Localization of a Yeast Early Golgi Mannosyltransferase, Och1p, Involves Retrograde Transport

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Abstract. To analyze the mechanism of integral membrane protein localization in the early Golgi apparatus of *Saccharomyces cerevisiae*, we have used Och1p, a cis-Golgi mannosyltransferase. A series of influenza virus hemagglutinin (HA) epitope-tagged fusion proteins was constructed in which invertase is appended to the Golgi-luminal carboxy terminus of full-length Oehlp. Several constructs included a Kex2p cleavage site between the Ochlp and invertase moieties to monitor transit to the Kex2p-containing TGN. Cells expressing an Ochlp-invertase fusion do not secrete invertase, but those expressing an Ochlp-Kex2p site-invertase fusion protein secrete high levels of invertase in a Kex2p-dependent manner. The Ochlp-Kex2p site-invertase fusion protein is cleaved with a half-time of 5 min, and the process proceeds to completion. Before cleavage the protein receives glycosyl modifications indicative of passage through the medial- and trans-Golgi, therefore cleavage occurs after ordered anterograde transport through the Golgi to the TGN. Transit to distal compartments is not induced by the invertase moiety, since noninvertase fusion constructs encounter the same glycosyltransferases and Kex2p as well. The Ochlp-HA moiety, irrespective of whether it is generated by cleavage of the fusion protein in the TGN or synthesized de novo, is degraded with a half-time of about 60 min. Thus, the half-time of degradation is 12-fold longer than the time required to reach the TGN. At steady state, de novo-synthesized and TGN-generated HA epitope-tagged Ochlp reside in a compartment with a buoyant density identical to that of wild-type Ochlp and distinct from that of the vacuole or the TGN. Finally, ochl null cells that express an Ochlp fusion construct known to rapidly encounter the TGN glycosylate invertase to the same extent as wild-type Ochlp and distinct from that of the vacuole or the TGN. These results lead us to propose a model for Ochlp-HA localization that involves movement to distal compartments, at least as far as the TGN, followed by retrieval to the cis compartment, presumably by vesicular transport.

The eukaryotic secretory pathway consists of a set of membrane-bound compartments that are involved in delivery of newly made proteins to their final destinations. The hub of this pathway is the Golgi apparatus, which is involved in the posttranslational modification and sorting of the proteins that pass through it. The Golgi is composed of several topologically distinct cisternae, each of which contains a unique set of resident proteins. These proteins must resist dispersion by the large flux of vesicular traffic to and from the Golgi apparatus. This process is as important as the initial delivery of proteins to this organelle, yet it is not well understood. For example, it is not known whether newly made Golgi proteins simply cease their forward movement upon reaching the correct cisterna, or whether they are localized by a more dynamic mechanism involving recycling through later compartments.

An active recycling mechanism has been shown to be operative for ER luminal proteins that possess the carboxy-terminal tetrapeptide sequence Lys-Asp-Glu-Leu (KDEL; HDEL in yeast) (63), as well as ER integral membrane proteins (IMP) that bear a cytoplasmic dityrosine motif K(X)KXX (42, 43, 101) at their carboxy termini. These proteins sometimes escape the ER in transport vesicles, perhaps because of failure of a static retention mechanism, and are delivered to the Golgi apparatus. A KDEL receptor resides in the Golgi (94), where it apprehends the escapees and escorts them back to the confines of the ER (52). Once in the ER, the protein is released, and the KDEL receptor returns to the early Golgi (109). In a similar fashion, the α subunit of the coatamer complex (41, 103) is likely to be involved in recovery of wayward K(X)KXX-bearing proteins (17, 51) from the Golgi to the ER.

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1. Abbreviations used in this paper: CPY, carboxypeptidase Y; DPAP A, dipeptidyl aminopeptidase A; HA, influenza virus hemagglutinin; IMP, integral membrane protein; IP, immunoprecipitation; ManT, mannosyltransferase; SD, synthetic dextrose.
Compared to the mechanism of ER protein retention, much less is known about the mechanism of protein retention in the Golgi apparatus. Although the scattered Golgi apparatus of *Saccharomyces cerevisiae* is morphologically distinct from its stacked mammalian counterpart, it is quite similar biochemically, and therefore provides an excellent model system for analysis of Golgi protein retention. Several lines of evidence indicate that the yeast Golgi, like that of mammalian cells, is composed of distinct compartments. For example, the sequential maturation of oligosaccharides in the yeast Golgi requires the *SEC18* gene product (yeast N-ethylmaleimide-sensitive fusion protein, NSF) at multiple stages, indicating that the modification enzymes are sequenced in functionally and topologically distinct compartments (31). Localization of Golgi resident proteins by indirect immunofluorescence (26) and subcellular fractionation (9, 11, 18, 28, 105) also reveals that different Golgi enzymes reside in separable compartments. Similarly, transport of pro-α-factor from the ER to the Golgi in vitro results in only the earliest glycosyl modification, indicating that the glycosyl transferases used at later steps are not contained within the acceptor compartment (4, 34). In sum, these and other studies indicate that the yeast Golgi can be subdivided into at least four compartments. The ER-proximal, or *cis*, compartment contains the initiating α-1,6-mannosyltransferase (ManT), Och1p (28, 65), which is responsible for addition of the first mannose residue to the core oligosaccharide obtained in the ER (64, 80). The next, or *medial*, compartment contains the elongating α-1,6-ManT activity (28). The terminal α-1,3-ManT, encoded by *MNN1* (77), is contained within the equivalent of the *trans*-Golgi and TGN (31, 33). The TGN, which is the most distal Golgi compartment, also contains the Kex2p, Kex1p, and DPAP A proteases (11, 18, 25, 31).

Most known residents of the *cis*, *medial*, and *trans*-Golgi are type II IMPs, with their amino termini in the cytoplasm and their carboxy termini in the Golgi lumen. A recently described exception is Emp47, which is a Type I IMP of the yeast *medial*-Golgi (88)). In contrast, TGN residents are generally type I IMPs with the opposite topology. Much effort has been directed towards identifying the "Golgi retention signal" of early type II proteins. Many studies indicate that the transmembrane domain is critical (2, 14, 32, 39, 57, 89, 98, 99, 111) with some researchers finding that the adjacent regions are also involved (15, 19, 58, 59, 62, 66, 83, 100, 104). However, several groups have argued that retention is conferred by multiple domains of the molecule (3, 7, 12, 32, 54, 90). For example, in yeast, the transmembrane domain of Mnn1p can localize a heterologous protein to the Golgi; nevertheless, a soluble form of Mnn1p, completely lacking a transmembrane domain, is also localized to the Golgi (32). Thus, there may be multiple signals involving cytoplasmic, membrane-spanning, and luminal domains that act together to affect the proper localization of Golgi proteins.

In contrast to the complex retention signals of type II *cis*, *medial*, and *trans* residents, the retention signals of three type I residents of the yeast TGN are well defined: they are located in the carboxy-terminal cytoplasmic tails, contain critical aromatic residues, and are similar to the signals within mammalian cell surface receptors that mediate clustering into clathrin-coated pits (107). Elimination of the cytoplasmic tails of the TGN proteases DPAP A, Kex2p, or Kex1p destroys retention and results in transport to the vacuole (16, 69, 106). In addition, loss of clathrin function in yeast results in mislocalization of Kex2p and DPAP A to the plasma membrane (91). It was suggested, therefore, that clathrin binding to the cytoplasmic tails of Kex2p or DPAP A was necessary for either direct retention or recycling of late Golgi enzymes (91).

Localization of the type I proteins to the TGN and type II proteins to the earlier compartments appears to occur via different mechanisms. First, clathrin may not be directly required for localization of early Golgi proteins because GDPase, a type II early Golgi protein, is not mislocalized in a clathrin mutant (92). In contrast, Mnn1p, a type II enzyme that seems to have dual citizenship in both the *trans*-Golgi and TGN of yeast, is mislocalized in a clathrin mutant (33). However, this effect is not mediated through the cytoplasmic tail of Mnn1p (33), and it may be due to a more indirect effect on TGN structure or function in clathrin mutants. Second, the early and late Golgi retention mechanisms differ in their saturability. For example, overexpression of the type I proteins Kex2p or DPAP A causes a significant fraction of the protein to mislocalize to the vacuole (69, 106), whereas the Golgi seems to be able to readily localize overexpressed Mnn1p, which is a type II protein (32). Behavior of Mnn1p is more similar to mammalian glycosyltransferases, which upon overexpression are predominately correctly localized, although ER accumulation is sometimes observed (62, 66, 97). Finally, early type II and late type I retention systems appear to be distinct because overexpression of Mnn1p does not alter the turnover of Kex2p, suggesting that the proteins are not competing for a common component (33).

Several mechanistic models have been proposed for Golgi protein localization (10, 55, 67). Probably the most widely held view, first proposed by Swift and Machamer (97), is that Golgi proteins form aggregates that are too large to enter forward-moving transport vesicles and are thus retained (53, 68, 90, 97, 104). This model requires that aggregation be induced at a particular point during Golgi transit, perhaps by some physiochemical property of particular Golgi subcompartments. A related model, called the "kin recognition model" (67, 68), posits that different resident proteins of a particular cisterna interact to form large heterooligomers that restrict forward movement, possibly by anchoring to a Golgi matrix (96). Yet a third model is based on two observations: first, that Golgi protein transmembrane domains are shorter, in general, than those of plasma membrane proteins (10), and second, that a cholesterol concentration gradient seems to exist in the mammalian secretory pathway (72), with the ER being relatively depleted and the plasma membrane being relatively enriched. Cholesterol tends to thicken lipid bilayers, hence the proposal that proteins transit the secretory pathway until their transmembrane domains "fit" the thickness (35) of the surrounding bilayer. Premature retention or continued movement forward would be energetically unfavorable due to the hydrophobic effect. One of the features common to all these models is that once a protein reaches its correct subcompartment within the Golgi, it is postulated to cease its forward movement and be retained.
We have investigated the localization mechanism of Ochlp, a cis-Golgi ManT, using a series of influenza virus hemagglutinin (HA) epitope-tagged Ochlp-invertase fusion proteins (see Fig. 1). In contrast to most current models, we have found that the Ochlp fusion proteins do not efficiently halt their forward movement upon encountering the cis compartment. Instead, they move to later Golgi compartments, at least as far as the TGN, after which they return to the cis compartment. Our results indicate that yeast possesses a retrograde transport pathway from the TGN to the cis compartment, and furthermore, that localization of HA epitope-tagged Ochlp to the cis-Golgi at steady state is due, at least in part, to recycling from later Golgi cisternae.

Materials and Methods

Strains and Media

The Escherichia coli strains used were XLI-Blue (supE44 thi-1 lac endA1 gyrA96 thi-1 rpsL761 damR16 relA1 supE44 lacI977 lacZAM15 Tn10) (Strategene, La Jolla, CA) and BMH 71-18 (ihi supE [lac-proAB] [mutS::Tnl0] [F' proAB, lacZAM15] [48]). Bacterial strains were grown on standard media (61). S. cerevisiae strains used were SEY6210 (MATa, ura3-52, leu2-3,112, his3-200, ade2-101, prb::LEU2) (Stewart, et al. 1981) (this study), SHY101 (MATa, ura3-52, leu2-3,112, his3-200, trpl-A901, lys2-801, suc2A9, kex2A2::HIS3-3) (this study), SHY103 (MATa, ura3-52, leu2-3,112, his3-200, trpl-A901, lys2-801, suc2A9, kex2A2::HIS3-3) (this study), SHY104 (MATa, ura3-52, leu2-3,112, ade2-101, trplA1, suc2A9, sec18A1) (this study). Yeast strains were grown on yeast extract, peptone, and dextrose (YPD) supplemented with 2% dextrose or on synthetic dextrose (SD) medium supplemented as necessary (81).

Plasmid Construction and Site-directed Mutagenesis

To create plasmid pSEC18 (URA3, CEN), the BamHI-HindIII SEC18-containing fragment from plasmid pSEY8-SEC18 (2 µm) (22), kindly provided by S. Emr (University of California, San Diego), was subcloned into the yeast CEN-containing shuttle vector pRS416 (95) (this study). Yeast strains were grown on SD medium supplemented with appropriate supplements. Cells were washed in water and then resuspended in 0.2 ml 5% TCA. Glass beads (0.45-p.m diam) were added to about three-fourths of the total sample volume. Cells were lysed by vortexing for five 1-min periods, alternating with 1-min incubations on ice. The purified recombinant protein was used for immunization of rabbits against the OCH1 ORF. Deletion of OCH1 was confirmed by PCR (84) using oligonucleotide primers flanking the deletion. SHY102 was created from SEY6210 by one-step gene replacement of KEX2 with the BamHI fragment containing the kex2A2::HIS3-3 allele from plasmid pKEX2 (kindly provided by R. Fuller (University of Michigan, Ann Arbor)). Deletion of KEX2 was confirmed by a decrease in mating efficiency of the null strain. SHY103 was created from SEY6210 by one-step gene replacement of PE44 with the BamHI fragment containing the pep4A::LEU2 allele from plasmid pTS17 (kindly provided by R. Schekl and Gietz (87) with the modifications of Eibele (23). To construct the S. cerevisiae strains, SHY101, a XhoI-HindIII och1::LEU2-containing fragment from plasmid pSH113 (described above) was integrated into diploid strain SHY100 (a/a, ura3-52, leu2-3,112, ade2-101, DE2, leu2-3,112, trpl-A901) by one-step gene replacement (82). After sporulation, temperature-sensitive Leu¹ haploid segregants were isolated that contained a complete deletion of the OCH1. Deletion of OCH1 was confirmed by PCR (84) using oligonucleotide primers flanking the deletion. SHY102 was created from SEY6210 by one-step gene replacement of KEX2 with the BamHI fragment containing the kex2A2::HIS3-3 allele from plasmid pKEX2 (kindly provided by R. Fuller (University of Michigan, Ann Arbor)). Deletion of KEX2 was confirmed by a decrease in mating efficiency of the null strain. SHY103 was created from SEY6210 by one-step gene replacement of PE44 with the BamHI fragment containing the pep4A::LEU2 allele from plasmid pTS17 (kindly provided by R. Schekl and Gietz (87) with the modifications of Eibele (23)). Deletion of PE44 was confirmed by the inability of the cells to proteolytically process carboxypeptidase Y to its mature form (27, 47).

Invertase activity was determined by the method of Goldstein and Lampen (29) with the modifications for measurement of periplasmic activity presented in Johnson et al. (44).

Production of Anti-Ochlp Antisera

The gene fragment ranging from the HpaI site to the NsiI site of OCH1 was cloned behind the gene encoding the maltose binding protein in the vector pMAL-c2 (New England Biolabs, Beverly, MA). The recombinant protein (71 to 480 of Ochlp from E. coli and purified on an amylose column according to the manufacturer's direction. The purified recombinant protein was used for immunization of a rabbit against standard procedures (37).

Protein Extraction and Immunoblotting

Yeast protein extracts for immunoblotting were prepared from 1 ml of mid-logarithmic phase (OD600 nm = 0.5–1.0) cells grown in SD media containing appropriate supplements. Cells were washed in water and then suspended in 0.2 ml 5% TCA. Glass beads (0.45-µm diam) were added to about three-fourths of the total sample volume. Cells were lysed by vortexing for five 1-min periods, alternating with 1-min incubations on ice. The lysates were removed from the beads and centrifuged for 15 min in a microfuge at 4°C. TCA-precipitated protein was resuspended in Laemmli
sample buffer (50), heated at 100°C for 4 min, and separated on an 8% SDS-polyacrylamide gel. Immunoblots, which were developed with the enhanced chemiluminescence detection kit (Amer sham Corp., Arlington Heights, IL), were performed according to standard methods (37). HA epitope-tagged Ochlp and Kex2p were detected with 12CA5 hybridoma serum-free culture supernatant at a dilution ranging from 1:250 to 1:1500. Membrane fractionation antibody 1D07-A7-B2 (21), which recognizes the yeast vacuolar H+-ATPase 100 kD subunit, was used at a concentration of 250 ng/ml (Molecular Probes, Eugene, OR).

**Radiolabeling and Immunoprecipitation**

Yeast were grown in SD media lacking uracil, cysteine, and methionine at 30°C (unless otherwise indicated) to an OD_{600} n = 0.5–1.0. 2 OD U of cells per time point were washed in SD lacking uracil, cysteine, and methionine and resuspended in 1 ml of the same media. Cells were washed with 150 μl of Triton X-100 (ICN Biochemicals, Irvine, CA) per 3 OD U of cells for 2 min at 30°C (unless otherwise noted). Labeling was quenched by addition of a 25X chase solution (250 mM methionine, 250 mM cysteine) to a final concentration of 1X. The chase was stopped by addition of NaCl, to 10 mM and cycloheximide to 100 μg/ml on ice. The cells were centrifuged, and the pellets were resuspended in 100 μl of bead buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 1% SDS, and 1 mM PMSF). Glass beads (0.45-μm diam) were added to about three-fourths of the total sample volume, and cells were disrupted by vortexing for five 1-min intervals separated by 1-min cooling periods on ice. The lysate was removed, and the beads were washed with 150 μl of bead buffer. The lysate and wash were combined and incubated at 100°C for 4 min, centrifuged (12,000 g) for 10 min to remove insoluble material, and added to 750 μl of immunoprecipitation (IP) dilution buffer (60 mM Tris-HCl, pH 7.4, 190 mM NaCl, 6 mM EDTA, 1.25% Triton X-100, and 1 mM PMSF). To precipitate HA epitope-tagged protein, 200 μl of IP buffer from 12CA5 hybridoma serum-free culture supernatant was added. Antibody to CPY was a gift from S. Emr. Immunoprecipitation of invertase used 9 μl of rabbit polyclonal anti-invertase, provided by S. Emr. The linkage-specific antibodies were added, and the immune complexes were allowed to form overnight at 4°C with gentle mixing. Complexes were washed with 150 μl of IP dilution buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, and 0.2% SDS) and 5 μl of polyclonal antisera specific to α-1,6- or α-1,3-linked mannose residues were added, and the immune complexes were allowed to form overnight at 4°C while slowly mixing. Sepharose-bound immune complexes were pelleted (12,000 g, 1 min) and washed sequentially in 1 ml each of high salt wash buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, 0.2% SDS), IP buffer, and detergent-free buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA). Immunoprecipitated protein was resuspended in Laemmli buffer (50), incubated at 100°C for 4 min, and separated on an 8% SDS-polyacrylamide gel. Gels were fixed in 25% isopropanol, 10% acetic acid for 15 min, treated with Amplify (Amersham Corp.) for 15 min, dried, and exposed to film (X-AR5; Eastman-Kodak Co., Rochester, NY) at −70°C.

**Results**

**Rationale**

Most models of protein retention within the Golgi apparatus propose a static mechanism whereby proteins fail to move forward upon reaching their resident compartment (10, 67), although retrieval mechanisms have been discussed (32, 40, 56). In the light of recent data suggesting a recycling component to the localization mechanisms of ER (28, 74, 101) and TGN resident proteins (108), as well as a type I IMP in the medial-Golgi of yeast (88), we thought it was possible that localization of type II IMPs within the Golgi stacks might also involve retrieval of proteins that have escaped to more distal compartments. To examine this question, we chose to use Ochlp because its activity represents the first post-ER enzymatic event required for synthesis of outer-chain mannose linkages on yeast secretory proteins, and thus Ochlp must reside in a more distal compartment. To localize wild-type Ochlp, SEY6210 cells were grown in SD media lacking cysteine and methionine at 30°C to an OD_{600} n of 0.5–1.0. 20 OD U of cells were washed in SD lacking cysteine and methionine and resuspended in 12 ml of the same media. Cells were pulse labeled with 50 μCi of Tran35S-label per OD of cells for 2 min at 30°C. Labeling was quenched by addition of a 25X chase solution (250 mM methionine, 250 mM cysteine) to a final concentration of 1X. Cells were incubated at 30°C for 60 min after which the chase was stopped by addition of NaCl, to 10 mM and cycloheximide to 100 μg/ml on ice. An additional 80 OD U of unlabeled SEY6210 cells that had been washed two times in spheroplast buffer were added to the labeled cells. The cells were then spheroplasted and lysed, and the protein was fractionated on a 25–40% sucrose gradient as described above. The protein in 0.65 ml of each fraction was immunoprecipitated using 1 μl of the rabbit polyclonal anti-Ochlp antiserum per fraction. Immunoprecipitations were performed as described above.

**Subcellular Fractionation**

Yeast strain SHY102 (Δkex2) expressing HA epitope-tagged Kex2 on a TRP CEN plasmid was transformed with either the pOH (URA3) or pOHKI (URA3) plasmid. The two strains were grown in 250 ml of SD lacking uracil and tryptophan at 30°C to an OD_{600} n of 0.5–0.8, harvested by centrifugation, and washed twice in spheroplast buffer (50 mM Tris-HCl, pH 7.5, 1.2 M sorbitol, 10 mM NaCl, 40 mM β-mercaptoethanol). Cells were resuspended in spheroplast buffer at 40 OD U/ml. Zymolyase 100T (ICN, Costa Mesa, CA) was added to 150 μg/ml, and cells were incubated at 30°C for 30 min with occasional mixing. The spheroplasts were washed twice in spheroplast buffer and resuspended in 0°C lysis buffer (20 mM Hepes/KOH, pH 7.4, 100 mM K-acetate, 5 mM Mg(acetate), 1 mM EDTA, 1 mM DTT) plus 2 mM EGTA, 1 mM PMSF, 5 mM l,10-phenan- throline, 2 μM pepstatin A, 2 μg/ml aprotinin, and 0.5 μg/ml leupeptin to the same volume in which they were spheroplasted. Cells were lysed in a 7 ml Dounce homogenizer (25 strokes of "B" pestle; Kontes Glass Co., Vine-

land, NJ) on ice, and the lysates were centrifuged in a rotor (SA600; Sor-

vall Instruments Div., DuPont Co., Newton, CT) at 4°C for 3 min at 3,300 rpm (1000 g). The resulting supernatants (S1) were centrifuged in a Sor-

vall SA600 rotor at 4°C for 15 min at 10,000 g to generate pellet (P10) and supernatant (S10) fractions. The S10 fractions (1.5 ml) were loaded onto the tops of 10.5 ml continuous 25–40% sucrose (wt/wt) gradients and cen-

trifuged in a rotor (SW41; Beckman Instruments, Fullerton, CA) at 40,000 rpm at 4°C for 18 h. 15 0.75-ml fractions were collected starting from the bottom of the gradient.

To monitor wild-type Ochlp, SEY6210 cells were grown in SD media lacking cysteine and methionine at 30°C to an OD_{600} n of 0.5–1.0. 20 OD U of cells were washed in SD lacking cysteine and methionine and resus-

pended in 12 ml of the same media. Cells were pulse labeled with 50 μCi of Tran35S-label per OD of cells for 2 min at 30°C. Labeling was quenched by addition of a 25X chase solution (250 mM methionine, 250 mM cysteine) to a final concentration of 1X. Cells were incubated at 30°C for 60 min after which the chase was stopped by addition of NaCl, to 10 mM and cycloheximide to 100 μg/ml on ice. An additional 80 OD U of unlabeled SEY6210 cells that had been washed two times in spheroplast buffer were added to the labeled cells. The cells were then spheroplasted and lysed, and the protein was fractionated on a 25–40% sucrose gradient as described above. The protein in 0.65 ml of each fraction was immunoprecipitated using 1 μl of the rabbit polyclonal anti-Ochlp antiserum per fraction. Immunoprecipitations were performed as described above.

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Figure 1. Diagrammatic representation of the Ochlp and Ochlp-invertase fusion proteins used in this study. All proteins were expressed from the OCH1 promoter on low copy number (CEN) plasmids. *aa*, amino acid residue.

Efficient Kex2p-dependent Invertase Secretion

Yeast strain SEY6210, which lacks a functional SUC2 gene and is therefore unable to utilize sucrose, was transformed with plasmids encoding the Ochlp-invertase fusion proteins diagrammed in Fig. 1. The nomenclature used for portions of the various fusion proteins is as follows: O refers to Ochlp, H symbolizes the HA epitope, K indicates the Kex2p cleavage site, and I represents invertase. Only the Ochlp-invertase fusion proteins that contained an intervening Kex2p cleavage site, i.e., OKI and OHKI, but not OI, were able to restore the ability of the suc2A9 strain to use sucrose as a carbon source (Table I). The ability to use sucrose was strictly dependent on the Kex2 protease because in an isogenic suc2A9 strain that lacks functional Kex2p, OKI and OHKI were no longer able to functionally complement the suc2A9 mutation (Table I). These data indicate that some Ochlp-invertase fusion protein is exposed to the Kex2p enzyme, resulting in invertase secretion. However, the level of invertase secretion does not need to be high to yield a Suc" phenotype, since as little as 1% of the wild-type level expressed from the SUC2 promoter suffices (46).

To determine the amount of invertase released from OKI by Kex2p and subsequently secreted, the invertase activity of intact cells and cell lysates was quantitated (Table II). In the absence of the Kex2 cleavage site (OI), ~1% of the total cellular invertase was secreted. Cells expressing either OKI or OHKI secreted ~70% of the invertase produced. The high level of invertase secreted from cells expressing either OKI or OHKI suggests that the majority of fusion protein present in these cells at steady state is cleaved by Kex2p. To examine this finding further, we determined the amount of cleaved OHKI present in cells at steady state by immunoblot analysis using the anti-HA epitope antibody to detect OH and OHKI. Indeed, uncleaved OHKI, which migrates at ~150 kD (see Fig. 3), is virtually undetectable in logarithmically growing cells (Fig. 2). Instead, the only HA-immunoreactive protein in the strain expressing OHKI appears as a single band at ~66 kD that comigrates on SDS-PAGE with HA epitope-tagged Ochlp. Only uncleaved OHKI is detected in a Δkex2 strain (data not shown). Together, these results indicate that the large majority of the OHKI fusion protein is cleaved by the late Golgi Kex2 protease.

Cleavage of the OHKI Fusion Protein Is Rapid

To determine the rate at which the OHKI fusion protein is cleaved by Kex2p, we performed a pulse-chase analysis. The yeast strain SEY6210 containing the pOHKI plasmid was labeled at 30°C for 2 min and chased for 20 min (Fig. 3). Aliquots of cells were removed at 0, 0.5, 1, 2, 5, 10, and 20 min of chase. Labeled proteins were immunoprecipitated with the anti-HA epitope antibody, separated by SDS-PAGE, and visualized by autoradiography. At the 0-min time point, the uncleaved OHKI fusion protein is evident migrating at ~150 kD. The fusion is rapidly cleaved to yield HA epitope-tagged Ochlp (OH) and invertase, with a half-time of ~5 min. By 20 min, there is no detectable fusion protein remaining in the cell. Therefore, OHKI is rapidly (Fig. 3) and completely (Figs. 2 and 3) cleaved by the Kex2 protease.

Table 1. Kex2p-dependent Secretion of Invertase

| Construct | Δoch1 @37°C | Suc+ in suc2Δ9 | Suc+ in suc2Δ9, Δkex2 |
|-----------|-------------|----------------|----------------------|
| O         | +           | −              | −                    |
| OH        | +           | −              | −                    |
| OI        | +           | −              | −                    |
| OKI       | +           | +              | −                    |
| OHKI      | +           | +              | +                    |

Δoch1 strains expressing the above constructs were tested for the ability to grow on rich media at 37°C. suc2Δ9 and suc2Δ9 Δkex2 strains expressing the above constructs were tested for the ability to grow on yeast extract peptone-sucrose-containing media at 30°C. The Δoch1 strain is temperature sensitive, being unable to grow at 37°C.
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**Table II. Quantitation of Kex2p-dependent Invertase Secretion**

| Construct | Periplasmic | Total | Percentage secreted |
|-----------|-------------|-------|---------------------|
| OI        | 30          | 3000  | 1                   |
| OKI       | 1105        | 1597  | 69                  |
| OHKI      | 1059        | 1565  | 68                  |

The invertase activity of strain SEY6210 expressing the above constructs was measured. Total activity from extracts prepared from lysed cells was determined by the method of Goldstein and Lampen (29). Whole cell extracts were prepared to measure periplasmic activity (44). Each value represents the mean of three experiments. The ~50% reduction in the total invertase activity in OKI and OHKI cells relative to OI cells is probably due to release of invertase into the media, which would have been undetected with the method used.

**Proteolytic Processing of Fusion Proteins Occurs in the TGN**

Although OHKI is not subject to the high degree of outer-chain glycosylation as seen with wild-type invertase (102), a portion of the fusion protein migrates as a heterogeneous population of higher molecular mass polypeptides before cleavage by Kex2p (see the 5-min time point in Fig. 3). To examine whether this higher molecular mass "smear" represents fusion protein that had received outer-chain glycosylation, we used mannoside linkage-specific antibodies (Fig. 4). Cells expressing OHKI were briefly pulse labeled as in Fig. 3 and then chased for 5 min before IP with anti-HA epitope antibody. The immunoprecipitated proteins were eluted from the anti-HA epitope antibody and subjected to a second IP with antibody specific for either the HA epitope, the $\alpha$-1,6-mannose linkage, which is added in the cis/medial Golgi, or the $\alpha$-1,3-mannose linkage, which is added in the trans/TGN compartments. The 150-kD OHKI fusion protein (Fig. 4) was immunoprecipitated by the $\alpha$-1,6-mannose-specific antisera, indicating that it had obtained at least the "initial" $\alpha$-1,6-mannose residue in the cis-Golgi. A portion of the heterogeneous smear that migrates just slightly slower than the 150-kD protein could be immunoprecipitated by the $\alpha$-1,6-mannose-specific antisera, but not the $\alpha$-1,3-mannose-specific antisera (Fig. 4, compare lanes 2 and 3). We believe this represents material that had progressed to the medial-Golgi, where the $\alpha$-1,6 mannose outer-chain backbone was elongated, but had not moved to the trans-Golgi, and therefore had not obtained terminal $\alpha$-1,3-mannose linkages. The higher molecular mass region of the smear was immunoprecipitable by both the $\alpha$-1,6- and $\alpha$-1,3-mannose-specific antibodies, indicating that this material had been exposed to the $\alpha$-1,3 ManT in the trans-Golgi. Finally, as indicated previously, OH is derived from OHKI by Kex2p cleavage in the TGN. This experiment indicated that within 5 min of synthesis, OHKI receives all the modifications indicative of transit through the cis, medial, trans, and TGN compartments. Some OH derived from OHKI is also immunoprecipitable by the $\alpha$-1,6- and the $\alpha$-1,3-mannose-specific antibodies, but to a lesser extent than the invertase-containing fusion protein. This may reflect the fact that Ochlp has only four potential glycosylation sites (65) compared to the 13 sites of invertase (13). Another possibility is that the oligosaccharides on the Ochlp moiety are less heavily modified than those on the invertase moiety and therefore less immunoreactive (6).

The experiments presented above indicate that OHKI rapidly receives outer-chain glycosyl modifications and is cleaved by Kex2p, but do not clearly show where this cleavage is occurring. Three possibilities have been considered: (a) OHKI is cleaved by Kex2p in the ER or in transport vesicles bound for the Golgi, (b) OHKI is statically retained in the cis-Golgi and is cleaved in situ by newly synthesized Kex2p or Kex2p that may be recycling from the TGN (if this, in fact, occurs), and (c) OHKI travels to the TGN where it encounters Kex2p and is cleaved.

If Ochlp is cleaved by Kex2p in the TGN, then the fusion protein should obtain outer-chain glycosylation in the medial- and trans-Golgi before cleavage. This appears to be the case in Fig. 3, but because the processing is so fast relative to the pulse time, the two modifications may be occurring simultaneously to different subpopulations of labeled molecules, making clear kinetic resolution of the processes difficult. To circumvent this difficulty, we have used a sec18-1 temperature-sensitive strain, where transport of vesicles from the ER to the Golgi, and within the Golgi, is blocked at the restrictive temperature (31, 71). Isogenic sec18 and sec18-1 strains expressing OHKI were labeled at 25°C for 7.5 min. Cells were chased at the restrictive temperature (37°C) for 0, 1, 2, and 5 min, and radiolabeled protein was immunoprecipitated with anti-HA epitope antibody. The antigens were then reimmunoprecipitated with the linkage-specific antibodies to determine how far the proteins had progressed through the secretory pathway.
pathway before the block in transport. In the wild-type (SEC18) strain (Fig. 5, top panel), OHKI obtained outer-chain glycosylation, and the Golgi-modified fusion protein was rapidly cleaved to OH. During the chase period the levels of the α-1,6- and α-1,3-modified OHKI diminished, with a concomitant increase in the level of OH. Thus, α-1,6- and α-1,3-modified OHKI is the precursor of OH.

The same experiment, done in a sec18-1 strain, revealed that less fusion protein had progressed to the Kex2p compartment in the sec18-1 strain than in the wild-type strain at the earliest timepoint (compare the levels of OH derived from OHKI at the 0-min time points). Therefore, the kinetics of vesicular transport in sec18-1 cells is slightly slower than in wild-type cells, even at the “permissive” temperature of 25°C. This probably reflects a reduction in the activity of Sec18p. Despite this kinetic effect, it is clear that in sec18-1 cells (Fig. 5, bottom panel), conversion of OHKI to OH ceases after blocking vesicular transport at the restrictive temperature. This result is inconsistent with the first two possibilities described above. OHKI is not processed in the ER, or in cis-Golgi either by newly synthesized Kex2p or by Kex2p recycling back from the TGN. If this were occurring, the Kex2p present in the OHKI-bearing compartments would be trapped there after the temperature shift and continue to be active. The result would be generation of OH from OHKI, which is not observed.

This leaves the third possibility mentioned above: that OHKI is processed in the TGN. In fact, in sec18-1 cells, the α-1,3-mannosylated fusion protein was not cleaved by Kex2p even after 1 h at 37°C (data not shown). This result indicates that processing by Kex2p only occurs after modification in the medial- and trans-Golgi. Furthermore, the processing must occur in a separate compartment, distinct from the medial- and trans-Golgi. Taken together, this analysis indicated that conversion of OHKI to OH does not take place in the ER, or the cis-, medial-, or trans-Golgi. We conclude that the processing of OHKI to OH occurs in the Kex2p compartment, the TGN.

Transit of the OHKI Fusion Protein to the TGN Is Not Induced by the Invertase Moiety

To examine whether transit of the OHKI fusion protein to the TGN is reflective of the traffic pattern of wild-type Ochlp or whether it was induced by the presence of the in-
vertase moiety in the fusion protein, we created a reporter molecule that more closely resembles wild-type Ochlp. This construct, termed OKH (Fig. 1), consists of Ochlp followed by the Kex2 cleavage site and the HA epitope tag, but lacks the invertase moiety. Cleavage of the OKH protein by Kex2p will result in the removal of the HA epitope causing the protein to be refractory to IP by the anti-HA epitope antibody. OKH was expressed in isogenic suc2Δ9 and suc2Δ9 Δkex2 strains, and pulse-chase experiments were performed to follow the transit of the OKH protein (Fig. 6). In the absence of Kex2p activity, the OKH is immunoprecipitable with the anti-HA epitope antibody for an extended period of time. However, in the presence of functional Kex2p, the OKH protein is rapidly cleaved by the protease with a half-time (~5 min) very similar to that seen for the OHKI fusion protein (Fig. 3). Thus, movement of the fusion protein to the late Golgi is not induced by the invertase moiety.

To test directly whether HA epitope-tagged Ochlp (OH) is exposed to late Golgi compartments, cells expressing OH were labeled briefly, chased for 30 min, and immunoprecipitated with the anti-HA epitope antibody followed by IP with the linkage-specific antibodies (Fig. 7). We found that de novo–synthesized OH (i.e., not generated by cleavage of OHKI) was immunoprecipitable with the α-1,6-mannose–specific antisera and, to a lesser degree, with α-1,3-mannose–specific antisera. Although only a small amount of OH is immunoprecipitable with the α-1,3-mannose–specific antisera, this amount is similar to the amount of α-1,3-mannose–bearing OH generated from OHKI, which is known to have reached the TGN (Fig. 4). The presence of comparable levels of α-1,3-Man linkages on de novo–synthesized OH and OH generated from OHKI suggests that at least some of the de novo–synthesized OH has been exposed to the α-1,3-ManT, which is resident in both the trans-Golgi and the TGN (33).

These results (Figs. 6 and 7) indicate that OKH and OH undergo a similar traffic pattern as OHKI, and by extrapolation, we suggest that wild-type Ochlp may do so as well.

**Figure 6.** Rapid transit to the TGN is not dependent on the invertase moiety. Isogenic KEX2 and Δkex2 strains expressing OKH were labeled with Tran35S-label for 2 min at 30°C and chased for the times indicated. The proteins were immunoprecipitated with the anti-HA epitope antibody, separated by SDS-PAGE, and visualized by autoradiography.

**Figure 7.** De novo–synthesized OH bears α-1,6-, and to a lesser degree, α-1,3-mannose linkages. SEY6210 cells expressing OH were labeled for 5 min at 30°C and chased for 30 min. Protein was immunoprecipitated with the anti-HA epitope antibody. The immune complex was dissociated by heating, and the precipitated protein was divided into equivalent aliquots. The protein was then subjected to a second immunoprecipitation with either the anti-HA epitope antibody or the α-1,6- or α-1,3-mannose linkage–specific antibodies. The proteins were separated by SDS-PAGE and visualized by autoradiography.

**Ochlp Is Not Rapidly Transported to the Vacuole after Encountering the TGN**

The experiments presented so far indicate that OH and OHKI rapidly travel to the TGN. To determine the fate of Ochlp after encountering the late Golgi, we first examined the stability of both de novo–synthesized OH and OH derived from OHKI (Fig. 8). Cells were labeled for 10 min at 30°C, chased for 180 min, and aliquots of cells were removed at various time points. OH (Fig. 8A) and OH derived from OHKI (Fig. 8B) have similar half-lives of ~60 min. This half-life is an order of magnitude longer than the half-time of transit of OHKI (Fig. 3) or OKH (Fig. 6) to the TGN. OH is stable in a Apep4 strain (data not shown) indicating that the eventual degradation of Ochlp occurs in the vacuole (38). These findings indicate that Ochlp is
not rapidly transported to the vacuole after reaching the late Golgi.

To confirm these results, we compared the subcellular fractionation behavior of an IMP of the vacuole, the 100-kD subunit of the H\(^{+}\)-ATPase (21) (Fig. 9 A), to that of OH, either synthesized de novo (Fig. 9 B) or generated from OHKI (Fig. 9 C). These strains also expressed HA epitope-tagged Kex2p that will be discussed below. A lysate (S1) was prepared from strains expressing both HA epitope-tagged Kex2p and OH, or both HA epitope-tagged Kex2p and OHKI. The S1 fractions were separated by differential centrifugation at 10,000 g into pellets (P10) and supernatants (S10). The S1, P10, and S10 fractions were then probed by immunoblotting with an antibody specific for the 100-kD subunit of the H\(^{+}\)-ATPase (21) and with the anti-HA epitope antibody to detect OH and HA epitope-tagged Kex2p. While the large majority of the HA epitope-tagged Ochlp and Kex2p fractionate into the 10,000-g supernatant (S10) (Fig. 9, B and C; left), the vacuolar marker was exclusively found in the 10,000-g pellets (P10) (Fig. 9 A). This again indicates that after OH encounters the TGN it does not reside in the vacuole.

**Ochlp Returns to the cis-Golgi after TGN Transit**

To examine whether OH simply remains in the TGN after transit there, or whether it returns to the cis-Golgi, the Golgi-enriched S10 supernatants were fractionated by isopycnic centrifugation on 25-40% sucrose gradients (Fig. 9, B-D), and the distribution of OH and HA epitope-tagged Kex2p, which is a marker of the TGN, was examined by immunoblotting. In both gradients Ochlp migrated in a peak (predominantly fractions 6-9) corresponding to \(\sim 28-32\% \) (wt/wt) sucrose, whereas a more defined peak of Kex2p (predominantly fractions 4-5) was evident at about 34\% sucrose (Fig. 9, B-D). Thus, at steady state, the bulk of OH, irrespective of whether it is synthesized de novo or derived from OHKI, resides in a compartment physically separable from both the Kex2p compartment and the vacuole.

To determine if the compartment that contained OH at steady state was indeed the cis-Golgi, we localized wild-type Ochlp. We used an antisera directed against the luminal domain of Ochlp for this purpose. When used to immunoprecipitate protein from whole cell extracts (Fig. 10 A), the antibody recognizes a protein with an apparent molecular mass of 66 kD in the wild-type strain that is absent in the \(\Delta ochl\) cells. To localize Ochlp, wild-type cells were labeled for 2 min at 30°C and chased for 60 min. A Golgi-enriched S10 supernatant was prepared from labeled cells and was fractionated on a 25-40\% sucrose gradient. Protein from each gradient fraction was immunoprecipitated with the anti-Ochlp antisera and separated by SDS-PAGE (Fig. 10 B). Ochlp was found in fractions 7-10 corresponding to 28-32\% sucrose. Thus, the wild-type Ochlp compartment migrates at the same density observed for the compartment bearing HA epitope-tagged Ochlp, suggesting that the epitope-tagged protein returns to the cis-Golgi after encountering the TGN.

In another approach to demonstrate that the Ochlp fusion proteins reside in the cis-Golgi at steady state, we examined whether cells expressing only an Ochlp construct that rapidly encounters the Kex2p compartment can glycosylate invertase to the same degree as wild-type cells. Invertase provides a sensitive measure of the outer-chain glycosylation because it is hyper-glycosylated as it passes through the Golgi (24). Since Ochlp is responsible for initiation of this extensive glycosylation (64, 80), a significant decrease in Ochlp activity should be evident as reduced glycosylation of invertase. Therefore, various strains (wild-type, \(\Delta ochl\) , and \(\Delta ochl\) expressing wild-type Ochlp [O], OH, or OKH) were labeled, and the glycosylation state of endogenous invertase was examined by immunoprecipitation and SDS-PAGE (Fig. 11). As expected, invertase is
Invertase glycosylation is normal in cells expressing an Ochlp construct that rapidly encounters the TGN. Wild-type (SEY6210) cells and Δochl cells expressing either wild-type Ochlp (pO), HA epitope-tagged Ochlp (pOH), or the OKH fusion construct were labeled with Tran35S-label for 30 min at 30°C. Protein was immunoprecipitated with an anti-invertase antibody, separated by SDS-PAGE, and visualized by autoradiography.

Hyper-glycosylated in the wild-type strain, migrating as a heterogeneous smear. In contrast, invertase in the Δochl strain migrates as a distinct band because outer-chain glycosylation cannot be initiated. Expression of either wild-type Ochlp (O) or HA epitope-tagged Ochlp (OH) in the Δochl strain results in wild-type glycosylation of invertase. Significantly, expression of OKH, which encounters the Kex2p compartment with a half-time of 5 min (Fig. 6), also results in hyper-glycosylation of invertase, suggesting that an Ochlp moiety known to transit to the late Golgi is fully active. Taken together, these data suggest a model in which the steady-state distribution of epitope-tagged Ochlp is in the cis-Golgi, yet the protein undergoes rapid movement through later compartments and is retrieved.

In an effort to determine if the behavior of HA epitope-tagged Ochlp is representative of the wild-type protein, we examined the stability of wild-type Ochlp and compared it to OH (Fig. 12). Wild-type cells expressing OH were labeled for 10 min at 30°C, chased for 240 min, and aliquots of cells were analyzed by IP with the anti-Ochlp antisera followed by SDS-PAGE. The anti-Ochlp antisera recognizes both wild-type Ochlp and OH, which migrates more slowly through the gel due to the presence of the HA epitope tag. The half-life of the wild-type protein is ~2.5-fold longer than the tagged protein. Although the HA epitope tag does not seem to perturb the steady-state distribution of the protein (compare Figs. 9 and 10), it may affect the kinetics of recycling, which will be discussed below.

**Discussion**

Most recent models for the retention of type II IMPs in the Golgi suggest that these proteins cease their forward movement when they reach the appropriate compartment. It has been suggested that this process could be mediated by entry into a large oligomer, interaction with a putative Golgi matrix, or by virtue of a cholesterol-mediated gradient of membrane thickness across the Golgi stack (see Introduction). In contrast to these models we have found that the cis-Golgi protein used in our analyses can move past the cis compartment and subsequently be retrieved.

The yeast Golgi apparatus can be biochemically subdivided into at least four compartments (see Introduction). By analogy to mammalian Golgi, we refer to these as cis,
medial, trans, and TGN. The ManT, Och1p, is thought to be a resident of the cis-Golgi in yeast. At the distal end of the Golgi is the TGN compartment that houses the Kex2p endoprotease responsible for the maturation of pro-α-factor and pro-killer toxin in S. cerevisiae.

To study Golgi localization in yeast, we have analyzed the traffic pattern of a series of Och1p-invertase fusion proteins. The most instructive protein consisted of full-length Och1p, an HA epitope tag, a Kex2p cleavage site, and full-length invertase (OHKI). Surprisingly, we have found that OHKI is not efficiently retained in the cis-Golgi. This protein moves to the TGN, where it is processed by Kex2p, resulting in invertase secretion. Remarkably, this cleavage event occurs with a half-time of ~5 min, and is so efficient that the uncleaved fusion protein is undetectable at steady state. The oligosaccharides of the fusion protein receive medial (elongated α-1,6-mannose) and trans (terminal α-1,3-mannose) modifications before Kex2p cleavage. This indicates that the cleavage of OHKI occurs after the fusion protein has encountered the cis-, medial-, and trans-Golgi. Newly synthesized Kex2p in transit through the cis compartment, or Kex2p recycling through the early Golgi, are not responsible for cleavage of OHKI. Thus, processing of OHKI occurs in the TGN.

OH is ultimately degraded in the vacuole in a PEP4-dependent fashion. We found that the half-life of OH degradation, irrespective of whether it is synthesized at the ER or generated in the TGN, is 60 min. This turnover time is 12-fold longer than the time required to reach the TGN, indicating that the OH does not move directly from the TGN to the vacuole, a result that was confirmed by subcellular fractionation. We have also shown that the bulk of both ER-synthesized OH and TGN-generated OH resides in a compartment with the same density as wild-type Och1p and a lower buoyant density than the TGN. This indicates that OH derived from OHKI in the TGN is correctly localized to the cis-Golgi at steady state despite its rapid transit through the TGN. Finally, we found that cells exclusively expressing an Och1p construct that rapidly moves through the TGN can glycosylate invertase as efficiently as wild-type cells. Taken together, these findings indicate that the cis-Golgi protein Och1p is capable of recycling from the TGN.

The rapid transit time (~5 min) to the TGN, although slower than the ~1.5 min required for invertase to acquire outer-chain oligosaccharide modification (86), is much too fast to represent efficient retention in the cis-Golgi. Nevertheless, the finding that OH recycles does not rule out the possibility that there is a "statically retained" state. It does imply, however, that entry into this putative retained state is not an efficient process. In fact, it is difficult to imagine a model that allows for distinct protein compositions in different cisternae that does not involve at least a transiently retained state. One can view the process of retention as a pause in a particular compartment. At one extreme, the protein "pauses" for its entire lifetime (that is, it has a high affinity for the retained state), after which it is released and degraded. In the other extreme, the pause is short compared to the lifetime of the protein (a low affinity for the retained state), and after continued movement the protein can be retrieved from distal compartments. This recycling continues until the protein finally escapes the retrieval system and is degraded. The degree to which different proteins pause may vary. In this kinetic retention model epitope-tagged Och1p would pause only briefly, possibly because of a low affinity for the putative retained state, but this effect would be sufficient to result in the predominantly cis-Golgi distribution of OH at steady state.

These findings are in accordance with a recent study of the localization of Mnn1p (32), which is the terminal α-1,3-ManT in yeast. Mnn1p is distinctive because of its approximately equal distribution between two physically separable compartments, one of which contains Kex2p (33); that is, Mnn1p resides in both the trans-Golgi and TGN compartments. Recently, Graham and Krasnov (32) used a fusion protein consisting of the Mnn1p transmembrane domain followed by a Kex2p site and invertase to examine the extent of exposure of this protein to the Kex2p enzyme. They found that 85% of the protein was rapidly processed by Kex2p, which is more than could be accounted for by the dual steady-state distribution of Mnn1p in the trans-Golgi and TGN. Therefore, they suggested that Mnn1p may cycle between the trans-Golgi and TGN. In the same study, however, it was also shown that the luminal domain of Mnn1p, which was not included in the fusion protein, also contains localization information. Accordingly, the authors pointed out that the disproportionately high exposure of the fusion protein to the TGN might be due to the absence of the Mnn1p luminal domain. Our results with full-length Och1p constructs tend to support Graham and Krasnov's suggestion that Mnn1p cycles between the trans and TGN compartments.

Another outcome of our analyses with Och1p is the clear demonstration of a retrograde transport pathway from the TGN to the cis-Golgi. This extends the recent demonstration by Schröder et al. (88) that a type I medial-Golgi protein, Emp47, has a recycling itinerary that extends from the trans-Golgi (i.e., the α-1,3-ManT compartment) to the ER. There are also several precedents for retrograde transport through the Golgi apparatus in mammalian cells. First, several residents of the secretory pathway have been shown to obtain oligosaccharide modifications that occur in later compartments. For example, the oligosaccharides of the ER resident calreticulin are terminally galactosylated, a modification that occurs in the trans-Golgi (76). Likewise, two medial-Golgi resident proteins, GIMP (112) and MG160 (30), have been shown to bear terminal sialic acid residues, which are added in the TGN. In addition, the N-acetylglucosaminyl-transferase 1 enzyme, a resident of the medial/trans-Golgi, can undergo retrograde transport to the early Golgi (40). Second, several toxins which are taken up by endocytosis, such as Pseudomonas exotoxin, ultimately gain access to the cytosol via the ER (73, 75) and their cytotoxicity can be increased by placing a KDEL sequence at their carboxy termini (93). And finally, it was recently shown that a short peptide that contains KDEL at the carboxy terminus can move via retrograde transport from the TGN to the ER (60).

Although an epitope-tagged version of Och1p has been used for many of the experiments presented, a series of experiments has been performed to determine if the traffic pattern of the fusion protein is representative of the wild-type protein. One could envision that the bulky invertase moiety appended to the carboxy terminus of Och1p could somehow interfere with retention, perhaps by inhibiting...
entry into a putative oligomer. We have shown that is not the case because a construct without the invertase moiety encounters the TGN with the same kinetics as the invertase-bearing fusion. In addition, we have shown that Och1p, altered only by the addition of an HA epitope tag (OH), obtained the α-1,6- and α-1,3-mannose linkages characteristic of transit through the medial and trans compartments. This indicates that neither the specific sequences present in the Kex2 cleavage site nor invertase are responsible for continued forward movement. Finally, Och1p–invertase fusion protein–dependent secretion of invertase is not dependent on the HA epitope tag (Tables I and II). Thus, movement to distal Golgi compartments is not dependent on any of the specific sequences appended to the carboxy terminus of Och1p. However, it is possible that modification of the carboxy terminus itself, independent of the protein sequence (HA epitope, Kex2 site, or invertase), is responsible for this traffic pattern.

Several experiments suggest that epitope-tagged Och1p behaves similarly to the native protein. First, epitope-tagged Och1p appears to be localized to the same compartment as wild-type Och1p at steady state (Figs. 9 and 10). In addition, the fusion constructs appear to be fully active, as measured by their ability to glycosylate invertase, again suggesting that the proteins are correctly localized at steady state. However, it is important to note that the half-life of wild-type Och1p is about 2.5-fold longer than the half-life of epitope-tagged Och1p. There are several possible reasons for this difference. One is that the wild-type protein may enter into the putative retained state with greater efficiency than the epitope-tagged protein. The majority of the Och1p would enjoy a lengthy retained state, and only a small percentage of the Och1p would travel to the late Golgi where it could be either retrieved by retrograde transport or shunted to the vacuole for degradation. If this is true, then the rapid flux demonstrated by the tagged molecule OKH, for example, could indicate that a retention signal exists at the carboxy terminus of Och1p that is disrupted by the addition of the HA epitope. Alternatively, both wild-type and epitope-tagged Och1p may enter into the putative retained state with a similar efficiency, but retrieval from the TGN may be compromised by the presence of the epitope tag, resulting in diversion to the vacuole.

Nevertheless, despite the kinetic difference between OH and wild-type Och1p, it is now apparent that the localization mechanism of Och1p involves a retrograde transport component. The majority of epitope-tagged Och1p is transported to the late Golgi and must be recycled back to the cis-Golgi using a retrograde transport system. It is likely that the wild-type protein uses the same transport system, at least to some extent. It remains to be determined whether recycling plays a major or supporting role in the localization of the wild-type protein.

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References

1. Achstetter, T., and D.H. Wolf. 1985. Hormone processing and membrane-bound proteases in yeast. EMBO (Eur. Mol. Biol. Organ.) 4:173-177.

2. Aoki, D., N. Lee, N. Yamagughi, C. Dubois, and M.N. Fukuda. 1992. Golgi retention of a transf-Golgi membrane protein, galactosyltrans-ferase, requires cytoplasmic and transmembrane anchor sequences in the membrane-anchoring domain. Proc. Natl. Acad. Sci. USA 89:4319-4323.

3. Armstrong, J., and S. Patel. 1991. The Golgi sorting domain of coronavirus E1 protein. J. Cell Sci. 98:567-575.

4. Brown, R.A., A. Salmia, H. Rusinga, P. Novick, and S. Ferro-Novick. 1985. The GTP-binding protein Ypt1p is required for transport to the Golgi apparatus is defective in ypt1 mutants. J. Cell Biol. 109:1015-1022.

5. Ballo, C.E. 1990. Isolation, characterization, and properties of Saccharomyces cerevisiae mutant with nonsensitive protein glycosylation defects. Methods Enzymol. 185:440-470.

6. Ballo, L., E. Alvarado, P.K. Tsai, A. Dell, and C.E. Ballou. 1989. Protein glycosylation defects in the Saccharomyces cerevisiae mutants 2c7, 2c2, 2c3, and 2c4. Proc. Natl. Acad. Sci. USA 86:11864-11868.

7. Banfield, D.K., M.J. Lewis, C. Rabouille, G. Warren, and H.R.B. Pelham. 1994. Localization of Sec5, a putative vesicle targeting molecule, to the cis-Golgi network involves both its transmembrane and cytoplasmic domains. J. Cell Biol. 127:357-371.

8. Bochm, J., H.D. Ulrich, R. Ossig, and H.D. Schmitt. 1994. Kex2-dependent invertase secretion as a tool to study the targeting of transmembrane proteins which are involved in ER to Golgi transport in yeast. EMBO (Eur. Mol. Biol. Organ.) 13:3696-3710.

9. Bowser, R., and P. Novick. 1991. Sec13 protein, an essential component of the exocystic apparatus, is associated with the plasma membrane and with a soluble 19S particle. J. Cell Biol. 112:1117-1121.

10. Bretscher, M.S., and A. Boyd. 1993. Immunoisolation of Kex2p-containing organelles from yeast demonstrates colocalization of three transport proteins into a single Golgi subcompartment. J. Cell Sci. 106:815-822.

11. Burke, J.M., E.U. Lee, and J.C. Paulson. 1992. The signal anchor and stem of the protein sequence (HA epitope, Kex2 site, or invertase), is responsible for this traffic pattern. The Journal of Cell Biology, Volume 132, 1996 996

12. Burke, J., J.M. Pettitt, D. Humphris, and P.A. Gleeson. 1994. Medial-Golgi retention of N-acetylglucosaminyltransferase. J. Compartmentalized assembly of the complex of the enzyme. J. Biol. Chem. 269:26310-26319.

13. Carlson, M., and D. Botstein. 1981. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell 28:145-154.

14. Chapman, R.E., and S. Munro. 1994. The function of the yeast Golgi apparatus requires an ER protein encoded by ANP1, a member of a new family of genes affecting the secretory pathway. EMBO (Eur. Mol. Biol. Organ.) 13:4896-4907.

15. Colley, K.J., E.U. Lee, and J.C. Paulson. 1992. The signal anchor and stem regions of the β-galactoside α-2,6-sialyltransferase may each act to localize the enzyme to the Golgi apparatus. J. Biol. Chem. 267:7784-7793.

16. Cooper, A., and H. Bushey. 1992. Yeast Kexlp is a Golgi-associated membrane protein: deletions in a cytoplasmic targeting domain result in mislocalization to the vacuolar membrane. J. Cell Biol. 119:1459-1468.

17. Cosson, P., and F. Letourneur. 1994. Coatamer interaction with di-lysine endoplasmic reticulum retention motifs. Science (Wash. DC). 263:1629-1631.

18. Cunningham, K.W., and W.T. Wickner. 1989. Yeast KEX2 protein and mannosyltransferase I are localized to distinct compartments of the secretory pathway. Yeast. 5:25-33.

19. Dahdal, R.Y., and K.J. Colley. 1993. Specific sequences in the signal anchor of the β-galactoside α-2,6-sialyltransferase are not essential for Golgi localization. Membrane flanking sequences may specify Golgi retention. J. Biol. Chem. 268:26310-26319.

20. Deng, W.P., and J.A. Nicklöffel. 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. 200:81-88.

21. Doherty, R.D., and P.M. Kane. 1993. Partial assembly of the yeast vacuolar H+-ATPase in mutants lacking one subunit of the enzyme. J. Biol. Chem. 268:16645-16651.

22. Eakle, K.A., M. Bernstein, and S.D. Emr. 1992. Characterization of a component of the yeast secretion machinery: identification of the MNN48 product. Mol. Cell. Biol. 8:4098-4109.

23. Eible, R. 1992. A simple and efficient procedure for transformation of yeasts. Biotechniques. 13:18-20.

24. Eise, B., P. Novick, and R. Scheekman. 1981. Compartimentalized assembly of oligosaccharides on exported glycoproteins in yeast. Cell. 25:451-460.
83. Russo, R.N., N.L. Sharper, D.J. Tsatsis, and J.H. Sharp. 1992. β-1,4-galactosyltransferase: a short NH2-terminal fragment that includes the cytoplasmic and transmembrane domain is sufficient for Golgi retention. J. Biol. Chem. 267:9204–9207.

84. Saiki, R., D.H. Gelfand, S. Stoffel, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science (Wash. DC). 239:487–491.

85. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463–5467.

86. Saiki, R., D.H. Gelfand, S. Stoffel, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science (Wash. DC). 239:487–491.

87. Schiestl, R.H., and R.D. Gietz. 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acid as a carrier. Curr. Genet. 16:339–346.

88. Schröder, S., F. Schimmlöller, B. Singer-Kriiger, and H. Riezman. 1995. The Golgi-localization of yeast Emp47p depends on its di-lysine motif but is not affected by the ret-1 mutation in α-COP. J. Cell Biol. 131:895–912.

89. Schweinert, T., C. Lorenz, and J.F. Ernst. 1995. Golgi localization in yeast is mediated by the membrane anchor region of rat liver sialyltransferase. J. Biol. Chem. 270:5483–5489.

90. Schweizer, A., J. Rohrer, H.-P. Hauri, and S. Kornfeld. 1994. Retention of p63 in an ER-Golgi intermediate compartment depends on the presence of all three of its domains and on its ability to form oligomers. J. Cell Biol. 126:25–39.

91. Seeger, M., and G.S. Payne. 1992. Selective and immediate effects of increased cytotoxic activity of non-clathrin-coated Golgi transport vesicles. Nature (Lond.). 349:248–251.

92. Seeger, M., and G.S. Payne. 1992. Selective and immediate effects of increased cytotoxic activity of non-clathrin-coated Golgi transport vesicles. Nature (Lond.). 349:248–251.

93. Seetharam, S., V.K. Chaudhary, D. FitzGerald, and I. Pastan. 1991. Invertase and mature sequence substitutions that delay intercompartmental transport of active enzyme. J. Cell Biol. 100:1664–1675.

94. Semenza, J.C., K.G. Hardwick, N. Dean, and H.R. Pelham. 1990. The transmembrane domain of β-galactosidase α-2,6-sialyltransferase is in the trans-membrane domain of N-glycosaminyltransferase I contains a Golgi retention signal. J. Biol. Chem. 267:10122–10126.

95. Teasdale, R.D., G. D’Agostaro, and P.A. Gleeson. 1992. The signal for Golgi retention of bovine β-1,4-galactosyltransferase is in the trans-membrane domain [erratum appears in 1992 J. Biol. Chem. 267:13113]. J. Biol. Chem. 267:4084–4096.

96. Teasdale, R.D., F. Matheson, and P.A. Gleeson. 1994. Post-translational modifications distinguish cell surface from Golgi-retained β-1,4-galactosyltransferase molecules. Golgi localization involves active retention. Glycobiology. 4:917–928.

97. Towneley, F.M., and H.R. Pelham. 1994. The KXXX signal mediates retrieval of membrane proteins from the Golgi to the ER in yeast. Eur. J. Cell Biol. 64:211–216.

98. Trimble, R.B., F. Maley, and F.K. Chu. 1983. Glycoprotein biosynthesis in yeast: Protein conformation affects processing of high mannose oligosaccharides on carboxypeptidase Y and invertase. J. Biol. Chem. 258:2562–2567.

99. Towneley, F.M., and H.R. Pelham. 1994. The KKXX signal mediates retrieval of membrane proteins from the Golgi to the ER in yeast. Eur. J. Cell Biol. 64:211–216.

100. Wilcox, C.A., K. Redding, R. Wright, and R.S. Fuller. 1992. Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole. Mol. Biol. Cell. 3:1353–1371.

101. Wilmsbach, K., and G.S. Payne. 1993. Dynamic retention of TGN membrane proteins in Saccharomyces cerevisiae. Trends Cell Biol. 3:426–431.

102. Wilmsbach, K., and G.S. Payne. 1993. Vpslp, a member of the dynamin GTPase family, is necessary for Golgi membrane protein retention in Saccharomyces cerevisiae. EMBO (Eur. Mol. Biol. Organ.) J. 12:3049–3059.

103. Waters, M.G., T. Serafini, and J.E. Rothman. 1991. ‘Coatomer’: a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. Nature (Lond.). 349:348–251.

104. Weisz, O.A., A.M. Smith, and C.E. Machamer. 1993. Oligomerization of a membrane protein correlates with its retention in the Golgi complex. J. Cell Biol. 122:1185–1196.

105. Whittier, E.A., T.P. McGee, and V.A. Bankaitis. 1994. Purification and characterization of a late Golgi compartment from Saccharomyces cerevisiae. J. Biol. Chem. 269:28106–28117.

106. Williams, C.A., K. Redding, R. Wright, and R.S. Fuller. 1992. Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole. Mol. Biol. Cell. 3:1353–1371.

107. Wilmsbach, K., and G.S. Payne. 1993. Dynamic retention of TGN membrane proteins in Saccharomyces cerevisiae. Trends Cell Biol. 3:426–431.

108. Wilmsbach, K., and G.S. Payne. 1993. Vpslp, a member of the dynamin GTPase family, is necessary for Golgi membrane protein retention in Saccharomyces cerevisiae. EMBO (Eur. Mol. Biol. Organ.) J. 12:3049–3059.

109. Wilson, D.W., M.J. Lewis, and H.R. Pelham. 1993. pH-dependent binding of KDEL to its receptor in vitro. J. Biol. Chem. 268:7465–7468.

110. Wilson, I.A., H.L. Niman, R.A. Houghten, M.L. Cherenson, M.L. Connolly, and R.A. Lerner. 1984. The structure of an antigenic determinant in a protein. Cell. 37:767–778.

111. Wong, S.H., S. Low, and W. Hong. 1992. The 17-residue transmembrane domain of β-galactosidase α-2,6-sialyltransferase is sufficient for Golgi retention. J. Cell Biol. 117:245–258.

112. Yuan, L., J.G. Barriocanal, J.S. Bonifacino, and I.V. Sandoval. 1987. Two integral membrane proteins located in the c/s-middle and trans-part of the Golgi system acquire sialylated N-linked carbohydrates and display different turnovers and sensitivity to cAMP-dependent phosphorylation. J. Cell Biol. 105:215–227.