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Protein glycosylation in *pmt* mutants of *Saccharomyces cerevisiae*. Influence of heterologously expressed cellobiohydrolase II of *Trichoderma reesei* and elevated levels of GDP-mannose and *cis*-prenyltransferase activity

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Abstract

Protein O-mannosylation has been postulated to be critical for production and secretion of glycoproteins in fungi. Therefore, understanding the regulation of this process and the influence of heterologous expression of glycoproteins on the activity of enzymes engaged in O-glycosylation are of considerable interest. In this study we expressed cellobiohydrolase II (CBHII) of *T. reesei*, which is normally highly O-mannosylated, in *Saccharomyces cerevisiae pmt* mutants partially blocked in O-mannosylation. We found that the lack of Pmt1 or Pmt2 protein O-mannosyltransferase activity limited the glycosylation of CBHII, but it did not affect its secretion. The *S. cerevisiae pmt1*Δ and *pmt2*Δ mutants expressing *T. reesei cbh2* gene showed a decrease of GDP-mannose level and a very high activity of *cis*-prenyltransferase compared to untransformed strains. On the other hand, elevation of *cis*-prenyltransferase activity by overexpression of the *S. cerevisiae RER2* gene in these mutants led to an increase of dolichyl phosphate mannose synthase activity, but it did not influence the activity of O-mannosyltransferases. Overexpression of the *MPG1* gene increased the level of GDP-mannose and stimulated the activity of mannosyltransferases elongating O-linked sugar chains, leading to partial restoration of CBHII glycosylation.

Keywords: O-mannosylation; GDP-mannose level; *cis*-prenyltransferase activity; *pmt* mutants

1. Introduction

The majority of *Trichoderma reesei* secretory proteins are highly glycosylated cellulases with both N- and O-linked glycans [1]. The O-glycosylation process has been suggested to be essential for protein secretion in *T. reesei* [2]. Protein secretion was shown to be influenced by choline and Tween 80 added to the culture medium. The same compounds affected the in vivo activity of dolichyl phosphate mannose synthase (DPM synthase), the key enzyme in the O-glycosylation pathway. Overexpression of the *Saccharomyces cerevisiae DPM1* gene, encoding DPM synthase, in *T. reesei* elevated the enzyme activity two-fold and resulted in an increased level of protein secretion. The secreted proteins were glycosylated to the same extent as in the control strain despite their seven-fold increased amount [3].

A correlation between glycosylation and secretion was also established in a *Hansenula polymorpha* conditional mutant partially blocked in GDP-mannose production [4]. Mutation in GDP-mannose pyrophosphorylase altered protein glycosylation and secretion. GDP-mannose is an essential sugar donor for the synthesis of both O- and N-linked oligosaccharides in fungi. Examination of glycosylation and secretion of the N-glycosylated invertase and the O-glycosylated chitinase revealed differences in the effect of both types of glycosylation on protein secretion. Invertase, a heavily N-glycosylated enzyme due to the presence of four N-glycosylation sites, was secreted in a larger amount by the mutant than by the wild type strain. The enzyme secreted by the mutant was unglycosylated and

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this state did not disturb secretion. The expression of the N-glycosylated human urinary type plasminogen activator (u-PA) in the *Hansenula* mutant and the control strain showed the same tendency. u-PA, even though unglycosylated, was secreted in a detectable amount, but only by the mutant strain. Moreover, the shortage of GDP-mannose influenced also O-glycosylation, and this defect in glycosylation decreased the secretion of a model O-glycosylated protein, chitinase. The secreted chitinase was underglycosylated because O-glycosylation was blocked only partially, and the enzyme was secreted in a very limited amount. The same situation was observed in the *Hansenula pmt1* mutant [5].

O-glycosylation was also reported to be required for stability, localization and function of some proteins in yeast. Axl2/Bud10p and Fus4p, integral plasma membrane proteins, need Pmt4 O-mannosyltransferase for their O-mannosylation. In the *pmt4*Δ mutant the Axl2/Bud10 protein remained un-O-glycosylated and was probably recognized as improperly folded and became degraded in the Golgi apparatus [6]. Un-O-glycosylated Fus1p was accumulated in the late Golgi structures suggesting that O-glycosylation functions as a sorting determinant for cell surface delivery of Fus1p [7].

Those experiments have attested to the important role of O-glycosylation in protein function and secretion in several species of fungi.

Protein O-glycosylation in yeast and other fungi is catalyzed by O-mannosyltransferases responsible for the transfer of the first mannosyl residue from dolichyl phosphate mannose (DPM) to the serine/threonine OH group of the protein. In *S. cerevisiae* these enzymes are encoded by seven *PMT* genes, and an involvement in protein O-mannosylation has been demonstrated for six of them (PMT1, 2, 3, 4, 5 and 6) [8,9]. The Pmt proteins are grouped in three subfamilies, PMT1, PMT2 and PMT4. The PMT1 and PMT2 subfamilies include two protein O-mannosyltransferases each, Pmt1 and Pmt5, and Pmt2 and Pmt3, respectively. The PMT4 subfamily has only a single Pmt4 protein. Protein O-mannosyltransferases are substrate-specific [9].

In this study we expressed *T. reesei cbh2* gene encoding cellobiohydrolase II (CBHII), a heavily O-glycosylated protein, in different *S. cerevisiae pmt* mutants. Our aim was to study the effects of CBHII protein expression on activities of enzymes engaged in O-mannosylation, especially under conditions of limited O-mannosyltransferases activity, and the possible regulation of O-glycosylation by the level of GDP-mannose and increased activity of dolichyl phosphate (Dol-P) synthesis.

We found that CBHII was glycosylated by O-mannosyltransferases from the PMT1 and PMT2 subfamilies, and that expression of CBHII protein caused changes in GDP-mannose level, and in activities of *cis*-prenyltransferase, protein O-mannosyltransferases and mannosyltransferases elongating O-linked sugar chain. Moreover, the *pmt1* and *pmt2* mutations were themselves characterized by increased intracellular concentration of GDP-mannose and higher activity of *cis*-prenyltransferase. Elevated level of GDP-mannose caused by overexpression of the *MPG1* gene mainly influenced the activity of elongation of O-linked sugar chains. At the same time, an additional increase of *cis*-prenyltransferase activity by *RER2* overexpression had no effect on the activity of O-mannosyltransferases in the *pmt* mutants, although the activity of DPM synthase was elevated.

2. Materials and methods

The *S. cerevisiae* strains used for transformation are described in Table 1. *Escherichia coli* DH5α (Bethesda Research Laboratories) was used as the plasmid host. Yeast strains were grown in SC medium [10] with the necessary supplements. For yeast transformation, the one-step transformation method of Chen et al. [11] was used.

2.1. Expression plasmid

Expression plasmid pMA91 [12] with *T. reesei cbh2* gene under the yeast *PGK* (phosphoglycerate kinase) promoter and terminator was constructed as described by Penttila et al. [13]. The resultant plasmid pMP29 with *LEU2* marker was used for transformation of yeast strains.

For expression of *T. reesei pmt* gene in *S. cerevisiae*, a cDNA fragment of 2400 bp containing the *pmt1* open reading frame was amplified by PCR and cloned into the pNEV plasmid (with *URA3* marker) between the *PMA1* (plasma membrane H^+ ATPase) promoter and terminator (gift from Prof. F. Karst of Laboratoire de Genetique de la Levure, Universite de Poitiers, France) as described by Zakrzewska et al. [14].

For expression of *S. cerevisiae MPG1* gene a PCR-amplified fragment of 2724 bp was ligated into the Yep352 vector as described by Janik et al. [15].

For expression of *S. cerevisiae RER2* gene an 860 bp DNA fragment obtained by PCR was cloned into pNEV as described for the *T. reesei pmt1* gene.

2.2. In situ assay of CBHII activity

Yeast strains were cultured for 2 days at 30 °C on SC medium supplemented with 0.1% barley β-glucan (ICN). The cells were washed off from the plates with 1 M Tris/HCl pH 7.5, and then the plates were stained with 0.1% Congo Red (Sigma) for 10 min. Extra dye was washed off with 1 M NaCl. The presence of a halo around colonies indicated hydrolysis of the substrate to fewer than seven glucose units [16].

2.3. Enzymatic activity of protein O-mannosyltransferases

S. cerevisiae strains were cultured at 30 °C in 1 l of SC-Leu or SC-Ura medium to $OD_{600}=1$; the cells were then harvested by centrifugation and resuspended into 25 ml of 150 mM Tris buffer pH 7.4 containing 15 mM $MgCl_2$

Table 1
S. cerevisiae strains used

Strain	Genotype
BY4741	(*Mata, his 3Δ1, leu2Δ0, met15Δ0, ura3Δ0*)
*pmt1*Δ	BY4741*(Mata, his 3Δ1, leu2Δ0, met15Δ0, ura3Δ0, pmt1::kanMX4)*
*pmt2*Δ	BY4741*(Mata, his 3Δ1, leu2Δ0, met15Δ0, ura3Δ0, pmt2::kanMX4)*
*pmt6*Δ	BY4741*(Mata, his 3Δ1, leu2Δ0, met15Δ0, ura3Δ0, pmt6::kanMX4)*
SEY6210	(*Matα, ura3-52, leu2-3,112, his3Δ200, trp1-Δ901, lys2-801, suc2-Δ9*)
*pmt4*Δ	SEY6210(*Matα, ura3-52, leu2-3,112, his3Δ200, trp1-Δ901, lys2-801, suc2-Δ9, pmt4::TRP1*)

SEY6210 and *pmt4* mutant are gifts from Prof. Sabine Strahl, Lehrstuhl fuer Zellbiologie und Pflanzenphysiologie, Universitaet Regensburg, Germany; BY4741 and BY-based mutants are from EUROSCARF.