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Research Article

Mass spectrometric identification of covalently bound cell wall proteins from the fission yeast *Schizosaccharomyces pombe*

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Abstract

The cell wall of *Schizosaccharomyces pombe* is bilayered, consisting of an inner layer of mainly polysaccharides and an outer layer of galactomannoproteins. We present a detailed analysis of the cell wall proteome. Six covalently-bound cell wall proteins (CWPs) were identified using tandem mass spectrometry, including four predicted GPI-dependent CWPs (Gas1p, Gas5p, Ecm33p and Pwp1p) and two alkali-sensitive CWPs (Psu1p and Asl1p). Gas1p and Gas5p belong to glycoside hydrolase family 72, and are believed to be involved in 1,3- β -glucan elongation. Ecm33p belongs to a ubiquitous fungal protein family with an unknown but crucial function in cell wall integrity. Pwp1p is an abundant protein with an unknown but probably non-enzymatic function. All four CWPs were present in HF-pyridine extracts, indicating that they are linked via a phosphodiester bridge to the glucan network. Psu1p is a homologue of the *Saccharomyces cerevisiae* Sun family, whereas Asl1p has no homologues in *S. cerevisiae* but is related to *Aspergillus fumigatus* and *Ustilago maydis* proteins. Finally, although the protein content of *Sz. pombe* cell walls is only slightly less than in *S. cerevisiae* and *Candida albicans*, the amount of carbohydrate added to the proteins was found to be two- to three-fold decreased, consistent with earlier reported differences in outer chain *N*-glycosylation. Copyright © 2007 John Wiley & Sons, Ltd.

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Introduction

The fungal cell wall is an essential organelle that enables cells to withstand osmotic pressure and cope with environmental stress, and also determines cell shape. In contrast to the oval-shaped budding cells of *Saccharomyces cerevisiae* and *Candida albicans* growing in the yeast form, *Schizosaccharomyces pombe* cells have a cylindrical rod-like shape and divide by medial fission.

In *S. cerevisiae* and *C. albicans*, the composition and architecture of cell walls have been studied in great detail (De Groot *et al.*, 2005; Klis *et al.*, 2001, 2006; Lesage and Bussey, 2006). The inner part of the cell wall in *S. cerevisiae* is a skeletal layer that is mainly composed of a polysaccharide network. This is surrounded by an external layer that is comprised of a variety of different manno-proteins. The major cell wall polysaccharide in *S. cerevisiae* and *C. albicans* is 1,3- β -glucan, which

forms a strong but flexible water-insoluble network and contains acceptor sites for covalent attachment of the polysaccharides 1,6- β -glucan and chitin, and cell wall mannoproteins. Studies of the cell wall composition in *Sz. pombe* have revealed similarities but also some important differences as compared to *S. cerevisiae* and *C. albicans*. In fission yeast, the major wall component is also 1,3- β -glucan (46–54%). However, the walls of *Sz. pombe* lack chitin and instead contain 1,3- α -glucan (28%), a component that has not been found in *S. cerevisiae* (Grün *et al.*, 2005; Magnelli *et al.*, 2005; Manners and Meyer, 1977; Pérez and Ribas, 2004). Also, instead of the branched 1,6- β -glucan found in *S. cerevisiae*, *Sz. pombe* contains short polymers termed diglucan (2%). These polymers consist of a backbone of 1,6- β -linked glucose residues, approximately 75% of which carry 1,3- β -linked glucose substituents (Magnelli *et al.*, 2005). Not much is known about the interpolymeric linkages between cell wall components and about the identity of the covalently-bound cell wall proteins (CWPs) in *Sz. pombe*.

Depending on the type of their linkages to the cell wall glucans, covalently-bound yeast CWPs are divided into two groups: (a) glycosylphosphatidylinositol (GPI)-modified proteins are the largest group of proteins; they are linked via a sugar remnant of the GPI anchor to 1,6- β -glucan which interconnects them to 1,3- β -glucan or, especially under cell wall stress conditions, to chitin; (b) a minor group of proteins is linked via a mild alkali sensitive linkage (ASL) to 1,3- β -glucan (De Groot *et al.*, 2005; Ecker *et al.*, 2006). Genome analysis indicates that genes encoding Pir proteins, the best-characterized ASL-CWPs in *S. cerevisiae*, are not present in *Sz. pombe* (data not shown). Whether other ASL-CWPs are present is not known. In *Sz. pombe* 33 proteins were predicted to be GPI-modified (De Groot *et al.*, 2003), and a table presenting these proteins can be downloaded from http://www.pasteur.fr/recherche/unites/Galar_Fungail/#pro. For one of the predicted GPI proteins, the predominantly membrane-localized α -amylase Aah3p, some immunological evidence has been presented recently indicating that it also might be partially localized in the cell wall (Morita *et al.*, 2006). These data, together with the visualization of a darkly stained outer mannoprotein layer by electron microscopy, similar to what has been observed in *S. cerevisiae*, *C. albicans* and other

fungi (Baba and Osumi, 1987; Humbel *et al.*, 2001; Osumi, 1998; Osumi *et al.*, 1998; Tokunaga *et al.*, 1986), suggest that covalently-bound GPI-CWPs are also present in *Sz. pombe*.

Previously, we have performed mass spectrometric analysis of proteins that are covalently bound to the cell wall polysaccharide network in *S. cerevisiae* and *C. albicans*, resulting in the identification of about 15–20 CWPs in both species under a given condition (De Groot *et al.*, 2004; Pardini *et al.*, 2006; Yin *et al.*, 2005). Intriguingly, many of the proteins identified were classified as carbohydrate-active enzymes, in particular members of the fungal-specific Gas/Phr [glycoside hydrolase (GH) family 72], Bgl2p (GH 17) and Crh (GH 16) families, and the *C. albicans* chitinase Cht2p (GH 18), which may all use cell wall polysaccharides as their natural substrates. The covalent incorporation of these enzymes raises important questions about their functionality, as continued hydrolysis of cell wall polymers might weaken the skeletal wall layer. Importantly, cell wall construction is tightly controlled, allowing the cell to grow, divide and adapt its wall to environmental changes. Cell wall-localized carbohydrate-active enzymes may be actively involved in these processes. In agreement with this, many of the genes encoding such enzymes are cell-cycle regulated or activated in response to cell-wall stress conditions, and the proteins are enriched at specific locations where their activity seems needed (Klis *et al.*, 2006; Rodríguez-Peña *et al.*, 2000).

Gas family members of various fungi have 1,3- β -transglucosidase activity *in vitro* (Mouyna *et al.*, 2000) and are thought to hydrolyse and extend newly formed 1,3- β -glucan chains. Fungal Bgl2p also has transglucosidase activity *in vitro* (Mouyna *et al.*, 1998). Crh proteins in *Yarrowia lipolytica* possess endo-1,3- β -glucosidase activity *in vitro* (Hwang *et al.*, 2006; Mrša *et al.*, 1993), but it is yet unknown whether they also have transglucosidase activity. Crh-family members in both *S. cerevisiae* and *C. albicans* are cell cycle-regulated and their cell-surface localization largely coincides with spots of chitin incorporation (Pardini *et al.*, 2006; Rodríguez-Peña *et al.*, 2000). Based on these results, it has been postulated that Crh proteins may be transglycosidases involved in the coupling of chitin to cell wall β -glucan. Consistent with this, *Sz. pombe* has chitin-less walls and lacks

Crh homologues but contains homologues of other CWP-encoding gene families, such as *BGL2*, *GAS* and *ECM33* (as indicated by BLAST analysis; this study).

In this study, we show that *Sz. pombe* contains multiple covalently-bound CWPs and we identified six CWPs by direct trypsin digestion of SDS-treated walls followed by tandem mass spectrometry. We also investigated the covalent linkages between the proteins and the cell-wall carbohydrates by extracting proteins using different chemical or enzymatic treatments.

Materials and methods

Strain, growth conditions and cell wall isolation

Sz. pombe wild-type strain 972 (h^-) was precultured on YEA plates (0.5% w/v yeast extract, 3% w/v glucose, 0.025% w/v adenine sulphate, solidified with 2% w/v agar) and then cultured at 30 °C in liquid YEA medium until $OD_{600} \approx 2$. Cells were harvested by centrifugation (5 min at 5000 r.p.m.) and washed with cold H₂O and with 10 mM Tris-HCl, pH 7.5. The cells were resuspended in 10 mM Tris-HCl, pH 7.5 (2×10^9 cells/tube in 200 μ l) and disintegrated with 0.25–0.50 mm glass beads (Emergo BV, Landsmeer, The Netherlands) in the presence of a protease inhibitor cocktail (Sigma, St. Louis, MO) using a Bio-Savant Fast Prep 120 machine (Qbiogene, Carlsbad, CA). Complete cell breakage was assessed by light microscopy. Preliminary mass spectrometric experiments with *Sz. pombe* indicated that the procedure for cell wall isolation as described for *C. albicans* in De Groot *et al.* (2004) required some modifications to obtain wall preparations that are free from non-covalently associated or cytosolic proteins. Broken cell material was collected and repeatedly washed (by resuspending them) with 1 M NaCl until the supernatant after centrifugation became clear. The walls were then extracted four times, instead of the usual two as described for *C. albicans* (De Groot *et al.*, 2004), for 5 min at 100 °C with 50 mM Tris-HCl, pH 7.8, containing 2% SDS, 100 mM Na-EDTA and 100 mM β -mercaptoethanol. SDS/ β -mercaptoethanol-treated walls are washed with milliQ water until all SDS is removed (no foam formation by resuspension) and freeze-dried.

Cell wall protein and carbohydrate content

Protein and chitin contents in the cell wall were determined following the protocols described by Kapteyn *et al.* (2001) using BSA and glucosamine, respectively, as standards. For determination of the glucan and galactomannan content, the polysaccharides in cell wall preparations were hydrolysed to monosaccharides using sulphuric acid (Dallies *et al.*, 1998) and analysed by HPLC analysis. Briefly, ~ 4 mg freeze-dried walls were incubated with 100 μ l 72% v/v H₂SO₄ for 3 h at room temperature, then diluted with 575 μ l distilled H₂O to get a 2 M H₂SO₄ solution and incubated further for 4 h at 100 °C. The samples were analysed on a REZEX organic acid analysis column (Phenomenex, Torrance, CA) at 40 °C with 7.2 mM H₂SO₄ as eluent using a RI 1530 refractive index detector (Jasco, Great Dunmow, UK). Chromatograms were analysed using AZUR chromatography software and compared with chromatograms of known amounts of mannose, glucose, galactose and glucosamine.

Cell wall protein extraction

Four different extraction methods were used to solubilize covalently-bound CWPs. Endo-1,6- β -glucanase (ProZyme, San Leandro, CA) digestion and hydrogen fluoride (HF)-pyridine treatment, both resulting in specific release of GPI-CWPs in *S. cerevisiae* and *C. albicans*, were performed as described previously (De Groot *et al.*, 2004; Kapteyn *et al.*, 2001; Yin *et al.*, 2005). ASL-CWPs can be extracted by treatment with 30 mM NaOH at 4 °C for 17 h (De Groot *et al.*, 2004; Mrša *et al.*, 1997; Yin *et al.*, 2005). Quantazyme (Qbiogene, Morgan Irvine, CA), is a recombinant endo-1,3- β -glucanase that releases both classes of CWPs in *S. cerevisiae* and *C. albicans* (De Groot *et al.*, 2004; Yin *et al.*, 2005). Digestion with Quantazyme was performed as described by Kapteyn *et al.* (2001). Protein extracts for mass spectrometric analysis were dialysed against MilliQ water and freeze-dried.

Lectin blot analysis

Extracted CWPs were separated on 3–8% NuPAGE® Tris-Acetate Gels (Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane (Millipore, Billerica, MA). The blot was incubated

with the mannoprotein-binding lectin concanavalin A (0.5 µg/ml; Sigma-Aldrich, Zwijndrecht, The Netherlands) and developed using ECL detection reagents (GE Healthcare, Buckinghamshire, UK) as described in Kapteyn *et al.* (2001).

MS analysis

Cell walls and protein extracts were reduced with dithiothreitol and *S*-alkylated with iodoacetamide as described previously (Shevchenko *et al.*, 1996; Yin *et al.*, 2005). Samples were digested with sequencing grade trypsin (Roche Applied Science, Mannheim, Germany) overnight at 37 °C at an estimated CWP: trypsin ratio of 50:1. Resulting peptides were analysed by nanoscale HPLC coupled to quadrupole time-of-flight tandem mass spectrometry (LC/MS–MS), using an Ultimate™ Nano-LC system (Dionex-LC Packings, Hercules, CA) interfaced with a Waters MicroMass Q-TOF mass spectrometer (Waters, Milford, MA). Instrument settings are described in Yin *et al.* (2005). Masslynx software automatically selected peptides from the survey spectrum for fragmentation by argon as the collision gas. Each LC/MS–MS run was repeated at least twice, thereby excluding identified ions from previous runs. MS–MS spectra were analysed with Masslynx software to generate peak lists. These data were used to search an in-house *Sz. pombe* proteome database using the Mascot search engine, version 2.0 (Matrix Science Ltd, London, UK). The *Sz. pombe* proteome is available at the website of the Sanger Institute (<http://www.sanger.ac.uk/Projects/S.pombe/>).

To identify *N*-terminal peptides, signal peptidase cleavage sites of putative GPI proteins (De Groot *et al.*, 2003) and identified ASL-CWPs were predicted using the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) and sequences of matured proteins were added to the proteome file. Database searching was restricted to tryptic peptides of *Sz. pombe*, used enzymes (trypsin and glucanases) and possible contaminations (keratin) — altogether 5049 protein sequences. Searching parameters were: *S*-carbamidomethyl (CAM) addition as a fixed modification, allowing one missed cleavage; a precursor tolerance of 2.0 Da; and a MS–MS tolerance of 0.8 Da. Probability-based Mascot scores were used to evaluate protein identifications (<http://www.matrixscience.com/>).

Only matches with $p < 0.05$ for random occurrence were considered to be significant. Peptide identifications with a Mascot score <25 were verified by manual inspection of the corresponding MS–MS spectra.

Bio-informatics

For functional assignment and homology studies, identified proteins were analysed using the BLAST tool of the *Sz. pombe* GeneDB (Sanger Institute: <http://www.genedb.org/genedb/pombe/>) and NCBI-BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). Conserved domains of carbohydrate-active proteins were retrieved from the CAZy database (<http://afmb.cnrs-mrs.fr/CAZY/>; Coutinho and Henrissat, 1999). Boundaries of the Sun domains were determined using CDD (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=pfam03856>). The presence of GPI-modification signal peptides was analysed using the bigPI server (http://mendel.imp.ac.at/gpi/fungi_server.html; Eisenhaber *et al.*, 2004). Pairwise alignments to calculate the percentage identity between two sequences were performed at <http://www.ebi.ac.uk/emboss/align/>, using default settings. The unrooted phylogenetic tree was calculated with ClustalX, with correction for multiple substitutions and excluding gaps, and was plotted using TreeView version 1.6.6 (Page, 1996).

Results and discussion

Cell wall composition in *Sz. pombe*

In contrast to *C. albicans* and *S. cerevisiae*, cell walls of *Sz. pombe* lack chitin, but contain a considerable amount of 1,3- α -glucan and also short polymers of an extremely branched 1,6- β -glucan (Magnelli *et al.*, 2005; Pérez and Ribas, 2004). To investigate whether these differences in cell wall composition may be reflected by differences in the CWP composition, and to gain insight into the role of CWPs in cell wall construction, we have performed a detailed proteomic analysis of the cell walls of *Sz. pombe*.

First, to enable efficient trypsin digestion of covalently-bound CWPs, we determined the protein content and the amount of glycan (galactomannan) additions. *Sz. pombe* cell walls contained $2.8 \pm 0.1\%$ (cell wall dry weight) protein

and $13.5 \pm 0.1\%$ galactomannan; the latter is in full agreement with previously reported values (Magnelli *et al.*, 2005; Pérez and Ribas, 2004). In the cell walls of *S. cerevisiae* and *C. albicans* (in the yeast form), 3–4% protein and about 30% mannan was measured (our unpublished results) using the same methods. Thus, the amount of glycan added to the CWPs in *Sz. pombe* seems to be relatively low, which is consistent with reported differences in outer-chain *N*-glycosylation (Ballou *et al.*, 1994; Gemmill and Trimble, 1999). Finally, also consistent with the detailed cell wall carbohydrate analysis by Magnelli *et al.* (2005), we found that chitin was absent in *Sz. pombe* cell walls and that the remaining 83–84% of the cell wall mass consisted of glucose originating from 1,3- β -glucan, 1,6- β -glucan and 1,3- α -glucan.

Sz. pombe cell walls contain covalently-bound CWPs

GPI-CWPs that are covalently-bound to the glucan network in *S. cerevisiae*, *C. albicans* and *Candida glabrata* can efficiently and specifically be released from cell wall preparations by hydrolysis of 1,6- β -glucan with 1,6- β -glucanase, or by breaking the phosphodiester bond between the protein and the GPI remnant using HF-pyridine or phosphodiesterase (De Groot *et al.*, 2004; Kapteyn *et al.*, 1996, 1997; Weig *et al.*, 2004; Yin *et al.*, 2005). To investigate whether cell walls of *Sz. pombe* also contain covalently-bound GPI-CWPs, we tested HF-pyridine and endo-1,6- β -glucanase treatments. Lectin blotting using the lectin concanavalin A of CWPs released by HF-pyridine revealed several distinct protein bands and a heavy smear in the size range 110–440 kDa (Figure 1). Lowering the amount of protein loaded onto the gel revealed that the smear actually comprises a highly prominent protein band of ~ 220 kDa. In contrast to *C. albicans* and *S. cerevisiae*, treatment with recombinant endo-1,6- β -glucanase hardly released visible protein bands, except for very faint bands at about 220 and 330 kDa. We surmise that the extremely branched nature of 1,6- β -glucan (therefore also termed diglucan; Magnelli *et al.*, 2005) in *Sz. pombe* may cause the observed resistance to the glucanase.

In *S. cerevisiae* and *C. albicans*, a mild alkali treatment solubilizes a second class of CWPs (De Groot *et al.*, 2005), the so-called ASL-CWPs. Mild

Figure 1. *Sz. pombe* cell walls contain covalently-bound (galacto)mannoproteins. Freeze-dried, SDS/ β -mercaptoethanol-treated walls were incubated with recombinant 1,3- β -glucanase (Quantazyme), 1,6- β -glucanase, HF-pyridine or 30 mM NaOH. Protein separation and lectin blotting with concanavalin A are described in Materials and methods. Protein bands that are described in the text are indicated by arrows

alkali extraction of *Sz. pombe* cell walls released two clearly visible protein bands with an apparent M_r of about 330 and 110 kDa, and two faint protein bands of M_r about 400 and 220 kDa, indicating that ASL-CWPs are also present in *Sz. pombe*. Finally, we performed an enzymatic digestion using recombinant endo-1,3- β -glucanase (Quantazyme). In *S. cerevisiae* and *C. albicans* Quantazyme digestion hydrolyses the 1,3- β -glucan backbone of the cell wall, resulting in the release of both classes of CWPs (De Groot *et al.*, 2004; Yin *et al.*, 2005). Quantazyme digestion in *Sz. pombe* solubilized four clearly visible protein bands, which

all seem to correspond to the mild alkali-extracted proteins, indicating that ASL-CWPs are efficiently released by Quantazyme. Fragments of 1,3- β -glucan chains remain attached to the solubilized proteins after Quantazyme treatment, explaining why the apparent M_r of the protein bands in this extract is slightly higher than the corresponding bands in the mild alkali extract (Kapteyn *et al.*, 1999). The protein band of about 220 kDa seems to correspond with the very abundant protein in the HF-pyridine extract, suggesting that it is a GPI protein that is partly solubilized by Quantazyme treatment, and that a minor amount is also liberated by mild alkali treatment. An additional very faint band at ~ 70 kDa is visible only in the 1,3- β -glucanase extract and probably originates from Quantazyme. From these data we conclude that *Sz. pombe* contains GPI-CWPs as well as ASL-CWPs, and that the high number of side-groups of the 1,6- β -glucan backbone limits the accessibility of the 1,6- β linkages for enzymatic hydrolysis, thereby preventing the release of GPI proteins by 1,6- β -glucanase treatment.

Identification of six covalently-bound CWPs

Until now, the identity of the CWPs in *Sz. pombe* was largely unknown. To identify covalently-linked CWPs, we have performed direct trypsin digestion of cell walls that were pretreated with hot SDS/ β -mercaptoethanol, followed by tandem mass spectrometry. A major advantage of this approach is that the identification of CWPs is independent of prior knowledge about the linkages between the proteins and the cell wall polysaccharides. Also, it avoids the need for protein separation on gel, which is often hampered by a high degree of protein glycosylation. Application of this method resulted in the unambiguous identification of six CWPs of *Sz. pombe*, listed in Table 1. Although the protein content in cell walls of *Sz. pombe* is only slightly lower than in *S. cerevisiae* and *C. albicans*, the number of identified proteins is significantly lower, which is in good agreement with the number of protein bands that are visible on the lectin blots. Consistent with this, the number of predicted GPI proteins in *Sz. pombe* (33) is lower than in *S. cerevisiae* (66) and *C. albicans* (104) (De Groot *et al.*, 2003). In those fungi, about half of the GPI proteins are predicted to be predominantly localized in the plasma membrane, rather

than in the cell wall (De Groot *et al.*, 2005). All four identified GPI-CWPs from *Sz. pombe* lack a dibasic motif and contain stretches of consecutive serine residues immediately upstream of the predicted GPI-attachment site, conforming to the rules for cell wall vs. plasma membrane localization (Caro *et al.*, 1997; Frieman and Cormack, 2004) (Table 2). However, we cannot exclude the possibility that some covalently-bound CWPs might escape our analysis because: (a) the expression of many cell wall protein-encoding genes is regulated during the cell cycle and is dependent on the growth conditions (Klis *et al.*, 2006); (b) proteins that lack unglycosylated tryptic peptides within the size range (~ 400 – 3500 Da) from which our instrument selects ions for fragmentation may remain undetected; (c) some evidence has been presented that the GPI protein Aah3p is partially cell wall-localized (Morita *et al.*, 2006); and (d) the amount of protein molecules present in the wall may be below the detection limit of our mass spectrometer. In the following sections the identified proteins are discussed in more detail. To determine how these CWPs are linked to the cell wall network, the fractions obtained by endo-1,3-glucanase, endo-1,6-glucanase, HF-pyridine and mild alkali treatments were also analysed by mass spectrometry.

Two identified CWPs, designated Gas1p and Gas5p after their closest *S. cerevisiae* homologues (Table 2), are GPI proteins belonging to the Gas family (GH 72) of transglucosidases. BLAST analysis indicated that in *Sz. pombe* this family consists of four proteins, three of which were predicted to be GPI proteins (De Groot *et al.*, 2003). Similar Gas (also designated Phr or Gel) protein families have been described in various ascomycetous fungi, e.g. *S. cerevisiae*, *C. albicans*, *C. glabrata* and *Aspergillus fumigatus* (Carotti *et al.*, 2004; Mouyna *et al.*, 2000; Popolo and Vai, 1999; Weig *et al.*, 2001). The most abundant Gas protein in *S. cerevisiae*, Gas1p, is predominantly localized in the plasma membrane; however, a minority of its protein molecules are covalently linked to the cell wall. In cell wall extracts of *S. cerevisiae* three (Gas1p, Gas3p and Gas5p) and in *C. albicans* two (Phr1p and Pga4p) Gas homologues have been identified (De Groot *et al.*, 2004; Yin *et al.*, 2005). In contrast to the *GAS* genes in *S. cerevisiae*, transcription of *PHR1* and *PHR2* in *C. albicans* is controlled by the external pH through the transcription factor

Table 1. Identification of covalently-linked CWPs in *Sz. pombe* by LC/MS–MS

Protein name (Accession No.)	Observed mass (<i>m/z</i>)	Calculated <i>M_r</i> (Da)	Charge state	Residues	Mascot score of sequenced peptide ^a					Peptide sequence ^b
					D ^c	HF ^c	A ^c	1,6 ^c	1,3 ^c	
GPI-CWPs										
Gas1p (SPAC19B12.02C)	526.84	1051.54	2+	20–29	53	13				SVSPVHVDGR ^d
	1072.28	3213.40	3+	43–72	27					GIAYQPNVDDSDTEGTLFV
	825.90	1649.84	2+	73–86	53					DPLSDGDACSR ^e
	854.52	1706.81	2+	189–203	55					DVPYFQELSVNAIR
Gas5p (SPAC11E3.13C)	686.38	1370.59	2+	33–44	50					QIPVGYSTNDEEVTR
	1190.73	2379.13	2+	49–71	48	85				GNAFFNSDTNER
	815.57	1628.89	2+	73–86	21					GVDYQPGGSSTLVDPLADTSICK ^e
	788.46	1574.79	2+	191–205	82	44				DLPYLQGLNINTIR
Ecm33p (SPAC1705.03C)	856.39, 1284.31	2566.26	3+, 2+	170–192	81					DIPVGYSAADVAEIR
	419.23, 837.39	836.44	2+, 1+	193–200	42	36			43	YIQEITMEGLESAQNIQISANSK
	409.73, 818.42	817.45	2+, 1+	314–321	49	39			44	GVSVNFSK
Pwp1p (SPCC1322.10)	423.73	845.46	2+	21–28	41	28	MS ^f	32	37	GALTVETK
ASL-CWPs										
Psl1p (SPAC1002.13C)	795.36	1588.71	2+	220–233	68		37		40	VSVTSPTR ^d
	565.78	1129.54	2+	313–322	72		63		28	TQWPSTQPSDGETR
Asl1p (SPAC13G6.10c)	1033.99	2066.01	2+	298–316	61	97	71	19	35	GTSAQYYVNK
	631.07, 945.94	1889.93	3+, 2+	477–494	65	115			91	GLAWIPGTDLGYSDFNVNK
										TLMTSALGFLDGHGSVER

^a Mascot score is reported as $-10 \cdot \log_{10}(P)$, where P is the absolute probability that the observed match between the experimental data and the database sequence is a random event. P value <0.05 can be reported as a score higher than 13.

^b Trypsin cleaves after K or R; the LC/MS–MS cannot discriminate between K or Q and I or L.

^c Analysed cell wall fractions. D, direct trypsin digestion of cell walls; HF, HF-pyridine; A, mild alkali extract; 1,6, 1,6- β -glucanase; 1,3, 1,3- β -glucanase CWP extracts.

^d N-terminus of the obtained peptide sequence is identical to the N-terminus of the mature protein after removing the signal peptide as predicted by SignalP 3.0.

^e Cysteine residues are S-alkylated by treatment with iodoacetamide.

^f MS₁ peptide is present but not selected for fragmentation.

Rim101p. *PHR1* expression is induced at neutral to alkaline pH and under those conditions the low pH-induced gene *PHR2* is repressed (Fonzi, 1999). The occurrence of these pH-controlled Gas homologues in *C. albicans* may reflect its evolutionary divergence into a species that can thrive in various niches (in terms of pH) within the human host. Recently, some Gas family members were reported to possess a carbohydrate-binding module (CBM 43) preceding their catalytic GH 72 domain, which may be important for substrate binding. *Sz. pombe* Gas1p does possess a CBM 43 domain, whereas Gas5p does not. This is similar to *S. cerevisiae* and *C. albicans*, where we also did not observe a direct relationship between the presence or absence of such a domain and cell-wall localization of the proteins. It is possible that functional subgroups exist

within this family. *Sz. pombe* Gas1p and Gas5p were both also identified in HF-pyridine extracts, suggesting that they are linked to 1,6- β -glucan via a GPI remnant. Both proteins were absent in endo-1,6- β -glucanase extracts, which is consistent with the absence of clear protein bands in our lectin blot experiments.

The *ECM33* family is a ubiquitous fungal gene family encoding GPI proteins that have an important role in cell wall biosynthesis, although their biochemical activity has not yet been elucidated. *ECM33* deletion mutants in *S. cerevisiae* and *C. albicans* produce cells that are spherical and swollen, show a dramatically increased sensitivity to cell wall-perturbing agents, such as Calcofluor white and Congo red, and a constitutively activated cell wall integrity pathway (Martinez-Lopez

Table 2. Functional characteristics of covalently bound CWP of *Sz. pombe*

Protein name	Closest (annotated) homologues	Putative biological function	Size (aa)	Signal peptide ^a	GPI ^b	Localization features of GPI proteins ^c	
						Dibasic motif	S/T-rich stretch
Gas1p	<i>S. cerevisiae</i> Gas1p (49% ^d)	1,3- β -Transglucosidase, GH 72 ^e , CBM 43 ^e	542	1–19	S ⁵¹⁶	No	Yes
Gas5p	<i>S. cerevisiae</i> Gas5p (48% ^d)	1,3- β -Transglucosidase, GH 72 ^e	510	1–22	S ⁴⁸⁵	No	Yes
Ecm33p	<i>Sz. pombe</i> Meu10p, <i>S. cerevisiae</i> Ecm33p (30% ^d)	Ecm33 family, unknown role in cell wall construction	421	1–19	S ³⁹⁸	No	Yes
Pwp1p	<i>C. neoformans</i> XP_001 221 446.1 <i>A. oryzae</i> BAE58538.1	Unknown but probably non-enzymatic	262	1–20	S ²³⁷	No	Yes
Psu1p	<i>S. cerevisiae</i> SUN family	Involved in septation, possibly 1,3- β -glucosidase activity	417	1–18	n/a	n/a	n/a
Asl1p	<i>Ustilago maydis</i> UM00102.1 <i>A. fumigatus</i> XP750728.1	Unknown	530	1–18	n/a	n/a	n/a

^a Predicted using the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>).

^b Most likely position for GPI anchoring as predicted using the bigPI server (http://mendel.imp.ac.at/gpi/fungi_server.html).

^c The absence of a dibasic motif (Vossen et al., 1997) and the presence of a stretch of serine and/or threonine residues (Frieman and Cormack, 2004) in the region preceding the GPI attachment site positively influence cell-wall localization of GPI proteins.

^d Percentage amino acid identity to closest *S. cerevisiae* homologue.

^e GH, glycoside hydrolase; CBM, carbohydrate-binding module families according to the carbohydrate-active enzyme classification by Coutinho and Henrissat (1999). n/a, not applicable.

et al., 2004; Pardo et al., 2004). Furthermore, *S. cerevisiae* *ecm33* Δ cells hypersecrete 1,6- β -glucosylated proteins into the growth medium (De Groot et al., 2001). These observed phenotypes strongly resemble those in *GAS* mutants, therefore the functions of these proteins might be related. The genome of *Sz. pombe* harbours two *ECM33* paralogues and both are likely to encode putative GPI proteins, although one, Meu10p, has a histidine in its hydrophobic tail. The other protein, encoded by SPAC1705.03C, was experimentally identified by mass spectrometry from trypsin-digested walls and also in HF-pyridine and Quantazyme extracts, and was termed Ecm33p. The presence of Ecm33p in the cell wall of *Sz. pombe* indicates that it has a function in cell wall construction which is not related to chitin incorporation. We hypothesize that Ecm33p-related proteins might be involved in the

modification or cross-linking of cell wall glucans, or in the covalent coupling of proteins to the glucan network. However, this still needs confirmation by biochemical means.

The fourth predicted GPI protein that was identified is encoded by SPCC1322.10. This gene encodes a protein of 262 amino acids with a very high content of serine (81) and threonine (29) residues, accounting for 42% of the protein. BLAST analysis indicated that SPCC1322.10 has no significant homology to known proteins (Table 2). It is therefore unlikely that this protein has any enzymatic activity, but it may possibly have an important function as a cell surface determinant and was named Pwp1p (*Sz. pombe* wall protein 1). The mature GPI protein lacks lysine residues and contains only one arginine. Fortunately, the latter enabled us to identify the

Figure 2. Identification of the *N*-terminal peptide of Pwp1p. Deconvoluted MS–MS spectrum of a peptide with a mass of 846.47 Da [$M + H^+$]. The corresponding peptide sequence, mainly based on assignment of abundant *y*-ions, is indicated

N-terminal end of the protein (Figure 2) and to confirm the predicted signal peptidase cleavage site (Table 1). Pwp1p was identified in a trypsin digest from isolated cell walls and in the extracts obtained with Quantazyme, HF-pyridine, and 1,6- β -glucanase, indicating that this GPI protein is likely to correspond to the abundant 220 kDa protein observed in the SDS–PAGE experiments (Figure 1). Although not identified by MS–MS fragmentation, the Pwp1p peptide was also present in the MS survey spectrum of the mild alkali extract, which is consistent with the faint 220 kDa band observed on the lectin blot of this fraction. Pwp1p, however, lacks Pir repeat-like sequences or other features of ASL-CWPs (see below) that could explain the partial alkali sensitivity. The apparent high M_r of Pwp1p can be explained by a high degree of *O*-glycosylation and *N*-glycan additions to two asparagine residues in the sequence Asn–Xaa–Ser/Thr. This would also tend to strengthen the signal when using the lectin concanavalin A for its detection.

The two remaining proteins, Psu1p and SPAC13G6.10C, were identified from mild alkali extracts (Table 1). They do not have *C*-terminal GPI anchor addition signals (Eisenhaber *et al.*, 2004), although Psu1p was originally marked as a putative GPI protein (De Groot *et al.*, 2003). In common with ASL-CWPs of *S. cerevisiae* and *C. albicans*, both proteins have a positively charged region, ²²RHPHH²⁶ in Psu1p and ²⁵HRHRR³⁰ in SPAC13G6.10C, immediately downstream of

the signal peptidase cleavage site, which might play a role in the covalent incorporation of ASL-CWPs (Yin *et al.*, 2005). Furthermore, they have a high content of serine and threonine residues in their *N*-terminal halves only, indicating that the *C*-terminal parts contain their functional domains, which is typical of ASL-CWPs but not GPI proteins (De Groot *et al.*, 2005). We conclude that both proteins are ASL-CWPs, and SPAC13G6.10c was therefore named Asl1p (alkali-sensitive linkage). *Psu1* has two paralogues (*SPBC2G2.17C* and *Adg1p*) and is homologous to the SUN family in *S. cerevisiae* (Sim1p, Uth1p, Nca3p, Sun4p) and *C. albicans* (Sim1p, Sun41p, Orf19.5896). All these proteins contain a conserved SUN domain (www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=pfam03856). In most cases this domain is found in the *C*-terminal part of the proteins, whereas the *N*-terminal part is predominantly S/T-rich. Exceptions to this are SpAdg3p, which has a reversed orientation, and Caorf19.5896, which lacks a S/T-rich part. Because of their homology to *BGLA* of *Candida wickerhamii*, *SUN* genes are frequently annotated as β -glucosidase-encoding genes. *BGLA*, together with *BGLB*, was isolated as a positive clone in an immunological screening using polyclonal antibodies against a purified extracellular exo- β -glucosidase (a GH 1 enzyme). *BGLB* turned out to encode the corresponding β -glucosidase (Skory and Freer, 1995) but is not significantly related to *BGLA* (Figure 3). Taken together, so far there is no direct evidence that

SUN proteins actually have β -glucosidase activity. Interestingly, however, inactivation of *SUN4* in *S. cerevisiae* (Mouassite *et al.*, 2000) and *PSU1* in *Sz. pombe* (Omi *et al.*, 1999) resulted in cells that were unable to complete cell division, although mitosis seemed to occur normally. *PSU1*-deficient cells were resistant to 1,3- β -glucanase digestion, indicating that their cell wall integrity was affected (Omi *et al.*, 1999). Furthermore, newly formed cells were able to divide several times before swelling and eventual lysis, indicating that the cell walls were weakened by subsequent cell divisions. From this we hypothesize that Psu1p is important to strengthen the glucan network of the bud scar during cytokinesis, but at this point it is unclear whether this involves (trans)glucosidase activity. Sun4p has been described as a 'soluble cell wall protein' that was isolated from cell walls of *S. cerevisiae* by extraction with dithiothreitol (Cappellaro *et al.*, 1998), but until now no SUN proteins have been identified in mild alkali extracts.

BLAST analysis of the putative functional domain of Asl1p indicated that it has no homologues in *S. cerevisiae* and *C. albicans* or other budding yeasts. On the other hand, significant homology (>30% identity) was found with unknown proteins from filamentous ascomycetes as well as filamentous and yeast-like basidiomycetes (Table 2). Surprisingly, this protein was identified in all protein fractions, including the 1,6- β -glucanase and HF-pyridine extracts, which both

specifically release GPI proteins in *S. cerevisiae* and *C. albicans*. Possibly the C-terminal end of Asl1p, which contains seven (out of 11) hydrophobic residues, has structural similarity with that of GPI proteins, enough to be recognized as a substrate for GPI anchor addition. Asl1p (32% S or T residues and three potential *N*-glycosylation sites) seems to correspond to the 330 kDa protein band identified on the lectin blot that is abundant in the mild alkali and Quantazyme extracts and faint in the 1,6- β -glucanase extract.

In contrast to *S. cerevisiae* and *C. albicans*, no alkali-sensitive Bgl2p or Tos1p homologues and no adhesin-like GPI proteins were identified in the cell wall of *Sz. pombe*. Although we have to keep in mind that proteins may escape identification due to technical limitations, it is interesting to speculate how differences in the cell wall proteome between these fungi may reflect differences in cell wall composition. The absence of the single Bgl2p homologue (SPAC26H5.08C) in cell walls of *Sz. pombe* may be related to the fact that it shares higher similarity with *S. cerevisiae* Bgl2p, a non-covalently-associated CWP, than with its covalently-bound paralogues, Scw4p and Scw10p. In the past, purified Bgl2p has been shown to bind to chitin as well as to 1,3- β -glucan (Mrša *et al.*, 1993), suggesting a possible role in cross-linking these polysaccharides. This would be consistent with the absence of SPAC26H5.08C in the chitin-less walls of *Sz. pombe*. Deletion of *TOS1* in *S. cerevisiae* causes a dramatically increased resistance to 1,3- β -glucanase activity. However, it is unknown what its function in cell wall integrity is and how this may be related to the absence of the Tos1p homologue (SPBP23A10.11C) in cell walls of *Sz. pombe*. Finally, the absence of adhesin-like proteins in *Sz. pombe* cell walls is consistent with the low number of proteins with adhesive properties among the predicted GPI proteins.

Figure 3. *Sz. pombe* Psu1p and the SUN family are not related to the β -glucosidase BglBp of *C. wickerhamii*. Unrooted radial tree showing the phylogenetic relationships between SUN domains of *Sz. pombe* Psu1p, SPBC2G2.17C and Adg1p, *S. cerevisiae* Sim1p, Uth1p, Nca3p and Sun4p, *C. albicans* Sim1p, Sun41p, and Orf19.5896, *C. wickerhamii* BglAp and the most homologous part of *C. wickerhamii* BglBp

General discussion

Our results show that *Sz. pombe* contains covalently bound GPI-CWPs and ASL-CWPs, similar to *S. cerevisiae* and *C. albicans*. All six proteins identified by direct trypsin digestion of the cell walls could also be extracted with either mild alkali or HF-pyridine, indicating a similar cell wall incorporation pattern of CWPs in *Sz. pombe*. Three

of the identified proteins are homologues of GPI-CWPs in *S. cerevisiae* and *C. albicans*, indicating that they are involved in the construction of parts of the cell wall network that these fungi have in common (β -glucan and proteins). On the other hand, the absence of homologues of certain CWPs from *S. cerevisiae* and *C. albicans* in the cell walls of *Sz. pombe* may underline important differences in the cell wall composition between these fungi.

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