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 Och1p. Several constructs included a Kex2p cleavage site between the Och1p and invertase moieties to monitor transit to the Kex2p-containing TGN. Cells expressing an Och1p–invertase fusion do not secrete invertase, but those expressing an Och1p-Kex2p site–invertase fusion protein secrete high levels of invertase in a Kex2p-dependent manner. The Och1p-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 Och1p-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 Och1p reside in a compartment with a buoyant density identical to that of wild-type Och1p and distinct from that of the vacuole or the TGN. Finally, *och1* null cells that express an Och1p fusion construct known to rapidly encounter the TGN glycosylate invertase to the same extent as wild-type cells, indicating that they have phenotypically wild-type Och1p activity. These results lead us to propose a model for Och1p-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 cisternae, 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)[1] that bear a cytoplasmic dilysine 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 coatomer 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 sequestered 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 oligosaccharides 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 cisternae 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 Och1p, a *cis*-Golgi ManT, using a series of influenza virus hemagglutinin (HA) epitope-tagged Och1p–invertase fusion proteins (see Fig. 1). In contrast to most current models, we have found that the Och1p 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 Och1p 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 XL1-Blue (*supE44 thi-1 lac endA1 gyrA96 hsdR17 relA1* [F′ *proAB lacI*^{q} ZΔM15 Tn*10*]) (Stratagene, La Jolla, CA) and BMH 71-18 (*thi supE Δ*[*lac-proAB*] [*mutS::Tn10*] [F′ *proAB, lacI*^{q} ZΔM15]) (48). Bacterial strains were grown on standard media (61). *S. cerevisiae* strains used were SEY6210 (*MATα, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901, lys2-801, suc2Δ9*) (79), SHY101 (*MATα, ura3-52, trp1Δ1, leu2-3,112, ade2-101, Δoch1::LEU2*) (this study), SHY102 (*MATα* ura3-52, *leu2-3,112, his3-Δ200, trp1-Δ901, lys2-801, suc2Δ9, kex2Δ2::HIS3-s*) (this study), SHY103 (*MATα, ura3-52, leu2-3,112, his3-Δ200, trp1-Δ901, lys2-801, suc2Δ9, pep4Δ::LEU2*) (this study) and SH104 (*MATa, lys2-801, ura3-52, leu2-3,112, ade2-101, trp1Δ1, suc2Δ9, sec18-1*) (this study). Yeast strains were grown on yeast extract, peptone, and 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) that had also been digested with BamHI and HindIII. Plasmid pKEX2-HA (*TRP1, CEN*) was made by subcloning the *KEX2-HA*–bearing SalI/EagI fragment from pSN218 (70), kindly provided by T. Stevens (University of Oregon, Eugene), into the yeast *CEN*-containing shuttle vector pRS414 (95) that had been digested with SalI and EagI. The *KEX2* gene on pKEX2-HA contains the sequence encoding three repeated copies of the 9-amino acid epitope (YPYDVPDYA) from HA protein (110). Plasmid pSH113 was created by digesting pBL-OCH1, generously provided by Y. Jigami (National Institute of Bioscience and Human Technology, Tsukuba, Japan) (65), with XcmI followed by incubation with T4 DNA polymerase to yield blunt ends. The linearized plasmid was then digested with NsiI and ligated to a SmaI-PstI *LEU2* fragment from pMR2253, kindly provided by M. Rose (Princeton University, Princeton, NJ). This created a complete deletion of the *OCH1* open reading frame.

Plasmid pO, which encodes Och1p, was created by inserting the 2.6-kb XhoI-HindIII *OCH1* fragment from pBL-OCH1 into pRS416. Plasmid pO-HindIII was made by introducing a HindIII site at the codon for amino acid 472 (of the 480 amino acid Och1p) by unique site elimination site-directed mutagenesis (20). Plasmid pOI, which encodes the first 472 amino acids of Och1p fused in frame to amino acid 2 of mature invertase, was created by ligating the *SUC2*-containing HindIII-PvuII fragment from plasmid pSEY306 (44) to pO-HindIII that had been digested with HindIII and SacII. The amino acid sequence in single letter code at the Och1p–invertase fusion joint encoded by pOI is KEAST (where K is amino acid 470 of Och1p and T is amino acid 2 of mature invertase). Plasmid pOKI encodes, at the junction between Och1p and invertase, six amino acids from pro-α-factor (MYKREA) that contain the dibasic recognition site for the Kex2 protease. Plasmid pOKI was created by site-directed mutagenesis of the pOI plasmid using a mutagenic oligonucleotide that spanned *OCH1* and *SUC2*. In one round of mutagenesis, the HindIII site at the junction of *OCH1* and *SUC2* was removed, amino acids 472–478 of Och1p were restored, the Kex2 (MYKREA) cleavage site was added, and a NheI site was created. The sequence present at the fusion junction encoded by pOKI is: G (amino acid 478 of Och1p) AS (encoded by the NheI site) MYKREA (Kex2p cleavage site) M (amino acid 1 of mature invertase).

To construct vectors encoding HA epitope-tagged proteins, we inserted a sequence encoding three repeated copies of the epitope (YPYDVPDYAG) from HA (110). First, a NheI site was added to the carboxy terminus of Och1p by site-directed mutagenesis of plasmid pO resulting in the replacement of amino acids 479 and 480 (HK) with the amino acids AS in pO-NheI. To make pOH, a 100-bp fragment encoding the triple HA epitope sequence was removed from plasmid pMR2654 (kindly provided by M. Rose) by digestion with XbaI. The XbaI fragment was ligated into the NheI site of pO-NheI. The sequence at the carboxy terminus of Och1p encoded by pOH is G (amino acid 478 of Och1p) AR YPYDVPDYAG YPYDVPDYAGSYPYDVPDYASS. Similarly, the same 100-bp HA epitope-encoding XbaI fragment was ligated into the NheI site of plasmid pOKI to create pOHKI (*URA3*). The fusion junction between Och1p and invertase encoded by pOHKI is G (amino acid 478 of Och1p) AR YPYDVPDYAG YPYDVPDYAG SYPYDVPDYASS MYKREA (cleavage site) M (amino acid 1 of mature invertase). pOHKI (*TRP1*) was created by digesting pOHKI (*URA3*) with XhoI and SacII and ligating the *OCH1-SUC2*–containing fragment into pRS414 (*TRP1 CEN*) (95) that had been digested with XhoI and SacII. Plasmid pOKH, created by site-directed mutagenesis of plasmid pOH, encodes an Och1p fusion protein whose carboxy terminus is appended with the Kex2 cleavage site followed by the triple HA epitope. The sequence at the carboxy terminus of Och1p encoded by pOKH is G (amino acid 478 of Och1p) SS MYKREA (cleavage site) YPYDVPDYAG YPYDVPDYAG SYPYDVPDYASS. All mutations were verified by DNA sequence analysis by the dideoxy method (85).

Yeast and Bacterial Methods

E. coli transformations were performed using the method of Hanahan (36). Yeast transformations were performed by the method of Schiestl and Gietz (87) with the modifications of Eible (23). To create the *Δoch1* strain, SHY101, a XhoI-HindIII *och1::LEU2*–containing fragment from plasmid pSH113 (described above) was integrated into diploid strain SHY100 (**a**/*a*, *ura3-52/ura3-52, ade2-101/ADE2, leu2-3,112/leu2-3,112, TRP1/trp1Δ1*) by one-step gene replacement (82). After sporulation, temperature-sensitive Leu$^+$ haploid segregants were isolated that contained a complete deletion of 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 *kex2Δ2::HIS3-s* allele from plasmid pKX::HIS3-s, 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 *PEP4* with the BamHI fragment containing the *pep4Δ::LEU2* allele from plasmid pTS17 (kindly provided by T. Stevens). The deletion of *PEP4* 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-Och1p 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 that included amino acids 71 to 480 of Och1p was expressed in *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 according to standard procedures (37).

Protein Extraction and Immunoblotting

Yeast protein extracts for immunoblotting were prepared from 1 ml of midlogarithmic phase ($OD_{600\ nm}$ = 0.5–1.0) cells grown in SD media containing appropriate supplements. Cells were washed in water and then resuspended 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 (Amersham Corp., Arlington Heights, IL), were performed according to standard methods (37). HA epitope-tagged Och1p and Kex2p were detected with 12CA5 hybridoma serum-free culture supernatant at a dilution ranging from 1:250 to 1:1000. Mouse monoclonal antibody 10D7-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 $OD_{600\ nm}$ = 0.5–1.0. 3 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 pulse labeled with 150 μCi of Tran^{35}S-label (ICN Radiochemicals, Irvine, CA) per 3 OD U of cells for 2 min at 30°C (unless otherwise noted). Labeling was quenched by addition of a 25× chase solution (250 mM methionine, 250 mM cysteine) to a final concentration of 1×. The chase was stopped by addition of NaN_3 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 per 3 OD U of cells of 12CA5 hybridoma serum-free culture supernatant was added. To precipitate CPY, 0.6 μl of rabbit polyclonal anti-CPY per 3 OD U of cells were added. Antibody to CPY was a gift from S. Emr. Immunoprecipitation of invertase used 9 μl of rabbit polyclonal anti-invertase, provided by M. Rose, per 3 OD U of cells. 20 μl of a 50% slurry of protein A–Sepharose CL-4B (Pharmacia, Piscataway, NJ) in IP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS) was 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.

Antigens to be reimmunoprecipitated with a second antibody were dissociated from the first antibody by incubation at 100°C for 10 min in the presence of 100 μl of 1% SDS. 900 μl of IP dilution buffer was added to the eluted antigen. 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 with gentle mixing. Complexes were washed and processed as described above. The linkage-specific antibodies (5) were provided by R. Schekman (University of California, Berkeley).

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 $OD_{600\ nm}$ of 0.5–0.8, harvested by centrifugation, and washed two times in spheroplast buffer (50 mM Tris-HCl, pH 7.5, 1.2 M sorbitol, 10 mM NaN_3, 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 two times 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 1,10-phenanthroline, 2 μM pepstatin A, 2 μg/ml aprotinin, 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., Vineland, NJ) on ice, and the lysates were centrifuged in a rotor (SA600; Sorvall 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 Sorvall SA600 rotor at 4°C for 15 min at 10,000 *g* to generate pellet (P10) and supernatant (S10) fractions. The S10 fractions (1.3 ml) were loaded onto the tops of 10.5-ml continuous 25–40% sucrose (wt/wt) gradients and centrifuged 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 localize wild-type Och1p, SEY6210 cells were grown in SD media lacking cysteine and methionine at 30°C to an OD_{600} 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 Tran^{35}S-label per OD of cells for 2 min at 30°C. Labeling was quenched by addition of a 25× chase solution (250 mM methionine, 250 mM cysteine) to a final concentration of 1×. Cells were incubated at 30°C for 60 min after which the chase was stopped by addition of NaN_3 to 10 mM and cycloheximide to 100 μg/ml on ice. An additional 60 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-Och1p antisera per fraction. Immunoprecipitations were performed as described above.

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 Och1p because its activity represents the first post-ER enzymatic event required for synthesis of outer-chain mannose linkages on yeast secretory proteins, and thus Och1p must reside in a very early Golgi compartment (64, 80). This *cis*-Golgi localization has recently been confirmed by Gaynor et al. (28). To monitor the trafficking of Och1p, it was fused to the reporter enzyme invertase, which is encoded by the *SUC2* gene (Fig. 1). Secretion of invertase into the yeast periplasm is necessary for cells to use sucrose as a carbon source (13). A recognition site for the Kex2 protease was included at the junction of Och1p and invertase to monitor passage of the fusion protein into the Kex2p compartment, or TGN. The amino acid sequence encoding the Kex2 cleavage site (MYKREA) was derived from the *MFα1* gene (49). *MFα1* encodes a precursor protein that transits the secretory pathway and is proteolytically processed by several enzymes in the TGN to yield mature α-factor pheromone, which is secreted from the cell (27, 78). The Kex2p endopeptidase cleaves on the carboxyl side of the KR sequence found in the α-factor peptide (1, 45).

We reasoned that if a static retention mechanism exists for Och1p, then the Och1p-Kex2 site-invertase fusion protein would remain in the *cis*-Golgi. This anchoring would result in a Suc$^-$ phenotype in *suc2Δ9* cells because the fu-

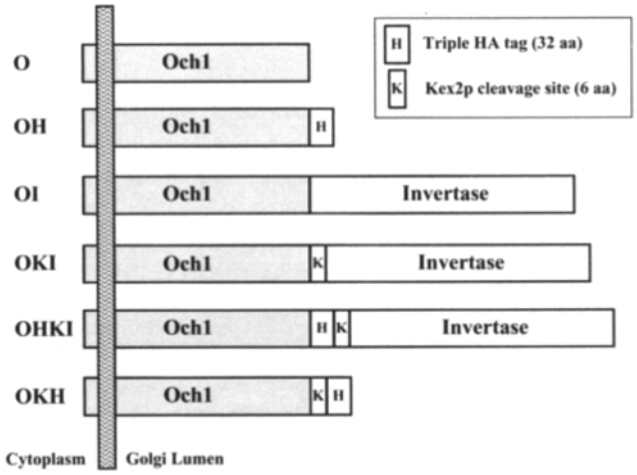

Figure 1. Diagrammatic representation of the Och1p and Och1p–invertase fusion proteins used in this study. All proteins were expressed from the *OCH1* promoter on low copy number (*CEN*) plasmids. *aa*, amino acid residue.

sion would not travel to the TGN. Thus, the invertase moiety could not be released by Kex2p, and subsequent movement to the cell surface would be prohibited. If, however, the fusion protein was capable of movement to the TGN, then Kex2p would release the invertase moiety, allowing the enzyme to travel to the cell surface resulting in a Suc^+ phenotype. Therefore, the ability of various Och1p-Kex2 site-invertase fusion proteins to confer a Suc^+ phenotype to *suc2Δ9* cells would be the first test of whether Och1p is capable of movement to more distal Golgi compartments. A similar method has recently been used to study localization of Sec12p (8), a protein that cycles between the ER and Golgi, as well as Mnn1p (32), a glycosyltransferase localized to the *trans*-Golgi and TGN.

To detect Och1p as well as the various fusion proteins by Western blot analysis and IP, we epitope tagged several of the proteins (Fig. 1) with the HA epitope recognized by the mAb, 12CA5 (110). All proteins were expressed from single-copy centromere-based (CEN) plasmids under the control of the wild-type *OCH1* promoter. All Och1p epitope-tagged molecules and fusion proteins used in this study are capable of complementing the temperature-sensitive defect (65) of the *och1* null strain (Table I), suggesting that the proteins are properly folded and correctly localized.

Table I. Kex2p-dependent Secretion of Invertase

Construct	Complementation		
	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.

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 Och1p–invertase fusion proteins diagrammed in Fig. 1. The nomenclature used for portions of the various fusion proteins is as follows: O refers to Och1p, H symbolizes the HA epitope, K indicates the Kex2p cleavage site, and I represents invertase. Only the Och1p–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 *suc2Δ9* 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 *suc2Δ9* strain that lacks functional Kex2p, OKI and OHKI were no longer able to functionally complement the *suc2Δ9* mutation (Table I). These data indicate that some Och1p–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 Och1p. 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 Och1p (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 II. Quantitation of Kex2p-dependent Invertase Secretion

Construct	Invertase activity (nmol/min/OD_{600} of cells)		
	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.

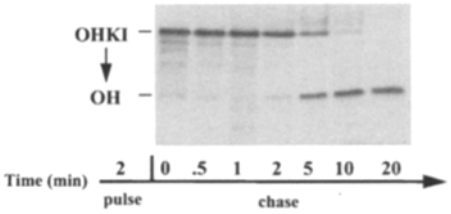

Figure 3. Cleavage of OHKI to OH occurs with a half-time of ~5 min. SEY6210 cells expressing OHKI were labeled with Tran^{35}S-label for 2 min at 30°C and chased for the times indicated. HA epitope-tagged proteins from each time point were immunoprecipitated with the anti-HA epitope antibody, separated by SDS-PAGE, and visualized by autoradiography. The OHKI and OH (generated from OHKI) proteins are indicated on the left.

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 mannose 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 α-1,6-mannose linkage, which is added in the *cis/medial* Golgi, or the α-1,3-mannose linkage, which is added in the *trans*/TGN compartments. The 150-kD OHKI fusion protein (Fig. 4) was immunoprecipitated by the α-1,6-mannose–specific antisera, indicating that it had obtained at least the "initial" α-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 α-1,6-mannose–specific antisera, but not the α-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 α-1,6 mannose outer-chain backbone was elongated, but had not moved to the *trans*-Golgi, and therefore had not obtained terminal α-1,3-mannose linkages. The higher molecular mass region of the smear was immunoprecipitable by both the α-1,6- and α-1,3-mannose–specific antibodies, indicating that this material had been exposed to the α-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 α-1,6- and the α-1,3-mannose–specific antibodies, but to a lesser extent than the invertase-containing fusion protein. This may reflect the fact that Och1p has only four potential glycosylation sites (65) compared to the 13 sites of invertase (13). Another possibility is that the oligosaccharides on the Och1p 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 Och1p 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

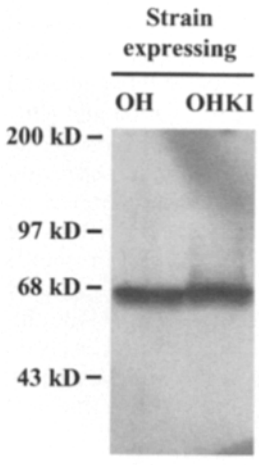

Figure 2. Uncleaved OHKI is undetectable at steady-state. Whole cell lysates prepared from an equivalent number of logarithmically growing cells from strain SEY6210 expressing either OH or OHKI were separated by 8% SDS-PAGE, transferred to nitrocellulose, and probed with anti-HA epitope hybridoma serum-free culture supernatant used at a 1,000-fold dilution. The immunoblot was developed using chemiluminescence detection (ECL; Amersham Corp.). Molecular mass markers are indicated on the left.

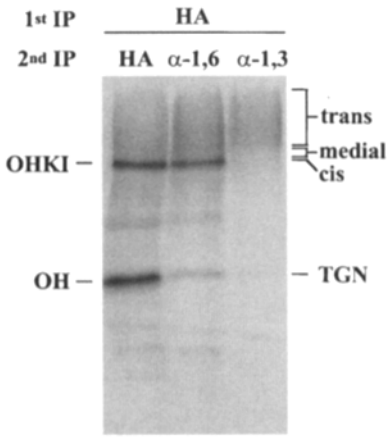

Figure 4. The oligosaccharides of OHKI receive α-1,6- and α-1,3-mannose linkages. SEY6210 cells expressing OHKI were labeled with Tran^{35}S-label for 2 min at 30°C and chased for 5 min at 30°C. Protein was immunoprecipitated with anti-HA epitope antibody. The immunoprecipitated protein was dissociated from the immune complex and divided into equivalent aliquots. The protein was then subjected to a second immunoprecipitation with either the anti-HA epitope antibody or with α-1,6- or α-1,3-mannose linkage–specific antisera. The precipitated proteins were separated by SDS-PAGE and visualized by autoradiography. The OHKI and OH (generated from OHKI) proteins are indicated on the left. Our interpretation of how far each protein has progressed through the secretory pathway (see text) is indicated on the right.

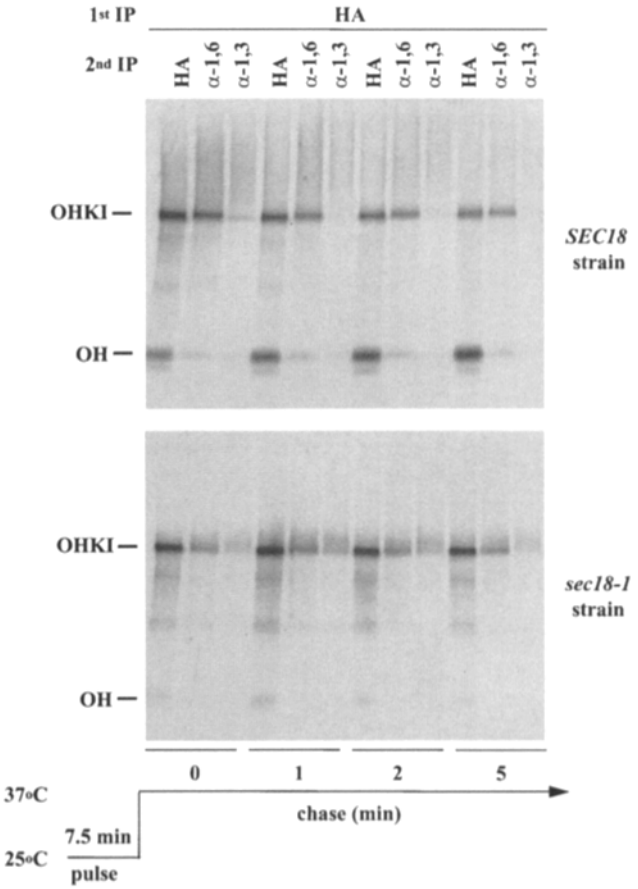

Figure 5. Cleavage of OHKI occurs in the TGN. Isogenic *SEC18* and *sec18-1* strains (SH104 with or without pSEC18) expressing OHKI were labeled with Tran^{35}S-label for 7.5 min at 25°C. At the 0-min time point, cells were shifted to 37°C and chased for the times indicated. Radiolabeled, HA epitope-tagged protein was immunoprecipitated with the anti-HA epitope antibody. The immune complexes were dissociated, and the protein was divided into equivalent aliquots and subjected to a second immunoprecipitation with either 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. The migration of the OHKI fusion protein and the cleaved OH are indicated on the left.

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, in ER-derived transport vesicles, or in the *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 support of this conclusion is the finding that neither the α-1,6- nor α-1,3-mannose–modified OHKI is converted to OH after imposition of the transport block (Fig. 5, *bottom panel*) in *sec18-1* cells. 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 Och1p 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 Och1p. This construct, termed OKH (Fig. 1), consists of Och1p 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 Och1p (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 Och1p may do so as well.

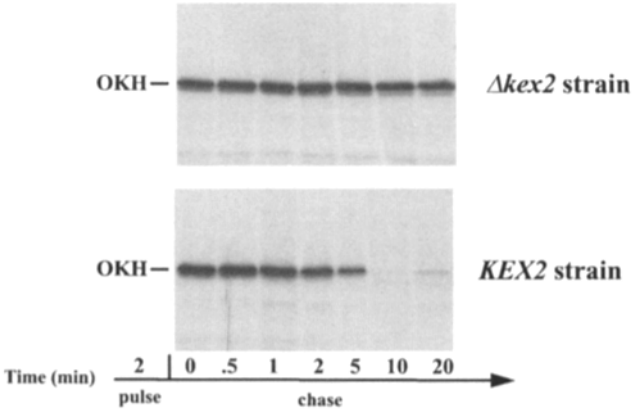

Figure 6. Rapid transit to the TGN is not dependent on the invertase moiety. Isogenic *KEX2* and *Δkex2* strains expressing OKH were labeled with Tran^{35}S-label for 2 min at 30°C and chased for the times indicated. The proteins were immunoprecipitated with the anti-HA epitope antibody, separated by SDS-PAGE, and visualized by autoradiography.

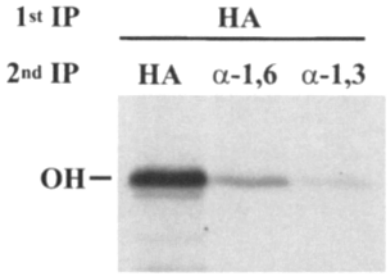

Figure 7. De novo–synthesized OH bears α-1,6-, and to a lesser degree, α-1,3-mannose linkages. SEY6210 cells expressing OH were labeled for 5 min at 30°C and chased for 30 min. Protein was immunoprecipitated with the anti-HA epitope antibody. The immune complex was dissociated by heating, and the precipitated 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.

Och1p 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 Och1p 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. 8 *A*) and OH derived from OHKI (Fig. 8 *B*) 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 *Δpep4* strain (data not shown) indicating that the eventual degradation of Och1p occurs in the vacuole (38). These findings indicate that Och1p is

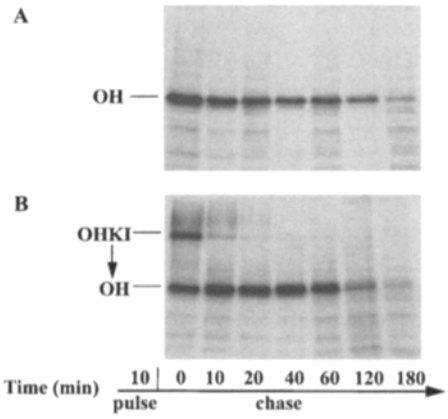

Figure 8. De novo–synthesized OH and OH derived from OHKI persist in the cell 12-fold longer than the time taken to reach the TGN. SEY6210 cells expressing either OH or OHKI were labeled with Tran^{35}S-label for 10 min at 30°C and chased for the times indicated. The proteins were immunoprecipitated with the anti-HA epitope antibody, separated by SDS-PAGE, and visualized by autoradiography. The migration of OH and OHKI are indicated.

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 Och1p 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.

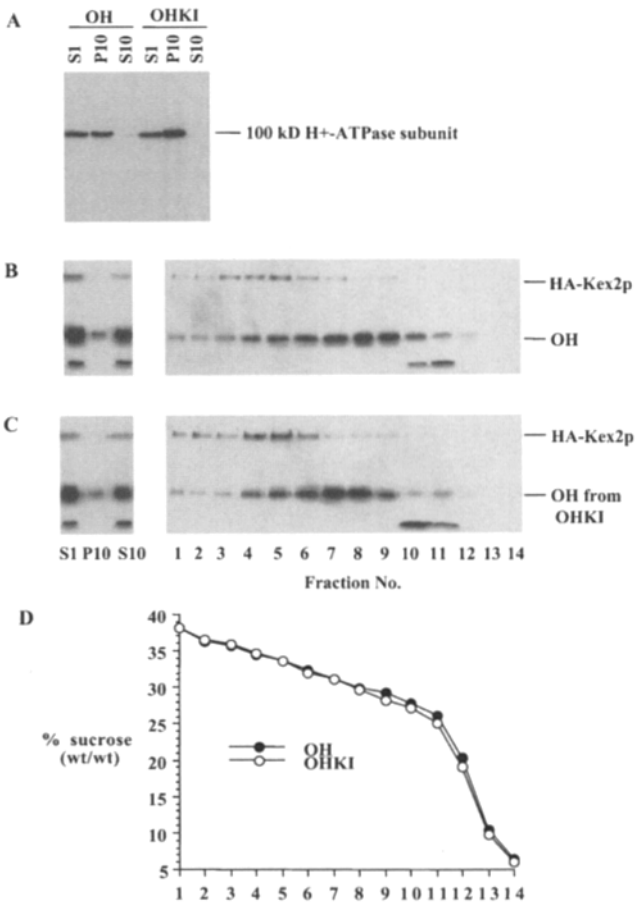

Figure 9. The compartment(s) bearing de novo–synthesized OH and OH derived from OHKI have similar buoyant densities, distinct from that of the vacuole and the Kex2-compartment. Lysates (S1) were prepared from *Δkex2* strains expressing HA epitope-tagged Kex2p and either (*A* and *B*) OH or (*A* and *C*) OHKI and separated by differential centrifugation into a pellet (*P10*) and a Golgi-enriched supernatant (*S10*). A portion of each S10 was centrifuged to equilibrium in 25–40% (wt/wt) sucrose gradients. The gradients were fractionated, and (*D*) the sucrose concentration of each gradient fraction was determined by refractometry. (*A*) The proteins in 2 μl of the S1, P10, and S10 were separated by 8% SDS-PAGE and analyzed by immunoblotting with the anti-yeast vacuolar H^+-ATPase 100-kD subunit at a 1:4,000 dilution. (*B* and *C*) The proteins in 2 μl of the S1, P10, and S10, as well as 15 μl of each gradient fraction, were separated by 8% SDS-PAGE and analyzed by immunoblotting with the anti-HA epitope antibody at a 1:1,000 dilution. Protein was visualized using the chemiluminescence detection system.

Och1p 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 Och1p migrated in a peak (predominantly fractions 6–9) corresponding to ~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 Och1p. We used an antisera directed against the luminal domain of Och1p 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 *Δoch1* cells. To localize Och1p, 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-Och1p antisera and separated by SDS-PAGE (Fig. 10 *B*). Och1p was found in fractions 7–10 corresponding to 28–32% sucrose. Thus, the wild-type Och1p compartment migrates at the same density observed for the compartment bearing HA epitope-tagged Och1p, suggesting that the epitope-tagged protein returns to the *cis*-Golgi after encountering the TGN.

In another approach to demonstrate that the Och1p fusion proteins reside in the *cis*-Golgi at steady state, we examined whether cells expressing only an Och1p 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 Och1p is responsible for initiation of this extensive glycosylation (64, 80), a significant decrease in Och1p activity should be evident as reduced glycosylation of invertase. Therefore, various strains (wild-type, *Δoch1* , and *Δoch1* expressing wild-type Och1p [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

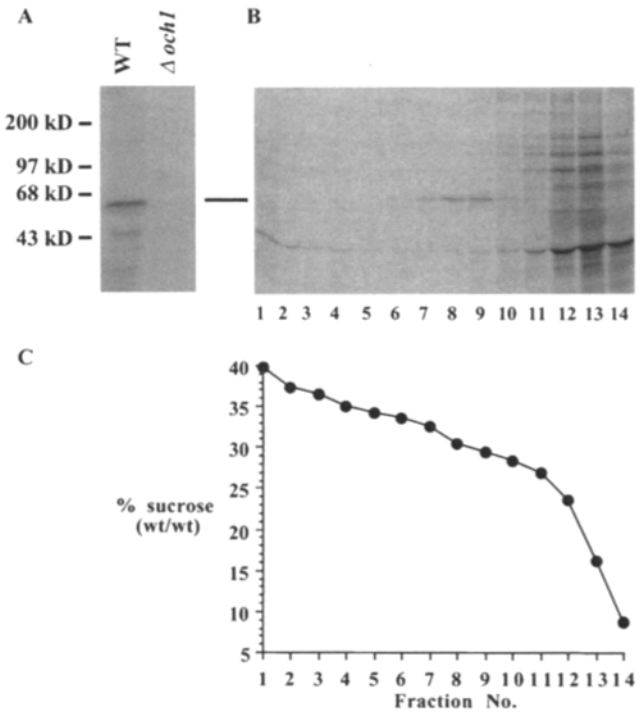

Figure 10. Wild-type Och1p resides in a compartment with a buoyant density between 28–32% sucrose. (*A*) Wild-type cells and *Δoch1* cells were labeled with Tran^{35}S-label for 2 min at 30°C and chased for 60 min. (*A*) Radiolabeled protein was immunoprecipitated with the rabbit polyclonal anti-Och1p luminal domain antibody, separated by SDS-PAGE, and visualized by autoradiography. (*B*) A Golgi enriched (S10) fraction was prepared from radiolabeled wild-type (SEY6210) cells and centrifuged to equilibrium in a 25–40% (wt/wt) sucrose gradient. Protein was immunoprecipitated from each gradient fraction with the anti-Och1p luminal domain antibody, separated by SDS-PAGE, and visualized by autoradiography. The migration of Och1p is indicated by a bar. (*C*) The sucrose concentration of each gradient fraction was determined by refractometry.

hyper-glycosylated in the wild-type strain, migrating as a heterogeneous smear. In contrast, invertase in the *Δoch1* strain migrates as a distinct band because outer-chain glycosylation cannot be initiated. Expression of either wild-type Och1p (O) or HA epitope-tagged Och1p (OH) in the *Δoch1* 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 Och1p 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 Och1p 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 Och1p is representative of the wild-type protein, we examined the stability of wild-type Och1p 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-Och1p antisera followed by SDS-PAGE. The anti-Och1p antisera recognizes both wild-type Och1p and OH, which migrates

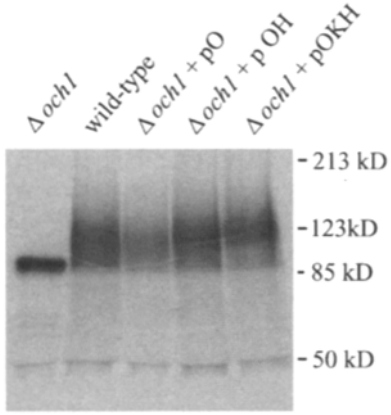

Figure 11. Invertase glycosylation is normal in cells expressing an Och1p construct that rapidly encounters the TGN. Wild-type (SEY6210) cells and *Δoch1* cells expressing either wild-type Och1p (*pO*), HA epitope-tagged Och1p (*pOH*), or the OKH fusion construct were labeled with Tran^{35}S-label for 30 min at 30°C. Protein was immunoprecipitated with an anti-invertase antibody, separated by SDS-PAGE, and visualized by autoradiography.

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*,

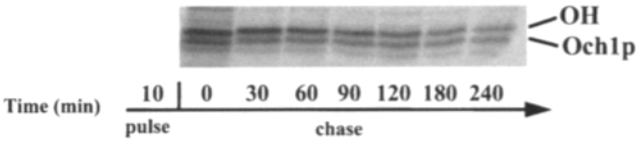

Figure 12. Wild-type Och1p has a slightly longer half-life than HA epitope-tagged Och1p. Wild-type (SEY6210) cells expressing the HA epitope-tagged Och1p construct (pOH) were labeled with Tran^{35}S-label for 10 min at 30°C and chased for the times indicated. Protein was immunoprecipitated with the anti-Och1p antibody, separated by SDS-PAGE, and visualized by autoradiography. The migration of Och1p and HA epitope-tagged Och1p is indicated. The amount of Och1p present at each time point was quantitated by Phosphorimager (Molecular Dynamics, Sunnyvale, CA) analysis.

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, $GIMP_c$ (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 I 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 proteinases in yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:173–177.
2. Aoki, D., N. Lee, N. Yamagughi, C. Dubois, and M.N. Fukuda. 1992. Golgi retention of a *trans*-Golgi membrane protein, galactosyltransferase, requires cysteine and histidine residues within 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. Bacon, R.A., A. Salminen, H. Ruohola, P. Novick, and S. Ferro-Novick. 1989. The GTP-binding protein Ypt1 is required for transport in vitro: the Golgi apparatus is defective in ypt1 mutants. *J. Cell Biol.* 109:1015–1022.
5. Ballou, C.E. 1990. Isolation, characterization, and properties of *Saccharomyces cerevisiae mnn* mutants with nonconditional protein glycosylation defects. *Methods Enzymol.* 185:440–470.
6. Ballou, L., E. Alvarado, P.K. Tsai, A. Dell, and C.E. Ballou. 1989. Protein glycosylation defects in the *Saccharomyces cerevisiae mnn7* mutant class. Support for the stop signal proposed for regulation of outer chain elongation. *J. Biol. Chem.* 264:11857–11864.
7. Banfield, D.K., M.J. Lewis, C. Rabouille, G. Warren, and H.R.B. Pelham. 1994. Localization of Sed5, a putative vesicle targeting molecule, to the *cis*-Golgi network involves both its transmembrane and cytoplasmic domains. *J. Cell Biol.* 127:357–371.
8. Boehm, 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.) J.* 13:3696–3710.
9. Bowser, R., and P. Novick. 1991. Sec15 protein, an essential component of the exocytotic apparatus, is associated with the plasma membrane and with a soluble 19.5*S* particle. *J. Cell Biol.* 112:1117–1131.
10. Bretscher, M.S., and S. Munro. 1993. Cholesterol and the Golgi apparatus. *Science (Wash. DC).* 261:1280–1282.
11. Bryant, N.J., and A. Boyd. 1993. Immunoisolation of Kex2p-containing organelles from yeast demonstrates colocalisation of three processing proteinases to a single Golgi subcompartment. *J. Cell Sci.* 106:815–822.
12. Burke, J., J.M. Pettitt, D. Humphris, and P.A. Gleeson. 1994. *Medial*-Golgi retention of *N*-acetylglucosaminyltransferase I. Contribution from all domains of the enzyme. *J. Biol. Chem.* 269:12049–12059.
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 functioning 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.) J.* 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. Bussey. 1992. Yeast Kex1p 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. Coatomer 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 protease 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. Nickoloff. 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.* 268:16845–16851.
22. Eakle, K.A., M. Bernstein, and S.D. Emr. 1988. Characterization of a component of the yeast secretion machinery: identification of the SEC18 gene 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. Esmon, B., P. Novick, and R. Schekman. 1981. Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. *Cell.* 25: 451–460.

25. Franzusoff, A., and R. Schekman. 1989. Functional compartments of the yeast Golgi apparatus are defined by the *sec7* mutation. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2695–2702.
26. Franzusoff, A., K. Redding, J. Crosby, R.S. Fuller, and R. Schekman. 1991. Localization of components involved in protein transport and processing through the yeast Golgi apparatus. *J. Cell Biol.* 112:27–37.
27. Fuller, R.S., R.E. Sterne, and J. Thorner. 1988. Enzymes required for yeast prohormone processing. *Annu. Rev. Physiol.* 50:345–362.
28. Gaynor, E.C., S. te Heesen, T.R. Graham, M. Aebi, and S.D. Emr. 1994. Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. *J. Cell Biol.* 127:653–665.
29. Goldstein, A., and J.O. Lampen. 1975. β-D-Fructofuranoside fructohydrolase from yeast. *Methods Enzymol.* 42:504–511.
30. Gonatas, J.O., S.G.E. Mezitis, A. Stiebert, B. Fleisher, and N.K. Gonatas. 1989. MG-160, a novel sialoglycoprotein of the *medial* cisternae of the Golgi apparatus. *J. Biol. Chem.* 264:646–653.
31. Graham, T.R., and S.D. Emr. 1991. Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast *sec18* (NSF) mutant. *J. Cell Biol.* 114:207–218.
32. Graham, T.R., and V.A. Krasnov. 1995. Sorting of yeast α-1,3 mannosyltransferase is mediated by a luminal domain interaction, and a transmembrane domain signal that can confer clathrin-dependent Golgi localization to a secreted protein. *Mol. Biol. Cell.* 6:809–824.
33. Graham, T.R., M. Seeger, G.S. Payne, V.L. MacKay, and S.D. Emr. 1994. Clathrin-dependent localization of α-1,3-mannosyltransferase to the Golgi complex of *Saccharomyces cerevisiae*. *J. Cell Biol.* 127:667–678.
34. Groesch, M.E., H. Ruohola, R. Bacon, G. Rossi, and S. Ferro-Novick. 1990. Isolation of a functional vesicular intermediate that mediates ER to Golgi transport in yeast. *J. Cell Biol.* 111:45–53.
35. Grove, S.N., C.E. Bracker, and D.J. Morré. 1968. Cytomembrane differentiation in the endoplasmic reticulum-Golgi apparatus-vesicle complex. *Science (Wash. DC).* 101:171–173.
36. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557–580.
37. Harlow, E., and D. Lane. 1988. Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
38. Henning, B.A., G.S. Zubenko, A. Halsilik, and E.W. Jones. 1981. Mutant defective in processing of an enzyme located in the lysosome-like vacuole of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 78:435–439.
39. Hobman, T.C., L. Woodward, and M.G. Farquhar. 1995. Targeting of a heterodimeric membrane protein complex to the Golgi: Rubella virus E2 glycoprotein contains a transmembrane Golgi retention signal. *Mol. Biol. Cell.* 6:7–20.
40. Hoe, M.H., P. Slusarewicz, T. Misteli, R. Watson, and G. Warren. 1995. Evidence for recycling of the resident *medial/trans* Golgi enzyme *N*-acetylglucosaminyltransferase I, in ldlD cells. *J. Biol. Chem.* 270: 25057–25063.
41. Hosobuchi, M., T. Kreis, and R. Schekman. 1992. *SEC21* is a gene required for ER to Golgi protein transport that encodes a subunit of a yeast coatomer. *Nature (Lond.).* 360:603–605.
42. Jackson, M.R., T. Nilsson, and P.A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3153–3162.
43. Jackson, M.R., T. Nilsson, and P.A. Peterson. 1993. Retrieval of transmembrane proteins to the endoplasmic reticulum. *J. Cell Biol.* 121:317–333.
44. Johnson, L.M., V.A. Bankaitis, and S.D. Emr. 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. *Cell.* 48:875–885.
45. Julius, D., R. Schekman, and J. Thorner. 1984. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. *Cell.* 36: 309–318.
46. Kaiser, C.A., D. Preuss, P. Grisafi, and D. Botstein. 1987. Many random sequences functionally replace the secretion signal sequence of yeast invertase. *Science (Wash. DC).* 235:312–317.
47. Klionsky, D.J., P.K. Herman, and S.D. Emr. 1990. The fungal vacuole: composition, function, and biogenesis. *Microbiol. Rev.* 54:266–292.
48. Kramer, B., W. Kramer, and H.-J. Fritz. 1984. Different base/base mismatches are corrected with different efficiencies by the methyl-directed DNA mismatch-repair system of *E. coli*. *Cell.* 38:879–887.
49. Kurjan, J., and I. Herskowitz. 1982. Structure of a yeast pheromone gene (*MFα*): a putative α-factor precursor contains four tandem copies of mature α-factor. *Cell.* 30:933–943.
50. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680–685.
51. Letourneur, F., E.C. Gaynor, S. Hennecke, C. Demolliere, R. Duden, S.D. Emr, H. Riezman, and P. Cosson. 1994. Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell.* 79:1199–1207.
52. Lewis, M.J., and H.R. Pelham. 1992. Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell.* 68:353–364.
53. Locker, J.K., D.-J.E. Opstelten, M. Ericsson, M.C. Horzinek, and P.J.M. Rottier. 1995. Oligomerization of a *trans*-Golgi/*trans*-Golgi network retained protein occurs in the Golgi complex and may be part of its retention. *J. Biol. Chem.* 270:8815–8821.
54. Lussier, M., A.-M. Sdicu, T. Ketals, and H. Bussey. 1995. Localization and targeting of the *Saccharomyces cerevisiae* Kre2p/Mnt1p α-1,2-mannosyltransferase to a *medial*-Golgi compartment. *J. Cell Biol.* 131:913–927.
55. Machamer, C.E. 1991. Golgi retention signals: do membranes hold the key? *Trends Cell Biol.* 1:141–144.
56. Machamer, C.E. 1993. Targeting and retention of Golgi membrane proteins. *Curr. Opin. Cell Biol.* 5:606–612.
57. Machamer, C.E., and J.K. Rose. 1987. A specific transmembrane domain of a coronavirus E1 glycoprotein is required for its retention in the Golgi region. *J. Cell Biol.* 105:1205–1214.
58. Masibay, A.S., P.V. Balaji, E.E. Boeggeman, and P.K. Quasba. 1993. Mutational analysis of the Golgi retention signal of bovine β-1,4-galactosyltransferase. *J. Biol. Chem.* 268:9908–9916.
59. Matsuoka, Y., S.-Y. Chen, and R.W. Compans. 1994. A signal for Golgi retention in the Bunyavirus G1 glycoprotein. *J. Biol. Chem.* 269:22565–22573.
60. Meisenböck, G., and J.E. Rothman. 1995. The capacity to retrieve escaped ER proteins extends to the *trans*-most cisterna of the Golgi stack. *J. Cell Biol.* 129:309–313.
61. Miller, J.H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
62. Munro, S. 1991. Sequences within and adjacent to the transmembrane segment of α-2,6-sialyltransferase specify Golgi retention. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3577–3588.
63. Munro, S., and H.R. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899–907.
64. Nakanishi-Shindo, Y., K.I. Nakayama, A. Tanaka, Y. Toda, and Y. Jigami. 1993. Structure of the *N*-linked oligosaccharides that show the complete loss of α-1,6-polymannose outer chain from *och1*, *och1 mnn1*, and *och1 mnn1 alg3* mutants of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 268:26338–26345.
65. Nakayama, K., T. Nagasu, Y. Shimma, J. Kuromitsu, and Y. Jigami. 1992. *OCH1* encodes a novel membrane bound mannosyltransferase: outer chain elongation of asparagine-linked oligosaccharides. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2511–2519.
66. Nilsson, T., J.M. Lucocq, D. Mackay, and G. Warren. 1991. The membrane spanning domain of β-1,4-galactosyltransferase specifies *trans* Golgi localization. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3567–3575.
67. Nilsson, T., P. Slusarewicz, M.H. Hoe, and G. Warren. 1993. Kin recognition. A model for the retention of Golgi enzymes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 330:1–4.
68. Nilsson, T., M.H. Hoe, P. Slusarewicz, C. Rabouille, R. Watson, F. Hunte, G. Watzele, E.G. Berger, and G. Warren. 1994. Kin recognition between *medial* Golgi enzymes in HeLa cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:562–574.
69. Nothwehr, S.F., C.J. Roberts, and T.H. Stevens. 1993. Membrane protein retention in the yeast Golgi apparatus: dipeptidyl aminopeptidase A is retained by a cytoplasmic signal containing aromatic residues. *J. Cell Biol.* 121:1197–1209.
70. Nothwehr, S.F., E. Conibear, and T.H. Stevens. 1995. Golgi and vacuolar membrane proteins reach the vacuole in *vps1* mutant yeast cells via the plasma membrane. *J. Cell Biol.* 129:35–46.
71. Novick, P., S. Ferro, and R. Schekman. 1981. Order of events in the yeast secretory pathway. *Cell.* 25:461–469.
72. Orci, L., R. Montesano, P. Meda, F. Malaisse-Lagae, D. Brown, A. Perrelet, and P. Vassalli. 1981. Heterogeneous distribution of filipin-cholesterol complexes across the cisternae of the Golgi apparatus. *Proc. Natl. Acad. Sci. USA.* 78:293–297.
73. Pastan, I., V. Chaudary, and D.J. FitzGerald. 1992. Recombinant toxins as novel therapeutic agents. *Annu. Rev. Biochem.* 61:331–354.
74. Pelham, H.R., K.G. Hardwick, and M.J. Lewis. 1988. Sorting of soluble ER proteins in yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1757–1762.
75. Pelham, H.R.B., L.M. Roberts, and J.M. Lord. 1992. Toxin entry: how reversible is the secretory pathway? *Trends Cell Biol.* 2:183–185.
76. Peter, F., P.N. Van, and H.-D. Söling. 1992. Different sorting of Lys-Asp-Glu-Leu proteins in rat liver. *J. Biol. Chem.* 267:10631–10637.
77. Raschke, W.C., K.A. Kern, C. Antalis, and C.E. Ballou. 1973. Genetic control of yeast mannan structure. Isolation and characterization of mannan mutants. *J. Biol. Chem.* 248:4660–4666.
78. Redding, K., C. Holcomb, and R.S. Fuller. 1991. Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* 113:527–538.
79. Robinson, J.S., D.J. Klionsky, L.M. Banta, and S.D. Emr. 1988. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* 8:4936–4948.
80. Romero, P.A., and A. Herscovics. 1989. Glycoprotein biosynthesis in *Saccharomyces cerevisiae*. Characterization of α-1,6-mannosyltransferase which initiates outer chain formation. *J. Biol. Chem.* 264:1946–1950.
81. Rose, M., F. Winston, and P. Hieter. 1990. Methods in yeast genetics. A laboratory course manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
82. Rothstein, R. 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* 194:281–301.

83. Russo, R.N., N.L. Sharper, D.J. Taatjes, and J.H. Sharper. 1992. β-1,4-galactosyltransferase: a short NH_2-terminal fragment that includes the cytoplasmic and transmembrane domain is sufficient for Golgi retention. *J. Biol. Chem.* 267:9241–9247.
84. Saiki, R., D.H. Gelfand, S. Stoffel, R. Higuuchi, 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. Schauer, I., S. Emr, C. Gross, and R. Schekman. 1985. Invertase signal and mature sequence substitutions that delay intercompartmental transport of active enzyme. *J. Cell. Biol.* 100:1664–1675.
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. Schimmöller, B. Singer-Krüger, and H. Riezman. 1995. The Golgi-localization of yeast Emp47p depends on its di-lysine motif but is not affected by the *ret1-1* mutation in α-COP. *J. Cell Biol.* 131: 895–912.
89. Schweintek, 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. A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2811–2818.
92. Seeger, M., and G.S. Payne. 1992. Selective and immediate effects of clathrin heavy chain mutations on Golgi membrane protein retention in *Saccharomyces cerevisiae. J. Cell Biol.* 118:531–540.
93. Seetharam, S., V.K. Chaudhary, D. FitzGerald, and I. Pastan. 1991. Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL. *J. Biol. Chem.* 266:17376–17381.
94. Semenza, J.C., K.G. Hardwick, N. Dean, and H.R. Pelham. 1990. *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell.* 61:1349–1357.
95. Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae. Genetics.* 122:19–27.
96. Slusarewicz, P., T. Nilsson, N. Hui, R. Watson, and G. Warren. 1994. Isolation of a matrix that binds *medial* Golgi enzymes. *J. Cell Biol.* 124:405–413.
97. Swift, A.M., and C.E. Machamer. 1991. A Golgi retention signal in a membrane-spanning domain of coronavirus E1 protein. *J. Cell Biol.* 115:19–30.
98. Tang, B.L., S.H. Wong, S.H. Low, and W. Hong. 1992. The transmembrane domain of *N*-glucosaminyltransferase I contains a Golgi retention signal. *J. Biol. Chem.* 267:10122–10126.
99. 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 transmembrane domain [erratum appears in 1992 *J. Biol. Chem.* 267:13113]. *J. Biol. Chem.* 267:4084–4096.
100. 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.
101. Townsley, 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.
102. 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.
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.).* 349:248–251.
104. Weisz, O.A., A.M. Swift, 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. Whitters, 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. 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.
107. Wilsbach, K., and G.S. Payne. 1993. Dynamic retention of TGN membrane proteins in *Saccharomyces cerevisiae. Trends Cell Biol.* 3:426–431.
108. Wilsbach, K., and G.S. Payne. 1993. Vps1p, 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.H. Low, and W. Hong. 1992. The 17-residue transmembrane domain of β-galactoside 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 *cis*-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.