O-Glycosylation as a Sorting Determinant for Cell Surface Delivery in Yeast

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Little is known about the mechanisms that determine localization of proteins to the plasma membrane in *Saccharomyces cerevisiae*. The length of the transmembrane domains and association of proteins with lipid rafts have been proposed to play a role in sorting to the cell surface. Here, we report that Fus1p, an O-glycosylated integral membrane protein involved in cell fusion during yeast mating, requires O-glycosylation for cell surface delivery. In cells lacking *PMT4*, encoding a mannosyltransferase involved in the initial step of O-glycosylation, Fus1p was not glycosylated and accumulated in late Golgi structures. A chimeric protein lacking O-glycosylation motif was missorted to the vacuole and accumulated in late Golgi in wild-type cells. Exocytosis of this protein could be restored by addition of a 33-amino acid portion of an O-glycosylated sequence from Fus1p. Our data suggest that O-glycosylation functions as a sorting determinant for cell surface delivery of Fus1p.

INTRODUCTION

In polarized epithelial cells, newly synthesized material is sorted in the *trans*-Golgi network and delivered via separate routes to the apical and basolateral domains on the plasma membrane (Keller *et al.*, 2001). Similar pathways exist in nonpolarized cells (Keller *et al.*, 2001). It has been shown that short motifs present on the cytoplasmic domain of transmembrane proteins are recognized as determinants for basolateral delivery (Matter and Mellman, 1994). The principles of sorting to the apical plasma membrane are less understood. It is known that association with lipid rafts plays a role and that protein glycosylation is involved in apical exocytosis (Scheiffele *et al.*, 1995; Simons and Ikonen, 1997; Yeaman *et al.*, 1997). Nonglycosylated secretory proteins are sorted both apically and basolaterally, whereas glycosylated proteins are preferentially sorted to the apical membrane. Also membrane proteins have been demonstrated to use N-glycans and O-glycans for apical delivery (Yeaman *et al.*, 1997; Gut *et al.*, 1998; Benting *et al.*, 1999; Spodisberg *et al.*, 2001).

Similar to mammalian cells, the budding yeast *Saccharomyces cerevisiae* has been shown to have at least two different routes for biosynthetic transport from the Golgi to the cell surface (Harsay and Bretscher, 1995; David *et al.*, 1998; Gurunathan *et al.*, 2002; Harsay and Schekman, 2002). Little is known about the sorting determinants, specifying surface delivery of proteins. The length of the transmembrane domain has been proposed to regulate transport to the plasma

membrane and to the vacuole (Rayner and Pelham, 1997; Levine *et al.*, 2000). For Pma1p, the major plasma membrane protein, it was shown that clustering and association with lipid rafts is required for cell surface delivery (Bagnat *et al.*, 2000, 2001, 2002b; Lee *et al.*, 2002). Similarly, plasma membrane localization of the amino acid permease Tat2p depends on its association with lipid rafts (Umebayashi and Nakano, 2003).

In this study, we have explored whether O-glycosylation plays a role in surface delivery in the yeast *S. cerevisiae*. In yeast, O-glycosylation is initiated in the endoplasmic reticulum (ER) and extended in the Golgi complex (Haselbeck and Tanner, 1983; Herscovics and Orlean, 1993). Dolichol-phosphate-mannose is used as a donor for the first mannose transfer to serine or threonine residues during O-glycosylation in the ER. GDP-mannose is the mannosyl donor for the elongation process in the Golgi (Babczinski and Tanner, 1973; Sharma *et al.*, 1974). A family of seven ER protein O-mannosyltransferases (PMTs) that initiates the assembly of O-glycosylation chains has been identified (Immervoll *et al.*, 1995; Gentzsch and Tanner, 1996, 1997; Strahl-Bolsinger *et al.*, 1999). The PMT family was classified into *PMT1*, *PMT2*, and *PMT4* subfamilies, which differ in their protein substrate specificity (Gentzsch and Tanner, 1997; Girrbach and Strahl, 2003). The PMT family is highly redundant and only the simultaneous deletion of *PMT1/PMT2* and *PMT4* subfamily members is lethal (Gentzsch and Tanner, 1996; Girrbach *et al.*, 2000). Unlike other PMT members, Pmt4p acts as a homomeric complex (Strahl-Bolsinger *et al.*, 1999). It was shown previously that Axl2, a protein involved in bud site selection, is incompletely glycosylated, mislocalized, and rapidly degraded in cells lacking *PMT4* (Sanders *et al.*, 1999). Here, we show that Fus1p, a raft associated and O-glycosylated protein involved in cell fusion during mating (Trueheart *et al.*, 1987; Trueheart and Fink, 1989; Bagnat and Simons, 2002a), requires O-glycosylation for its cell surface delivery. In *pmt4Δ* mutant cells, Fus1p is not glycosylated and mostly accumulated in late Golgi structures. A chimeric protein lacking an O-glycosylated sequence is mis-

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Abbreviations used: ER, endoplasmic reticulum; GFP, green fluorescent protein.

Table 1. Yeast strains used in this study

Yeast strain	Genotype	Source
RH690-15D	Mata <i>his4 leu2 ura3 lys2 bar1</i>	H. Riezman laboratory
MBY249	RH690-15D <i>pmt5::LEU2</i>	This study
MBY254	RH690-15D <i>pmt4::LEU3</i>	This study
SEY6210	Mat alpha <i>ura3-52 leu2-3,112 his3D200 trp1D90 lys2-801 suc2D9</i>	W. Tanner laboratory
1403	SEY6210 <i>pmt1::HIS3</i>	W. Tanner laboratory
1405	SEY6210 <i>pmt2::LEU2</i>	W. Tanner laboratory
1407	SEY6210 <i>pmt3::HIS3</i>	W. Tanner laboratory
1409	SEY6210 <i>pmt4::TRP1</i>	W. Tanner laboratory
1421	SEY6210 <i>pmt6::LEU2</i>	W. Tanner laboratory
SFNY28-6C	Mata <i>sec53 ura3-52</i>	S. Ferro-Novick laboratory
H891	Mata <i>sec18-1 trp1-289 leu2-3,112 ura3-52 his-</i>	S. Keranen
NY430	Mata <i>sec14-3 ura3-52</i>	Ch. Walch-Sollmena
W303	Mata <i>ade2::ADE2 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL psl+</i>	W. Zachariae laboratory
MBY114	W303 TRP-1::SEC7-DsRed	This study
MBY119	MBY114 <i>pmt4::LEU2</i>	This study
AAY1017	Mata <i>his1</i>	CSH laboratory course

sorted to the vacuole and accumulated in late Golgi in wild-type cells, but its secretion can be restored after addition of a portion of a Fus1p-glycosylated domain. Our data suggest that O-glycosylation functions as a sorting determinant for cell surface delivery of Fus1p.

MATERIALS AND METHODS

Strains, Growth Conditions, and Reagents

Yeast strains used in this study are listed in Table 1, and their construction is described below. Yeast cells were grown in yeast extract/peptone/dextrose (YPD) medium or extract/peptone (YP) medium containing 2% raffinose (YPRaf) as a carbon source at 24°C or at the indicated temperature. For induction of expression from the GAL5 promoter, cells from YPD media were washed twice with YP media containing 2% galactose (YPGal) and incubated for 3 h (or as indicated) in YPGal; cultures in YPRaf was supplemented with 2% galactose (final concentration).

Plasmid and Strain Construction

Plasmids used in this study are listed in Table 2, and expressed constructs are shown in a cartoon in Figure 1. All proteins used in this study were expressed from the centromeric plasmid p416 (Mumberg *et al.*, 1994). Polymerase chain reaction (PCR) products were integrated into the pGEM-T vector by using the TA ligation kit (A3600; Promega, Madison, WI), cut out using restriction sites introduced on primers, and subcloned by the triple ligation method into *XbaI/HindIII* linearized p416. The *FUS1* sequence was amplified from genomic DNA by using primers containing *XbaI* and *BamHI* restriction sites, and the TAP-tag sequence was PCR-amplified from pBS1539 (Puig *et al.*, 2001) with primers containing *BamHI* and *HindIII* sites. Restriction digested *FUS1* and TAP-tag sequences were coligated into p416 to generate the TPQ67 plasmid. pMBQ30 has been described previously (Bagnat and Simons, 2002a).

MBQ35 containing Mid2-GFP construct was cloned by coligation of the PCR-amplified *MID2* sequence from genomic DNA by using primers containing *XbaI* and *BamHI* sites, and the green fluorescent protein (GFP) sequence was PCR amplified with primers containing *BamHI* and *HindIII* sites. TPQ53, containing the MidFus construct (Figure 1) fused to GFP, was generated by coligation of a sequence encoding the extracellular domain of Mid2p amplified from MBQ35 by using primers containing *XbaI* and *BglII*, and the Fus1p-GFP sequence was amplified from MBQ30 with primers containing *BamHI* and *HindIII* sites. TPQ55, containing Fus1-Mid construct (Figure 1), was generated by coligation of the DNA encoding the extracellular domain of Fus1p amplified from MBQ30 with primers containing *XbaI* and *BglII* sites and part of the *MID2-GFP* sequence amplified from MBQ35 with primers containing *BglII* and *HindIII* sites. The sequence encoding the N-terminal part of invertase (*SUC2*), present in four constructs (Figure 1), was amplified from genomic DNA by using primers containing *XbaI* and *BamHI* sites. To generate TPQ34, the Inv-Fus (Figure 1) portion of the invertase sequence was coligated with a sequence encoding portion of Fus1-GFP amplified from genomic DNA from the MBY229 strain (Bagnat and Simons, 2002a) with primers containing *BamHI* and *HindIII* sites. To generate TPQ80, containing Inv-Mid, the invertase sequence was coligated with the part of *MID2-GFP* used to generate Fus-Mid construct described above. To generate TPQ52, containing Inv33Fus construct, part of invertase was coligated to portion of *FUS1-GFP* amplified from MBQ30 with primers containing *BglII* and *HindIII* sites. To generate TPQ79, containing the Inv33Mid construct, the sequence encoding the extracellular part of Inv33Fus was amplified from TPQ52 with primers containing *XbaI* and *BglII* and coligated with portion of *MID2-GFP* prepared as described above to generate Fus-Mid. All protein constructs were membrane associated as determined by density gradient centrifugation.

Disruption of *PMT4* and *PMT5* was done by integrating PCR-amplified *Candida glabrata* *LEU2* into the *PMT4* or *PMT5* locus. To express SEC7-DsRed, a Ylplac204/-SEC7DsRed plasmid, kindly provided by Benjamin Glick (University of Chicago, Chicago, IL), was integrated into the genome of wild-type or *pmt4Δ* cells in the W303 background.

Table 2. Plasmids used in this study

Plasmid name	Expressing	Plasmid type	Source	Original name
TPQ67	Fus1-TAP	Centromeric	This study	No
MBQ30	Fus1-GFP	Centromeric	This study	No
MBQ38	Mid2-GFP	Centromeric	This study	No
TPQ53	Mid-Fus	Centromeric	This study	No
TPQ55	Fus-Mid	Centromeric	This study	No
TPQ34	Inv-Fus	Centromeric	This study	No
TPQ80	Inv-Mid	Centromeric	This study	No
TPQ52	Inv33Fus	Centromeric	This study	No
TPQ79	Inv33Mid	Centromeric	This study	No
TPQ1	(Vector)	Centromeric	W. Zachariae laboratory	p416
TPQ61	(Source of TAP-tag)	Centromeric	B. Seraphin laboratory	pBS1539
TPQ76	Sec7-DsRed	Integration	B. Glick laboratory	Ylplac204/SEC7DsRed

Figure 1. Schematic representation of constructs used in this study. The amino acid sequence of the extracellular domain of Fus1p is shown at the bottom. In red are shown potentially O-glycosylated amino acids. Green arrows and numbers indicate the length of the segment from the extracellular domain of Fus1p added to the invertase fusion constructs. Colors represent sequence from different proteins: yellow, Fus1p; red, Mid2p; and white, invertase. All protein construct contain a C-terminal added GFP tag. Additionally, Fus1p was also tagged with the TAP-tag (see Figures 2A and 3).

Western Blot

Western blot analysis was performed according to standard procedures. Cells were disrupted by beating with glass beads for 5 min at 4°C and boiled with SDS-PAGE sample buffer containing 5% mercaptoethanol. To detect protein A present in the TAP-tag, we used a peroxidase-anti-peroxidase antibody (P-2026; Sigma-Aldrich, St. Louis, MO). For Western blot analysis of GFP fusion proteins, we used a mouse monoclonal anti-GFP antibody (B-2) (sc-9996; Santa Cruz Biotechnology, Santa Cruz, CA).

Metabolic Labeling and Immunoprecipitation

Cells were grown to mid-log phase in complete synthetic medium without methionine containing 2% raffinose as carbon source and expression of Fus1-GFP was induced for 15 min by addition of 2% galactose. Then, the cells were pulse labeled with 1 mCi of [³⁵S]methionine for 5 min and chased for various times. At the indicated times, samples were taken, and cells were killed in 0.2% sodium azide on ice. Then, the cells were lysed in lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and CLAP protease inhibitors mix: 0.1% chymostatin, 0.1% leupeptin, 0.1% antipain, and 0.1% pepstatin) by shaking with glass beads for 5 min at 4°C. After removing cell debris, lysates were adjusted to 1% NP-40 and 0.1% SDS and warmed at 37°C for 5 min. Insoluble material was removed by centrifugation (1 min, 6000 × g), and samples were diluted twofold in IP buffer (10 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 1% NP-40, and 0.1% SDS). Then, samples were incubated with protein A and a rabbit anti-GFP antibody (sc-9996; Santa Cruz Biotechnology) for 3 h at room temperature. Immunoprecipitates were washed four times with IP buffer, once with Tris 20 mM, pH 7.4, and subjected to SDS-PAGE. Protein bands were analyzed by autoradiography.

Tunicamycin Treatment and Mating Assay

Cells were grown overnight in raffinose-containing medium, and expression was induced by addition of galactose (2%). Tunicamycin was added (10 μg/ml), and cells were incubated for 3 h at 24°C. Mating assay was performed as described previously (Bagnat and Simons, 2002a). Then, 0.5 ml of the overnight culture was diluted 20 times in YPD and incubated for 3 h. The density of cells in the culture was assessed by cell counting in the counting chamber, and 1 × 10⁷ of each mating type cells were mixed together and collected on a nitrocellulose filter. Filters were incubated for 3 h at 24°C on YPD plates to allow for cell fusion. Finally, cells were resuspended in water, diluted 100 times, and an equal amount was plated on SD (selection for diploids) and YPD (nonselective) plates. Mating efficiency was calculated as a ratio of number of colonies from SD plates to number of colonies from YPD plates. MBY1102 (AAY1017) was used as a wild-type Mat alpha strain for mating with either RH690-15D (wild-type) or MBY249 (*pmt4Δ*).

Microscopy

Microscopy was performed on live cells that were washed twice in water and resuspended in water for imaging using an Olympus BX61 microscope, RT

Slider SPOT camera (Diagnostic Instruments, Sterling Heights, MI) and MetaMorph software.

Quantification of the Fluorescence Microscopy

Cells were grown until mid-log phase in YPD media, washed twice, and resuspended in YPGal media followed by incubation for 3 h at 24°C to induce protein expression. Images of cells expressing different constructs were taken using the same conditions. Stacks of the pictures were converted to the TIFF format by using ImageJ program (Wayne Rasband, National Institutes of Health, Bethesda, MD), and the fluorescence intensity was analyzed using the IpLab (Scanalytics, Fairfax, VA) software. For each image, the background fluorescence was measured and subtracted. The area representing plasma membrane of each cell was marked as a ring-like segment. The differential interference contrast images were used to localize the periphery of the cells when fluorescence from the plasma membrane was too weak to localize it. The total fluorescence of the plasma membrane was measured from the encircled area. The fluorescence corresponding to the intracellular space was measured from the area inside the ring-like segment. The bars represent the ratio of the total fluorescence measured for the plasma membrane and for the intracellular area. The data are standardized to O-glycosylated constructs (Fus1-GFP and Fus-Mid) expressed in wild-type cells.

RESULTS

Unglycosylated Fus1p Accumulates Intracellularly in *pmt4Δ* Mutant Cells

Fus1p is an O-glycosylated type I integral membrane protein, which is raft associated and is required for cell fusion during yeast mating (Trueheart *et al.*, 1987; Trueheart and Fink, 1989; Bagnat and Simons, 2002a). The function of the O-glycosylation of Fus1p is not known. To gain insight into the role of O-glycosylation, we first determined which particular member of the PMT gene family is responsible for the glycosylation of Fus1p. We expressed an epitope-tagged version of the protein in all *pmt* mutants under the control of the inducible GAL-S promoter. In Western blot analysis of Fus1 expressed in wild-type cells, we detected four specific bands (Figure 2A). These different forms are due to the processing of the protein (Trueheart and Fink, 1989; Bagnat and Simons, 2002a) (see below). A similar pattern was observed when the protein was expressed in *pmt1Δ*, *pmt2Δ*, *pmt3Δ*, *pmt5Δ*, and *pmt6Δ* mutants, indicating that in these mutants the protein was processed normally (Figure 2A). In contrast, in *pmt4Δ* cells Fus1 was detected as a single band,

Figure 2. Fus1 processing and its surface delivery is blocked in *pmt4*Δ cells. (A) Western blot analysis of Fus1-TAP expressed in different *pmt* mutants. (B) Cellular localization of Fus1-GFP in wild-type and *pmt4*Δ cells. In wild-type cells, the protein is localized to the plasma membrane and the vacuole (arrow). In *pmt4*Δ cells, protein accumulates in dot-like structures inside the cell and in the vacuole (arrow). Quantitative analysis of fluorescence from plasma membrane and inside of the cell (see MATERIALS AND METHODS) demonstrated that Fus1-GFP is much more efficiently delivered to the cell surface in wild-type cells than in the *pmt4*Δ cells (see Figure 9).

suggesting that in this mutant Fus1 was not properly processed. We observed variation in the relative amount of the different forms of the protein produced in different *pmt* mutants; however, similar variations were observed for the protein produced in wild-type cells in different experiments.

Next, we tested whether altered processing of Fus1 in *pmt4*Δ mutant cells affected its cellular localization. A green fluorescent protein fusion (Fus1-GFP) was expressed from the GAL-S promoter (Bagnat and Simons, 2002) in wild-type and *pmt4*Δ cells. In wild-type cells, 3 h after induction of expression, Fus1-GFP was localized to the plasma membrane of the bud and to vacuoles (Figure 2B). Fus1p has a very fast turnover (half-life <1 h), and after delivery to the cell surface it is rapidly endocytosed and transported to the vacuole for degradation. In *pmt4*Δ cells, transport of Fus1-GFP to the cell surface was inhibited (only 3.6% of cells had surface staining; N = 300); instead, the protein accumulated

Figure 3. Unglycosylated Fus1 accumulates in *pmt4*Δ cells. Western blot analysis of Fus1-TAP expressed in wild-type, *pmt4*Δ, and different secretory mutant cells. The form of Fus1 that is produced in the *pmt4*Δ mutant migrates at the level of the unglycosylated protein produced in *sec53* cells at 37°C (*sec53* form). In *sec18* cells at 37°C, the protein accumulated in the ER and migrated as a partially glycosylated precursor (p) form. In *sec14* cells at 37°C, the protein accumulated in the Golgi as a fully glycosylated mature form (m1). In the Golgi complex, a second mature form (m2) was generated. The m2 form migrated more rapidly than all the other forms. In *pmt4*Δ cells, minute amounts of m1, p, and m2 forms were also detected.

intracellularly in dot-like structures and in vacuoles (Figure 2B). These structures resembled Golgi or endosomal elements.

To examine which form of Fus1 is produced in *pmt4*Δ cells, we expressed the protein in thermosensitive mutants that block biosynthetic traffic along the secretory pathway at different stages. *SEC53* encodes a phospho-mannomutase, necessary for the production of dolichol-P-mannose and GDP-mannose, donors of sugars for both N- and O-glycosylation (Babczinski and Tanner, 1973; Sharma *et al.*, 1974; Ruohola and Ferro-Novick, 1987; Kepes and Schekman, 1988). When *sec53* cells were incubated at 37°C (the restrictive temperature), both N- and O-glycosylation were blocked. Fus1 expressed in *sec53* cells at the restrictive temperature migrated as a single band with the same mobility as the band generated in *pmt4*Δ cells (Figure 3), suggesting that Fus1 was not glycosylated in the *pmt4*Δ mutant. In *sec18* cells, when the protein accumulated in the ER, Fus1 migration on SDS-PAGE was shifted compared with the *sec53* form (Figure 3), due to partial glycosylation of the protein. This precursor (p) form was transported to the Golgi where elongation of the mannose chains takes place. The fully glycosylated form of Fus1, accumulating in the Golgi in the *sec14* mutant cells, migrated as the slowest band, mature1 (m1). A second mature form of Fus1, mature2 (m2 that migrates as the fastest band) was also generated in the Golgi complex (Figure 3). The big shift in migration between the m1 and m2 forms suggested that the protein was proteolytically cleaved. In *sec14* cells, both mature forms were generated, indicating that the cleavage occurred in the Golgi complex.

We also followed the maturation of Fus1-GFP in wild-type and *pmt4*Δ mutant cells in a pulse-chase experiment. Cells were grown in medium containing raffinose as carbon source and expression of Fus1-GFP was induced for 15 min by addition of galactose. Then, cells were pulse labeled with [³⁵S]methionine for 5 min and chased for various times. In wild-type cells at the beginning of the chase, the unglycosylated (*sec53* form) form and the ER precursor form (p) of the protein were detected (Figure 4). After 5 min of chase, the mature form (m1) was visible and after 30 min of chase the m2 form was generated. As the protein matured the amount of the precursor form was reduced. In contrast, in the *pmt4*Δ mutant Fus1-GFP migrated as the unglycosylated form throughout the whole chase period. Only faint bands that

Figure 4. Processing of Fus1-GFP in wild-type and *pmt4Δ* cells. Cells were grown in medium containing raffinose as the carbon source and expression of Fus1-GFP was induced for 15 min by addition of galactose. The cells were then pulse labeled with [³⁵S]methionine for 5 min and chased for various times as indicated. In wild-type cells, at the beginning of chase the sec53 and p forms of Fus1 were present. After 5 min, m1 form was visible and after 30 min the m2 form was generated. Throughout the chase period, the p and sec53 forms were also detected. In *pmt4Δ* cells throughout the chase period Fus1 migrated with the same mobility as the unglycosylated sec53 form.

represent other forms of Fus1 were detected. Thus, in the *pmt4Δ* mutant, Fus1 was not degraded and remained unglycosylated throughout the chase period.

pmt4Δ Cells Do Not Show a General Secretory Defect

To investigate whether the secretory block, which we observed in the *pmt4Δ* mutant, is specific for Fus1-GFP or also affects other proteins, we examined the cell surface delivery of Mid2p. Mid2p, a cell wall integrity sensor, is a type I, O-glycosylated membrane protein (Rajavel *et al.*, 1999; Philip and Levin, 2001). Several members of the PMT family are responsible for the glycosylation of Mid2p (Lommet *et al.*, 2004). Mid2-GFP was efficiently delivered to the cell surface in wild-type and *pmt4Δ* cells (Figure 5), indicating that in the *pmt4Δ* mutant there is no general block in exocytosis.

O-Glycans on the Extracellular Domain of Fus1p Are Important for Surface Localization.

Because the extracellular parts of both Fus1p and Mid2p are O-glycosylated and surface delivery of Fus1p but not Mid2p is affected in *pmt4Δ*, we decided to swap the extracellular domains of Fus1p and Mid2p to evaluate the role of the ectodomains in protein exocytosis. The chimeric protein containing the extracellular domain from Mid2p fused to the transmembrane domain and cytoplasmic tail from Fus1p, followed by GFP was named Mid-Fus (see cartoon in Figure 1). Similar to Mid2, Mid-Fus was efficiently delivered to the cell surface, but weak GFP fluorescence was also localized to the vacuole (Figure 5). Conversely, the GFP-tagged chimeric protein containing the extracellular part from Fus1p fused to

the transmembrane domain and cytoplasmic part of Mid2p was named Fus-Mid. Fus-Mid expressed in *pmt4Δ* mutant, accumulated intracellularly in dot-like structures and in the vacuole (Figure 5). In SDS-PAGE, Fus-Mid expressed in the *pmt4Δ* mutant cells migrated with the same mobility as the protein expressed in *sec53* cells at the restrictive temperature (our unpublished data), indicating that Fus-Mid was O-glycosylated in a PMT4-dependent manner.

Next, we asked in which compartments were the unglycosylated forms of Fus1 and Fus-Mid accumulated. To do so, we introduced either Fus1-GFP or Fus-Mid GFP in *pmt4Δ* cells expressing Sec7-DsRed, a late Golgi marker (Franzusoff *et al.*, 1991; Rossanese *et al.*, 1999; Rossanese *et al.*, 2001). Most of the intracellular structures that accumulated GFP fusion proteins also showed intensive DsRed staining (Figure 6), indicating that the unglycosylated Fus1 and Fus-Mid accumulated in the Golgi complex. Together, our results indicate that the O-glycosylation of the extracellular part of Fus1p is required for surface delivery of Fus1p.

pmt4Δ Mutant Cells Have a Strong Unilateral Mating Defect

Because in the *pmt4Δ* cells Fus1p, a protein involved in cell fusion during yeast mating, is not delivered efficiently to the cell surface, we checked whether the *pmt4Δ* cells show a defect in mating. The *pmt4Δ* *Mat a* cells crossed to wild-type *Mat α* cells showed an 18-fold reduced mating efficiency compared with control wild-type *Mat a* cells. *fus1Δ* cells show a bilateral mating phenotype that is pronounced only when *FUS1* is deleted in both parental strains (Berlin *et al.*, 1991; Gammie *et al.*, 1998). However, the mating defect of the *pmt4Δ* cells was unilateral and strongly pronounced when only one of the mating strains was missing the *PMT4* gene. Probably, in *pmt4Δ* cells not only Fus1p but also other proteins involved in mating were not glycosylated and therefore mislocalized and/or not functional.

Fus1 and Mid2 Chimeric Proteins Lacking O-Glycans Are Retained Intracellularly

Because Fus1p and Fus-Mid lacking O-glycans were blocked in their transport to the cell surface in *pmt4Δ* cells, we analyzed whether the intracellular accumulation of both markers is due to the lack of O-glycosylation or to an effect of the *PMT4* deletion. To do so, we replaced the extracellular domain of both proteins with a sequence, similar in length, from the N-terminal part of invertase (Suc2p) (Figure 1). Invertase is a soluble secreted protein that is N-glycosylated, but not O-glycosylated. The protein has been used as a reporter fused to different membrane proteins to study membrane sorting (Darsow *et al.*, 2000). Invertase contains an N-terminal signal sequence, which allows the correct translocation of the fusion constructs into the secretory pathway (Li *et al.*, 2002). We expressed the chimeric proteins named Inv-Fus and Inv-Mid containing a GFP tag in wild-type cells. Unlike the O-glycosylated Fus1 and Fus-Mid, both Inv-Fus and Inv-Mid were inhibited in their transport to the plasma membrane and were instead missorted to the vacuole and accumulated in Golgi-like structures (Figure 7A). Out of >300 cells, we could not find any cell with surface staining.

Because invertase is an N-glycosylated protein, we checked whether our fusion constructs containing 70 amino acids from invertase, including three potential N-glycosylated sites, were N-glycosylated. We expressed Inv-Fus and Inv-Mid in wild-type cells in the presence or absence of tunicamycin, an inhibitor that

Figure 5. Cellular localization of Fus-GFP, Mid2-GFP, and chimeric proteins in wild-type and *pmt4* cells. In wild-type cells, Fus1-GFP was delivered to the cell surface and to the vacuole (arrow). In *pmt4*Δ cells, protein accumulated in dot-like structures (arrowhead) inside the cell and in the vacuole (arrow). Mid2-GFP was efficiently delivered to the cell surface in wild-type and *pmt4*Δ mutant. Similarly, Mid-Fus was delivered to the plasma membrane both in wild-type and *pmt4*Δ cells. In the *pmt4*Δ mutant, the protein was also detected in the vacuole (arrow). Fus-Mid surface delivery was dependent on *PMT4*. In the *pmt4*Δ mutant, the Fus-Mid protein accumulated in dot-like structures (arrowhead) inside the cell and in the vacuole (arrow). Quantitative fluorescence analysis confirmed increased intracellular accumulation of Fus-Mid in *pmt4*Δ cells compared with wild-type cells (see Figure 9).

specifically blocks *N*-glycosylation (Lehle and Tanner, 1976). By Western blotting, both proteins migrated more slowly when tunicamycin was added to the cell culture compared with control cells not treated with the inhibitor (Figure 7B). In tunicamycin-

treated cells, both proteins migrated with the same mobility as proteins produced in *sec53* cells cultured at the restrictive temperature, indicating that both Inv-Fus and Inv-Mid were *N*-glycosylated but not *O*-glycosylated.

Figure 6. In *pmt4*Δ cells Fus1 and Fus-Mid accumulates in the late Golgi structures. In *pmt4*Δ mutant, Fus1-GFP and Fus-Mid GFP colocalized with Sec7-DsRed, a late Golgi marker.

Figure 7. Inv-Fus and Inv-Mid are not delivered to the cell surface in wild-type cells and accumulate in late Golgi compartments. (A) Surface delivery of Fus1-GFP, Mid2-GFP and chimeric invertase constructs in wild-type cells. Both Fus1-GFP and Mid2-GFP were efficiently delivered to the cell surface. GFP fusion constructs of Fus1 and Mid2 carrying a portion of invertase sequence instead of their extracellular domain accumulated in dot-like structures (arrowhead) inside the cell and in vacuole (arrow). Quantitative analysis showed strongly reduced surface delivery of both invertase fusion constructs (Inv-Fus and Inv-Mid) compared to *O*-glycosylated constructs (Fus-GFP and Fus-Mid in wild-type) (see Figure 9). (B) Inv-Fus and Inv-Mid are *N*-but not *O*-glycosylated. Western blot analysis showed that Inv-Fus and Inv-Mid expressed in wild-type cells in presence of tunicamycin (tun) migrated faster than the proteins expressed in the absence of tunicamycin. After inhibition of *N*-glycosylation (WT + tunicamycin) both proteins migrated with the same mobility as the proteins produced in *sec53* cells at the restrictive temperature. (C) Inv-Fus was missorted to the vacuole (double arrowhead) and also accumulated in late Golgi structures (marked by Sec7-DsRed). Note, that two different strains were used in A–C (see MATERIALS AND METHODS).

Next, we asked whether the dot-like structures that accumulated the invertase fusion construct were Golgi structures. We expressed Inv-Fus in wild-type cells producing Sec7-DsRed as before. As shown in Figure 7C, there was partial colocalization of GFP and DsRed staining. Inv-Fus was missorted to the vacuole and also localized in Golgi structures. Most likely, dots that were stained with GFP and did not colocalize with Sec7-DsRed marker were in intermediate structures on the way to the vacuole.

In summary, these results show that Fus1 and Mid2 proteins fused to the *N*-terminal part of the invertase sequence containing *N*-glycans accumulated in Golgi structures and were also missorted to the vacuole.

Surface Delivery of Invertase Chimeric Proteins Is Rescued by Addition of an *O*-Glycosylated Sequence from Fus1p

The invertase fusion proteins were not delivered to the cell surface. This defect could be due to the lack of *O*-glycosylation. To test this hypothesis, we tried to rescue surface delivery of Inv-Fus and Inv-Mid by addition of a portion (33

amino acids) of the *O*-glycosylated domain from Fus1p containing 15 potential *O*-glycosylation sites (Figure 1). We generated “rescue” constructs containing the same *N*-terminal part of invertase as used before fused to the 33-amino acid sequence of the juxtamembrane part of the extracellular domain of Fus1p, followed by the transmembrane domain and the cytoplasmic tail from either Fus1p or Mid2p with the GFP tag at the C terminus (Figure 1). The new constructs were named Inv33Fus and Inv33Mid. In wild-type cells, unlike the parent invertase fusion proteins, Inv33Fus and Inv33Mid were efficiently delivered to the cell surface (Figure 8A). Most of cells expressing Inv33Mid showed not only GFP fluorescence on the plasma membrane but also a weak fluorescence signal was observed in the ER-like structures. The expression level of Inv33Fus was very low, but similar to Mid2, most of the cells (64.1%; *N* = 256) expressing Inv33Fus showed fluorescence signal on the plasma membrane.

Interestingly, Inv33Fus and Inv33Mid expressed in *pmt4Δ* mostly accumulated inside cells in dot-like structures and also in vacuoles (Figure 8A), indicating that the surface

*pmt4*Δ mutant, wild-type, and *sec53* cells (temperature as indicated) in the presence or absence of tunicamycin (tun-/- +). Differences in protein migration due to *N*- and *O*-glycosylation are indicated on the right side of the figure.

Figure 8. Surface delivery of invertase constructs can be rescued by addition of portion of the Fus1p *O*-glycosylated extracellular domain and is dependent on *PMT4*. (A) Cellular localization of invertase rescue constructs containing portions from the extracellular domain of Fus1p. The constructs were expressed in wild-type and *pmt4*Δ cells as indicated. Quantification showed that the addition of 33 amino acids from the extracellular part of Fus1p could restore the plasma membrane localization of Inv33Mid (see Figure 9). Cells were grown in medium containing a raffinose as a carbon source, and proteins expression was induced overnight by addition of galactose. Western blot analysis of Inv33Mid (B) and Inv33Fus (C) expressed in

delivery of “rescue” constructs was at least partially dependent on *PMT4*. Next, we checked whether the rescue constructs were *O*-glycosylated. The Inv33Mid protein expressed in *pmt4*Δ cells migrated in SDS-PAGE with a slower mobility than the protein produced in wild-type cells (Figure 8B), suggesting that *PMT4* is involved in the *O*-glycosylation of Inv33Mid. The protein was also *N*-glycosylated, because we observed a shift in migration of the protein expressed in wild-type cells in the presence of tunicamycin compared with the protein expressed in control cells without tunicamycin (Figure 8B). There was an additional shift in migration of the protein between the form expressed in wild-type cells in presence of tunicamycin and the unglycosylated form produced in *sec53* at 37°C (Figure 8B). These experiments show that Inv33Mid contains *N*-glycans and is also *O*-glycosylated in a *PMT4*-dependent manner. Similar results were obtained for Inv33Fus protein (Figure 8C).

Therefore, addition of 33 amino acids from the glycosylated domain of Fus1p allowed surface delivery of the invertase fusion constructs. When we further shortened the 33-amino acid fragment by 10 amino acids (from the N terminus) and inserted a 22-amino acid portion into Inv-Fus and Inv-Mid, these chimeric proteins were not delivered to the plasma membrane and were not *O*-glycosylated (our unpublished data).

Together, these experiments show that surface delivery of Inv-Fus and Inv-Mid could be rescued by addition of the 33-amino acid segment from the glycosylated domain of Fus1p. Inv33Fus and Inv33Mid were *O*-glycosylated, and surface delivery of both proteins depended on *PMT4*. Thus, we conclude that *O*-glycosylation is required for surface delivery of Fus1p.

DISCUSSION

In this study, we have analyzed the potential role of *O*-glycans as sorting determinants for surface delivery in yeast. We used Fus1p as our marker protein. Fus1p is an *O*-glycosylated integral membrane protein (Trueheart and Fink, 1989). Its *O*-glycosylation starts in the ER through the action of the *O*-mannosyl transferase Pmt4p (Figure 3). Further mannose groups are added to Fus1p in the Golgi complex and then the processed protein is transported to the plasma membrane (Trueheart and Fink, 1989) (Figure 3). Even when the *PMT4* is deleted, a small amount of glycosylated Fus1 was detected (Figures 2–4). Probably, in the absence of Pmt4p, other members of the

PMT family can glycosylate Fus1p, but it occurs with low efficiency. We found that when *O*-glycosylation is blocked in *pmt4*Δ cells Fus1p accumulates intracellularly in punctate structures that colocalize with Sec7p, a Golgi marker (Figure 6). The surface transport of another *O*-glycosylated plasma membrane protein, Mid2p, was not affected in *pmt4*Δ cells. Using chimeric constructs consisting of parts of Fus1p and Mid2p, we demonstrated that the ectodomain of Fus1p was responsible for the blocked transport to the cell surface in *pmt4*Δ cells (Figure 5). To find out whether *O*-glycans were indeed involved in Fus1 sorting and delivery to the cell surface, we used a strategy previously used by Rose and coworkers in mammalian cells (Guan *et al.*, 1985). They demonstrated that a nonglycosylated secretory protein, rat growth hormone, was blocked in the Golgi complex when it was anchored to the membrane by fusing the hormone to the transmembrane and cytosolic domains of vesicular stomatitis virus G protein. However, when one or two *N*-glycosylation sites were introduced into the growth hormone ectodomain, the *N*-glycosylated membrane protein was transported to the cell surface. Similarly, when we fused the N-terminal part of the invertase sequence to the transmembrane and cytosolic domains of Fus1p or of Mid2p, these chimeric proteins accumulated intracellularly (Figure 7). Next, we inserted segments from the Fus1p ectodomain between the invertase and the Fus1 transmembrane anchor (see cartoon in Figure 1) in an attempt to find out whether addition of *O*-glycans could influence transport to the plasma membrane. When a 33-amino acid segment containing several potential *O*-glycosylation sites was added to the fusion protein construct, the chimeric protein was transported to the plasma membrane (Figures 8 and 9). In *pmt4*Δ cells, the protein was blocked in the Golgi complex. Biochemical analysis demonstrated that *O*-glycans were present on the chimeric protein that was delivered to the cell surface in wild-type cells, whereas *O*-glycans were lacking in *pmt4*Δ cells (Figure 8). Our data suggest that *O*-glycosylation can serve as a signal for protein transport to the plasma membrane in *S. cerevisiae*.

The first suggestive evidence for carbohydrate side chains of proteins acting as sorting determinants for cell surface delivery came from the work of Rose and coworkers in fibroblasts (Guan *et al.*, 1985; Machamer *et al.*, 1985). Further support for a role of glycans as signals for transport from the Golgi complex to the plasma membrane emerged from studies of epithelial cells. Scheiffele *et al.* (1995) demonstrated

Figure 9. Quantification of the plasma membrane delivery of different protein constructs. Images of cells expressing different constructs were taken using the same conditions and the intensity of the fluorescence were measured for >40 cells in each experiment using the IpLab software (see MATERIALS AND METHODS). The bars (with SEM) represent the ratio of the total fluorescence measured for the plasma membrane area and for the intracellular area of each cell.

that in epithelial Madin-Darby canine kidney cells *N*-glycosylated rat growth hormone was secreted apically, whereas the nonglycosylated native form of the protein was secreted randomly, both apically and basolaterally. This was also the case when a glycosyl-phosphatidylinositol-anchor was added to the rat growth hormone (Benting *et al.*, 1999). Gut and coworkers showed that a protein lacking its basolateral sorting determinants accumulated in the Golgi complex (Gut *et al.*, 1998). Addition of *N*-glycans to this mutant protein promoted its delivery to the apical surface. Several reports have also demonstrated that *O*-glycans can serve as apical sorting determinants (Yeaman *et al.*, 1997; Alfalah *et al.*, 1999; Spodsborg *et al.*, 2001). In fact, also glycosylated basolateral proteins will be delivered apically if the basolateral sorting determinants in their cytosolic protein domains are mutated or deleted (Gut *et al.*, 1998). These data demonstrate that it is difficult to analyze sorting determinants for surface delivery in cells with two (or more) pathways from the Golgi complex to the plasma membrane. A protein can switch from one pathway to another.

Yeast also has two pathways to the cell surface (Harsay and Bretscher, 1995; David *et al.*, 1998; Gurunathan *et al.*, 2002; Harsay and Schekman, 2002), both using the same fusion machinery comprising the exocyst (TerBush *et al.*, 1996), the GTPase Sec4p (Guo *et al.*, 1999), and the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor complexes Snc1/2p and Sso1/2p (Aalto *et al.*, 1993; Protopopov *et al.*, 1993; Jahn *et al.*, 2003). Despite numerous genetic screens for secretion mutants little is known of how sorting of surface proteins occurs in the Golgi complex. Inactivation of one pathway may be rescued by routing to the other pathway (Gurunathan *et al.*, 2002; Harsay and Schekman, 2002). It was shown that blocking of the vacuolar pathway leads to exocytosis of vacuolar proteins (Marcusson *et al.*, 1994; Nothwehr *et al.*, 1995).

Another problem complicates the analysis of the role of glycosylation in secretion. Inhibition of glycosylation often leads to misfolding of the protein, and accumulation and degradation in the ER (Ng *et al.*, 2000; Hampton, 2002; Ellgaard and Helenius, 2003). In *S. cerevisiae*, the Golgi complex also seems to be a site for quality control (Hong *et al.*, 1996; Jorgensen *et al.*, 1999). Proteins that are not folded correctly or are incompletely oligomerized are sin-

gled out for the vacuolar delivery in the Golgi complex. Obviously, therefore, one has to differentiate between effects due to quality control mechanisms or to sorting for surface delivery in the Golgi complex. It was reported that Axl2p, involved in bud site selection has altered glycosylation in *pmt4Δ* cells and is rapidly degraded before reaching cell surface, most probably in the Golgi, (Sanders *et al.*, 1999). The kinetics of the degradation process of misfolded proteins is usually fast. The chimeric proteins composed of Fus1p ectodomain and of the Mid2p transmembrane and cytosolic domains in *pmt4Δ* cells were in fact more long lived than the wild-type Fus1p. Thus, we had no indication of misfolding or increased vacuolar delivery. Instead, the chimeric protein mostly accumulated in the Golgi complex. We did not observe accumulation of our markers in ER structures (except for Inv33Mid, which showed a faint staining of the ER but most of the fluorescence was localized to the plasma membrane), and we did not observe enhanced degradation as was the case for Axl2p (Sanders *et al.*, 1999).

If *O*-glycans of Fus1p function as sorting determinants for delivery to the cell surface, the question arises how this signal functions mechanistically. Two models have been proposed for how carbohydrate-sorting determinants function in apical transport in epithelial cells. Rodriguez-Boulton and Gonzalez (1999) have suggested that glycans change the biophysical properties of an apical protein such that the presentation of a proteinaceous sorting signal to a hypothetical sorting receptor is facilitated. Alternatively, the glycans contribute to a transport-permissive conformation of the apical protein that facilitate its incorporation into lipid rafts and thus into the apical targeting pathway. The latter possibility seems unlikely because it has been shown that raft association by itself is not sufficient for apical delivery (Simons and Ikonen, 1997; Rietveld and Simons, 1998). Rafts are also routed basolaterally. The second model postulates the existence of apical lectins that bind to the glycans and sort the apical proteins into transport carriers in the *trans*-Golgi network (Scheiffele *et al.*, 1995). However, such a lectin has not yet been identified. According to this model *O*-glycosylated Fus1p would be bound by a lectin, which facilitates its surface delivery. Our findings that *O*-glycans promote plasma membrane transport of proteins in yeast provide all the tools that this model organism supplies for identification of the underlying sorting mechanisms.

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