Protein *O*-glycosylation in *Saccharomyces cerevisiae*: the protein *O*-mannosyltransferases Pmt1p and Pmt2p function as heterodimer

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Abstract The protein *O*-mannosyltransferases Pmt1p and Pmt2p are catalyzing the *O*-glycosylation of serine and threonine residues in the endoplasmic reticulum of yeast. Deletion of each of these proteins by disruption of the corresponding gene leads to a dramatic decrease of mannosyltransferase activity in vitro. With an anti-Pmt1p immunoaffinity column a complex of Pmt1p and a second protein was purified; this protein turned out to be Pmt2p. Overexpression of Pmt1p or Pmt2p, respectively, does not increase mannosyltransferase activity in vitro. Overexpression of both mannosyltransferases together, however, raises in vitro activity threefold. These data indicate that Pmt1p and Pmt2p function as a complex catalyzing protein *O*-glycosylation in yeast.

Key words: Dolichyl phosphate-D-mannose; Immunoaffinity chromatography; Glycoprotein; Protein *O*-glycosylation; Mannosyltransferase; *Saccharomyces cerevisiae*

1. Introduction

Glycosylation is a complex and common modification of proteins. Glycoproteins carry their carbohydrate chains N-linked to special asparagine residues or O-linked to hydroxy amino acids [1]. In *Saccharomyces cerevisiae*, mannose transfer to specific serine and threonine residues is catalyzed in the endoplasmic reticulum and requires dolichyl phosphate-D-mannose as immediate sugar donor [2]. The enzyme Dol-P-Man:protein *O*-mannosyltransferase1 (Pmt1p) catalyzing the initial reaction of protein *O*-glycosylation has been purified to homogeneity and the corresponding gene *PMT1* has been cloned [3,4]. By sequencing chromosome I of *Saccharomyces cerevisiae*, an open reading frame (YAL023/*FUN25*) was identified, the gene product of which possessed significant similarity to Pmt1p and was also shown to be involved in protein *O*-glycosylation [5]. Pmt1p and Pmt2p consist of 817 and 758 amino acids, respectively, and share 33% identity. The predicted proteins have almost identical hydropathy profiles which indicate integral membrane proteins with a tripartite structure. The N- and C-terminal thirds are lipophilic with multiple potential transmembrane helices, whereas the central part is hydrophilic. Recently, four more *PMT* genes (*PMT3–6*) have been detected ([6,7], Dommaschk et al., unpubl.).

In this study, we report that Pmt1p and Pmt2p form a heterodimer. Using an anti-Pmt1p immunoaffinity column a complex composed of Pmt1p and Pmt2p could be isolated. It will be shown that concurrent overexpression of both proteins leads to a significant increase in mannosyltransferase activity, whereas the individual overexpression of each protein has no effect.

2. Materials and methods

2.1. Yeast strains and media

The following strains were used: SEY6210 (*Matα leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9*), SEY6210 *pmt1Δ::URA3* [4], SEY6210 *pmt2Δ::LEU2* [5], SEY6210 *pmt1Δ::URA3 pmt2Δ::LEU2* [5], SEY6210 *pmt3::HIS3* [6], SEY6210 *pmt4::TRP1* [6], SEY6210 *pmt3::HIS3 pmt4::TRP1* [6], GFUII-4B (*Mata ura3-52 leu2-3,112 his*), BYS232-31-4 (*Mata prb1-1 prc1-3 cps1-1 ade2-1 ura3-52 leu2-3,112*). Standard yeast media and yeast DNA technology methods have been used as described [8].

2.2. Mannosyltransferase assay

The peptide assay for Dol-P-Man:protein *O*-mannosyltransferase activity was performed as described by Strahl-Bolsinger and Tanner [3]. The blocked peptides Ac-SSSSS-NH_2 and Ac-YNPTSV-NH_2 were added to a concentration of 4.5 mM and 3 mM, respectively, in a total test volume of 70 μl. To measure enzyme activity in yeast extracts overproducing Pmt1p and Pmt2p, 5 μg of membrane protein and 4.5 mM of the blocked peptide Ac-YATAV-NH_2 were used.

2.3. Isolation of membranes

Cells were grown to the middle of the log phase, washed in TM buffer (50 mM Tris-HCl, pH 7.5, 50 mM $MgCl_2$, 1 mM PMSF) and broken with glass beads in the same buffer. After centrifugation at $3000 \times g$, membranes were collected by centrifugation at $48{,}000 \times g$ for 45 min and resuspended in TM buffer.

2.4. Immunoaffinity chromatography

Solubilizing of membranes, coupling of anti-Pmt1p antiserum to protein A-Sepharose and incubation of solubilized extracts with the affinity resin were carried out as described [4]. The complex was eluted with 100 mM glycine-HCl, pH 3.0, 0.05% Lubrol PX, 15% glycerol.

2.5. Overexpression of PMT1 and PMT2

The *LEU2* gene and the *GAL10* promotor were placed immediately upstream of the open reading frame of the chromosomal *PMT1* gene of GFUII-4B by homologous recombination (Strahl-Bolsinger, unpubl.). The resulting strain TF1.8 was Leu^+ and showed galactose-dependent growth [9]. Transformation of the yeast multicopy vector YEp352 containig *PMT2* (YEp352-*PMT2*) [5] into TF1.8 and GFUII-4B led to overexpression of *PMT2*. The same strains were transformed with YEp352 and used as a control. Cells were grown in selective medium containing 2% galactose or 2% glucose and membranes prepared as described.

2.6. Other procedures

Proteins were separated by SDS-PAGE in Laemmli system [10]. For immunoblots, proteins were transferred to nitrocellulose and visualized using enhanced chemoluminescence (Amersham). For production of polyclonal antibodies, a rabbit was immunized with purified protein, which was isolated after separation by SDS-PAGE.

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Abbreviations: Ac, acetyl; Dol-P, dolichyl phosphate; Man, mannose; PMSF, phenylmethylsulfonyl fluoride; Tris, Tris-hydroxymethylaminomethane; PAGE, polyacrylamide gel electrophoresis; WT, wild type.

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3. Results

3.1. Pmt1p and Pmt2p are necessary for O-mannosylation activity in vitro

With wild type membrane extracts of *S. cerevisiae* the blocked peptides Ac-SSSSS-NH_2 and Ac-YNPTSV-NH_2 function as suitable mannosyl acceptors for the reaction:

Dol-P-Man + Peptide (Ser/Thr) →
Peptide (Ser/Thr)-Man + Dol-P.

Less than 11% of the mannosyl transfer activity is found in extracts of the single knock out mutants *pmt1* or *pmt2* (Table 1) The data indicate that at least with these two peptides and under the test conditions used the deletion of Pmt2p or of Pmt1p results in the loss of most of the in vitro activity. Therefore, both proteins seem to be essential to reach wild type activity. If this is the case, the low activity in a single knock out mutant *pmt1* or *pmt2* could be due to a minor activtiy of the remaining mannosyltransferase. Indeed, Pmt1p expressed solely in *E. coli* possesses a low mannosyl transfer activity, which was less than 5% of the in vitro activity obtained with a comparable amount of Pmt1p in extracts of yeast wild type cells [4].

3.2. Isolation of a Pmt1p-Pmt2p complex

To prove that Pmt1p and Pmt2p form a complex, an anti-Pmt1p antibody was used to prepare an immunoaffinity column. After elution of the column and following SDS-PAGE, the Pmt1p with a molecular mass of 92 kDa but also a second protein with an apparent molecular mass of approximately 78 kDa could be visualized (Fig. 1). This 78 kDa protein was isolated and used to immunize a rabbit. The analysis of the rabbit serum is shown in Fig. 2. Membrane extracts of wild type and mutants were transferred to nitrocellulose and probed against the anti-78 kDa protein antibody. Only membranes from *pmt2* and *pmt1 pmt2* strains turned out to show no reaction with the antibody. Since these strains do not produce Pmt2p it is evident that the antibody is directed against Pmt2p. Therefore, it had to be concluded that Pmt2p was co-purified with Pmt1p using the anti-Pmt1p affinity column. It is important to mention that the Pmt1p antibody does not cross-react with Pmt2p. Thus, the reason for co-purifcation Pmt2p is that Pmt1p and Pmt2p are interacting in a complex.

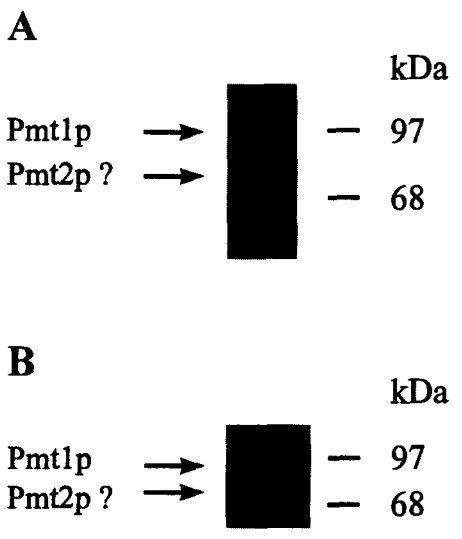

Fig. 1. Solubilized membrane extracts were prepared and purified by an anti-Pmt1p affinity column as described [4]. The eluate of the affinity column was separated by 8% SDS-PAGE for analytical purpose and visualized by silverstaining (A) or separated in preparative scale by 10% SDS-PAGE and visualized by Coomassie staining (B). Besides the 92 kDa protein Pmt1p a 78 kDa protein is visible.

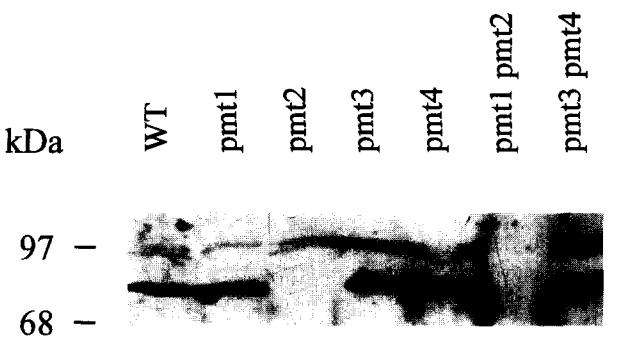

Fig. 2. Immunoblot analysis of the antibody directed against the 78 kDa protein that co-purifies with Pmt1p. Membranes were isolated from the wild type (WT, SEY6210) and various disruptants and analysed by Western blotting.

3.3. Overproduction of Pmt1p together with Pmt2p results in increased O-mannosylation activity in vitro

The *GAL10* promotor was inserted upstream of the chromosomal *PMT1* gene and, in addition, yeast cells were transformed with a multicopy vector to overexpress *PMT2* (see section 2). To verify the overexpression, yeast membrane extracts were subjected to SDS-PAGE and analysed by Western blotting using an anti-Pmt1p and an anti-Pmt2p antibody. In addition, the same membrane preparations were used to measure enzyme activity in vitro (Fig. 3). Overproduction of Pmt2p (lane 4) or Pmt1p (lane 5) alone in wild type cells does not significantly increase the in vitro activity in comparison to the wild type level (lane 1). Concurrent overexpression of both proteins (lane 6), however, results in a threefold increase in mannosyltransferase activity. These data indicate that Pmt1p and Pmt2p each are limiting for the activity of the complex. The observation that overproduction of Pmt2p in the absence of Pmt1p (lane 3) does not raise the in vitro activity when compared with the control level of Pmt2p in the absence of Pmt1p (lane 2) also agrees with the conclusion that only the heterodimer shows high activity.

4. Discussion

In this study, it has been shown that the protein *O*-mannosyltransferase Pmt2p co-purifies with Pmt1p using an anti-

Table 1
Dol-P-Man:protein *O*-D-mannosyltransferase activity in vitro

	[^{14}C]Mannose transferred (cpm) to acceptor peptide	
	Ac-SSSSS-NH_2	Ac-YNPTSV-NH_2
Wild type	3008	13974
pmt1	310	215
pmt2	295	1247
pmt1 pmt2	22	16

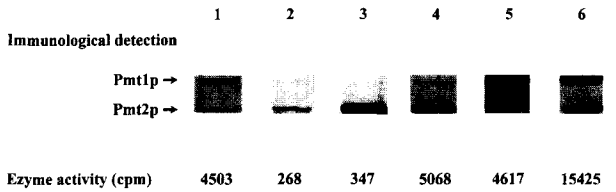

Fig. 3. Overproduction of Pmt1p and/or Pmt2p yeast membrane extracts were subjected to SDS-PAGE and analysed by Western blotting using an anti-Pmt1p and an anti-Pmt2p antibody. The corresponding mannosyltransferase activities measured in vitro are indicated below. Lane 1, wild type GFUII-4B transformed with YEp352; lane 2, TF1.8 transformed with YEp352 and grown on glucose to repress *PMT1*; lane 3, TF1.8 transformed with YEp352-*PMT2* and grown on glucose to repress *PMT1*; lane 4, GFUII-4B transformed with YEp352-*PMT2*; lane 5, TF1.8 transformed with YEp352 and grown on galactose to overexpress *PMT1*; lane 6, TF1.8 transformed with YEp352-*PMT2* and grown on galactose to overexpress *PMT1*.

Pmt1 affinity column. Moreover, it has been assessed that both mannosyltransferses are necessary for in vitro mannosyltransferase activity and that the activity is increased only by concurrent overexpression of both enzymes. These data strongly indicate that Pmt1p and Pmt2p are forming a complex to catalyze protein *O*-glycosylation. Since both proteins appear in approximately equal amounts (Fig. 1) it is likely that the proteins form a 1:1 heterodimer.

For *N*-glycosylation, it is known that the oligosaccharyltransferase catalyzing the en bloc transfer of $Glc_3Man_9GlcNAc_2$ to asparagine residues is a heterooligomeric membrane protein complex. Related oligosaccharyltransferase complexes have been isolated from yeast and mammalian cells [11–14].

Although a number of reports on glycosyltransferases catalyzing *O*-glycosylation in eukaryotic cells exist [15–17], to our knowledge this is the first one presenting evidence for a heterodimer. Whereas the initial reactions of *N*-glycosylation are highly conserved in all eukaryotes, protein *O*-glycosylation differs among organisms. In mammalian cells, protein *O*-glycosylation is generally initiated within the Golgi by the transfer of GalNAc from UDP-GalNAc [18]. In yeast, mannose is attached to the glycoproteins in the endoplasmic reticulum using dolichyl phosphate-D-mannose as immediate sugar donor [2,19,20]. To date, no homolgy between yeast protein *O*-mannosyltransferases and protein *O*-glycosyltransferases of higher eukaryotes has been found.

So far, the protein *O*-mannosyltransferase family of *S. cerevisiae* consists of 6 members. In this study, the interaction of Pmt1p and Pmt2p has been analysed. Since all protein *O*-mannosyltransferases possess almost identical hydropathy profiles pointing to integral membrane proteins, they consequently should have similar secondary structures. Thus, it is conceivable that also other Pmt proteins function as heterodimers. Future work will show whether and in what combination they interact and what functional consequences this may have.

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