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In silicio Identification of Glycosyl-Phosphatidylinositol-Anchored Plasma-Membrane and Cell Wall Proteins of *Saccharomyces cerevisiae*

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Use of the Von Heijne algorithm allowed the identification of 686 open reading frames (ORFs) in the genome of *Saccharomyces cerevisiae* that encode proteins with a potential N-terminal signal sequence for entering the secretory pathway. On further analysis, 51 of these proteins contain a potential glycosyl-phosphatidylinositol (GPI)-attachment signal. Seven additional ORFs were found to belong to this group. Upon examination of the possible GPI-attachment sites, it was found that in yeast the most probable amino acids for GPI-attachment are asparagine and glycine.

In yeast, GPI-proteins are found at the cell surface, either attached to the plasma-membrane or as an intrinsic part of the cell wall. It was noted that plasma-membrane GPI-proteins possess a dibasic residue motif just before their predicted GPI-attachment site. Based on this, and on homologies between proteins, families of plasma-membrane and cell wall proteins were assigned, revealing 20 potential plasma-membrane and 38 potential cell wall proteins. For members of three plasma-membrane protein families, a function has been described. On the other hand, most of the cell wall proteins seem to be structural components of the wall, responsive to different growth conditions.

The GPI-attachment site of yeast slightly differs from mammalian cells. This might be of use in the development of anti-fungal drugs. © 1997 John Wiley & Sons, Ltd.

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KEY WORDS — GPI-anchor; GPI-attachment site; yeast; Ascomycetes; fungi

INTRODUCTION

Glycosyl-phosphatidylinositol-anchored proteins (GPI-proteins) are found in all eukaryotic cells. The addition of GPI-anchors to newly synthesized proteins occurs at the membrane of the endoplasmic reticulum. Subsequently, the GPI-proteins are transported to the cell surface via the secretory route.

Precursors of proteins to be GPI-anchored contain two hydrophobic sequences: one at their amino-terminus, which is a signal sequence that

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Table 1. Putative GPI-proteins in *S. cerevisiae* and their expected ω-site (depicted in bold).

Gene name	Protein name	GPI-signal
YAL063c	Flo9	STASLEISTYA**G** SANSLLAGSGLSVFIASLLLAII
YAR050w	Flo1**	STASLEISTYA**G** SANSLLAGSGLSVFIASLLLAII
YBR067c	Tip1**	VETASNAGQRV**N** AGAASFGAVVAGAAALLL
YBR078w		SSSSSSSKKSK**G** AAPELVPATSFMGVVAAVGVAYYKIKATICVSII TLISSLMISLPFLFYYETVGSSLNFICR
YCL048w		DSKKRVISKYA**N** SANPSMQLDPLLFGTCLVAMLLF
YCR089w		WITTPIVSTYA**G** SASKFLCSKFFMIMVMVINFI
YDR055w		ASSSSSKSSKG**N** AAIMAPIGQTTPLVGLLTAIIMSIM
YDR077w	Sed1**	SASSHSVVINS**N** GANVVVPGALGLAGVAMLFL
YDR144c	Mkc7	SPTSSSSPRKE**N** GGHNLNPPFFARFITAIFHHI
YDR261c	Exg2*	LSSTTTSRKSK**N** AAISNKLTTSQLLPIKNMSLTWKASVCALAITIAALCASL
YDR349c		SNSTNRTSSAS**G** AGVRLSSPYTFNKDPAGHVTRIASLLLLSIFSILIVL
YDR522c	Sps2	AKSQGSSKKME**N** SAPKNIFIDAFKMSVYAVFTVLFSIIF
YDR534c		SSTTPQIVNYT**G** AADSIAAGTGLMGAALAAVIFL
YEL040w		ASSSTSSMSGN**N** AGANVAANWRLTVLCVILGYVL
YER011w	Tir1/Srp1**	ATKAVSEQTEN**G** AAKAFVGMGAGVVAAAAMLL
YER150w		VSSTHDVETNS**N** AANARAIPGALGLAGAVMMLL
YGR189c		EASSTNSVQIS**N** GADLAQSLPREGKLFSVLVALLALL
YHR126c		PSSTANVSVYE**G** AGMKVESKNMGYIVGVAALLFL
YHR211w	Flo5	STASLEISTYA**G** SANSLLAGSGLSVFIASLLLAII
YIL011w	Yib1	STNSSSSATSK**N** AGAAMDMGFFSAGVGAAIAGAAAMLL
YIR019c	Flo11	YSVPSISSTYQ**G** AANIKVLGNFMWLLLALPVVF
YJL078c		TSTTAKLSAYE**G** AATPLSIFQCNSLAGTIAAFVVAVLFAF
YJL171c		LSNGVRLTNMQ**N** GVWYYILAIFTAFTQVVLI
YJR004c	Agα1/Sag1**	TSTSLMISTYE**G** KASIFFSAELGSIIFLLLSYLLF
YJR150c		SSASRVIDVTT**N** GANKFNNGVFGAAAIAGAAALLL
YJR151c		SSASYTVSINT**N** GAYNFDKDNIFGTAIVAVVALLLL
YKL046c		APLNITKGSKA**G** AGIITAVIGISIVACALWLVF
YKL096w	Cwp1**	QAPNTVYEQTE**N** AGAKAAVGMGAGALAVAAAYLL
YKL097w-a	Cwp2**	SSTETISQQTE**N** GAAKAAVGMGAGALAAAMLL
YKR102w	Flo10	STASLEMSSYL**G** IANHLLTNSGISIFIASLLLAIV
YLR040c		SSSTSRTSQSQ**N** GAHAKSLYFPMALFGIFAVAL
YLR042c		TNTISSSTSTG**G** VGSVKPCLYFVLMLETIAYLFS
YLR110c		AAPTHSVTSYT**G** AAAKALPAAGALLAGAAALLL
YLR120c	Yap3*	TASATSTSSKR**N** VGDHIVPSLPLT LISLLFAFI
YLR121c		KSKRALQRAAT**N** SASSIRSTLGLLLVPSLLILSVFFS
YLR194c		GKVASVMSNST**N** GAFAGTHIAYGAGAFAVGALLL
YLR343w	Gas2	NVKYPSSEERE**N** DGTIAFKTSGFVILLISMIAAGILL
YLR391w-a	Icwp**	AVISTFSEGSG**N** VLEAGKSVFIAAVAAMLI
YMR006c		ARSSSSTANKA**N** AAAISYANTNTLMSLLGAITALFGLI
YMR008c	Plb1	ASASGSSTHKK**N** AGNALVNYSNLNTNTFIGVLSVISAVFGLI

directs the protein into the secretory pathway, and another at the carboxy-terminus, which is cleaved off and replaced by a preformed GPI-anchor by a putative GPI-protein transamidase complex (Hamburger *et al*., 1995; Benghezal *et al*., 1996).

The GPI-attachment signal is composed of a cleavage/attachment domain, a spacer domain of approximately 8–12 amino acids, and a terminal hydrophobic domain of at least 11 amino acids. The attachment site, the ω-site, has to be a small

Table 1. *Continued*.

Gene name	Protein name	GPI-signal
YMR200w	Rot1	NRHKTNAIKRQ**N** TSFLTSNAIWYISAGMLGVGSLLFLAF
YMR215w	Gas3	SSKSKGVGNIV**N** VSFSQSGYLALFAGLISALL
YMR307w	Gas1*	SSASSSSSSKK**N** AATNVKANLAQVVFTSIISLSIAAGVGFALV
YNL190w		TYGPGEKARKNN**N** AAPGPSNFNSIKLFGVTAGSAAVAGALLLL
YNL300w		NTTTHEISTYV**G** AAVKGSVAGMGAIMGAAAFALL
YNL322c	Kre1*	IKSAIKKTVSH**N** EAQHLGMSSFTSILGGLLTVLIWFL
YNL327w	Egt2	TIKPPSISTYS**G** AAGQLTIRIGSLLLGLISFLL
YNR044w	Aga1**	TSSMVTISQYM**G** SGSQTRLPLGKLVFAIMAVACNVIFS
YOL030w	Gas5	TSSSQSSSKSK**G** AAGIIEIPLIFRALAELYNLVL
YOL132w	Gas4	EDKDDLKRKHR**N** SASISGPLLPLGLCLLFFTFSLFF
YOL155c		KTSTGIIVQSE**G** IAAGLNANTLNALVGIFVLAFFN
YOR009w		SKTTGIVEQTE**N** GAAKAVIGMGAGALAAVAAMLL
YOR010c	Tir2	QATSTVSEQTE**N** GAAKAVIGMGAGVMAAAAMLL
YOR214c		PGNITTIGGYE**N** SSSSLMPSMGILSFLFGLYLLLHP
YOR382w		SSSSSSSASSS**G** AAPAAFQGASVGALALGLISYLL
YOR383c		SSSTAELSSYT**G** AADAITAGTGLMGAALAAVMLL
YPL130w		SNISSLNEDYD**N** ASNFLTPTTVALAVLLTILLFIQAY
YPL261c		LGPLPDDKKLK**N** DAKYSFMNYFIITCIGIIM

Dibasic motifs just prior to the putative ω-site are underlined (see text). Ybr078p is also shown here, although an extra transmembrane domain following the GPI-attachment signal is encoded by the ORF.
*Known plasma-membrane protein; **known cell wall protein.

amino acid, and is followed by two small amino acids at the carboxyl side, the ω+1 and the ω+2 sites. The requirement for the ω- and the ω+2 sites are the most stringent (Coyne *et al*., 1993; Nuoffer *et al*., 1993). The structure requirements, however, are not identical between mammalian cells and yeast cells (Udenfriend and Kodukula, 1995).

In *Saccharomyces cerevisiae*, GPI-proteins are found not only attached to the plasma-membrane, but also as an intrinsic part of the cell wall. Whereas GPI-proteins linked to the plasma-membrane possess an intact GPI-anchor, GPI-proteins in the cell wall have their GPI-anchor trimmed at the plasma-membrane, prior to incorporation into the cell wall (Lu *et al*., 1994, 1995; Müller *et al*., 1996). The exact structure of the GPI-remnant present in mature cell wall proteins is still unknown, but it lacks at least the phospholipid part. Therefore, in the case of cell wall proteins (CWPs), the phospholipid of the GPI-moiety is not the anchoring structure. Instead, the glycan part of the GPI-remnant has been shown to be bound to the cell wall glucans (Kapteyn *et al*., 1996; Van Der Vaart *et al*., 1997a; F. Fujii, pers. commun.). We therefore suggest the name GPI-protein, instead of GPI-anchored protein.

With the complete genome of *S. cerevisiae* sequenced, we sought to identify the GPI-proteins in yeast, and to determine which GPI-proteins are destined for the cell wall and which for the plasma-membrane.

METHODS

The non-redundant open reading frames (ORFs) from the *S. cerevisiae* genome were retrieved from MIPS: Martinsrieder Institut fur Protein Sequenzen (http://www.mips.biochem.mpg.de/yeast/). These sequences were first screened for the presence of a signal sequence, using PSIGNAL (Von Heijne, 1986), with a cut-off value of 3·5.

In sequences containing a signal sequence, the presence of potential transmembrane spans was calculated according to the KKD algorithm (Klein *et al*., 1985) with the threshold value of 15 for the peripheral/integral odds (Nelissen *et al*., 1995).

The amino acid sequences of the potential CWPs and of families of potential plasma-membrane proteins were aligned with the multiple alignment program PILEUP, of the Wisconsin

Sequence Analysis Package (Version 8, (1994) Program Manual, Genetics Computer group, 575 Science Drive, Madison, WI 53711, U.S.A.).

The evolutionary distance *D* between two proteins was calculated as described in Nelissen *et al*. (1995). Phylogenetic trees were constructed using the free PHYLIP package: Phylogeny Interference Package (version 3.57c).

RESULTS

All 6218 known ORFs in the *S. cerevisiae* genome were analysed for the presence of a putative signal sequence in the encoding protein. The algorithm predicts the presence of a signal peptide with an accuracy of 75–80% (Von Heijne, 1986). This calculation identified 686 potential secretory proteins. Within this subset, 55 ORFs that encode proteins containing only one additional hydrophobic domain at the extreme C-terminus were found. In this set it was determined whether a potential GPI-attachment signal could be found according to the consensus rules described by Nuoffer *et al*. (1993) and by Udenfriend and Kodukula (1995). This revealed 51 proteins with a potential GPI-attachment signal. Four ORFs do not predict a clear GPI-attachment site: *YAL058w/CNE1*, *YDR506c*, *YKR032w* and *YPR157w*. To the group of GPI-proteins seven ORFs were added that were missed in the original screen for proteins with a secretion signal, or that were not present in the database searched (*YBR078w*, *YDR144c*, *YDR349c*, *YDR534c*, *YKL097w-a*, *YLR391w-a* and *YOR382w*). These were either known GPI-proteins or were found through homology searches with known GPI-proteins. *YBR078w* might represent a pseudogene, because it has a GPI-attachment signal, which is followed by an extra transmembrane domain. In Table 1, the 58 different GPI-proteins and their putative ω-sites for GPI-attachment are presented. In *S. cerevisiae* the most probable amino acids for GPI-attachment are asparagine and glycine.

GPI-proteins in yeast have been found both in the plasma-membrane and as an intrinsic part of the cell wall. All GPI-proteins that are known not to be covalently linked to the cell wall, Exg2p, a β1,3-exoglucanase (Cid *et al.*, 1995), Gas1p, which is involved in cell wall construction (Nuoffer *et al.*, 1991; Ram *et al.*, 1995), Yap3p, an aspartyl protease (Ash *et al.*, 1995), and Kre1p, which presumably is involved in coupling GPI-proteins to glucan (Lu *et al.*, 1995; Roemer and Bussey, 1995), contain a dibasic amino acid motif just prior to their ω-site (see Table 1; Vossen *et al.*, 1997). The function of these basic amino acids at that location is not known (see Discussion for an hypothesis). It is, however, tempting to postulate that proteins with a dibasic motif amino-terminal to their GPI-signal are destined for the plasma-membrane (see Table 2). Other proteins, of unknown localization, with strong sequence similarities to any of these putative plasma-membrane proteins but lacking this motif are also listed in Table 2.

Several families of GPI-anchored plasma-membrane proteins were assigned. The Gas-family, in which Gas1p is known to be involved in cell wall construction, consists of five homologs (Figure 1a), only two of which contain the dibasic motif. Deletion of *GAS1* renders the cell hypersensitive to Calcofluor White, due to a weakened cell wall (Ram *et al.*, 1995). Deletions of *GAS2,3,4,5* also renders the cells hypersensitive to Calcofluor White (A. F. J. Ram, unpublished results), indicating that the homologs of Gas1p may have a function in cell wall construction as well.

The Yap3-family of GPI-anchored aspartyl proteases consists of four members (Figure 1b). For two members, Yap3p and Mkc7p, proteolytic activity has been demonstrated. *In vitro*, Yap3p proteolytically cleaved several pro-hormones at di- and monobasic sites (Cawley *et al.*, 1993; Azaryan *et al.*, 1993); whereas *in vivo*, pro-hormones were cleaved under conditions of either overexpression of Yap3p or overexpression of the pro-hormone (Egel-Mitani *et al.*, 1990; Bourbonnais *et al.*, 1993). The physiological substrates of Yap3p and Mkc7p have not been identified.

The Sps2-family consists of four members (Figure 1c), three of which contain the dibasic motif. Sps2p is a sporulation-specific protein (Percival-Smith and Segall, 1986, 1987).

Plb1p is a lysophospholipase. In a *plb1* deletion strain no residual lysophospholipase/phospholipase B activity could be detected in culture supernatants or cell extracts. However, the mutant had no apparent phenotypic defect, suggesting that Plb1p is functionally redundant with another protein (Lee *et al.*, 1994). This could be the product of *YMR006c*, which, interestingly, is located almost next to *PLB1* on chromosome XIII.

Five potential plasma-membrane GPI-proteins do not show a strong homology to any of the other plasma-membrane proteins. For two of these, Kre1p (Lu *et al.*, 1995; Roemer and Bussey, 1995)

Table 2. Known and putative plasma-membrane proteins and related proteins, containing a GPI-anchor attachment signal. The proteins are grouped in families.

Gene name	Protein name	Dibasic motif	AA no.	*N*-sites no.	S/T (%)	Reference
Gas-family:						
						Vai *et al*. (1991)
						Nuoffer *et al*. (1991)
YMR307w	Gas1p	+	559	10	24	Ram *et al*. (1995)
YLR343w	Gas2p	−	555	1	13	
YMR215w	Gas3p	−	524	7	22	
YOL132w	Gas4p	+	471	2	13	
YOL030w	Gas5p	−	484	6	23	
Yap3-family:						
YDR144c	Mkc7	+	596	9	26	Komano and Fuller (1995)
YDR349c		−	596	15	25	
YLR120c	Yap3	+	569	10	26	Ash *et al*. (1995)
Ylr121c		+	508	11	21	
Sps2-family:						
YBR078w		+	478	11	28	
YCL048w		+	463	3	13	
YDR055w		−	444	15	29	
YDR522c	Sps2	+	502	5	14	
Plb1-family:						
YMR006c		−	706	25	23	
YMR008c	Plb1	+	664	20	18	
Others:						
YDR261c	Exg2	+	562	15	17	Cid *et al*. (1995)
YMR200w	Rot1	+	256	5	19	
YNL190w		+	204	11	29	
YNL322c	Kre1	+	313	0	41	Roemer and Bussey (1995)
YPL261c		+	102	0	14	

and Exg2p (Cid *et al*., 1995) a function has been described.

In *S. cerevisiae*, 13 genes encoding CWPs have been described: *AGα1* (Lipke *et al*., 1989), *AGA1* (Roy *et al*., 1991), *CWP1, CWP2* (Van Der Vaart *et al*., 1995), *TIP1* (Kondo and Inouye, 1991; Van Der Vaart *et al*., 1995), *TIR1/SRP1* (Marguet *et al*., 1988), *TIR2* (Kowalski *et al*., 1995), *FLO1* (Teunissen *et al*., 1993), *FLO5* (Bidard *et al*., 1994), *FLO11* (Lo and Dranginis, 1996), *SED1* (Van Der Vaart *et al*., 1996), *YCR089w* (Van Der Vaart *et al*., 1997b) and *YLR391w-a* (Moukadiri *et al*., 1997). Furthermore Flo9p and Flo10p have been described as potential CWPs (Teunissen and Steensma, 1995). They all possess an N-terminal signal peptide and a putative GPI-anchor addition signal at their C-terminus. Furthermore, they all contain serine- and threonine-rich regions, which probably become heavily *O*-glycosylated with short mannose side chains, thereby conferring a rod-like structure on these regions (Jentoft, 1990; Klis *et al*., 1997). The serine- and threonine-rich stretch usually covers the C-terminal part of the protein, but sometimes the whole protein.

In addition to the 15 CWPs described so far, 23 additional ORFs encode potential CWPs (Table 3). They all meet the criteria for having a GPI-attachment signal, stretches rich in serine and threonine and lacking the dibasic motif. In addition, many show sequence similarity to known CWPs.

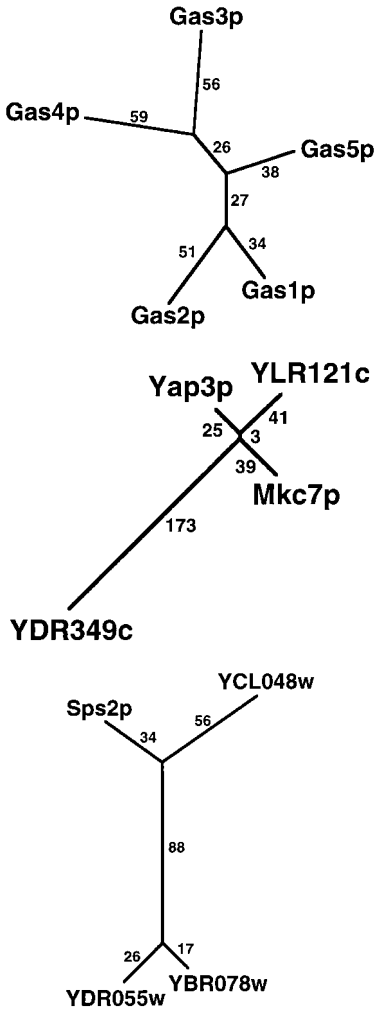

Figure 1. Phylogenetic trees of GPI-proteins potentially localized in the plasma-membrane. Each number corresponds to the phylogenetic distance *D* multiplied by 100. (a) Gas-family; (b) Yap3-family; (c) Sps2-family.

Based on sequence homology, several families of CWPs can be assigned. The Tir-family, some members of which were described by Marguet *et al.* (1988), Kondo and Inouye (1991), Van Der Vaart *et al.* (1995), and Kowalski *et al.* (1995), is depicted in Figure 2a. Our search revealed five new members of this family (Tir3p–Tir7p). The Flo-family, involved in flocculation of cells, consists of five members (Figure 2b), but also includes some pseudogenes (Teunissen and Steensma, 1995). The sexual agglutinins were grouped as a family based mostly on their functional homology (Table 3). Seventeen (potential) CWPs do not show a strong homology with any of the other CWPs, and for one a function is known: Egt2p is involved in cell separation after cytokinesis and is expressed in the G1-phase of the cell cycle (Kovacech *et al.*, 1996).

Another striking feature of many CWPs is that they contain repeats. In general, the repeats are very rich in serine and threonine, and to a lesser extent in alanine, valine, proline and glutamate. For many of these proteins the repeats may be a means of spanning the cell wall and exposing the functional domain to the outside of the cell. The repeats are strongly conserved within families, as described by Teunissen *et al.* (1995) for the Flo-family and by Marguet *et al.* (1988) for some members of the Tir-family. Although plasma-membrane GPI-proteins also often have a serine/threonine-rich stretch, no repeats are found in these proteins.

DISCUSSION

All ORFs encoded by the genome of *S. cerevisiae* were analysed for the presence of a signal peptide and a GPI-attachment signal in the predicted proteins. The 58 candidate proteins all have serine/threonine-rich stretches. The serine/threonine-rich domain, which will be heavily *O*-glycosylated, probably functions in protruding the protein in or partially through the cell wall. GPI-proteins are found in the plasma-membrane or in the cell wall and some differences between these proteins were noted (Table 4). Plasma-membrane proteins contain a dibasic residue motif just N-terminal to the ω-site for GPI-attachment. Cell wall proteins often have repeats in their serine/threonine-rich domain. Furthermore, mature CWPs have trimmed GPI-anchors and are glucosylated, as opposed to plasma-membrane proteins.

None of the GPI-proteins that have been found to be covalently linked to the cell wall, contain a dibasic residue motif N-terminal to the ω-site for GPI-attachment. The function of such a dibasic residue motif in plasma-membrane proteins is not known. It may be a targeting signal for e.g. receptor-mediated endocytosis. Such a motif has been described for endoplasmic reticulum-membrane proteins as interacting with the coatomer in vesicle-mediated transport (Cosson and Letourneur, 1994). Alternatively, this motif may function as a cleavage-site for plasma-membrane-localized proteases. In this way proteins can be removed from the cell surface, thereby

Table 3. Known and putative cell wall proteins containing a GPI-anchor attachment signal, rich in serine and threonine and lacking a dibasic residue motif.

Gene name	Protein name	AA no.	*N*-sites no.	S/T (%)	Reference
Tir-family:					
YBR067c	Tip1	210	0	31	Van der Vaart *et al.* (1995) Kowalski *et al.* (1995)
YER011w	Tir1/Srp1	254	0	31	Marguet *et al.* (1988) Kowalski *et al.* (1995)
YIL011w	Yib1	269	1	35	
YJR150c	Tir3*	298	2	34	
YJR151c	Tir4*	1161	7	48	
YKL096w	Cwp1	239	1	29	Van der Vaart *et al.* (1995)
YKL097w-a	Cwp2	92	0	27	Van der Vaart *et al.* (1995)
YLR040c	Tir5*	224	3	25	
YOR009w	Tir6*	487	5	41	
YOR010c	Tir2	251	0	36	Kowalski *et al.* (1995)
Flo-family:					
YAL063c	Flo9	1322	17	41	Teunissen and Steensma (1995)
YAR050w	Flo1	1537	14	41	Teunissen *et al.* (1993)
YHR211w	Flo5	1075	6	40	Bidard *et al.* (1994)
YKR102w	Flo10	1169	12	41	Teunissen and Steensma (1995)
YIR019c	Flo11	1367	2	50	Lo and Dranginis (1996)
Sed1-family:					
YDR077w	Sed1	338	5	43	Van der Vaart *et al.* (1996)
YER150w		148	3	29	
Yel040-family:					
YEL040w		467	9	29	
YGR189c		507	3	36	
Agglutinin family:					
YJR004c	Aga1/Sag1	650	11	30	Lipke *et al.* (1989)
YNR044w	Aga1	725	0	54	Roy *et al.* (1991)
Others:					
YCR089w		1609	15	45	Van der Vaart *et al.* (1996)
YDR534c		528	3	40	
YHR126c		159	7	21	
YIL171c		396	11	21	
YJL078c		881	8	42	
YKL046c		449	12	13	
YLR042c		161	3	32	
YLR110c		133	3	30	
YLR194c		254	1	41	
YLR391w-a	Icwp	238	1	42	Moukadiri *et al.* (1997)
YNL300w		102	1	37	
YNL327w	Egt2	1041	19	38	Kovacech *et al.* (1996)
YOL155c		967	5	42	
YOR214c		236	4	20	
YOR382w		153	1	42	
YOR383c		204	0	41	
YPL130w		223	3	17	

*Proposed synonym.

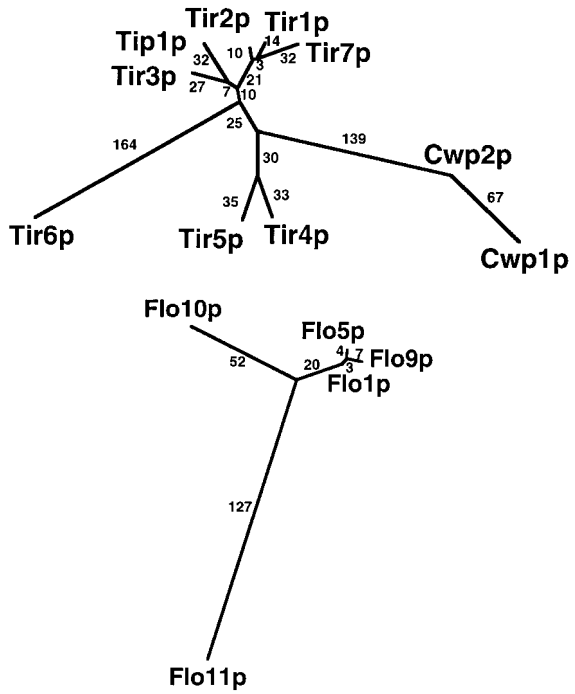

Figure 2. Phylogenetic trees of GPI-proteins potentially localized in the cell wall. Each number corresponds to the phylogenetic distance *D* multiplied by 100. (a) Tir-family; (b) Flo-family.

preventing their incorporation into the cell wall, or they can be removed after incorporation into the cell wall. The plasma-membrane proteases Yap3p or Mkc7p might be involved in this cleavage step. This may at the same time define substrates for these plasma-membrane proteases, for which so far no substrates have been found (Egel-Mitani *et al.*, 1990; Azaryan *et al.*, 1993; Bourbonnais *et al.*, 1993; Cawley *et al.*, 1993; Ash *et al.*, 1995; Komano and Fuller, 1995). However, when a dibasic motif was introduced before the ω-site for GPI attachment in Cwp2p (in the Cwp2-α-galactosidase chimeric protein) the protein was still found in the cell wall (M. J. Van Der Vaart, pers. commun.). One should realize that this was only determined in cells overexpressing the mutated protein. In these cells, much of the overproduced substrate could have escaped proteolysis, or receptor-mediated endocytosis.

The cell wall of *S. cerevisiae* has a layered structure, consisting of about equal amounts of glucan and heavily mannosylated proteins. Glucan and some chitin form the inner skeletal layer, which is interspersed with and surrounded by mannoproteins. Some glycoproteins are non-covalently linked to the cell wall as demonstrated by their extractability with hot sodium dodecyl sulfate, but the bulk of the wall proteins can be extracted only by β-glucanases, suggesting that they are tightly linked to the β-glucan skeleton of the cell wall (Klis, 1994). To date, all genes that code for glucanase-extractable CWPs have been found to contain a GPI-anchor attachment signal (Lipke *et al.*, 1989; Kondo and Inouye, 1991; Roy *et al.*, 1991; Teunissen *et al.*, 1993; Van Der Vaart *et al.*, 1995). For Agα1p (Wojciechowicz *et al.*, 1993), Cwp1p (Shimoi *et al.*, 1995), Cwp2p (Van Der Vaart *et al.*, 1997a), and Tip1p (F. Fujii, pers. commun.), the addition of a GPI-anchor has been biochemically confirmed. Van Berkel *et al.* (1994) showed that addition of the C-terminal 30 amino acids of Agα1p, which includes the GPI-attachment signal, to α-galactosidase from a plant (guar) is sufficient for incorporation of the chimeric protein into the cell wall.

Table 4. Characteristics of yeast GPI-proteins. The proposed characteristics are based on the analysis of known representatives of each group.

Characteristics	GPI-proteins	
	Plasma-membrane	Cell wall
Signal peptide	+	+
GPI-attachment signal	+	+
Serine/threonine-rich	+	+
Repeats	−	Often
Dibasic motif preceding ω-site	+	−
GPI-anchor trimmed	−	+
β-Glucosylated	−	+

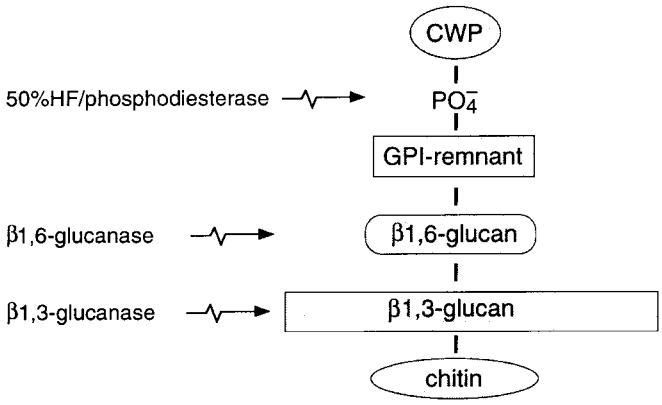

Figure 3. The cell wall building block: incorporation of cell wall proteins (CWPs) into the cell wall. On the left-hand side treatments to disrupt specific linkages are depicted (Kapteyn *et al.*, 1996).

The model explaining how the GPI-remnant is involved in the incorporation of CWPs into the cell wall is shown in Figure 3. Biochemical studies have shown that the GPI-moiety of wall-bound Agα1p is modified, and lacks at least the inositol and the phospholipid part (Lu *et al.*, 1994). Cell wall anchorage of Agα1p was accompanied by addition of β1,6-glucan (Lu *et al.*, 1995). Like Agα1p, other CWPs could be released from the cell wall by β1,3-glucanases. These proteins were shown to contain β1,6-glucan (Montijn *et al.*, 1994; Van Berkel *et al.*, 1994; Van Der Vaart *et al.*, 1995, 1996; Kapteyn *et al.*, 1996). From these proteins, the β1,6-glucan could be released by treatment with aqueous HF, which is known to cleave phosphodiester bonds, suggesting that β1,6-glucan is attached to the GPI-anchor remnant (Kapteyn *et al.*, 1996). For Cwp2p and Tip1p it was shown that β1,6-glucan is attached to the GPI-remnant, most probably to what is left of its glycan part (Van Der Vaart *et al.*, 1997a; F. Fujii, pers. commun.). Recently it was established that Agα1p, Cwp1p, and other CWPs form a complex with β1,3-glucan through their β1,6-glucan moiety, and that the attachment of this β1,3-/β1,6-glucan heteropolymer is responsible for anchoring CWPs (Kapteyn *et al.*, 1996). In its turn, β1,3-glucan may become covalently linked to chitin in the cell wall (Hartland *et al.*, 1994; Kollár *et al.*, 1995). In this way the glucans and proteins form an entity, constituting the cell wall.

Similar β-glucosylated CWPs have been found in various filamentous and yeast-like members of the Ascomycetes, suggesting that this attachment-structure is a common feature of the outer layer of the cell wall of the Ascomycetes (Kapteyn *et al.*, 1994; Bailey *et al.*, 1996; Schoffelmeer *et al.*, 1996; Staab *et al.*, 1996; Montijn *et al.*, 1997).

Why does a yeast cell have the information for the synthesis of almost 40 CWPs of similar structure? For the agglutinins and at least three of the flocculins (Flo1p, Flo5p, Flo11p), as well as for Egt2p, a function has been identified. Most of the other CWPs are probably structural proteins functioning as building block. It has been found that mutants with a deletion of one or more CWPs have a weakened cell wall (Van Der Vaart *et al.*, 1995), although they have no growth phenotype under many conditions (Kowalski *et al.*, 1995). It is possible that different proteins are required under different growth conditions. Indeed, for some CWPs, especially members of the Tir-family, it has been shown that their synthesis is induced upon stress conditions. Tip1p was identified as a cold and heat shock-inducible protein (Kondo and Inouye, 1991). *SRP1/TIR1* expression is increased when cells are grown in glucose (Marguet and Lauquin, 1986), by cold shock and under anaerobic growth conditions (Donzeau *et al.*, 1996). Interestingly, *CWP1* transcripts and protein levels are induced in mutants with a weakened cell wall such as *fks1*Δ and *gas1*Δ (Ram *et al.*, 1996). *SED1*, which belongs to another CWP-family, also responds to several stress conditions, such as a weakened cell wall or a temperature shock (L. H. P. Caro and A. F. J. Ram, unpublished results). Apart from determining the strength of the wall, CWPs might determine the permeability of the cell wall. Deleting *CWP2* results in cells with increased permeability, as demonstrated by their

hypersensitivity to Zymolyase (Van Der Vaart *et al*., 1995). Furthermore, it is tempting to speculate that a different set of CWPs is incorporated into cell walls of pseudohyphally growing cells, as has been described for *Candida albicans* (Kapteyn *et al*., 1994), or in spore walls. Flo11p is produced only in haploid cells (Lo and Dranginis, 1996).

The Tir-family of CWPs has homology to the *PAU*-family (Viswanathan *et al*., 1994), encoding serine-poor proteins (seripauperins). The *PAU*-genes encode proteins with a signal sequence, but no additional hydrophobic regions. The gene products are homologous to the N-terminus of Tir1p, which does not include the serine/threonine-rich part of Tir1p. Since the *PAU*-products do not have a GPI-attachment signal, it seems unlikely that they are members of the Tir-family.

Analysis of the GPI-signal containing proteins revealed some interesting characteristics of the yeast genome. Often duplications are found of the serine/threonine-rich parts within CWPs. On several occasions two very homologous genes are located next to each other on the chromosome, indicative of gene-duplication. In the Tir-family of CWPs this was found for *TIR3/YJR150c* and *TIR4/YJR151c* on chromosome X, for *CWP1/YKL096w* and *CWP2/YKL097w-a* on chromosome XI, and for *TIR6/YOR009w* and *TIR2/YOR010c* on chromosome XV. Furthermore, many genes were found on two copies of duplicated chromosomal regions, as described by Wolfe and Shields (1996).

Some pseudogenes have been identified in the Flo-family containing frame-shifts and sometimes missing the C-terminus: *YAL065*, *YAR061w/062w* and *YHR213w* (Teunissen and Steensma, 1995). In the Sps2 family of plasma-membrane GPI-proteins *YBR078w* might be a pseudogene since after its putative GPI-attachment site, an additional membrane-spanning domain is found.

As reported here, yeast seems to preferentially use asparagine and glycine as GPI-attachment sites, whereas mammalian cells use serine and asparagine (Udenfriend and Kodukula, 1995). This indicates that there is a difference in the specificity of yeast and mammalian transamidase, which might be important for anti-fungal drug development.

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