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The *PMT* gene family: protein *O*-glycosylation in *Saccharomyces cerevisiae* is vital

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The transfer of mannose to seryl and threonyl residues of secretory proteins is catalyzed by a family of protein mannosyltransferases coded for by seven genes (*PMT1–7*). Mannose dolichylphosphate is the sugar donor of the reaction, which is localized at the endoplasmic reticulum. By gene disruption and crosses all single, double and triple mutants of genes *PMT1–4* were constructed. Two of the double and three of the triple mutants were not able to grow under normal conditions; three of these mutants could grow, however, when osmotically stabilized. The various mutants were extensively characterized concerning growth, morphology and their sensitivity to killer toxin K1, caffeine and calcofluor white. *O*-Mannosylation of gp115/Gas1p was affected only in *pmt4* mutants, whereas glycosylation of chitinase was mainly affected in *pmt1* and *pmt2* mutants. The results show that protein *O*-glycosylation is essential for cell wall rigidity and cell integrity and that this protein modification, therefore, is vital for *Saccharomyces cerevisiae*.

Keywords: glycoprotein/mannosyltransferase/osmotic stability/*PMT* gene family/yeast

Introduction

The covalent attachment of oligo- and polysaccharides to proteins constitutes the most extensive and complex modification of proteins. The sugar moieties are either linked to the protein via the amide group of an asparagyl residue (*N*-glycosylation) or via the hydroxy group of a seryl or threonyl residue (*O*-glycosylation). The biosynthetic pathway of *N*-glycosylation starts in all eukaryotic cells at the endoplasmic reticulum (ER) and involves the dolichol cycle (Kornfeld and Kornfeld, 1985; Lehle and Tanner, 1995). Protein *O*-glycosylation on the other hand proceeds differently in lower and higher eukaryotes. Whereas sugar nucleotides are substrates for protein *O*-glycosylation, which in general proceeds in the Golgi apparatus of mammalian cells (Roth *et al.*, 1994; Roth, 1995), dolichylphosphate-activated mannose is the sugar donor for the corresponding reaction taking place in the ER of the yeast *Saccharomyces cerevisiae* (Lehle and Tanner, 1995).

The integral membrane protein Dol-P-Man:protein-(Ser/Thr) mannosyltransferase has been purified to homogeneity from yeast and the corresponding gene (*PMT1*) has been cloned (Strahl-Bolsinger and Tanner, 1991; Strahl-Bolsinger *et al.*, 1993). Disruption of the *PMT1* gene showed on the one hand that the gene product is not essential for yeast cells to grow and divide, on the other hand, however, that more than one gene for protein *O*-glycosylation exists in *S.cerevisiae* (Strahl-Bolsinger *et al.*, 1993). Six more genes have been identified, either accidentally through the yeast genome project (Lussier *et al.*, 1995; Guerreiro *et al.*, 1996) or by PCR (Immervoll *et al.*, 1995); the whole gene family, consisting of *PMT1* to *PMT7*, is compiled in Table I. Their involvement in protein *O*-glycosylation has so far definitely been demonstrated only for the gene products of *PMT1*, *PMT2* and *PMT4* (Strahl-Bolsinger *et al.*, 1993; Immervoll *et al.*, 1995; Lussier *et al.*, 1995). In addition it has recently been shown that for Pmt1p and Pmt2p to be enzymatically active *in vitro* and *in vivo* they have to exist together as a heterodimer (Gentzsch *et al.*, 1995b).

The remaining open question is whether protein *O*-glycosylation is a vital protein modification in *S.cerevisiae*. Concentrating on the four genes *PMT1–4* and their gene products, this question has been investigated by constructing all possible double and triple mutants. It will be shown that two of the double and three of the triple mutants are not able to grow under normal conditions, however, three of these five mutants can be saved by osmotic stabilization. The triple disruptions *pmt1pmt2pmt4* and *pmt2pmt3pmt4*, however, are lethal under all conditions. Thus either a minimal requirement of protein *O*-glycosylation in general or the *O*-glycosylation of one or more specific proteins is vital for *S.cerevisiae* cells. The surviving multiple disruptions partly possess conspicuous phenotypes.

Results

Multiple PMT disruptions

As shown previously (Lussier *et al.*, 1995), the double disruptant *pmt1pmt2* is still viable, although its generation time is increased >2-fold and the cells grow in clumps; it also shows residual protein *O*-glycosylation activity. To test whether the disruption of additional *PMT* genes might demonstrate a vital role of protein *O*-glycosylation, the *pmt1pmt2* double disruptant was crossed with *pmt3*, *pmt4* and with *pmt3pmt4* disruptants (Table II). Since indications had already been obtained that *pmt* mutants are osmotically labile (see also below), all dissected spores were germinated in the presence of 1 M sorbitol. The number of dead spores obtained, especially from the crosses *pmt1pmt2* with *pmt4* and with *pmt3pmt4*, clearly indicated that spores of certain genotypes were not able to germinate. By testing for the disruption markers and by Southern analysis of the predicted double and triple disruptants among the survivors the genotypes of the dead spore(s) of each tetrad were inferred (Table III). From the four possible triple

Table I. The *PMT* gene family of *S.cerevisiae*

Gene	Accession number (coding sequence)	Reference	Chromosome	No. of amino acid residues
PMT1	L19169 (532–2985)	Strahl-Bolsinger *et al.* (1993)	IV	817
PMT2	L05146 (2409–133)	Lussier *et al.* (1995)	I	758
PMT3	X83797 (37–2298)	Immervoll *et al.* (1995)	XV	753
PMT4	X83798 (101–2389)	Immervoll *et al.* (1995)	X	762
PMT5	X92759 (182–2413)	Dommaschk (unpublished)	IV	743
PMT6	Z49133 (213–2492)	Gueirreiro *et al.* (1996)	VII	759
PMT7	U28374 (24180–26168)	Ding (unpublished)	IV	662

Table II. Spore analysis of various crossings

Crossing	Separated spores	Dead spores (%)
SEY6210 × SEY6211	64	0
pmt1pmt2 × SEY6211	328	4
pmt1pmt2 × *pmt3*	316	12
pmt1pmt2 × *pmt4*	344	25
pmt1pmt2 × *pmt3pmt4*	380	32

Table III. Genotypes of the analyzed spores (*pmt1pmt2* × *pmt3pmt4*)

Strain	Viable spores (%)	Dead spores (%)
Wild-type	100	0
pmt1	100	0
pmt2	100	0
pmt3	100	0
pmt4	100	0
pmt1pmt2	100	0
pmt1pmt3	100	0
pmt1pmt4	78	22
pmt2pmt3	100	0
pmt2pmt4	29	71
pmt3pmt4	100	0
pmt1pmt2pmt3	33	67
pmt1pmt2pmt4	0	100
pmt1pmt3pmt4	89	11
pmt2pmt3pmt4	0	100
pmt1pmt2pmt3pmt4	0	100

mutants two, *pmt1pmt2pmt4* and *pmt2pmt3pmt4*, seemed to be lethal. On the other hand, a small percentage of spores with the genotype *pmt1pmt2pmt3* germinated and the corresponding cells subsequently grew, although extremely slowly, whereas the triple disruptant *pmt1pmt3pmt4* was little affected; even the double disruptant *pmt2pmt4* was considerably more vulnerable.

To exclude the possibility that the two triple mutated spores have a defect in spore germination but would otherwise be able to grow and divide as cells, these triple mutants were constructed under conditions of rescue by the *PMT2* gene on a YEp352 plasmid. When tested for the ability to lose the plasmid in the presence of 5-fluoroorotic acid (5-FOA), the results shown in Figure 1 clearly demonstrate that the mutants were not able to lose the rescuing plasmid and therefore could not grow in the presence of 5-FOA. Thus *O*-glycosylation is an essential protein modification in *S.cerevisiae* for cell growth and division. When this glycosylation is decreased to an extent beyond that occuring in double disruptants, but not necessarily abolished altogether, then at least the

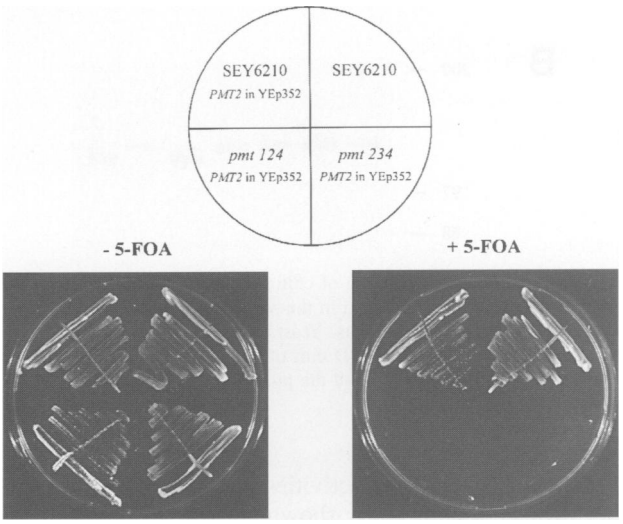

Fig. 1. Lethality of two *pmt* mutants. *pmt1pmt2pmt4* (*pmt124*) and *pmt2pmt3pmt4* (*pmt234*) are rescued by YEp352(*PMT2*). Both triple mutants are unable to grow in the presence of 0.1% 5-FOA.

genotypes *pmt1pmt2pmt4* and *pmt2pmt3pmt4* are not able to survive.

Protein O-glycosylation measured in vivo and in vitro in the various PMT disruptants

Chitinase, an extracellular protein exclusively *O*-glycosylated (Kuranda and Robbins, 1991), has been taken as a good indicator protein to demonstrate the degree of glycosylating activity *in vivo*. As shown in Figure 2A, chitinase shifts to a lower molecular weight when synthesized in cells carrying *pmt1* and *pmt2* mutations, whereas only a slight shift is seen in *pmt4* (see also Immervoll *et al.*, 1995). Surprisingly, results obtained with another *O*-glycosylated protein, gp115/Gas1p (Nuoffer *et al.*, 1991; Vai *et al.*, 1991), differ completely from those with chitinase: the *O*-glycosylation of this protein is unaffected by mutations in *PMT1* and *PMT2* and it only shifts to a lower molecular weight in cells carrying the *pmt4* mutation (Figure 2B). Since it has been shown that this shift is due to a reduced amount of *O*-linked sugars (T.Seidl, unpublished results), these data to our knowledge show conclusively that different protein *O*-glycosyltransferases differ in their specificity towards protein substrates.

Protein mannosyl transfer activity *in vitro* can be measured by the transfer of [^{14}C]mannose from Dol-P-[^{14}C]Man to various peptides (Strahl-Bolsinger and Tanner, 1991). A test which allows measurement of residual enzyme activity in *pmt1* mutants has been developed using the peptide YATAV as acceptor (Gentzsch *et al.*, 1995a).

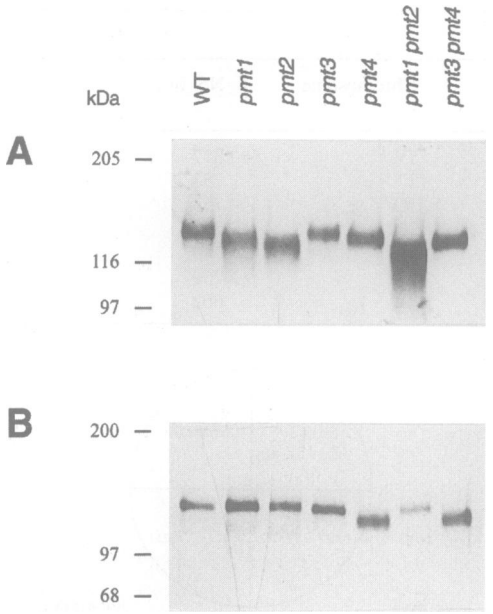

Fig. 2. Immunological detection of chitinase (**A**) and gp115/Gas1p (**B**) synthesized in the wild-type and in the *pmt1*, *pmt2*, *pmt3*, *pmt4*, *pmt1pmt2* and *pmt3pmt4* mutants. Yeast total membrane and cell wall extracts were isolated from 1 OD unit of a stationary culture, separated by 6% SDS–PAGE and the proteins detected by immunoblotting.

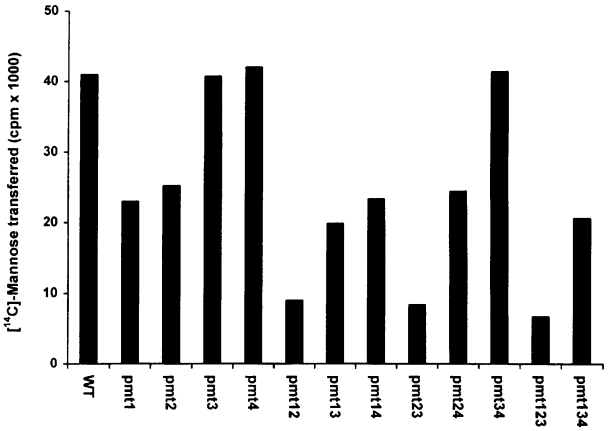

Fig. 3. *In vitro* Dol-P-Man:protein *O*-mannosyltransferase activities of *pmt* mutants. [^{14}C]Mannose was transferred from Dol-P-[^{14}C]Man to the peptide YATAV as described in Materials and methods.

In Figure 3 the enzyme activities measured in extracts of the various mutants are shown. These results can be summarized as follows. (i) The *in vitro* test with the pentapeptide is not suitable for measuring *PMT3* and *PMT4* activity. The single as well as the double disruptions show wild-type activity. (ii) Single disruptions of either *PMT1* or *PMT2*, as well as all combinations where one of these two genes is missing, with the only exception of *pmt2pmt3*, yield ~50% of wild-type activity. (iii) Only in three mutants was the activity decreased to ~20%. These are the *pmt1pmt2* and the *pmt1pmt2pmt3* disruptions and in addition *pmt2pmt3*, i.e. the exception mentioned above.

Since it has been demonstrated (Gentzsch *et al.*, 1995b) that Pmt1p and Pmt2p have to form a heterodimer to be fully active, the enzyme activity data suggest that heterodimers between other gene product partners are also possible. Thus the three lowest activities, which have been reproducibly obtained, can be explained if the existence of a Pmt1p Pmt3p dimer with transfer activity towards the peptide were assumed. The *pmt1pmt2* double disruptant would then result in lower enzyme activity than the single disruptions, since in the absence of *PMT2* part of the observable *in vitro* activity will be caused by Pmt1p Pmt3p; since this cannot form in the *pmt2pmt3* double disruptant, the lowest activity again will show up in this mutant (Figure 3). Obviously, in the absence of *PMT1* an additional heterodimer with Pmt2p would also have to be assumed; this has indeed been indicated by analogous data concerning a *pmt1pmt6* double disruption (T.Seidl, unpublished results).

Phenotypes of the various PMT disruptants

The various *pmt* mutants did not only show a decreased growth rate in liquid culture (except for the *pmt3* and *pmt4* single and the *pmt3pmt4* double disruptants), the more obvious phenotype of some of them was their osmolability. As can be seen in Figure 4, the double mutants *pmt2pmt3* and *pmt2pmt4* and the triple mutant *pmt1pmt2pmt3* only grow when osmotically stabilized with 1 M sorbitol. The third plate in Figure 4 also shows that five of the mutants are temperature sensitive, however the temperature sensitive phenotype and osmolability do not coincide for *pmt1pmt4* and *pmt1pmt3pmt4*. Double disruptants which do not show an osmolabile phenotype in the plate assay, like *pmt1pmt2*, nevertheless show higher susceptibility in deionized water as compared with the wild-type (Figure 5). To see whether the cell wall composition is changed in the osmolabile mutants, the glucan:mannan:chitin ratio of the *pmt2pmt3* and the *pmt1pmt2* double mutants was checked. Whereas carefully washed and completely hydrolyzed cell walls of logarithmically growing wild-type cells contain 49.5% D-mannose, 48% D-glucose and 2.3% D-glucosamine (Table IV), the most obvious change in cell walls of osmolabile mutants was a significant decrease in the glucan fraction; the D-glucose content amounting to 39% in the *pmt2pmt3* mutant, which corresponds to a 20% decrease when compared with the amount of glucan in the wild-type. The relative amounts of D-mannose and D-glucosamine increased (Table IV), the D-glucosamine (chitin + *N*-linked sugars), although a minor fraction, even doubling or tripling.

The growth morphology of the various *pmt* mutants also changed drastically. Examples are shown in Figures 6 and 7. In liquid culture all double and triple mutants, with the exception of *pmt3pmt4*, divided but did not separate and formed large clumps; on plates a tendency to hyphae formation was observed (Figure 6). In the triple mutant *pmt1pmt3pmt4* up to five nuclei could be observed per cell (Figure 7), which indicates that cell division, but not however nuclear division, was severely inhibited when protein *O*-glycosylation was reduced.

Another phenotype associated with *pmt* mutations is killer toxin resistance. It has been shown that for the killer toxin K1 to be active it has to interact with 1,6-β-glucan components of the cell wall; mutants defective in glucan synthesis are often killer toxin resistent (*kre* mutants) (Boone *et al.*, 1990). Surprisingly, one *kre* mutant turned out to be identical to *mnt1*, a mutant deleted in a

mannosyltransferase attaching the third sugar residue to saccharide chains *O*-linked to protein (Häusler *et al.*, 1992). This, as well as the observations that *pmt1* and *pmt2* mutants are partly and the double mutant fully resistant to killer toxin K1 (Strahl-Bolsinger *et al.*, 1993; Lussier *et al.*, 1995), suggests that protein *O*-glycosylation is in some way connected with the synthesis or the final structure of the cell wall glucans which are required for killer toxin action. In Figure 8 the resistances to killer toxin of the various *pmt* mutants are shown. Whereas the *pmt3pmt4* mutant was almost as sensitive as the wild-type and the *pmt1pmt3* mutant had a reduced sensitivity, not however different from *pmt1* alone (data not shown), *pmt1pmt4* and *pmt2pmt3* were almost fully resistant. Both the triple mutants were fully resistant, as was *pmt1pmt2*. The most interesting phenotype was shown by *pmt2pmt4*, which on the one hand was as sensitive as the wild-type (inhibitory zone) but, on the other hand, did not seem to be killed by the toxin (no dark zone of dead cells which accumulated dye). It seems possible therefore to distinguish between a growth/cell division inhibitory and a killing effect of killer toxin.

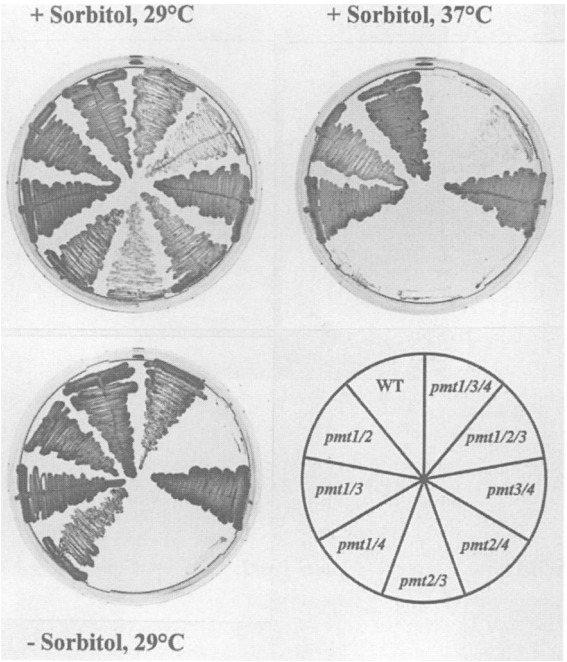

Fig. 4. The wild-type and the *pmt* double and triple mutants were grown on YPD plates in the presence and absence of 1 M sorbitol at 29°C and in the presence of 1 M sorbitol at 37°C.

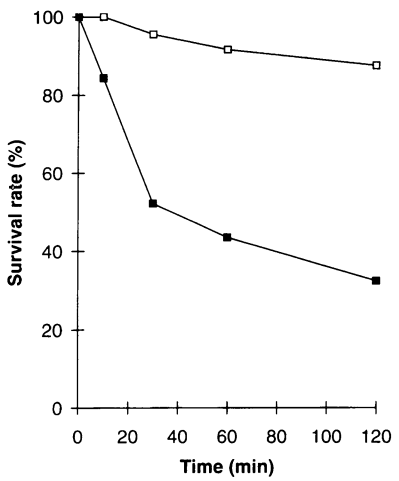

Fig. 5. Survival rate of logarithmically grown yeast cells in distilled water. Wild-type cells (□) and *pmt1pmt2* mutant cells (■) were incubated in distilled water and aliquots were taken at different times and spread on YPD plates. The number of colonies was determined after incubation for 3 days.

Calcofluor white is known to negatively affect glucan synthesis and stability and has been used, for example, to select for cell wall mutants (Ram *et al.*, 1994). Cells with weakened cell walls are sensitive to calcofluor white concentrations which do not affect wild-type cells. To see whether *pmt* mutants also show increased sensitivity to calcofluor white, three cell concentrations were plated in the absence and presence of the inhibitor (Figure 9). All *pmt* mutants were more sensitive, those mutants which grew more slowly in the absence of calcofluor white, like *pmt2pmt3*, being most sensitive.

Finally the effect of caffeine on growth of the *pmt* mutants was investigated. Evidence had been obtained (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992) that a protein kinase C of *S.cerevisiae* is involved in some way in cell wall stability and osmolability. The corresponding mutants showed an increased sensitivity to the diesterase inhibitor caffeine (Costigan *et al.*, 1992). Some of the *pmt* mutants are also severely growth inhibited by 5 mM caffeine (Figure 10) and those most sensitive (*pmt1pmt4*, *pmt2pmt3*, *pmt2pmt4*, *pmt1pmt2pmt3* and *pmt1pmt3pmt4*) are conspicuously the ones which are also temperature sensitive (see Figure 4).

Discussion

Evidence is presented here for the first time that protein *O*-glycosylation is vital for a unicellular eukaryotic organism. Whereas single mutations in the four *PMT* genes *PMT1–4* did not result in a conspicious phenotype nor in a pronounced decrease in growth rate (Strahl-Bolsinger *et al.*, 1993; Immervoll *et al.*, 1995; Lussier *et al.*, 1995), the double knockout *pmt1pmt2* did show a severe defect in growth (Lussier *et al.*, 1995). The triple mutants *pmt1pmt2pmt4* and *pmt2pmt3pmt4* were not able to survive

Table IV. Sugar composition of cell walls

Strain/mutant	Carbohydrates[a] (mg)	D-Mannose (%)	D-Glucose (%)	D-GlcNH$_2$ (%)	Man/Glc
SEY6210 (WT)	3.8	49.5	48.0	2.3	1.03
pmt1pmt2	3.3	49.0	44.0	7.2	1.12
pmt2pmt3	3.2	55.7	39.0	5.3	1.43

[a]Starting material in each case 18 ml cells of 2 OD_{578}.

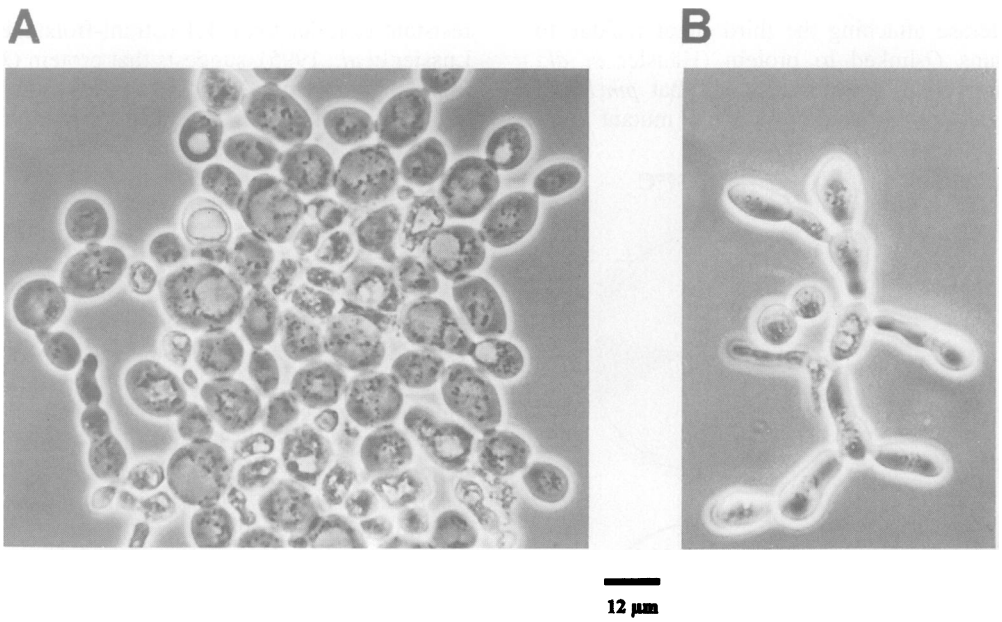

Fig. 6. Phenotype of a *pmt2pmt4* mutant. Yeast cells were grown in liquid YPD medium (**A**) or on YPD plates (**B**) in the presence of 1 M sorbitol and examined by phase contrast microscopy.

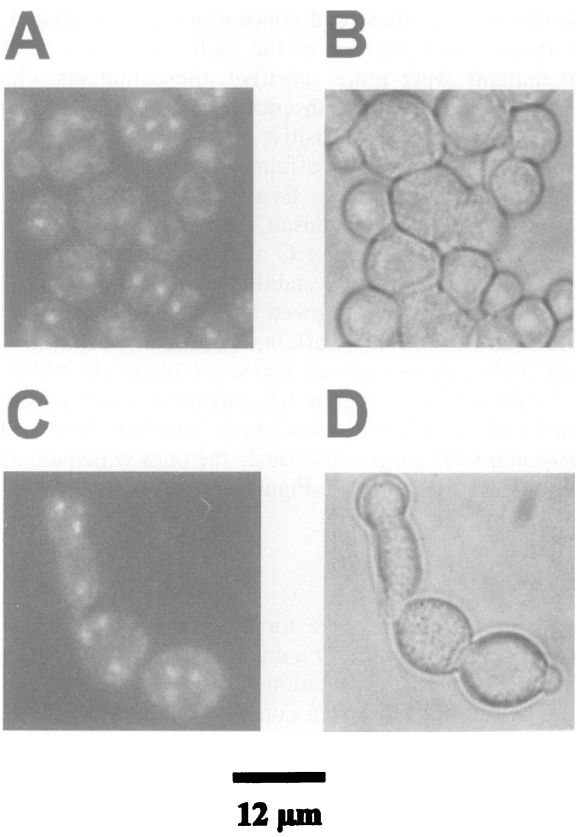

Fig. 7. Phenotype of a *pmt1pmt3pmt4* triple mutant. Cells were examined by phase contrast microscopy (**B** and **D**). The nuclei were visualized (**A** and **C**) by staining with 4,6-diamidino-2-phenylindole (DAPI).

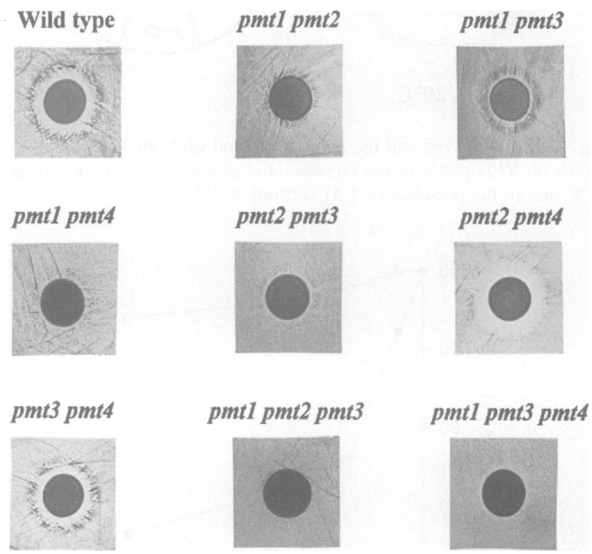

Fig. 8. Resistance of *pmt* mutants to killer toxin K1. Yeast strain T158/S14a producing killer toxin K1 was dropped on a lawn of 1×10^5 cells from a fresh culture of each strain. After subsequent incubation toxin-sensitive cells are killed and an inhibition zone is detectable in the growth lawn.

under any growth conditions (Table III and Figure 1). Also, the double mutants *pmt2pmt3* and *pmt2pmt4* are only viable when osmotically stabilized, which is also true for the triple mutant *pmt1pmt2pmt3*. Under such growth conditions all mutants show a peculiar phenotype: growth is very slow and clumpy and the cells frequently contain several nuclei (Figure 7). All these features indicate that the cell wall structure may be affected and its rigidity severely decreased. Also, budding and cytokinesis does not proceed normally, most likely as a consequence of these cell wall defects. An analysis of the cell wall composition showed that *pmt1pmt2* and especially the more highly osmolabile *pmt2pmt3* showed a significant change in the mannan:glucan ratio (Table IV). One possible interpretation of these data is that part of the glucan is cross-linked to cell wall proteins via sugars *O*-linked to protein; in the absence of the latter the corresponding glucan fraction may be released into the medium. The existence of cell wall proteins covalently linked to

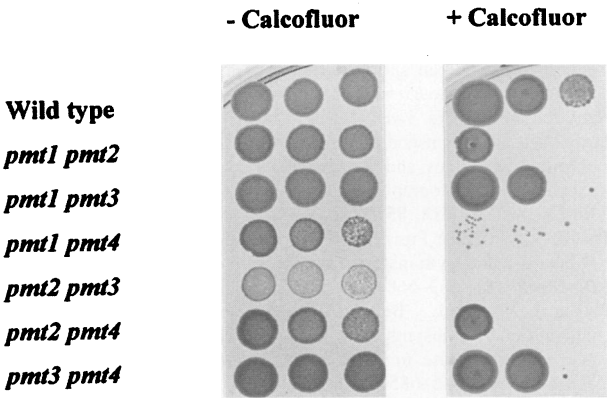

Fig. 9. Sensitivity of the *pmt* mutants to calcofluor white. Different dilutions of freshly grown yeast cultures (from left to right 50 000, 5000 and 500 cells) were spotted on a YPDS plate containing 50 μg/ml calcofluor white.

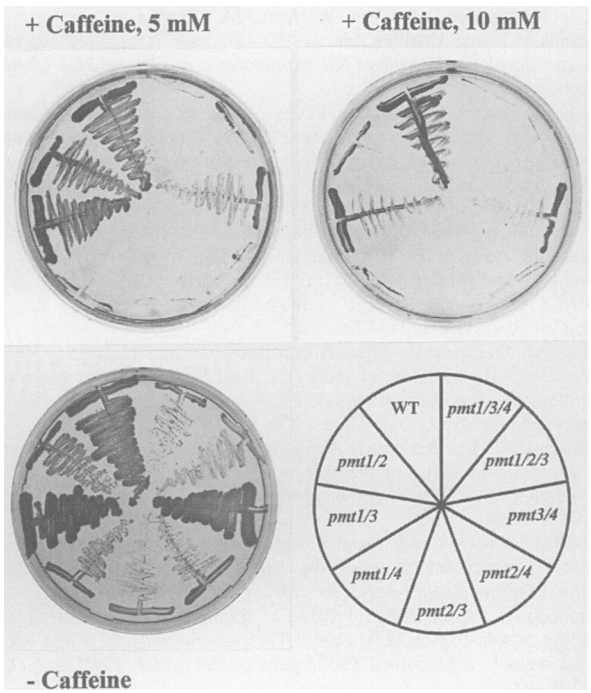

Fig. 10. The wild-type and the *pmt* double and *pmt* triple mutants were grown on YPDS plates in the presence of 5 and 10 mM caffeine.

1,6-β-glucans has been demonstrated (Montijin *et al.*, 1994), but protein-linked mannose did not seem to be involved in this type of cross-linking.

Recently observations that a defect in a protein kinase C gene (*PKC1*) causes an osmolabile phenotype of *S.cerevisiae* (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992) and that a number of protein kinases partially compensate for the defect led to the postulation of a protein phosphorylation cascade being involved in cell integrity (Lee *et al.*, 1993). The target reaction finally leading to cell wall instability is not known and so far hardly any candidates, i.e. enzymes involved in the biosynthesis of cell wall components essential for osmostability, are obvious. Therefore the possibility has to be considered that *O*-glycosylation of cell wall proteins may be such a reaction. Indeed, some of the features of the *PKC1* pathway, like the temperature sensitive phenotype of some of its deletion mutants and the increased sensitivity to caffeine, are reminiscent of the results described herein. However, although the *PMT1–7* gene products possess three highly conserved protein kinase C phosphorylation sites (SXR/K and TXR/K), neither the *pkc1*^{ts} mutant nor a deletion mutant showed decreased activity in the *in vitro* Pmtp activity test (data not shown).

The fact that deletion of the *PMT4* gene only affects *O*-mannosylation of plasmalemma-bound gp115/GAS1p, whereas Pmt1p and Pmt2p mainly affect chitinase mannosylation, is considered an important finding. Although this might indicate different *O*-glycosylation pathways in relation to topology or possible variations in the secretory pathway for corresponding proteins, it is considered to be more likely that these data support the notion that different amino acid sequences specify protein *O*-glycosylation and that they are recognized by specific glycosyltransferases.

A protein *O*-glycosylation mutant has been described for *Dictyostelium discoideum* (Gerisch *et al.*, 1993). This mutation is not lethal; it is not known, however, whether in *Dictostelium* a number of isogenes may also be active. On the other hand, *D.discoideum* does not have to establish a rigid cell wall. In mammalian cells protein *O*-glycosylation, although of a different type, is also brought about by more than one gene product (Sorensen *et al.*, 1995). The establishment of rigid 'stalk regions' of membrane-bound proteins is considered as a general function of protein *O*-glycosylation (Jentoft, 1990). For fungal cells an additional more direct involvement of *O*-linked saccharides in wall structure and stability is suggested by the results described in this paper.

Materials and methods

Yeast strains and media

The wild-type strains used were SEY6210 (MATα *leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9*) and SEY6211 (MATa *leu2-3,112 ura3-52 his3-Δ200 ade2-101 trp1-Δ901 suc2-Δ9*). Sensitivity to killer toxin K1 was tested with the help of diploid killer strain T158C/S14a (MATa/MATα *his4C-864/HIS4 ade2-5/ADE2*) (Boone *et al.*, 1990). Standard yeast media were used (Sherman, 1991).

Construction of multiple pmt mutants

SEY6211 *pmt1::URA3 pmt2::LEU2* (Lussier *et al.*, 1995) was crossed with SEY6210 *pmt3::HIS3*, SEY6210 *pmt4::TRP1* and SEY6210 *pmt3::HIS3 pmt4::TRP1*. The plasmids used for disruption of *PMT1* and *PMT2* are discribed in Strahl-Bolsinger *et al.* (1993) and Lussier *et al.* (1995) respectively. Disruption plasmids for knocking out *PMT3* and *PMT4* were a gift from Thomas Immervoll and are described in Immervoll *et al.* (1995). The disruptions were checked by Southern blot analysis. Mating, sporulation, dissection of the progeny and tetrad analysis were carried out as described (Sherman and Hicks, 1991).

Construction of pmt triple mutants rescued by YEp352(PMT2)

A *pmt2pmt4* mutant was transformed with *PMT2* in YEp352 (Lussier *et al.*, 1995) and disrupted in *PMT1* to create a *pmt1pmt2pmt4* triple mutant rescued by *PMT2*. A *pmt2pmt3pmt4* triple mutant was constructed by transforming a *pmt2pmt3* mutant with *PMT2* in YEp352, followed by disruption of *PMT4*. All disruptions were checked by Southern blot analysis. 5-FOA was used to verify the inability to lose the plasmid YEp352(*PMT2*). 5-FOA plates contained standard medium supplemented with 0.1% 5-FOA (Boeke *et al.*, 1987).

Immunological methods

Yeast cells were grown to late logarithmic growth phase, washed with 50 mM Tris–HCl, pH 7.5, 50 mM $MgCl_2$ and broken with glass beads in the same buffer. Cell walls were collected by centrifugation at 3000 *g*. Cell wall proteins were separated by SDS–PAGE (6%). Chitinase

was detected by immunoblotting with antibody produced against the deglycosylated protein (Immervoll *et al.*, 1995); anti-gp115 antiserum was kindly provided by Dr L.Popolo.

Determination of Dol-P-Man protein O-mannosyltransferase activity in vitro

Dol-P-[^{14}C]Man was enzymatically synthesized following the instructions of Haselbeck (1989). The assay for measuring Dol-P-Man protein *O*-mannosyltransferase activity contained 0.04 μCi Dol-P-[^{14}C]Man (sp. act. 284 Ci/mol), 10 mM KH_2PO_4:Na_2HPO_4, pH 6.5, 7 mM $MgCl_2$, 0.14% Triton X-100, 3.5 mM acetyl-YATAV-NH_2 and 250 μg membrane protein in a total volume of 140 μl. After incubation for 30 min at 22°C the *in vitro* tests were stopped according to Strahl-Bolsinger and Tanner (1991).

Preparation of membranes

Yeast cells were grown to mid logarithmic phase, washed with 50 mM Tris–HCl, pH 7.5, 50 mM $MgCl_2$ and broken with glass beads in the same buffer. After centrifugation at 3000 *g*, membranes were collected from the supernatant by centrifugation at 48 000 *g*.

Preparation of cell walls and sugar analysis

Yeast cells were harvested in the exponential phase of growth, washed three times with 10 mM Tris–HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium azide and broken with glass beads in the same buffer. Cell walls were sedimented by centrifugation at 1000 *g* and washed five times with 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride and once with H_2O. Five milligrams of cell wall were hydrolysed in 0.5 ml 4 N trifluoracetic acid (TFA) at 100°C for 4 h. TFA was evaporated under nitrogen and monosaccharides released were analyzed by HPAEC on a CarboPac PA1 column (Hardy and Townsend, 1988). Elution was performed with 16.5 mM NaOH at a flow rate of 1 ml/min; detection was carried out by pulsed amperometry with a gold electrode. Reference monosaccharides were used for identification.

Plate assays

Calcofluor sensitivity was tested according to Ram *et al.* (1994), sensitivity to killer toxin K1 was tested according to Fink and Styles (1972) and caffeine sensitivity was tested according to Posas *et al.* (1993).

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