Transmembrane Topology of Pmt1p, a Member of an Evolutionarily Conserved Family of Protein O-Mannosyltransferases

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The identification of the evolutionarily conserved family of dolichyl-phosphate-α-mannose:protein O-mannosyltransferases (Pmts) revealed that protein O-mannosylation plays an essential role in a number of physiologically important processes. Strikingly, all members of the Pmt protein family share almost identical hydrophilic profiles; a central hydrophilic domain is flanked by amino- and carboxy-terminal sequences containing several putative transmembrane helices. This pattern is of particular interest because it diverges from structural models of all glycosyltransferases characterized so far. Here, we examine the transmembrane topology of Pmt1p, an integral membrane protein of the endoplasmic reticulum, from Saccharomyces cerevisiae. Structural predictions were directly tested by site-directed mutagenesis of endogenous N-glycosylation sites, by fusing a topology-sensitive monitor protein domain to carboxy-terminal truncated versions of the Pmt1 protein and, in addition, by N-glycosylation scanning. Based on our results we propose a seven-transmembrane helical model for the yeast Pmt1p mannosyltransferase. The Pmt1p amino terminus faces the cytoplasm, whereas the carboxyl terminus faces the lumen of the endoplasmic reticulum. A large hydrophilic segment that is oriented toward the lumen of the endoplasmic reticulum is flanked by five amino-terminal and two carboxy-terminal membrane spanning domains. We could demonstrate that this central loop is essential for the function of Pmt1p.

Glycosylation is one of the most elaborate covalent protein modifications known. The carbohydrate chains can be coupled to the protein through either an N- or O-glycosidic bond. Protein O-mannosylation, originally observed in fungi (1), is initiated at the endoplasmic reticulum by protein mannosyltransferases (Pmts) that catalyze the transfer of a mannosyl residue from dolichyl phosphate-activated mannose (Dol-P-Man) to serine or threonine residues of nascent proteins entering the secretory pathway; in the Golgi apparatus additional sugars are added to the O-linked mannose with GDP-mannose serving as carbohydrate donor (2, 3). Dol-P-Man-dependent O-glycosylation of secreted proteins is a general feature of yeasts and filamentous fungi (4). The key enzyme of protein O-mannosylation, the Dol-P-Man:protein O-mannosyltransferase Pmt1p, was purified from S. cerevisiae following the enzyme activity, and the corresponding gene was cloned (5, 6). Pmt1p is an integral membrane glycoprotein located at the ER (5, 7–9). Based on homology to Pmt1p, a family of seven protein O-mannosyltransferases (Pmt1p–Pmt7p) has been identified (10–13). Thus far, protein O-mannosyltransferase activity has been demonstrated for Pmt1p, Pmt2p, Pmt3p, Pmt4p, and Pmt6p (13, 14). The individual mannosyltransferases recognize specific protein substrates that might explain the presence of more than one transerase in S. cerevisiae (14). Moreover, Pmtp orthologues have been identified from other yeasts (4), from the opportunistic fungal pathogen Candida albicans (15), and from Drosophila melanogaster (16) suggesting that protein O-mannosylation may be common among eucaryotes.

The isolation of pmt mutants showed that protein O-mannosylation plays a substantial role in a number of physiologically important processes. In the yeast S. cerevisiae, protein O-mannosylation is an indispensable modification for the maintenance of cell integrity (13). Deletion of the PMT1 homologue in C. albicans results in defects in morphogenesis, a significant loss of virulence, and reduced adherence to host cells (15). In addition, mutations at the Drosophila PMT1 orthologous locus, rotated abdomen, alter muscle structures and the alignment of adult cuticle (16). Despite the functional importance of the evolutionarily conserved Pmtp mannosyltransferases, the initial steps of protein O-mannosylation are still very poorly understood.

Pmtp family members are, on average, 50–55% homologous overall with most variation occurring in the length and sequence of amino and carboxyl termini. Most interestingly, all of the Pmts share a nearly identical hydrophathy profile, wherein an integral membrane protein with a tripartite structure (amino- and carboxy-terminal regions, each with several putative transmembrane helices, and a central hydrophilic segment) is predicted (6, 10, 11, 15, 16). Strikingly, this pattern diverges from structural models of other ER glycosyltransferases as well as from the common type II model of glycosyltransferases of the Golgi apparatus.

In the present study we report the mapping of the membrane topology of S. cerevisiae Pmt1p using site-directed mutagenesis, carboxy-terminal reporter fusions, and N-glycosylation scanning. These topology-sensitive monitors can distinguish between the lumen of the ER and the cytoplasm. We propose a structural model indicating that Pmt1p spans its cognate membrane seven times. In addition, we demonstrate that a large luminally oriented hydrophilic loop is essential for Pmt1p function.
**EXPERIMENTAL PROCEDURES**

**YEAST STRAINS**

The S. cerevisiae strain STY50 (MATa, his3-401, leu2-3, 112, trp1-1, ura3-52, HOLL-1, suc2::LEU2) was derived from the strain FC2a (17) by disruption of the UCC2 gene by homologous recombination. For this purpose, FC2a was transformed with the plasmid pRR8.01 (kindly provided by L. Lehle, University of Regensburg) digested with HindIII. Yeast shuttle vectors YEp352 (2 μm, URA3) (18), pR90 (PMT1<sup>78A</sup>, 2 μm, URA3) (21), YEp371 (PMT1<sup>533A</sup>, 2 μm, URA3; see below) were transformed into the yeast strain STY50. The pmt1 deletion strain pmt1Δ (MATa, his3Δ200, leu2-3, 112, lys2-s801, trp1-901, ura3-52, suc2-2α, his3Δ113, URA3<sup>his3-401</sup>) (13) was transformed with the shuttle vectors YEp352, pSB53 (PMT1, 2 μm, URA3; see below), pSB57 (PMT1<sup>NT78A</sup>, 2 μm, URA3; see below), pSB60 (PMT1<sup>NT85A</sup>, 2 μm, URA3; see below), pSB52 (PMT1<sup>NT78A</sup>, 2 μm, URA3; see below), pSB62 (PMT1<sup>NT78A</sup>-loop 1, 2 μm, URA3; see below), pSB59 (PMT1<sup>NT85A</sup>-loop 4, 2 μm, URA3; see below), pSB61 (PMT1<sup>NT85A</sup>-loop 6, 2 μm, URA3; see below), pSB73 (PMT1, 2 μm, URA3; see below) or pSB79 (PMT1<sup>N104-S131</sup>, 2 μm, URA3; see below). All yeast transformations were performed following the method of Gietz et al. (19). UCC2 gene disruption was confirmed by measuring invertase activity (20).

**Plasmid Constructions**

Standard procedures were used for all DNA manipulations (21). All cloning and transformations were carried out in Escherichia coli host DH5α. PCR fragments were routinely checked by sequence analysis.

**PMT1-HIS4C Fusion Plasmids**—A 1.26-kilobase pair of genomic DNA encoding the coding region from bp 918 to 1379 was amplified by PCR with the primer pairs oligo Al6 (5'-gtg-3') and oligo 105 (5'-actactagcatgcggatccaccttcag-3'). The PCR fragment was digested with BglII and SacI and subcloned into pR157 (cut with the same). The HA epitope sequence (22) was subcloned into pBluescript KS<sup>II</sup>-<sup>−</sup>-I-, resulting in pSB52. This plasmid was used as template DNA to produce pSB60 (PMT1<sup>NT85A</sup>-loop 1), pSB59 (PMT1<sup>NT85A</sup>-loop 4), pSB61 (PMT1<sup>NT85A</sup>-loop 6). pSB62 was created by using the primer pair oligo 138 (5'-cctccttgaaagaatgttgctgtctgacctgttggtg-3')/oligo 139 (5'-ccacaagatggtataagcagagcattttgaaagaccggg-3'), pSB59 the primer pair oligo 151 (5'-ccacaagatggtataagcagagcattttgaaagaccggg-3')/oligo 139; pSB61 the primer pair oligo 150 (5'-gcggcaactttggtagttgaacttactacaaactaaac-3')/oligo 141 (5'-ccacaagatggtataagcagagcattttgaaagaccggg-3'). To create pSB79 (PMT1<sup>NT104–S131</sup>-loop 3'), the plasmid pSB53 was digested with SpHl, NotI, and religated. The resulting plasmid (pSB79) was digested with HindIII and religated, thereby deleting the PMT1 coding sequence coding for aa 304 to aa 531.

**Computer Analyses**

Structural predictions of Pmt1p were made using the programs TMAP (23) and TMREPD. The latter uses an algorithm based on the statistical analysis of TMbase (24). Furthermore, structural models made by Martinsried Institute for Protein Sequences accession number A47716 and SWISS-PROT accession number P37775, were used.

**Analysis of the Pmt1-His4C Fusion Proteins**

**Growth on Histidinol**—The strain STY50 was transformed with the plasmids pR90 to pC731. Transformants were selected for the URA3-containing plasmids on SD plates supplemented with the amino acids and bases required at 20–30 µg/ml, lacking uracil and containing 2% glucose. Ura<sup>−</sup> transformants were streaked on supplemented minimal medium lacking histidine but containing 6 µM histidinol. The plates were incubated at 30°C for 3–5 days.

**Immunoprecipitation from Whole-cell Extracts**—Yeast cells were grown on SD medium to a concentration of 2.0 × 10<sup>7</sup> cells/ml. Cells (50 ml) were harvested and whole-cell extracts prepared as described previously (25). 10 µl of anti-invertase antibody (26) were added to 400 µl of cell extract and cell extract was washed and incubated for 5 h at 4°C. Thereafter, 400 µl of lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% sodium deoxycholate, 2% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.25 mM 1-chloro-3-tosylamido-7-aminoo-2-heptanone, 50 µg/ml 1,1-tosylamide-2-phenylethyl chloromethyl ketone, 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) were added and the extracts disincubated. 15 µl of antipeptide protein A-Sepharose 4B beads (Pharmacia) were added, and the incubation was continued for 1 h at 4°C. The immunoprecipitates were washed five times with 1.4 ml of lysis buffer and once with 1.4 ml of 50 mM potassium phosphate buffer, pH 5.5, 0.02% SDS, protease inhibitors as above. Subsequently, the precipitates were subjected to endoglycosidase H digestion or mock treated.

**Preparation of Crude Membranes**

Yeast cells were grown on SD medium. At a concentration of 2.0 × 10<sup>7</sup> cells/ml, 20 ml of cells were harvested, washed with 10 ml of 50 mM Tris-HCl, pH 7.5, 0.3 mM MgCl<sub>2</sub>, and resuspended in 100 µl of the same buffer plus protease inhibitors (see above). An equal volume of glass beads was added, and the cells were lysed by vortexing, for 1 min, four times (with 1-min intervals on ice). The bottom of the tube was punctured and the lysate collected. Cell debris were removed by centrifugation for 5 min at 5,000 rpm at 4°C. Membranes were collected from the supernatant by centrifugation for 30 min at 20,000 rpm at 4°C and resuspended in 100 µl of 50 mM Tris-HCl, pH 7.5, 7.5 mM MgCl<sub>2</sub>.

**Isolation of Chitinase**

Yeast cells were grown on SD medium to 2.0 × 10<sup>8</sup> cells/ml. Chitinase (Cts1p) was isolated from cell walls as described in Lentz and Tanner (14).

**Deglycosylation by Endoglycosidase H Digestion**

Immunoprecipitates or 5 µl of crude membranes were suspended in 25 µl of Endo H buffer (50 mM potassium phosphate buffer pH 5.5, 0.02% SDS, 0.1 mM 2-mercaptoethanol, protease inhibitors as above) and digested with 1–5 units/µl Endo H for 1 to 2 h at 37°C. Mock samples were incubated without Endo H. The reaction was stopped by adding 10 µl of 5× SDS sample buffer.

**Western Blot Analyses**

Proteins were fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose (27). Anti-Pmt1p and anti-invertase polyclonal antibodies were used at 1:1,000, anti-Cts1p polyclonal antibody at 1:2,500, and anti-HA monoclonal antibody (16B12; Babco) at 1:5,000.

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FIG. 1. Carboxyl-terminal Pmt1-His4C reporter fusion constructs. The hydropathy profile of Pmt1p using a window of 17 amino acids is shown (60). Solid diamonds indicate N-glycosylation sites. Potential transmembrane spanning domains are marked by roman numerals. The terminal amino acids of the Pmt1 portion in the individual Pmt1-His4C fusion proteins are shown.

dilution. Protein-antibody complexes were visualized by enhanced chemiluminescence using the Amersham Pharmacia Biotech ECL system.

In Vitro Dol-P-Man:Protein O-Mannosyltransferase Assay

5–30 μg of membrane protein were incubated in the in vitro assay for Dol-P-Man:protein O-mannosyltransferase, as described previously (5). The pentapeptide acetyl-YATAV-NH₂ was used at a final concentration of 3.5 mM.

RESULTS

The Central Hydrophilic Loop and the Carboxyl-terminal End of Pmt1p Are Facing the ER Lumen—S. cerevisiae Pmt1p is a protein of 817 amino acids with three potential N-glycosylation sites as follows: two are located in the central hydrophilic loop (aa Asn-390 and Asn-513) and one at the carboxyl-terminal end (aa Asn-743) of the protein (Fig. 1). Treatment with endoglucosidase H (Endo H) reduces the molecular mass of the protein from 92 to 84 kDa (Fig. 2, lanes 2 and 3) (5). Considering the fact that Pmt1p resides in the ER (7–9) where only core glycosylation takes place, this difference in molecular mass indicates that all three N-glycosylation sequences (NXS/T) are glycosylated in vivo. Since N-glycosylation is carried out exclusively on the luminal side of the ER, these data indicate that the N-glycosylation sites are exposed to the ER lumen. To verify this predicted orientation, Pmt1p mutant proteins were constructed wherein the N-glycosylation sequons N390LT and N743QT were destroyed individually by changing the asparagines to alanine. The mutant mannosyltransferases were analyzed in the S. cerevisiae pmt1 deletion strain pmt1Δ/pSB53, pmt1Δ/pSB60, and pmt1Δ/pSB57 (PMT1N390A, lane 4) and treated with Endo H as indicated. Proteins (25 μg) were resolved on 8% SDS-polyacrylamide gels and analyzed by Western blot using an anti-Pmt1p antibody.

Table I

| Strain | [14C]Mannose transferred in vitro | cpm/mg/min |
|--------|----------------------------------|------------|
| pmt1Δ | 99 ± 20                          | 220 ± 99   |
| pmt1Δ/PMT1 | PMT1N390A | 23 ± 7 | 3500 ± 23 |
| pmt1Δ/PMT1 | PMT1N743A | 19 ± 7  | 3650 ± 19 |
| pmt1Δ/PMT1 | PMT1N743A | 7 ± 1   | 3660 ± 7  |

When fused to a signal sequence (28). Yeast hisΔ mutant strains expressing a His4C fusion protein are able to grow on minimal medium containing histidinol when the catalytic domain is present on the cytoplasmic side of the ER membrane. In this case histidinol is metabolized to histidine, resulting in a His⁺ phenotype. When the catalytic domain is targeted to the ER lumen histidinol cannot be converted to histidine, resulting in a histidinol resistant phenotype. In addition, the protein becomes extensively glycosylated due to the presence of several N-glycosylation sites.

We designed a series of fusion proteins consisting of carboxyl-terminal truncated versions of Pmt1p and the His4C protein domain (Pmt1R90 to Pmt1C731, Fig. 1 and Table II) which allowed us to distinguish between distinct numbers of transmembrane domains as well as their orientation. In addition, the constructs contained a part of the yeast invertase introducing an epitope for immunopurification. The fusion constructs were transformed into a hisΔ mutant background (STY50), and the transformants were tested for the ability to grow on selective medium supplemented with histidinol. Furthermore, using a polyclonal antibody directed against an unglycosylated form of invertase (26), the fusion proteins were immunoprecipitated from whole-cell extracts, treated with Endo H, and analyzed by Western blot.

First we wanted to distinguish between an odd versus an even number of membrane spanning domains. The number of transmembrane helices dictates the luminal or cytoplasmic orientation of the amino and carboxyl termini. The 10 transmembrane helical model requires that both termini face the ER lumen. On the other hand, in case of a protein with 7 or 11 transmembrane helices where the carboxyl terminus faces the ER lumen, the amino terminus would face the cytoplasm.

To determine the orientation of the amino terminus the deletion construct Pmt1R90 carrying the first putative transmembrane domain (Fig. 1) was analyzed. As shown in Fig. 3, Pmt1R90 does not support growth on histidinol indicating that
TABLE II
Pmt1-His4C fusion proteins

| Pmt1-His4C fusion | Calculated mass | Growth on histidine | Glycosylation of His4C |
|-------------------|-----------------|---------------------|-----------------------|
| Pmt1R90          | 132,201         | –                   | –                     |
| Pmt1R157         | 139,562         | +                   | –                     |
| Pmt1V175         | 141,548         | –                   | –                     |
| Pmt1L221         | 146,862         | –                   | –                     |
| Pmt1T306         | 151,448         | –                   | –                     |
| Pmt1F306         | 156,281         | –                   | +                     |
| Pmt1G355         | 161,565         | –                   | –                     |
| Pmt1P306         | 191,600         | +                   | –                     |
| Pmt1H655         | 196,247         | +                   | –                     |
| Pmt1C731         | 205,034         | –                   | +                     |

Fig. 3. Growth phenotypes of Pmt1-His4C fusions. The yeast strain STY50 was transformed with the plasmid YEps32 or plasmids coding for the Pmt1-His4C fusions Pmt1R90 to Pmt1C731 (Arg-90 to Cys-731). Transformants were streaked on selective media supplemented with histidine (left panels) or histidinol (right panels) and incubated for 3–5 days at 30°C. WWII-II

His4C is facing the lumen of the ER. This is validated by the fact that the fusion protein is highly glycosylated in vivo (Fig. 4, lanes 3 and 4). Pmt1R90 shows an apparent molecular mass of 167 kDa which decreases after Endo H treatment to 132 kDa. This is in agreement with the calculated mass of the unglycosylated protein (Table II). These results indicate that the amino terminus of this construct resides on the cytoplasmic side of the ER membrane. A very minor fraction of Pmt1R90, which varied in its abundance from experiment to experiment, is not glycosylated (Fig. 4, lane 3). We presume that this fraction is either oriented with the His4C domain in the cytoplasm or not translocated to the ER at all, remaining misfolded in the cytoplasm. The latter possibility would explain why this protein does not provide any growth on histidinol (Fig. 3).

To confirm that the carboxyl terminus faces the ER lumen we used the reporter fusion Pmt1C731, which contains all the potential transmembrane domains. Pmt1C731 produces a His-phenotype (Fig. 3) and is extensively glycosylated (Fig. 4, lanes 21 and 22) indicating that the His4C domain is oriented toward the ER lumen. Since the amino and carboxyl termini are located on different sides of the ER membrane we exclude the 10 transmembrane helical model. Furthermore, Pmt1C731 shows the same phenotype as Pmt1C731 confirming the lumenal orientation of the hydrophilic central part of Pmt1p (Fig. 3 and Fig. 4, lanes 15 and 16). Taken together, these results indicate that TM IX does not cross the membrane. These data confirm that the predicted transmembrane domains TM I, TM II, TM III, and TM IV (Fig. 1) are spanning the membrane in vivo. Unexpectedly, the catalytic domain of the fusion Pmt1C731 is also facing the cytoplasm (Fig. 3 and Fig. 4, lanes 11 and 12) implying that TM V does not cross the membrane. The His4C domains of both fusion proteins Pmt1P306 and Pmt1G355 are located in the ER lumen (Fig. 3 and Fig. 4, lanes 7 and 8) and Pmt1L221 is the cytoplasmic side of the membrane (Fig. 3 and Fig. 4, lanes 9 and 10). These data confirm that the predicted transmembrane domains TM I, TM II, TM III, and TM IV (Fig. 1) are spanning the membrane in vivo. These data argue for the presence of five membrane spanning domains in the amino-terminal half of Pmt1p.

How many transmembrane helices are present between the amino and carboxyl termini? To answer this question the fusion proteins Pmt1P306 and Pmt1G355 were analyzed. Pmt1P306 is oriented with the His4C catalytic domain on the cytoplasmic side of the ER membrane (Fig. 3). Therefore, TM VIII (Fig. 1) does span the membrane in vivo. A minor increase in the mobility of the protein after Endo H treatment is due to the removal of two N-linked carbohydrate chains at positions Asn-390 and Asn-513 in the Pmt1p portion of the fusion (Fig. 4, lanes 17 and 18). A very similar result was obtained for the fusion protein Pmt1H655 (Fig. 3 and Fig. 4, lanes 19 and 20) showing that TM IX does not cross the mem-
thus is consistent with the orientation of the central hydrophilic loop (aa 295–580) of the Pmt1p protein. Therefore, we concluded that this protein reflects the transmembrane topology of the native Pmt1p protein.

To confirm that the amino terminus faces the cytoplasm, we used Pmt1G355HA and introduced two N-glycosylation sequons into the loop region between the transmembrane helices TM I and TM II by site-directed mutagenesis (Fig. 5A and Table III). The N-glycosylation sites were placed at least 12 amino acids away from adjacent membrane helices to ensure they could become N-glycosylated (30). The resulting construct Pmt1G355HA/loop 1 was expressed from a high copy 2-μm plasmid in the pmt1 mutant strain pmt1Δ. A crude membrane fraction was isolated and treated with Endo H to verify N-glycosylation. The proteins were then analyzed by Western blot using a monocular antibody directed against the HA epitope. Our data show that the loop region between TM I and TM II is N-glycosylated in vivo. Core-glycosylated species of Pmt1G355HA/loop 1 were detected in addition to the unglycosylated protein (Fig. 5B, lanes 3 and 4). It is likely that this partial glycosylation is due to varied numbers of N-glycosylation sites being used. Similar effects were observed when Pmt1p was expressed from a high copy 2-μm plasmid.2 From these results we conclude that the loop region between TM I and TM II is located in the ER lumen and, consequently, that the amino terminus of Pmt1G355HA is oriented toward the cytoplasm, confirming the results obtained by His4C reporter fusions.

To answer the question as to whether TM V is used as a transmembrane span in vivo, we independently introduced two N-glycosylation consensus sequences in the loop regions between TM IV and TM V (Pmt1G355HA/loop 4; Table III) and between TM VI and TM VII (Pmt1G355HA/loop 6; Table III). Considering the odd number of transmembrane helices predicted to form between the amino terminus and the hydrophilic middle region, it may be expected that either both TM V and TM VII serve as transmembrane spans or neither of them. In the first case loop 4 and loop 6 would be localized in the cytoplasm (Fig. 6A); in the second case loop 4 and loop 6 had to be on opposite sides of the membrane wherein loop 6 is facing the ER lumen (Fig. 6B). The mutant proteins Pmt1G355HA/loop 4 and Pmt1G355HA/loop 6 were expressed in the strain pmt1Δ. To examine the state of glycosylation crude membranes were isolated, treated with Endo H, and analyzed by Western blot using a monocular anti-HA antibody. Fig. 6C shows that Pmt1G355HA/loop 6 (lanes 5 and 6) but not Pmt1G355HA/loop 4 (lanes 3 and 4) is glycosylated in vivo providing further evidence that TM V does not serve as a membrane spanning helix. These results demonstrate the presence of only five transmembrane spanning domains in the amino-terminal half of Pmt1p.

**The Central Hydrophilic Loop Is Crucial to Pmt1p Function**—Summarizing, our data ascertain five transmembrane
spans between the amino terminus and a large central hydrophilic loop region which is facing the ER lumen and two membrane spanning domains between the latter and the carboxyl terminus. The luminaly oriented middle loop (aa 295–580) is the largest hydrophilic segment in Pmt1p. Since it is almost certain that the transfer of the mannoes from Dol-P-Man to proteins occurs in the ER lumen (31, 32), this loop might be essential for Pmt1p function. To test this, we examined whether the large hydrophilic loop is crucial for Pmt1p enzymatic activity.

We created a mutant version of Pmt1p (Pmt1<sup>304–531</sup>; see “Experimental Procedures”) that lacks the amino acid residues 304–531 including the N-glycosylation sequons Asn-390 and Asn-513 (Fig. 1). Pmt1<sup>304–531</sup> was analyzed in a pmt1 mutant background. On SDS-PAGE Pmt1<sup>304–531</sup> shows an apparent mass of 64 kDa (Fig. 7A, lane 4), being similar to the predicted size of 66.9 kDa. Endo H treatment reduces the mass to 62 kDa (Fig. 7A, lane 5). This decrease in mass of 2 kDa indicates that the only N-glycosylation site (Asn-743; see Fig. 1) present in Pmt1<sup>304–531</sup> bears one N-linked core carbohydrate chain. From these results we conclude that (i) Pmt1<sup>304–531</sup> resides in the ER membrane and (ii) Pmt1<sup>304–531</sup> mirrors the membrane topology of native Pmt1p.

To test whether Pmt1<sup>304–531</sup> still has mannosyltransferase activity, we analyzed the in vivo glycosylation status of the highly O-mannosylated protein chitinase (Cts1p; see Ref. 33) in a yeast pmt1 mutant expressing Pmt1<sup>304–531</sup>. Confirming previous results (14) we found that in the pmt1 deletion strain Cts1p is less glycosylated as compared with a strain where Pmt1p is present (Fig. 7B, lanes 1, 2, and 4). The mutant protein Pmt1<sup>304–531</sup> does not repeal the underglycosylation of Cts1p (Fig. 7B, lane 3). Furthermore, Pmt1<sup>304–531</sup> did not show significant in vitro mannosyltransferase activity (data not shown). Since the amounts of Pmt1<sup>304–531</sup> and native Pmt1p protein are very similar in the strain pmt1Δ (Fig. 7A, lanes 2 and 4), these data definitively prove that the luminaly oriented hydrophilic loop is essential for Pmt1p function.

DISCUSSION

In this study, we present the first analysis of the transmembrane topology of a Pmt-mannosyltransferase, an enzyme crucial to initiating protein O-mannosylation at the ER. Our data provide strong genetic and biochemical evidence for a seven-transmembrane helical model, summarized in Fig. 8. The Pmt1p amino terminus faces the cytoplasm, the carboxyl terminus the ER lumen. A large hydrophilic region, located in the ER lumen, is separated from the amino terminus by five and from the carboxyl terminus by two membrane spanning domains. By using deletion mutagenesis we show that the ER luminaly oriented central loop is crucial for mannosyltransferase activity.

The successful use of heterologous protein fusions as topology-sensitive monitors (17, 34) encouraged us to use His4C as a reporter of the topological location which discrete portions of Pmt1p acquire in the membrane. The results obtained with Pmt1-His4C fusions are supported by several other lines of evidence as follows. (i) N-glycosylation scanning demonstrates that TM I integrates into the membrane with its carboxyterminal region reaching into the ER lumen. As a consequence the Pmt1p amino terminus is cytoplasmic. In agreement with these data TM I shows the features of a “type II signal anchor sequence” (reviewed in Ref. 35); TM I (aa 51–70) is amino-terminally flanked by three positive (Arg-42, Lys-48, and Lys-50) and carboxy-terminally by three negative (Asp-72, Asp-77, and Glu-78) charges. (ii) Mutation of the endogenous Pmt1p N-glycosylation sequons N390LT and N743QT demonstrates that the central hydrophilic loop and the carboxyl terminus of Pmt1p are oriented toward the ER lumen. Earlier results resting upon Endo H digestion of Pmt1p isolated from yeast and heterologous expression of Pmt1p in E. coli already suggested that all three N-glycosylation sites (Asn-390, Asn-513, and Asn-743) are used in the yeast Pmt1p protein (5, 6). (iii) Our N-glycosylation scanning data corroborates the prediction that
Fig. 8. Predicted seven-transmembrane helical structure of yeast Pmt1p. The amino and carboxyl termini face the cytoplasm and the ER lumen, respectively. The central hydrophilic loop is oriented toward the lumen of the ER and is flanked by five amino-terminal and two carboxyl-terminal membrane spanning domains. N-glycosylation sites are marked with solid diamonds.

The complex organization of yeast Pmt1p contrasts the structure of the mammalian UDP-GlcNAc:polypeptide N-acetylglucosaminyltransferases that initiate mucin-type O-glycosylation at the Golgi apparatus of higher eucaryotes (38). The Golgi-localized transferases show small cytosolic amino-terminal domains, single transmembrane segments, luminal stem regions, and large carboxyl-terminal luminal domains responsible for catalysis. This type II model structure is common to glycosyltransferases of the Golgi apparatus, for example, the yeast mannosyltransferases Mnt1/Kre2p (39, 40) or Mnn1 (41).

ER resident glycosyltransferases showing multiple putative transmembrane helices were found in S. cerevisiae as well as in higher eucaryotes. These include the glycosyltransferases involved in the synthesis of the lipid oligosaccharide precursor for N-glycans (3, 42) and the glycosylphosphatidylinositol (GPI) anchor (3, 43). The topological organization of these transferases is not well characterized, but for the ones investigated so far the putative transmembrane topology does not resemble the structure of Pmt1p. A scarce example where the structure-function relationship has been examined in detail is the hamster UDP-GlcNAc:dolichol-P GlcNAc-1-P transferase (GPT) (44, 45). This enzyme initiates N-linked glycosylation by catalyzing the synthesis of GlcNAc-P-P-dolichol. Lehrman and co-workers (42, 46) demonstrated that GPT is a multimeric enzyme with multiple, most likely 10 transmembrane spans. The largest hydrophilic segment, located between TM 9 and TM 10, is facing the cytoplasm. This loop region most likely bears the catalytic site consistent with the fact that GlcNAc-P-P-dolichol is synthesized on the cytoplasmic side of the ER. GPT seems to be highly conserved between higher eucaryotes and yeast since the human GlcNAc-1-P transferase complements a S. cerevisiae alg7 (asparagine linked glycosylation) mutant defective in GPT activity (47). Recently, several ER-localized glycosyltransferases have been identified which, like Pmt1p, use dolichol phosphate-activated sugars as donor substrates. These include Alg3p (48), Alg6p (49), Alg8p (50), Alg9p (51), and Alg10p (52) from S. cerevisiae which participate in the assembly of the dolichol pyrophosphate-linked oligosaccharide at the luminal side of the ER, the human PIG-B protein (53) required for GPI anchor synthesis, and its functional homologue Gpi10p from yeast (54). Computer analyses predict the presence of several transmembrane domains in the Dol-P-sugar-utilizing transferases, but only in the case of PIG-B has the membrane topology been investigated. Takahashi et al. (53) provided evidence that despite its hydrophobic nature PIG-B shows the topological structure of a type II membrane protein. PIG-B consists of a short amino-terminal cytoplasmic segment, a transmembrane domain, and a large carboxyl-terminal region facing the ER lumen. The discrepancy between the putative and experimentally determined structure of PIG-B demonstrates how cautious computer-based analyses should be interpreted and how important it is to obtain direct structural information.

Our analyses elucidate an elaborate structure for yeast Pmt1p with seven transmembrane domains and a number of loop regions. The large hydrophilic central loop (aa 295–580) is essential for Pmt1p activity, suggesting that the catalytic site is facing the ER lumen. This is in good agreement with previous data showing that Dol-P-Man is used as donor on the luminal side of the ER membrane for the mannosylation of the N-glycan precursor intermediates, for the synthesis of GPI anchors as well as for protein O-mannosylation (55). Like for Pmt1p the catalytic domain of PIG-B is also facing the ER lumen since deletion of the cytoplasmic domain does not abolish enzymatic activity (53).

Since protein O-mannosylation is an essential modification in yeast (13), Pmt1p may be subject to stringent regulation. Thus, it is possible that some regions are involved in catalysis, whereas others could interact with various proteins or regulators. This assumption is sustained by the observation that Pmt1p interacts with Pmt2p in vivo, and the formation of this complex is required for maximum transferase activity (56). Interactions with different regulators are also conceivable and even suggested by the finding that Pmt proteins possess three highly conserved phosphorylation sites for protein kinase C (S/X/R/K) and TX(R/K) and pmt mutants display phenotype similar to those observed in protein kinase C mutants (13, 57). In addition, S. cerevisiae and C. albicans Pmt1p can be activated by phospholipids in vitro (58). That Pmt O-mannosyltransferases are the subject of various regulation mechanisms is emphasized by the fact that yeast PMT1–PMT6 are also transcriptionally regulated during the cell cycle and during diauxic shift from fermentation to respiration (59).

Future work will be necessary to learn more about the mechanism how Pmts transfer mannos from Dol-P-Man to specific acceptor proteins at the endoplasmic reticulum. This study provides the basis for the identification and characterization of structural and functional important domains of Pmt O-mannosyltransferases.

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