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Sorting of Yeast α1,3 Mannosyltransferase Is Mediated by a Lumenal Domain Interaction, and a Transmembrane Domain Signal that Can Confer Clathrin-dependent Golgi Localization to a Secreted Protein

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α1,3 mannosyltransferase (Mnn1p) is a type II integral membrane protein that is localized to the yeast Golgi complex. We have examined the signals within Mnn1p that mediate Golgi localization by expression of fusion proteins comprised of Mnn1p and the secreted protein invertase. The N-terminal transmembrane domain (TMD) of Mnn1p is sufficient to localize invertase to the Golgi complex by a mechanism that is not saturable by ~15–20 fold overexpression. Furthermore, the TMD-mediated localization mechanism is clathrin dependent, as an invertase fusion protein bearing only the Mnn1p TMD is mislocalized to the plasma membrane of a clathrin heavy chain mutant. The Mnn1-invertase fusion proteins are not retained in the Golgi complex as efficiently as Mnn1p, suggesting that other signals may be present in the wild-type protein. Indeed, the Mnn1p lumenal domain (Mnn1-s) is also localized to the Golgi complex when expressed as a functional, soluble protein by exchanging its TMD for a cleavable signal sequence. In contrast to the Mnn1-invertase fusion proteins, overexpression of Mnn1-s saturates its retention mechanism, and results in the partial secretion of this protein. These data indicate that Mnn1p has separable Golgi localization signals within both its transmembrane and lumenal domains.

INTRODUCTION

The Golgi complex of plant and animal cells is comprised of a number of stacked, flattened cisternae with dilated rims and reticular networks emanating from each face of the stack (Palade, 1975). This organelle maintains a specific polarity with respect to the ER, and is divided into regions referred to as follows: the *cis* Golgi network (ER proximal); the *cis*, *medial*, and *trans* cisternae of the Golgi stack; and the *trans* Golgi network (ER distal) (Farquhar and Palade, 1981; Mellman and Simons, 1992). Each successive Golgi cisterna contains partially overlapping sets of enzymes (Nilsson *et al.*, 1993a) that form an assembly line for the production of glycoconjugates (Farquhar, 1985; Pfeffer and Rothman, 1987). Transport of newly synthesized proteins through the Golgi complex is thought to be mediated by vesicles that bud from one Golgi cisterna and fuse with the next in the *cis-trans* direction (Rothman, 1994). This organelle is also the primary site in the secretory pathway where proteins with different destinations are sorted into the appropriate vesicular carriers (Mellman and Simons, 1992). Stacked Golgi cisternae are rarely seen in electron micrographs of the yeast *Saccharomyces cerevisiae*. Instead, the Golgi complex appears as isolated, disk-shaped compartments scattered throughout the cytoplasm (Preuss *et al.*, 1992). In spite of the apparently unordered structure, the yeast Golgi complex is functionally organized into ordered compartments, within which are catalyzed distinct post-translational modifications of glycoproteins (Krasnov and Graham, 1995). A functional de-

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scription of the compartmental organization of the yeast Golgi complex has been inferred from analysis of protein transport in *sec* mutants that exhibit a temperature-conditional defect in intercompartmental protein transport (Franzusoff and Schekman, 1989; Graham *et al.*, 1992). Immunofluorescent localization of Golgi marker proteins (Franzusoff *et al.*, 1991) and subcellular fractionation (Cunningham and Wickner, 1989; Bowser and Novick, 1991; Bryant and Boyd, 1993; Gaynor *et al.*, 1994; Whitters *et al.*, 1994) also suggest that many Golgi enzymes are preferentially localized to distinct compartments. These studies indicate that the Golgi complex is minimally divided into compartments where glycoproteins traversing the secretory pathway first encounter, from *cis* to *trans*, an initiating α1,6 mannosyltransferase (Och1p), an elongating α1,6 mannosyltransferase, an α1,3 mannosyltransferase (Mnn1p), and Kex2 endopeptidase.

To gain a better understanding of how proteins are localized to the Golgi complex, we have begun to characterize the biosynthesis and localization of Mnn1p, a type II integral membrane glycoprotein that resides in medial, and perhaps late (Kex2p-containing) compartments of the yeast Golgi complex. Mnn1p is modified with both *N*- and *O*-linked oligosaccharides in the endoplasmic reticulum (ER), which are subject to slow, inefficient modification by the Golgi mannosyltransferases (Graham *et al.*, 1994). There is little known about the mechanisms used to sort early (*cis* to *medial*) Golgi proteins in yeast. However, for the late Golgi enzymes Kex2p and dipeptidylaminopeptidase A (DPAP A), a signal containing a critical aromatic amino acid located within the cytoplasmic tail of each protein has been defined by mutagenesis to specify Golgi localization (Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993, respectively). These Golgi localization motifs are similar to the cytoplasmic tail signals that mediate clustering of mammalian plasma membrane receptors into clathrin-coated pits (Nothwehr and Stevens, 1994), apparently by a direct interaction with the clathrin-associated protein AP-2 (Pearse, 1988; Beltzer and Spiess, 1991). The similarities in these motifs are significant in light of the observation that both Kex2p and DPAP A are mislocalized to the plasma membrane of cells that harbor a mutation in the clathrin heavy chain gene (Payne and Schekman, 1989; Seeger and Payne, 1992b), although a direct interaction between the cytoplasmic tails of yeast Golgi enzymes with Golgi-specific–associated protein AP-1/clathrin coat complexes has not yet been reported. The cytoplasmic tail of Mnnlp does not appear to be necessary for Golgi localization, suggesting that this protein employs a different type of localization signal than the late Golgi enzymes. However, Mnn1p is also mislocalized to the plasma membrane of a clathrin mutant to a similar extent as is Kex2p (Graham *et al.*, 1994). The latter observation suggests that the mechanism for Golgi localization or the trafficking patterns of Mnn1p may share some features with late Golgi enzymes.

In the case of most mammalian Golgi glycosyltransferases thus far characterized, it has been found that a transmembrane domain (TMD) and associated flanking amino acids are sufficient to localize a reporter enzyme to the Golgi complex (Munro, 1991; Nilsson *et al.*, 1991; Aoki *et al.*, 1992; Burke *et al.*, 1992; Russo *et al.*, 1992; Tang *et al.*, 1992; Teasdale *et al.*, 1992; Wong *et al.*, 1992). We suspected that this localization mechanism may have been conserved in evolution, so to test this idea we expressed invertase fusion proteins bearing the Mnn1p TMD in yeast. We found that these fusion proteins are localized to the Golgi complex, although not as efficiently as Mnn1p. However, the Mnn1p lumenal domain is also localized to the Golgi complex when expressed as a soluble protein by exchanging its TMD for a cleavable signal sequence. This suggests that both the lumenal and transmembrane domains contain signals that contribute toward Golgi localization of the intact protein. In addition, we show that Golgi localization of an invertase fusion protein containing only the TMD of Mnnlp is clathrin dependent.

MATERIALS AND METHODS

Strains and Media

The yeast strains used were XCY42–30D (*MATα ura3 leu2–3, 112 trp1 lys2 ade2–101 adeX suc2-Δ9*), XCY42–30D *Δmnn1::LEU2* (*MATα ura3 leu2–3, 112 trp1 lys2 ade2–101 adeX suc2-Δ9 Δmnn1::LEU2* [Graham *et al.*, 1994]), SEY6210 (*MATα ura3–52 leu2–3, 112 his3-Δ200 trp1-Δ901 lys2–801 suc2-Δ9* [Robinson *et al.*, 1988]), TVY1 (SEY6210 *Δpep4::LEU2* [Stack *et al.*, 1993]), CRY2 *kex2Δ2::HIS3-s* (*MATa, can1–100 ade2–1^{oc} his3–11,-15, leu2–3,-112 trp1–1 ura3–1 kex2Δ2::HIS3-s* [R. Fuller, University of Michigan]), GPY396.1 (*MATα ura3–52 leu2–3, 112 trp1 prb1 chc1–521^{ts}*) *GPY55–10B* (*MATα ura3–52 leu2–3, 112 trp1 prb1* [G. Payne, University of California at Los Angeles]).

Standard rich (YPD) and synthetic-defined (SD) minimal media were used for culturing yeast (Sherman, 1991). The SD medium was supplemented with 0.2% yeast extract (SD-YE) and other supplements as needed for growing cells in liquid culture before labeling experiments. Standard rich medium for growth of *Escherichia coli* (Miller, 1972) was used.

Reagents

Zymolyase-100T (Kirin Brewery) was obtained from Seikagako Kogyo Co. (Tokyo, Japan), DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA), endoglycosidase H and proteinase K were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), Protein A-Sepharose was obtained from Pharmacia (Piscataway, NJ), EXPRE^{35}S^{35}S protein labeling mix was obtained from Dupont NEN (Wilmington, DE), the Enhanced Chemiluminescence detection kit was obtained from Amersham (Arlington Heights, IL), 0.2- to 0.3-mm glass beads were obtained from Glen Mills (Maywood, NJ), and Boc-QRR-MCA Kex2p substrate was obtained from Peptides International (Louisville, KY). All other chemicals were purchased from Sigma Chemical (St. Louis, MO). Antiserum to α1–3 mannose linkages was prepared at Scantibodies Laboratory (Ramona, CA) by immunizing a male New Zealand white rabbit with heat-killed XCY42–30D cells and subsequent absorption of the antiserum with heat-killed cells of strain XCY42–30D *Δmnn1::LEU2*. Antisera to Mnn1p, invertase, and carboxypep-

tidase Y have been previously described (Graham *et al.*, 1994; Gaynor *et al.*, 1994; and Klionsky *et al.*, 1988, respectively).

Plasmid Construction

pTG104 (Graham *et al.*, 1994) is M13mp19 harboring a 1.4-kb *Bam*HI–*Eco*RI fragment containing 664 bp of the 5′-untranslated region of the *MNN1* gene and up to codon 244 of the open reading frame. Site-directed oligonucleotide mutagenesis was performed on pTG104 as previously described (Kunkel, 1985) to introduce *Bam*HI restriction sites after codons 39, 73, and 166 of the *MNN1* gene, which generated pTG104–39–pTG104–166. To prepare the *MNN1-SUC2* gene fusions, *Bam*HI fragments from the modified pTG104 replicative forms were subcloned in the proper orientation into pSEYC308 (*CEN4 ARS1 URA3*) (Klionsky *et al.*, 1988) or pSEY304 (2 μ *URA3*) (Johnson *et al.*, 1987) to produce pM39S-308, pM39S-304, pM73S-308, pM166S-308, and pM166S-304. To prepare an *MNN1-SUC2* gene fusion encoding only the TMD of Mnn1p, site-directed mutagenesis of pTG104 was performed with two mutagenic oligonucleotides to delete the sequences encoding amino acids 2–18 of *MNN1* and replace them with sequences derived from the cytoplasmic tail of dipeptidylpeptidase IV (Hong and Doyle, 1988) encoding amino acids 2–6, and to simultaneously introduce a *Bam*HI site after codon 39 of *MNN1*. The resulting 5′ *Bam*HI fragment was subcloned into pSEYC308 to produce pM39S-XCT (exchange cytoplasmic tail).

pαS-308 was constructed by subcloning a 63-bp *Hin*dIII fragment (originally from the *MFα1* gene) from pCαI-50 (Graham and Emr, 1991) into the *Hin*dIII site of pSEYC308 such that the correct open reading frame was maintained between the *MFα1* segment and *SUC2*. This *MFα1* segment encodes a Kex2p cleavage site preceding one copy of mature α-factor. pM39αS was constructed by subcloning the *Bam*HI fragment from pM39S into pαS-308 such that the open reading frame was maintained between all three gene segments.

To prepare pMNN1-s, a 1.4-kb *Sal*I–*Bam*HI fragment of the *PRC1* gene was subcloned from pCYI-20 (Johnson *et al.*, 1987) into pPHYC18 (*CEN4 ARS1 URA3*) (Herman and Emr, 1990) to produce pTG48. A 3.7-kb *Bam*HI–*Bgl*II fragment carrying the intact *MNN1* gene was subcloned into the *Bam*HI site of pTG48 such that the 5′ *Bam*HI site of the *MNN1* gene was apposed to the 3′ *Bam*HI site of *PRC1* (pTG49). Then, the *Bam*HI–*Eco*RI fragment encoding amino acids 40–244 of Mnn1p was excised from pTG104–39 and used to replace the *Bam*HI–*Eco*RI fragment of pTG49 to construct pMNN1-s. These manipulations replaced the *MNN1* sequences encoding the promoter and open reading frame up to codon 39 with that encoding the promoter and CPY cleavable signal sequence from *PRC1*. A multi-copy vector harboring the MNN1-s construct was prepared by subcloning a *Kpn*I–*Sac*I fragment from pMNN1-s into pRS426, resulting in pRS426 MNN1-s.

Cell Labeling and Immunoprecipitation

For labeling experiments, yeast cells were grown to mid-logarithmic phase in SD-YE, then washed twice in water and resuspended in SD media with appropriate supplements at 5 OD_{600}/ml. To initiate labeling, EXPRE^{35}S^{35}S-label was added to a final concentration of 150 μCi/ml and labeling was quenched by adding a 50× chase solution (50 mM methionine, 10 mM cysteine, and 5% yeast extract) to a 1× concentration. The chase was subsequently terminated by adding trichloroacetic acid (TCA) to a final concentration of 10%. Processing of TCA pellets for immunoprecipitation with anti-Mnn1p antiserum and size fractionation of proteins in sodium dodecyl sulfate (SDS)-polyacrylamide gels were done as previously described (Graham *et al.*, 1993). Double immunoprecipitations were done for all experiments using the anti-invertase antiserum. For this procedure, the primary immunoprecipitates were resuspended in 100 μl of 1% SDS, 20 mM Tris-Cl, pH 7.5, and heated for 5 min at 95°C. The samples were then diluted with 900 μl of Tween-20 IP buffer and the immunoprecipitation protocol was repeated as previously described (Graham *et al.*, 1993). Endoglycosidase H (endo H) treatment of immunoprecipitates was done as previously described (Graham *et al.*, 1994). An LKB laser densitometer was used to quantitate band intensities on autoradiograms. The half-times for Golgi modification were determined by plotting the disappearance of bands representing the core glycosylated forms over time. The corresponding endo H-treated samples were used to correct for sample recovery at each time point. The half-time for *PEP4*-dependent cleavage was determined from endo H-treated samples by plotting the percentage of total invertase in the 59-kDa form over time (0, 15, 30, and 60 min), and extrapolating to the time required for 50% cleavage.

Subcellular Fractionation

The methods used for differential centrifugation analysis and sucrose gradient fractionation of Golgi membranes have been previously described in greater detail (Graham *et al.*, 1994). Briefly, spheroplasts (300 OD_{600}) were lysed by dilution in hypo-osmotic buffer followed by Dounce homogenization. The lysate was centrifuged at 1000 × *g* for 6 min to generate the P1 (pellet) and the S1 (supernatant) fractions, and the latter was centrifuged at 13,000 × *g* to generate the P13 and S13 fractions. The S13 was layered onto a two-step sucrose cushion consisting of 1 ml of 66% sucrose and 1 ml of 20% sucrose, then centrifuged in an Sorvall TH641 rotor at 31000 rpm for 2 h at 4°C. The membranes present at the 20–66% sucrose interface (P120) were collected in as small a volume as possible and the refractive index of the sample was measured using a Bausch and Lomb refractometer. The P120 sample was then adjusted to ~48% sucrose using a 66% sucrose solution and 1.5 ml of the membrane sample was layered on top of a 0.5-ml 61% sucrose cushion in a 14 × 89 mm ultracentrifuge tube. A sucrose step gradient that consisted of 47.5% (1.5 ml), 45% (1.0 ml), 42% (2.0 ml), 40% (2.0 ml), 38% (1.0 ml), 36% (1.0 ml), and 32% (1.5 ml) sucrose was layered on top of the sample. All sucrose solutions were prepared weight/weight in 10 mM Na *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5, 1 mM sodium azide (gradient buffer). The gradients were centrifuged in a TH641 rotor at 31,000 rpm for 17 h at 4°C. Sixteen fractions (~0.75 ml) were collected starting from the top of the gradient using a Buchler Auto Densi-Flow IIc and a Gilson FC80 fraction collector. Each fraction was diluted to 2.5 ml with gradient buffer and centrifuged again in a Beckman 50Ti or Sorvall T1270 rotor with tube adapters at 40,500 rpm for 1.5 h at 4°C. After aspirating off the supernatants, the pellets were resuspended in 0.2 ml of gradient buffer and stored at −75°C.

Invertase, Kex2p, and GDPase were assayed as previously described (Johnson *et al.*, 1987, Graham *et al.*, 1994; Yanagisawa *et al.*, 1990, respectively). All assays were done in duplicate and were repeated if the standard deviation was >10% of the average value, such that all assay points have a standard deviation of less than 10% of the value reported. Protein concentration was determined using the bicinchoninic acid protein assay kit from Sigma. Western blot analysis of Mnn1p in gradient fractions was done by overnight incubation of the blot at 4°C with affinity-purified α-Mnn1p antibodies, followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibodies, and detection using the Enhanced Chemiluminescence detection kit from Amersham as previously described (Graham *et al.*, 1994).

RESULTS

Expression and Analysis of Mnn1-Invertase Fusion Proteins

Mnn1p is a type II integral membrane protein with a short N-terminal cytoplasmic tail (amino acids 1–18) followed by an uncleaved, hydrophobic signal/anchor domain (or TMD) of approximately 18 amino

acids (Graham *et al.*, 1994; Figure 1). To test whether the TMD of Mnnlp contains a Golgi localization signal, we prepared gene fusions between *MNN1* and *SUC2* (the invertase gene) that allowed for the expression of membrane spanning invertase fusion proteins (Figure 1). Invertase is a heavily glycosylated protein that is normally transported rapidly through the secretory pathway and secreted into the periplasmic space. Therefore, this protein lacks any sorting signals for retention within the ER, the Golgi complex, or for transport to the vacuole. Diversion of the invertase activity derived from an Mnn1-invertase fusion protein from this pathway of secretion to an intracellular destination suggests the presence of a sorting signal within the Mnn1 sequences present in the chimera. We refer to the *MNN1-SUC2* gene fusions as MXXS where XX is the 3′-most *MNN1* codon number in the chimera. The proteins encoded by the gene fusions are

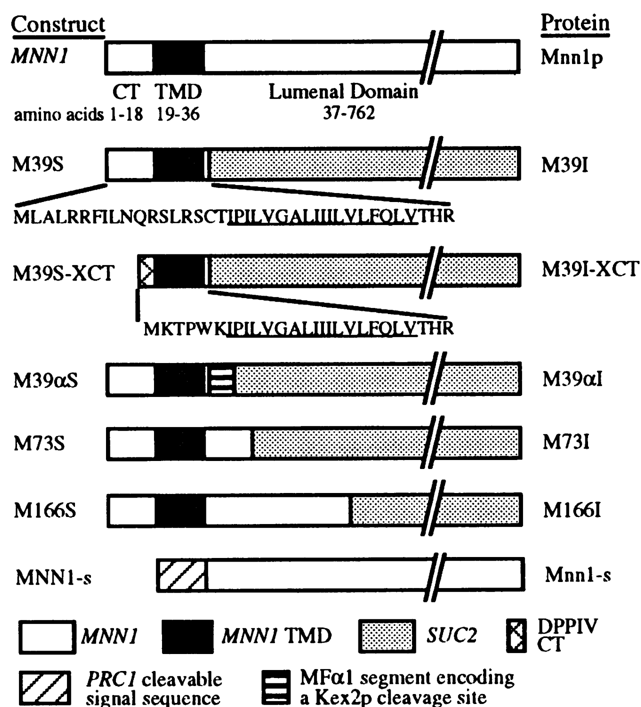

Figure 1. Diagrams showing the open reading frame of the constructs used in this study. The amino acid sequences of the cytoplasmic tail (CT) and transmembrane domain (TMD) of M39I and M39I-XCT are shown below the respective diagrams. Sequences encoding the cytoplasmic tail of Mnn1p were deleted up to the hydrophobic core of the TMD (underlined) in the construct pM39S-XCT and were replaced with that of rat dipeptidylpeptidase IV (DPPIV) to maintain the type II integral membrane topology. All constructs that contain *SUC2* sequences encode amino acids 3–511 of mature invertase. Expression of constructs that contain the *PRC1* signal sequence was driven from the *PRC1* promoter, which results in a 4- to 5-fold increase in expression level relative to the rest of the constructs that contain the *MNN1* promoter. The preparation of these constructs are described under MATERIALS AND METHODS.

referred to as MXXI (I for invertase). For example, the construct M39S contains the 5′ promoter region and first 39 codons of *MNN1* fused in frame to the region of *SUC2* coding for amino acids 3–511 of mature invertase.

M39S was expressed from a low-copy number vector in a Δ*suc2* yeast strain (SEY6210) and aliquots of cells in log-phase growth were divided equally to prepare a detergent lysate from one-half of each sample. The intact cells and lysates were assayed for invertase activity to determine the amount present on the cell surface and the total activity, respectively. We found that this strain expressed only 10% (± 5%) of the invertase activity at the cell surface (Table 1). This result suggested that the cytoplasmic tail and TMD of Mnn1p could divert invertase from its normal pathway of secretion to an intracellular destination. Cells harboring M39S on a high-copy number plasmid were also assayed for invertase activity to determine whether overexpression would saturate the intracellular retention mechanism and lead to an increase in the level of cell surface expression. These cells expressed 15- to 20-fold more invertase activity, but we found essentially the same percentage of invertase activity at the cell surface (12%, Table 1), indicating that this level of overexpression had no effect on the efficiency with which M39I was retained intracellularly.

ER to Golgi Protein Transport Kinetics of M39I

A common problem with using fusion proteins to analyze sorting signals specific to the secretory pathway is that these proteins may be retained in the ER if not folded properly. A pulse-chase experiment was performed to assess the kinetics of M39I transport from the ER to the Golgi complex by monitoring the mobility shift in SDS-polyacrylamide gels associated with modification of *N*-linked oligosaccharides of this fusion protein in the Golgi complex. Invertase is modified with 10–13 *N*-linked oligosaccharides as it is translocated into the ER. Upon transport to the Golgi complex, these oligosaccharides undergo extensive glycosyl modification resulting in a decreased and more heterogeneous mobility in SDS-polyacrylamide gels. This modification event is a useful marker, in that it is strictly dependent on protein transport from the ER to the Golgi complex (Esmon *et al.*, 1981; Graham and Emr, 1991). A wild-type strain harboring M39S on a multi-copy plasmid was pulse-labeled with [^{35}S]methionine for 10 min and chased for 60 min. Aliquots of cells were removed at 0, 15, 30, and 60 min of chase and the fusion protein was recovered by immunoprecipitation with anti-invertase antiserum. One-half of each sample was treated with endo H before electrophoresis to remove *N*-linked oligosaccharides (Trimble and Maley, 1977), so the molecular mass of the glycosylated and deglycosylated fusion proteins

Table 1. Cell surface expression and half-times for post-translational modification of Mnn1-invertase fusion proteins

Fusion protein	Plasmid copy-number	Strain	% Invertase at cell surface	Half-time for Golgi modification (min)	Half-time for *PEP4*-dependent cleavage (min)
M39I	Low	Wild type	10	7.5′	105′
M39I	High	Wild type	12	9′	90′
M39I	Low	Δ*mnn1*	10	9′	90′
M39I-XCT	Low	Δ*mnn1*	9	8′	110′
M39αI	Low	Wild type	83	7.5′	NA
M39αI	Low	Δ*kex2*	13	ND	ND
M73I	Low	Δ*mnn1*	10	9′	90′

The strains used were XCY42-30D (wild type), XCY42-30D Δ*mnn1* (Δ*mnn1*), and CRY2 *kex2Δ2::HIS3-s*. The % invertase at the cell surface is an average from three to four experiments and is ±5% or less for each reported value. The half-times for Golgi modification and *PEP4*-dependent cleavage were determined as described under MATERIALS AND METHODS. NA = not applicable, ND = not determined.

could be compared. M39I was initially synthesized as an 85-kDa form that migrated as a distinct band in the gel (Figure 2, lane 1). However, the 85-kDa form was rapidly converted to a larger, more heterogeneous form with a half-time of approximately 9 min (lanes 3, 5, and 7). At the early time points, both the 85-kDa and larger forms were reduced to a 62-kDa band by treatment with endo H (lanes 2 and 4), indicating that post-translational glycosyl modification of *N*-linked oligosaccharides was responsible for the larger forms of M39I. These data indicate that the M39I fusion protein was efficiently transported from the ER to the Golgi complex.

Interestingly, M39I was not subject to the same extent of hyperglycosylation as wild-type invertase. Most of the Golgi-modified fusion protein migrated as a broad band with an average molecular mass of 98 kDa, which corresponds to an increase in molecular mass of only 1–2 kDa per *N*-linked oligosaccharide upon arrival in the Golgi complex. Secreted invertase is much more heavily glycosylated in the Golgi complex and migrates in SDS-polyacrylamide gels as a heterogeneous smear with an average molecular mass of greater than 130 kDa (Trimble *et al.*, 1983).

Golgi to Vacuole Protein Transport Kinetics of M39I and M39I-XCT

Treatment of the samples with endo H (Figure 2, lanes 2, 4, 6, and 8) to examine the integrity of the M39I polypeptide revealed a slow proteolytic cleavage event that converted the deglycosylated 62-kDa form of M39I to a 59-kDa form that comigrated with deglycosylated, secreted invertase (labeled as Inv in Figure 2). The half-time for this proteolytic processing event was extrapolated to be approximately 90 min in this experiment (Table 1). Proteolysis of M39I may have occurred within the vacuole because this organelle is a primary site of proteolytic enzymes within the cell (Klionsky *et al.*, 1990), and it has been proposed that the vacuole is the default pathway for membrane protein transport in yeast (Roberts *et al.*,

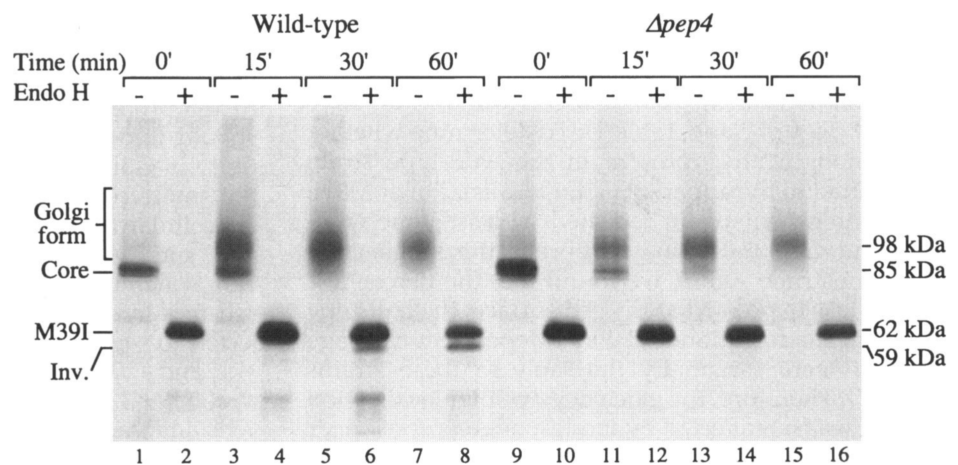

Figure 2. Post-translational glycosylation and *PEP4*-dependent proteolytic processing kinetics of the Mnn1-invertase fusion protein M39I. Isogenic strains SEY6210 (wild type) and TVY1 (Δ*pep4*) harboring M39S on a multicopy vector (pM39S-304) were labeled for 10 min with ^{35}S amino acids and chased for 60 min at 30°C. At the indicated time points, equal aliquots of cells were removed and killed in 10% TCA. The fusion protein M39I was recovered from each sample by immunoprecipitation with anti-invertase antiserum, then one-half of each sample was treated with endoglycosidase H (Endo H) to remove *N*-linked oligosaccharides before SDS-PAGE as described in MATERIALS AND METHODS.

1992). To test this hypothesis, we repeated this pulse-chase experiment in an isogenic Δ*pep4* strain (Figure 2, lanes 9–16) that is pleiotropically deficient in the major proteolytic enzyme activities of the vacuole. The *PEP4*-encoded vacuolar protease, proteinase A, cleaves inactive zymogens of several proteases (including itself) to their enzymatically active form upon arrival in the vacuole (Jones *et al.*, 1982; Zubenko *et al.*, 1983; Woolford *et al.*, 1986). Although the kinetics of post-translational glycosyl modification of M39I was identical in the wild-type and Δ*pep4* strain, the proteolytic cleavage event was not observed in the Δ*pep4* strain (Figure 2, lanes 12, 14, and 16). This experiment demonstrated that the 59-kDa proteolytic cleavage product of M39I was formed in the vacuole by the action of *PEP4*-dependent proteases. The junction between the Mnn1 segment and invertase is encoded by plasmid sequences and has been found to be protease sensitive in the context of other invertase fusion proteins that are transported to the vacuole (Johnson *et al.*, 1987; Graham and Emr, 1991). In addition, after cleavage in the vacuole, the invertase was no longer membrane associated and fractionated as a soluble protein (described below), indicating that the cleavage site was between the Mnn1 TMD and invertase.

The pulse-chase experiments and steady-state invertase assays suggested that the Mnn1-invertase fusion proteins are rapidly transported into the Golgi complex and slowly leak from this organelle to both the plasma membrane and vacuole. We reasoned that the kinetics of *PEP4*-dependent cleavage of the fusion proteins and the percentage of invertase activity at the cell surface could provide a measure of the efficiency with which these proteins are retained within the Golgi complex. For example, it was possible that overexpression of M39I saturated the Golgi localization mechanism and resulted in the slow transport to the vacuole shown in Figure 2. To test whether this was the case, the pulse-chase experiments described above were repeated with a wild-type strain harboring M39S on a single-copy vector. We found that the half-time for Golgi modification and *PEP4*-dependent cleavage of M39I were essentially the same in this strain (Table 1) as that shown for M39I expressed from a multi-copy plasmid (Figure 2 and Table 1). To determine whether endogenous Mnn1p present in the wild-type strain contributed to the apparent Golgi localization of M39I, these experiments were repeated with isogenic wild-type and Δ*mnn1* strains harboring the single-copy M39S construct. Again, we found that the percentage of invertase expressed at the cell surface, the half-time for Golgi modification and *PEP4*-dependent cleavage of M39I were essentially the same in these strains (Table 1). Therefore, the efficiency of Golgi localization of M39I was not affected by modest overexpression, or the absence of endogenous Mnn1p. Furthermore, the expression level had no discernible affect on the extent of M39I post-translational glycosylation.

We had previously found that the Mnn1 cytoplasmic tail could be exchanged for the cytoplasmic tail of a rat plasma membrane protein (dipeptidylaminopeptidase IV) without affecting Golgi localization or function of Mnn1p (Graham *et al.*, 1994). However, a role for the Mnn1p cytoplasmic tail could have been masked if either the TMD or the lumenal domain also contributed to Golgi localization. To determine whether the cytoplasmic tail (amino acids 1–18) contributed toward Golgi localization of M39I, we exchanged sequences encoding the Mnn1p cytoplasmic tail (up to the hydrophobic core of the TMD) for that of the 6-amino acid cytoplasmic tail of dipeptidyl peptidase IV to produce the construct M39S-*XCT* (Figure 1). If the Mnn1p cytoplasmic tail contributed to Golgi localization, we would expect the M39I-XCT fusion protein to show faster kinetics of *PEP4*-dependent cleavage or an increase in cell surface expression. However, the M39I-XCT protein showed a similar level of cell surface expression as compared with M39I and by pulse-chase analysis showed somewhat slower kinetics of *PEP4*-dependent cleavage (Table 1). These data suggest that the Mnn1p TMD alone is sufficient to confer Golgi localization to the reporter enzyme invertase, and that the cytoplasmic tail does not contribute significantly to M39I or Mnn1p localization.

Localization of Mnn1-Invertase Fusion Proteins to the Golgi Complex by Subcellular Fractionation

Mnn1p is a very stable enzyme in wild-type cells and does not appear to traffic to the vacuole over a 3-h time period after synthesis. In addition, we cannot detect any Mnn1p on the plasma membrane by immunofluorescence or cell surface iodination of wild-type cells (Graham *et al.*, 1994). The pulse-chase experiments described above suggest that M39I is not localized to the Golgi complex as efficiently as Mnn1p; however, it does appear to be retained as well as the resident Golgi enzyme Kex2p, which is subject to *PEP4*-dependent degradation in the vacuole with a half-life of ~80 min (Wilcox and Fuller, 1991). If the Mnn1-invertase fusion proteins are retained in the Golgi complex, we should find a significant enrichment of invertase activity in Golgi membranes. A Δ*mnn1* strain harboring the single copy M39S-XCT plasmid was subjected to subcellular fractionation by differential centrifugation and sucrose gradient fractionation of an enriched Golgi membrane preparation. Spheroplasts were lysed in a hypo-osmotic buffer and the lysate was subjected to sequential centrifugation at 1000 × *g*, 13,000 × *g*, and 120,000 × *g* as previously described (Graham *et al.*, 1994). This protocol resulted in >90% lysis of cells and vacuoles such that soluble vacuolar proteins remain in the supernatant after the high speed centrif-

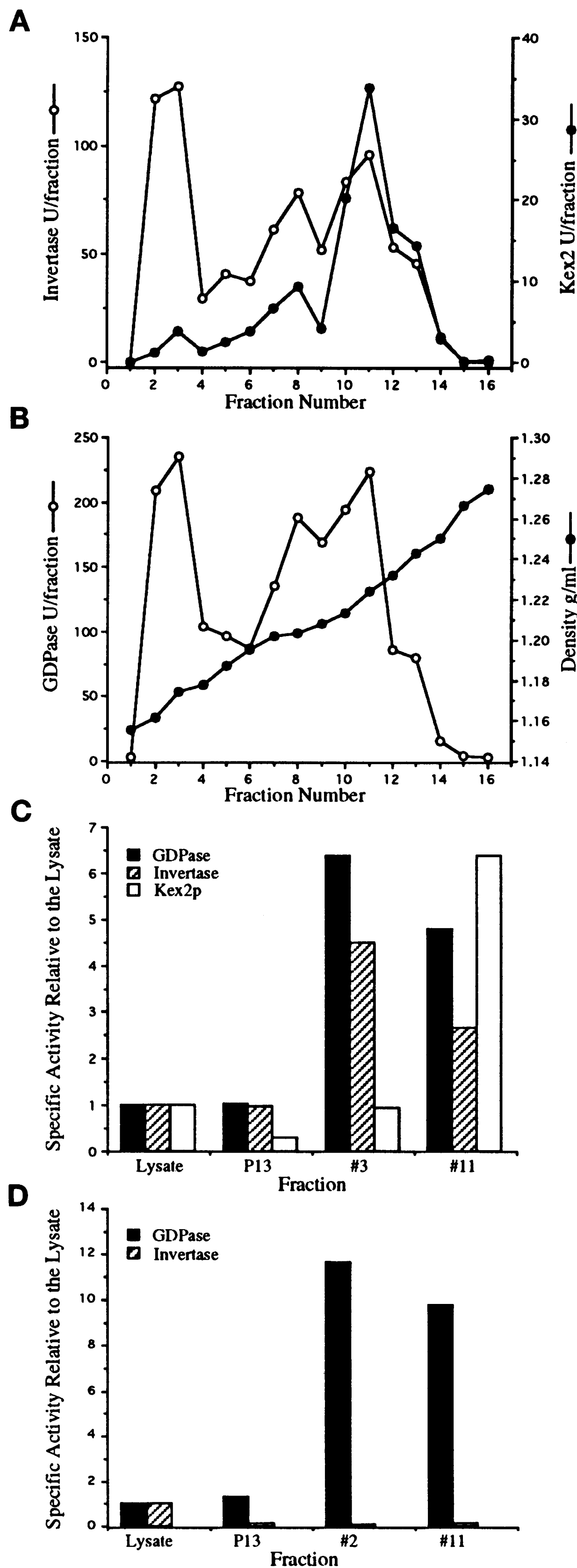

Figure 3. Localization of M39I-XCT to Golgi compartments by differential centrifugation and sucrose gradient fractionation. An enriched Golgi membrane fraction (P120) was prepared from strain XCY42–30D Δ*mnn1* harboring pM39S-XCT by differential centrifugation, and was subjected to equilibrium centrifugation in a sucrose gradient as described in MATERIALS AND METHODS. Fractions were collected starting from the top of the gradient and assayed for invertase (M39I-XCT), Kex2p (A), and GDPase (B) activities. The refractive index of each fraction was measured and used to calculate the sucrose density of each fraction (B). (C) The enzyme activities and protein concentration of the starting lysate, the p13 fraction, and the peak fractions from the sucrose gradients were measured and used to calculate the specific activities of the enzymes shown above. (D) A strain that expressed secreted invertase, 6210 pCYI20 (Johnson *et al.*, 1987), was subjected to subcellular fractionation and the specific activities of invertase and GDPase in Golgi fractions were determined as described above.

ugation step (S120). Most of the ER, vacuole membrane, and plasma membrane are found in the 13,000 $\times$ *g* pellet (Marcusson *et al.*, 1994), whereas the 120,000 $\times$ *g* pellet fraction (P120) typically contains >75% of Mnn1p, the Golgi enzyme guanosine diphosphatase (GDPase), and Kex2p (Graham *et al.*, 1994; Whitters *et al.*, 1994). We found 60–65% of the invertase activity was in the S120 fraction. This is consistent with the fact that invertase is a very stable enzyme, even in the vacuole, such that the 90- to 110-min half-time for *PEP4*-dependent cleavage of M39I-XCT should result in more than one-half of the enzyme residing in the vacuole as a soluble protein under steady-state conditions. The P120 fraction contained ~30% of the invertase activity with the remaining 10% fractionating in the P13. The P120 fraction was applied to the bottom of a sucrose step gradient which was centrifuged to equilibrium as described in MATERIALS AND METHODS. We typically found two or three peaks of GDPase activity in these gradient fractions. In the fractionation profile shown in Figure 3, the first GDPase peak in the early, lighter fractions contained very little Kex2 activity, whereas the third GDPase peak was found in denser fractions that contained most of the Kex2 activity (Figure 3, A and B). Mnn1p cofractionates with GDPase in these gradients (Graham *et al.*, 1994). Likewise, we found that M39I-XCT cofractionated with GDPase (Figure 3, A and B) and was enriched in these Golgi fractions to a similar extent as GDPase as measured by the specific activity of invertase relative to the starting lysate (Figure 3C). Golgi-enriched fractions prepared from a strain that expressed secreted invertase did not show an enrichment of invertase activity within fractions that contained GDPase (Figure 3D). These results indicate that the TMD of Mnn1p can direct the localization of invertase to the Golgi complex, and therefore must contain a specific Golgi localization signal. In addition, this fractionation data support our contention that the *PEP4*-dependent cleavage of the fusion proteins is an indicator of Golgi to vacuole transport kinetics. If the

fusion proteins were rapidly transported to the vacuole and slowly cleaved, we would have expected a larger percentage of invertase activity in the P13 fraction and no co-enrichment of invertase with Golgi markers in the sucrose gradients.

Mnn1-Invertase Fusion Proteins Rapidly Encounter Kex2p in the Golgi Complex

The fractionation data described above indicate that the Mnn1-invertase fusion protein was localized to the Golgi complex, and suggest that it might be concentrated in more than one Golgi compartment. However, co-localization of a portion of M39I-XCT with Kex2p in sucrose gradients could indicate that these proteins reside in the same Golgi cisternae, or that they reside in different Golgi cisternae with a similar density. To determine whether the Mnn1-invertase fusion proteins are trafficking into the late Golgi compartment that contains Kex2p, we prepared an Mnn1-invertase fusion protein that is a substrate for Kex2p. A 60-nucleotide portion of the *MFα1* (α-factor) gene that encodes a Kex2p cleavage site was subcloned into the junction between the Mnn1 TMD and *SUC2* to produce M39αS (Figure 1). If this fusion protein is transported into the Kex2p compartment, then invertase should be cleaved free of the TMD Golgi localization signal and secreted from the cell. We had previously found that it was possible to trap pro-α-factor in transit through the ER as well as the early and medial Golgi compartments for up to 1 h in a temperature-sensitive *sec18* mutant without detectable cleavage by Kex2p (Graham and Emr, 1991). These data suggest that early compartments of the Golgi complex are markedly deficient in Kex2p activity toward exogenous substrates, and that cleavage of the M39αI fusion protein by Kex2p would be a valid indicator that the fusion protein was trafficking into the late Golgi.

We found that strains harboring single-copy pM39αS secrete 80 ± 5% of the expressed invertase (Table 1), suggesting that most of the fusion protein encountered Kex2p in the late Golgi. A strain that harbors a Δ*kex2* null allele and pM39αS only expressed 10–15% of the invertase at the cell surface, demonstrating that this event was *KEX2* dependent. To analyze the kinetics of the *KEX2*-dependent cleavage event, wild-type cells harboring M39αS on a low copy number plasmid were labeled and chased as described above. As with M39I, the M39αI protein was efficiently transported into the Golgi where it was subject to limited modification by the Golgi mannosyltransferases (Figure 4, odd numbered lanes). However, the M39αI protein was subject to a rapid proteolytic cleavage event that converted the 62-kDa form to a 59-kDa form. This proteolytic event occurred with a half-time of ~5 min and went to ~90% completion (Figure 4, even numbered lanes). The endo H–treated

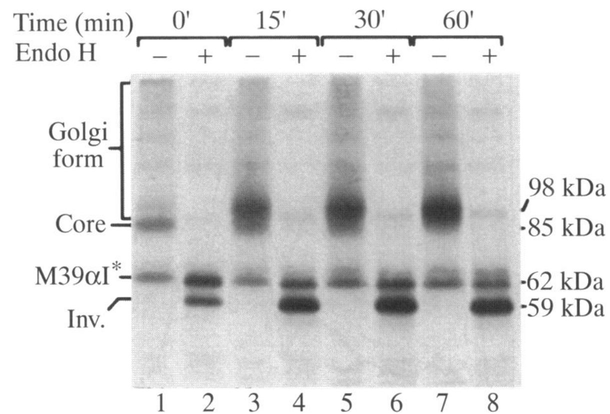

Figure 4. Post-translation glycosylation and *KEX2*-dependent proteolytic processing kinetics of M39αI. Strain SEY6210 harboring M39αS on a single-copy vector (pM39αS-308) was subjected to the same pulse-chase protocol described in the legend to Figure 2. The fusion protein M39αI was recovered from each sample by immunoprecipitation with anti-invertase antiserum and one-half of each sample was treated with endo H to remove *N*-linked oligosaccharides before SDS-PAGE. * Endo H–treated M39αI comigrates with a 62-kDa background band that is also recovered in anti-invertase immunoprecipitates from the parent strain (SEY6210) that does not express any invertase protein, and is more prevalent in experiments using strains that harbor low copy-number plasmids. This background band also migrates as a 62-kDa protein in the non-endo H–treated samples.

M39αI protein comigrated with a 62-kDa nonspecific protein that contaminates the immunoprecipitates (Figure 4, even numbered lanes). This contaminant also migrates as a 62-kDa protein in the untreated samples, so the amount of the 62-kDa band in the untreated samples was subtracted from the corresponding endo H–treated sample for quantitation. Had the cleavage event occurred in the ER or early Golgi, we would have expected the soluble invertase product to be extensively modified on *N*-linked oligosaccharides like wild-type invertase. However, the extent of *N*-linked modification of M39I and M39αI was very similar, even though the latter protein was converted into a soluble form that was secreted from the cell. This observation suggests that most of M39αI was cleaved by Kex2p late in the Golgi complex, subsequent to modification by the Golgi mannosyltransferases. These data suggest that the Mnn1-invertase fusion proteins were rapidly transported into the late Golgi compartment that contains Kex2p and were not appreciably retained in earlier Golgi compartments. This is a surprising result in light of the substantial steady-state localization of Mnn1-invertase fusion proteins to early Golgi compartments that lack Kex2p and will be discussed further below.

The Mnn1-invertase fusion proteins were not retained in the Golgi complex as well as intact Mnn1p, which suggested the presence of additional Golgi localization signals within the Mnn1p lumenal domain.

In the case of the mammalian Golgi enzyme α-1,2 sialyltransferase, sequences flanking the TMD on the lumenal side can function as an independent Golgi localization signal (Colley *et al.*, 1992), and can improve Golgi localization of fusion proteins containing the α-1,2 sialyltransferase cytoplasmic tail and TMD (Munro, 1991; Dahdal and Colley, 1993). To test whether additional lumenal sequences adjacent to the Mnn1 TMD could improve retention of our fusion proteins, we prepared constructs encoding the N-terminal 73 and 166 amino acids of Mnn1p fused to *SUC2* (Figure 1). Pulse-chase analysis indicated that these fusion proteins were not retained more efficiently in the Golgi complex as compared with M39I-XCT (Table 1 and unpublished observations).

Saturable Intracellular Retention of the Soluble Mnn1p Lumenal Domain

We have shown that the Mnn1p TMD was sufficient to confer Golgi localization to a reporter enzyme. To examine the effect of deleting the Mnn1p TMD, we exchanged the 5′ end of the *MNN1* gene that encodes the cytoplasmic tail and TMD of Mnn1p for that of the *PRC1* gene, which encodes the carboxypeptidase Y cleavable signal sequence (Figure 1, pMNN1-s). This construct was expressed in a strain harboring a Δ*mnn1* null allele so that pMNN1-s would be the only source of Mnn1 protein in the cell. The Δ*mnn1* pMNN1-s cells were converted to spheroplasts, then labeled for 10 min and chased for 90 min. Aliquots of cells were removed at the times indicated in Figure 5A, then the cells and media were separated by centrifugation and Mnn1-s was recovered from each sample by immunoprecipitation. We found that Mnn1-s was initially synthesized as a 97-kDa form that was converted to an aberrant, ~108-kDa form over time, but was not secreted into the media (Figure 5A). Treatment of one-half of these samples with endo H converted the 97- and 108-kDa forms to a closely spaced doublet of ~92 and 93 kDa, respectively (unpublished observations), which indicates that increased glycosyl modification of *N*-linked oligosaccharides in the Golgi complex was primarily responsible for generating the high molecular weight form of Mnn1-s. We found that most of the 97-kDa form of Mnn1-s was in the P13 (ER fraction) by differential centrifugation analysis of a lysate prepared from pulse-chased cells (analogous to the sample shown in Figure 5A, lane 5; unpublished observation). This suggests that the Mnn1-s protein was slowly transported from the ER to the Golgi complex, where it was subject to efficient glycosyl modification of its *N*-linked oligosaccharides. This is in contrast to wild-type Mnn1p, which appears to be efficiently transported from the ER to the Golgi complex where it undergoes a slow, incremental glycosyl modification, primarily on *O*-linked oligosaccharides (Graham *et al.*, 1994). For comparison, a pulse-chase analysis of wild-type Mnn1p is shown in Figure 5C.

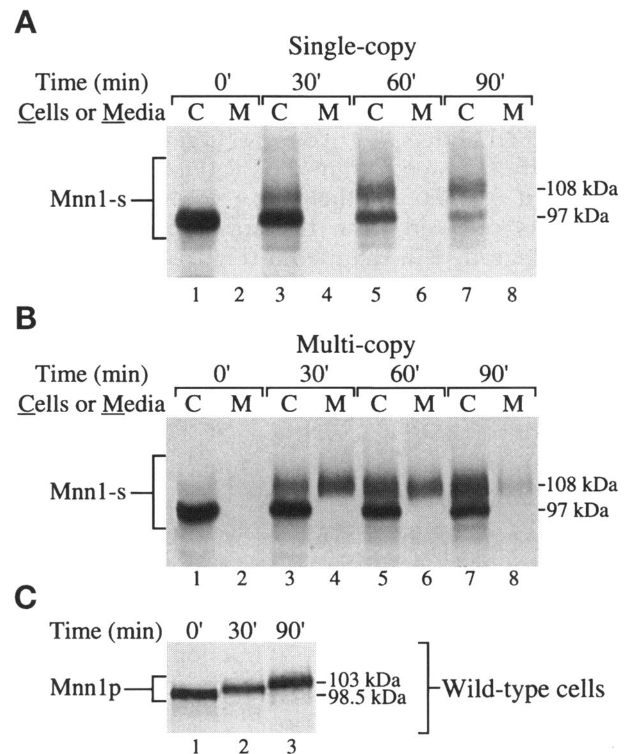

Figure 5. Saturable intracellular retention of the soluble lumenal domain of Mnn1p (Mnn1-s). Spheroplasts prepared from strain XCY42–30D Δ*mnn1* harboring MNN1-s on a low-copy number vector (A) or a high-copy number vector (B) were pulse-labeled for 10 min, and then chased for 90 min at 30°C. Aliquots were removed at the time points indicated and centrifuged briefly to separate the cells (C) from the media (M). Mnn1-s was recovered from each sample by immunoprecipitation with anti-Mnn1p antiserum and then subjected to SDS-PAGE. (C) A pulse-chase analysis of wild-type Mnn1p from strain XCY42–30D is shown for reference.

We considered the possibility that the carboxypeptidase Y signal sequence was not cleaved from Mnn1-s and acted as a signal/anchor domain. This was not the case, because extraction of pulse-labeled, lysed spheroplasts with carbonate buffer (pH 11.0) caused nearly 100% of Mnn1-s to fractionate in the supernatant after a 120,000 × *g* centrifugation, whereas the membrane spanning protein alkaline phosphatase (Klionsky and Emr, 1989) remained in the pellet (unpublished observations). These data suggest that the CPY signal sequence was cleaved to produce a soluble form of the Mnn1 lumenal domain. As a soluble protein, the Mnn1-s *N*-linked oligosaccharides were apparently more accessible to the mannosyltransferases in the Golgi complex, which resulted in the abnormal post-translational glycosylation of this protein. Although Mnn1-s was not quite as stable as the wild-type protein, we did not detect a difference in stability or modification of Mnn1-s in isogenic wild-type and

Δ*pep4* cells (unpublished observations). To determine whether the intracellular retention of Mnn1-s was saturable, Δ*mnn1* cells harboring MNN1-s on a multicopy plasmid were subjected to a pulse-chase analysis as described above. A greater fraction of Mnn1-s was apparently retained within the ER (Figure 5B, 97-kDa form), but ~40–50% of the fully glycosylated 108-kDa form was secreted from the spheroplasts and was slowly degraded in the media (Figure 5B). Although overexpression of M39I from a multi-copy plasmid had no apparent affect on its subcellular distribution, a portion of Mnn1-s was secreted from the overexpressing strain, indicating that retention of the soluble lumenal domain of Mnn1p was saturable.

Localization of Mnn1-s to the Golgi Complex by Subcellular Fractionation

To determine whether Mnn1-s was localized to the Golgi complex, we purified Golgi compartments from the Δ*mnn1* pMNN1-s (single copy) strain using the cell fractionation protocol described above. We did not detect any Mnn1-s in the S120 fraction where mature carboxypeptidase Y fractionated, indicating that Mnn1-s was not present in significant amounts within the lumen of the vacuole. However, there was a substantial co-fractionation of the 108-kDa form of Mnn1-s in sucrose gradient fractions marked by GDPase (Figure 6, A and C). A Western blot from a gel loaded with equal amounts of protein from the cell lysate (L), low speed pellets (p13), and peak fractions from the sucrose gradient (4 and 10) showed a substantial enrichment of Mnn1-s in the sucrose gradient peak fractions that were enriched for GDPase activity (Figure 6, B and D, fractions 4 and 10).

In addition, pMNN1-s complemented the Δ*mnn1* strain, further indicating that Mnn1-s is localized to the correct Golgi compartment. The p2 and mature forms of carboxypeptidase Y are normally modified with α1,3-linked mannose residues on *N*-linked oligosaccharides that allow immunoprecipitation of these forms with antibodies specific for α1,3-linked mannose epitopes. Carboxypeptidase Y from the Δ*mnn1* strain could not be precipitated with anti-α1,3 mannose linkage-specific antiserum as expected, because this strain lacks the α1,3 mannosyltransferase (Figure 7, Δ*mnn1*). However, carboxypeptidase Y from a Δ*mnn1* strain harboring MNN1-s on a single-copy plasmid (Δ*mnn1* pMNN1-s) was precipitated with α1,3 mannose linkage-specific antiserum nearly as efficiently as that from a wild-type (*MNN1*) strain (Figure 7). We conclude from these data that Mnn1-s is localized by a saturable mechanism to the Golgi complex, where it can function to modify other glycoproteins with α1,3-linked mannose residues.

TMD-Mediated Golgi Localization of an Mnn1-Invertase Fusion Protein Is Clathrin Dependent

A TMD-mediated localization mechanism for proteins that reside in early compartments of the Golgi complex is emerging as a conserved, universal mechanism. Currently, there is little known about the cellular components that are required to facilitate this localization mechanism. We had previously shown that Mnn1p was partially mislocalized to the plasma

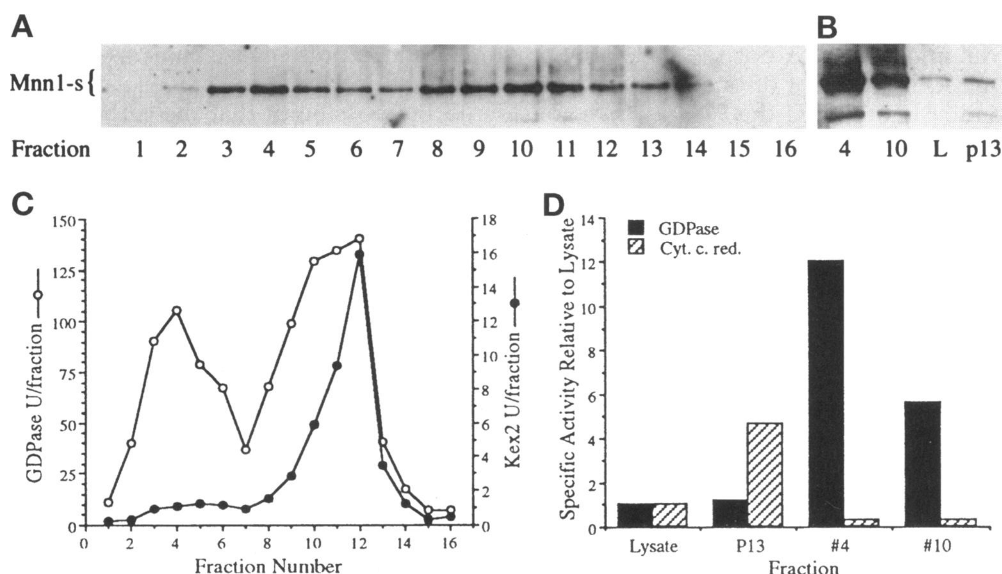

Figure 6. Localization of Mnn1-s to Golgi compartments by differential centrifugation and sucrose gradient fractionation. An enriched Golgi membrane fraction (P120) was prepared from strain XCY42–30D Δ*mnn1* harboring pMNN1-s by differential centrifugation, and was subject to equilibrium centrifugation in a sucrose gradient. (A) Western blot of the sucrose gradient fractions probed with affinity-purified anti-Mnn1p antibodies. (B) Western blot containing equal amounts of protein (3 μg) from the starting lysate (L), the p13 fraction obtained by differential centrifugation, and the peak fractions 4 and 10 from the sucrose gradient, probed with affinity-purified anti-Mnn1p antibodies. (C) Equal aliquots from each sucrose gradient fraction were assayed in duplicate for Kex2p and GDPase. (D) The protein concentration of the fractions shown was determined and used to calculate the specific activities of GDPase and the ER enzyme NADPH cytochrome c reductase. The fractionation protocol and enzyme assays are described in greater detail in MATERIALS AND METHODS.

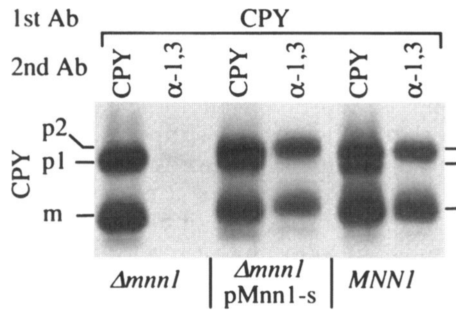

Figure 7. Complementation of a Δ*mnn1* strain with pMnn1-s. Strains XCY42–30D Δ*mnn1*, XCY42–30D Δ*mnn1* pMnn1-s, and XCY42–30D were labeled for 15 min at 30°C and the cells were killed by the addition of TCA to a final concentration of 10%. Carboxypeptidase Y (CPY) was recovered from each sample by immunoprecipitation with anti-CPY antiserum (1st Ab, CPY). CPY was eluted from the primary antibody and each sample was split in half and immunoprecipitated a second time with either anti-CPY antiserum (2nd Ab, CPY) or anti-α1,3 mannose antiserum (2nd Ab, α1,3). The samples were then subjected to SDS-PAGE and autoradiography.

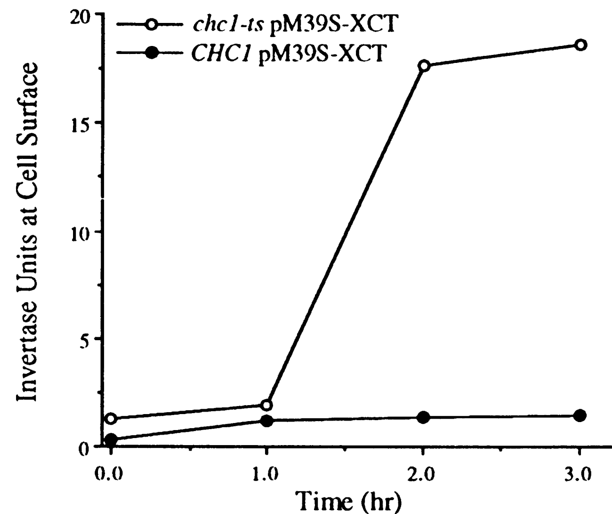

Figure 8. Mislocalization of the Mnn1-invertase fusion protein M39I-XCT to the plasma membrane of cells harboring a temperature-sensitive mutation in the clathrin heavy chain gene (*chc1-ts*). Congenic strains GPY396.1 (*chc1-ts*) and GPY55–10B (*CHC1*) harboring pM39S-XCT were gown in YPD at 24°C to a density of ~0.8 OD_{600}/ml. The cells were shifted to 37°C and aliquots were removed at the time points indicated. The 0-min time point was removed just before the temperature shift and indicates the amount of cell surface invertase activity of cells growing at permissive temperature. Intact cells were assayed for cell surface expression of invertase activity as previously described. The parent strains lacking pM39S-XCT were also assayed and subtracted as background from their transformed counterparts. Invertase activity is expressed as units/OD_{600} of cells and the standard error was less than 5% the value of each point.

membrane when the clathrin heavy chain was thermally inactivated in a strain harboring a temperature-sensitive *chc1-ts* (clathrin heavy chain) allele. Congenic wild-type and *chc1-ts* strains harboring pM39S-XCT were prepared to determine whether this fusion protein, that has only the Mnn1 TMD, requires clathrin for Golgi localization. These strains were grown to mid-logarithmic phase at the permissive temperature (24°C), then shifted to the nonpermissive temperature (37°C) and incubated for an additional 3 h. Aliquots of cells taken at 1 h intervals after temperature shift were divided to prepare a lysate from one-half of each sample. The intact cells and lysates were assayed to determine the amount of invertase activity present on the cell surface and the total activity, respectively. We could detect very little invertase on the plasma membrane of these cells that were grown at the permissive temperature (Figure 8, 0 min). However, there was a substantial increase in invertase activity at the cell surface of the *chc1-ts* mutant, but not the wild-type control, after 2 h at 37°C (Figure 8). The total invertase activity per cell was comparable at each time point. The amount of invertase activity at the cell surface of the *chc1-ts* cells at the 2-h time point was 20% of the total activity and did not increase significantly at the 3-h time point. In other experiments, we found up to 8% of the total invertase on the cell surface at the 1-h time point. Therefore, the phenotypic lag period after temperature shift appeared to be slightly less than 1 h.

We expected that at least one-half of the invertase present in the *chc1-ts* cells would be in the vacuole and would be unlikely to contribute toward mislocalization to the plasma membrane after the temperature shift. To estimate the true extent of mislocalization of M39I-XCT from the Golgi to the plasma membrane, the wild-type and *chc1-ts* strains harboring pM39S-XCT were grown at permissive temperature and then subjected to cell fractionation by differential centrifugation to determine the steady-state distribution of M39I-XCT in these cells. For both strains, we found ~2% at the plasma membrane and ~63% of the invertase was in the S120 (vacuolar) fraction, indicating that ~35% of the fusion protein was in the Golgi complex (or ER) at the time of the temperature shift. Therefore, we estimate that more than 50% (20%/35%) of the available pool of M39I-XCT was mislocalized to the plasma membrane when the clathrin mutant was shifted to 37°C for 2 h. This is consistent with estimates for the extent of mislocalization of Mnn1p in the clathrin mutant as determined by cell surface iodination (Graham *et al.*, 1994). Moreover, the kinetics of mislocalization after temperature shift were similar to that observed for wild-type Mnn1p. We have not yet been able to do the analogous experiment with the *chc1-ts* strain harboring pMNN1-s, because at 37°C, Mnn1-s does not appear to be transported out of the ER, and steady-state pools within the Golgi appear to be unstable. Transport of Mnn1-s out of the ER in spheroplasts prepared from a Δ*chc1* strain was also

very inefficient, so the extent of its secretion could not be reliably determined (unpublished observations).

DISCUSSION

Glycoproteins traversing the yeast secretory pathway first encounter Mnn1p (α1,3 mannosyltransferase) in a compartment analogous to the *medial* or *trans* cisternae of the mammalian Golgi complex (Graham and Emr, 1991). Cell fractionation studies support the physical localization of Mnn1p to early Golgi compartments that lack Kex2p, although a significant portion of Mnn1p also co-fractionates with Kex2p. These data suggest that a portion of Mnn1p may also reside in a late Golgi compartment analogous to the mammalian *trans* Golgi network (Graham *et al.*, 1994). We have analyzed the contribution of the Mnn1p cytoplasmic tail, TMD, and lumenal domain to the Golgi localization of this protein. In this study, we demonstrate the presence of a Golgi localization signal within the Mnn1 TMD that is sufficient to localize a reporter enzyme (invertase) to the yeast Golgi complex. However, the Mnn1 lumenal domain (Mnn1-s) is not secreted when expressed as a soluble protein lacking its TMD. Rather, Mnn1-s is retained in the Golgi complex and can function there to modify other glycoproteins with α1,3-linked mannose. These data indicate that Mnn1p has separable Golgi localization signals in both its membrane spanning (TMD) and lumenal domains. The localization mechanisms employed by these signals appear to be different. Localization of Mnn1-s is easily saturable by overexpression, resulting in secretion of the excess protein. This suggests that there are a limited number of specific binding sites for the Mnn1 lumenal domain within the yeast Golgi complex. However, modest overexpression (15- to 20-fold) of an invertase fusion protein containing the Mnn1 TMD (M39I) has no apparent effect on the efficiency of Golgi localization. Furthermore, we could not detect a contribution of the Mnn1p cytoplasmic tail to Golgi localization and neither the TMD nor lumenal domain signal required the presence of wild-type Mnn1p within the cell.

A Golgi localization signal within a TMD was first observed with the avian coronavirus M protein (Swift and Machamer, 1991), and has subsequently been found to be the case for several mammalian Golgi glycosyltransferases that are type II integral membrane proteins (Munro, 1991; Nilsson *et al.*, 1991; Aoki *et al.*, 1992; Burke *et al.*, 1992; Russo *et al.*, 1992; Tang *et al.*, 1992; Teasdale *et al.*, 1992; Wong *et al.*, 1992). One distinguishing feature of TMD-mediated protein sorting in mammalian cells is that overexpression does not lead to significant mislocalization to the plasma membrane or lysosome (Munro, 1991; Nilsson *et al.*, 1991; Swift and Machamer, 1991). This also appears to be the case for the invertase fusion proteins containing the Mnn1p TMD in that overexpression does not appear to affect their subcellular distribution. Our data suggest that the TMD-mediated mechanism for Golgi protein sorting is conserved in the yeast *S. cerevisiae*. Recent work by other groups supports this conclusion. Chapman and Munro have presented evidence that the TMD of Mnt1p, a Golgi α1,2 mannosyltransferase involved in the modification of *O*-linked oligosaccharides, is sufficient to localize a reporter enzyme to the yeast Golgi complex (Chapman and Munro, 1994). In addition, Banfield *et al.* (1994) have found that the yeast Golgi protein Sed5p is primarily localized to the *cis* Golgi when expressed in COS cells. Although it has not been demonstrated that the TMD of yeast Sed5p can mediate Golgi localization, it was shown that the TMD of a closely related *Drosophila* Sed5p homologue could mediate localization of a protein chimera to the mammalian Golgi complex (Banfield *et al.*, 1994). Together, these studies strongly imply that a TMD-mediated Golgi protein localization mechanism is conserved among eukaryotic organisms.

The essential feature of the Golgi localization signal within the first TMD of avian coronavirus appears to be uncharged polar residues that line one face of a predicted α-helix (Machamer *et al.*, 1993). This TMD can mediate the formation of detergent insoluble aggregates in vitro and mutations that disrupt aggregate formation correspond to mutations that disrupt the Golgi localization signal (Weisz *et al.*, 1993). In addition, the TMDs of the *medial* Golgi enzymes *N*-acetylglucosaminyltransferase and mannosidase II have been shown to interact. *N*-acetylglucosaminyltransferase was localized to the ER of HeLa cells by grafting on a cytoplasmic tail ER retention signal from the p33 invariant chain. Remarkably, wild-type mannosidase II was also localized in the ER of cells expressing the *N*-acetylglucosaminyltransferase/p33 chimera. This interaction was dependent on the TMDs of these two proteins and no interaction was found with the *trans* Golgi enzyme β-1,4-galactosyltransferase (Nilsson *et al.*, 1994). These TMD-mediated interactions have been termed "kin recognition" because the interactions appear restricted to enzymes localized to the same Golgi compartment (Nilsson *et al.*, 1993b). It has been proposed that the TMDs mediate a localized oligomerization of Golgi membrane spanning proteins in response to the specific environment of the appropriate Golgi cisternae, such that complexes are formed that are too large to enter into budding transport vesicles (Pfeffer and Rothman, 1987; Nilsson *et al.*, 1993). However, the p33 invariant chain ER localization signal apparently does not cause static retention in the ER, but instead mediates recycling from the Golgi complex back to the ER via a retrograde protein transport pathway (Jackson *et al.*, 1993; Schutze *et al.*, 1994). The ability of the *N*-acetylglucosaminyltransferase/p33 chimera to cause ER localization of

mannosidase II might suggest efficient packaging of these Golgi enzyme hetero-oligomers into retrograde transport vesicles for return to the ER, rather than exclusion from vesicles of this type.

Although we have not yet obtained direct evidence for the formation of Mnn1p oligomers, aspects of our data do suggest that the Mnn1p TMD can mediate a certain level of organization within the lumen of Golgi compartments. Invertase is normally hyperglycosylated on *N*-linked oligosaccharides such that its average molecular weight is >130,000 Da. The membrane-associated Mnn1-invertase fusion protein M39I shows a substantial underglycosylation of *N*-linked oligosaccharides on invertase. The *N*-linked oligosaccharides of Mnn1p are also poorly modified by Golgi mannosyltransferases (including auto-mannosylation), but expression of the Mnn1p lumenal domain as a soluble protein free of its TMD results in an abnormal increase in the modification of its *N*-linked oligosaccharides, even though the protein is still efficiently localized to the Golgi complex. These data suggest that the Mnn1p TMD is capable of recruiting either invertase or its own lumenal domain into an environment (perhaps loose oligomeric protein complexes) in which the *N*-linked oligosaccharides on these proteins have restricted access to the active sites of the Golgi mannosyltransferases. These observations are not unique to Mnn1p. The lumenal domains of Kex1p and DPAP A are also subject to an aberrant increase in glycosyl modification when expressed as soluble proteins (Cooper and Bussey, 1992; Roberts *et al.*, 1992).

It is not known whether the TMD of a Golgi protein mediates static retention within the appropriate cisternae as suggested by the kin recognition hypothesis, or whether it serves as a signal for retrieval from later compartments of the Golgi complex. There are reports of mammalian *cis* and *medial* Golgi proteins that acquire sialic acid on *N*-linked oligosaccharides, which suggests that they may traffic into the *trans* Golgi network and are subsequently returned by a retrograde transport pathway (Yuan *et al.*, 1987; Gonatas *et al.*, 1989). We found that the Mnn1-invertase fusion proteins (M39I and M39I-XCT) are distributed among two to three peaks in sucrose gradient fractions containing Golgi membranes. The lighter fractions are marked by the Golgi enzymes, GDPase, Mnn1p, and Och1p, but lack Kex2p activity, and therefore contain the early Golgi compartments (Graham *et al.*, 1994). The denser Golgi membrane fractions are marked by Kex2p, but also contain a variable amount of the early Golgi enzymes ranging from 20–50% of the total GDPase present in different gradients. Co-fractionation of M39I with Kex2p does not necessarily indicate coresidence in the same Golgi compartment, as different compartments may fortuitously migrate to the same position in these sucrose gradients. In fact, others have found that more highly enriched preparations of the late Golgi that contained 61% of the initial Kex2p contained only 3% and 11% of the initial GDPase and Mnn1p, respectively (Whitters *et al.*, 1994). However, an Mnn1-αf-invertase fusion protein is rapidly and efficiently cleaved by Kex2p, suggesting that these fusion proteins are trafficking into the late Golgi, and are not subject to significant retention as they are transported through early compartments of the Golgi complex. Yet, the fusion proteins appear to maintain a high steady-state concentration within both early and late Golgi compartments. This could be achieved by a constant cycle of forward transport through the Golgi complex, and retrieval from the late Golgi via a retrograde transport pathway. By this model, the Mnn1p TMD would mediate sorting of invertase into retrograde vesicles that are targeted for fusion with the appropriate Golgi cisternae. Interactions of the Mnn1p lumenal domain with other constituents of the Golgi cisternae may play a role in a static retention mechanism that could slow forward transport of Mnn1p. At this time we cannot rule out the possibility that there is a small amount of active Kex2p in earlier compartments of the Golgi that catalyzes the cleavage of our fusion proteins. However, we had previously shown that pro-α-factor could be trapped in transit through the α1,6 and α1,3 mannosyltransferase Golgi compartments for up to 1 h in a *sec18* mutant without noticeable cleavage by Kex2p. Therefore, it seems unlikely that this low level of Kex2 processing activity in early Golgi compartments could account for *KEX2*-dependent cleavage of the Mnn1-αf-invertase fusions with a half-time of ~5 min.

A number of yeast mutants have been isolated that exhibit a defect in Golgi function (Novick *et al.*, 1980; Ballou, 1990; Ballou *et al.*, 1991; Semenza *et al.*, 1990; Robinson *et al.*, 1991; Chapman and Munro, 1994); however, only strains harboring mutations within the clathrin heavy chain gene (*CHC1*) and the dynamin-related *VPS1* gene have been shown to mislocalize Golgi proteins (Payne and Schekman, 1989; Wilsbach and Payne, 1993b). We had previously shown that Mnn1p is mislocalized to the plasma membrane of strains harboring a temperature-sensitive clathrin heavy chain allele (Graham *et al.*, 1994). Here, we show that an Mnn1-invertase fusion containing only the TMD from Mnn1p is also mislocalized to the plasma membrane upon loss of clathrin function. Therefore, the TMD-mediated Golgi localization mechanism for Mnn1p is clathrin dependent. The way in which clathrin mediates the localization of yeast Golgi enzymes is not completely understood, although prevalent models suggest a dynamic role for clathrin. For example, one model suggests that Kex2p (and other late Golgi proteins) are collected in clathrin-coated pits on the late Golgi and routed to the endosome, where Vps1p acts to facilitate recycling of Golgi proteins back to the Golgi complex (Wilsbach

and Payne, 1993a). Yeast strains harboring a *vps1* mutation secrete vacuolar proteins and exhibit a marked increase in the rate of Kex2p degradation in the vacuole (Wilsbach and Payne, 1993b). Similarly, a simple explanation for the role of clathrin in localization of Mnn1p is that clathrin mediates the formation of retrograde transport vesicles from late Golgi compartments (analogous to the TGN) that are targeted to earlier compartments. This would be consistent with the apparent lack of retention for Mnn1-invertase fusion proteins in early compartments of the Golgi complex and our argument for a dynamic, retrieval mechanism for Golgi localization of these fusion proteins. Others have recently found that a late Golgi protein and the vacuolar membrane protein alkaline phosphatase are initially misrouted to the plasma membrane of Δ*vps1* cells and are subsequently endocytosed and delivered to the vacuole (Nothwehr *et al.*, 1995). These data suggest that clathrin and Vps1p act together at the late Golgi in the formation of vesicles that are targeted to the endosome. There is no indication that Mnn1p traffics to the endosome, vacuole, or plasma membrane in wild-type cells, but it is possible that Mnn1p follows the same route as Kex2p to the endosome and is subsequently retrieved from this organelle. However, in preliminary experiments we found that Mnn1p is stable and modified with normal kinetics in a *vps1* mutant, which suggests that this protein is not trafficking into the endosome/vacuolar system (our unpublished observations).

These explanations for the clathrin mutant phenotype are based on the assumption that mislocalization of Mnn1p is a direct consequence of loss of clathrin function. The relatively long lag period before Golgi proteins appear on the plasma membrane might suggest that this is a secondary consequence of inactivating the clathrin heavy chain. Vacuolar sorting of CPY (Seeger and Payne, 1992a) and endocytosis (Tan *et al.*, 1993) are disrupted immediately after shifting *chc1-ts* cells to the nonpermissive temperature, indicating that a slow inactivation of clathrin function is not a general feature of this mutant. In wild-type cells, the Mnn1-invertase fusion proteins leak from the Golgi, which result in their transport to the plasma membrane and the vacuole. Accumulation of the Mnn1-invertase fusion protein on the plasma membrane of the clathrin mutant could result from loss of vacuolar transport along either the biosynthetic or the endocytic pathways. However, the long lag period observed before invertase starts to accumulate at the plasma membrane after inactivation of clathrin is much more consistent with mislocalization of the Mnn1-invertase fusion protein present within the Golgi complex to the plasma membrane, as occurs for intact Mnn1p. Changes in the physical characteristics of the late Golgi in the clathrin mutant, such as lipid composition, lumenal pH, and concentration of ions could disrupt the TMD-mediated localization mechanism indirectly. Although the clathrin requirement for Golgi localization of Mnn1p may be indirect, it is a specific requirement because Golgi function in glycosylation and protein transport is relatively unperturbed in the clathrin mutant (Payne and Schekman, 1989), and a protein with an apparently similar distribution in the Golgi complex, GDPase, is not mislocalized to the plasma membrane of the clathrin mutant (Seeger and Payne, 1992b; Graham *et al.*, 1994). Clathrin is clearly an important component of the mechanism for localizing Mnn1p to the Golgi, and as additional components are characterized we should be able to resolve the nature of the role that clathrin plays in the TMD-mediated Golgi localization of Mnn1p.

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