A CONSERVED ACIDIC MOTIF IS CRUCIAL FOR ENZYMATIC ACTIVITY OF PROTEIN O-MANNOSYLTRANSFERASES*

Mark Lommel¹, Andrea Schott¹, Thomas Jank², Verena Hofmann³, Sabine Strahl

From the Centre for Organismal Studies Heidelberg, University of Heidelberg, Im Neuenheimer Feld 360, D-69120 Heidelberg, Germany

*Running title: PMT loop1 domain

¹ Authors contributed equally to this work
² Current address: Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität Freiburg, D-79104 Freiburg, Germany
³ Current address: Roche Diagnostics GmbH, Nonnenwald 2, D-82377 Penzberg, Germany

To whom correspondence should be addressed: Sabine Strahl, Centre for Organismal Studies Heidelberg, University of Heidelberg, Im Neuenheimer Feld 360, D-69120 Heidelberg, Germany, Tel.: 49-6221-546286; FAX: 49-6221-545859; E-mail: sabine.strahl@cos.uni-heidelberg.de

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Background: Dimerization is a prerequisite for protein O-mannosyltransferase activity.

Results: Transferase activity depends on an intact Asp-Glu motif located in a region crucial for acceptor binding/catalysis in both complex partners.

Conclusion: Complex formation leads to the assembly of a composite catalytic center.

Significance: Defining the role of complex formation is crucial for understanding the catalytic mechanism of protein O-mannosyltransferases.

SUMMARY

Protein O-mannosylation is an essential modification among fungi and mammals. It is initiated at the endoplasmic reticulum (ER) by a conserved family of dolichyl phosphate-mannose:protein O-mannosyltransferases (PMTs). PMTs are integral membrane proteins with two hydrophilic loops (loop1 and loop5) facing the ER lumen. Formation of dimeric PMT complexes is crucial for mannosyltransferase activity, but the direct cause is not known to date. In baker’s yeast, O-mannosylation is largely catalyzed by heterodimeric Pmt1p/Pmt2p and homodimeric Pmt4p complexes. To further characterize Pmt1p/Pmt2p complexes we developed a photoaffinity probe based on the artificial mannosyl acceptor substrate Tyr-Ala-Thr-Ala-Val. The photoreactive probe was preferentially crosslinked to Pmt1p, and deletion of the loop1 but not the loop5 region abolished this interaction. Analysis of Pmt1p loop1 mutants revealed that especially Glu-78 is crucial for binding of the photoreactive probe. Glu-78 belongs to an Asp-Glu motif that is highly conserved among PMTs. We further demonstrate that single amino acid substitutions in this motif completely abolish activity of Pmt4p complexes. In contrast, both acidic residues need to be exchanged to eliminate activity of Pmt1p/Pmt2p complexes. Based on our data, we propose that the loop1 regions of dimeric complexes form part of the catalytic site.

Protein O-mannosylation is a conserved modification among fungi, animals and some bacteria (1). O-mannosyl glycans have been implicated in a variety of physiological processes such as stability, sorting and localization of proteins, and ligand interactions. In fungi, reduced O-mannosylation affects cell polarity, morphogenesis, cell wall integrity and ER protein quality control, while loss of O-mannosylation is lethal (1-3). In higher eukaryotes, impaired O-mannosylation results in severe developmental defects and in humans, mutations that affect synthesis of O-mannosyl glycans lead to congenital muscular dystrophies with neuronal migration defects (4,5).
Protein O-mannosylation is initiated in the endoplasmic reticulum (ER) by the transfer of a mannosyl residue from dolichyl monophosphate-activated mannosyl to hydroxyl groups of serine and threonine residues of nascent polypeptide chains. This transfer reaction is catalyzed by the family of dolichyl phosphate mannosyl-dependent protein O-mannosyltransferases (PMTs). PMT family members have been characterized throughout the fungal and animal kingdoms and in some bacterial species (6-12).

PMTs have been best characterized in *Saccharomyces cerevisiae*, where the PMT family comprises at least six members (Pmt1p-Pmt6p) (1). Topological analysis of ScPmt1p revealed seven transmembrane domains (TMDs) with the N- and C-terminus situated in the cytosol and the ER lumen, respectively (13). Two prominent hydrophilic loops situated between TMD 1 and TMD 2 (loop1) and TMD 5 and TMD 6 (loop5) are facing the ER lumen. Based on highly similar hydrophathy profiles of PMT proteins this topology seems to be applicable to all PMT family members.

Phylogenetic analyses indicate that the PMT family is subdivided into PMT1, PMT2 and PMT4 subfamilies, whose members include transferases closely related to *S. cerevisiae* Pmt1p, Pmt2p and Pmt4p, respectively (5). In baker's yeast PMT1 (ScPmt1p and ScPmt5p) and PMT2 (ScPmt2p and ScPmt3p) subfamily members form heterodimeric complexes, whereas Pmt4p the sole member of the PMT4 family forms homodimeric complexes. Besides Pmt4p complexes, Pmt1p/Pmt2p complexes account for the major transferase activities in yeast, although alternative complexes can be formed (14).

Based on amino acid sequence similarities, glycosyltransferases (GT) have been classified into more than 90 GT families (15). According to their structural features, GT families have been further grouped into the GT-A and GT-B superfamilies (16). A third superfamily was predicted by iterative sequence searches (GT-C; 16). Due to their predicted architecture, GT-C members are large polytopic integral membrane proteins located in the ER or the plasma membrane (16,17). The vast majority of these enzymes utilize lipid phosphate-activated sugar donors and glycosyltransfer leads to an inversion of the stereochemistry of the glycosidic bond. PMTs that define the GT39 family represent generic GT-C superfamily members. To date, very little information on three-dimensional structures of GT-C transferases is impeding access to the molecular mechanism also of PMTs. Thus, in this study we used biochemical and genetic methods to elucidate structure-function relationships of PMTs.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids---**The following *S. cerevisiae* strains were used for this study: pmt1Δ (MATα, his3Δ200, leu2-3, -112, lys2-801, trpl-Δ901, ura3-52, suc2-Δ9, pmt1Δ::HIS3) (18), pmt2Δ (MATα, his3Δ200, leu2-3, -112, lys2-801, trpl-Δ901, ura3-52, suc2-Δ9, pmt2Δ::LEU2) (18), pmt4Δ (MATα, his3Δ200, leu2-3, -112, lys2-801, trpl-Δ901, ura3-52, suc2-Δ9, pmt4Δ::TRPI) (19), pmt1Δpmt2Δ (MATα, his3Δ200, leu2-3, -112, lys2-801, trpl-Δ901, ura3-52, suc2-Δ9, pmt1Δ::HIS3, pmt2Δ::LEU2) (18) and pmt2Δpmt3Δ (MATα, his3Δ200, leu2-3, -112, lys2-801, trpl-Δ901, ura3-52, suc2-Δ9, pmt2Δ::LEU2, pmt3Δ::HIS3) (7). Yeast strains were grown under standard conditions and transformed according to Ref. (20) with the following plasmids: YEp352 (21), pSB53 (PMT1) (13), pSB6 (PMT1HA) (22), pSB101 (PMT1ΔΔ6-124; Δloop1HA) (22), pVG13 (PMT1ΔΔ304-53; Δloop5HA) (22), pSB112 (PMT1ΔΔ-E78A) (22), pSB113 (PMT1ΔΔ-D96A) (22), pVG80 (PMT2HA) (14), pJK4-Bl (PMT4FLAG) (14) and the plasmids listed below. Standard procedures were used for all DNA manipulations (23). All cloning steps were carried out in *Escherichia coli* host SURE® (Stratagene). PCR fragments were routinely checked by sequence analysis. Oligonucleotide sequences will be made available upon request. Amino acid substitutions were constructed by site-directed mutagenesis using recombinant PCR as described in Ref. (24).

**PMT1 mutant versions.** PMT1HA point mutations were generated using the following mutagenic oligonucleotide pairs 580/581 (F76A), 582/583 (D77A), 584/585 (H80A), 586/587 (F81A), 588/589 (Y88A), 590/591 (V97A), 592/593 (H98A), 594/595 (P99A), and 596/597 (P100A) in combination with the outer primers vg1 and oligo195. The resulting PCR 1463 bp fragments were subcloned into pGEM®-T-Easy (Promega). A 1323 bp *Pml1-Kpnl* fragment was then excised and subcloned into pSB56 cut with the same enzymes resulting in the following plasmids: pAS40 (F76A), pAS41 (D77A), pAS42 (H80A), pAS43 (F81A), pAS44 (Y88A), pAS45 (V97A), pAS46 (H98A), pAS47 (P99A), and pAS48 (P100A).
To generate mutant \textit{PMT1-D77A/E78A} the mutagenic primer pair 1649/1650 was used in combination with oligo1516 and oligo1651. The resulting 1028 bp PCR fragment was cut with \textit{PshhI} and \textit{PflII} and subcloned into pSB53 cut with the same enzymes resulting in plasmid pScML5 (\textit{PMT1-D77A/E78A}).

\textbf{PMT2 mutant versions.} To generate plasmid pScML6 (\textit{PMT2\textsuperscript{174A}}) a 2905 bp \textit{PsrI-SalI fragment containing PMT2 promoter and coding regions} fused to three copies of the hemagglutinin epitope was excised from pVG80 and subcloned into vector pRS424 cut with \textit{XhoI} and \textit{XhoI}. To obtain mutant \textit{PMT2\textsuperscript{174A-D92A/E93A}} the mutagenic primer pairs 1649/1650 was used in combination with the outer primers oligo1518 and oligo1654 were used to generate a 1028 bp PCR fragment as described above. Plasmid pVG80 was used as template. The PCR product was cut with \textit{XhoI} and \textit{MluI} and subcloned into pScML6 cut with the same enzymes resulting in plasmid pScML7 (\textit{PMT2\textsuperscript{174A-D92A/E93A}}).

\textbf{PMT4 mutant versions.} For the generation of \textit{PMT4} mutants the mutagenic primer pairs 1470/1471 (\textit{D80A}), 1697/1698 (\textit{D80A}), 1699/1700 (\textit{E81D}), and 1701/1702 (\textit{D80E/81E}) in combination with the outer primers oligo1469 and oligo1694 were used to produce mutated PCR fragments as described above. For homologous recombination 100 ng of pJK4-BI (linearized with \textit{SphI} and \textit{ClaI}) and 500 ng of PCR product were co-transformed into yeast strain pmt4\textit{Δ} as described elsewhere (25).

pVG12 (\textit{hyPMT1\textsuperscript{174A}}), pSB53 was cut with \textit{SphI} and \textit{NarI}, treated with DNA polymerase I (Klenow fragment), and ligated to remove a \textit{HindIII} site from the multiple cloning site. The resulting plasmid (pSB73) was ligated via a \textit{HindIII} site with a PCR fragment encoding \textit{PMT4-loop5} that had been amplified on genomic DNA using oligo175B and oligo176. Subsequently, the created plasmid (pSB74) was digested with \textit{RsrII} and \textit{XhoI} and ligated with a 432 bp \textit{RsrII-XhoI} fragment isolated from pSB56 fusing six copies of the HA epitope to the \textit{PMT1/PMT4-loop5} hybrid.

\textbf{Computer Analyses---} Multiple sequence alignments of \textit{PMT} family members were prepared using ClustalW2 (26) and rendering was done using BoxShade (http://www.ch.embnet.org/software/BOX_form.html).

\textbf{Preparation of Crude Membranes---} Yeast cells from exponentially growing cultures were harvested and cell fractionation was performed as described previously (22).

\textbf{Preparation of Cts1p---} Yeast strains were grown to stationary phase (OD\textsubscript{600} = 5) and culture medium was collected by centrifugation at 3,000 g, 4°C for 5 min. Ice-cold trichloroacetic acid was added to a final concentration of 14% (v/v) to the medium and proteins were allowed to precipitate for 30 min at 4°C. The protein precipitate was recovered by centrifugation at 20,000 g, 4°C for 30 min. Protein pellets were washed with 500 µl of ice-cold acetone and air-dried. Protein pellets corresponding to 2 OD\textsubscript{600} of cells were resuspended in 10 µl of 10x SDS sample buffer and analyzed by SDS-PAGE and Western blot.

\textbf{Immunoprecipitation---} Protein extracts were prepared as described (22) substituting the DOC buffer by Triton buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3 mM MgCl\textsubscript{2}, 10% glycerol, 0.5% Triton X-100, 1 mM PMSF, 1 mM benzamidine, 0.25 mM TLCK, 50 mg/ml TPCK, 10 mg/ml antipain, 1 mg/ml leupeptin, and 1 mg/ml pepstatin). Immunoprecipitation of HA-tagged proteins was performed using 400 µl of Triton-extracts and 15 µl of anti-HA affinity matrix (clone 3F10, Roche) for 1-2 hours at 4°C. Immunoprecipitates were washed four times with 1 ml of pre-chilled Triton buffer and once with 1 ml of Triton-buffered saline.

\textbf{Photoaffinity labeling---} The photoreactive peptide substrate *Tyr-Ala-Thr-Ala-Val-Lys-Btn (*YATAVK-Btn) was generated by incubating 2 µl of 50 mM biotinylated peptide (NH\textsubscript{2}-YATAVK(Btn)-COOH; Thermo Hybaid) with 1 µl of 200 mM of N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS; Pierce) for 1 hour in the dark. Immunoprecipitates of Pmt1p\textsuperscript{HA}, Pmt1p\textsuperscript{HA} mutants and Pmt2p\textsuperscript{HA} were prepared as described above and suspended in 27 µl of buffer (120 mM Tris-HCl, pH 7.5, 7.5 mM MgCl\textsubscript{2}, 0.15% Triton X-100) including 0.3 mM HA-peptide (Sigma) to release HA-tagged PMTs from the affinity matrix, and the photoreactive probe. Peptides AcNH-Tyr-Ala-Thr-Ala-Val-Lys-Btn were added as required (final concentrations between 1.7 mM and 7.5 mM). To induce crosslinking the reaction mixture was exposed for 10 min to UV-light (312 nm). 5 µl of 5x SDS sample buffer were added, proteins separated by SDS-PAGE and analyzed by Western blot.

\textbf{Western Blot Analyses---} Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Polyclonal anti-Pmt1p (27) and...
anti-Gas1p (kindly provided by L. Popolo). Antibodies were used at 1:2,000 dilution. Polyclonal anti-Pmt2p (28) and anti-Cts1p (7) antibodies were diluted 1:1,000. Monoclonal anti-HA (16B12; Convance) and anti-FLAG (M2, Sigma) antibodies were used at a 1:8,000 dilution. Peroxidase-coupled goat anti-biotin antibody (Sigma), peroxidase-coupled rabbit anti-mouse-IgG antibody (Sigma) and peroxidase-coupled goat anti-rabbit-IgG antibody (Sigma) were used at a dilution of 1:5,000. Protein-antibody complexes were visualized by enhanced chemiluminescence using the SuperSignal West Pico Chemiluminescent System (Pierce).

In Vitro Dol-P-Man:Protein O-Mannosyltransferase Assay---Activity of Pmt1p/Pmt2p was analyzed using the donor substrate Dol-P-[1^4]C]mannose or Dol-P-[3H]mannose (American Radiolabeled Chemicals), 15–50 µg of membrane protein, and 3.5 mM acceptor substrate AcNH-YATAV-CONH$_2$ as described in Ref. (27).

Alternatively, Pmt1p/Pmt2p activity was analyzed using 100 nM Dol-P-[3H]mannose, 5-10 µg of membrane protein, 120 µM acceptor substrate Btn-NH-YATAV-CONH$_2$ (Thermo-Fischer), 20 mM Tris-HCl, pH 8, 10 mM EDTA, 0.5% 6-octylthioglucosid (AppliChem), 2 mM β-mercaptoethanol, and 1 mg/ml phosphatidylcholine from egg yolk (Sigma) in a total volume of 20 µl. After incubation at 20°C for 10 min, the reaction was stopped by adding 250 µl phosphate-buffered saline containing 1% Triton X-100 and incubating the reaction mixture at 95°C for 3 min. The acceptor substrate was precipitated using 40 µl slurry of avidin-agarose (Pierce) for 1 hour at 4°C. Precipitates were washed at least five times with 1 ml of PBS containing 1% Triton X-100 and suspended in 200 µl of PBS. Radioactivity adsorbed to the beads was measured using a liquid scintillation counter (Beckman Coulter).

In vitro activity of Pmt4p was based on the amount of mannose transferred from Dol-P-[3H]mannose to an α-dystroglycan glutathione S-transferase fusion protein (α-DG-GST) (29,30) according to Jank et al. (in preparation).

RESULTS

Photoaffinity labeling of Pmt1p and Pmt2p with an in vitro mannose acceptor substrate---In order to identify regions in Pmt1p/Pmt2p that are involved in binding of mannose acceptors and/or catalysis we developed a peptide-based photoaffinity probe. We used the biotinylated peptide NH$_2$-YATAV-(Biotin)-COOH (YATAV-Btn) that serves as in vitro mannosyl acceptor substrate of Pmt1p/Pmt2p and is O-mannosylated to a similar extent as the known acceptor peptide AcNH-YATAV-CONH$_2$ (YATAV; Table 1). YATAV-Btn was conjugated via its primary amino group with the heterobifunctional NHS-ester and photoactivatable crosslinker N-5-Azido-2-nitrobenzoyloxysuccinimide (spacer arm length 7.7 Å) as detailed in “Experimental Procedures”. The photoreactive peptide probe *YATAV-Btn (Fig. 1A) was incubated with Pmt1p$^{HA}$/Pmt2p complexes that were purified by co-immunoprecipitation (co-IP). We have previously shown that incorporation of six HA epitopes at the C-terminus of Pmt1p (Pmt1p$^{HA}$; apparent MW 98 kDa) followed by cell rupture, membrane isolation, solubilization and then IP under native conditions using a monoclonal anti-HA antibody, results in precipitation of Pmt1p$^{HA}$/Pmt2p complexes (14) that are enzymatically active (data not shown). After photoactivation at 312 nm, samples were resolved on SDS-polyacrylamide (PA) gels and visualized by enhanced chemiluminescence using the SuperSignal West Pico Chemiluminescent System (Pierce). As shown in Figure 1, a predominant crosslinking product could be detected which represents Pmt1p$^{HA}$ (Fig. 1B, lanes 3 and 4). In control reactions where photoactivation was omitted (Fig. 1B, lanes 2 and 5) or where heterobifunctional crosslinkers with different spacer arm length were used (up to 19.9 Å; data not shown), no crosslinking products were obtained. To further control the specificity of the labeling, crosslinking reactions were performed in the presence of varying effective in vitro mannosyl acceptor peptides which should compete with *YATAV-Btn for Pmt1p$^{HA}$ binding (Table 1). As expected, increasing amounts of the peptide YATAV were efficiently hampering *YATAV-Btn labeling (Fig. 1C). Compared to YATAV, this effect was less pronounced when the poorer mannosyl acceptor peptide YASAV was used (Fig. 1D, right panel; Table 1). Accordingly, the pentapeptide SSSSS which is not mannosylated by Pmt1p/Pmt2p did...
not interfere with *YATAVK-Btn binding (Fig. 1D, left panel; Table 1) further proving the specificity of the photoaffinity labeling.

Our data show that *YATAVK-Btn is preferentially crosslinked to Pmt1p. Only when exposure times of the blots were significantly increased, minor signals became evident suggesting labeling of Pmt2p (Figs 1B and D, triangle). To further follow that issue, we analyzed Pmt1p HA in the absence of known complex partners. Pmt1p interacts with Pmt2p and, mainly when Pmt2p is absent, also with Pmt3p (14). Thus, we expressed Pmt1p HA in the absence of Pmt2p (pmt2Δ) as well as Pmt2p and Pmt3p (pmt2Δ pmt3Δ), and performed photoaffinity labeling as described above. As shown in Figure 2A, Pmt1p HA still interacts with the peptide substrate. Further, we immunoprecipitated Pmt2p HA (apparent MW ~88 kDa) that was expressed in a pmt2Δ and a pmt1Δ pmt2Δ mutant background. As shown previously, IP under native conditions using a monoclonal anti-HA antibody, results in precipitation of Pmt1p/Pmt2p HA complexes (Fig. 2B, lanes 4 and 6; (14)). Photoaffinity labeling of Pmt1p/Pmt2p HA revealed that the photoreactive probe predominantly reacts with Pmt1p (Fig. 2B, lanes 2, 4 and 6). Only when Pmt1p is absent, Pmt2p HA is significantly labeled (Fig. 2B, lane 1).

In summary, our data demonstrate that both, Pmt1p and Pmt2p have the capacity to bind the peptide substrate. However, under the experimental conditions used, crosslinking of the *YATAVK-Btn probe to Pmt1p is favored.

Pmt1p loop1, especially Glu-78 is crucial for crosslinking of the photoreactive peptide---To further narrow down regions involved in peptide binding, we analyzed Pmt1p HA mutants where the major ER-oriented loop regions, loop1 (aa 71-135) and loop5 (aa 294-586) have been deleted (Fig. 3A). Following the transfer of mannose from Dol-P-Man to the pentapeptide YATAV we previously demonstrated that compared to Pmt1p HA in vitro activity of mutant Δloop1 HA (A76-124) and Δloop5 HA (Δ304-531) is diminished to 0.7% and 7%, respectively. In vivo both mutants resemble a pmt1Δ phenotype (22). Although mutant Δloop1 HA was stably expressed and efficiently immunoprecipitated, crosslinking to the *YATAVK-Btn probe was lost (Fig. 3B, lane 2; and supplemental Fig. 1A). In contrast, deletion of loop5 did not affect binding of the photoreactive probe (Fig. 3B, lane 3). The same was true for a Pmt1p Δ loop1 HA hybrid protein (~15% residual in vitro activity; data not shown) where the Pmt1p loop5 has been replaced by the Pmt4p loop5 region (Fig. 3B, lane 4).

Our data suggest that the loop1 region contains specific amino acids that are involved in peptide substrate binding and/or catalysis. The loop1 domain is highly conserved amongst eukaryotic as well as prokaryotic PMTs. By protein sequence alignments with ClustalW we identified peptide motifs that are conserved in loop1 of all PMTs analyzed (Fig. 4A; and supplemental Fig. 2). To test the role of the conserved amino acids we used site-directed mutagenesis and replaced them individually with alanine. The mutant Pmt1p HA proteins were expressed and characterized in a pmt1Δ background. Mutations did not alter protein stability and only mutants Y88A and P100A moderately decreased complex formation with Pmt2p (supplemental Fig. 3). Surprisingly only mutation E78A resulted in reduction of in vitro mannosyltransferase activity by more than 50% when compared to Pmt1p HA (Table 2). Accordingly, photoaffinity labeling of this mutant protein was decreased (Fig. 4B).

In summary, our data show that Glu-78 is important for acceptor binding and/or catalysis.

The loop1 Asp-Glu motif is essential for activity of Pmt1p/Pmt2p complexes---In agreement with our findings a recent study suggested that the loop1 Asp-Glu (DE) motif of the *Mycobacterium tuberculosis* PMT homologue is crucial for mannosyltransferase activity (11). But, in contrast to the Pmt1p mutations D77A (~72% residual activity; Table 2) and E78A (~47% residual activity; Table 2), the corresponding single mutations in the bacterial PMT homologue resulted in complete loss of enzymatic activity. We decided to go further into these differences, and generated a Pmt1p double mutant where both Asp-77 and Glu-78 were replaced by alanine (Pmt1pDE→AA). Pmt1pDE→AA was first characterized in a pmt1Δ background. Change of the DE motif did not alter stability of the mutant protein and its interaction with Pmt2p (supplemental Fig. 3B). To access in vivo activity, we analyzed the glycosylation status of the specific Pmt1p/Pmt2p substrate chitinase (Cts1p; (31)) that is highly O-mannosylated. As shown in Figure 5 Cts1p isolated from strain pmt1Δ is less glycosylated as compared with pmt1Δ expressing Pmt1p HA (lanes 1 and 2). No significant differences in the glycosylation of
Cts1p could be detected between strains expressing Pmt1HA or mutants D77A and E78A (Fig. 5, lanes 3 and 4), whereas Pmt1pDE→AA failed to restore Cts1p to its normal glycosylation levels (Fig. 5, lane 5). Further, when compared to the single mutants, the double mutant Pmt1pDE→AA showed basically no in vitro mannosyltransferase activity (Table 2). We further created a Pmt2pHA→AA mutant protein with amino acids Asp-92 and Glu-93 replaced by alanine (Fig. 4A) and measured in vitro mannosyltransfer activity of wild-type and mutant proteins in a pmt1Δpmt2Δ mutant background. Both the Pmt1pDE→AA/Pmt2pHA complex, as well as the Pmt1p/Pmt2pHA→AA complex, were inactive (Table 3). The residual in vitro activity detected (~3% when compared to Pmt1p/Pmt2HA) is most likely due to alternative Pmt1p/Pmt3p or Pmt2pHA/Pmt5p complexes (14). When mutant proteins Pmt1pHA→DE and Pmt2pHA→DE→AA were co-expressed mannosyltransferase activity was completely abolished (Table 3).

Taken together, our results demonstrate that the DE motif is important for enzymatic activity of yeast Pmt1p/Pmt2p complexes. But why is there a discrepancy between eukaryotic Pmt1p/Pmt2p and the bacterial PMT homologue where mutation of one individual residue of the DE motif already abolishes mannosyltransferase reaction?

Characterization of the loop1 DE motif of homodimeric Pmt4p complexes—For various glycosyltransferases it has been suggested that acidic amino acid residues contribute to the substrate binding/catalytic domain (17). Thus, two individual loop1 regions could contribute to such a catalytic site (Fig. 4A). This is one possibility to explain why complex formation is obligatory for PMT activity (1,30), although complex formation of bacterial PMTs has not been analyzed yet. It might also explain why mutation of a single amino acid of the loop1 DE motif is less deleterious for heteromeric complexes. To track that possibility, we characterized the loop1 DE motif of Pmt4p which forms homomeric complexes (14).

For that purpose, we generated the Pmt4p mutant proteins D80A (DE→AE), D80E (DE→EE), E81A (DE→DA), E81D (DE→DD); and D80E/E81D (DE→ED), and characterized them in a pmt4Δ background. Individual changes in the DE motif did not alter stability of the mutant proteins (supplemental Fig. 3). The glycosylation status of the β-1,3-glucansyltransferase Gas1 (Gas1p; (31)) that is O-mannosylated specifically by Pmt4p was analyzed to access in vivo activity of the mutant proteins. As shown in Figure 5B Gas1p isolated from strain pmt4A is hypoglycosylated as compared with pmt4A expressing Pmt4p (compare lanes 1, 8 and 2, 9). Both mutant proteins, Pmt4pDE→AE and Pmt4pDE→DA failed to restore Gas1p glycosylation (Fig. 5B, lanes 3 and 4). In contrast, Gas1p glycosylation was partially re-established when the acidic character of the DE motif (DD or EE) was maintained (Fig. 5B, lanes 5 and 6). No significant differences in the glycosylation of Gas1p could be detected between the strain expressing Pmt4pDE→ED and wild-type Pmt4p (Fig. 5B, lane 7).

To further quantify Pmt4p activity in more detail, we established an assay to monitor Pmt4p-specific mannosyltransferase activity in vitro. As shown in Table 4, exchange of each, Asp-80 or Glu-81 with alanine resulted in a drop of Pmt4p activity by more than 96%. Is the negative character of the exchanged amino acid conserved (DE→DD and DE→EE), enzymatic activity is less severely affected (~50% compared to wild-type Pmt4p activity), whereas turning round the DE motif (DE→ED) does not change activity (Table 4).

In agreement with our assumption, our data demonstrate that a non-conservative exchange of one amino acid of the DE motif causes a dramatic drop of Pmt4p activity. In addition, our results show that not only the charge but also the nature of the acidic amino acids of the DE motif are important for mannosyltransferase activity.

**DISCUSSION**

Among the well over 90 glycosyltransferases families annotated in the CAZY database two general folds GT-A and GT-B have been observed among all structures of nucleotide-sugar-dependent glycosyltransferases (17). Additionally a third fold of glycosyltransferases termed GT-C was recently predicted. But, the GT-C fold is merely speculative and only limited structural information is available. The predicted architecture of the GT-C fold is that of a large hydrophobic integral membrane protein having between 7 and 13 transmembrane helices (16). Protein O-mannosyltransferases (GT39) have been suggested to adopt a GT-C fold. PMTs are polytopic transmembrane proteins bearing seven transmembrane domains. Two major hydrophilic regions loop1 and loop5 reside on the luminal
side of the ER membrane (Fig. 3A; (13)). Deletion of either loop region of \textit{S. cerevisiae} Pmt1p results in highly reduced transferase activity (22) therefore the catalytic site could not be clearly assigned to one of the loop regions. Here we used a photoreactive peptide substrate (*YATAVK-Btn; Fig. 1A) to further address this issue. Photocross-linking was observed for wild-type Pmt1p and the Δloop5 mutant protein, but strongly reduced for a Pmt1p loop1 deletion mutant (Fig. 3B). Our data indicate that the loop1 region constitutes or is part of the acceptor binding and/or catalytic site.

A highly conserved DE motif is present in the loop1 domains of Pmt proteins ranging from bacteria up to humans. It is also found in Pmt homologous sequences of archaea that we identified by protein BLAST analyses using Pmt1p from \textit{S. cerevisiae} (Fig. 4A and supplemental Fig. 2). Here we demonstrate that the DE motif is crucial for Pmt activity. Interestingly, single mutations in the DE motif of \textit{S. cerevisiae} Pmt1p did only result in a moderate inhibition of enzymatic activity (Table 2; (22)) whereas in bacteria single mutations of the conserved acidic residues abolished transferase activity (11). This discrepancy might be explained by complex formation of O-mannosyltransferases that is obligatory for enzymatic activity in eukaryotes (22,30) although Pmt complexes have not been studied in bacteria. To date it is unclear in which way the physical interaction of two Pmt molecules contributes to their function. Two possible scenarios are conceivable: i) each monomer possesses an intrinsic transferase activity but interaction with the complex partner synergistically stimulates mannosyl transfer. ii) Only upon complex formation a composite catalytic center is formed while the monomers are not able to catalyze sugar transfer.

Mutation of the entire DE motif (DE \(\rightarrow\) AA) in either \textit{S. cerevisiae} Pmt1p or Pmt2p resulted in repression of \textit{in vitro} transferase activity by \(>95\%\). The extremely slight residual activity (\(\sim 4\%\); Table 3) is most likely due to less abundant Pmt1p/Pmt3p and Pmt2p/Pmt5p complexes (14). These findings support the latter assumption. Furthermore, participation of both loop1 regions within the complex leads to the presence of four acidic amino acids derived from the two DE motifs. Removal of one of this negatively charged amino acid affects enzymatic activity. Deletion of two negative charges in either Pmt1p or Pmt2p results in complete loss of complex function. For homodimeric Pmt4p complexes, the same effect is observed when one charge is eliminated in the DE motif of the loop1 region (Table 4; D80A or E81A). These data suggest that all four acidic amino acids are needed to grant full enzymatic activity. Conservative exchange of either Asp-80 or Glu-81 in Pmt4p to some extent diminishes mannosyl transfer while upon inversion of the motif (DE \(\rightarrow\) ED) Pmt4p activity is retained (Table 4).

In summary our findings highly support that in Pmt complexes two loop1 regions contribute to the formation of the catalytic site. The catalytic site of GT-C superfamily members was suggested to reside in a luminal oriented loop region of the enzyme containing conserved variations of a DxD motif corresponding to ExD, DxE, DDx, or DEx residues (16,32). This motif is located close to the C-terminus of the first TMD and is often followed by a small patch of hydrophobic amino acids (16). Mutational analyses of the acidic amino acids revealed their importance for the function of GT-C family members (33,34). Very recently, the 3D structure of a GT-C family member has been solved that sheds light on the role of acidic motifs. \textit{Campylobacter lari} PglB is a homolog of the STT3 subunit of the eukaryotic oligosaccharyltransferase involved in the N-glycosylation of Asn-X-Ser/Thr sequons (35). The structure revealed that PglB features two major domains, an N-terminal transmembrane region and a C-terminal periplasmic region. The transmembrane region consists of thirteen TMDs connected by short loops, with the exception of two long external loops situated between TMD 1 and 2 and TMD 9 and 10 (35). The transmembrane segment constitutes the substrate binding and catalytic sites. The catalytic pocket features four acidic amino acids including Asp-154, Asp-156 that derive from a conserved DxD motif and Asp-56 and Glu-319 that are also highly conserved in STT3 homologues. With regard to the enzyme function, Asp-56, Asp-154, and Glu-319 seem to coordinate a divalent cation that on the one hand properly positions Asp-56, Asp-154, and Glu-319 to interact with the substrate Asn and on the other hand electrochemically stabilizes the lipid-phosphate leaving group. Mutational analyses of Asp-56 and Glu-319 led to more than \(90\%\) reduction of enzymatic activity, while exchange of Asp-154 resulted in more than \(50\%\) inhibition, confirming their crucial function for sugar transfer (35). STT3 has been suggested to be distantly related to PMTs (A. Bateman, http://pfam.sanger.ac.uk/family/PF02366) suggesting similarities in either
architecture of the enzyme or the catalytic mechanism might be present. Like PglB/STT3 Pmts are inverting GTs changing the stereochemistry at the anomeric center of the carbohydrate (35, 36). At least three acidic amino acids are often involved in the reaction mechanism of sugar transferring enzymes. Inverting GTs employ a direct S₆₂-like reaction mechanism. Thereby, an active side chain serves as a base catalyst that deprotonates the nucleophile of an acceptor facilitating direct SN₂-like displacement of the leaving group (37). Asp and Glu often function as such a general base in GTs. However, the moderate decrease in enzymatic activity of Pmt1p mutants D77A and E78A does not support a role as direct catalyst for one of the residues of the DE motif. It has to be noted that within the loop5 region of eukaryotic PMTs at least three acidic amino acids are present that are conserved, too (22). But, alanine substitutions of these residues did not severely affect Pmt1p/Pmt2p activity in S. cerevisiae (data not shown). Since bacterial PMTs, that highly likely employ a similar reaction mechanism, feature a truncated loop5 lacking these acidic residues, their involvement in the transfer reaction is unlikely. Acidic amino acids can also be involved in facilitating the departure of the phosphate leaving group by the coordination of divalent cations (Mn²⁺ or Mg²⁺) that electrostatically stabilize the developing negative charge (17,35). Previous work has shown that protein O-mannosyltransferase activity is significantly stimulated by the presence of divalent Mg²⁺ cations (30,38,39). But, chelating agents such as EDTA do not inhibit in vitro mannosyltransferase activity suggesting that either PMTs have a stronger affinity for divalent cations than many other glycosyltransferases, or that assay conditions used hamper the accessibility of the chelator (30,38,39). At the moment it cannot be ruled out that the DE motif is involved in the coordination of Mg²⁺ ions within PMT complexes, but this issue needs to be further investigated.

The exact function of the DE motif cannot be clearly defined yet. Hence structural analysis on PMT complexes will be necessary in the future to elucidate the molecular mechanisms underlying the mannosyl transfer reaction.

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FOOTNOTES

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The abbreviations used are: ANB-NOS, N-5-azido-2-nitrobenzoyloxyxsuccinimide; Btn, biotin; cpm, counts per minute; ER, endoplasmic reticulum; GT, glycosyltransferase; IP, immunoprecipitation; PMT, protein O-mannosyltransferase; TMD, transmembrane domain.

FIGURE LEGENDS

Figure 1: Crosslinking of Pmt1p with a photoreactive mannosyl acceptor peptide.
A, Structure of the photoreactive probe *YATAVK-Btn. B-D, Pmt1pHA/Pmt2p complexes isolated from the yeast strains pmt1Δ/pSB56 (Pmt1pHA) and pmt1Δ/pSB53 (Pmt1p) were labeled using the photoprobe *YATAVK-Btn as detailed in "Experimental Procedures". Photoactivation of the affinity-probe was induced by UV light at 312 nm. Samples were resolved on 8% SDS-PAGE gels. Western blots were sequentially probed with anti-biotin and anti-HA antibodies. Triangles highlight labeling products with the apparent molecular mass of Pmt2p. B, *YATAVK-Btn predominantly reacted with Pmt1pHA (lanes 3 and 4). In control reactions without Pmt1p/Pmt2p (lane 1) and where photoactivation was omitted (lanes 2 and 5) no labeling was observed. A sample identical to that shown in lanes 3 and 4 was analyzed using polyclonal anti-Pmt1p (not shown) and anti-Pmt2p antibodies (lane 6). C, Crosslinking reactions were performed in the absence (lane 1) or presence of 1- and 4.5-fold molar excess (lanes 2 and 3) of the mannosyl acceptor peptide YATAV. D, Crosslinking...
reactions were performed in the absence (lanes 1 and 4) or presence of 4.5-fold molar excess of YATAV (lanes 2 and 5), YASAV (lane 6) and SSSSS (lane 3).

Figure 2: *YATAVK-Btn preferentially reacts with Pmt1p.
A, Pmt1pHA interacts with the photoreactive probe in the absence of Pmt2p. Pmt1pHA was isolated in the presence of Pmt2p and Pmt3p (lane 1; strain pmt1Δ/pSB56), in the absence of Pmt2p (lane 2; strain pmt1Δpmt2Δ/pSB56), as well as in the absence of both, Pmt2p and Pmt3p (lane 3; strain pmt2Δpmt3Δ/pSB56). Crosslinking reactions and analyses of the samples were performed as described in Figure 1. B, *YATAVK-Btn preferentially reacts with Pmt1p. Pmt2pHA was immunoprecipitated from strains pmt1Δpmt2Δ/pVG80 (lanes 1, 3 and 5; pmt1Δ) and pmt2Δ/pVG80 (lanes 2, 4 and 6; Pmt1p) and labeled with the photoprobe as detailed in “Experimental Procedures”. Samples were resolved on 8% SDS-PAGE gels and Western blots sequentially probed with anti-biotin (lanes 1 and 2) and anti-Pmt2p (lanes 3 and 4) antibodies. Blots were stripped and re-probed with anti-Pmt1p antibodies (lanes 5 and 6).

Figure 3: Pmt1p loop1 is crucial for crosslinking of the photoreactive peptide.
A, Schematic representation of the ER membrane topology of yeast PMTs. The conserved DE motif is indicated. B, Wild-type and mutant versions of Pmt1pHA were isolated from strain pmt1Δ transformed with pSB56 (lane 1; Pmt1pHA), pSB101 (lane 2; Δloop1HA), pVG13 (lane 3; Δloop5HA), and pVG12 (lane 4; hyPmt1pHA). After photoaffinity labeling samples were resolved on 8% SDS-PAGE gels. Western blots were sequentially probed with anti-biotin and anti-HA antibodies.

Figure 4: Photocrosslinking of Pmt1p loop1 point mutants.
A, Alignment of *S. cerevisiae* Pmt1p loop1 with other PMT family members. Protein sequences are from *S. cerevisiae* (Sc), *Aspergillus nidulans* (An) and human (Hs). Loop1 of ScPmt1p from position Arg-64 to Ala-79 is aligned with members of the three PMT subfamilies. Residues showing at least 80% identity (black) or similarity (gray) are shaded. B, Immunoprecipitations using anti-HA antibody were performed on extracts isolated from the yeast strain pmt1Δ expressing Pmt1pHA and individual point mutants thereof. Photoaffinity labeling and analysis of the samples was performed as in Figure 1.

Figure 5: In vivo mannosyltransferase activity of DE motif mutants.
A, Chitinase was precipitated from culture medium as described in “Experimental Procedures”. PMT1HA and the indicated point mutants were individually expressed in strain pmt1Δ. B, Gas1p was analyzed in microsomal membrane preparations. PMT4FLAG and the indicated point mutants were individually expressed in strain pmt4Δ. Proteins were resolved on 6% SDS-PAGE gels and analyzed by Western blot using anti-Cts1p (A) or anti-Gas1p (B) antibodies.
### TABLES

**Table 1: YATAVK-Btn is a Pmt1p/Pmt2p in vitro mannosyl acceptor substrate.**

25 µg of membrane proteins from strain pmt1Δ expressing Pmt1pHA were incubated in the *in vitro* mannosyltransferase assay following the transfer of [14C]mannose from Dol-P-[14C]Man to the indicated peptides (final concentration 1.8 mM). Values are corrected against the activity detected in a pmt1Δ strain, which is less than 1% of the activity detected when pSB56 (PMT1HA) is expressed. Results of a representative experiment are shown.

| Peptide     | cpm/µg/min |
|-------------|------------|
| YATAV       | 1.76       |
| YATAVK-Btn  | 1.40       |
| YASAV       | 0.34       |
| SSSSS       | 0.08       |

**Table 2: In vitro mannosyltransferase activity of Pmt1p loop1 mutant proteins.**

20 µg of membrane proteins from strain pmt1Δ expressing wild-type and individual Pmt1p mutant proteins were incubated in the *in vitro* mannosyltransferase assay following the transfer of [3H]mannose from Dol-P-[3H]Man to the mannosyl acceptor peptide Btn-NH-YATAV-CONH2. Values are corrected against the activity detected in a pmt1Δ strain. Average values of two biological replicates with at least four technical replicates each are shown.

| Mutant       | Relative activity % |
|--------------|---------------------|
| Pmt1pHA      | 100                 |
| F76A         | 78.79 ± 4.36        |
| D77A         | 71.95 ± 5.39        |
| E78A         | 46.68 ± 11.97       |
| H80A         | 83.03 ± 13.27       |
| F81A         | 71.71 ± 2.82        |
| Y88A         | 73.79 ± 5.06        |
| D96A         | 63.21 ± 4.97        |
| V97A         | 80.23 ± 8.97        |
| H98A         | 62.77 ± 8.52        |
| P99A         | 61.77 ± 9.72        |
| P100A        | 59.67 ± 8.97        |
| D77A/E78A    | 0.19 ± 1.53         |

1) Mutant described in Ref. 23.
Table 3: *In vitro* mannosyltransferase activity of loop1 DE motif mutants.
25 µg of membrane proteins from strain pmt1Δpmt2Δ expressing wild-type and individual Pmt1p and Pmt2p mutant proteins were incubated in the *in vitro* mannosyltransferase assay following the transfer of [3H]mannose from Dol-P-[3H]Man to the mannosyl acceptor peptide NH-YATAV-CONH₂. Values are corrected against the activity detected in a pmt1Δpmt2Δ strain. Average values of two biological replicates with three technical replicates each are shown.

|                         | Relative activity % |
|-------------------------|---------------------|
| Pmt1p, Pmt2p            | 100                 |
| Pmt1p, pmt2Δ            | 3.02 ± 2.28         |
| pm1Δ, Pmt2p             | 5.93 ± 3.64         |
| Pmt1p, Pmt2p-D92A/E93A  | 3.63 ± 1.69         |
| Pmt1p-D77A/E78A, Pmt2p  | 3.59 ± 0.48         |
| Pmt1p-D77A/E78A, Pmt2p-D92A/E93A | 0.24 ± 0.97 |

Table 4: *In vitro* mannosyltransferase activity of Pmt4p loop1 mutant proteins.
25 µg of membrane proteins from strain pmt4Δ expressing wild-type and individual Pmt4p and mutant proteins were incubated in the *in vitro* mannosyltransferase assay following the transfer of [3H]mannose from Dol-P-[3H]Man to α-DG-GST. Average values of two biological replicates with three technical replicates each are shown.

|                         | Relative activity % |
|-------------------------|---------------------|
| Pmt4p^{FLAG}            | 100                 |
| Pmt4p-D80A              | 3.21 ± 0.94         |
| Pmt4-E81A               | 1.43 ± 0.52         |
| Pmt4p-D80E              | 52.98 ± 3.83        |
| Pmt4p-E81D              | 46.59 ± 3.75        |
| Pmt4p-D80E/E81D         | 116.54 ± 12.20      |
Figure 1

A

B

C

D
Figure 2

A

B
Figure 4

A

| ScPmt1p   | 64 ALHGASARLSDLTVSDTSAPFSCLTSVRGRLVULDHSIPRGNLVMPYQPQGMDGQVHSSVGSNGKVRGKLKKELNNL-DSPSSTTPKAVPGDS|
| AnPmt1    | 71 RIGKNCSSVYVSDEWAPRPRENYTICKPSNRVHPLYREKLLELLQGARGFKNKDLEKPGYFVAKMLTA|
| ScPmt2p   | 79 RYKGGQNhRRVYQDEADFOCRPSYMLMMFHEYRSLPHGKMLVCLQGLMLAEEQGSDPSGRF--IYPDYLIYVQKRLPN|
| AnPmt2    | 74 RYKHLRSDPIYDEARFSDPRTSHYRKKYFRTP-VHPKEMKMLVCLQGLMLAEEQGSDPSGRF--KYPEDVNLPSIFIN|
| HsPOMT2   | 72 RPHRDEXEDPEKDEDFGQMSYYLRNNTSGFD-VHPKEMKMLIGCGLGQLLGSGDQKLF---DKYEHSHSFGHGRFO|
| ScPmt4p   | 67 RYKHLRSDPIYDEARFSDPRTSHYRKKYFRTP-VHPKEMKMLIGCGLGQLLGSGDQKLF---KYPEDVNLPSIFIN|
| AnPmt4    | 57 RPHRDEXEDPEKDEDFGQMSYYLRNNTSGFD-VHPKEMKMLIGCGLGQLLGSGDQKLF---DKYEHSHSFGHGRFO|
| HsPOMT1   | 50 RPHRDEXEDPEKDEDFGQMSYYLRNNTSGFD-VHPKEMKMLIGCGLGQLLGSGDQKLF---DKYEHSHSFGHGRFO|

| consensus | R ffl p vvIDE hfg fas Yi yf D vhPP akmlia gWL G f f g yy mR A |

B

[Image of a gel with bands labeled as anti-Btn and anti-HA]
Figure 5
A

B

Pro400 | ProMAA | DE-EE | DE-ED | DE-DD | DE-DE | ProMAA

kDa
100
75

1 2 3 4 5 6

Pro400 | ProMAA | DE-EE | DE-ED | DE-DD | DE-DE | ProMAA

kDa
116
100

1 2 3 4 5 6 7 8 9
A conserved acidic motif is crucial for enzymatic activity of protein \(O\)-mannosyltransferases

Mark Lommel, Andrea Schott, Thomas Jank, Verena Hofmann and Sabine Strahl

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