Amino Acid Sequence Requirement for Efficient Incorporation of Glycosylphosphatidylinositol-associated Proteins into the Cell Wall of *Saccharomyces cerevisiae*

Kenji Hamada, Hiromichi Terashima, Mikio Arisawa, and Kunio Kitada†‡

From the Department of Mycology, Nippon Roche Research Center, Kamakura, Kanagawa 247, Japan

During cell wall biogenesis in *Saccharomyces cerevisiae*, some glycosylphosphatidylinositol (GPI)-attached proteins are detached from GPI moieties and bound to β-1,6-glucan of the cell wall. The amino acid sequence requirement for the incorporation of GPI-attached proteins into the cell wall was studied by using reporter fusion proteins. Only the short ω-minus region composed of five amino acids, which is located upstream of the ω site for GPI attachment, determined the cellular localization of the GPI-associated proteins. Within the ω-minus region, amino acid residues at the ω-4 or -5 and ω-2 sites were important for the cell wall incorporation. Yap3p, a well characterized GPI-anchored plasma membrane aspartic protease, was localized in the cell wall when the ω-minus region was mutated to sequences containing Val or Ile at the ω-4 or -5 site and Val or Tyr at the ω-2 site.

Mannoproteins, one of the components of the yeast cell wall, can be divided into three groups: SDS-extractable mannoproteins, reducing agent-extractable mannoproteins, and glucanase-extractable mannoproteins (1, 2). The glucanase-extractable mannoproteins are covalently bound to β-1,6-glucan of the cell wall and are released only by glucanase treatment (3–6). Many genes encoding glucanase-extractable cell wall mannoproteins have been isolated in *Saccharomyces cerevisiae*, and so far all of them have been identified as GPI-dependent cell wall proteins (7–15).

GPI-associated proteins have been isolated from various organisms from yeasts and protozoa to mammals (16–18). These proteins are structurally related in that they all contain a signal sequence for secretion in the N terminus and a GPI signal for an attachment to a GPI in the C terminus. The GPI-signal region is composed of three domains: a GPI attachment region comprising ω, ω+1, and ω+2 sites; a spacer of 5–10 amino acids; and a hydrophobic stretch of 10–15 amino acids. A protein containing the GPI-signal is cleaved at the ω site, and the resulting carboxyl terminus of the protein becomes covalently bound to a GPI moiety. This reaction occurs in the luminal face of the endoplasmic reticulum and, in yeast, requires GAA1 (19) and GPI8 (20) gene products. The GPI-associated proteins are then transported to the cell surface, where they are exposed on the extracytoplasmic face of the plasma membrane. During the transportation from the endoplasmic reticulum to the plasma membrane, the proteins are mannosylated and become GPI-anchored mannoproteins.

In protozoa and mammals, the GPI-anchored mannoproteins remain on the plasma membrane and take biological functions related to cell-cell and cell-environment interactions (18, 21, 22). In addition to the above functions, some of the GPI-anchored mannoproteins in the yeast are further processed and are incorporated into the cell wall. The incorporation of GPI-associated proteins into the cell wall is thought to occur in two steps: detachment of a GPI moiety from the protein and linking of the GPI-detached protein to β-1,6-glucan of the cell wall (3). Our knowledge of this process is very rudimentary. The addition of 30–40 amino acids from the C terminus of GPI-dependent cell wall proteins to a reporter protein directs incorporation of the chimeric proteins into the cell wall (23–25). This suggests that the short C-terminal sequences contain all the information necessary for the incorporation of proteins into the cell wall. Therefore, it is possible that there is a gene product that would recognize a sequence or structural motif in the C-terminal region of GPI-associated proteins and direct their final cellular localization. A sequence alignment from our previous study on GPI-dependent cell wall proteins (24) suggested that there is some sequence similarity among the ω-minus regions, corresponding to regions upstream of the ω site, of GPI-dependent cell wall proteins identified in the yeast. In this study, we examined, in detail, the amino acid sequences of the ω-minus regions and determined the sequence requirement for the incorporation of GPI-attached proteins into the cell wall.

**EXPERIMENTAL PROCEDURES**

*Strains and Media*—All experiments were carried out in *S. cerevisiae* strain YPH499 (*Mata, his3 leu2 trpl1 ura3; Ref. 26*). Selective medium supplemented with the appropriate nutrients except uracil (SC-Ura; Ref. 27) was used for plasmid maintenance. *Escherichia coli* strain DH5 (28) was used for the construction and amplification of plasmids. Bacterial medium (LB broth) was prepared as described by Maniatis *et al.* (28).

*Plasmids and DNA Manipulation*—pEoGalHA (24) containing a reporter gene comprising the signal sequence of yeast invertase, α-galactosidase from guar (*Cyamus tetragonoloba*), and a hemagglutinin (HA) epitope was used as a host vector. The reporter gene is transcribed through the *PGK1* promoter. A DNA fragment covering the C-terminal amino acids of a GPI-attached protein and about 200 base pairs of its 3′-noncoding region was amplified by polymerase chain reaction (PCR) with primers containing a *SalI* or *BamHI* restriction site. The resulting fragment was inserted into the *SalI* and *BamHI* sites of pEoGalHA and then used to transform the yeast strain YPH499. Replacement of amino acid residues and construction of chimeric fusion genes were carried out by PCR-based site-directed mutagenesis (29). A 3′ HA epitope sequence was inserted in-frame into the 5′-position of the Yap3p amino acid sequence (30). A fragment containing the 400 base pairs of the 5′-noncoding region and the N-terminal 514

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amino acids of Yap3p was amplified by PCR and inserted into the SpH1 and SalI sites of pEHA, generating pEHAAnYAP3. pEHA contains the 3 × HA epitope sequence (31) between the BamHI and SalI sites of the multicloning site of YEplac195 (32). Another fragment containing the C-terminal 55 amino acids of Yap3p and the 460 base pairs of its 3′-noncoding region was amplified and inserted into the BamHI and PstI on pEHAAnYAP3, generating pEHAAYAP3.

Treatment of Membranes with Phosphatidylinositol-specific Phospholipase C (PI-PLC)—Yeast transformant cells grown at 30 °C in the SC-Ura medium were harvested by centrifugation and washed with TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA). Membrane proteins were isolated from the transformant cells and subjected to phase separation with Triton X-114 as described previously (24, 33, 34). 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 protease inhibitor mixture (Complete; Boehringer Mannheim). After removal of debris by centrifugation (in a microcentrifuge, 1 min, 2000 rpm, 4 °C), a 10% Triton X-114 solution was added to the crude lysate to a final concentration of 1.4%, and the mixture was incubated on ice for 40 min. The solution was centrifuged (in a microcentrifuge, 5 min, 10,000 rpm, 4 °C), and the supernatant was transferred to a new tube. After incubation at 33 °C for 3 min, the solution was centrifuged (in a microcentrifuge, 20 s, 10,000 rpm, room temperature) and separated into an aqueous phase and a detergent phase. The aqueous phase was removed, and the remaining detergent phase was twice extracted with 1 volume of LM buffer. Membrane proteins in the aqueous phase were treated with PI-PLC as described previously (35). Membrane proteins resolved in LY buffer without NaCl were incubated with 0.1 unit of PI-PLC from Bacillus thuringiensis (Oxford GlycoSciences) at 33 °C for 30 min and then separated into a detergent phase and an aqueous phase. Proteins in both phases were separately precipitated with trichloroacetic acid and analyzed by SDS-polyacryl-

amide gel electrophoresis and Western blotting (36). An anti-HA monoclonal antibody (12CA5, Boehringer Mannheim) was used for detection of the fusion proteins on blots.

Treatment of Cell Walls with Laminarinase—Cell walls were isolated from yeast transformant cells as described previously (24, 37, 38). Yeast cells were broken with glass beads in LY buffer containing 2% SDS, and the enzyme solution was centrifuged (10,000 rpm, 1 min, room temperature). The resulting pellet was twice treated with a 2% SDS-containing LY buffer by heating for 10 min at 95 °C each time and washed 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 (Sigma L5144) at a final concentration of 0.25 unit/ml twice for 1 h each at 37 °C as described previously (24, 39). The treated sample was centrifuged (in a microcen-

trifuge, 2 min, 10,000 rpm, 4 °C), and the resulting supernatant was analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Detection of the proteins on blots was carried out using the anti-HA monoclonal antibody.

Endoglycosidase H (Endo H) Digestion—Mannoproteins were di-
gested with Endo H as described previously (51). Mannoproteins liberated from the cell wall with the laminarinase treatment were heated at 100 °C for 10 min in LM buffer containing 0.1% SDS and 0.5% 2-mercaptoethanol. Recombinant Endo H (Boehringer Mannheim) was added to the solution to a final concentration of 0.3 milliunit/ml with protease inhibitor mixture (Complete; Boehringer Mannheim). The reaction so-

lution was incubated overnight at 37 °C and then further incubated at 37 °C for a further 3 h after the addition of the same amount of Endo H.

RESULTS

Determination of ω Sites for GPI Attachment—Potential ω sites for GPI attachment are predictable based on the consensus rules described by Nuoffer et al. (33) and by Udenfriend and Kodukula (17). A sequence analysis of putative GPI-associated proteins has suggested that in S. cerevisiae two amino acid residues, Asn and Gly, are preferentially used as ω site resi-
dues (24, 40). A mutational analysis of the ω site residue in Gas1p, a well characterized yeast GPI-anchored plasma mem-
brane protein (41, 42), has shown selectivity of the ω site residue. Substitution of the ω site residue from Asn, an authen-
tic ω site residue, with Ser, Gly, Ala, Asp, or Cys slightly decreased the efficiency of GPI attachment, while replacement of Asn with other amino acid residues including Gln, Glu, and Thr resulted in an almost complete loss of the GPI attachment efficiency (33). Based on this selectivity of the ω site residue, we determined the ω sites of two GPI-dependent cell wall proteins, Yjr151c (24) and Ydr077w/Seq1p (15), and two GPI-anchored plasma membrane proteins, Yir039c (24) and Yir120c/Yap3p (43–45). The C-terminal sequences, shown in Fig. 1A, with mutated amino residues at the putative ω site were fused to the reporter gene, and the resulting fusion proteins were expressed in yeast cells. GPI anchoring of the fusion proteins was exam-

ined by using phase separation of membrane proteins with Triton X-114 and digestion with PI-PLC (Fig. 1B). In the C-terminal sequences of Ydr077w and Yir120c containing the wild type residue Asn at the putative ω site, the fusion proteins were detected in the detergent phase and were released into the aqueous phase by the PI-PLC treatment, indicating GPI-dependent anchoring in membranes. Mutant fusion proteins with replacement of Asn with Gly or Asp at the ω site were also extracted in the detergent phase. The amounts of these proteins were comparable with that of the wild type, and they were also PI-PLC-sensitive. On the other hand, mutant fusion proteins with replacement of Asn with Gln, Glu, or Thr at the ω site were not detected in the detergent phase as much as the wild type. The amounts of these proteins detected were quite low, and most of them were not PI-PLC-sensitive, indicating loss of GPI anchoring efficiency. All these effects on GPI an-
choring efficiency suggest the same selectivity of the ω site residue for GPI anchoring to that previously reported in Gas1p (33). Similar results were also obtained with amino acid changes at the ω sites for the C-terminal sequences of Yjr151c and Yir039c (data not shown). From these results, it could be concluded that for all four proteins the N residues function as the ω sites for GPI attachment.

Identification of ω-Minus Regions Determining Cellular Lo-
calization of the Fusion Proteins—Our previous study (24) has shown that the addition of the most C-terminal 40 amino acids of Yjr151c or Ydr077w to a reporter protein directs the result-
ing fusion proteins into the cell wall and that the addition of the C-terminal amino acids of Yir039c or Yir120c directs their fusion proteins to the plasma membrane. This indicates that the C-terminal amino acid sequences determine the final cellular localization of GPI-associated proteins. These C-terminal amino acid sequences can be divided into two regions: the ω-plus and ω-minus regions with the ω site as a center (Fig. 1A). The ω-plus region contains the residues of ω, ω+1, and ω+2 that function as the sequence required for GPI attachment; a spacer sequence of 5–10 amino acids; and a hydropho-

bic stretch of 10–15 amino acids. The ω-minus region is N-
terminal to the ω site. To determine which of the two regions is responsible for determination of cellular localization, chimeric genes between the two regions were made and expressed in yeast cells, and the cellular localization of their corresponding chimeric fusion proteins was examined (Fig. 2). The chimeric proteins of the ω-minus region from either Yjr151c or Ydr077w and the ω-plus region from either Yir039c or Yir120c were detected in the cell wall as much as those of the wild type Yjr151c and Ydr077w (Fig. 2B). They were released only by digestion of the cell wall with glucanase. On the other hand, the chimeric proteins of the ω-minus region from either Yir039c or Yir120c and the ω-plus region from either Yjr151c or Ydr077w were slightly detected in the cell wall (Fig. 2B). Their amounts were as low as those observed in the wild types of Yir039c and Yir120c. All chimeric proteins were determined to be mem-
brane-associated GPI-associated proteins, because they were released from membrane fractions by the PI-PLC treatment (Fig. 2C). From these results, it could be concluded that only the ω-minus region is responsible for determination of the final cellular localization of GPI-associated proteins. Short ω-minus
sequences of 15 amino acids were used in these experiments (Figs. 1A and 2A). Therefore, a signal determining the final cellular localization could be located within these short \( \omega \)-minus sequences.

**Mutational Analysis of \( \omega \)-Minus Regions**—To identify amino acid sequence required for the cell wall incorporation, we further examined the \( \omega \)-minus regions of the GPI-dependent cell wall proteins examined are Ser- and/or Thr-rich (24, 40), we replaced each amino acid residue in the sequence with Ser and examined the effects of the mutations on the incorporation of Yjr151c and Ydr077w-derived fusion proteins into the cell wall (Fig. 3A). All fusion proteins examined were expressed in almost the same levels (data not shown, but see Fig. 3C, lanes 7). For the C-terminal sequence of Yjr151c, changes only within the sequence VSINT corresponding to the \( \omega \)-5 to \( \omega \)-1 sites reduced the efficiency for the cell wall incorporation (Fig. 3B, CT22-M11). Similarly, the C-terminal sequence of Ydr077w, amino acid replacements only in the sequence VVINS corresponding to the \( \omega \)-5 to \( \omega \)-1 sites reduced the efficiency for the cell wall incorporation. Single changes of the \( \omega \)-5 or \( \omega \)-2 site residue in the sequence showed a partial reduction of the cell wall incorporation efficiency (Fig. 3B, CT26-M04 and -M08), while double replacements of both \( \omega \)-5 and \( \omega \)-2 site residues caused a severe reduction of cell wall incorporation (Fig. 3B, CT26-M09). From these results, it could be concluded that the signal for the cell wall incorporation would be located in the sequence of the \( \omega \)-5 to \( \omega \)-1 sites and that within the sequence the \( \omega \)-5 and \( \omega \)-2 sites are the most important.

**Identification of Amino Acid Residues Required for the Cell Wall Incorporation**—A sequence alignment of the \( \omega \)-minus regions in the GPI-dependent cell wall proteins (24) indicates that residues Ile and Val preferentially appear at the \( \omega \)-5 site and residues Tyr and Asn are predominant at the \( \omega \)-2 site. We examined the requirement of these amino acid residues for the cell wall incorporation by using a model construct. It contains a stretch of 15 serines followed by the \( \omega \)-site and the \( \omega \)-plus region of Ylr120c/Yap3p, a well characterized GPI-anchored plasma membrane protein (43–45), fused to a HA-tagged \( \alpha \)-galactosidase (Fig. 4A, SCT01). As expected, this model fusion protein was mostly localized in membranes as a GPI-anchored protein (data not shown). However, a small amount of the
Chimeric fusion proteins. YPH499 cells carrying plasmids with chimeric sequences were constructed by connecting a representation of chimeric proteins examined. Chimeric C-terminal sequences of Yjr151c, Ydr077w, Yir039c, and Ylr120c, respectively (see Fig. 1), were used to construct the reporters for their sequences.

A schematic representation of the reporter gene on pE-GALHA. CT22, CT26, CT04, and CT05 represent the C-terminal sequences of either VSSVS, TVSVS, or ISSYS in the GPI-attached membrane proteins. YPH499 cells carrying plasmids with chimeric fusion proteins were then fused to the reporter gene on pE-GALHA. CT22, CT26, CT04, and CT05 represent the C-terminal sequences of Yjr151c, Ydr077w, Yir039c, and Ylr120c, respectively (see Fig. 1A for their sequences). B, analysis of cell wall-bound forms of chimeric fusion proteins. YPH499 cells carrying plasmids with chimeric fusion proteins were grown in SD-Ura media to a concentration of A500 = 1.5–2.0. Cell wall fractions were prepared and digested with laminarinase. The proteins released from the cell wall were analyzed by Western blotting using anti-HA monoclonal antibody. C, analysis of membrane-bound forms of chimeric fusion proteins by phase separation and PI-PLC. Results for the two chimeric fusion proteins, CTX(26:05) that was abundantly incorporated into the cell wall and CTX(05:26) that was slightly incorporated into the cell wall, are shown here. See the legend of Fig. 1B for further explanation.

We replaced Ser with Val at each residue between the ω-6 and ω-1 sites and examined their effects on the cell wall incorporation of the fusion proteins (Fig. 4B, SCT07-12). Only a change at the ω-2 site increased the incorporation efficiency of these fusion proteins. A similar result was obtained when each Ser was changed to Ile (data not shown). Next, Ser at the ω-2 site was replaced with Val, Ile, Tyr, Asn, or Lys (Fig. 4B, SCT11-16). The efficiency of the cell wall incorporation was increased strongly by the replacement with Val or Ile and weakly by the replacement with Tyr or Asn. No increase was observed by the replacement with Lys. Finally, in addition to the change of Ser to Tyr at the ω-2 site, a change of Ser to Ile at the other sites was introduced (Fig. 4B, SCT14-20). The additional changes at the ω-5 or ω-4 site further increased the efficiency of the cell wall incorporation. In SCT01, only 4.1 ± 0.9% of the total fusion protein was detected in the cell wall, while in SCT18 and SCT19, 26.6 ± 4.2 and 24.9 ± 3.7%, respectively, of the total fusion proteins were detected in the cell wall. The efficiency of the cell wall incorporation was enhanced 6-fold by combining the mutation of Ser to Ile at the ω-5 or ω-4 site with a mutation of Ser to Tyr at the ω-2 site. Similar enhancement of the cell wall incorporation was observed by the replacement of Ser with Val at the ω-5 or ω-4 site and of Ser with Asn at the ω-2 site (data not shown). A slight enhancement of the cell wall incorporation effect was observed by a change of Ser to Val at the ω-4 or ω-5 site in addition to the change of Ser to Val at the ω-2 site (data not shown). From all these results, it could be concluded that combinations of Ile or Val at the ω-4 or -5 site and Tyr, Asn, or Val at the ω-2 site act to promote the incorporation of fusion proteins into the cell wall.

Cell Wall Incorporation of Mutated Yap3p GPI-anchored Plasma Membrane Protein—S. cerevisiae Yap3p, an aspartic protease, is a GPI-anchored plasma membrane protein (43–45). Using this protein, we tested whether the replacement of amino acid residues at the ω-4 or -5 and ω-2 sites directs the protein into the cell wall. First, the C-terminal sequence of Yap3p including the ω-minus region was used (Fig. 5A). The fusion protein with this C-terminal sequence was not detected in the cell wall as a glucanase-extractable protein (Fig. 5B, CT05). Changes of the ω-minus region from the wild type TSSSKR to VSSVS, SVSVS, ISSYS, or VSSS resulted in the fusion protein being incorporated into the cell wall (Fig. 5B, CT05-M02-05). These sequences correspond to the ω-5 to ω-1 regions of GPI-dependent cell wall proteins. Interestingly, a change from TSSKR to SSSSS also led to the incorporation of the fusion protein in the cell wall, but its amount was very low (Fig. 5B, CT05-M01). Second, the intact Yap3p tagged with HA was used. The Yap3p protein prepared from membrane fractions was detected as a broad band around 70 kDa after digestion with Endo H (Fig. 6B). Yap3p was extracted in a detergent phase of two-phase separation with Triton X-114 and was released from membrane fractions by treatment with PI-PLC (Fig. 6B), indicating that Yap3p is a GPI-anchored membrane protein. Mutant Yap3p proteins examined were also detected as GPI-attached membrane proteins (Fig. 6C). Next, incorporation of the wild type and mutant Yap3p proteins into the cell wall was examined. Yap3p proteins liberated from the cell wall by glucanase were further digested with Endo H, because without the Endo H treatment they formed diffused bands near the origin of gels in SDS-polyacrylamide gel electrophoresis. Compared with the wild type Yap3p, large amounts of three mutant Yap3p proteins were released from the cell wall fractions by the glucanase treatment (Fig. 6D). They contain mutated sequences of either VSSVS, TVS VS, or ISSYS in the ω-5 to ω-2 region (Fig. 6A). A mutant Yap3p containing SSSSS in the ω-5 region of gels in SDS-polyacrylamide gel electrophoresis. Comparison of membrane-bound forms of chimeric fusion proteins by phase separation and PI-PLC. Results for the two chimeric fusion proteins, CTX(26:05) that was abundantly incorporated into the cell wall and CTX(05:26), that was slightly incorporated into the cell wall, are shown here. See the legend of Fig. 1B for further explanation.

The efficiency of the cell wall incorporation was enhanced 6-fold by combining the mutation of Ser to Ile at the ω-5 or ω-4 site with a mutation of Ser to Tyr at the ω-2 site. Similar enhancement of the cell wall incorporation was observed by the replacement of Ser with Val at the ω-5 or ω-4 site and of Ser with Asn at the ω-2 site (data not shown). A slight enhancement of the cell wall incorporation effect was observed by a change of Ser to Val at the ω-4 or ω-5 site in addition to the change of Ser to Val at the ω-2 site (data not shown). From all these results, it could be concluded that combinations of Ile or Val at the ω-4 or -5 site and Tyr, Asn, or Val at the ω-2 site act to promote the incorporation of fusion proteins into the cell wall.
to ω-1 region was slightly detected in the cell wall, and its amount was comparable with that of the wild type (Fig. 6D, 05HA-M01).

**DISCUSSION**

We have studied the sequence requirement for incorporation of GPI-attached proteins into the cell wall and reached the conclusion that only the short ω-minus region composed of five amino acids (called the ω-minus 5-1 sequence), which is located upstream of the ω site for GPI attachment, determines the cellular localization of the GPI-associated proteins and that within the ω-minus 5-1 sequence amino acid residues at the ω-4 or -5 and ω-2 sites are important for the determination (called the ω-4/5 and ω-2 rule). Yap3p, a well characterized GPI-anchored plasma membrane protein (43–45), became localized in the cell wall when the ω-minus 5-1 sequence of Yap3p was mutated to sequences containing Val or Ile at the ω-4 or -5 site and Val or Tyr at the ω-2 site. In the previous study (24), we showed some sequence similarity in the ω-minus regions of 15 GPI-dependent cell wall proteins where either Val or Ile occupies the ω-5 site and Tyr is predominant at the ω-2 site. This study demonstrated their importance for the cell wall incorpo-
Although a single residue of Val, Ile, Tyr, or Asn at the \( \omega-2 \) site has some tendency to direct fusion proteins into the cell wall, the combination of the Val, Ile, Tyr, or Asn at the \( \omega-2 \) site with Val or Ile at the \( \omega-5 \) site strongly directed the fusion protein into the cell wall. In fact, out of 15 GPI-dependent cell wall proteins identified, seven, two, and three proteins possess combinations of Ile and Tyr, Val and Tyr, and Val and Asn, respectively, at the \( \omega-5 \) and \( \omega-2 \) sites (24).

The fusion protein containing the wild type sequence of TSSKR as the \( \omega-5 \)-minus 1 sequence of Yap3p was not localized in the cell wall, while the fusion protein containing the mutated sequence of SSSSS was partially localized in the cell wall (Fig. 5B). The sequence difference of KR in TSSKR and SS in SSSSS is important, because a changing of TSSKR to SSSKR led to no detectable change in the localization of the fusion protein (data not shown). It is possible that the Lys and Arg residues at the \( \omega-2 \) and \( \omega-1 \) sites may act negatively for the cell wall incorporation. Dibasic residues like this have been identified in the \( \omega-5 \)-minus region in some of putative GPI-anchored plasma membrane proteins (24, 40, 46). These dibasic residues may be a motif acting as a negative signal. A protein containing the dibasic motif may not be processed for incorporation into the cell wall and will then remain on the plasma membrane with a GPI. If this is the case, the action of the \( \omega-4/5 \) and \( \omega-2 \) rule for the cell wall incorporation would be interpreted as removal of the negative signal by interrupting the dibasic motif. However,
this interpretation would not be enough to explain the positional and additional effects by the $\omega$-5 and $\omega$-2 rule on the cell wall incorporation. Sequences meeting the rule clearly incorporated the fusion proteins into the cell wall more efficiently than that of the SSSSSS sequence. Therefore, although the rule may not be indispensable for the cell wall incorporation, it would be required for efficient incorporation of GPI-attached proteins into the cell wall.

The incorporation of GPI-associated proteins into the cell wall has been thought to occur in two steps: detachment of a GPI-moiety from a GPI-attached protein and binding of the GPI-detached protein to $\beta$-1,6-glucan of the cell wall (3). This protein-bonded $\beta$-1,6-glucan is covalently associated with $\beta$-1,3-glucan as well as chitin and forms a large complex involving cell wall components (6, 47, 48). Since $\beta$-1,6-glucan assembly is assumed to be intracellularly initiated (49), it is possible that the binding of a GPI-detached protein to $\beta$-1,6-glucan occurs somewhere in intracellular space during or after the $\beta$-1,6-glucan synthesis. However, the study using an anti-$\beta$-1,6-glucan antibody refuted this possibility. The antibody reacted with proteins collected from the cell wall, indicating association with $\beta$-1,6-glucan (4, 47–50), while it did not react with proteins intracellularly collected (5). Therefore, the removal of the GPI moiety and the linking to $\beta$-1,6-glucan would occur in the extracellular space. This suggests that GPI-dependent cell wall proteins are transported to the plasma membrane as a form of GPI-attached proteins that are associated with membranes by the GPI moiety. As expected, the GPI-attached form of the cell wall proteins was extracted in the detergent phase of Triton X-114 and showed PI-PLC sensitivity. The cell wall incorporation reaction would require an enzymatic activity, and at least the first step of the incorporation

![Figure 5](image)

**FIG. 5.** Cell wall incorporation of Yap3p-derived fusion proteins. A, schematic representation of amino acid sequences. CT05 is the wild type C-terminal sequence of Ylr120c/Yap3p. Mutant sequences were constructed by replacing the $\omega$-5 to $\omega$-1 sequence of CT05 with the sequences shown by using PCR-based mutagenesis. The CT05 and mutant sequences were fused to the reporter gene for analysis. B, analysis of cell wall-bound forms of the wild type and mutant fusion proteins from CT05. Numbers at the bottom represent ratio of the amounts of fusion proteins in the cell wall to that of CT05-M01. See the legend of Fig. 3B for further explanation.

![Figure 6](image)

**FIG. 6.** Cell wall incorporation of mutated Yap3p proteins. A, schematic representation of amino acid sequences. 05HA represents the entire sequence of Ylr120c/Yap3p tagged with the HA epitope sequence. Mutations in the $\omega$-5 to $\omega$-1 region of Yap3p were constructed by two-step PCR-based mutagenesis. B, analysis of membrane-bound forms of the wild type Yap3p protein by phase separation and PI-PLC. Yap3p extracted in a detergent phase of phase separation with Triton X-114 was treated with (+) and without (−) PI-PLC and then separated into a detergent (D) and an aqueous phase (A). Proteins in each fraction were treated with (1) and without (2) Endo H and then precipitated with trichloroacetic acid. The proteins were analyzed by Western blotting using anti-HA antibody. T, total cell lysates. C, analysis of membrane-bound forms of the wild type and mutant Yap3p proteins. The same procedure as described in B was carried out for the mutant Yap3p proteins. Only parts of results are shown here. + and − represent +, A, + and −, A, −, respectively, for treatment with PI-PLC, aqueous/detergent phase in two-phase separation and treatment with Endo H, respectively, as explained in B. D, analysis of cell wall-bound forms of the wild type and mutant Yap3p proteins. The Yap3p proteins released from the cell wall with laminarinase were treated with Endo H and then analyzed by Western blotting using anti-HA monoclonal antibody.
reaction, detachment of a GPI moiety, would require proper binding of the enzyme to the GPI-attached protein. The ω-4/5 and ω-2 rule may be related to this binding. Mutations in the ω-minus 5-1 sequence that reduced the cell wall incorporation did not increase the amount of the fusion proteins secreted into the medium (data not shown). This suggests that the low cell wall incorporation observed in these mutants is probably due to reduction of the GPI detachment efficiency caused by insufficient binding between the enzyme and the GPI-attached protein.

A structural analysis of GPI-dependent cell wall proteins has demonstrated that β-1,6-glucan is attached to the protein through a GPI remnant. Several lines of evidence indicate that the GPI remnant contains ethanolamine but does not contain glucosamine and phosphatidylinositol, suggesting that the GPI moiety is removed by cleavage either through a GPI remnant. Several lines of evidence indicate that the GPI moiety may be lipolytically cleaved by an enzyme (38), the GPI moiety may be lipolytically cleaved by an enzyme.

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