O-Mannosylation precedes and potentially controls the N-glycosylation of a yeast cell wall glycoprotein

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Secretory proteins in yeast are *N*- and *O*-glycosylated while they enter the endoplasmic reticulum. *N*-glycosylation is initiated by the oligosaccharyl transferase complex and *O*-mannosylation is initiated by distinct *O*-mannosyltransferase complexes of the protein mannosyl transferase Pmt1/Pmt2 and Pmt4 families. Using covalently linked cell-wall protein 5 (Ccw5) as a model, we show that the Pmt4 and Pmt1/Pmt2 mannosyltransferases glycosylate different domains of the Ccw5 protein, thereby mannosylating several consecutive serine and threonine residues. In addition, it is shown that *O*-mannosylation by Pmt4 prevents *N*-glycosylation by blocking the hydroxy amino acid of the single *N*-glycosylation site present in Ccw5. These data prove that the *O*- and *N*-glycosylation machineries compete for Ccw5; therefore *O*-mannosylation by Pmt4 precedes *N*-glycosylation.

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INTRODUCTION

Glycosylation is an essential protein modification in yeast and higher eukaryotes (te Heesen *et al.*, 1992; Metzler *et al.*, 1994; Gentzsch & Tanner, 1996). There are two main types of glycosylation, *N*- and *O*-glycosylation, in which saccharides are attached either to the amide group of an asparagine residue or to hydroxy amino acids, which are mainly serine and threonine residues. In all eukaryotes, *N*-glycosylation is initiated in the endoplasmic reticulum (ER) and is dependent on the reactions of the dolichol cycle (Kornfeld & Kornfeld, 1985; Lehle & Tanner, 1995; Knauer & Lehle, 1999; Helenius & Aebi, 2001). The oligosaccharide GlcNAc₂Man₉Glc₃, which is preformed on dolichol pyrophosphate, is transferred by an oligosaccharyl

transferase (OST) to an Asn residue within the sequence Asn-X-Ser/Thr, where X indicates any amino acid except proline. In yeast, the OST complex consists of at least ten proteins (Knauer & Lehle, 1999; Helenius & Aebi, 2001). *O*-Glycosylation takes place in several ways. The main pathway in higher eukaryotes is located in the Golgi and directly uses sugar nucleotides (Brockhausen, 1995). In yeasts, *O*-mannosylation starts in the ER lumen and, like *N*-glycosylation, requires dolicholphosphate-activated sugar residues. The initial reaction is catalysed by the evolutionarily conserved protein mannosyl transferase (Pmt) family (Strahl-Bolsinger *et al.*, 1999). The Pmt family is divided into the Pmt1, the Pmt2 and the Pmt4 subfamilies. Pmt1 subfamily members form specific complexes with members of the Pmt2 subfamily, whereas Pmt4 functions as a homomeric complex (Girrbach & Strahl, 2003). In addition, Pmt1/Pmt2 subfamily members differ from Pmt4 in their specificity for their protein substrates (Gentzsch & Tanner, 1997; Girrbach *et al.*, 2000). Recently, it has been reported that the type of *O*-mannosylation that takes place in yeast also occurs in mammalian cells (Chiba *et al.*, 1997; Yuen *et al.*, 1997; Jurado *et al.*, 1999; Yoshida *et al.*, 2001; Willer *et al.*, 2002), and is probably also initiated in the ER (Willer *et al.*, 2002). Mutations in a corresponding human gene (protein *O*-mannosyl transferase 1; *POMT1*) give rise to a severe neuronal disease (Beltran-Valero de Bernabé *et al.*, 2002).

Secretory proteins in yeast cells are usually heavily *N*- and/or *O*-glycosylated. As *N*-glycosylation proceeds alongside *O*-mannosylation in the ER of fungal and mammalian cells, several new, general questions arise. It is not known, for example, whether *N*-glycosylation precedes *O*-mannosylation, or vice versa, in those cases in which proteins carry both types of modification. Neither is it known whether *O*-mannosylation that takes place in the ER is able to prevent *N*-glycosylation, although evidence for the opposite situation has been reported (Harty *et al.*, 2001).

Both of these questions can be answered by studying the biosynthesis of the yeast covalently linked cell-wall protein 5 (Ccw5). This protein is highly *O*-mannosylated and has one *N*-glycosylation site. We show that the *O*-mannosyltransferase Pmt4 specifically glycosylates a cluster of serine and threonine residues that is not used by any other Pmt family member. In

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1 MQFKNVALAA SVAALSATAS AEGYTPGEPW STLTPGSGIS CGAAEYTTTF
51 GIAVQAITSS KAKRDVISOI GDGOVQATSA ATAAQATDSQA QATTATPTTS
101 SEKISSSASK TSTNATSSSC ATPSLKDSSC KNSGTLELTL KDGVLTDAGK
151 RIGSIVANRQ FQFDGPPPPQA GAIYAAGWSI TEDGYLALGD SDVFYQCLSG
201 NFYNLYDQNV AEQCSAIHLE AVSLVDC

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Fig. 1 | The amino-acid sequence of the *Saccharomyces cerevisiae* covalently linked cell-wall protein 5. The amino-acid sequence was deduced from the DNA sequence (Mrsa *et al.*, 1997). The arrow indicates the Kex2 processing site; the potential *N*-glycosylation site is underlined. The first boxed sequence (solid lines) corresponds to the typical repeat motif of the proteins with internal repeats (Pir) family. Another such motif is also present (dashed box), although it is less conserved than the first motif.

addition, we show that Ccw5 is only *N*-glycosylated in a *pmt4Δ* mutant, demonstrating competition between *O*- and *N*-glycosylation. Finally, these results prove that in yeast, for at least one protein, *O*-mannosylation precedes *N*-glycosylation.

RESULTS AND DISCUSSION

The *Saccharomyces cerevisiae* cell-wall protein Ccw5/Pir4 is a member of the Pir (proteins with internal repeats) family, and is covalently linked to β -1,3-glucan (Toh-e *et al.*, 1993; Mrsa *et al.*, 1997; Kapteyn *et al.*, 1999). Deletion of this protein family results in a loss of cell-wall stability and an altered cell morphology (Mrsa & Tanner, 1999). Ccw5 consists of 227 amino acids; the protein is processed by Kex2 (Mrsa *et al.*, 1997) and is 64 amino acids shorter in its mature form (Fig. 1). It contains a single *N*-glycosylation site at Asn 114, and in wild-type yeast the apparent relative molecular mass of the mature protein is ~37 kDa, whereas the calculated molecular mass of the mature protein is 17 kDa, the difference being due to glycosylation (Fig. 2). It was previously shown that Ccw5 is *O*-mannosylated by Pmt1 and Pmt2 (Mrsa *et al.*, 1997). In a *pmt4Δ* mutant, the protein could no longer be detected in an extract of total cell-wall proteins, and is thought to be specifically degraded in this mutant (Mrsa *et al.*, 1997). To study the fate of the Ccw5 protein, a protein-A-epitope-tagged version was transformed into wild-type yeast and into *pmt* mutants, and Ccw5–ProtA was analysed. We found that the protein was not degraded, but instead its relative molecular mass is greatly increased in the *pmt4Δ* mutant (Fig. 2).

As shown in Fig. 2, this increase in size occurs only in the *pmt4Δ* mutant. In the *pmt1Δ* mutant, the relative molecular mass of the protein decreases slightly, and a similar decrease is seen in the *pmt2Δ* mutant (data not shown; Mrsa *et al.*, 1997). How can the increase in molecular weight in the *pmt4Δ* mutant be explained? Ccw5 contains one potential *N*-glycosylation site at Asn 114. The most likely explanation for the increase in the mass of the protein shown in Fig. 2 is, therefore, that the protein is not normally *N*-glycosylated, but is modified in this way when the *pmt4* gene is deleted. To test this possibility, Ccw5–haemagglutinin (HA) from *pmt4Δ* cells was treated with endoglycosidase H (Endo H) to remove any *N*-linked sugar chains. As shown in Fig. 3, the protein then had the same electrophoretic mobility as in wild-type cells. Endo H treatment does not influence the size of the wild-type protein, showing that the *N*-glycosylation site of

Fig. 2 | Western blot of Ccw5–ProtA. The protein was isolated from *Saccharomyces cerevisiae* wild-type (WT) cells and from protein mannosyl transferase (*pmt*) mutants, as indicated. Ccw5–ProtA, a fusion of covalently linked cell-wall protein 5 (Ccw5) with two protein A (ProtA) epitopes.

Fig. 3 | Covalently linked cell-wall protein 5 is *N*-glycosylated in the *pmt4Δ* mutant. Ccw5–HA (covalently linked cell wall protein 5 tagged with haemagglutinin) obtained from cell walls of wild-type (WT) and *pmt4Δ* cells was immunodetected after SDS–polyacrylamide gel electrophoresis (see the Methods section). Endoglycosidase H (Endo H) treatment was carried out using the method of Sanders *et al.* (1999). *pmt4*, protein mannosyl transferase 4D.

Ccw5 is not used in wild-type cells. The difference in size of ~2 kDa between the Endo-H-treated protein from *pmt4Δ* cells and that from wild-type cells must be due to the *O*-linked mannosyl residues added by Pmt4.

These results can be most easily understood by combining the essential mechanistic role of the neighbouring hydroxyl in the *N*-glycosylation sequon Asn-X-Ser/Thr (Imperiali & Hendrickson, 1995) with the specific *O*-glycosylation of this hydroxy group. The -OH residue is required for specific hydrogen bonding within the Asx turn, which has been shown to be a prerequisite for the efficient transfer of the oligosaccharide by the OST complex

Fig. 4 | *N*-glycosylation of covalently linked cell wall protein 5 is prevented in wild-type cells by the action of protein mannosyl transferase 4. M, mannosyl residue; OST, oligosaccharyl transferase; Pmt4, protein mannosyl transferase 4; WT, wild-type.

(Imperiali & Hendrickson, 1995). Fig. 4 shows schematically the competition between the *O*- and *N*-glycosylation of Ccw5 in yeast. In the wild type, the threonine hydroxyl in the sequence Asn-Ala-Thr is expected to be blocked by a mannosyl residue and thus not to be available to the OST. Because, in the absence of Pmt1 (Fig. 2) and Pmt2 (Mrsa et al., 1997), *N*-glycosylation does not occur, the *O*-mannosylation of the threonine should be catalysed specifically and solely by Pmt4.

To prove this, the Ccw5 protein was isolated from wild-type and *pmt4Δ* cells. Two protein-A tags were fused to the carboxyl terminus of Ccw5, and the fusion protein was expressed in wild-type and *pmt4Δ* cells and purified by IgG affinity-column chromatography. The protein sequence was determined by Edman degradation. In Fig. 5, the deduced amino-acid sequence is compared with the sequence of the wild-type protein and with that of the protein from *pmt4Δ* cells. Whereas in wild-type cells no free hydroxy amino acids were detected within the amino-terminal half of the Ccw5-ProtA protein because of mannosylation, this was not the case for the protein from *pmt4Δ* cells. As expected, the threonyl residue of the Asn-Ala-Thr sequon in the mutant protein was not mannosylated; however, in addition, the seven hydroxy amino acids preceding the Asn-Ala-Thr sequon, as well as the two seryl residues after it, were not modified. The latter was unexpected, although the decrease in molecular weight of Endo-H-treated Ccw5 from *pmt4Δ* cells (Fig. 3) indicated that Pmt4 contributes more than one mannose residue. It is clear that a cluster of hydroxy amino acids is recognized by Pmt4, and not by any of the other yeast *O*-mannosyl transferases. As the signals of the three hydroxy amino acids Thr 99, Ser 100 and Ser 101 from sequencing reactions were too weak to be unequivocally identified as being modified or unmodified in the protein synthesized in the *pmt4Δ* cells, it is unclear as to where Pmt4 initiates mannosylation. The results show, however, that the hydroxy amino acid of the Asn-Ala-Thr sequon, as well as the neighbouring seryl and threonyl residues, is specifically and exclusively recognized and mannosylated by Pmt4, thus preventing *N*-glycosylation.

Whether the prevention of *N*-glycosylation by *O*-mannosylation is a cellular regulatory mechanism is an open question. Physiological conditions might exist that, by inhibiting Pmt4 activity, would allow *N*-glycosylation in a yes/no decision at

Based on DNA 65 DVISQIGDGQ VQATSAATAQ ATDSQAQATT
Ccw5-ProtA WT 65 DVIXQIGDGQ VQAXXAAXAQ AXDXQAQAXX
Ccw5-ProtA *pmt4Δ* 65 DVIXQIGDGQ VQAXXAAXAQ AXDXQAQAXX

Based on DNA 95 TATPTSSEKI SSSASKTSTNATSSSCATPS
Ccw5- WT 95 XAXPXXXXEKI XXXAXXXXXNAXXX
Ccw5-ProtA *pmt4Δ* 95 XAXP. SSSASKTSTKATSS

Fig. 5 | Analysis of the protein sequence of Ccw5-ProtA isolated from wild-type and *pmt4Δ* mutant cells. The ten hydroxy amino acids shown in red are mannosylated by Pmt4. The *N*-glycosylation site Asn-Ala-Thr (NAT) is shown in blue, except for the positions modified in *pmt4Δ*. Ccw5, covalently linked cell wall protein 5; *pmt4*, protein mannosyl transferase 4; ProtA, protein A epitope; WT, wild type. The *N*-glycosylation sites are shown in the boxes.

specific sites. A previous report suggests that the results obtained here for the cell-wall protein Ccw5 may also apply to other proteins (Sanders et al., 1999). The bud-site selection protein, Ax12 (protein required for axial budding 2), is specifically degraded in the *pmt4Δ* mutant; no other *pmt* mutants show this effect. The *pmt4Δ*-dependent protein degradation was prevented by tunicamycin, which suggests that Pmt4 also prevents *N*-glycosylation at specific sites in this case. In the *pmt4* mutant, *N*-glycosylation might take place, and could then be the signal for glycosylation-induced glycoprotein degradation (Jakob et al., 2001).

The results in this paper can only be explained by assuming that *O*-mannosylation precedes *N*-glycosylation. At least for Pmt4, this has to be the case. Evidence has been obtained for the existence of *O*-mannosylated proteins that are still attached to polysomes in yeast (Larriba et al., 1976). As it is known from *in vivo* studies (for example, with hen oviduct and Chinese hamster ovary cells) that *N*-glycosylation also proceeds cotranslationally (Glabe et al., 1980; Chen et al., 1995), specific protein mannosyl transferases would be expected to be located even closer to the Sec61 channel (Matlack et al., 1998) than the multi-meric OST (oligosaccharyl transferase) complex (Kelleher et al., 1992; Knauer & Lehle, 1999). A close association of Pmt2 with the Sec61 channel in yeast has been suggested (Harty et al., 2001). In any case, with a space of 30–40 Å as estimated from *in vitro* studies with dog pancreas membranes, there seems to be sufficient space between the luminal side of the ER membrane and the active site of the OST (Nilsson & Heijne, 1993). The Pmt proteins are integral, seven-transmembrane helix proteins (Strahl-Bolsinger et al., 1999; Strahl-Bolsinger & Scheinost, 1999), and their active sites could therefore be located close to the surface of the ER membrane.

METHODS

Yeast strains and growth conditions. *S. cerevisiae* wild-type strain SEY6210 (*MATα*, *ura3-52*, *leu2-3, 112*, *his3-Δ200*, *trp1-Δ901*, *lys2-801*, *suc2-Δ9*; Robinson et al., 1988) and its derivatives XMA0-1U (*pmt1Δ::URA3*), XMA0-3H (*pmt3::HIS3*), XMA0-4T (*pmt4::TRP1*), XMA0-6U (*pmt6Δ::URA3*; Gentzsch & Tanner, 1997) and *pmt5Δ* (*pmt5Δ::URA3*; Mrsa et al., 1997) were used. Yeast cells were grown in synthetic complete medium.

Plasmids. To obtain the plasmid pCCW5–ProtA, the *CCW5/PIR4* gene and its promotor region from nucleotides –484 to +681 were amplified by PCR using genomic DNA as a template and with oligonucleotides CCW5protA-1 and CCW5protA-3. *Pst*I and *Sac*II restriction sites were thereby added at the 5' and 3' ends of the PCR product. A protein-A tag was obtained as a *Sac*II–*Spe*I fragment from YCplac33-ZZ (Whyte & Munro, 2001). Both fragments were three-way ligated into a pRS425 vector that was digested with *Pst*I and *Spe*I.

For pCCW5–HA, a fragment of *CCW5* consisting of nucleotides –168 to +785 was made by PCR using oligonucleotides CWP4B and CCW5B as primers and using genomic DNA as a template. A 111-bp *Not*I fragment that encodes three copies of the HA epitope was isolated from pAx12 (Roemer et al., 1996) and cloned into the C-terminal *Sa*I restriction site of *CCW5* to give pCCW5–HA. To make plasmid pCCW5–ProtA–I104M, the Ile 104 amino acid was exchanged for Met by PCR. The primer pairs Ccw5protA1/I104M2 and I104M1/Ccw5protA3 were used to generate overlapping megaprimers I104M-A and I104M-B, respectively, using pCCW5–ProtA as a template. A second PCR reaction was performed using megaprimers I104M-A and I104M-B and the flanking oligonucleotides, CCW5protA-1 and CCW5protA-3.

Isolation of Ccw5–HA from cell walls. Cells were grown overnight to a concentration of 2.0×10^7 cells ml^{–1}. 50 ml of cells were harvested and resuspended in 150 µl 50 mM KPO₄ buffer, pH 8.0, with a protease inhibitor mix. Cell lysis was performed in a Hybaid Rybolyzer for 30 s at 4 °C. After removal of the glassbeads, the cell walls were pelleted for 20 s at 14,000 r.p.m. and washed twice with 1 ml KPO₄ buffer. The cell walls were then resuspended in 500 µl Laemmli buffer and boiled for 10 min at 95 °C. This extraction was repeated once. After centrifugation, the pellet was washed and Ccw5 was released from the SDS–extracted walls using 50 µl 30 mM NaOH overnight at 4 °C (Mrsa et al., 1997). For western blot analysis, 10 µl of the neutralized supernatant was loaded. An anti-HA monoclonal antibody (16B12; Babco) was used at a 1:8,000 dilution; anti-mouse IgG peroxidase conjugate was used at a 1:5,000 dilution (A9044; Sigma).

Purification of Ccw5–ProtA from the medium. 1,000 ml of medium containing 8×10^7 cells ml^{–1} was lyophilized, dialysed, and concentrated to a volume of 500 µl. For affinity purification of Ccw5–ProtA, we used Mobicol columns (MoBiTec) and 150 µl IgG Sepharose 6 Fast Flow (Amersham). The protein was eluted twice with 120 µl 500 mM HAc, pH 3.5, and lyophilized.

Protein sequence analysis. For Edman degradation, the following samples were prepared: first, Ccw5–ProtA was prepared from the medium of wild-type cells as described, separated by SDS–PAGE and blotted onto Immobilon membrane. Second, an elastase/subtilisin fragment of Ccw5–ProtA from wild-type cells, starting at Ala 85 and comprising the potential *N*-glycosylation site, was prepared. This fragment was generated by cleaving the protein in 0.2% NH₄HCO₃ with elastase (1 µg 100 µl^{–1}) and isolating a fragment containing the N-terminal half of the protein by reverse-phase chromatography using a Vydac 218TP52 column (250 × 2.1 mm; C18). The lyophilized fragment was redissolved in 0.2% NH₄HCO₃ and cleaved further using subtilisin (1 µg 100 µl^{–1}). The desired fragment was isolated by reverse-phase chromatography. Last, the BrCN (cyanogen bromide) fragment of the mutated Ccw5–ProtA protein (I104M) isolated from *pmt4Δ* cells was prepared. This fragment starts at Ser 105. BrCN digestion was carried out in 200 µl of 80% formic

acid containing 500 µg of the reagent. The required peptide was isolated by SDS–PAGE and blotting onto Immobilon membrane. By sequencing the corresponding peptide of Ccw5–ProtA (I104M) obtained from protein isolated from wild-type yeast cells, we checked that the BrCN digestion conditions do not remove sugar moieties from hydroxy amino acids.

Peptides were sequenced using a Procise 492A sequencer (PE Biosystems), with on-line detection of the phenylthiohydantoin amino acids, in accordance with the manufacturer's instructions.

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