

# Amino Acid Residues in the $\omega$ -Minus Region Participate in Cellular Localization of Yeast Glycosylphosphatidylinositol-Attached Proteins

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**The final destination of glycosylphosphatidylinositol (GPI)-attached proteins in *Saccharomyces cerevisiae* is the plasma membrane or the cell wall. Two kinds of signals have been proposed for their cellular localization: (i) the specific amino acid residues V, I, or L at the site 4 or 5 amino acids upstream of the GPI attachment site (the  $\omega$  site) and Y or N at the site 2 amino acids upstream of the  $\omega$  site for cell wall localization and (ii) dibasic residues in the region upstream of the  $\omega$  site (the  $\omega$ -minus region) for plasma membrane localization. The relationships between these amino acid residues and efficiencies of cell wall incorporation were examined by constructing fusion reporter proteins from open reading frames encoding putative GPI-attached proteins. The levels of incorporation were high in the constructs containing the specific amino acid residues and quite low in those containing two basic amino acid residues in the  $\omega$ -minus region. With constructs that contained neither specific residues nor two basic residues, levels of incorporation were moderate. These correlations clearly suggest that GPI-attached proteins have two different signals which act positively or negatively in cell wall incorporation for their cellular localization.**

Mannoproteins, which are one type of the components of the *Saccharomyces cerevisiae* cell wall, can be divided into three groups: sodium dodecyl sulfate (SDS)-extractable, reducing-reagent-extractable, and glucanase-extractable mannoproteins (5, 12, 23). Glucanase-extractable mannoproteins are covalently bound to  $\beta$ -1,6-glucan of the cell wall, which forms a large complex with  $\beta$ -1,3-glucan and chitin (9, 10, 11, 13, 17, 19, 20). Many genes encoding glucanase-extractable cell wall mannoproteins have been isolated from *Saccharomyces cerevisiae*, and so far most of them have been identified as glycosylphosphatidylinositol (GPI)-dependent cell wall proteins (15, 16, 18, 21, 24, 26, 28, 31, 32).

GPI-associated proteins have several common structural characteristics: a signal sequence for secretion in the N terminus and a GPI signal for attachment to GPI in the C terminus. The GPI signal itself has three domains: the region containing the GPI attachment site (the  $\omega$  site) plus the first and second amino acids downstream of the  $\omega$  site, a spacer of 5 to 10 amino acids, and a hydrophobic stretch of 10 to 15 amino acids (22, 29). A protein containing the GPI signal is cleaved at the  $\omega$  site, and the resulting carboxy terminus of the protein is covalently bound to a GPI moiety (23, 30). This reaction occurs in the endoplasmic reticulum. Being associated with membranes by means of the GPI moiety, GPI-attached proteins are then transported to the cell surface and remain on the plasma membrane as GPI-anchored proteins; however, some of them are further processed. They are incorporated into the cell wall by detaching themselves from the GPI moiety and then by linking themselves to  $\beta$ -1,6-glucan of the cell wall (6, 30). These different processes suggest that each GPI-attached protein has a signal for selecting either to be incorporated into the cell wall or to remain on the plasma membrane. Two kinds of amino acid sequences in the region upstream of the  $\omega$  site (the

$\omega$ -minus region) have been proposed as being responsible for the selection: dibasic residues for remaining on the plasma membrane (3, 33) and specific amino acid residues at sites 4 or 5 and 2 amino acids upstream of the  $\omega$  site ( $\omega$ -4/5 and  $\omega$ -2 sites, respectively) for incorporation into the cell wall (7). The former residues were determined by sequence analysis, and the latter residues were determined by mutational analysis mainly by using synthetic model sequences. We now need to evaluate these proposals with more authentic sequences of GPI-attached proteins. A number of open reading frames (ORFs) encoding putative GPI-attached proteins have been selected from previous studies (3, 8). In this study, we quantitatively measured the cell wall incorporation of fusion reporter proteins that were constructed from these ORFs and examined the relationship between efficiencies of cell wall incorporation and amino acid residues in their  $\omega$ -minus regions.

## MATERIALS AND METHODS

**Nucleotide sequences.** The *Saccharomyces* Genome Database was used as a source of nucleotide and amino acid sequences of all the ORFs examined, except for YBR078W. Our sequence analysis of YBR078W identified an insertion of C at nucleotide position 1610, resulting in reduction of the ORF size from 468 to 429 amino acids. The C-terminal amino acid sequence used for YBR078W is shown in Table 1.

**Expression of a fusion reporter protein.** A DNA fragment covering the most C-terminal 40 amino acids of a GPI-attached protein and about 200 bp of its 3' noncoding region was amplified by PCR with primers containing a *SalI* or *BamHI* restriction site. The resulting fragment was inserted into the *SalI* and *BamHI* sites of a multicopy plasmid, pE $\alpha$ GALHA (8). pE $\alpha$ GALHA contains a reporter gene comprising the signal sequence of yeast invertase, the  $\alpha$ -galactosidase from *Cyamopsis tetragonoloba*, and a hemagglutinin (HA) epitope. The plasmid containing the fusion reporter gene was used to transform the yeast strain YPH499 (27).

**Construction of genes encoding GPI proteins tagged with HA.** A fragment containing the 400 bp of the 5' noncoding region and the N-terminal 25 amino acids of Yer150w was amplified by PCR and inserted into the *SacI* and *BamHI* sites of a plasmid, pEHA. pEHA contains the triple-HA epitope sequence between the *BamHI* and *SalI* sites of the multicloning site of a multicopy plasmid, YEplac195 (7). Another fragment containing the C-terminal 122 amino acids of Yer150w and the 300 bp of its 3' noncoding region was amplified by PCR and inserted into the *SalI* and *HindIII* sites, generating pEHAYer150w. Similarly, the triple-HA epitope sequence was inserted in frame into the 25th amino acid position of the Yor382w sequence on pEHAYor382w.

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TABLE 1. Efficiencies of incorporation of the fusion reporter proteins into cell walls

ORF <sup>a</sup> /GPI-attached protein	Amino acid sequence fused to the reporter protein <sup>b</sup>	Cell wall incorporation <sup>c</sup>
YDR534C	I FTNGKSSSTTPQIVNYT G AADSIAAGTGLMGAALAAVIFL*	0.421 ± 0.045
YIR019C/FLO11	SGSAVATYSVPSISSTYQ G AANIKVLGNFMWLLLLALPVVF*	0.409 ± 0.122
YNL327W/ETG2	ITWSSSTIKPPSISTYS G AAGQLTRIGSLLGLLISFLL*	0.380 ± 0.060
YOR214C	PTITPGNITIGGYE N SSSSLMPSMGLSFLFGLYLLLHP*	0.338 ± 0.065
YDR134C	TEKPTQQGSSTQTVTSYT G AAVKALPAAGALLAGAAALLL*	0.325 ± 0.025
YPL130W	TPYSNIISSLNEDYD N ASNFLTPTTVALAVLLTILLFIQAY*	0.315 ± 0.099
YOR009W	TVVSVQSKTTGIVEQTE N GAAKAVIGMGAGALAAVAAML*	0.308 ± 0.074
YER150W	KPTSETSVSSTHDTVETNS N AANARAIPGALGLAGAVMMLL*	0.299 ± 0.022
YDR077W/SED1	STVVPVSSASSHSVVINS N GANVVVPALGLAGVAMFL*	0.297 ± 0.062
YOR383C	TSSSATSSSTAELSSYT G AADAITAGTGLMGAALAAVMLL*	0.291 ± 0.062
YJR151C	SIHSSASYSVTISINT N GAYNFDKDNIFGTAIIVAVALLL*	0.280 ± 0.053
YJR004C/Agα1	IQQNFSTSTSLMISTYE G KASIFFSAELGSIIFLLSYLLF*	0.265 ± 0.049
YJL078C/PRY3	TSTTAKLSAYE G AATPLSIFQCNSLAGTIAAFVAVLFAF*	0.263 ± 0.053
YLR110C	APKNTTSAAPTHSVTSYT G AAKALPAAGALLAGAAALLL*	0.220 ± 0.040
YNL300W	SISSQANTTTHEISTYV G AAVKGSVAGMGAIMGAAAFALL*	0.214 ± 0.024
YEL040W/UTR2	SSVSGSASSSTSSMSGN N AGANVAANWRLTVLCVILGYVL*	0.197 ± 0.060
YLR042C	TKSSQTNTIISSTSTG G VGSVKPCLYFVLMLETIAYLFS*	0.179 ± 0.018
YOR382W	TSSSSSSSSSSSSASSS G AAPAAFQASVAGALALGLISYLL*	0.136 ± 0.026
YLR040C	ALISANSSTSRSTSQSQ N GAHAKSLYFPALFGIFAVAL*	0.123 ± 0.008
YMR006C	SSSTARRSSSTANKA N AAAISYANTNTLMSLLGAITALFGLI*	0.116 ± 0.038
YJL171C	SATAPSTTSKS N GVALTKMQNGVWYIILAIFTAFTQVVL*	0.105 ± 0.028
YIL011W/YIB1	EKSTNSSSSATS K N AGAAMDGMFFSAGVGAIAAGAAAML*	0.085 ± 0.033
YOL030W/GAS5	SLLKSAASATSSSSQSSSKSK G AAGIIEIPLIFRALAELYNLVL*	0.081 ± 0.028
YDR055W	SSGASSSSSKSKG N AAIMAPIGQTTPVLGLLTAIIMSIM*	0.060 ± 0.028
YBR078w/ECM33	AQANVSASASSSSSSSKSK G AAPPELVPATSPMGVVAAGVALL*	0.059 ± 0.003
YNL190W	GPGEKARKN N AAPGPSNFNSIKLFGVTAGSAAVAGALLL*	0.057 ± 0.038
YDR144C/YAP2	SSTGMLSPSTSSSTRKE N GGHNLNPPFFARFITAIFHHI*	0.056 ± 0.026
YIR039C/YAP6	SSFSSSGGSSESTTKKQ N AGYKYRSSFSFLLSFIYFLL*	0.055 ± 0.014
YLR194C	YKSTVNGKVASVMSNST N GATAGTHIAYGAGAFVAGALLL*	0.052 ± 0.055
YLR120C/YAP3	SGNLTTSTASATSTSSKR N VGDHIVPSLPLTLISLLFAFI*	0.051 ± 0.029
YDR522C/SPS2	GKNGAKSQSSSKKME N SAPKNIFIDAFKMSVYAVFTVLFSEIF*	0.051 ± 0.057
YMR215W/GAS3	TGSSASSSSKSK G VGNIVNVFSQSGLALFAGLISALL*	0.046 ± 0.030
YMR008C/PLB1	AGSSSTHKK N AGNALVNYSNLNTNTFIGVLSVISA VFGLI*	0.045 ± 0.025
YOL132W/GAS4	EDADEDKDDLKRKHR N SASISGPLPLGLCLLFFTFSLFF*	0.042 ± 0.031

<sup>a</sup> Six ORFs, YMR006C, YOL030W, YBR078W, YLR194C, and YDR522C, were originally identified to encode putative GPI-attached proteins through an in silico sequence analysis by Caro et al. (3). Three ORFs, YIR019C, YOR383C, and YOR382W, were not studied for cell wall localization in our previous study (8).

<sup>b</sup> The potential GPI attachment site (the ω site) of each sequence (3, 8) is indicated in the middle, sandwiched between spacer regions. The amino acid residues I, V, and L at the ω-5 site and Y and N at the ω-2 site are marked in bold, and the basic amino acid residues K and R in the short ω-minus region are marked in bold italic. The potential N-glycosylation sites are double underlined.

<sup>c</sup> Cell wall incorporation is represented as ratios of the amounts of laminarinase-extractable cell wall HA-tagged proteins to total amounts of HA-tagged proteins in crude cell lysate, including the cell wall. Values are means with standard deviations from measurements of four different transformants.

**Analysis of cell wall-associated proteins.** Cell walls were isolated from yeast transformant cells grown in a selection medium. The yeast cells were broken with glass beads in LY buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) containing 2% SDS. The pellet generated by centrifugation was twice treated with hot 2% SDS in LY buffer by heating it for 10 min at 95°C each time, followed by washing it five times with LM buffer (100 mM sodium acetate [pH 5.5], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The washed cell walls were incubated with laminarinase (product no. L5144; Sigma) at a concentration of 0.25 U/ml twice for 1 h each time at 37°C. The treated sample was centrifuged in a microcentrifuge at 10,000 rpm for 2 min at 4°C, and the resulting supernatant was analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Protein bands on the blots were detected with the anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim). The band intensities were measured with an Image Master ID (Pharmacia Biotech) and were represented as mean values with standard deviations from measurements of four different transformants.

## RESULTS

The most C-terminal 40 amino acid sequence of each ORF was fused to a reporter protein comprising the invertase signal sequence, an α-galactosidase from guar, and an HA epitope. The fusion reporter gene is transcribed by the *PGK1* promoter (8). A plasmid containing the fusion reporter gene was intro-

duced into the yeast strain YPH499. Cell wall fractions were isolated from each transformant, digested with glucanase, and analyzed on Western blots. The amounts of glucanase-extracted fusion reporter proteins were measured, and the ratios of their amounts to the total amounts of fusion reporter proteins in cells were calculated (Table 1). Table 1 also shows the amino acid sequences of ORFs used to construct the fusion reporter genes. Among the 34 fusion reporter proteins examined, 15 have amino acid residues of I, V, or L at the ω-4/5 site and Y or N at the ω-2 site, 11 have the basic amino acid residues K and R or K alone in the short ω-minus region, and the remaining 8 have none of these distinguishing residues. These fusion reporter proteins were incorporated into the cell wall in various ratios from 0.421 for the fusion reporter protein of Ydr534c (FP-Ydr534c) to 0.042 for FP-Yol132w. Fusion reporter proteins constructed from two well-characterized GPI-dependent cell wall proteins, Sed1p (also called Ydr077w) (25, 32) and Agα1p/Yjr004c (19, 34), showed ratios of 0.297 and 0.265, respectively, while that of a well-characterized GPI-dependent plasma membrane protein, Yap3p/Ylr120c (1, 4, 14), showed a ratio of 0.051. A histogram based on the results

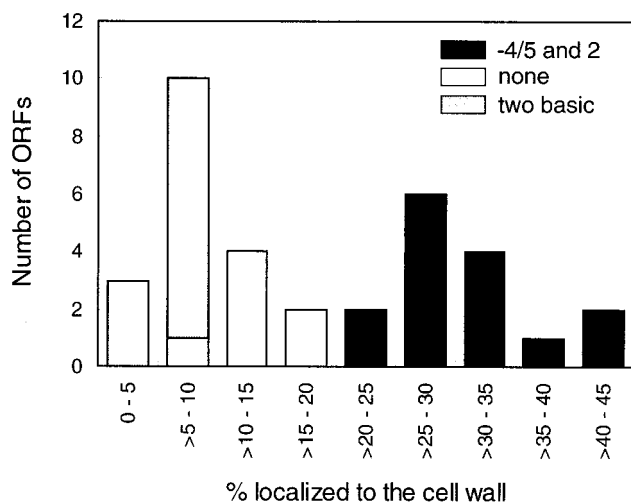


FIG. 1. Relationship between efficiencies of cell wall incorporation and sequences of the  $\omega$ -minus region. The data of Table 1 were used for this histogram representation. Results shown are for sequences with the specific amino acid residue of I, V, or L at the  $\omega$ -4/5 site and Y or N at the  $\omega$ -2 site (-4/5 and 2); sequences with two basic amino acid residues R and/or K in the  $\omega$ -minus region (two basic); and sequences with neither the specific amino acid residues nor the two basic residues (none).

in Table 1 clearly indicates the correlation between efficiencies of cell wall incorporation and sequences in the  $\omega$ -minus region (Fig. 1): cell wall incorporation was higher in the fusion reporter proteins containing the specific amino acid residues I, V, or L at the  $\omega$ -4/5 site and Y or N at the  $\omega$ -2 site than in those containing no such specific amino acid residues.

Two proteins, Yer150w, which has V at the  $\omega$ -5 site and N at the  $\omega$ -2 site, and Yor382w, which has a stretch of serine residues in the  $\omega$ -minus region, were examined for their cell wall incorporation. An HA epitope was inserted at the 25th amino acid position from the first methionine of each protein, generating the HA-tagged proteins Yer150w-HA and Yor382w-HA, and their cellular localization was examined. Of the total amount of Yer150w-HA protein, 12.7% was detected as glucanase-extractable cell wall protein (Fig. 2A). This level was much higher than that of Yor382w-HA. The amount of Yor382w-HA released from the cell wall by glucanase was only 2.9% of the total amount of Yor382w-HA protein in the cells (Fig. 2B). Yer150w-HA has three potential N-glycosylation sites, while Yor382w-HA has not. Consistent with these characteristics, the Yer150w-HA bands appeared differently on Western blots before and after endoglycosidase H treatment (Fig. 2A, lanes 3 and 4), while the Yor382w-HA bands appeared to be unchanged before and after the treatment (data not shown). These results suggested that N glycosylation occurs at least at one of the three potential sites in Yer150w-HA.

## DISCUSSION

We demonstrated in a previous study that the amino acid sequences at the  $\omega$ -minus region are important for the determination of the final cellular localization of GPI-attached proteins and that the amino acid residues V or I at the  $\omega$ -4/5 site and V, I, N, or Y at the  $\omega$ -2 site are required for the efficient incorporation of GPI-attached proteins into the cell wall (7). This requirement, called the  $\omega$ -4/5 and  $\omega$ -2 rule, was identified through a mutational analysis of synthetic  $\omega$ -minus regions. In this study, this rule was verified, as shown in the histogram of Fig. 1. Fusion reporter proteins which have the specific amino acid residues of I, V, or L at the  $\omega$ -5 site and Y or N at the  $\omega$ -2

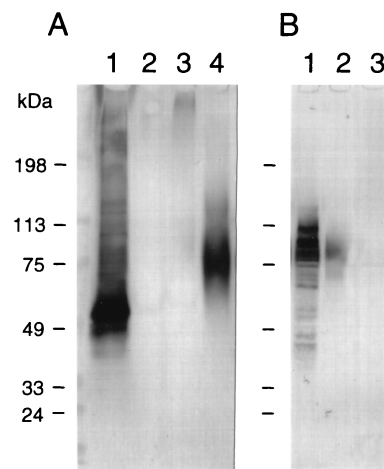


FIG. 2. Cell wall incorporation of HA-tagged proteins. Cell wall fractions were prepared from transformant cells and analyzed by Western blotting. (A) Ypl130w-HA. Lane 1, crude cell lysate; lane 2, cell wall fraction; lane 3, cell wall fraction treated with laminarinase; lane 4, cell wall fraction treated with laminarinase and endoglycosidase H (7). The sample amounts in lanes 2 to 4 were five times more than that in lane 1. (B) Yor382w-HA. Lane 1, crude cell lysate; lane 2, cell wall fraction treated with laminarinase and endoglycosidase H; lane 3, cell wall fraction. The sample amounts in lanes 2 and 3 were five times more than that in lane 1.

site were incorporated into the cell wall with greater efficiency than those which have no specific amino acid residues in the short  $\omega$ -minus region. A similar correlation was also observed for intact proteins tagged with HA. The level of cell wall incorporation of Yer150w-HA was much higher than that of Yor382w-HA. Yer150w meets the rule, while Yor382 does not. Our results clearly indicate that these specific residues function as a signal that enhances the incorporation efficiency of GPI-attached proteins into the cell wall. Furthermore, in keeping with the previous findings that replacement of S with Y in a synthetic sequence comprising a serine stretch partially enhances cell wall incorporation (7), efficiencies of the cell wall incorporation of FP-Yir019c and FP-Ypl130w, both of which have only Y at the  $\omega$ -2 site, were comparable to those of fusion reporter proteins containing the specific amino acid residues in both of the  $\omega$ -4/5 and  $\omega$ -2 sites. This suggests that specific amino acid residues only at the  $\omega$ -2 site in some sequences may be enough for efficient incorporation of GPI proteins into the cell wall. To reach a conclusion, however, further examination will be required.

Levels of glucanase-extractable cell wall proteins varied among the fusion reporter proteins with the specific amino acid residues, suggesting that some factors other than those of the  $\omega$ -4/5 and  $\omega$ -2 rule may modify the efficiency of cell wall incorporation. These factors are probably governed by the amino acid sequences themselves, and it is possible that glycosylation of proteins may be one of these factors. However, N glycosylation would be excluded from the possibility. Among the 15 ORFs, YOR214C, YPL130W, YLR110C, and YNL300W have potential N-glycosylation sites within the sequences used for the construction of the fusion reporter proteins (Table 1). In fact, their fusion reporter proteins were detected as broad and smeared bands at considerably higher molecular-mass positions on Western blots (data not shown). Ratios of cell wall incorporation were 0.338, 0.315, 0.220, and 0.214 for FP-Yor214c, FP-Ypl130w, FP-Ylr110c, and FP-Ynl300w, respectively, indicating that there is no correlation between N glycosylation and levels of cell wall incorporation. In our previous study, Ypl130w was not classified as a putative

GPI-dependent cell wall protein, because the fusion protein was not detected in the cell wall fraction treated with glucanase (8). In this study, it was clearly detected. This difference in detection may be due to difficulties in detecting N-glycosylated proteins on Western blots. On the other hand, it is possible that O glycosylation of proteins may partly explain the diversity of cell wall incorporation. Considering the S/T richness of the  $\omega$ -minus regions, it is likely that the fusion reporter proteins are O glycosylated to various extents. Deletion of several genes required for O glycosylation has been known to affect the incorporation efficiency of GPI-attached proteins into the cell wall (2). Further examinations are needed to reach a conclusion.

Vossen et al. have suggested from sequence analysis that a dibasic motif in the  $\omega$ -minus region may participate in a signal for detaching GPI-attached proteins on the plasma membrane (33). Our results support this proposal. As clearly shown in Fig. 1, incorporation ratios of all the fusion reporter proteins that contain two basic amino acid residues in the  $\omega$ -minus region into the cell wall were quite low. These ratios were lower than those of the fusion reporter proteins that did not have the two basic amino acid residues. Furthermore, all of the fusion reporter proteins examined in this study were detected in a detergent phase by phase separation with Triton X-114, and the proteins extracted in the detergent phase were phosphatidylinositol-phospholipase C sensitive (data not shown), indicating GPI attachment. Therefore, it can be concluded that with fusion reporter proteins with two basic amino acid residues in the  $\omega$ -minus region, nearly all the amounts of their proteins remain on the plasma membrane in a GPI-associated form. These two basic residues probably function as a negative factor in the process of cell wall incorporation.

Taken together, these results indicate that GPI-attached proteins have two different signals for determining their cellular localization. These are the amino acid residues V, I, or L at the  $\omega$ -4/5 site and Y or N at the  $\omega$ -2 site and the basic residue(s) R and/or K in the short  $\omega$ -minus region. The former residues act as a positive factor for cell wall incorporation, while the later residues act as a negative factor for incorporation, such that the proteins remain on the plasma membrane as GPI-anchored proteins.

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