Activity of recycling Golgi mannosyltransferases in the yeast endoplasmic reticulum

Leena Karhinen¹ and Marja Makarow¹,²,*

¹Program in Cellular Biotechnology, Institute of Biotechnology, University of Helsinki, Viikinkaari 9, 00710 Helsinki, Finland
²Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland

*Author for correspondence (e-mail: marja.makarow@helsinki.fi)

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Summary
In yeast primary N- and O-glycans are attached to proteins in the endoplasmic reticulum (ER), and they are elongated in the Golgi. Thus, glycan extension by Golgi enzymes has been taken as evidence for arrival of a protein in the Golgi. Two α1,6-mannosyltransferase activity-containing multiprotein complexes have been reported to recycle between the Golgi and the ER, but since resident ER proteins are not Golgi-modified, Golgi enzymes were not thought to function in the ER. Here we show that when protein exit from the ER was blocked in COPII-defective yeast mutants, the N-glycans of vacuolar carboxypeptidase Y and a set of unidentified glycoproteins were decorated with an α1,6-mannose residue, normally added in the Golgi by Och1p. Immunofluorescent staining demonstrated that Och1p accumulated in the ER under these conditions. Concomitantly, primary O-glycans of a secretory protein were extended, apparently by the medial Golgi transferase Mnt1p. Similar O-glycan extension occurred in wild-type cells when an HDEL-tagged protein was allowed to encounter glycosyltransferases in the Golgi during recycling between ER and Golgi. Golgi-specific glycosylation in the ER was reduced when Golgi-to-ER traffic was blocked, confirming that glycan extension in the ER was mainly due to recycling, rather than newly synthesized transferases.

Key words: Yeast, ER, Golgi, Glycosyltransferases, Activity, Recycling

Introduction
In yeast and mammalian cells, the pre-assembled primary N-glycan consisting of two N-acetylgalactosamine, nine mannose and three glucose residues is transferred from dolichol pyrophosphate to an asparagine residue of a consensus tripeptide, usually during translocation of the polypeptide into the lumen of the endoplasmic reticulum (ER). Thereafter the biosynthetic pathways of N-glycans differ in these two organisms. In S. cerevisiae, the glucose residues and only one mannose are removed in the ER. Once the glycoprotein reaches the Golgi, one α1,6-linked mannose residue is added to the core glycan by Och1p. Thereafter the N-glycans of proteins to be externalised are extended with a linear α1,6-linked mannose homopolymer by two enzyme complexes. The one acting first is composed of Mnn9p and Van1p (M-Pol I), and the other of Mnn9p, Anp1p, Mnn10p, Mnn11p and Hoc1p (M-Pol II) (Jungmann and Munro, 1998; Jungmann et al., 1999). Many of these residues then obtain branches of one or two α1,2-mannose residues, added by Mnn2p and Mnn5p, respectively. Mnn1p terminates the branches with single α1,3-mannose residues. Mnn4p and Mnn6p add phosphomannose residues to inner α1,2-mannose residues. The total number of mannose residues of an N-glycan can exceed two hundred (Munro, 2001). The N-glycans of many proteins of organelles, like vacuolar carboxypeptidase Y (CPY), do not acquire the α1,6-mannose homopolymer, and obtain only a few more mannose residues, including α1,6-mannose added by Och1p (Stevens et al., 1982).

The protein-bound O-glycans of S. cerevisiae consist of linear arrays of up to five mannose residues. Their assembly is initiated in the ER by protein mannosyltransferases Pmt1p and Pmt2p on serine and threonine residues of newly translocated proteins (Strahl-Bolsinger et al., 1993; Lussier et al., 1995a). Then, an α1,2-linked residue is added, and to it another one by α1,2-mannosyltransferase Mnt1p in the Golgi (Haselbeck and Tanner, 1993; Häusler et al., 1992; Lussier et al., 1995b). Finally, two successive α1,3-linked mannose residues are added by Mnn1p, the same transferase that terminates the branches of N-glycans.

The Golgi glycosyltransferases are type 2 transmembrane proteins with a short cytosolic N-terminal fragment. They have been thought to be resident proteins of Golgi subcompartments. Och1p and the M-Pol I and M-Pol II complexes are believed to reside in the cis-Golgi (Jungmann and Munro, 1998; Jungmann et al., 1999), and Mnn1p in the medial/trans-Golgi (Lussier et al., 1995b; Harris and Waters, 1996). Thus, decorations by these enzymes have been generally taken as an indication of arrival of the substrate protein in the Golgi. As resident ER proteins lack Golgi-specific glycan decorations, newly synthesized transferases en route to the Golgi have been thought not to function in the ER. However, recently it was shown that components of M-Pol I and M-Pol II recycle back and forth between the Golgi and the ER. The complexes were found to be incorporated into COPII and COPI vesicles in vitro (Todorow et al., 2000). Exit of membrane-bound and soluble proteins from the ER occurs in vesicles whose cytosolic face is covered with the COPII coat consisting of four structural proteins, whereas Golgi-derived...
vesicles recycling proteins back to the ER are covered with the COPI coat assembled from an unrelated set of seven proteins (Barlowe, 1998). We show here that Golgi glycosyltransferases were able to extend N- and O-glycans on newly synthesized exocytic proteins, which were blocked in the ER in COPII-defective mutants. Relocation of Och1p from the Golgi to the ER in COPII-defective mutants was demonstrated by indirect immunofluorescence. Our data suggest that O-glycosylating enzymes travel back and forth between the Golgi and the ER, and that both they, and Och1p, do function in the ER when allowed to accumulate there together with substrate proteins.

Materials and Methods

Strain construction

Yeast cells were grown in YPD medium containing 2% glucose, or SC medium lacking appropriate amino acids or nucleotides and containing 2% glucose, unless otherwise stated. Transformations were done with the lithium acetate method (Hill et al., 1991). For genotypes of yeast strains see Table 1. Strains H606 and H610 were constructed by transforming strains H1 and H4, respectively, with the integrative plasmid pKTH4628. To construct this plasmid, the sec23-1 leu2-3,112 ura3-52 his3-11,15, leu2-3,112 trpl-1, ade2-1, can1-100 gene was synthesized with PCR with oligonucleotide A (GCT TAT ACC CCA), containing a sec13-1 leu2-3,112 suc2-99 gal2 URA3::HISP150Δ-β-lactamase sequence. The PCR product was ligated into an SCW4 gene by PCR from the genomic DNA of strain H1. The 5′ primer was C1819 (TTC AAT GCA TCT CTC TAA CTT AAC ATG), containing an Ndel site and resulting in conversion of the second amino acid from Arginine to Histidine. The 3′ primer was C1820 (TTG ATG ATG ATG ATG TGT ATG TAT AAT GGA), containing a HindIII site and a pentahistidine-coding sequence. The PCR product was ligated into an Ndel site of plasmid pKTH4700 (Paunola et al., 2001), creating pKTH4934. The XbaI/Nhel fragment of pKTH4934 containing the SCW4 gene was ligated into the XbaI site of the integrative yeast vector pFL26, resulting in plasmid pKTH4939.

Table 1. Yeast strains

| Strain | Genotype | Reference/source |
|--------|----------|------------------|
| H1 | (SEy2101a) MATa ade2-101 ura3-52 leu2-3,112 suc2-99 gal2 | R. Schekman, University of California, Berkeley, CA, USA |
| H4 | (mBY12-6D) MATet,sec18-1 trpl-1,188,189 leu2-3,112 ura3-52 his3-11,15 | R. Schekman |
| H230 | (HMSF163) MATa sec13-1 | Novick et al., 1980 |
| H388 | (HMSF190) MATa sec23-1 | Novick et al., 1980 |
| H245 | (W303-1A) Mata, ura3-1, his3-11,15, leu2-3,112, trpl1-1, ade2-1, can1-100 | K. Kučerová and J. Thorner, University of California, Berkeley, CA, USA |
| H335 | MATa ade2-101 ura3-52 leu2-3,112 suc2-99 gal2 URA3::HISP150Δ-β-lactamase | Simonen et al., 1994 |
| H480 | (RSY640) Mata sec23-1 leu2-3,112 ura5-2 | R. Schekman |
| H481 | (RSY282) MATa sec23-1 ura3-52 ura3-52 | R. Schekman |
| H606 | MATa ade2-101 ura3-52 leu2-3,112 suc2-99 gal2 URA3::HISP150Δ-β-lactamase-HDEL | This study |
| H610 | MATet sec18-1 trpl-1,188,189 leu2-3,112 ura3-52 his3-11,15, leu2-3,112 trpl1-1, ade2-1, can1-100 | This study |
| H830 | (RH 239-SC) MATa sec21-1 ura3 his4 leu2 lys2 bar1-1 | H. Riezman, University of Geneva, Geneva, Switzerland |
| H1065 | MATa sec13-1 leu2-3,112 ura3-52 SEC23-1 ura3-52 HIS3-11,15, leu2-3,112 trpl1-1, ade2-1, can1-100 | Fatal et al., 2002 |
| H1488 | MATa ade2-101 ura3-52 leu2-3,112 suc2-99 gal2 URA3::OCH1-HA LEU2::MNN1-myc | This study |
| H1489 | MATa sec13-1 his3-11,15, ura3-52 ura3-52 trpl1-1,188,189 URA3::OCH1-HA LEU2::MNN1-myc | This study |
| H1490 | MATa sec23-1 ura3-52 ura3-52 GAL2 URA3::OCH1-HA LEU2::MNN1-myc | This study |
| H1495 | MATa ade2-101 ura3-52 leu2-3,112 suc2-99 gal2 LEU2::SCW4-His6 | This study |
| H1496 | MATa sec23-1 ura3-52 leu2-3,112 ura3-52 gal2 LEU2::SCW4-His6 | This study |
| H1497 | MATa sec21-1 ura3-52 leu2-3,112 his3-11,15, leu2-3,112 bar1-1 LEU2::SCW4-His6 | This study |
| H1628 | MATa sec18-1 trpl-1,188,189 leu2-3,112 ura3-52 his3-11,15, leu2-3,112 bar1-1 | This study |
| H1691 | (BY7472) MATa his3-11,15, leu2Δ0 lys2Δ0 ura3Δ0 VGL083::kanMX4 | EUROSCARF, Johann Wolfgang Goethe – University Frankfurt, Germany |
| H1791 | Mata sec3-1,188,189 leu2-3,112 ura3-52 URA3::OCH1-HA LEU::cyth(5)-opsin | This study |

Other methods

Metabolic labelling with [35S]methionine/cysteine (1000 Ci/mmol) and [3H]mannose (11.5 Ci/mmol; Amersham International, Buckinghamshire, UK), as well as immunoprecipitation with antisera against Hsp150 (1:400, 2 hours), β-lactamase (1:400, 2 hours), CPY (1:400, 2 hours), and monoclonal antibody against pentahistidine (Qiagen, USA; 1:400, overnight), as well as SDS-PAGE in 8% gels were described before (Paunola et al., 1998). Quantitation of the radioactive signals was performed using TINA 2.0 software. Lectin precipitation was performed with 0.5% Concanavalin A-Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) in 20 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl and 2% Triton X-100, overnight at 4°C. The lectin beads were released by boiling for 3 minutes in 1% SDS. Indirect immunofluorescent staining was according to Suntio et al. (Suntio et al., 1999), with a 1:100 dilution of antiserum against the EP epoxide (Santa Cruz, USA), or of monoclonal antibody against opsins (Adamus et al., 1991). In double staining anti-mouse-Alexa488 and anti-rabbit-Alexa568 (Molecular Probes, USA) were used as secondary antibodies. DAPI, CHX and NaN3 (Sigma, USA) were used.
used in final concentrations of 2 μg/ml, 100 μg/ml and 10 mM, respectively.

Results
Pro-CPY acquires α1,6-mannose in the ER
Pro-carboxypeptidase Y is synthesized as a 59 kDa precursor, which upon translocation into the ER acquires primary N-glycans, resulting in the p1 form of 67 kDa. Once pro-CPY arrives in the cis-Golgi, the glycans are decorated by Och1p with one α1,6-mannose residue, which can be immunologically recognized, followed by addition of a few more mannose residues to yield the p2 form (69 kDa). This intermediate is then targeted to the vacuole, where the pro-fragment is cleaved off, resulting in mature catalytically active CPY (m; 62 kDa) (Stevens et al., 1982). We used CPY as a reporter to study whether Och1p activity could be detected in vivo in the ER. ER exit of newly synthesized pro-CPY was blocked by preventing the assembly of the COP II coat, and hence budding of pro-CPY-containing vesicles from the ER membrane. This was achieved by using sec13-1 and sec23-1 mutants, where the structural proteins Sec13p and Sec23p of the COP II coat are defective at 37°C (Novick et al., 1980). The cells were preincubated at 37°C to impose the ER exit block, pulse-labelled with [35S]-methionine/cysteine and chased at 37°C. Immunoprecipitation with antiserum against CPY revealed in both mutants the ER-specific form of 67 kDa (Fig. 1A, lanes 1 and 4), and small amount of the untranslocated form of 59 kDa (pre). When the sec13-1 mutant was labelled and chased at permissive temperature 24°C, mature CPY was detected (m, lane 2), together with a small amount of the Golgi form p2. Next, CPY antiserum was released from parallel immunoprecipitates, and re-immunoprecipitation was performed with an antiserum which specifically recognizes the α1,6-mannose residue that is normally added by Och1p in the Golgi (Franzusoff and Schekman, 1989). Pro-CPY from sec13-1 (Fig. 1B, lane 1) and sec23-1 (lane 4) mutants could be re-precipitated, as well as mature CPY (lane 2). Quantitation showed that 44% and 39% of pro-CPY could be immunoprecipitated with α1,6-mannose antiserum as compared to CPY antiserum from strains sec13-1 and sec23-1, respectively (compare lanes 1 and 4 in panels A and B), and 46% of mature CPY (compare lanes 2 in panels A and B).

To rule out the possibility that the α1,6-mannose decoration of pro-CPY we observed in the ER-blocked molecules could be due to other enzymes than Och1p, we repeated the above experiment using a wild-type strain from which the OCH1 gene had been deleted. Mature CPY was immunoprecipitated with CPY antiserum, but none of it was re-immunoprecipitated with the α1,6-mannose-antiserum (Fig. 1C). The parental strain H245 gave similar results as shown in lanes A2 and B2 for the sec13-1 mutant at permissive temperature 24°C (not shown). We conclude that recycling Och1p molecules were under restrictive conditions in the sec13-1 and sec23-1 mutants trapped in the ER, where they decorated newly synthesized pro-CPY molecules with α1,6-mannose.

To study whether newly synthesized Och1p molecules, which had not yet left the ER when the COP II pathway was blocked, had a role in α1,6-mannose decoration of pro-CPY, the above experiment was performed in a sec18-1 mutant. In these cells at 37°C pro-CPY, together with newly synthesized Och1p, was allowed to leave the ER lumen but was blocked in transport vesicles which could not fuse with the Golgi due to non-functional N-ethyl-maleimide-sensitive factor NSF (Sec18p). At the same time, recycling Golgi transferases were blocked in a different compartment, namely Golgi-derived vesicles. Golgi-to-ER transport was inhibited, because NSF is required for all vesicle fusion events of the secretory pathway. Under these conditions, only 4% of pro-CPY was recognized by the α1,6-mannose antiserum (Fig. 1B, lane 3), as compared to the amount immunoprecipitated with CPY antiserum (Fig. 1A, lane 3). Thus, apparently mostly recycling Och1p molecules, rather than their de novo synthesized counterparts, were responsible for α1,6-mannose decoration of pro-CPY in the ER.

Fig. 1. α1,6-Mannosylation of pro-CPY in the ER. Sec13-1 (lanes 1 and 2; H230), sec18-1 (lanes 3; H4), sec23-1 (lanes 4; H238) and Δoch1 (panel C, H1691) were preincubated for 15 minutes, pulse-labelled with [35S]methionine/cysteine for 5 minutes and chased in the presence of CHX for 30 minutes (A,B) or 20 minutes (C) at the indicated temperatures. The cell lysates were subjected to immunoprecipitation with CPY antiserum alone, or first with CPY antiserum followed by re-immunoprecipitation with antiserum against α1,6-mannose residue as indicated, followed by SDS-PAGE analysis. The untranslocated form (pre), ER form (p1), Golgi form (p2) and mature CPY (m) are indicated on the right.

Other glycoproteins acquiring α1,6-mannose residues in the ER
Next, we searched for α1,6-mannose decoration in the ER of other glycoproteins. Sec13-1, sec23-1 and Δoch1 strains were 35S-labeled after 15 minutes pre-incubation at 37°C to impose the ER exit block in the COP II mutants. The cells were lysed and subjected to concanavalin A precipitation, to collect the newly synthesized N-glycosylated proteins. One half of the samples were subjected directly to SDS-PAGE analysis (Fig. 2, uneven lanes). The other half was released from the concanavalin A-Sepharose beads and immunoprecipitated with α1,6-mannose antiserum (even lanes). In the COP II mutants, a subset of similar bands as detected by concanavalin A precipitation alone, was recognized by the α1,6-mannose antiserum, suggesting that they were decorated by Golgi-specific Och1p, though arrested in the ER. In the case of the Δoch1 deletion strain the quantity of the proteins precipitated by the lectin (lane 5) was much less than in the COP II mutants and migrated in the gel differently, apparently due to lack of
extension of primary N-glycans. This set of proteins appeared not to be recognized by \( \alpha \)-1,6-mannose antiserum (lane 6).

**Extension of O-glycans in the ER**

Next we studied whether Golgi-specific O-glycan extension could take place in the ER. To this end we used a fusion protein consisting of a 321 amino acid long N-terminal fragment of the secretory yeast glycoprotein Hsp150 (Hsp150Δ) plus the mature portion of \( E. \ coli \) β-lactamase (Hsp150Δ-β-lactamase). The Hsp150Δ fragment consists of a signal peptide, subunit I (54 a.a.) and 11 repeats of a 19 amino acid peptide (Simonen et al., 1994). The signal peptide is lost upon ER translocation, and subunit I cleaved in the late Golgi at a Kex2p recognition site (Simonen et al., 1994). Twenty percent of the amino acids of the Hsp150Δ portion are serines and threonines, most of which normally are O-glycosylated, whereas the β-lactamase portion has no, or very few O-glycans (Jämsä et al., 1995; Suntio et al., 1999; Holkeri et al., 1996). The entire fusion protein lacks N-glycosylation sites (Russo et al., 1992). The O-glycans of mature authentic Hsp150, secreted to the medium from normal cells, are di-, tri-, tetra- and pentamannosides, occurring in the ratio of 4:1:1:1 (Jämsä et al., 1995). As the Hsp150Δ fragment carries most of the glycans of Hsp150, mature Hsp150Δ-β-lactamase probably has a similar set of O-glycans as mature Hsp150.

We pulse-labelled the sec13-1 mutant for 5 minutes after 15 minutes pre-incubation at 37°C to block Hsp150Δ-β-lactamase in the ER. We confirmed recently biochemically and morphologically that Hsp150Δ-β-lactamase does not leave the ER at 37°C in the sec13-1 mutant (Fatal et al., 2002). Immunoprecipitation with β-lactamase antiserum revealed in the lysate the cytoplasmic form of 66 kDa plus the primary O-glycosylated ER form of 110 kDa (Fig. 3, lane 1) (Paunola et al., 1998; Fatal et al., 2002). No protein was found in the medium (lane 5). The Hsp150 signal peptide confers slow post-translational translocation, and this is why some cytosolic form can be detected after a 5 minutes pulse (Paunola et al., 1998). With increasing chase time, the cytosolic form disappeared with concomitant increase of the glycosylated form, the migration of which became slower. After a chase of 60 minutes all of the cell-associated form migrated like the mature 145 kDa protein (lane 4). A small amount of Hsp150Δ-β-lactamase appeared in the medium (lanes 7 and 8), serving as a marker for the fully glycosylated protein. Next we repeated the above experiment by labelling with \[^{3}H\]mannose instead of \[^{35}S\]methionine/cysteine. After a 5 minutes pulse, no signal was detected from the immunoprecipitated cell lysate (lane 9). After chase for 10-60 minutes, protein variants could be detected, which comigrated with the respective \[^{35}S\]-labeled forms, and were more and more \[^{3}H\]-labeled (lanes 10-12). As
expected, the untranslocated form of 66 kDa could not be labelled with [3H]mannose. In the sec18-1 mutant Hsp150Δ-β-lactamase reached only a size of 120 kDa after an hour of chase (lanes 13-16), indicating that it had incomplete O-glycans. This must have been due to failure of the transferase-carrying Golgi-derived vesicles to fuse with the ER, while Hsp150Δ-β-lactamase accumulated in ER-derived vesicles. Thus, the reporter protein indeed acquired more mannose residues upon prolonged residence in the ER when exit was blocked in the sec13-1 mutant. Since the ER form of Hsp150Δ-β-lactamase contains subunit I, but the secreted 145 kDa form does not, we suggest that the O-glycans of the 145 kDa form of Hsp150Δ-β-lactamase, retained in the ER in the sec13-1 mutant, had been extended up to tri-mannosides, but not further.

O-glycosylation of HDEL-tagged Hsp150Δ-β-lactamase

If mannosyltransferases of the Golgi can extend O-glycans in the ER, once they recycle from the Golgi to the ER and remain there together with the substrate protein, then conversely, they should perform the same extensions in the Golgi, if the substrate protein recycles between the ER and the Golgi. To study this, we expressed an HDEL-tagged Hsp150Δ-β-lactamase variant in normal cells. C-terminally HDEL-tagged

proteins exit the ER, but are recognized by the Erd2p receptor, which returns them to the ER (Semenza et al., 1990). When Hsp150Δ-β-lactamase-HDEL was 35S-labeled in normal cells, immunoprecipitation showed that it remained cell-associated (Fig. 4, lane 2), and none could be immunoprecipitated from the medium (lane 1). Most of the cell-associated protein (lane 2) comigrated with Hsp150Δ-β-lactamase lacking HDEL, which was mostly secreted to the medium in the same strain (lanes 3 and 4). A small amount of cell-associated Hsp150Δ-β-lactamase-HDEL (lane 2) co-migrated with the same variant trapped in the pre-Golgi compartment in the sec18-1 mutant (110 kDa, lane 6). As Hsp150Δ-β-lactamase-HDEL probably does not reach the latest Golgi subcompartment where Kex2 protease is located, it was likely to contain subunit I, which adds more than 10 kDa to the molecular mass (Suntio et al., 1999). Thus, when Hsp150Δ-β-lactamase-HDEL was allowed to encounter Golgi transferases multiple times by recycling between the ER and the Golgi, its O-glycans were matured, but apparently not to full length.

Effect of absence of COPI traffic on Golgi-specific N-glycan extension in the ER

To confirm that recycling rather than de novo synthesized Och1p was responsible for decoration of N-glycans in the ER, we used as a reporter the soluble cell wall protein Scw4p, which is N-glycosylated at a single potential site, and runs in SDS-PAGE like a 66 kDa protein (Cappellaro et al., 1998). A Scw4 variant encoding a C-terminally histidine-tagged version of the protein was integrated into the genome of control cells and sec mutants. The control cells were preincubated for 15 minutes at 37°C and 35S-labeled for 5 minutes, and a parallel sample was thereafter chased for 30 minutes. Immunoprecipitation with antibody against pentahistidine revealed a protein migrating at 59 kDa (Fig. 5, lane 1). During chase it was converted to a 66 kDa form (lane 2), apparently the mature form arisen by glycan extension during transport to the cell wall. In the presence of tunicamycin (TM), which inhibits N- but not O-glycosylation, the increase in apparent molecular weight of Scw4p during secretion. The above experiment was repeated in a sec23-1 mutant to block Scw4p in the ER. During the chase the apparent molecular weight increased from 59 kDa (lane 5) to only 61 kDa (lane 6), suggesting that some extension of glycans occurred in the ER. Part of the glycan addition was on the N-glycan, because after chase with TM, the increase of

![Fig. 4. Electrophoretic migration of Hsp150Δ-β-lactamase-HDEL.](image)

Normal cells expressing Hsp150Δ-β-lactamase-HDEL (H606; lanes 1 and 2) or Hsp150Δ-β-lactamase (H335; lanes 3 and 4), and a sec18-1 mutant expressing Hsp150Δ-β-lactamase-HDEL (H610; lanes 5 and 6) were preincubated for 15 minutes and 35S-labeled for 30 minutes at 37°C. The medium (m) and respective cell lysate (c) samples were immunoprecipitated with β-lactamase antiserum and analysed by SDS-PAGE. The figures on the right indicate biosynthetic intermediates of the reporter proteins and those on the left molecular weight markers.

![Fig. 5. Scw4p glycosylation in a COPI mutant.](image)

Control (H1495), sec23-1 (H1496) and sec21-1 (H1497) cells were preincubated at 37°C for 15 minutes, and pulse-labelled with [35S]methionine/cysteine for 5 minutes. Parallel samples were chased with CHX for 30 minutes, as indicated. TM was present from the preincubation onwards, as indicated. The cell lysates were immunoprecipitated with antibody against pentahistidine only (lanes 1-4), or reimmunoprecipitated with antiserum against α1,6-mannose (lanes 13-15), followed by SDS-PAGE analysis. Apparent molecular weights of Scw4p forms are indicated on the right.
Fig. 6. Immunofluorescent staining of Och1p-HA in the sec23-1 mutant. (A,B,E,F) Och1p-HA in sec23-1 (H1490) or (C,D) Och1p-HA plus cytochrome b(5)-opsin in sec23-1 (H1791) were grown at 24°C, followed by a 1 hour incubation with CHX at 37°C. The cells were fixed and immunostained with polyclonal antibody against HA (A,C,E). The cell samples on the right were viewed through Nomarski optics (B), or double stained with monoclonal antibody against opsin (D), or with DAPI (F). The arrows point to ER-like structures (C,D,E) or nuclei (F).

apparent molecular weight was much less (lane 8), than in the absence of TM.

To study whether the moderate glycan extension in the sec23-1 cells was due to Golgi glycosyltransferases recycling to the ER, and not de novo synthesized transferases, we repeated the experiment in sec21-1 cells to block recycling from the Golgi to the ER. The apparent molecular weight of Scw4p increased similarly in the absence (lane 10) and presence of TM (lane 12), indicating little or no N-glycan extension. When this experiment was repeated using the sec18-1 mutant, similar results as shown in lanes 9-12 were obtained (not shown, strain H1628). Parallel samples labelled and chased in the absence of TM and immunoprecipitated with antibody against pentahistidine were subjected to reimmunoprecipitation with antiserum against α1,6-mannose. Scw4p from control cells (lane 13) and sec23-1 cells (lane 14) were recognized by the antiserum indicating glycosylation by Och1p. By contrast, Swp4p from sec21-1 cells appeared not to be decorated with α1,6-mannose (lane 15). Thus, in the absence of COPII traffic the glycans of Scw4p were not extended to the same extent than under conditions where transferases were allowed to relocate to the ER and remain there together with the substrate protein. These data substantiate the notion that it was the recycling Och1p molecules, rather than de novo synthesized ones, which performed the decoration of N-glycans in the ER.

Relocation of Och1p to the ER in the absence of COPII function

Finally, we wanted to verify morphologically that Och1p accumulates in the ER when ER exit of proteins is blocked. An Och1p version tagged with the hemagglutinin epitope (Och1p-HA) (Harris and Waters, 1996) was expressed under its own promoter in a sec23-1 mutant. Cells were incubated at 24°C in the presence of CHX in order to stop the synthesis of new proteins, and thereafter at 37°C to block ER exit. Then, cells were fixed and subjected to indirect immunofluorescent staining using antibody against the HA epitope. Mostly an ER-like staining was observed (Fig. 6A). Nomarski optics revealed the vacuole (Fig. 6B). Next we constructed a strain which co-expressed Och1p-HA and opsin-tagged mammalian cytochrome b(5), which is an ER-resident protein both in mammalian and S. cerevisiae cells (Yabal et al., 2003). In double staining experiments HA polyclonal antibody (Fig. 6C) and opsin monoclonal antibody (Fig. 6D) stained similar structures (arrows). A similar HA antibody-stained cell sample as in Fig. 6A (Fig. 6E) was co-stained with DAPI (Fig. 6F) to reveal the nucleus. For the above experiments we performed the following controls using DAPI as nuclear marker. After growth of sec23-1 cells at 24°C, mostly dots, plus some nuclear membrane-like staining was detected (Fig. 7A), suggesting Golgi plus some ER localization. After chase at permissive temperature in the presence of CHX mostly dots were observed, suggesting that most of Och1p-HA was in the Golgi (Fig. 7B). Similar staining was observed when Och1p-HA was stained in a sec-7-1 mutant, where at 37°C membrane traffic is blocked in the Golgi (Fig. 7C). In normal cells Golgi-like staining was obtained for Och1p-HA at 24°C (Fig. 7D). These data support the conclusion based on our biochemical data above, that Och1p normally recycles between the Golgi and the ER. It is able to decorate protein-bound N-glycans in the ER, once it has access to the substrate glycoprotein for a sufficiently long time, which is the case when membrane traffic from the ER is blocked by mutations in COPII components.

Discussion

We found here that once newly synthesized proteins were blocked in the ER lumen by preventing the assembly of the COPII coat, their protein-bound N-glycans were nevertheless decorated with an α1,6-mannose residue. Normally this glycosylation step is carried out in the Golgi by Och1p. We reasoned that Och1p might in fact recycle between the Golgi and the ER under normal conditions. In COPII mutants it then would accumulate together with substrate glycoproteins in the ER, and exert its function in this organelle. Indeed, we could detect relocation of Och1p to the ER by indirect immunofluorescence microscopy in sec23-1 mutant cells. The M-Pol I and II multienzyme complexes of the Golgi have been found to recycle between the ER and the Golgi. By contrast,
Och1p was reported by Todorow et al. to remain in the Golgi according to immunofluorescent staining of sec12-4 cells at restrictive temperature after CHX treatment (Todorow et al., 2000). We applied in our immunofluorescent staining experiments the same protocol, except that the sec23-1 mutant was used. The reason for the different results remained unclear. Peyroche et al. demonstrated by immunofluorescent staining that Och1p localized to the ER in some wild-type cells and in mutants defective in ARF exchange factors Gea1/2p, which regulate the Golgi structure (Peyroche et al., 2001). Moreover, in subcellular fractionation of normal cells 38% and 60% of Och1p was found in fractions enriched in ER and Golgi, respectively (Schleip et al., 2001). In mammalian cells resident Golgi proteins have been shown to recycle through the ER (Storrie et al., 1998; Miles et al., 2001). Under conditions where ER exit sites were disrupted by inactivating Sar1p, a number of Golgi proteins accumulated in the ER, promoting the conclusion that most of the integral membrane proteins of the Golgi recycle through the ER (Miles et al., 2001; Ward et al., 2001).

Here we could extend the finding of retrograde transport in yeast cells of Golgi enzymes from N-glycan-specific transferases to transferases elongating O-glycans. We found that the O-glycans of the reporter protein Hsp150Δ-β-lactamase, blocked in the ER in COPII mutants, were extended. In normal cells the Hsp150Δ fragment is O-glycosylated at nearly all of its almost 70 serine and threonine residues with di-, tri-, tetra- and pentamannosides, occurring in the ratio of 4:1:1:1 (Jämsä et al., 1995; Suntio et al., 1999), whereas the β-lactamase portion is not detectably glycosylated (Holkeri et al., 1996). Lack of the 4th and 5th residues of the O-glycans of Hsp150Δ-β-lactamase would correspond to a decrease of about 7 kDa (calculated molecular mass). However, subunit I remains attached to the fusion protein in the ER, adding a molecular mass of 9.7-13.5 kDa (determined by mass spectrometry) (Suntio et al., 1999). Since ER-retained Hsp150Δ-β-lactamase and the secreted fully glycosylated variant lacking subunit I co-migrated in SDS-PAGE, we suggest that the O-glycans of ER-retained Hsp150Δ-β-lactamase were extended up to tri-mannosides. Such extensions are normally accomplished by Mnt1p, which is suggested to reside in the medial Golgi (Lussier et al., 1995b).

The recycling of Golgi transferases, rather than de novo synthesized transferases en route to the Golgi, appeared to be responsible for the Golgi-specific glycan extensions in the ER. This is based on the finding that the extensions could be diminished by two ways, by accumulation of the substrate protein in ER-derived vesicles and the transferases in Golgi-derived vesicles by blocking all vesicle fusion events with target membranes in an NSF-deficient sec18-1 mutant, and by abolishing specifically Golgi-to-ER traffic in a COPI-defective sec21-1 mutant, under which conditions also ER-to-Golgi traffic is blocked.

In summary, we found that the glycosyltransferase Och1p, responsible of starting the extension of the primary N-glycans in the Golgi by addition of an α1,6-mannose residue, recycles between the Golgi and the ER, and not only between the early and late Golgi as is currently thought. Moreover, Och1p was found to be functional in the ER, as shown by decoration by α1,6-mannose of several ER-blocked glycoproteins. Primary O-glycans were also extended in the ER, apparently by Mnt1p of the medial Golgi. By contrast, the N-glycans of ER-blocked invertase were not extended beyond the α1,6-mannose residue (Kaiser and Scheckman, 1990). Nor appeared Mnn1p, adding the 4th and 5th mannose residues on O-glycans in the medial/trans Golgi, to elongate O-glycans of our ER-blocked reporter glycoprotein. Perhaps only the glycosyltransferases of early Golgi subcompartments recycle between the Golgi and the ER.

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