked to the 1,3- β -glucan network, either through 1,6- β -glucan or through an alkali-sensitive linkage, and that the cell-wall content of these proteins is markedly reduced in the *Cagpi7* mutant.

CWPs are released into the medium of *gpi7* disrupted strains

The above results show that mutant cell walls generally contain less GPI-CWP than the wild-type, suggesting that these proteins are either repressed, degraded or mislocalized. Preliminary results (not shown) indicate that transcription of eight *ALS* genes tested was not modified in the mutant. We thus checked whether GPI-CWPs were released into the medium. Culture supernatants of the *C. albicans* strains were concentrated by TCA precipitation and analysed by Western blotting. Anti-Als protein and anti-Pir protein antisera revealed increased levels of putative CWPs in the culture medium of the mutant (Fig. 5a). These results indicate that these proteins are not normally linked to the cell wall in the *Cagpi7* null mutant.

A similar analysis was performed for CWPs of *S. cerevisiae*, using anti-Ssr1p, anti-Cwp1p and anti-Pir2p antisera. Ssr1p is a GPI-dependent CWP, which is probably associated with glucan in a structural role, and Cwp1p is a GPI cell-wall mannoprotein up-regulated by cell-wall stress. In both cases, an increased release into the growth medium of these proteins, as well as of Pir2p, was observed in the *gpi7* mutant (Fig. 5b). The behaviour of Pir2p confirms previous data reporting an increase of Pir protein secretion in the case of cell-wall defects in *S. cerevisiae* (de Groot *et al.*, 2001).

Taken together, these data demonstrate that disruption of *GPI7* in *C. albicans* or in *S. cerevisiae* results in a defective cell-wall linkage of GPI-CWPs and Pir-CWPs.

GPI membrane proteins are not affected by the *GPI7* deletion

We then wanted to test whether the *GPI7* deletion specifically disrupted localization of GPI-CWPs, or whether it also affected proteins anchored by GPI to the cell membrane (GPI-MP). We followed the behaviour of one GPI-MP in each organism.

In *S. cerevisiae* we chose Gas1p, a GPI-modified 1,3- β -glucanosyltransferase, predominantly anchored to the plasma membrane with only traces in the cell wall (Popolo *et al.*, 1993). SDS washes of cell-wall preparations (see Methods), known to be heavily contaminated by cell membranes (Schreuder *et al.*, 1993), were used as a non-purified plasma membrane fraction. This crude fraction, as well as culture supernatants from *S. cerevisiae* mutant and wild-type cells, were probed with an

Fig. 5. Protein composition of the culture medium of wild-type and *gpi7* mutants of *S. cerevisiae* (a) or *C. albicans* (b). Western analysis of protein from the culture supernatant from (a) wild-type (SC5314) and two independent *Cagpi7* null mutants (MLR2A42 and MLR4A2) using peroxidase-labelled ConA, anti-Als and anti-Pir2 antisera; (b) wild-type (FY23) and *gpi7* null mutant ($\Delta gpi7$) using anti-Ssr1p anti-Cwp1 and anti-Pir2 antisera. Each lane was loaded with proteins precipitated by TCA from 1 ml culture medium at an OD₆₀₀ of 2.

Fig. 6. GPI-MPs are not affected by the *GPI7* deletion in *S. cerevisiae* (a) and *C. albicans* (b). Western analysis of protein from the culture medium from (a) wild-type (FY23) and *gpi7* null mutant ($\Delta gpi7$) using anti-Gas1p antiserum; (b) wild-type (SC5314) and *Cagpi7* null mutant (MLR2A42) using anti-Gas1p antiserum. Each lane was loaded with proteins either precipitated by TCA from 1 ml culture medium at an OD₆₀₀ of 2 or SDS-extracted from 0.2 μ g wet weight of cell walls.

anti-Gas1p antiserum (Fig. 6a). No signal was detected in the culture supernatants from wild-type and mutant strains. In contrast, a protein band was present in the membrane fraction of both strains at the expected size of 125 kDa and in nearly identical amounts. These data suggest normal anchorage of at least one GPI-MP to the cell surface in the *S. cerevisiae gpi7* mutant.

A previous study (Popolo & Vai, 1998) demonstrated that anti-Gas1p antiserum cross-reacted with the homologues of Gas1p in *C. albicans*, the Phr1/2/3 proteins. To extend the results obtained in *S. cerevisiae* to *C. albicans*, an SDS extract of *C. albicans* cell walls was analysed using the same anti-Gas1p antiserum (Fig. 6b).

In both wild-type and mutant similar patterns were obtained: no signal was detected in the culture supernatants, whereas in the SDS extract two clear protein bands and a third, fainter band were detected at the expected positions for Phr proteins. These results suggest that in both organisms membrane anchorage of at least Gas1p and its homologues, and probably other GPI-MPs, is not affected by the deletion of *GPI7*.

DISCUSSION

In this study we investigated the potential role of the EtN-P moiety added by Gpi7p in GPI-anchoring in two

Fig. 7. Alignment of the highly conserved domain in the predicted amino acid sequences of *MCD4* and *GPI3* (A) and *GPI7* (B) with homologues from the non-redundant sequence database.

different organisms, *S. cerevisiae* and *C. albicans*. In a first step, we scanned the *C. albicans* sequence database and found homologues for the three genes, *MCD4*, *GPI7* and *GPI13*, involved in the addition of EtN-P to the GPI core structure. Interestingly, the *C. albicans* database contains a single homologue for each of these three genes. Although we confirmed the conservation of the biochemical function only for CaGpi7p (Richard *et al.*, 2002), we speculate that the function of CaMcd4p and CaGpi13p is also conserved in view of their strong similarity to their homologues in *S. cerevisiae*. Within the conserved phosphodiesterase/nucleotide pyrophosphatase domain shared by all these proteins, a motif of about 30 aa is highly conserved between each of the three pairs of homologues and permits a clear discrimination of the three paralogues. An attractive hypothesis is that these motifs may determine the binding specificity of the proteins for each of the three mannose groups of the GPI core structure: Mcd4p on the first, Gpi7p on the second and Gpi13p on the third. Several potential homologues of the three genes can be found in lower and higher eukaryotes. As shown in Fig. 7(A), the 30 aa motif is well conserved in most of them, permitting identification of four excellent matches with proteins from *Schizosaccharomyces pombe*, *Drosophila melanogaster*, mice and humans for both the Mcd4p and Gpi13p motifs, from *Arabidopsis thaliana* for the Mcd4p motif and from *Caenorhabditis elegans* for the Gpi13p motif. Interestingly, one EtN-P is added on the third mannose of GPI anchors in all eukaryotes and in most cases on the first one, paralleling conservation of Gpi13p and Mcd4p. On the other hand, the modification of the second mannose carried out by Gpi7p in yeast is more

variable. We have noticed that Gpi7p tends to be more divergent. Closely related relatives are found only among proteins of fungal origin, from *Yarrowia lipolytica*, *Saccharomyces bayanus* and *Schizosaccharomyces pombe* (Fig. 7B). More distant ones are detected among humans, mice, *Drosophila* and *Caenorhabditis* proteins, where the Gpi7p motif is interrupted by two insertions of 9 and 5 aa (not shown). This suggests that Gpi7 proteins have a conserved specific function in fungi, whereas their homologues in higher eukaryotes carry out different GPI-anchor modifications.

It has been demonstrated that many GPI proteins of yeast are CWPs or plasma-membrane proteins participating in the building of the cell wall (Caro *et al.*, 1997; Hamada *et al.*, 1998). The phenotype of *GPI7*-depleted cells strongly suggests that Gpi7 affects cell-wall structure. Increased cell-wall fragility, a common feature of all *gpi* mutants, already suggested by previous tests with Calcofluor White (Richard *et al.*, 2002), was confirmed by SDS hypersensitivity (Benghezal *et al.*, 1995; Vossen *et al.*, 1997) and we observed direct evidence of a difference in chitin content in the cell wall of mutant *C. albicans* and *S. cerevisiae* cells.

This defect in cell-wall structure correlates with a lower protein content of the cell wall in *C. albicans* and an increased release of CWPs in the growth medium of the mutant. Moreover, mutations in *GPI7* do not affect localization of the cell membrane-attached GPI protein Gas1p.

The lower levels of GPI-dependent CWPs can be explained in different ways. First, the lack of EtN-P on the second mannose of the GPI core structure might affect the transport of GPI-dependent CWPs through the secretory pathway. This does not seem to be the case since the growth rate and the quantity of Gas1p in the plasma membrane were not affected. Second, the lack of EtN-P might block the release of the plasma-membrane-bound intermediate form of GPI proteins destined for the cell wall. This seems unlikely because in the mutant more soluble forms were found in the culture medium. Third, this side-chain might be essential for efficient linkage of GPI-CWPs to other cell-wall components. Thus, the lack of this side-chain may trigger mislocalization of GPI-CWPs, including some proteins involved in cell-wall synthesis and in the linkage of Pir proteins. Consequently, a defective cell wall is formed, the cell aggregates, morphogenesis is affected and virulence and the resistance to macrophages are also affected (Richard *et al.*, 2002).

Finally, although *gpi7* mutants of *S. cerevisiae* and *C. albicans* share common phenotypes, like secretion of GPI-CWPs, resistance to Quantazyme and increased chitin deposition, they also differ. In particular, no SDS hypersensitivity was detected in *S. cerevisiae* mutants and the amount of proteins in the cell wall was negligibly affected. Possibly, these phenotypic differences are due to the different growth temperatures used for *S. cerevisiae* (30 °C) and *C. albicans* (37 °C).

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