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O-Mannosylation Protects Mutant Alpha-Factor Precursor from Endoplasmic Reticulum-associated Degradation

Carol Harty,* Sabine Strahl,† and Karin Römisch*‡

*University of Cambridge, Wellcome Trust Center for Molecular Mechanisms in Disease, Cambridge Institute for Medical Research, and Department of Clinical Biochemistry, Cambridge CB2 2XY, United Kingdom; and †Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, 93053 Regensburg, Germany

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Secretory proteins that fail to fold in the endoplasmic reticulum (ER) are transported back to the cytosol and degraded by proteasomes. It remains unclear how the cell distinguishes between folding intermediates and misfolded proteins. We asked whether misfolded secretory proteins are covalently modified in the ER before export. We found that a fraction of mutant alpha-factor precursor, but not the wild type, was progressively *O*-mannosylated in microsomes and in intact yeast cells by protein *O*-mannosyl transferase 2 (Pmt2p). *O*-Mannosylation increased significantly in vitro under ER export conditions, i.e., in the presence of ATP and cytosol, and this required export-proficient Sec61p in the ER membrane. Deletion of *PMT2*, however, did not abrogate mutant alpha-factor precursor degradation but, rather, enhanced its turnover in intact yeast cells. In vitro, *O*-mannosylated mutant alpha-factor precursor was stable and protease protected, and a fraction was associated with Sec61p in the ER lumen. Thus, prolonged ER residence allows modification of exposed *O*-mannosyl acceptor sites in misfolded proteins, which abrogates misfolded protein export from the ER at a posttargeting stage. We conclude that there is a limited window of time during which misfolded proteins can be removed from the ER before they acquire inappropriate modifications that can interfere with disposal through the Sec61 channel.

INTRODUCTION

Secretory proteins fold in the lumen of the endoplasmic reticulum (ER; Ellgaard *et al.*, 1999). Only fully folded proteins are packaged into ER-to-Golgi transport vesicles and transported through the secretory pathway (Ellgaard *et al.*, 1999). Proteins that fail to acquire their native conformation are retained in the ER, at least initially by interaction with ER-resident chaperones (Ellgaard *et al.*, 1999; Römisch, 1999). Many misfolded proteins are subsequently transported back across the ER membrane to the cytosol where they are degraded by proteasomes (Ellgaard *et al.*, 1999; Römisch, 1999). It remains unclear how the cell distinguishes between chaperone-associated folding intermediates and misfolded proteins and at which point the decision is made to target an aberrant protein to export and degradation (Römisch, 1999; Parodi, 2000). For *N*-glycosylated misfolded proteins, the "timer" that triggers retrograde transport may be trimming of the *N*-glycan mannoses (Ellgaard *et al.*, 1999; Cabral *et al.*, 2000; Parodi, 2000). In the cases studied, only mannose-trimmed glycoproteins were subject to retrograde transport and degradation; inhibition of mannose-trimming resulted in the retention of the misfolded glycoproteins in the ER lumen, suggesting that a mannose-specific lectin recognizes export substrates and targets them to degradation (reviewed by Parodi, 2000).

Retrograde protein transport across the ER membrane to the cytosol is mediated by a channel that is formed by the same core component as the protein translocation channel that mediates secretory protein import into the ER lumen (Römisch, 1999). Specific mutations in this protein, Sec61p, interfere differentially with protein import and protein export, and *sec61* mutants that specifically block retrograde protein transport have recently been isolated by Zhou and Schekman (Pilon *et al.*, 1997, 1998; Plemper *et al.*, 1997; Zhou and Schekman, 1999). The location of these point mutations suggests that the mutated sites are interaction sites of Sec61p with ER luminal or transmembrane proteins that may be

‡ Corresponding author. E-mail: kbr20@cam.ac.uk.

Abbreviations used: ConA, concanavalin A; ER, endoplasmic reticulum; Δgpαf, *N*-glycosylation site mutant alpha-factor precursor; mΔgpαf, mannosylated Δgpαf; PDI, protein disulfide isomerase; Pmt, protein mannosyl transferase; PNGase, peptide *N*-glycosidase; TCA, trichloroacetic acid.

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involved in targeting of substrates to the export channel (Römisch, 1999; Zhou and Schekman, 1999). Specific interaction partners of Sec61p during retrograde protein transport across the ER membrane are still unknown.

The heterotrimeric Sec61 complex in the ER membrane consists of Sec61p, a 53-kDa protein with 10 transmembrane domains, and two smaller tail-anchored proteins, Sbh1p, and Ssh1p (Johnson and van Waes, 1999). The Sec61 channel is formed by four or five Sec61 complexes that assemble in the ER membrane for protein import into the ER in response to the presence of a functional signal peptide or in the presence of the Sec63 complex, which is required for post-translational protein import into the yeast ER (Hanein *et al.*, 1996). The Sec61 channel is sealed at both ends to maintain the permeability barrier across the ER membrane (Hamman *et al.*, 1998). Channel opening for protein import is triggered by functional signal peptides that are recognized by the Sec61 channel itself (Jungnickel and Rapoport, 1995). The signal peptide of most misfolded secretory proteins, however, is cleaved off before retrograde transport is initiated. Channel opening from the ER lumen must therefore be triggered by a fundamentally different mechanism (Römisch, 1999).

To identify a targeting signal for retrograde protein transport, we asked whether mutant secretory proteins are covalently modified in the ER lumen before export. As a substrate we used a mutant form of the yeast pheromone precursor prepro alpha-factor, which had its *N*-glycosylation acceptor sites removed by site-directed mutagenesis (Mayinger and Meyer, 1993). The *N*-glycosylation site mutant alpha-factor precursor protein (Δgpαf) is subject to ER-associated degradation in vivo and in a cell-free assay based on yeast microsomes and cytosol (McCracken and Brodsky, 1996). We found that a fraction of Δgpαf, but not the wild-type precursor, was covalently modified in microsomes and in intact yeast cells. This modification was significantly enhanced in vitro under ER export conditions, i.e., in the presence of ATP and cytosol, and required the presence of export-competent Sec61p in the membranes. We identified the modification as *O*-mannosylation and Pmt2p in the ER as the responsible enzyme. Deletion of the corresponding gene, *PMT2*, however, did not abrogate Δgpαf degradation but rather increased its turnover both in vitro and in vivo. In wild-type microsomes, the *O*-mannosylated fraction of Δgpαf was stable, protease protected, and accumulated in the ER lumen over the time course of a degradation reaction. A fraction of mΔgpαf could be cross-linked to Sec61p. Our data suggest that *O*-mannosylation of Δgpαf interferes with its retrograde transport across the ER membrane at a post-targeting stage.

MATERIALS AND METHODS

Strains and Growth Conditions

The following strains were used: RSY255 (*MATα leu2-3,-112 ura3-52*; (Stirling *et al.*, 1992), RSY1293 (*MATα can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura3-1 ade2-1 sec61::HIS3* pDQ1[*sec61-his6*] or pDQ1 expressing the indicated *sec61* mutants (Pilon *et al.*, 1997), WCG4a (*MATa leu2-3,-112 ura3 his3-11,-15* (Hiller *et al.*, 1996), WCG4-2 (*MATa leu2-3,-112 ura3 his3-11,-15 pre1 pre2* [Hiller *et al.*, 1996]); RSY281 (*MATα sec23-1 ura3-52 his4-619* [Hicke *et al.*, 1992]); SEY6210 (*MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801, suc2-Δ9* [Gentzsch and Tanner, 1996]); SEY6211 (*MAT a ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 ade2-101, suc2-Δ9* [Gentzsch and Tanner, 1996]); SEY6210 *pmt5::URA3*, SEY6210 *pmt6::URA3*, SEY6210 *pmt2::LEU2*; all *pmt* double and triple mutants used were progeny from crosses of SEY6210 *pmt3::HIS3 pmt4::TRP1* with SEY6211 *pmt1::URA3 pmt2::LEU2* (Gentzsch and Tanner, 1996). *TF1.8 (MATa ura3 leu2 his3 GAL1-PMT1-LEU2* (Gentzsch *et al.*, 1995) transformed with YEp352 or YEp352[PMT2]. Yeast were grown in YPD (1% yeast extract, 2% peptone [Difco, Detroit, MI], 2% dextrose) or synthetic media with the appropriate additions (Sherman, 1991).

Pulse-Chase Experiments

Cells were radiolabeled and lysed, and proteins were immunoprecipitated or precipitated with concanavalin A (ConA)-Sepharose as in described by Gillece *et al.* (1999). Lectin-precipitated samples were eluted with 1 M α-methyl mannoside in 20 mM Tris-HCl, pH7.5, 150 mM NaCl, 2 mM EDTA for 1 h at 30°C. Where indicated, samples were treated with 2 mU peptide *N*-glycosidase (PNGase) F (Roche Diagnostics Ltd., Lewes, United Kingdom) for 2 h at 37°C before electrophoresis.

[^{3}H]Mannose Labeling

The method used was modified from Orlean *et al.* (1991). Cells were grown to OD_{600} = 0.5 in full or selective medium with 0.6% sucrose as a carbon source, washed once in fresh medium labeled with 1 mCi of [^{3}H]mannose (Amersham, Arlington Heights, IL) per 1.5 OD_{600} of cells in 500 μl of medium for 90 min at the indicated temperature. Cells were lysed, and proteins were immunoprecipitated as above, resolved on 16% or 7.5% SDS-PAGE, and detected by fluorography.

Cell Fractionation

Microsomes for in vitro degradation assays were prepared from cells grown to OD_{600} = 1 as described by Pilon *et al.* (1997). Microsomes from *PMT* wild-type and mutant strains for the experiment shown in Figure 3A were prepared using the same method, but the sucrose gradient purification was omitted. Cytosol was prepared from WCG4a by liquid nitrogen lysis as described by Pilon *et al.* (1997).

Translocation and ConA Precipitation

In vitro translated, [^{35}S]methionine-labeled nonglycosylated alpha-factor precursor (pΔgpαf) or wild-type precursor (ppαf) were translocated into wild-type or *pmt* mutant microsomes for the indicated periods of time in the presence of ATP and an ATP-regenerating system (40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 1 mM ATP, 50 μM GDP-mannose) in B88 (20 mM HEPES, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM sorbitol) at 24°C. At the end of the incubation period, SDS was added to 1%, samples were heated to 95°C for 5 min, and mannosylated signal-cleaved Δgpαf precipitated with ConA-Sepharose (Pharmacia, Piscataway, NJ). Wild-type pαf was immunoprecipitated, de*N*-glycosylated with PNGase F (Boehringer), and subsequently precipitated with ConA-Sepharose.

ER Degradation Assay and Cross-Linking

ER degradation of Δgpαf was assayed at 24°C as described by Pilon *et al.* (1997). Briefly, 20-μl translocation reactions contained 2 μl of microsomes of OD_{280} = 30, B88, ATP, a regenerating system, and 2 μl of in vitro translated, ^{35}S-labeled pΔgpαf (500,000 cpm). Translocation reactions were incubated for 50 min at 24°C, and the membranes were washed twice in B88. Membranes containing Δgpαf were resuspended in B88 with ATP and the regenerating system, and degradation reactions were started by adding cytosol to 6 mg/ml final concentration in a 20-μl/reaction final volume. Degradation reactions were incubated at 24°C for the indicated periods

of time. At the end of the incubation, samples were precipitated with trichloroacetic acid (TCA) and analyzed after electrophoresis on 18% polyacrylamide, 4 M urea SDS gels with a Cyclone phosphorimager (Hewlett-Packard, Bracknell, United Kingdom). The Δgpαf and mannosylated Δgpαf (mΔgpαf) bands were quantified and expressed as percentages of Δgpaf at the beginning of the reaction (time 0). Cross-linking was performed with DSP as described by Pilon et al. (1997). Individual samples were 10× scaled up reactions as described above. Cross-linking was initiated after 10 min of incubation in the presence of ATP, the regenerating system, and 6 mg/ml yeast cytosol at 24°C.

RESULTS

Mutant Alpha-Factor Precursor Is O-Mannosylated in the ER

Misfolded secretory proteins are exported from the ER to the cytosol through the Sec61 channel after their signal sequences have been cleaved off (Römisch, 1999). We therefore asked whether misfolded proteins in the ER acquire a covalent modification that serves as an export signal during prolonged residence in the ER lumen. As substrate proteins we used [^{35}S]methionine-labeled, in vitro translated wild-type alpha-factor precursor (ppαf) or a mutant counterpart in which the three *N*-glycosylation acceptor sites in the proregion had been destroyed by site-directed mutagenesis (pΔgpαf) (Mayinger and Meyer, 1993; McCracken and Brodsky, 1996). In the presence of ATP and an ATP-regenerating system, both proteins can be translocated into yeast microsomes posttranslationally, resulting in signal cleaved mutant alpha-factor precursor (Δgpαf) and signal-cleaved, triply *N*-glycosylated wild-type alpha-factor precursor (3gpαf), respectively. Posttranslational protein import into the ER is essentially complete after a 10-min incubation at 24°C; nevertheless, we had observed previously that retrograde transport of a mutant secretory protein from the ER in vitro was more efficient after extended import reactions (K.R., unpublished data). We therefore suspected that the export substrate might be modified during its prolonged residence in the ER lumen. When we incubated yeast microsomes containing Δgpαf for 50 min at 24°C, we detected a small increase in molecular weight of a fraction of Δgpαf (mΔgpαf, Figure 1A, compare lanes 1, 2 and 5, 6). Concomitantly with the molecular weight shift, this form of Δgpαf acquired an affinity for the mannose-specific lectin ConA (mΔgpαf, Figure 1A, compare lanes 3, 4 and 7, 8). Because Δgpαf no longer contains any *N*-glycosylation sites, these data suggest that the protein might be *O*-mannosylated. In yeast, protein *O*-mannosylation is initiated in the ER lumen by the transfer of a single mannosyl residue from dolichol-P-mannose to specific serine and threonine residues of the substrate (Strahl-Bolsinger *et al.*, 1999). So far, *O*-mannosylation of alpha-factor precursor has not been reported. To investigate whether the wild-type precursor acquired the same modification, after incubation of yeast microsomes containing wild-type 3gpαf for 50 min at 24°C, we removed the *N*-glycans of the wild-type precursor with PNGase F before precipitation with ConA-Sepharose (Figure 1A, lanes 9–12). We found that none of de-*N*-glycosylated wild-type precursor (pαf) bound to the lectin (Figure 1A, lanes 11, 12 versus 13, 14). We conclude that the mutant, but not the wild-type precursor, is likely to be *O*-mannosylated after prolonged residence in the ER lumen in vitro.

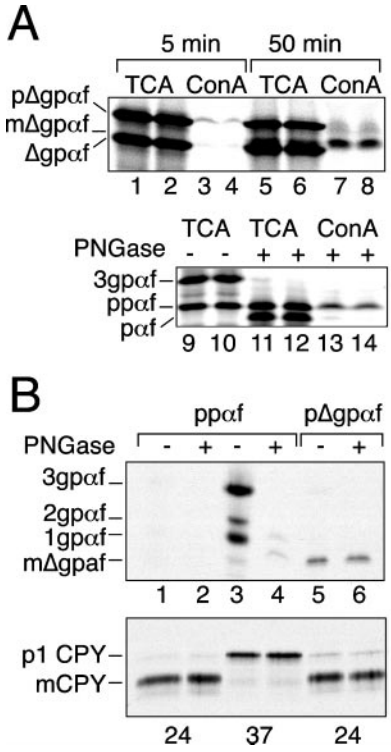

Figure 1. Mutant alpha-factor precursor is *O*-mannosylated in the ER. (A) Prolonged ER residence of Δgpαf in vitro results in *O*-mannosylation. In vitro translated, [^{35}S]methionine-labeled pΔgpαf (top) or ppαf (bottom) was translocated into wild-type yeast microsomes at 24°C in the presence of ATP and an ATP-regenerating system for the indicated periods of time. Duplicate samples were either TCA precipitated or membranes lysed and proteins precipitated directly with ConA-Sepharose (lanes 3, 4, 7, and 8) or first treated with PNGase and then precipitated with ConA-Sepharose as indicated (lanes 13 and 14). Proteins were analyzed by SDS-PAGE on 18% 4 M urea gels and autoradiography. Note that the nonspecific binding of ppαf to ConA-Sepharose is higher than that of pΔgpαf; this is an intrinsic property of ppαf. (B) Δgpαf is *O*-mannosylated in vivo. RSY281 (*sec23* ts) was labeled with [^{3}H]mannose for 90 min at the permissive (24°C) or at the restrictive (37°C) temperature. Cells were lysed, and CPY and alpha-factor precursor were immunoprecipitated. Alpha-factor immunoprecipitates shown in lanes 2, 4, and 6 were PNGase digested before SDS-PAGE and autoradiography. Note that a small amount of 1gpαf in lane 4 was refractory to PNGase digestion.

We next asked whether Δgpαf was also modified in intact yeast cells and whether again the modification was specific to the mutant precursor. To this end, we labeled intact yeast cells with [^{3}H]mannose for 90 min. Steady-state labeling is required to be able to detect mannose incorporation into secretory proteins (Orlean *et al.*, 1991). We found, however, that at steady state there was no detectable amount of wild-type alpha-factor precursor present inside the cells, even if the protein was overexpressed from a 2μ plasmid, because of its efficient processing and secretion. To compare wild-type and mutant alpha-factor precursor in vivo, we therefore performed the experiment in a strain that carries a temperature-sensitive mutation in Sec23p, a protein essential for ER-to-Golgi transport vesicle budding from the ER

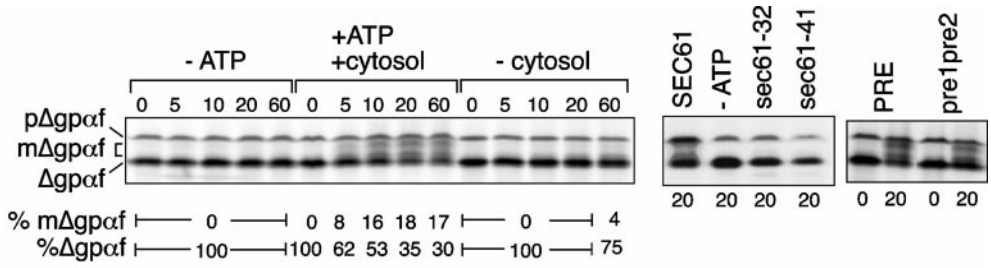

Figure 2. *O*-mannosylation of Δgpαf in vitro is stimulated by ATP and cytosol and requires export-proficient Sec61p. Left, in vitro translated, [^{35}S]methionine-labeled pΔgpαf was translocated into wild-type yeast microsomes, and the washed membranes were incubated at 24°C for the indicated periods of time (minutes) in the presence of ATP and an ATP-regenerating system, cytosol or both. At the end of the incubation period, samples were TCA precipitated and analyzed by SDS-PAGE and autoradiography. Δgpαf and mΔgpαf were quantified using a phophorimager and expressed as percentages of Δgpαf at 0 min. Note that a fraction of Δgpαf is degraded after 60 min, even in the absence of cytosol; the degree of cytosol-independent degradation varies between microsome preparations and is likely due to proteasomes associated with the cytoplasmic faces of the microsomes. Middle, samples contained *SEC61* wild-type or the indicated mutant microsomes and were incubated for 20 min in the presence of ATP and cytosol before TCA precipitation. Right, samples contained wild-type microsomes and were incubated with ATP and either wild-type (*PRE*) or proteasome-mutant (*pre1pre2*) cytosol for 0 or 20 min before TCA precipitation.

(RSY281; Hicke *et al.*, 1992). After [^{3}H]mannose labeling of RSY281-overexpressing ppαf at the permissive temperature (24°C), as in wild-type cells we were unable to detect intracellular wild-type alpha-factor precursor because of its rapid transport to the cell surface (Figure 1B, lanes 1 and 2). In contrast, after [^{3}H]mannose labeling of RSY281-expressing pΔgpαf at 24°C, we were able to immunoprecipitate a mannose-labeled form of the mutant precursor that was refractory to PNGase digestion and that migrated in the appropriate position for mΔgpαf (Figure 1B, lanes 5 and 6). To investigate whether the wild-type precursor could be *O*-mannosylated in vivo if its residence time in the ER was increased, we performed the [^{3}H]mannose labeling of RSY281 overexpressing ppαf at the restrictive temperature for vesicle budding from the ER (37°C). At 37°C, ER-to-Golgi transport in RSY281 ceases, as evident from the accumulation of the ER-specific form of carboxypeptidase Y in the cells (p1CPY, Figure 1B, bottom, compare lanes 24 and 37). Under these conditions we were indeed able to detect intracellular, mannose-labeled forms of wild-type alpha-factor precursor (Figure 1B, lane 3). The majority of the incorporated [^{3}H]mannose, however, could be removed from the wild-type precursor by PNGase F digestion, suggesting that the label had been primarily incorporated into the *N*-glycans of singly, doubly, and triply *N*-glycosylated wild-type precursor (1gpαf, 2gpαf, 3gpαf, Figure 1B, compare lanes 3 and 4). The small fraction of wild-type precursor whose position in the gel suggested that it had not been *N*-glycosylated after translocation into the ER, however, incorporated [^{3}H]mannose in a PNGase-resistant manner like the mutant precursor (mΔgpαf, Figure 1B, lanes 3 and 4). Our data suggest that in the absence of *N*-glycans alpha-factor precursor is *O*-mannosylated during prolonged residence in the ER.

O-*Mannosylation of Mutant Alpha-Factor Precursor In Vitro Is Stimulated by ATP and Cytosol and Requires Export-Proficient Sec61p*

Export of misfolded secretory proteins from the ER through the Sec61 channel and their degradation by proteasomes can be reconstituted in a cell-free system based on yeast microsomes and cytosol (McCracken and Brodsky, 1996). Incubation of wild-type yeast microsomes containing Δgpαf in the presence of ATP, an ATP-regenerating system, and 6 mg/ml wild-type yeast cytosol results in a disappearance of the Δgpαf band with a half life of ~12 min (Figure 2, left; McCracken and Brodsky, 1996). Concomitantly with the decrease in Δgpαf, we also observed a further molecular weight increase of a fraction of Δgpαf (mΔgpαf, Figure 2, left). This molecular weight shift was due to an increased mannosylation of Δgpαf under export conditions, as demonstrated by ConA precipitation of the newly appearing bands (mΔgpαf, Figure 4, top). Like Δgpαf export and degradation, increased *O*-mannosylation was dependent on the presence of ATP and cytosol (Figure 2, left). In contrast to Δgpαf degradation, however, *O*-mannosylation did not require functional proteasomes in the cytosol (Figure 2, right). Surprisingly, we found that *O*-mannosylation of Δgpαf in the ER was dramatically reduced in microsomes derived from export-deficient *sec61* mutant strains, suggesting that the *O*-mannosylation of this substrate required at least the initiation of its export to the cytosol (Figure 2, middle, compare *SEC61* to *sec61-32* and *sec61-41*; Pilon *et al.*, 1997). We conclude that, in vitro, conditions that promote misfolded protein export from the ER stimulate *O*-mannosylation of mutant alpha-factor precursor.

Pmt2p Is Responsible for Mutant Alpha-Factor Precursor O-*Mannosylation but Not Required for Its Degradation*

Based on the stimulation of Δgpαf *O*-mannosylation under ER export conditions and its *SEC61* dependence, we assumed that *O*-mannosylation was intimately linked to Δgpαf export through the Sec61 channel and that the *O*-mannosylated bands might be export intermediates. We therefore sought to identify the protein *O*-mannosyl transferase (Pmt) responsible for Δgpαf *O*-mannosylation. There are seven *PMT* genes in the *Saccharomyces cerevisiae* genome. For Pmt1p-Pmt4p and Pmt6p, mannosyl transferase activity has been demonstrated (Strahl-Bolsinger *et al.*, 1999). The individual transferases show distinct specificities toward their protein substrates (Gentzsch and Tanner, 1996, 1997). The active sites of the Pmts are predicted to be on the luminal face of the ER membrane (Girrbach *et al.*, 2000). The transferases Pmt1p and Pmt2p have been shown to act as

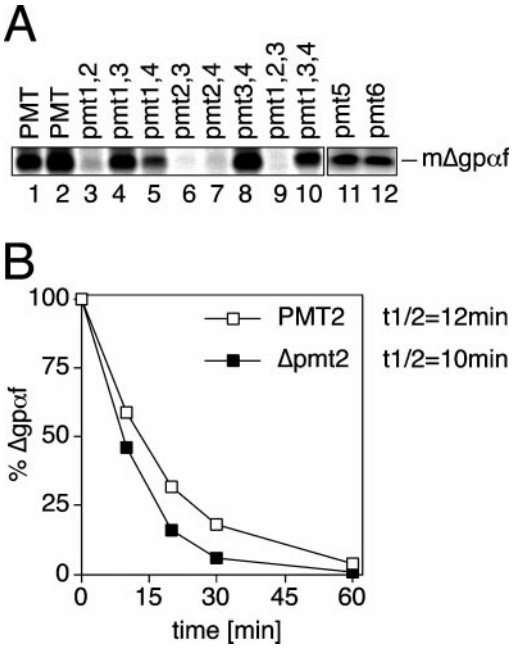

Figure 3. Pmt2p is required for Δgpαf mannosylation but not for its degradation. (A) Microsomes were prepared from *PMT* wild-type SEY6210 (lane 1) and SEY6211 (lane 2) and the indicated mutant strains. Equal amounts of in vitro translated, [^{35}S]methionine-labeled pΔgpαf were translocated into wild-type yeast microsomes at 24°C in the presence of ATP and an ATP-regenerating system for 50 min, followed by membrane lysis and ConA precipitation. Translocation efficiencies were similar for all microsome preparations tested. Lectin-bound material was analyzed by SDS-PAGE and autoradiography. (B) *PMT2* wild-type and Δ*pmt2* cells expressing pΔgpαf were pulse-labeled for 5 min with [^{35}S]methionine/cysteine and chased for the indicated periods of time. At each time point, cells were lysed and alpha-factor precursor immunoprecipitated and quantified by SDS-PAGE and phosphorimager analysis. The experiment was repeated twice.

heterodimers in vitro and in vivo (Girrbach *et al.*, 2000). We prepared microsomes from all viable *pmt1-4* double and triple mutants and from strains with individual deletions of *PMT5* and *PMT6* (Gentzsch and Tanner, 1996); we subsequently translocated pΔgpαf into wild-type and *pmt* mutant microsomes for 50 min at 24°C, lysed the membranes, and assessed Δgpαf mannosylation by precipitation with ConA-Sepharose. We found that all mutants with a deletion of *PMT2* were defective in Δgpαf *O*-mannosylation (Figure 3A). Deletion of *PMT2* on its own also resulted in lack of Δgpαf *O*-mannosylation, and no stimulation of Δgpαf *O*-mannosylation was seen in the presence of ATP and cytosol in Δ*pmt2* microsomes.

We then investigated whether *O*-mannosylation was required for Δgpαf degradation. We transformed *PMT2* wild-type and Δ*pmt2* strains with a plasmid expressing pΔgpαf and performed pulse-chase experiments. As shown in Figure 3B, we found that mannosylation by Pmt2p was dispensable for Δgpαf turnover and that, in fact, the half-life of Δgpαf was slightly reduced in the Δ*pmt2* strain, suggesting that *O*-mannosylation of Δgpαf interfered with its degradation (Figure 3B, half-time of Δgpαf in *PMT2* = 12 min, in Δ*pmt2* =10 min). This observation was confirmed in vitro where we observed a slightly reduced half-life of Δgpαf in *pmt2pmt4* mutant microsomes. The expression levels of pΔgpαf were similar in *PMT2* wild-type and Δ*pmt2* cells, but by quantitative immunoblotting we found that the increased turnover of Δgpαf in Δ*pmt2* cells resulted in a reduction to ~50% of intracellular Δgpαf compared with wild-type cells. We observed no effects of *PMT2* deletion on vesicular transport of wild-type or mutant alpha-factor precursor and CPY and no effects on alpha-factor secretion. Deletion of *PMT2* also did not affect the ER export and degradation of a mutant form of CPY, CPY*; nor did we observe *O*-mannosylation of CPY*. We conclude that Δgpαf is specifically *O*-mannosylated by Pmt2p in the ER and that this modification is not required for Δgpαf degradation.

O-Mannosylation Protects Mutant Alpha-Factor Precursor from Export to the Cytosol

The increased turnover of Δgpαf in Δ*pmt2* cells raised the possibility that mannosylation interfered with the degradation of Δgpαf. We therefore investigated the fate of mΔgpαf during a degradation time course in vitro. We incubated wild-type microsomes containing Δgpαf in the presence of ATP, an ATP-regenerating system, and 6 mg/ml wild-type cytosol for up to 60 min at 24°C and at each time point precipitated one aliquot with TCA and ConA-precipitated mΔgpαf from twice the amount of material. We found that over time Δgpαf was increasingly *O*-mannosylated, which resulted in an increased molecular weight close to the position on pΔgpαf on 18% polyacrylamide 4 M urea gels (Figure 4A, top). The highly *O*-mannosylated form was not subject to degradation, however, but accumulated during the time course of the reaction (Figure 4A, top).

To clarify whether mannosylation interfered with degradation of Δgpαf by cytosolic proteasomes, or whether the modification prevented export of mΔgpαf from the ER lumen to the cytosol, we performed protease protection experiments. At each time point during a degradation reaction. we transferred samples to ice and digested them with 0.1 mg/ml proteinase K for 20 min. We found that the mannosylated forms of Δgpαf were refractory to proteinase K digestion in contrast to pΔgpαf, which is associated with the cytosolic face of the microsomes and was protease sensitive (Figure 4A, bottom). In the presence of 0.1% Triton X-100, mΔgpαf was fully digested by proteinase K. Our data suggest that mΔgpαf resides in the lumen of the ER and, thus, that *O*-mannosylation of Δgpαf interferes with its export through the Sec61 channel to the cytosol.

We next asked whether overexpression of PMT2 would lead to retention of a higher proportion of Δgpαf in the ER. We found that to increase Pmt2p activity, we needed to co-overexpress *PMT1* and *PMT2*. Expression of *PMT2* from a 2μ plasmid in a strain that had *PMT1* integrated under control of the *GAL1* promoter resulted in a threefold increase of mannosyl-transferase activity in vitro (S.S., unpublished data). In microsomes derived from this strain, Δgpαf was maximally O-mannosylated with a half-life of <10 min (Figure 4B, top) in contrast to wild-type microsomes, in which the maximally *O*-mannosylated form appeared with a half-life of 20–30 min (Figure 4A, top, ConA). The proportion of Δgpαf that was *O*-mannosylated did not change significantly upon *PMT1/2* overexpression, however, and consis-

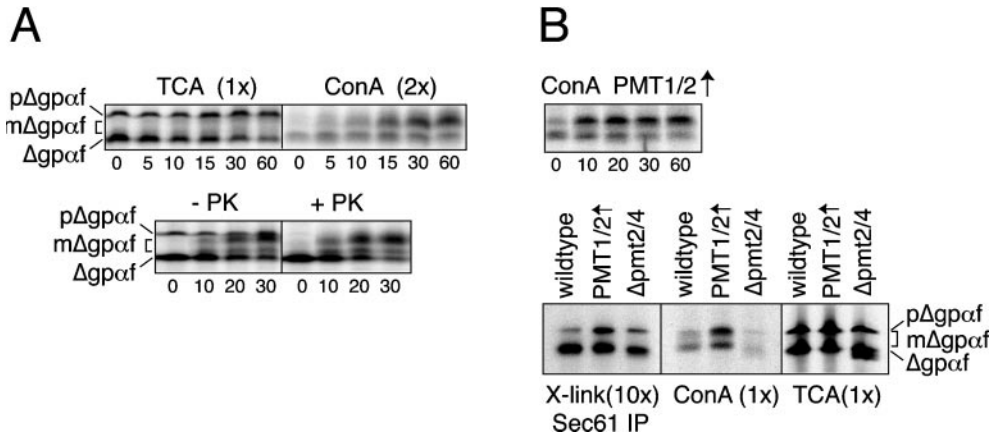

Figure 4. *O*-mannosylation protects Δgpαf from export to the cytosol. (A) Wild-type yeast microsomes containing Δgpαf were incubated in the presence of ATP, an ATP-regenerating system, and 6 mg/ml wild-type yeast cytosol at 24°C for the indicated periods (minutes). Top, At each time point, samples were either TCA precipitated or membranes lysed and mΔgpαf ConA precipitated; note that lectin precipitation was done from twice the amount of material as the TCA precipitation. Bottom, at each time point, samples were transferred to ice and either mock incubated (−PK) or incubated with 0.1 mg/ml proteinase K for 20 min (+PK) before TCA precipitation and gel electrophoresis. (B) Top, microsomes from *PMT1/2*-overexpressing cells containing Δgpαf were incubated as in A and mΔgpαf ConA precipitated. Bottom, wild-type, *PMT1/2*-overexpressing, and Δ*pmt2/4* microsomes containing Δgpαf were incubated the presence of ATP, an ATP-regenerating system, and 6 mg/ml wild-type yeast cytosol at 24°C for 10 min; proteins were cross-linked by the addition of DSP, membranes were lysed, and Sec61p and associated proteins were immunoprecipitated. Cross-links were cleaved with dithiothreitol before gel electrophoresis. ConA and TCA precipitates of 10% of the material used for cross-linking are shown in the middle and right. All samples were run on the same gel. Left panel was exposed 10× longer than the right panel. The reason for the faster migrating bands below Δgpαf in the Δ*pmt2/4* sample is unknown but specific to this strain.

tent with this observation there was no effect of turnover of Δgpαf (Figure 4, A and B, top, and data not shown).

We suspected that *O*-mannosylation might stabilize a nonproductive interaction of mΔgpαf with the Sec61 channel, which would thus lead to increased mannosylation if higher Pmt2 activity was present in the ER membrane. We investigated whether mΔgpαf could be cross-linked to Sec61p. The cross-linking efficiency of Δgpαf to Sec61p is low (1–2%; Pilon et al., 1997), and the maximal proportion of Δgpαf that was mannosylated in the ER was <20% (Figure 2). To maximize our chances for detecting mΔgpαf interacting with Sec61p, we performed the cross-linking experiment in microsomes derived from *PMT1/2*-overexpressing cells, in which most mΔgpαf is present as a single, highly mannosylated band that migrates close to the signal sequence containing pΔgpαf in our gel system (Figure 4A, bottom). Wild-type microsomes and microsomes derived from a Δ*pmt2/4* deletion strain, in which Δgpαf mannosylation is reduced (Figure 3A), were included as controls. Mutant alpha-factor precursor was translocated into the microsomes; the membranes were washed and incubated with ATP, an ATP-regenerating system, and 6 mg/ml cytosol for 10 min at 24°C before cross-linking was initiated. Membranes were lysed, and Sec61p and associated proteins were immunoprecipitated with affinity-purified Sec61 antibodies. Cross-links were cleaved with dithiothreitol before electrophoresis on 18% 4 M urea SDS gels. The amount of signal-cleaved Δgpαf associated with Sec61p was proportional to the amount of Δgpαf in the ER lumen and identical for wild-type and *PMT1/2*-overexpressing microsomes (Figure 4B, bottom, compare X-link and TCA). In wild-type and Δ*pmt2/4* microsomes, a small amount of cytosolic pΔgpαf was found cross-linked to Sec61p (Figure 4B, bottom). In *PMT1/2*-overexpressing microsomes, the intensity of this upper band was increased approximately threefold (Figure 4B, bottom, X-link). This increase was dependent on the presence of cross-linking reagent and Sec61 antibodies and corresponds well to the increased amount of maximally mannosylated Δspαf present in these microsomes (ConA, bottom, Figure 4B). Sec61p itself is not glycosylated, but a fraction of the Sec61 complex in the ER membrane is bound to the glycoprotein-containing Sec63 complex. We were therefore unable to determine the association of Sec61p with mΔgpαf by lectin precipitation. Because we observed no change in the amount of pΔgpαf associated with the cytoplasmic face of *PMT1/2*-overexpressing microsomes, however, we conclude that the upper band found associated with Sec61p in the cross-linking experiment in *PMT1/2*-overexpressing microsomes consists primarily of highly mannosylated Δgpαf. Interestingly, this interaction was specific for the highly mannosylated form of Δgpαf; the fast migrating mΔgpαf band could not be cross-linked to Sec61p (Figure 4B, bottom, ConA versus X-link).

Competition between N*-Glycosylation and* O*-Mannosylation in the ER*

Do *N*-linked and *O*-linked glycosylation compete in proteins other than alpha-factor precursor? *N*-glycosylation acceptor sites are well defined (N-X-S/T), but there is currently no known consensus acceptor site for *O*-linked mannosylation; thus, the general proximity or the degree of overlap between the two types of acceptor sites could not be determined by studying the available protein data bases (Strahl-Bolsinger et al., 1999). We therefore addressed the question experimentally. We metabolically labeled wild-type yeast with [^{35}S]methionine/cysteine for 10 min at 30°C in the absence or presence of the *N*-glycosylation inhibitor tunicamycin. Cells were lysed, and a protein with five *N*-glycosylation acceptor sites, protein disulfide isomerase (PDI), was immunoprecipitated. Immunocomplexes were digested with PNGase F or mock incubated, followed by precipitation with the mannose-specific lectin ConA. In the absence of tunicamycin, PDI is heterogeneously *N*-glycosylated on four or

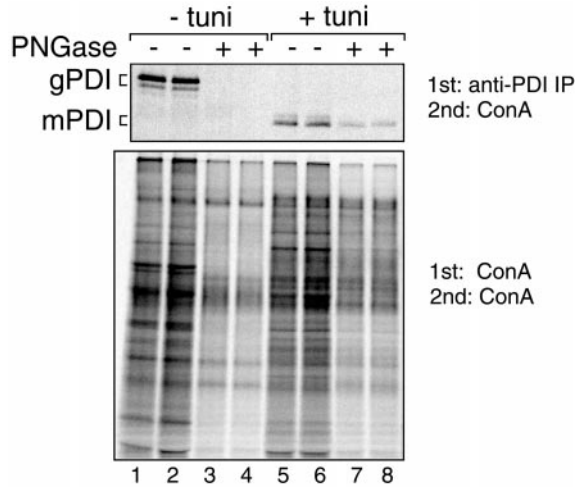

Figure 5. Competition between *N*-glycosylation and *O*-mannosylation in the ER. Wild-type cells were incubated in the presence (+) or absence (−) of 10 μg/ml tunicamycin (tuni) for 20 min before radiolabeling for 10 min with [^{35}S]methionine/cysteine. Cells were lysed and proteins either immunoprecipitated (IP) with a polyclonal anti-PDI antiserum (top) or ConA precipitated (bottom). Precipitates were digested with PNGase or mock incubated, followed by a second round of ConA precipitation. Proteins were eluted from ConA-Sepharose with α-methyl mannoside before electrophoresis on 10% (top) and 12.5% gels (bottom).

five of its five acceptor sites and therefore binds to ConA (Figure 5, top, lanes 1 and 2; gPDI). PNGase treatment removes the *N*-glycosyl side chains and results in complete loss of affinity of PDI for the lectin (Figure 5, top, lanes 3 and 4). PDI isolated from tunicamycin-treated cells migrates at a lower mobility, consistent with lack of *N*-glycosyl side chains, but a fraction of non-*N*-glycosylated PDI nevertheless binds to ConA (Figure 5, top, lanes 5 and 6; mPDI). Digestion of PDI isolated from tunicamycin-treated cells with PNGase does not alter its mobility on the gel or its ability to bind to ConA (Figure 5, top, lanes 7 and 8; mPDI). Our data suggest that like alpha-factor precursor PDI can be *O*-mannosylated in the absence of *N*-glycosylation.

How general is the competition of *N*-glycosylation and *O*-mannosylation in the ER? To answer this question, we labeled wild-type cells in the presence or absence of tunicamycin, as above, and precipitated glycoproteins with ConA-Sepharose. Lectin precipitates were PNGase treated or mock incubated and subsequently subjected to a second round of ConA precipitation. We found that an increased proportion of glycoproteins isolated from tunicamycin-treated cells bound to ConA-Sepharose in a PNGase-resistant manner (Figure 5, bottom, compare lanes 3, 4 to 7, 8; 35% increase in total signal). Our data confirm that the competition of *N*- and *O*-linked glycosylation that we documented for two specific proteins, alpha-factor precursor and PDI, is a general phenomenon in the ER that affects a large number of proteins.

DISCUSSION

We have shown that a misfolded secretory protein, Δgpαf, is specifically *O*-mannosylated in the ER lumen by Pmt2p. This *O*-mannosylation is stimulated in vitro by the presence of ATP and cytosol and requires functional Sec61p, suggesting that *O*-mannosylation is linked to the initiation of misfolded protein export from the ER through the Sec61 channel. The *O*-mannosylated fraction of Δgpαf, however, is not transported to the cytosol but remains protease protected in the ER lumen and is therefore stable. In addition, a fraction of mΔgpαf is associated with Sec61p. Our work demonstrates that there is a limited window of time during which misfolded proteins can be removed from the ER before they acquire inappropriate modifications that can interfere with protein disposal through the Sec61 channel.

The yeast ER contains several *O*-mannosyl transferases with distinct substrate specificities (Gentzsch and Tanner, 1996, 1997). The basis of substrate recognition has been difficult to characterize; the correlation between data derived from peptide substrates and protein substrates is poor, suggesting that protein conformation contributes significantly to the recognition of *O*-mannosyl acceptor sites by Pmts (Strahl-Bolsinger *et al.*, 1999). Wild-type alpha-factor precursor is *N*-glycosylated at three sites in its proregion (Kurjan and Herskowitz, 1982). Conversion of the asparagine residues in the *N*-glycosyl acceptor sites to glutamine residues results in a protein that is recognized in the ER as misfolded and subsequently transported to the cytosol for disposal by the proteasomes (Mayinger and Meyer, 1993; McCracken and Brodsky, 1996). Most serine and threonine residues in alpha-factor precursor that could serve as *O*-mannosyl acceptors are clustered around the *N*-glycosylation sites (Kurjan and Herskowitz, 1982). Our finding that only non-*N*-glycosylated alpha-factor precursor is *O*-mannosylated suggests that access to the *O*-mannosyl acceptor sites in the *N*-glycosylated precursor is sterically blocked by the *N*-glycans. Alternatively, *N*-glycosylation may introduce a conformational change into the gpαf proregion that prevents its *O*-mannosylation by Pmt2p.

N-glycosylation of wild-type alpha-factor precursor is rapid and efficient and is initiated during import into the ER (Figure 1A, K.R. unpublished data). By contrast, *O*-mannosylation of Δgpαf is inefficient and slow (Figure 1A). The fact that significant O-glycosylation occurs only after prolonged incubation of yeast microsomes containing Δgpαf at physiological temperature suggests that it is a posttranslocational event (Figure 1A). Similarly, a polytopic ER membrane protein fused to a degradation signal, Deg1-Hmg1p, is only glycosylated under conditions that prevent its degradation and therefore prolong its residence in the ER (Wilhovsky *et al.*, 2000); the nature of the glycosylation, however, was not identified in this case. The addition of ATP and cytosol, which promote targeting of Δgpαf to the Sec61 channel for export, stimulates Δgpαf *O*-mannosylation (Figure 2). This increased *O*-mannosylation is dependent on export-competent Sec61 channels, suggesting that the substrate may be in contact with the export channel when it is mannosylated (Figure 2). This was confirmed by cross-linking mΔgpαf to Sec61p (Figure 4B). The Pmts in the ER are polytopic transmembrane proteins with large ER-luminal domains containing the active sites (Girrbach *et al.*, 2000). It is therefore conceivable that Pmts modify membrane-associated substrates more efficiently; Pmts may even be located in close proximity to the translocon, which would ensure that

wild-type secretory proteins with *O*-mannosyl acceptor sites are modified efficiently and without interference from protein folding during entry into the ER through the Sec61 channel. This notion is supported by the observation that in vitro only short peptides and partially hydrolyzed proteins are modified by Pmts, but fully folded proteins are not substrates for *O*-mannosylation ((Strahl-Bolsinger *et al.*, 1999; S.S., unpublished data). In their preference for unfolded substrates, Pmts resemble glucosyl transferase in the ER lumen, which specifically tags unfolded proteins (Parodi, 2000).

Because alpha-factor precursor is not normally a substrate for *O*-mannosylation, it is possible that its orientation in the translocation channel during import is incompatible with *O*-mannosylation but that it exposes the appropriate sites during initiation of export (Figures 1 and 2). An alternative explanation may be that import of pΔgpαf into the ER is extremely rapid and efficient, whereas export proceeds with much slower kinetics and may therefore result in prolonged exposure of *O*-mannosyl acceptor sites in the appropriate vicinity of Pmt2p. A third possibility is that the Sec63 complex associated with the Sec61 channel during posttranslational pΔgpαf import into the ER interferes with access of Pmt2p to proteins in the translocon (Panzner *et al.*, 1995). The Sec63 complex is not required for misfolded protein export from the ER and thus most likely absent from Sec61 channels engaged in export, which may allow mannosylation of export substrates by Pmt2p (Pilon *et al.*, 1997).

We have demonstrated that mΔgpαf remains in the ER lumen, which suggests that the *O*-mannosyl moieties prevent export through the Sec61 channel (Figure 4). *O*-mannosylation is largely Sec61 dependent, suggesting that the modification occurs after the export substrate has made contact with the Sec61 channel (Figures 2 and 4B); *O*-mannosylation therefore aborts export at a posttargeting step (Figure 4B). Protease digestion of microsomes containing mannosyl-Δgpαf did not result in any loss of signal or in the occurrence of partially protease-protected intermediate bands (Figure 4A); because our gel system resolves size differences of <10 amino acids, our data suggest that mΔgpαf is still fully contained inside the ER lumen. It is so far unknown whether proteins insert into the Sec61 channel for export with their N or their C termini first. The lack of protease-protected, *O*-mannosylated export intermediates combined with the fact that all serine and threonine residues of Δgpαf that can serve as *O*-mannosyl acceptor sites are contained in the proregion of the protein—the first is close to the extreme N terminus of the signal-cleaved protein at amino acid position 5—may suggest that the N terminus of Δgpαf interacts with the Sec61 channel first and that *O*-mannosylation of the N terminus aborts its subsequent insertion into the channel (Figure 4; Kurjan and Herskowitz, 1982). Given that misfolded proteins with several *N*-glycans attached, such as CPY*, can be exported through the Sec61 channel to the cytosol, we consider it unlikely that *O*-mannosylation sterically interferes with protein transport through the channel (Plemper *et al.*, 1997). *O*-mannosylation may, however, introduce a conformational change into or increase the rigidity of the alpha-factor precursor proregion and thus abort export of the protein. One intriguing alternative explanation for the effect of *O*-mannosylation on mutant alpha-factor precursor export is that the *O*-mannosyl acceptor sites in Δgpαf precursor overlap with an essential recognition motif for misfolded protein transport through the Sec61 channel to the cytosol, which may be recognized by the so far uncharacterized machinery that drives misfolded protein export from the ER.

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