Members of the Evolutionarily Conserved PMT Family of Protein O-Mannosyltransferases form Distinct Protein Complexes Among Themselves

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1The abbreviations used are: aa, amino acid; BN-PAGE, blue native polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; DOC, sodium deoxycholate; Dol-P-Man, dolichyl phosphate-activated mannose; HA, hemagglutinin; MW, molecular weight; OPA, o-phtaldialdehyde; NP40, Nonidet P40; PCR, polymerase chain reaction; Pmt, PMT, protein O-mannosyltransferase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.
SUMMARY

Protein O-mannosyltransferases (PMTs) initiate the assembly of O-mannosyl glycans, an essential protein modification. Since PMTs are evolutionarily conserved in fungi but are absent in green plants, the PMT family is a putative target in order to develop new antifungal drugs, particularly fighting the threat of phytopathogenic fungi. The PMT family is phylogenetically classified into PMT1, PMT2 and PMT4 subfamilies, which differ in protein substrate specificity. In the model organism Saccharomyces cerevisiae as well as in many other fungi the PMT family is highly redundant and only the simultaneous deletion of PMT1/PMT2 and PMT4 subfamily members is lethal. In this study we analyzed the molecular organization of the PMT family members in S. cerevisiae. We show that members of the PMT1 subfamily (Pmt1p and Pmt5p) interact in pairs with members of the PMT2 subfamily (Pmt2p and Pmt3p), and that Pmt1p-Pmt2p and Pmt5p-Pmt3p complexes represent the predominant forms. Under certain physiological conditions, however, Pmt1p interacts also with Pmt3p, and Pmt5p with Pmt2p, suggesting a compensatory cooperation that guarantees the maintenance of O-mannosylation. Unlike the PMT1/PMT2 subfamily members, the single member of the PMT4 subfamily (Pmt4p) acts as a homomeric complex. Using mutational analyses we demonstrate that the same conserved protein domains underlie both heteromeric and homomeric interactions, and we identify an invariant arginine residue of transmembrane domain two as essential for the formation and/or stability of PMT complexes in general. Our data suggest that protein-protein interactions between the PMT family members offer a point of attack to shut down overall protein O-mannosylation in fungi.
INTRODUCTION

Protein O-mannosylation is an evolutionarily conserved protein modification of fundamental importance in many eukaryotes. In yeasts and fungi, the attachment of O-linked mannosyl residues to proteins of the secretory pathway is essential for cell viability (1). In particular it is indispensable for cell wall integrity and normal cellular morphogenesis (2-4). Impairment of O-mannosylation also affects the stability, localization, and/or proper function of individual proteins (5-10). Furthermore, aberrant O-mannosylation can interfere with the retrograde transport of misfolded proteins across the membrane of the endoplasmic reticulum (ER) (11). O-mannosylation is not only important in yeast, but also in mammals. It was recently shown that in humans, O-mannosyl glycosylation represents a new pathomechanism for muscular dystrophy and neuronal migration disorders (12, 13).

In yeast and fungi, O-mannosylation is initiated in the lumen of the ER by an essential family of protein O-mannosyltransferases (PMTs). These enzymes catalyze the transfer of mannose from dolichyl phosphate-activated mannose (Dol-P-Man) to serine or threonine residues of secretory proteins (2). In Saccharomyces cerevisiae, a total of seven PMT family members (Pmt1-7p) have been identified, which share almost identical hydropathy profiles that predict the PMTs to be integral membrane proteins with multiple transmembrane domains. Pmt1-6p feature an overall protein sequence identity of 57.5%. Pmt7p is less conserved. Protein O-mannosyltransferase activity has been demonstrated for Pmt1-4p and Pmt6p (14). Aside from S. cerevisiae PMTs, orthologues are known from many other yeasts and fungi, for example, Candida albicans (CaPMT1-2 and CaPMT4-6) and Schizosaccharomyces pombe (SpPMT1, SpPMT3 and SpPMT4) (3, 4; Willer T. and S.S., unpublished data). Moreover, PMT homologues have been also identified in many multicellular eukaryotes such as Drosophila melanogaster, mouse and, humans (15-17). Despite their evolutionarily conservation in fungi and throughout the animal kingdom (with
the exception of *Caenorhabditis elegans*, PMTs are not present in green plants (S.S., unpublished data). This makes the PMT family in fungi especially attractive as target for the development of new antifungal drugs in order to combat phytopathogenic fungi.

The protein O-mannosyltransferases can be divided into three subfamilies: the *PMT1*, *PMT2* and *PMT4* subfamily, which include transferases closely related to *S. cerevisiae* Pmt1p, Pmt2p and Pmt4p, respectively (17, 18). Members of the *PMT1* and *PMT2* subfamilies show marked similarities and distinctions from *PMT4* subfamily members. First, all PMT family members share three conserved sequence motifs but, these show significant variations between *PMT1/PMT2* and *PMT4* subfamily members (18). Second, the *PMT1/PMT2* and *PMT4* subfamilies use distinct acceptor protein substrates in vivo (9, 14). Third, in fungi the *PMT1/PMT2* subfamily is highly redundant, whereas the *PMT4* subfamily has only one representative per species (17, 18).

Among the PMT family members, Pmtp1 from *S. cerevisiae* has been most extensively characterized. Pmt1p is an integral ER membrane glycoprotein with seven transmembrane spanning domains (19). Its amino terminus faces the cytoplasm whereas the carboxyl terminus faces the lumen of the ER. Two major hydrophilic domains that are located between transmembrane spans one and two (loop 1) and transmembrane spans five and six (loop 5), respectively, are oriented towards the ER lumen and are essential for Pmt1p activity (18, 19). The replacement of invariant amino acid residues in these regions suggested that these segments are involved in the recognition and/or binding of protein substrates and/or catalysis (18). Comparison of PMTs from different organisms defined highly conserved peptide motifs present in loop 5 (18), which are also found in IP3 - and ryanodine receptors. Their common function is unknown (20). Pmt1p forms a heteromeric complex with Pmt2p in vivo and this complex formation is essential for maximal mannosyltransferase activity (18, 21). N- and C-terminal regions of Pmt1p are involved in Pmt1p-Pmt2p interactions (18). Other than Pmt1p,
very little is known about the molecular organization of the rest of the O-mannosylation machinery.

In the present study we analyzed the molecular assembly of the PMT family in yeast. We demonstrate that complex formation is of general validity for all members of the PMT family in S. cerevisiae. Strikingly, members of the PMT1 subfamily form specific heteromeric complexes with members of the PMT2 subfamily in vivo, while Pmt4p acts as a homomeric complex. Despite the differences between PMT1/PMT2 and PMT4 complexes, we show that the same rules and residues govern Pmtp protein-protein-interactions.
EXPERIMENTAL PROCEDURES

Strains and Plasmids--The *S. cerevisiae* strains are listed in Table I. Yeast strains were grown under standard conditions and transformed following the method of Gietz *et al.* (27) with the yeast shuttle vectors pRS423 (28), YEp352 (29), pSB53 (19), pSB56 (18), PMT2-YEp352 (23), pVG13 (18), pSB114 (18) and the plasmids listed below. Standard procedures were used for all DNA manipulations (30). All cloning and transformations were carried out in *Escherichia coli* host SURE®2 (Stratagene). Polymerase chain reaction (PCR)\(^1\) fragments were routinely checked by sequence analysis. Oligonucleotide sequences are available upon request.

Plasmid pVG80 (PMT2\(^{HA}\))-A SalI site was introduced downstream of the PMT2 coding region by cloning a 2.96 kb *Pst*I/*Hind*III fragment from PMT2/YEp352 (23) into pBluescript SK\(^+\) (Stratagene) digested with the same enzymes. From the resulting plasmid pVG70 a 2.97 kb *Pst*I/SalI fragment was isolated and cloned into YEp352 (cut with *Pst*I and SalI), resulting in plasmid pVG76. A total of six copies of the hemagglutinin (HA)\(^1\) epitope were fused to the C terminus of PMT2 by recombinant PCR (31). Two separate PCR products that overlap in sequence were produced. One was amplified by PCR on pVG76 with the oligonucleotides vg65 and vg66, the other on plasmid pHA-kanMX (gift of U. Schermer) with the oligonucleotides vg67 and vg68. The overlapping, primary PCR products were combined into one longer product using oligonucleotides vg65 and vg68. The resulting 730 bp fragment was cloned into pGEM T-easy (Promega). A 695 bp *Bgl*II/SalI fragment of the resulting plasmid pVG78 was subcloned into pVG76 (cut with *Bgl*II and SalI). DNA sequence analysis of the resulting plasmid pVG80 (PMT2\(^{HA}\)) was performed. In the course of this analysis, we realized a discrepancy between the PMT2 sequence we obtained and the yeast database entry (GenBank Acc.No. AAC04934). To verify the PMT2 sequence, we amplified a 664 bp
genomic DNA fragment of *PMT2* from the yeast strains S288c, BY4742, W303-1A and SEY6210 using the oligonucleotides vg63 and vg69. PCR products were cloned into pGEM T-easy and several independent clones were sequenced. These analyses showed that in contrast to the database entry, the *PMT2* ORF contains three additional base pairs (bp +400 to +429 is tgggacttccCttctggGGaaatttaccca; additional bases in capital letters). The insertions result in the predicted amino acid sequence of Pro-Ser-Gly instead of Leu-Leu at position 137 of Pmt2p.

*Plasmid pJK4-B1 (PMT4FLAG)*—A copy of the FLAG sequence (32) was obtained by annealing oligonucleotides oligo211 with oligo212. The annealed oligo pair features BamHI and NotI overhang sequences. The FLAG sequence was joined by a three-piece ligation with a 0.7 kb SacI/NotI fragment (isolated from plasmid SAP/EN; 33) that contains the yeast plasma membrane ATPase terminator (34) and the yeast shuttle vector pRS423 (digested with SacI and NotI) resulting in plasmid pRS423/TER/FLAG. The *PMT4* promoter and coding region (bp –675 to +2286) was amplified from *S. cerevisiae* genomic DNA using oligonucleotides oligo213 and oligo214. The PCR fragment was digested with BamHI and SalI, and cloned into pRS423/TER/FLAG. Additional FLAG sequences were cloned into the BamHI site of the resulting plasmid pJK4, using the annealed oligonucleotide pair oligo233/oligo234. In the resulting plasmid (pJK4-B1) four copies of the FLAG epitope were fused to the C terminus of *PMT4*.

*Plasmid pVG37 (PMT4 Δloop5)*—To remove aa 394-521 of Pmt4p, plasmid pJK4-B1 was digested with PflMI/HpaI and religated using the adapter oligonucleotides vg23 and vg24.

*Plasmid pVG45 (PMT4 R142EFLAG)*—An arginine residue at position 142 of Pmt4p was exchanged for a glutamate by site-directed mutagenesis (GeneEditor™, Promega) using the oligonucleotide vg30 and plasmid pVG43 (pUC18, containing bp +24 to +1165 of...
Mutations were confirmed by DNA sequence analysis of the resulting plasmid pVG44. A 476 bp MunI fragment of pVG44 was cloned into pVG42 (pUC18, containing bp +307 to +1165 of PMT4) digested with the same enzyme. From the resulting plasmid pVG69 a 1.87 kb SphI/HpaI fragment was isolated and cloned into pJK4-B1 resulting in pVG45.

Plasmid pVG36 (PMT1HA R138K)–Amino acid Arg-138 of Pmt1pHA was changed to lysine by site-directed mutagenesis using the oligonucleotide vg16 and plasmid pVG26 (pUC19, containing bp +61 to +537 of PMT1). Mutations were confirmed by DNA sequence analysis. A 335 bp Ncol/BsrGI fragment of the resulting plasmid pVG29 was cloned into plasmid pVG20 (pUC19, containing a 990 bp EcoRI/PstI fragment of pSB56 (18)) cut with Ncol/BsrGI. From the resulting plasmid pVG28 a 594 bp PmlI/BsrGI fragment was isolated and cloned into pSB56 to generate pVG36.

Production of Polyclonal Anti-Pmt3-6p Antibodies in Rabbits--Rabbits were immunized with recombinant fusion proteins consisting of glutathione S-transferase and the amino acids (aa)\(^1\) Met-1 to Arg-78 of S. cerevisiae Pmt3p, aa Met-1 to Ala-45 of Pmt4p, aa Asp-10 to Thr-112 of Pmt5p and aa Met-1 to Gln-66 of Pmt6p, respectively. The corresponding DNA fragments were amplified by PCR using genomic S. cerevisiae DNA as template and adapter oligonucleotides oligo151/oligo152 for PMT3, oligo106/oligo107 for PMT4, oligo143b/oligo144 for PMT5, oligo147/oligo148 for PMT6. The respective DNA fragments were combined with the glutathione S-transferase sequence by EcoRI/BamHI subcloning into a pGEX-2TK expression vector (Pharmacia). The fusion proteins were expressed in E. coli host BL21. The recombinant proteins were excised from SDS-polyacrylamide gels and injected into rabbits. Pineda Antikoerper-Service, Berlin, Germany, performed immunizations. Antibodies were affinity purified by binding to nitrocellulose derivatized with the glutathione S-transferase fusion protein (35).

Preparation of Crude Membranes--Crude membranes were isolated as described (18).
Immunoprecipitation--Sodium deoxycholate (DOC) extracts were prepared as described (18). Immunoprecipitation of Pmt1p\(^{HA}\), Pmt2p\(^{HA}\) and Pmt3-6p was performed using 300 µl DOC extract made from 10\(^9\) cells of the appropriate yeast strains. Pmt1p\(^{HA}\) was immunoprecipitated with 10 µl anti-HA monoclonal antibody covalently coupled to Protein A-Sepharose (16B12, Babco) for 1-2 h at 4°C. To precipitate Pmt3-6p, affinity purified polyclonal anti-Pmt3-6p antibodies were covalently coupled to Protein A-Sepharose (Pmt3-6p beads) as described (36). Pmt3-6p were immunoprecipitated with 20-50 µl Pmt3-6p beads for 1-2 h at 4°C. Immunoprecipitates were washed four times with 1 ml of cold lysis buffer (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.3 mM MgCl\(_2\), 10 % (v/v) glycerol, 0.35 % DOC, 0.5 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.25 mM TLCK, 50 µg/ml TPCK, 10 µg/ml leupeptin, and 1 µg/ml pepstatin) and once with 1 ml Tris-buffered saline. Subsequently, precipitates were resuspended in 20 µl of 3x SDS-sample buffer.

Pmt4p\(^{FLAG}\) and FLAG-tagged Pmt4p mutant proteins were solubilized from crude membranes, prepared as described in (18) using 500 µl solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3 mM MgCl\(_2\), 0.5 % Triton X-100, plus protease inhibitors) by vortexing for 30 min at 4°C. The suspension was clarified by centrifugation for 30 min at 20,000 rpm (Sorvall SS34 rotor) to obtain the Triton extract. FLAG-tagged proteins were immunoprecipitated for 1-2 h at 4°C from 300 µl Triton extract, using 30 µl of anti-FLAG M2 affinity Gel (Sigma). Precipitates were washed four times with 1 ml of cold solubilization buffer, once with 1 ml Tris-buffered saline and resuspended in 20 µl of 3x SDS-sample buffer.

Blue Native Polyacrylamide Gel Electrophoresis--Yeast cells were grown to 2-4 x 10\(^7\) cells/ml in YPD medium (37). A total of 10\(^10\) cells were harvested and crude membranes were isolated (18) using a modified buffer (50 mM Tris-HCl, pH 7.5, 15 % (v/v) glycerol,
plus protease inhibitors). Membranes were resuspended in 1 ml Nonidet P40 (NP40)\(^1\) solubilization buffer (50 mM Tris-HCl, pH 7.5, 0.5 % NP40, 750 mM aminocaproic acid, 15 % (v/v) glycerol, plus protease inhibitors). Membrane proteins were solubilized by vortexing for 20 min at room temperature. The suspension was clarified by ultracentrifugation at 100,000 xg at 4°C for 60 min. Samples (40 µg protein) were loaded onto polyacrylamide gradient gels. Just prior to electrophoresis, Coomassie Brilliant Blue G (Serva; dissolved in 500 mM aminocaproic acid) was added from a 5 % stock solution to adjust to a detergent/Coomassie ratio of 4/1 (g/g). Blue native polyacrylamide gel electrophoresis (BN-PAGE)\(^1\) was performed using a polyacrylamide gradient from 4-13 %, with a 4 % stacking gel, as described (38). 0.02 % Nonidet P40 was added to both polyacrylamide gel and cathode buffer (50 mM Tricine, 15 mM Bistris). The cathode buffer also contained 0.02 % Coomassie Brilliant Blue G. This buffer was replaced after electrophoresis for 1 h at 80 V, 4°C, by cathode buffer without Coomassie. Electrophoresis was continued for ~6 hours at 100 V, 4°C. Gels were blotted onto nitrocellulose membranes using 20 mM Tris, 150 mM Glycine, 20 % MeOH, 0.02 % SDS.

**Chemical Cross-Linking of Yeast Membrane Proteins**

2.0 \(\times 10^9\) yeast cells from a logarithmically growing culture were harvested at 3,000 g, 4 °C and washed once with 20 ml OPA buffer (50 mM boric acid-sodium tetraborate, pH 8.0). The cell pellet was resuspended in 200 µl OPA buffer plus protease inhibitors. Crude membranes were prepared as described (18). The membrane pellet was resuspended in 20 ml OPA buffer without protease inhibitors and centrifuged for 30 min at 20,000 rpm (Sorvall, SS34 rotor). Subsequently, membranes were resuspended in 500 µl OPA buffer. For cross-linking, o-phtaldialdehyde (OPA\(^1\); Sigma) was added to final concentrations of 25–200 µM to 100 µl of membrane suspension and incubated in the dark for 30 min at 25°C. The reaction was quenched with 100 mM Tris-HCl, pH 6.8.
**Western Blot Analysis**--Proteins were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)\(^1\) or BN-PAGE and transferred to nitrocellulose. Polyclonal anti-Pmt1p (39) and anti-Wbp1p (40) antibodies were used at a dilution of 1:2000, and anti-Pmt2p (21) at a dilution of 1:1000. Affinity-purified antibodies anti-Pmt4p, anti-Pmt5p and anti-Pmt6p were used at a dilution of 1:2500; anti-Pmt3p at a dilution of 1:500. The anti-HA (16B12, Babco) and anti-FLAG (M2; Sigma) monoclonal antibodies were used at 1:8000 and 1:5000 dilutions, respectively. Protein-antibody complexes were visualized by enhanced chemiluminescence using the Amersham ECL system.

**In Vitro Dol-P-Man:Protein O-Mannosyltransferase Assay**--Dol-P-Man:protein O-mannosyltransferase activity was measured as described (14).
RESULTS

To uncover common principles that underlie the functionality of Pmtps we investigated whether complex formation is a general feature of the yeast PMT family members. We generated polyclonal antibodies that specifically recognize *S. cerevisiae* Pmt3p (predicted molecular weight (MW) 86.2 kDa), Pmt4p (predicted MW 87.8 kDa), Pmt5p (predicted MW 84.8 kDa), and Pmt6p (predicted MW 87.9 kDa) in wild type yeast as shown by Western blot analyses (Fig. 1, lanes 1, 3, 5 and 7). Extracts from the corresponding *pmt* deletion strains contained no cross-reactive material, proving that the antibodies are highly specific (Fig. 1, lanes 2, 4, 6 and 8). We also created epitope-tagged versions of Pmt2p (Pmt2p<sup>HA</sup>) and Pmt4p (Pmt4p<sup>FLAG</sup>) (described in “Experimental Procedures”). These tools enabled us to detect PMT complexes isolated by coimmunoprecipitation, BN-PAGE, and chemical cross-linking.

*Members of the PMT1 subfamily interact in pairs with members of the PMT2 subfamily*—To characterize PMT complexes, we performed coimmunoprecipitation experiments using a HA-epitope tagged version of *S. cerevisiae* Pmt1p (Pmt1p<sup>HA</sup>; 18). Pmt1p<sup>HA</sup> was expressed in a *pmt1* deletion strain and solubilized from crude membranes using Triton X-100 and sodium deoxycholate (DOC extract). Pmt1p<sup>HA</sup> was immunoprecipitated from DOC extracts with monoclonal anti-HA antibodies (see “Experimental Procedures”). The immunoprecipitate and an aliquot of the DOC extract were resolved on 8% SDS-polyacrylamide gels and analyzed by Western blotting and sequentially probing the blots with polyclonal antibodies to Pmt1p to Pmt6p (Fig. 2A, lanes 1 and 2). To ensure the specificity of the immunoprecipitation reaction the same experiment was performed using strain *pmt1Δ* expressing Pmt1p without the HA-tag (Fig. 2A, lanes 3 and 4). As shown previously (21) we confirmed that Pmt2p is the major interacting partner of Pmt1p (Fig. 2A, lane 2). Moreover, a weak signal for Pmt3p could be specifically detected in the
Pmt1p<sup>HA</sup> immunoprecipitate (Fig. 2A, compare lanes 2 and 3). The amount of coimmunoprecipitated Pmt3p was small, but this result was highly reproducible. In contrast, neither Pmt4p, Pmt5p or Pmt6p could be detected (Fig. 2A, lane 2). Coimmunoprecipitation of Pmt1p<sup>HA</sup> from <i>pmt3</i> (Fig. 2B, lane 4) and <i>pmt2</i> (Fig. 2B, lane 6) deletion strains showed that Pmt1p<sup>HA</sup> interacts with Pmt2p independently of Pmt3p and vice versa.

To circumstantiate the existence of distinct Pmt1p-Pmt2p and Pmt1p-Pmt3p complexes a HA-tagged version of Pmt2p was expressed in strain <i>pmt2Δ</i> and coimmunoprecipitation experiments were performed as described above. Figure 2C shows that Pmt2p<sup>HA</sup> specifically binds to Pmt1p but not Pmt3p, Pmt4p or Pmt6p (lane 2) corroborating that Pmt1p interacts with Pmt3p independently of Pmt2p. In addition, in the Pmt2p<sup>HA</sup> immunoprecipitate a weak signal for Pmt5p could be detected (Fig. 2C, lane 2). The amount of coimmunoprecipitated Pmt5p was little, however, the result was specific (data not shown) and highly reproducible. Furthermore, immunoprecipitation of Pmt2p<sup>HA</sup> from a <i>pmt1</i> deletion strain showed that Pmt2p<sup>HA</sup> interacts with Pmt5p independently of Pmt1p (data not shown).

So far our data indicated that in wild type yeast Pmt1p and Pmt2p form a dominant protein complex. In addition, we found that Pmt1p also interacts with Pmt3p, and that Pmt2p interacts with Pmt5p. Next we addressed the question of which is the major interacting partner of Pmt3p. Pmt3p was immunoprecipitated from DOC extracts of wild type and <i>pmt3</i> mutant strains using polyclonal anti-Pmt3p antibodies (see “Experimental Procedures”). In the Pmt3p immunoprecipitate a small amount of Pmt1p, but not Pmt2p, Pmt4p or Pmt6p was present (Fig. 2D, lane 2). Only Pmt5p was highly enriched when compared to the input material (Fig. 2D, compare lanes 1 and 2), demonstrating that Pmt3p predominantly interacts with Pmt5p. Again, the association between Pmt3p and Pmt5p was independent of other Pmt proteins (Fig. 2A, C, D and data not shown).
Summarizing, our data show that distinct Pmt1p-Pmt2p, Pmt1p-Pmt3p, Pmt5p-Pmt2p and Pmt5p-Pmt3p complexes are present in *S. cerevisiae*. Of these, however, Pmt1p-Pmt2p and Pmt5p-Pmt3p complexes represent the predominant forms.

The Pmt1p-Pmt3p and Pmt5p-Pmt2p complexes which we detected in our coimmunoprecipitation experiments are less abundant when compared to Pmt1p-Pmt2p or Pmt5p-Pmt3p complexes. To substantiate that the minor complexes are not formed artificially during coimmunoprecipitation, and to confirm that Pmt1p-Pmt2p and Pmt5p-Pmt3p represent the predominant PMT complexes in wild type yeast, we performed BN-PAGE (38) that separates native protein complexes. NP40 extracts derived from wild type and *pmt1-6* mutant strains were resolved on 4 - 13 % polyacrylamide gels to separate native PMT complexes (see “Experimental Procedures”). Pmtp-containing complexes were detected by Western blots probed with polyclonal anti-Pmtp antibodies.

Analyses of Pmt1p-containing complexes showed that in NP40 extracts from wild type yeast two protein bands with an apparent MW of ~170 kDa and ~310 kDa, respectively, were specifically detected by anti-Pmt1p antibodies (Fig. 3A, compare lanes 1 and 7). The ~170 kDa band highly likely represents the monomeric Pmt1p. The discrepancy in MW of ~170 kDa in BN-PAGE versus 92 kDa in SDS-PAGE (19) is probably due to an abnormal migration behavior of Pmt1p caused by the hydrophobic nature of the protein, an unusual Coomassie Blue to protein ratio and/or the charge to mass ratio, which is variable in BN-PAGE (41). The formation of Pmt1p homodimers was excluded by coimmunoprecipitation experiments using Pmt1p<sup>HA</sup> and untagged Pmt1p (data not shown). In addition to monomeric Pmt1p, specific Pmt1p containing protein complexes with an apparent molecular weight of ~310 kDa could be detected (Fig. 3A, lane 1). The ratio of monomeric Pmt1p to Pmt1p-containing high molecular weight complexes varied to some extend in independent experiments (Figs 3A and 3B, lane 1), which is highly likely due to disaggregation of PMT
complexes during solubilization. Protein complexes with molecular weights of ~300-320 kDa were also detected by polyclonal anti-Pmt2p, anti-Pmt3p and anti-Pmt5p antibodies (data not shown).

Assuming that in wild type yeast *in vivo* Pmt1p-Pmt3p complexes are only present in minor amounts when compared to Pmt1p-Pmt2p, one would expect that in a *pmt2* but not in a *pmt3* deletion mutant the amount of the ~310 kDa complexes recognized by anti-Pmt1p antibodies should decrease dramatically. As shown in Figure 3A (lanes 2 and 3), this is exactly what we observed. Nevertheless, in the absence of Pmt2p a very small amount of protein complexes with an apparent MW of ~310 kDa could be detected after raising the limit of detection of the Western analysis (Fig. 3B, lane 2). These complexes were not only recognized by anti-Pmt1p but also by anti-Pmt3p antibodies (data not shown). In *pmt4-6* mutants Pmt1p containing complexes were not affected (Fig. 3A, lanes 4-6). These data corroborate our finding that Pmt1p interacts individually with Pmt2p and Pmt3p; however, Pmt2p represents the major interacting partner. BN-PAGE also showed that Pmt3p-containing protein complexes vanished almost completely only in the absence of Pmt5p (data not shown), consistent with Pmt3p forming an abundant heteromeric complex with Pmt5p.

The amount of Pmt1p-Pmt3p and Pmt5p-Pmt2p complexes in wild type yeast appeared very minor. Therefore, the question comes up whether these complexes are physiologically relevant. One possibility is that these Pmtp complexes are mainly formed in the absence of their major interacting partners to compensate partially for a lack of protein O-mannosyltransferase activity. To investigate this hypothesis, we analyzed *pmt1pmt3* and *pmt2pmt5* deletion mutant strains where the preferred interacting partners of Pmt2p/Pmt5p, and Pmt1p/Pmt3p, respectively, are missing. Western analysis of crude membranes isolated from a *pmt1pmt3* mutant showed that the amount of both Pmt2p and Pmt5p is increased when compared to wild type (Fig. 4A, compare lanes 1 and 2). Accordingly, in the *pmt2pmt5*
mutant Pmt3p is more abundant (Fig. 4A, compare lanes 1 and 3). The amount of Pmt1p is not obviously changed, suggesting that Pmt1p is not limiting (Fig. 4A, lane 3). These observations suggested that in the absence of the favored partners Pmt1p-Pmt3p and Pmt5p-Pmt2p complexes accumulate.

To substantiate these data we analyzed Pmt5p-Pmt2p complex formation by chemical cross-linking using o-phtaldialdehyde (OPA)\(^1\). Under the conditions we applied (50 µM OPA) in wild type yeast only upon overexpression of Pmt2p a protein complex with an apparent MW of ~152 kDa could be detected which is specifically recognized by polyclonal anti-Pmt5p (Fig. 4B, compare lanes 1 and 3) as well as anti-Pmt2p antibodies (data not shown). In contrast, Pmt5p-Pmt2p complexes could be easily detected in the \( \text{pmt1pmt3} \) mutant even without overexpression of Pmt2p (Fig. 4B, lane 4).

To test whether the formation of Pmt5p-Pmt2p and Pmt1p-Pmt3p complexes results in increased O-mannosyltransfer, we determined \textit{in vitro} O-mannosyltransferase activity in \( \text{pmt1pmt3} \Delta \) and \( \text{pmt2pmt5} \Delta \) mutant strains. The \textit{in vitro} assay system we used preferentially detects O-mannosyltransferase activity of Pmt1p- and Pmt2p-containing complexes (14, 24; Table II). As shown in Table II \textit{in vitro} O-mannosyltransferase activity is dramatically decreased in \( \text{pmt1} \Delta \) and \( \text{pmt1pmt2} \Delta \) mutants when compared to wild type yeast. Western analysis of crude membranes revealed an equal abundance of Pmt3-6p in \( \text{pmt1} \Delta, \text{pmt1pmt2} \Delta \) and wild type strains (data not shown). In \( \text{pmt1pmt3} \Delta \) and \( \text{pmt2pmt5} \Delta \) mutants, in which Pmt5p-Pmt2p and Pmt1p-Pmt3p complexes are formed (see Fig. 4), \textit{in vitro} O-mannosyltransferase activity is increased by 68.2 % and 42.6 %, respectively, when compared to \( \text{pmt1pmt2} \Delta \) mutants (Table II).

In summary, our data show that members of the \textit{PMT1} subfamily (Pmt1p and Pmt5p) form distinct complexes with members of the \textit{PMT2} subfamily (Pmt2p and Pmt3p), and that
Pmt1p-Pmt2p and Pmt5p-Pmt3p pairs are the predominant forms. Under specific conditions Pmt1p-Pmt3p and Pmt5p-Pmt2p complexes can be formed to perpetuate O-mannosyltransfer.

*Pmt4p forms homomeric complexes in vivo*—We could not detect interactions between members of the *PMT1* or *PMT2* subfamily and Pmt4p, the only member of the *PMT4* subfamily. In agreement with these results, no other Pmt proteins could be copurified when Pmt4p was precipitated from DOC extracts from a yeast wild type strain using polyclonal anti-Pmt4p antibodies (data not shown). However, BN-PAGE revealed the presence of larger Pmt4p complexes, which were not influenced by the absence of any other *PMT* family member (data not shown). We wished to determine whether these complexes were due to Pmt4p-Pmt4p homotypic interactions or whether Pmt4p is associated with other proteins. Therefore, an epitope-tagged version of Pmt4p was constructed by fusing four copies of the FLAG epitope to the C terminus of Pmt4p (see “Experimental Procedures”). Complementation of the temperature sensitive phenotype of a *pmt1pmt4* mutant by Pmt4p^FLAG^ proved that this construct is fully functional in vivo (Fig. 7). To test for homotypic interactions, *PMT4^FLAG^* was expressed in a *S. cerevisiae* wild type strain and crude membranes were prepared. Proteins were solubilized with Triton X-100 (Triton extract) and immunoprecipitation of Pmt4p^FLAG^ was performed using monoclonal anti-FLAG antibodies covalently linked to Protein A-Sepharose. Immunoprecipitates were resolved on 8% SDS-polyacrylamide gels. Wild type Pmt4p and Pmt4p^FLAG^, which differ in molecular weight by 4.8 kDa, were detected on a Western blot probed with polyclonal anti-Pmt4p antibodies. As shown in Figure 5A Pmt4p^FLAG^ specifically coimmunoprecipitates wild type Pmt4p (compare lane 4 with lanes 5 and 6), indicating that Pmt4p is present in homomeric complexes in vivo. Chemical cross-linking experiments corroborated these results. Pmt4p^FLAG^ was expressed in a *pmt4* deletion strain, crude membranes were prepared, and cross-linking was performed using
OPA at final concentrations of between 25-100 µM. Proteins were resolved on 8 % SDS-polyacrylamide gels and analyzed by Western blotting using anti-Pmt4p antibodies as probe. Figure 5B (lane 2) shows that in the absence of OPA, Pmt4p\textsuperscript{FLAG} migrates with an apparent MW of ~90 kDa which is in agreement with a deduced MW of 92.6 kDa. Upon addition of OPA, larger complexes with an apparent molecular weight of ~165 kDa could be detected (Fig. 5B, lanes 3-5), consistent with the formation of homodimeric Pmt4p complexes.

Summarizing, our data suggest that Pmt4p forms homomeric complexes, however, the association with other smaller molecular weight molecules cannot be ruled out completely.

Conserved protein domains underlie heteromeric Pmt1p-Pmt2p and homomeric Pmt4p-Pmt4p interactions--Since, in contrast to the other PMT family members, Pmt4p forms homomeric complexes, we asked whether common principles underlie homomeric and heteromeric PMT complex formation and/or stability. We previously showed that a large hydrophilic endoplasmic-reticulum-oriented segment of Pmt1p (loop 5, aa 294-586) is crucial for mannosyltransferase activity but not for Pmt1p-Pmt2p complex formation (18; see also Fig. 7). To test whether the same may be the case for Pmt4p we deleted the large predicted luminal loop 5 region of Pmt4p\textsuperscript{FLAG} (aa 394-521; Fig. 6A) and expressed the internal deletion construct (\textDelta loop5) in \textit{pmt4} mutant and wild type yeast strains. Pmt4p complex formation was assayed by chemical cross-linking. Fig. 6B shows that in the presence of OPA, larger complexes with an apparent MW of ~140 kDa (compare lanes 1 and 2) can be detected in the \textit{pmt4} mutant strain in addition to monomeric \textDelta loop5. Furthermore, when \textDelta loop5 and wild type Pmt4p are expressed simultaneously, additional complexes varying in size from ~156 kDa to ~170 kDa appeared (Fig. 6B, lane 4). From these data we conclude that Pmt4p\textsuperscript{FLAG} \textDelta loop5 is able to interact with itself as well as with wild type Pmt4p. When Pmt4p\textsuperscript{FLAG} \textDelta loop5 is expressed in a temperature sensitive \textit{pmt1pmt4} mutant strain it does not restore the
growth defect at 35°C (Fig. 7), indicating that this large hydrophilic segment is essential for Pmt4p activity even though it does not obviously affect Pmt4p dimerization. Our data show that loop 5 domain of Pmt4p appears to behave in the same way as loop 5 of Pmt1p.

For *S. cerevisiae* Pmt1p, amino acid residue Arg-138, located in transmembrane domain two at the water-membrane interface, is essential for the formation of heteromeric Pmt1p-Pmt2p complexes (18; see also Fig. 6C, lane 3). In addition, exchange of Pmt1p Arg-138 for alanine results in a complete loss of mannosyltransferase activity (18; see also Fig. 7). When Arg-138 is exchanged for lysine, Pmt1p-Pmt2p complexes (Fig. 6C, compare lanes 1 and 4) as well as O-mannosyltransferase activity (Fig. 7) are partially restored, indicating that a positive charged amino acid at that position is important for the establishment of functional Pmt1-Pmt2p complexes. Because this arginine residue is highly conserved between all *PMT* family members, we asked whether this residue also affects Pmt4p’s homomeric interactions. We therefore replaced Arg-142 of Pmt4p<sub>FLAG</sub> (the equivalent of Arg-138 in Pmt1p) with glutamate using site-directed mutagenesis. The Pmt4p mutant protein R142E<sub>FLAG</sub> was expressed and characterized in a yeast wild type, a *pmt4* and a *pmt1pmt4* mutant strain. SDS-PAGE and Western blotting of Triton extracts with polyclonal anti-Pmt4p antibodies revealed that in wild type yeast Pmt4p<sub>FLAG</sub> and the mutant protein R142E<sub>FLAG</sub> show an identical MW and are expressed at similar levels (data not shown). However, Pmt4p-R142E<sub>FLAG</sub> failed to complement the temperature-sensitivity of the *pmt1pmt4* mutant, indicating that Arg-142 is essential for Pmt4p activity in *vivo* (Fig. 7). In addition, coimmunoprecipitation experiments were performed on Triton extracts of a wild type strain coexpressing wild type Pmt4p and, alternatively, Pmt4p<sub>FLAG</sub>, or Pmt4p-R142E<sub>FLAG</sub> using monoclonal anti-FLAG antibodies. In contrast to Pmt4p<sub>FLAG</sub>, which efficiently coimmunoprecipitates wild type Pmt4p, Pmt4p-R142E<sub>FLAG</sub> almost completely fails to
precipitate wild type Pmt4p (Fig. 6D, compare lanes 1 and 2), consistent with a critical role for Arg-142 in Pmt4p-Pmt4p complex formation.

Taken together, our data show that similar principles underlie the formation of heteromeric complexes between members of the PMT1 and PMT2 subfamilies and homomeric Pmt4p complexes.
DISCUSSION

In yeast the *PMT1* and *PMT2* subfamilies but not the *PMT4* subfamily are highly redundant. In this study we demonstrate that the formation of specific protein complexes is a common feature of *PMT* family members in yeast. We found that, in general, members of the *PMT1* subfamily (Pmt1p and Pmt5p) interact in pairs with members of the *PMT2* subfamily (Pmt2p and Pmt3p). As schematically shown in Figure 8A, Pmt1p-Pmt2p and Pmt5p-Pmt3p are the predominant complexes formed between *PMT1* and *PMT2* subfamily members in wild type *S. cerevisiae* cells. Under certain conditions, however, Pmt1p can interact with Pmt3p, and Pmt5p with Pmt2p, respectively. This can occur, for example, when one of the principle partners is absent, as is the case in *pmt* mutant strains. In contrast, the unique representative of the *PMT4* subfamily forms homomeric complexes (Fig. 8B). Interestingly, we further uncovered that the same conserved protein domains influence both heteromeric and homomeric Pmt-protein interactions.

*Heteromeric protein complexes between *PMT1* and *PMT2* subfamily members might have evolved by gene duplication and fulfill similar tasks in *S. cerevisiae*. Within the *PMT* family in *S. cerevisiae*, Pmt1p is most closely related to Pmt5p (53% identity and 72% homology), and Pmt2p to Pmt3p (65% identity and 81% homology), respectively. In view of this high degree of conservation, the proteins Pmt1p and Pmt5p, as well as Pmt2p and Pmt3p might have evolved by gene duplication. This is supported by the fact that Pmt1p (YDL095w) and Pmt5p (YDL093w) are located directly next to each other on chromosome IV. Further, the report of Wolfe and Shields (42) suggests that Pmt2p (YAL023c; chr. I) and Pmt3p (YOR321w; chr. XV) constitute a protein pair that derives from an ancient duplication of the entire yeast genome. As a consequence of these gene duplication events, the ability to form specific protein complexes between individual members of the *PMT1* and the *PMT2* subfamily (Pmt1p-Pmt2p and Pmt5p-Pmt3p) evolved in *S. cerevisiae*. This connection also
explains why Pmt1p-Pmt3p and Pmt5p-Pmt2p complexes can be formed, even though only under certain physiological conditions.

A series of observations suggest that Pmt1p-Pmt2p and Pmt5p-Pmt3p O-mannosyltransferase complexes act on the same protein substrates, albeit Pmt1p-Pmt2p complexes represent the predominant mannosyltransferase activity. Analyses of in vitro mannosyltransfer from Dol-P-Man to specific synthetic acceptor peptides have shown that the simultaneous deletion of *PMT1* and *PMT2* results in loss of >80% of in vitro O-mannosyltransferase activity, when compared with wild type yeast (1, 24). In addition, in vivo O-mannosylation of the same protein substrates is dramatically decreased in *pmt1* and *pmt2* mutant strains, such as the cell wall proteins Kre9p, chitinase (Cts1p), Bar1p, Ccw4p and Ccw5p (14, 25). In contrast, in vitro and in vivo O-mannosylation is not obviously affected in *pmt3* or *pmt5* mutants (1, 14, 25). However, deletion of *PMT3* in a *pmt1pmt2* mutant further decreases in vitro mannosyltransfer from Dol-P-Man to a synthetic Pmt1p/Pmt2p-acceptor peptide by ~25%, when compared to the in vitro activity measured in *pmt1pmt2* strains (1). In analyses of the O-mannosylation state of chitinase in *pmt1pmt2* and *pmt1pmt2pmt3* mutants, Gentzsch and Tanner (1) showed that in vivo chitinase is O-mannosylated by Pmt1p, Pmt2p as well as Pmt3p. Pmt3p therefore, mannosylates the same proteins as Pmt1p and Pmt2p, but comes into operation mainly in the absence of Pmt1p and Pmt2p. The notion that Pmt1p-Pmt2p and Pmt5p-Pmt3p complexes fulfill similar tasks in vivo is further supported by the fact that transcription of *PMT1*, *PMT2*, *PMT3* and *PMT5*, but not *PMT4* and *PMT6* is enhanced in response to cell stress conditions that cause the accumulation of misfolded proteins in the ER (43). In summary, Pmt1p-Pmt2p and Pmt5p-Pmt3p are likely to O-mannosylate the same set of substrate proteins, yet, Pmt5p-Pmt3p complexes might, for example, exhibit lower substrate affinities and, therefore, play only a minor role in wild type yeast cells. In the absence of Pmt1p and Pmt2p, however, Pmt5p-
Pmt3p might compensate for O-mannosylation deficiency in pmt mutant strains. Similar functions might be assigned to the Pmt1p-Pmt3p and Pmt5p-Pmt2p complexes, which we could detect only in small amounts in wild type strains (Figs 2 and 3) or under specific physiological conditions such as in pmt mutants (Fig. 4). The observed increase of in vitro O-mannosyltransferase activity in pmt1pmt3 and pmt2pmt5 mutants (Table II) substantiates that Pmt1p-Pmt3p and Pmt5p-Pmt2p act as mannosyltransferases and feature substrate specificities similar to Pmt1p-Pmt2p. Our data suggest a compensatory cooperation between PMT1/PMT2 subfamily members, which might explain why in S. cerevisiae only the simultaneous deletion of several PMT subfamily members results in a substantial decrease in O-linked oligomannose chains and finally causes cell death (1). This possibility is supported by the fact that overexpression of PMT2 rescues the growth defect of a temperature sensitive pmt1pmt4 mutant (data not shown). Because overexpression of PMT2 results in the formation of Pmt5p-Pmt2p complexes (Fig. 4), Pmt5p-Pmt2p might at least partially compensate the lack of Pmt1p-Pmt2p. Concordantly, Gentzsch et al. (21) showed that overexpression of PMT2 causes a slight increase of in vitro O-mannosyltransferase activity in wild type yeast and in pmt1 deletion mutants. A compensatory cooperation between the redundant members of the PMT1/PMT2 subfamily in S. cerevisiae is also supported by the observation that in the fission yeast Schizosaccharomyces pombe where only one member of each PMT subfamily is present, the deletion of the single PMT2 subfamily member is lethal (Willer T. and S.S.; unpublished results).

The third member of the S. cerevisiae PMT2 subfamily, Pmt6p, interacts with none of the other PMT family members. In S. cerevisiae Pmt6p shares an overall sequence identity of 46% with Pmt2p and of 45% with Pmt3p. In the course of our analyzes, BN-PAGE, coimmunoprecipitation and chemical cross-linking experiments showed that Pmt6p interacts neither with Pmt1-5p nor with itself (data not shown), and therefore behaves differently from
all other Pmtps in yeast. Nevertheless, BN-PAGE suggested that Pmt6p interacts with other proteins, even though not with Pmtps (data not shown). To identify potential interacting partners, we analyzed whether selected components of the N-glycosylation machinery or the translocon are stably associated with Pmt6p. Again, no evidence was obtained that Wbp1p (40), Ost1p (44), Stt3p (45), or Sec61p (46) interact with Pmt6p (data not shown). Further studies are needed to elucidate the nature of these Pmt6p containing complexes.

*Common principles underlie heteromeric Pmt1p-Pmt2p and homomeric Pmt4p-Pmt4p interactions.* Considering Pmtps as antifungal targets it is noteworthy that in *S. cerevisiae* and in *C. albicans* only deletion of Pmt4p in combination with *PMT1/PMT2* subfamily members causes lethality (1, 47). Therefore, to eliminate protein O-mannosylation both *PMT1/PMT2* and *PMT4* subfamily members must be inhibited. Pmt4p differs in several respects from *PMT1/PMT2* subfamily members, such as substrate specificity and conserved signature sequence motifs (9, 14, 18). In addition, Pmt4p forms homomeric protein complexes as demonstrated in this study (Fig. 5). In view of these variances, it is of particular importance that common principles form the basis of the formation, structure and/or stability of PMT complexes in both *PMT1/PMT2* and *PMT4* subfamilies. Our mutational analyses showed that Pmt4p Arg-142, which is highly conserved between all PMT family members, is crucial for Pmt4p-Pmt4p complexes and enzyme activity (Figs 6 and 7). Analogical, the corresponding mutation in Pmt1p affects Pmt1p-Pmt2p complexes and results in loss of mannosyltransferase activity (18). Thus, *PMT* complexes offer a point of attack to abolish protein O-mannosylation in fungi.

*PMT complexes might ensure efficient O-mannosylation.* There are a number of reasons why protein O-mannosyltransferases form homo- or heteromeric protein complexes. One is that these complexes ensure an efficient O-glycosylation of a wide range of proteins. A common feature of O-glycosylated proteins is that O-linked carbohydrate chains are clustered
in distinct serine/threonine rich regions (48). Such areas are thought to adopt rod-like structures important for protein function. With a few exceptions O-mannosylation occurs while proteins are translocated in the lumen of the ER (49, 11, Hagen I. and S.S., unpublished data). Thus, the clustering of O-linked sugars requires high-efficiency sugar transfer, which might be provided by mannosyltransferase complexes. That oligomerization enhances enzyme function has been proven for other glycosyltransferases such as UDP-GlcNAc:dolichol-P GlcNAc-1-P transferase (50) or the mannosyltransferase complexes M-Pol I and M-Pol II (51-53). Furthermore, even though members of the PMT1 and PMT2 subfamily act on the same protein substrate (14, 25), they might actually O-mannosylate different serine and threonine residues within one and the same protein. Since to date no specific consensus sequences are known which are required for O-mannosylation this assumption remains to be verified. However, this hypothesis is further supported by the fact that mutant alpha-factor precursor is O-mannosylated by Pmt2p but no other PMT family member (11). To understand the functioning of PMT complexes it will be important to learn more about their different substrate specificities and what features of PMTs determine specificity.
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FIGURE LEGENDS

FIG. 1. Specificity of polyclonal anti-Pmt3-6p antibodies. NP40 extracts (10 µg protein) from yeast wild type strain SEY6210 (WT) and pmt3-6Δ deletion mutants (pmt3-6) were resolved on 8 % SDS-polyacrylamide gels and analyzed by Western blotting using affinity purified polyclonal anti-Pmt3p (lanes 1 and 2), anti-Pmt4p (lanes 3 and 4), anti-Pmt5p (lanes 5 and 6) and anti-Pmt6p (lanes 7 and 8) antibodies.

FIG. 2. Coimmunoprecipitation experiments reveal specific interactions between members of the PMT1 and members of the PMT2 subfamily. A-D, DOC extracts were prepared and immunoprecipitation was performed as indicated below. Precipitates (IP) and aliquots (35 µg protein) of the DOC extracts prior to immunoprecipitation (input) were resolved on 8 % SDS-polyacrylamide gels and analyzed by Western blotting. Blots were sequentially probed with polyclonal anti-Pmt1p (Pmt1p), anti-Pmt2p (Pmt2p), anti-Pmt3p (Pmt3p), anti-Pmt4p (Pmt4p), anti-Pmt5p (Pmt5p), anti-Pmt6p (Pmt6p) antibodies. A, Immunoprecipitation of Pmt1pHA reveals Pmt1p-Pmt2p and Pmt1p-Pmt3p complexes. Pmt1pHA and Pmt1p were individually expressed in strain pmt1Δ from plasmid pSB56 and pSB53, respectively. Immunoprecipitation was performed using monoclonal anti-HA antibody coupled to Protein A-Sepharose (anti-HA). B, Pmt1pHA interacts with Pmt2p in the absence of Pmt3p and vice versa. Pmt1pHA was expressed in strain pmt1Δ (WT), pmt3Δ (pmt3), and pmt2Δ (pmt2) and immunoprecipitated using anti-HA beads. C, Immunoprecipitation of Pmt2pHA reveals Pmt5p-Pmt2p complexes. Pmt2pHA was expressed from plasmid pVG80 in strain pmt2Δ. Immunoprecipitation was performed using monoclonal anti-HA beads. C, Immunoprecipitation of Pmt3p reveals Pmt5p-Pmt3p complexes. Pmt3p
was immunoprecipitated from wild type SEY6210 (WT) and \textit{pmt3}\Delta (pmt3) strains using polyclonal anti-Pmt3p antibodies coupled to Protein A-Sepharose.

Fig. 3. \textbf{Pmt1p-Pmt2p represent the predominant Pmt1p containing complexes.} \textit{A, B,} NP40 (40 µg protein) extracts from yeast wild type (WT) and mutant strains \textit{pmt1-6}\Delta (\textit{pmt1-6}) were resolved by BN-PAGE using 4-13 % polyacrylamide gels and analyzed by Western blotting using anti-Pmt1p antibodies. Monomeric Pmt1p shows an apparent MW of \sim 170 kDa, Pmt1p containing complexes of \sim 310 kDa. \textit{A,} In the absence of Pmt2p (lane 2) mainly monomeric Pmt1p is detected. \textit{B,} The arrow indicates Pmt1p containing high molecular weight complexes, which could be detected even in the absence of Pmt2p (lane 2). Improved sensitivity in visualizing proteins was achieved using Hyperfilm MP (Amersham Pharmacia).

Fig. 4. \textbf{Formation of Pmt1p-Pmt3p and Pmt5p-Pmt2p complexes.} \textit{A,} Western blots of crude membranes (30 µg protein) isolated from the yeast strains SEY6210 (WT; lane 1), \textit{pmt1pmt3}\Delta (lane 2) and \textit{pmt2pmt5}\Delta (lane 3). Blots were sequentially probed with polyclonal anti-Pmt1p, anti-Pmt2p, anti-Pmt3p, anti-Pmt5p and anti-Wbp1p antibodies. \textit{B, Chemical cross-linking of Pmt5p-Pmt2p complexes using OPA.} Membranes were prepared from yeast strains SEY6210 (WT; lane 1) and \textit{pmt1pmt3}\Delta (lanes 4 and 5); further from strain SEY6210 (WT; lanes 2 and 3) overexpressing \textit{PMT2} from the multi-copy plasmid YEp352 (\textit{PMT2}\↑). Membranes were treated with 50 µM OPA (+) or mock (-) treated as described in “Experimental Procedures”. Proteins were resolved on 8% SDS-polyacrylamide gels and analyzed by Western blotting using polyclonal anti-Pmt5p antibodies. An arrow indicates Pmt5p-Pmt2p complexes (MW \sim 152 kDa).
Fig. 5. Pmt4p forms homomeric complexes. A, Coimmunoprecipitation of Pmt4p\textsuperscript{FLAG} and Pmt4p. Pmt4p\textsuperscript{FLAG} was expressed from plasmid pJK4-B1 in wild type SEY6210 (\textit{PMT4}/\textit{PMT4}\textsuperscript{FLAG}; lanes 1 and 4) and \textit{pmt4}\textsuperscript{Δ} (\textit{PMT4}\textsuperscript{FLAG}; lanes 2 and 5) strains. In addition, SEY6210 was transformed with plasmid pRS423 (\textit{PMT4}; lanes 3 and 6). Triton extracts were prepared and immunoprecipitation of Pmt4p\textsuperscript{FLAG} was performed using monoclonal anti-FLAG antibodies, coupled to Protein A-Sepharose (anti-FLAG). Aliquots (35 µg protein) of the Triton extracts (input) and precipitates (IP) were resolved on 8 % SDS-polyacrylamide gels. B, Chemical cross-linking of Pmt4p\textsuperscript{FLAG}. Membrane extracts were isolated from strain \textit{pmt4}\textsuperscript{Δ} expressing Pmt4p\textsuperscript{FLAG} (\textit{PMT4}\textsuperscript{FLAG}; lanes 2-5) and \textit{pmt4}\textsuperscript{Δ} transformed with pRS423 (\textit{pmt4}; lane 1). 50 µg protein was treated with OPA (final concentrations 25-100 µM) or mock (-) treated and resolved on 8 % SDS-polyacrylamide gels. An arrow indicates Pmt4p dimers (MW ~165 kDa). A, B, Proteins were identified by Western blotting using anti-Pmt4p antibodies.

Fig. 6. Conserved protein domains mediate Pmt4p-Pmt4p interactions. A, Schematic presentation of Pmt4p. By analogy to Pmt1p (18) we established a topological model for Pmt4p. Invariant amino acids and conserved signature motifs are indicated in gray. Scissors mark the internal deletion Δloop5 (aa 394-521). The conserved Arg-142 residue is indicated. B, Chemical cross-linking of Pmt4p\textsuperscript{FLAG}–Δloop5. The internal deletion construct Δloop5 was expressed from plasmid pVG37 in \textit{pmt4}\textsuperscript{Δ} (\textit{pmt4}; lanes 1 and 2) and wild type (WT; lanes 3 and 4) yeast strains. Chemical cross-linking using OPA (75 µM final concentration) was performed as described in Figure 5B. Asterisks mark Δloop5-Δloop5 (lane 2) and Δloop5-Δloop5, Pmt4p-Δloop5 and Pmt4p-Pmt4p (lane 4) complexes. C, Coimmunoprecipitation of HA-tagged Pmt1p mutant proteins with Pmt2p. Pmt1p (\textit{PMT1}; lane 2), Pmt1p\textsuperscript{HA} (\textit{PMT1}\textsuperscript{HA}; lane 1) and Pmt1p\textsuperscript{HA} mutants R138A (lane 3) and R138K (lane 4) were expressed in strain
Coimmunoprecipitation was performed using anti-HA beads and analyzed as described in Figure 2A. Blots were sequentially probed with polyclonal anti-Pmt1p and anti-Pmt2p antibodies. 

**D. Coimmunoprecipitation of Pmt4p-R142E-FLAG.** In wild type SEY6210 (*PMT4*; lanes 1, 2 and 4) and *pmt4Δ* (lane 3) strains Pmt4p-FLAG (*PMT4* FLAG; lane 1) and Pmt4p-R142E-FLAG (*R142E* FLAG; lanes 2 and 3) were expressed from plasmid pJK4-B1 and pVG45, respectively. Immunoprecipitation of FLAG-tagged proteins was performed as described in Figure 5A. Blots were probed with polyclonal anti-Pmt4p antibodies.

**FIG. 7.** Similar domains of Pmt1p and Pmt4p affect their functionality *in vivo*. The temperature sensitive *pmt1Δpmt4Δ* mutant strain (CFY3; 25) was transformed with *PMT1*HA (pSB56), the *PMT1* mutants Δloop5 (pVG13), *R138A*HA (pSB114) and *R138K*HA (pVG36), vector pRS423, *PMT4* FLAG (pJK4-B1) and the *PMT4* mutants *R142E*FLAG (pVG45) and Δloop5 (pVG37). Strains were grown for 4 days on YPD medium at 35°C and 30°C, respectively.

**FIG. 8.** Protein-protein interactions between the *PMT* family members in yeast. A. Members of the *PMT1* subfamily interact in pairs with members of the *PMT2* subfamily. Thereby, Pmt1p-Pmt2p and Pmt5p-Pmt3p are the predominant complexes present in wild type cells. Under certain premises (for instance when one of the principal partners is absent) Pmt1p can interact with Pmt3p, and Pmt5p with Pmt2p, respectively. B. The single member of the *PMT4* subfamily forms homomers.
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| Strain   | Genotype                                                                 | Reference/Source |
|----------|--------------------------------------------------------------------------|------------------|
| SEY6210  | MATα, his3-Δ200, leu2-3, -112, lys2-801, trp1-Δ901, ura3-52, suc2-Δ9     | 22               |
| pmt1Δ    | SEY6210 except pmt1Δ::HIS3                                               | 23               |
| pmt2Δ    | SEY6210 except pmt2Δ::LEU2                                                | 23               |
| pmt3Δ    | SEY6210 except pmt3Δ::HIS3                                               | 24               |
| pmt4Δ    | SEY6210 except pmt4Δ::TRP1                                                | 24               |
| pmt5Δ    | SEY6210 except pmt5Δ::URA3                                               | 25               |
| pmt6Δ    | SEY6210 except pmt6Δ::LEU2                                                | 25               |
| pmt1pmt2Δ | SEY6210 except pmt1Δ::HIS3, pmt2Δ::LEU2                                  | 23               |
| pmt1pmt3Δ | SEY6210 except pmt1Δ::URA3, pmt3Δ::HIS3                                  | 1                |
| CFY3     | MATα, ade2-1, his3-Δ200, leu2-3, -112, trp1-Δ901, ura3-52, suc2-Δ9, pmt1Δ::HIS3, pmt4Δ::TRP1 | 26               |
| pmt2pmt5Δ | MATα, his3-Δ200, leu2-3, -112, lys2-801, trp1-Δ901, ura3-52, suc2-Δ9, pmt2Δ::LEU2, pmt5Δ::URA3 | T. Seidl, unpublished |
**TABLE II**

*In vitro O-mannosyltransferase activity of pmt1pmt3 and pmt2pmt5 deletion mutants*

Crude membranes were isolated from yeast strains SEY6210 (WT), *pmt1Δ*, *pmt1pmt2Δ*, *pmt1pmt3Δ* and *pmt2pmt5Δ*. 250 µg of membrane proteins were incubated in the *in vitro* mannosyltransferase assay following the transfer of [14C]mannose from Dol-P-Man to the peptide Ac-YATAV-NH₂. Average values of three independent experiments are shown.

| Strain       | cpm/mg/h       | Relative activity % |
|--------------|----------------|---------------------|
| WT           | 107,448 ±1894  |                     |
| *pmt1Δ*      | 12,012 ±1025   |                     |
| *pmt1pmt2Δ*  | 13,720 ± 732   | 100.0              |
| *pmt1pmt3Δ*  | 23,080 ± 780   | 168.2              |
| *pmt2pmt5Δ*  | 19,564 ±1156   | 142.6              |
Figure 1

[Image of a gel electrophoresis diagram with lanes labeled Pmt3p, Pmt4p, Pmt5p, and Pmt6p. The lanes are labeled WT, pmt3, WT, pmt4, WT, pmt5, WT, and pmt6. The molecular weight markers are 116, 97, and 66 kDa. The bands are visible for WT and pmt3, WT and pmt4, and WT and pmt5.]

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Figure 2

A

IP with anti-HA

Pmt1p

Pmt6p

Pmt3p

Pmt5p

Pmt4p

Pmt2p

1 2 3 4

B

IP of Pmt1p

WT

pmt3

pmt2

input IP input IP input IP

Pmt1p

Pmt3p

Pmt2p

1 2 3 4 5 6

C

IP with anti-HA

Pmt2p

input IP

Pmt2p

Pmt1p

Pmt3p

Pmt4p

Pmt5p

Pmt6p

1 2

D

IP of Pmt3p

WT

pmt3

input IP input IP input

Pmt3p

Pmt1p

Pmt2p

Pmt3p

Pmt4p

Pmt5p

Pmt6p

1 2 3 4
Figure 3

A

| WT | pmt2 | pmt3 | pmt4 | pmt5 | pmt6 | pmt1 |
|----|------|------|------|------|------|------|
| 1  | 2    | 3    | 4    | 5    | 6    | 7    |

kDa

160
240
450

B

| WT | pmt2 |
|----|------|
| 1  | 2    |

kDa

160
240
2450
Figure 5

A

IP with anti-FLAG

|       | MT4/MT4 | MT4 | MT4/MT4 | MT4 | MT4/MT4 | MT4 |
|-------|---------|-----|---------|-----|---------|-----|
| input | 1       | 2   | 3       | 4   | 5       | 6   |
| IP    |         |     |         |     |         |     |

Pmt4p^{FLAG} → Pmt4p

B

pmt4 - - - 25 50 100 μM OPA

PMT4^{FLAG}

kDa 205
116
97
66

1 2 3 4 5
Figure 6

A

B

C

D

ER-lumen

loop1

loop5

loop4

loop6

Cytoplasm

PMT1\textsuperscript{HA}  PMT1  PMT1\textsuperscript{HA} R138A  PMT1\textsuperscript{HA} R138K

1  2  3  4

Pmt1p  Pmt2p

IP with anti-HA

Pmt4p\textsuperscript{FLAG}  Pmt4p

1  2  3  4

IP with anti-FLAG

\Delta loop5

\Delta loop5

\Delta loop5

\Delta loop5

pmt4  WT

OPA

116

97

66

kDa
Figure 7

35°C

30°C

PMT1\(^{HA}\) R138A

PMT1\(^{HA}\) Δloop5

PMT4\(^{FLAG}\) Δloop5

PMT4\(^{FLAG}\) R142E

PMT1\(^{HA}\)

PMT1\(^{HA}\)

PMT1\(^{HA}\)

pRS423

PMT4\(^{FLAG}\)
Members of the evolutionarily conserved PMT family of protein O-mannosyltransferases form distinct protein complexes among themselves
Verena Girrbach and Sabine Strahl

J. Biol. Chem. published online January 27, 2003

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