Multiple sequence signals determine the distribution of glycosylphosphatidylinositol proteins between the plasma membrane and cell wall in *Saccharomyces cerevisiae*

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Glycosylphosphatidylinositol (GPI)-anchored cell wall proteins (GPI-CWPs) play an important role in the structure and function of the cell wall in *Saccharomyces cerevisiae* and other fungi. While the majority of characterized fungal GPI-anchored proteins localize to the cell wall, a subset of GPI proteins are thought to reside at the plasma membrane and not to traffic significantly to the cell wall. The amino acids immediately upstream of the site of GPI anchor addition (the ω site) are the primary signal determining whether a GPI protein localizes to the cell wall or to the plasma membrane. Here, evidence was found that in addition to this ω -proximal signal, other sequences in the protein can impact the distribution of GPI proteins between cell wall and membrane. In particular, it was found that long regions rich in serine and threonine residues (a feature of many cell wall proteins) can override the ω -proximal signal and redirect a model GPI plasma membrane protein to the cell wall.

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INTRODUCTION

Glycosylphosphatidylinositol (GPI)-anchored cell wall proteins (GPI-CWPs) are key protein constituents of the yeast cell wall. GPI proteins are modified by addition of a GPI anchor in the endoplasmic reticulum, which is required for trafficking through the normal secretory pathway to the plasma membrane (Doering & Schekman, 1996). GPI-CWPs are then liberated from the plasma membrane and covalently cross-linked to 1,6- β -glucan in the cell wall through a remnant of the GPI anchor (Klis *et al.*, 1997; Lu *et al.*, 1994). The domain structure of GPI proteins is conserved. All contain a hydrophobic signal sequence at the N terminus and a second hydrophobic sequence at the C terminus that signals GPI anchor addition to the so-called ω amino acid, located between 17 and 25 aa from the C terminus of the protein (Caras & Weddell, 1989; Caras *et al.*, 1989; Moran & Caras, 1991). In addition, many GPI-CWPs contain regions rich in serine and threonine residues. GPI-CWPs are found in many fungal species in addition to *Saccharomyces cerevisiae*, including *Candida albicans* (Kapteyn *et al.*, 1994), *Candida tropicalis* (Hoyer *et al.*, 2001) and *Candida glabrata* (Frieman *et al.*, 2002). Functionally, GPI-CWPs have diverse roles. In *S. cerevisiae*,

described functions for GPI-CWPs range from enzymic roles in cell wall biosynthesis [for example, Gas1p (reviewed by Popolo & Vai, 1999)] to roles in structural rigidity of the cell wall [for example, Cwp2p (van der Vaart *et al.*, 1995)] and non-structural roles in surface variation [for example, Flo1p (Teunissen *et al.*, 1993; Watari *et al.*, 1994) or Flo11p (Lo & Dranginis, 1996)]. In the fungal pathogens *C. albicans* and *C. glabrata* some GPI-CWPs are adhesins mediating adherence to host cells [for example, Epa1p in *C. glabrata* (Cormack *et al.*, 1999) and Hwp1p, Als1p and Als5p in *C. albicans* (reviewed by Sundstrom, 2002)].

While the majority of GPI proteins in *S. cerevisiae* are thought to be GPI-CWPs (incorporated into the cell wall), there are some that are largely retained at the plasma membrane. In several instances, this difference in plasma membrane or cell wall localization has been shown to be important for function. For the protein Ecm33p, correct localization of the GPI protein to the membrane is important for viability. If Ecm33p is mis-localized to the cell wall by changing its GPI anchor to that of a GPI-CWP, the cells are non-viable (Terashima *et al.*, 2003). In experiments from our own lab, we found that optimal function of the *C. glabrata* Epa1p adhesin also depends on the nature of the GPI anchor signal. Chimeric constructs in which the ligand-binding domain of Epa1p was fused to GPI signal sequences were fully functional to mediate adherence when the fusions were made to GPI anchor signals from

Abbreviations: GPI, glycosylphosphatidylinositol; GPI-CWP, GPI-anchored cell wall protein; GPI-PMP, GPI plasma membrane protein; HA, haemagglutinin.

GPI-CWPs, but were significantly compromised for function when the fusions were made to GPI anchor signals from GPI plasma membrane proteins (GPI-PMPs) (Frieman & Cormack, 2003).

How does the cell recognize two classes of GPI proteins and ensure that GPI-PMPs remain primarily membrane-associated while GPI-CWPs are removed from the plasma membrane, eventually to be cross-linked to glucan? Klis proposed, based on bioinformatic considerations, that GPI-PMPs contain at least two basic amino acids in the region upstream of the ω site, while GPI-CWPs do not have such a dibasic motif (Caro *et al.*, 1997). There is considerable experimental support that the dibasic motif does act to retain GPI proteins at the plasma membrane. Using fusions of the GPI signal sequences from *S. cerevisiae* to α -galactosidase, Hamada *et al.* (1998) found a good correlation between presence or absence of the dibasic motif and partitioning of the fusion protein to the plasma membrane or cell wall. Analysis of various point mutations in specific GPI anchor signal sequences also supported the importance of the dibasic motif in GPI protein localization. Experiments from our own lab also support the overall importance of the amino acids upstream of the ω -proximal site in GPI protein targeting. If a GPI-CWP, such as Cwp2p, was mutated to carry a dibasic motif preceding the ω site, it no longer trafficked to the cell wall, but was retained at the plasma membrane. Conversely, if we mutated the dibasic motif in a model GPI-PMP to other amino acids, the mutant protein was efficiently targeted to the cell wall (Frieman & Cormack, 2003).

In contrast to these studies, other work suggests that the dibasic motif model for GPI protein distribution may be too simplistic (De Sampaio *et al.*, 1999). Importantly, this paper showed that Gas1p or a fusion protein between α -galactosidase and the GPI signal of Gas1p were found primarily in the cell wall even though, according to the dibasic motif model, these should have been localized primarily to the plasma membrane. This result, combined with the demonstrated role of the ω -proximal site in determining distribution of protein between the plasma membrane and cell wall (Frieman & Cormack, 2003; Hamada *et al.*, 1998), suggests that more than one signal in the protein may impact on the ultimate distribution of GPI proteins in the cell.

In the current paper we begin to define a second signal that affects the distribution of GPI proteins between membrane and cell wall, and which in fact is able to override the ω -proximal signal. We find that amino acid stretches rich in serine and threonine residues promote localization to the cell wall even in proteins whose ω -proximal signal contains a dibasic motif (and which therefore would be expected to localize to the plasma membrane). These data show that the cell can integrate multiple sequence signals in determining the ultimate distribution of GPI proteins between cell wall and cell membrane.

METHODS

Strains and media. The *S. cerevisiae* strain used for the experiments was BY4742 *MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0*. Media were prepared as described by Sherman *et al.* (1986). YP media contained glucose (2%, w/v); synthetic complete media (SC) was supplemented with Casamino acids (0.6%) and, where appropriate, uracil (25 mg l⁻¹). For galactose induction, strains were grown in 2% raffinose and induced with 2% galactose for 2 h.

Transformation of *S. cerevisiae*. Transformation of *S. cerevisiae* was carried out using the modified lithium acetate protocol (Gietz *et al.*, 1992). Cells were grown in YPD to early exponential phase and collected by centrifugation. They were washed twice with water and resuspended in 0.1 M lithium acetate, 1 mM EDTA and 10 mM Tris, pH 7.5. Transforming DNA (1 μ g) with 100 μ g denatured salmon sperm DNA was added to the cells in 0.5 ml 0.1 M lithium acetate, 40% PEG 3350, 1 mM EDTA and 10 mM Tris, pH 7.5. The mix was incubated at 30 °C for 20 min, heat-shocked at 42 °C for 20 min and plated on SC-Ura plates for selection of transformants.

Plasmid constructs. The *YPS1* constructs were made by cloning the C terminus of *YPS1* into pEpa1p(33-933) (Frieman & Cormack, 2002) whose expression is under the control of a galactose-inducible (*GALS*) promoter. The *YPS1* fragment was amplified by PCR with primers 905 (5'-AACCGCTCGAGTAAGGCACCAGGCTATACAA-CACTTG-3') and 616 (5'-GCGAATTCTTATCAGATGAATGCAAA-AAGAAGAGAAATTAATG-3'), digested with *Xho*I and *Eco*RI and ligated into an appropriately digested vector. The resulting plasmid was named pBC570. Constructs ST₈₆₁₋₉₆₀ ω YPS1, ST₇₆₁₋₉₆₀ ω YPS1, ST₆₃₃₋₉₆₀ ω YPS1, ST₅₈₃₋₉₆₀ ω YPS1 and ST_{FLO1} ω YPS1 were made by PCR amplification of the appropriate fragments and cloning into the *Xho*I site of pBC570. Construct ST₈₆₁₋₉₆₀ ω YPS1 was made with primers 1915 (5'-CCGGCTCGAGGCTATTTCCAAAAATGGTAAG-GTGT-3') and 1912 (5'-CCGGCTCGAGTATAACAAGCTTAACA-ATCCAGGT-3'). ST₇₆₁₋₉₆₀ ω YPS1 was made with primers 1915 (see above) and 1913 (5'-CCGGCTCGAGTCTTGATTATGACTGTTCG-TTAGTC-3'). ST₆₃₃₋₉₆₀ ω YPS1 was made with primers 1915 (see above) and 498 (5'-AACCGCTCGAGTACCTCTATAAGCGTACC-CTTC-3'), and ST₅₈₃₋₉₆₀ ω YPS1 was made with primers 1915 (see above) and 1914 (5'-CCGGCTCGAGTGTGCGCTCAACACTTC-CTTCATCA-3'). ST_{FLO1} ω YPS1 was made with primers 2395 (5'-ACGCTCTCGAGAGAACTGTGATTGTTATC-3') and 1065 (5'-CTCGAGAACTTTGTTGTCTCTGTAGTAGAAATAGGGC-3'). The pHA- ω CWP2 vector was made by amplification by PCR of a piece of CWP2 with primers 903 (5'-AACCGCTCGAGTGAATCCGCT-GCCGCCATTCTCAAAT-3') and 621 (5'-GCGAATTCTTATTAT-AACAACATAGCAGCAGCAGCTAGAGCACC-3'), which was then cloned into plasmid pBC214 (Frieman & Cormack, 2002) as an *Xho*I- and *Eco*RI-digested fragment. The same *FLO1* fragment amplified to make ST_{FLO1} ω YPS1 was cloned into the pHA- ω CWP2 vector as an *Xho*I-digested fragment to make ST_{FLO1} ω CWP2. ST₆₃₃₋₉₆₀ ω CWP2 was made by amplification of a region of *EPAL* with the primers 1915 (see above) and 498 (see above) and cloned into the *Xho*I site in pHA- ω CWP2 plasmid.

FACS analysis. Constructs were transformed into the yeast strain BY4742 selecting for growth in the absence of uracil. For analysis, strains were grown overnight in media lacking uracil, supplemented with 2% raffinose. These cells were induced for expression of the fusion proteins by addition of 2% (w/v) galactose and subsequent culture for 2 h. No agglutination of yeast was observed for any of the constructs analysed. The yeast cells were collected by centrifugation and labelled with mouse anti-haemagglutinin (HA) antibody (50 μ g ml⁻¹) (Sigma-Aldrich) for 30 min at 4 °C on a rotating wheel. The yeast cells were then washed three times with PBS and

labelled with secondary FITC-conjugated goat anti-mouse antibody ($50 \mu\text{g ml}^{-1}$) (Santa Cruz Biotechnology). Yeast cells were again washed three times in PBS and resuspended in 1 ml PBS for FACS analysis.

Protein extraction. Cells were grown overnight in SC-Ura medium plus 2% raffinose. For galactose induction, 2% galactose (final concn) was added to the culture and induced for 2 h. Cells were collected by centrifugation and resuspended in 1 ml 50 mM Tris/HCl, pH 7.5. Then ~ 0.5 ml glass beads were added and placed in a Bead Beater (Biospec) for 2 min. Broken cells were washed from the glass beads and the extract was pelleted at $15\,000\text{ g}$ for 10 min to spin down all cell wall and membrane material. After removal of supernatant the pellet was then boiled for 10 min in 50 mM Tris/HCl, pH 7.5, containing 2% SDS. The SDS-extractable material was saved and the remaining pellet was boiled again in 50 mM Tris/HCl, pH 7.5, containing 2% SDS. The cell debris was collected by centrifugation and washed two times in 1 ml H_2O . The cell debris was again pelleted and the pellet was resuspended in 100 μl 67 mM potassium phosphate. This is the washed cell wall material. Proteins were released from the washed cell wall material by treatment with 1,3- β -glucanase (Quantazyme; Qbiogene) as follows. A sample of the washed cell wall material (20 μl) was added to 75 μl phosphate buffer with 2 units 1,3- β -glucanase and 0.1 μl β -mercaptoethanol. The reaction was incubated at 30°C for 2 h.

The reaction was then pelleted for 5 min at $15\,000\text{ g}$ and the supernatant removed for analysis. For all Westerns comparing cell wall and membrane fractions, we loaded amounts of 1,3- β -glucanase releasable material and SDS-extractable material corresponding to the same number of yeast cells.

Western analysis. Proteins from cell extracts were separated by SDS-PAGE on 3–8% gradient gels (Invitrogen). After transfer to PVDF membrane (Amersham Pharmacia Biotech), membranes were incubated overnight with mouse monoclonal IgG horseradish peroxidase-conjugated anti-HA antibody (Santa Cruz) at a concentration of 1:10 000 in 5% non-fat milk/TBS. The blots were washed two times in 2% Tween/TBS for 30 min and antibody was detected using ECL-Plus (Amersham Pharmacia Biotech).

RESULTS

Full-length Epa1p is targeted to the cell wall independent of the ω -proximal targeting signal

The ligand-binding domain of Epa1p, an adhesin of *C. glabrata*, is contained within the N-terminal 280 aa (Fig. 1a and Frieman *et al.*, 2002). In *S. cerevisiae*, protein chimeras in which this domain is fused to GPI anchor

Fig. 1. Full length Epa1 is targeted to the cell wall independent of the ω -proximal signal. (a) Schematic of full-length Epa1p showing domains of the protein. The N-terminal domain contains a signal peptide (SP) followed by the ligand-binding domain (LBD) which mediates binding to *N*-acetylglucosamine. The white box immediately downstream of the signal peptide is the HA epitope tag, engineered into our constructs. The C terminus of Epa1p includes the Ser/Thr-rich region and the GPI anchor signal. A line (aa 332) represents the somewhat arbitrary demarcation between the N-terminal ligand-binding domain and the C-terminal Ser/Thr-rich domain. The black triangles represent a 40 aa Ser/Thr-rich repeat present four times. ω , the omega site (aa 1010), is the site of GPI anchor attachment. Indicated amino acid positions correspond to the amino acid positions of the native Epa1p. (b) FACS analysis of expressed fusion proteins. The N-terminal LBD of Epa1p was fused to the GPI anchor region of *YPS1* or *CWP2*. Full length Epa1p (excluding the GPI anchor) was also fused to the GPI anchor region of *YPS1* or *CWP2*. All constructs are under the control of the *GAL1* promoter. Surface expression was monitored using FACS after labelling with anti-HA antibody and FITC-conjugated secondary goat anti-mouse antibody. Average fluorescence of the population is indicated. C-terminal grey boxes represent the *YPS1* ω region; C-terminal black boxes represent the *CWP2* ω region.

signal sequences from Cwp2p (or other GPI-CWPs) are localized primarily to the cell wall where they are competent to mediate adherence. By contrast, fusions of this N-terminal ligand-binding domain to GPI anchor signal sequences from predicted GPI-PMPs are localized primarily to the membrane fraction where they are incompetent to mediate adherence (Fig. 1b and Frieman & Cormack, 2003). However, when we fused full-length Epa1p to GPI signals from GPI-CWPs or GPI-PMPs, we found no significant difference in cell wall localization; rather, both fusion proteins were efficiently delivered to the cell wall where both could mediate adherence (Fig. 1b). This raised the

possibility that the Ser/Thr domain (which is present in the full-length Epa1p, but absent in constructs fusing the ligand-binding domain directly to the GPI anchor signal) directly impacts distribution between cell wall and membrane fractions.

To test this possibility, we examined the ability of Ser/Thr regions of different lengths to influence the distribution of a model GPI-PMP between the plasma membrane and the cell wall. These constructs are shown in Fig. 2(a). Each construct contains an HA tag at its N terminus just downstream of the secretion signal. The C-terminal domain in

Fig. 2. Surface expression of ω_{YPS1} fusion constructs. (a) Schematic of ω_{YPS1} fusion constructs. Amino acid numbers correspond to positions in the full-length protein from which they were derived. The N-terminal white bar represents the HA epitope tag. C-terminal light grey boxes represent the *YPS1* GPI region, and dark grey boxes represent the *CWP2* GPI region. (b) FACS analysis of expressed fusion proteins. Surface expression was monitored by FACS after labelling with anti-HA antibody and FITC-conjugated secondary goat anti-mouse antibody. The mean fluorescence of the population is indicated.

all constructs consists of the last 72 aa of the *S. cerevisiae* protein Yps1p. These 72 aa contain the GPI anchor addition signal and 50 aa upstream of the ω site. Different lengths of the Epa1p Ser/Thr region were engineered between the epitope tag and the GPI anchor site. Each construct was expressed in *S. cerevisiae* from the *GAL1* promoter. As shown in Fig. 2(b), the small parent construct (ω YPS1) was not efficiently delivered to the cell surface as measured by surface fluorescence using FITC-conjugated antibody specific for the HA epitope tag. As increasing amounts of the Ser/Thr domain were added, however, surface localization of the fusion proteins increased dramatically.

Interpretation of these fluorescence data is not straightforward because in the assay accessibility of GPI proteins to antibodies is affected by two parameters: first, GPI proteins that are membrane-localized rather than cell-wall-localized are inaccessible to antibodies (Frieman & Cormack, 2003); second, even for cell wall GPI proteins, surface fluorescence depends on a minimum length of the Ser/Thr region that acts essentially as a spacer between the site of cross-linking in the cell wall and the outer surface of the cell to which the antibodies have access. In the absence of a sufficient spacer region, surface fluorescence is low, even though the protein is demonstrably in the cell wall (Frieman *et al.*, 2002). Thus, the low surface fluorescence shown by some of the constructs in Fig. 2(b) could in principle be due either to localization of the epitope to the plasma membrane or alternatively to internal layers of the cell wall.

To distinguish between these two possibilities, we carried

out Western analysis on membrane and cell wall fractions from cells expressing the various constructs in Fig. 2. Cells expressing each construct were grown in parallel and fractionated into cell wall and membrane fractions. Both fractions were separated by SDS-PAGE gel electrophoresis and analysed by Western blotting. The cell wall fractions contain very high molecular mass species that are both glycosylated and modified by covalent attachment to glucan. In the membrane fractions, we have previously shown that the fast-migrating species (Fig. 3, lanes 6, 8, 10, 12 and 14) are endoplasmic reticulum intermediates. The slower species found in the membrane fractions have been further glycosylated in the Golgi (Frieman & Cormack, 2003). As shown in Fig. 3, there is very little if any material found in the cell wall lanes for ω YPS1 and ST_{861–960} ω YPS1, even though the protein is abundantly expressed in the membrane fraction. A very small fraction of protein can be seen in the cell wall lane for ST_{761–960} ω YPS1, although most of the protein is in the membrane fraction. Constructs ST_{633–960} ω YPS1 and ST_{583–960} ω YPS1, which contain 328 and 378 aa of Ser/Thr, respectively, are primarily localized to the cell wall. As a control, we analysed constructs ω CWP2 and ST_{633–960} ω CWP2, both of which contain a GPI anchor signal of the GPI-CWP class; as expected, both are primarily localized to the cell wall (Fig. 3). This effect was not specific to the Ser/Thr region derived from the *FLO1* gene of *S. cerevisiae* as also able to redirect the GPI-PMP ω YPS1 to the cell wall (Fig. 2b). These experiments demonstrate that the addition of 200–300 aa rich in serine and threonine residues can redirect a model GPI-PMP to the cell wall.

Fig. 3. Western blot of fusion proteins analysed in Fig. 2. Fusion proteins expressed from a galactose-inducible promoter for 2 h with 2% galactose. Cell wall and membrane fractions were prepared as described in Methods. C denotes the 1,6- β -glucanase-released cell wall fraction and M denotes the total membrane fraction. Molecular mass markers are indicated (kDa).

GPI protein localization in glycosylation mutants

The structure of the Ser/Thr-rich regions found in many cell wall proteins has been modelled to assume a linear rigid rod-like configuration when glycosylated (Jentoft, 1990; Stratford, 1994). We wished to know whether the effect the Ser/Thr-rich region had on protein distribution between membrane and cell wall was related to glycosylation of the Ser/Thr-rich region. To test whether mutations in genes affecting glycosylation have an effect on surface expression of GPI-PMP and GPI-CWP, we expressed ST₆₃₃₋₉₆₀ω_{CWP2} and ST₆₃₃₋₉₆₀ω_{YPS1} in *S. cerevisiae* strains deleted for various genes important in *N*-linked and *O*-linked glycosylation. We expressed the ST₆₃₃₋₉₆₀ω_{YPS1} and ST₆₃₃₋₉₆₀ω_{CWP2} fusion

proteins in *pmt1*Δ, *pmt2*Δ, *pmt3*Δ, *pmt5*Δ, *pmt6*Δ, *mnv1*Δ, *mnv9*Δ, *ktr1*Δ, *ktr3*Δ and *mnt1*Δ strains. As shown in Fig. 4, we used antibodies against the HA epitope present in both fusion proteins to assess surface fluorescence in the various mutant backgrounds. We found that only *pmt2*Δ strains showed any significant reduction in surface fluorescence (Fig. 4 and data not shown). The reduction of surface expression of both ST₆₃₃₋₉₆₀ω_{YPS1} and ST₆₃₃₋₉₆₀ω_{CWP2} in the *pmt2*Δ background could be due to failure to localize the proteins to the cell wall or to a failure of protein in the cell wall to display the epitope at the cell surface in the *pmt2*Δ background. We therefore analysed cell wall and plasma membrane localization of the ST₆₃₃₋₉₆₀ω_{YPS1} and ST₆₃₃₋₉₆₀ω_{CWP2} constructs in the *pmt2*Δ background

Fig. 4. FACS analysis of expressed fusion proteins in *pmt*Δ backgrounds. Fusion proteins ST₆₃₃₋₉₆₀ω_{YPS1} and ST₆₃₃₋₉₆₀ω_{CWP2} were expressed in the wild-type (BY4742) background and the indicated *pmt*Δ backgrounds. The ST domains are derived from the Ser/Thr-rich C-terminal domain of Epa1p. Labelling and FACS analysis were performed as described in Methods. The mean fluorescence of the population is indicated.

Fig. 5. Western blot of fusion proteins in wild-type and *pmt2Δ* backgrounds. Fusion proteins expressed from a galactose-inducible promoter for 2 h with 2% galactose. Cell wall and membrane fractions were prepared as described in Methods. Proteins were visualized with a horseradish peroxidase-conjugated anti-HA antibody (Santa Cruz). C denotes the 1,6- β -glucanase-released cell wall fraction and M denotes the total membrane fraction. Note decrease in the high mobility band in cell wall lane between wild-type and *pmt2Δ* lanes. Also note the reduction in the size of the lower molecular mass band in the membrane lanes. Molecular mass markers are indicated (kDa).

and found in both cases that in the *pmt2Δ* background there was a reduction of protein in the cell wall fraction relative to the wild-type parental strain (Fig. 5).

We then asked whether the role of *PMT2* in trafficking of these constructs was specific for the Ser/Thr region derived from *EPA1* or whether there was a general requirement for *PMT2* in GPI protein trafficking. We therefore made fusions of ω_{YPS1} and ω_{CWP2} to Ser/Thr regions derived from *FLO1*

and *FLO9*, GPI-CWPs of *S. cerevisiae*. When $ST_{FLO1}\omega_{YPS1}$, $ST_{FLO1}\omega_{CWP2}$ and $ST_{FLO9}\omega_{CWP2}$ were expressed in the *pmt2* background we saw only a modest decrease in surface fluorescence compared to the decrease of $ST_{633-960}\omega_{YPS1}$ and $ST_{633-960}\omega_{CWP2}$ (Table 1). Thus, the requirement for *PMT2* in trafficking of GPI proteins is not general, but reflects a specific role for proteins with a Ser/Thr region derived from *EPA1*.

Endogenous GPI protein Ser/Thr content

Since there are at least two signals in GPI proteins (the ω -proximal signal and the Ser/Thr signal described above) that can affect distribution of GPI proteins between the cell wall and the plasma membrane, we examined the sequences of all GPI proteins in *S. cerevisiae* for the presence of putative signals of both types. De Groot *et al.* (2003) identified GPI proteins from *S. cerevisiae* based on bioinformatic analysis. This analysis was refined by Eisenhaber *et al.* (2004) who, using an alternative algorithm for GPI protein identification, identified a total of 59 putative GPI proteins in *S. cerevisiae*. We examined this list to determine the relationship between Ser/Thr content and predicted GPI-PMP or GPI-CWP ω -proximal sites. We noticed that those GPI proteins with a dibasic motif (GPI-PMPs) have significantly less total Ser/Thr content than those GPI proteins with predicted GPI-CWP ω -proximal sites. When we divided the 59 GPI proteins there into two groups, those containing a dibasic motif (18 proteins) and those that do not (41 proteins), we found that GPI-PMPs have a Ser/Thr content of less than 30% while most GPI-CWPs (28/41) have a Ser/Thr content above 30% (Fig. 6).

DISCUSSION

GPI proteins in yeast can be divided into two classes: GPI-PMPs, whose primary localization is at the plasma membrane; and GPI-CWPs, whose primary localization is the outer layers of the cell wall, where they are conjugated to 1,6- β -glucan. The existence of these two classes of GPI proteins is somewhat controversial, in part because localization of actual GPI proteins to either cell wall or membrane

Table 1. Surface fluorescence of fusion proteins expressed in *PMT* mutant backgrounds

Numbers shown are the mean surface FACS fluorescence for a given construct in the indicated *pmtΔ* strain normalized to the fluorescence for that construct in the wild-type strain. Numbers are the means for three independent experiments. Note the effect of *pmt2Δ* on surface expression for constructs containing the Ser/Thr region from *Epa1p* but not *Flo1p* or *Flo9p*.

Protein	4742	<i>pmt1Δ</i>	<i>pmt2Δ</i>	<i>pmt3Δ</i>	<i>pmt5Δ</i>	<i>pmt6Δ</i>
$ST_{633-960}\omega_{YPS1}$	100	115 $\pm$ 9	17 $\pm$ 8	113 $\pm$ 19	116 $\pm$ 10	106 $\pm$ 32
$ST_{633-960}\omega_{CWP2}$	100	99 $\pm$ 3	11 $\pm$ 2	103 $\pm$ 22	101 $\pm$ 15	82 $\pm$ 8
$FLO1\omega_{CWP2}$	100	75 $\pm$ 15	73 $\pm$ 10	108 $\pm$ 3	105 $\pm$ 7	64 $\pm$ 21
$FLO9\omega_{CWP2}$	100	70 $\pm$ 8	61 $\pm$ 8	88 $\pm$ 8	90 $\pm$ 6	89 $\pm$ 11
$FLO1\omega_{YPS1}$	100	104 $\pm$ 3	58 $\pm$ 3	111 $\pm$ 1	121 $\pm$ 10	115 $\pm$ 6

Fig. 6. Plot of serine and threonine content of GPI-anchored proteins in *S. cerevisiae*. The 59 GPI proteins described by Eisenhaber *et al.* (2004) were divided into two groups: those that contain a dibasic motif (GPI-PMPs) and those that do not (GPI-CWPs). The distribution of overall Ser/Thr content for the two groups is shown.

is not absolute. Rather, there is a spectrum of distribution between cell wall and membrane for natural as well as chimeric GPI proteins.

GPI proteins can be conveniently divided into two groups based on a sequence feature first noticed by Klis (Caro *et al.*, 1997) – proteins with a dibasic motif in the amino acids immediately upstream of the ω site are typically destined for the plasma membrane while GPI proteins lacking that signal are destined for the cell wall. The validity of this rule has been largely borne out by mutagenesis studies on model GPI proteins which clearly show that the distribution between cell wall and plasma membrane can be dramatically altered by point mutations in the region immediately upstream of the ω site (the ω -proximal site), including introduction or elimination of a dibasic motif. That said, for certain natural proteins, the rule is an imperfect predictor of ultimate localization. For example, even though Gas1p has traditionally been annotated as a GPI-PMP, it can clearly be found at the cell wall (De Sampaio *et al.*, 1999). One complication in using the ω site sequence to predict cell wall versus plasma membrane localization is that there can be multiple potential ω sites in one protein. Without protein sequence data, it is not possible to predict which of the multiple ω sites is used. It is possible, therefore, that GPI protein species in cell wall or membrane

fractions may in fact have different sites of GPI anchor attachment, and therefore different ω -proximal signals.

In addition to the above considerations, localization of natural GPI proteins to the cell wall or membrane can clearly be affected by other features of the protein. In this paper, we show that for a constant GPI anchor addition sequence, the distribution between membrane and cell wall can be strongly affected by the sequence of the protein attached to the GPI anchor. For minimal GPI constructs (consisting essentially of an epitope tag and the GPI signal sequence), our previous work, as well as work from other labs (Frieman & Cormack, 2003; Hamada *et al.*, 1998, 1999), found that localization to the plasma membrane or the cell wall is determined by the ω -proximal signal and largely by the presence or absence of a dibasic motif. For larger chimeric proteins in which the GPI anchor is fused to the N-terminal domain of Epa1p (aa 1–440), localization is again largely determined by the ω site-proximal signal, since a fusion to the *YPS1* anchor sequence is largely membrane-localized while a fusion to the *CWP2* anchor sequence is largely cell-wall-localized. However, full-length Epa1p or chimeric fusions that contain approximately 300 aa of the Ser/Thr region of Epa1p (or Flo1p) are localized to the cell wall independent of the GPI-proximal signal, since fusions to both the *YPS1* and *CWP2* GPI anchor signals are cell-wall-localized. Thus, in addition to the ω site proximal signals, the ultimate distribution of GPI proteins between cell wall and membrane is affected by the sequence of the protein (which we term the ‘cargo’) attached to the GPI anchor. In the case of regions derived from the Ser/Thr regions of Epa1p or Flo1p, this cargo signal can override the ω -proximal site signal. The ability of the cargo to override the ω -proximal site signal is not wholly determined by size, since the N-terminal domain of Epa1p (aa 1–440) which does not override the ω -proximal site signal (Fig. 1b and Frieman & Cormack, 2003) is larger than the Ser/Thr region (approx. 300 aa) which does override the ω -proximal site signal. We have shown only that the Ser/Thr regions of Epa1p and Flo1p are sufficient to redirect a GPI-PMP to the cell wall. We think it is unlikely that a Ser/Thr region is the only domain that can override a GPI-PMP ω -proximal site signal. Indeed, De Sampaio *et al.* (1999) showed that Gas1p and fusions of Gas1p to α -galactosidase are both primarily cell-wall-localized. Neither Gas1p nor α -galactosidase is particularly rich in serine and threonine residues.

We tested whether mannosylation of the Ser/Thr regions of Epa1p or Flo1p by any single *PMT*-encoded mannosyl transferase was required for its ability to redirect a GPI-PMP to the cell wall. We found that only deletion of *PMT2* had any effect at all. However, while deletion of *PMT2* did result in a modest decrease in the total amount of epitope-tagged GPI protein as well as some decrease in the ratio of tagged protein in the cell wall versus the membrane fractions (Fig. 5), this effect was really significant only for GPI proteins containing the Epa1p Ser/Thr region; by contrast,

deletion of *PMT2* had much less effect on surface expression for constructs containing the Ser/Thr region from Flo1p or Flo9p (Table 1). Even more importantly, loss of *PMT2* affected trafficking of constructs containing the Ser/Thr region of Epa1p regardless of whether the GPI anchor was derived from *YPS1* or *CWP2*. Thus, while *PMT2* affects trafficking of constructs containing the Epa1p Ser/Thr region, we have no evidence that *PMT2* plays a role in the ability of Ser/Thr regions to override the GPI-PMP ω -proximal signal. An important caveat of our experiments with the mannosylation mutants is that there is only a partial loss of mannosylation in any of the *pmt* backgrounds as measured by change in mobility on SDS-PAGE gels (data not shown). This is not surprising, given the overlapping functions of mannosyl transferases, but it remains possible that in strains deleted for multiple *PMT* genes, for example, there would be an effect on the ability of the Ser/Thr-rich regions to override a GPI-PMP ω -proximal signal.

The reduction in overall protein levels seen in the *pmt2* background for the chimeras containing the Epa1p-derived Ser/Thr region (Fig. 5) could be due to reduced stability of the proteins in the *pmt2* background, perhaps as a result of degradation of undermannosylated protein. Indeed, we could demonstrate a small reduction in the molecular mass of the chimeric Epa1-derived proteins in a *pmt2* background, consistent with a role for *PMT2* in mannosylating the Epa1p Ser/Thr domain (Fig. 5). Interestingly, we found no equivalent effect of *PMT2* on the Ser/Thr region derived from Flo1p or Flo9p, perhaps suggesting some specificity of *PMT2* for the Epa1p Ser/Thr domain. Mannosylation of the Mid2p cell wall integrity sensor which has an extracellular domain rich in serine and threonine residues also specifically requires *PMT2* (Philip & Levin, 2001). The specific requirement for *PMT2* in mannosylation of Mid2p or of the Epa1p Ser/Thr region is not understood.

We have shown that multiple, separable signals can be integrated to determine the ultimate localization of GPI proteins to the cell wall or plasma membrane. We found that a long Ser/Thr region could override the ω -proximal GPI signal of a predicted GPI-PMP to localize it to the cell wall. This raises the question as to the fate of actual GPI proteins in the cell, since GPI proteins apparently can contain at least two signals that in theory could direct different localizations. To begin to address this question, we examined the list of predicted GPI proteins in *S. cerevisiae* and found that the overall Ser/Thr content is low for predicted GPI-PMPs and high for the majority of predicted GPI-CWPs. It may be that this correlation simply reflects a functional role of Ser/Thr residues in GPI-CWPs rather than GPI-PMPs. It also suggests, however, that for the majority of GPI proteins in *S. cerevisiae*, the two signals we describe in this paper (the ω -proximal signal and Ser/Thr content) might act in concert rather than antagonistically. While at least some of the *cis*-requirements directing localization of GPI proteins to the plasma membrane or cell wall are now

understood, it remains to be determined how the cell actually interprets those sequence signals and sorts the two classes of GPI proteins.

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