Compartmental Organization of Golgi-specific Protein Modification and Vacuolar Protein Sorting Events Defined in a Yeast *sec18* (NSF) Mutant

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Abstract. The *sec18* and *sec23* secretory mutants of *Saccharomyces cerevisiae* have previously been shown to exhibit temperature-conditional defects in protein transport from the ER to the Golgi complex (Novick, P., S. Ferro, and R. Schekman. 1981. *Cell*. 25:461–469). We have found that the Sec18 and Sec23 protein functions are rapidly inactivated upon shifting mutant cells to the nonpermissive temperature (<1 min). This has permitted an analysis of the potential role these SEC gene products play in transport events distal to the ER. The *sec*-dependent transport of α -factor (α f) and carboxypeptidase Y (CPY) biosynthetic intermediates present throughout the secretory pathway was monitored in temperature shift experiments. We found that Sec18p/NSF function was required sequentially for protein transport from the ER to the Golgi complex, through multiple Golgi compartments and from the Golgi complex to the cell surface. In contrast, Sec23p

function was required in the Golgi complex, but only for transport of α f out of an early compartment. Together, these studies define at least three functionally distinct Golgi compartments in yeast. From *cis* to *trans* these compartments contain: (a) An α 1 $\rightarrow$ 6 mannosyltransferase; (b) an α 1 $\rightarrow$ 3 mannosyltransferase; and (c) the Kex2 endopeptidase.

Surprisingly, we also found that a pool of Golgi-modified CPY (p2 CPY) located in a compartment distal to the α 1 $\rightarrow$ 3 mannosyltransferase does not require Sec18p function for final delivery to the vacuole. This compartment appears to be equivalent to the Kex2 compartment as we show that a novel vacuolar CPY- α f-invertase fusion protein undergoes efficient Kex2-dependent cleavage resulting in the secretion of invertase. We propose that this Kex2 compartment is the site in which vacuolar proteins are sorted from proteins destined to be secreted.

THE Golgi complex plays an important role in the post-translational modification of glycoproteins and in the sorting of these proteins to their appropriate destination. In mammalian cells the Golgi complex is divided into at least four spatially and functionally distinct regions: *cis*, medial, *trans*, and the *trans*-Golgi network (TGN)¹ (Palade, 1975; Farquhar, 1985; Pfeffer and Rothman, 1987). These Golgi compartments differ in their content of specific enzymes involved in the posttranslational modification of glycoproteins. In addition, lysosomal enzymes appear to be sorted from secreted proteins in the TGN, indicating that protein sorting reactions are also restricted to specific compartments of the Golgi complex (Griffiths and Simons, 1986; Dahms et al., 1989). Although *Saccharomyces cerevisiae* has become an important model organism for studying the secretory pathway, the compartmental organization of the

yeast Golgi complex has not been extensively characterized. Reasons for this include: (a) The wild-type yeast Golgi has been difficult to identify by EM, possibly because it lacks the customary stacked cisternal morphology of the mammalian Golgi complex; (b) subcellular fractionation of yeast organelles has not yet resulted in the clean separation of Golgi enzyme activities; and (c) few mutants (*sec7* and *sec14*) that affect protein transport through the yeast Golgi have been isolated (Esmon et al., 1981; Novick et al., 1981). Recently however, Franzusoff and Schekman (1989) have presented evidence that the enzyme(s) which catalyze α 1 $\rightarrow$ 6 linked mannose addition to yeast glycoproteins appear to be sequestered in an early Golgi compartment that lacks α 1 $\rightarrow$ 3 mannosyltransferase and Kex2 endopeptidase activities (Franzusoff and Schekman, 1989). In addition, Cunningham and Wickner (1989) have reported that membranes enriched for an α 1 $\rightarrow$ 3 mannosyltransferase activity and Kex2 endopeptidase activity can be partially separated in a Percoll density gradient (Cunningham and Wickner, 1989). The implication from these studies is that, like the mammalian Golgi complex, the yeast Golgi complex may be composed of discrete cisternae with specific functions in glycoprotein modification and sorting.

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1. **Abbreviations used in this paper:** α f, α factor; CPY, carboxypeptidase Y; m α f, mature α factor; NSF, N-ethylmaleimide sensitive factor; TGN, *trans*-Golgi network; WiMP, Wickerman minimal proline media; WiM-PYE, WiMP yeast extract.

Both genetic and biochemical approaches are being used to study how proteins are transported through the secretory pathway. In yeast, a number of temperature-conditional secretory (*sec*) mutants have been isolated in which protein secretion is blocked at the nonpermissive temperature (Novick et al., 1980; Novick and Schekman, 1979). These *sec* mutants were classified into groups that exhibited defects either in protein transport from the ER, or from the Golgi complex, and those that accumulated 80–100-nm secretory vesicles (Esmon et al., 1981; Novick et al., 1981). Mutants defective in transport from the ER have been further divided into those that accumulated 50-nm transport vesicles (class II, e.g. *sec18*) and those that did not (class I, e.g. *sec23*). The class I *SEC* gene products are thought to be required for budding transport vesicles from the ER and the class II gene products for catalyzing the transport and/or fusion of these vesicles with the target membrane (Kaiser and Schekman, 1990). Using an in vitro reconstitution assay, Rothman and colleagues (1989) have shown that protein transport between adjacent cisternae of the mammalian Golgi complex involves several obligate subreactions which appear to include: the budding of coated transport vesicles from the donor membrane, targeting and attachment of these vesicles to the appropriate acceptor compartment, uncoating of the vesicles, and the subsequent fusion of these vesicles with the acceptor compartment membranes (Orci et al., 1989). The first protein to be purified using this assay was called *N*-ethylmaleimide sensitive factor (NSF) and was found to act in the fusion stage of the reaction (Block et al., 1988; Malhotra et al., 1988). Subsequently, NSF was also found to be required in vitro for ER to Golgi protein transport and for fusion of endocytic vesicles with endosomes suggesting that NSF has a crucial role in many intercompartmental protein transport events (Beckers et al., 1989; Diaz et al., 1989). The genetic approach described above and this biochemical approach have recently converged. The *SEC18* gene and NSF cDNA have been cloned, sequenced and found to encode proteins 48% identical in amino acid sequence (Eakle et al., 1988; Wilson et al., 1989). In addition, *Sec18p* can replace NSF in the mammalian intra-Golgi transport assay indicating that these two proteins share a common biochemical function (Wilson et al., 1989). In yeast, it is not known if *Sec18p* or any of the early *SEC* gene products are required for intercompartmental protein transport events distal to the ER to Golgi transport step.

In this work we have used a novel approach to examine the potential requirement for early *SEC* gene products in protein transport through compartments of the yeast secretory pathway in vivo. We found that protein transport through the yeast Golgi was rapidly and completely blocked when *sec18* mutant cells were shifted to the nonpermissive temperature and that multiple steps in the biosynthesis and secretion of α factor (α f) were blocked. We also found that *Sec18p* was required for transport of carboxypeptidase Y (CPY) through the yeast Golgi complex but not for final delivery of CPY to the vacuole. On the other hand, *Sec23p* was only required for protein transport from an early Golgi compartment in addition to its requirement for ER to Golgi transport. Further, we show that vacuolar proteins are sorted from the secretory pathway at, or beyond a late Golgi compartment that contains *Kex2p*. We discuss these observations in terms of the compartmental organization of the yeast Golgi complex.

Materials and Methods

Strains and Media

Yeast and *E. coli* strains used in this study are listed in Table I. Standard rich media for yeast (Sherman et al., 1979) and *Escherichia coli* (Miller, 1972) was used. The minimal proline media of Wickerman (WiMP) (Wickerman, 1946) was supplemented with 0.2% yeast extract (WiMPYE) and other supplements as needed.

Reagents

Zymolyase-100T was obtained from Seikagako Kogyo Co. (Tokyo, Japan), endoglycosidase H was from New England Nuclear (Boston, MA), DNA modifying enzymes were from New England Biolabs, Inc. (Beverly, MA) and were used as instructed, Trans ³⁵S-label was from ICN Radiochemicals (Irvine, CA) and 0.2–0.3-mm glass beads were from Glen Mills Inc. (Maywood, NJ). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Antisera to α f, α 1 $\rightarrow$ 6 mannose linkages and α 1 $\rightarrow$ 3 mannose linkages were gifts from R. Schekman (University of California, Berkeley, CA). The specificity of these antibodies has been described elsewhere (Franzoso and Schekman, 1989). Antisera to CPY and invertase have been previously described (Klionsky et al., 1988).

Plasmid Construction

The pC α I constructs were made by subcloning the 63 bp HindIII fragment, containing one α f repeat, from pSE200 (Emr et al., 1983) into the HindIII site of pCYI-50 (Johnson et al., 1987). To construct pTG-*SEC18*, pSEY8-*SEC18* (Eakle et al., 1988) was digested with HindIII, the ends were made blunt using the Klenow fragment of DNA polymerase I, then cut with BamHI and the resulting 3-kbp fragment containing the *SEC18* gene was subcloned into the BamHI, EcoRV sites of pRS313 (Sikorski and Hieter, 1989).

Cell Labeling and Immunoprecipitation

Yeast cells were grown to mid-logarithmic phase in WiMPYE and then prepared for radiolabeling as described (Klionsky et al., 1988). To initiate labeling, Trans ³⁵S-label was added to a final concentration of 250 μ Ci/ml to cells at 10 OD₆₀₀ U/ml in WiMP, 1 mg/ml BSA. Cells were chased by adding an equal volume of labeling media containing 2 mM cysteine and 5 mM methionine and the chase was subsequently terminated by adding TCA to a final concentration of 5%. The TCA precipitates were washed twice with acetone, dried, resuspended in 100 μ l of breaking buffer (50 mM Tris-Cl, pH 7.5, 6 M urea, 1% SDS, and 1 mM EDTA), and vortexed 1 min in the presence of glass beads. After boiling for 4 min, 100 μ l of a CB018 extract (a glass bead lysate of strain CB018, equivalent to 100 OD₆₀₀ U/ml yeast in 1% SDS, 1% NP40, 50 mM Tris-Cl, pH 7.5, and 1 mM EDTA) and 800 μ l of immunoprecipitation dilution buffer (50 mM Tris-Cl, pH 7.5, 1.25% Triton X-100, 190 mM NaCl, 6 mM EDTA) were added and the lysates cleared by centrifugation in a microfuge for 15 min. The appropriate antisera was added to the supernatant and a single immunoprecipitation was done as previously described (Klionsky et al., 1988). CPY and α f samples were electrophoresed in 9 and 17% SDS-polyacrylamide gels, respectively, as previously described (Laemmli, 1970). After electrophoresis the gels were fixed and treated with sodium salicylate as previously described (Julius et al., 1984b). Cells were converted to spheroplasts as described (Vida et al., 1990) except Zymolyase 100T was used at 10 μ g/OD₆₀₀ U of cells. Spheroplasts were labeled as described above except the labeling and chase media were made 1.0 M in sorbitol.

Results

Rationale

The role of NSF in Golgi transport in vitro suggested that *SEC18* may be required for similar steps in vivo. Other *SEC* gene products might also be expected to function in multiple steps along the secretory pathway. In the original experiments designed to characterize the position of the secretory block in *sec* mutants, mutant cells were shifted to the non-

Figure 1. Transport and posttranslational modification of α f and CPY. The proteins analyzed in this study, CPY, α f, and invertase are subject to core glycosylation and signal sequence cleavage in the ER (Fuller et al., 1988; Klionsky et al., 1990). The core oligosaccharides are extended in the Golgi complex by the sequential addition of α 1 $\rightarrow$ 6, α 1 $\rightarrow$ 2, and α 1 $\rightarrow$ 3 linked mannose residues (Esmon et al., 1981; Ballou, 1982). CPY receives only limited mannose extension which results in a discrete mobility shift when analyzed by SDS-PAGE (p1 CPY to p2 CPY transition). Invertase and α f receive more extensive mannose addition due to elongation of branched α 1 $\rightarrow$ 6 mannose chains. This results in the addition of 50–100 mannose residues per N-linked oligosaccharide (Ballou et al., 1990) and in these hyperglycosylated forms migrating as a heterogeneous smear in SDS-polyacrylamide gels. The pro- α f polypeptide contains four repeated copies of the mature 13 amino acid peptide (Kurjan and Herskowitz, 1982). These peptides are excised from the precursor, presumably in the Golgi complex, by the concerted action of Kex2 endopeptidase (Kex2p), Kex1 carboxypeptidase, and dipeptidylaminopeptidase A (Ste13p) (Bussey, 1988; Fuller et al., 1988). While invertase and maf are secreted from the cell, CPY is efficiently removed from the secretory pathway and delivered to the vacuole where it is proteolytically processed by proteinase A (the *PEP4* gene product) and proteinase B to the mature CPY (mCPY) (Mechler et al., 1987). The migration pattern of precursor and mature forms of α f and CPY in SDS-polyacrylamide gels are shown. These proteins were immunoprecipitated from wild-type cells after a brief labeling period as described in Materials and Methods.

permissive temperature before labeling and the extent of posttranslational modification of the labeled secretory glycoproteins was determined (Esmon et al., 1981; Novick et al., 1981). In the case of an early *sec* mutant, like *sec18*, a requirement for this gene function in transport events late in the secretory pathway would be masked in such experiments because *sec18* acts at the early event of protein transport out of the ER. We reasoned that if the mutant early *sec* gene products were susceptible to rapid thermal inactivation, we could test for a role of these gene products in transport events subsequent to protein transport out of the ER. By briefly labeling mutant cells first at the permissive temperature, then shifting the cells to the nonpermissive temperature, we could ask if labeled glycoprotein precursors present in the Golgi complex require *SEC* gene function to continue transport through the secretory pathway. We chose α f and CPY as model secreted and vacuolar proteins, respectively, because both of these glycoproteins undergo specific modifications in the yeast Golgi complex (Fuller et al., 1988; Klionsky et al., 1990). These modifications can be easily monitored by electrophoretic mobility differences in the precursor forms of these proteins during SDS-PAGE and by using mannose linkage-specific antibodies (Fig. 1). This permitted us to determine the fate of α f and CPY intermediate

forms present in the Golgi complex in the experiments described below.

Requirement for *Sec18p* and *Sec23p* in the Processing and Secretion of α f

We tested all of the early acting *sec* mutants for a rapid onset of the secretion defect by labeling mutant cells immediately after shifting the cultures to the nonpermissive temperature (see Table I for a list of *sec* mutant strains which were tested). Only the *sec12-4*, *sec18-1*, and *sec23-1* mutants accumulated the ER form of CPY (p1 CPY) exclusively. All of the other *sec* mutants displayed a slower phenotypic onset as judged by the appearance of mCPY in this experiment (data not shown). We found that the *sec23-1* and *sec18-1* mutants displayed the most rapid and tightest phenotypic onset (Figs. 2 and 3). These two mutants were chosen for further study.

To mark compartments of the secretory pathway with radio-labeled transport intermediates of α f, we briefly labeled mutant (*sec18* and *sec23*) and wild-type yeast cells at a permissive temperature (20°C) slightly below that which is normally used. The reduced rate of protein transport at this temperature allowed us to easily detect α f biosynthetic intermediates and to follow multiple modification events simultaneously.

Table I. Strains Used

Yeast strains	Genotype	Source
SEY6210	<i>MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson et al., 1988
SEY5188	<i>MATα sec18-1 suc2-Δ9 leu2-3,112 ura3-52</i>	This study
DKY6183	<i>MATα sec18-1 Δpep4::LEU2 ura3-52 leu2-3,112 his3-Δ200 ade2-10</i>	D. Klionsky (University of California, Davis, CA)
DKY6224	<i>MATα ura3-52 leu2-3,112 trp1-Δ901 his3-Δ200 ade2-101 suc2-Δ9 Δpep4::LEU2</i>	Klionsky et al., 1988
X2180 IB	<i>MATα gal2</i>	Novick et al. 1980
SF274-3A	<i>MATα gal2 sec12-4</i>	C. Field and R. Schekman (University of California, Berkeley, CA)
SF276-6C	<i>MATα gal2 sec13-1</i>	C. Field and R. Schekman
SF280-3C	<i>MATα gal2 sec16-2</i>	C. Field and R. Schekman
SF281-1C	<i>MATα gal2 sec17-1</i>	C. Field and R. Schekman
SF284-2A	<i>MATα gal2 sec20-1</i>	C. Field and R. Schekman
SF286-4B	<i>MATα gal2 sec21-1</i>	C. Field and R. Schekman
SF303-1A	<i>MATα gal2 sec22-3</i>	C. Field and R. Schekman
SF309-2C	<i>MATα gal2 sec23-1</i>	C. Field and R. Schekman
XMC36-10d	<i>MATα kex2-1 ura3-52 leu2-3,112 his4-580 trp1-289 gal</i>	J. Thorner (University of California, Berkeley, CA)
CBO18	<i>MATα can1-100 ade2-1^{oc} his3-11,15 leu2-3,112 trp1-1 ura3-1 Δpep4::HIS3 Δprb1::hisG Δprc1::hisG</i>	R. Fuller (Stanford University, Palo Alto, CA)
<i>E. coli</i> strain		
DH5α	<i>F⁻φ80dlacZΔM15 Δ(lacZYA-argF)U169 endA1 recA1 hsdR17(r_K⁻m_K⁺) deoR thi-1 supE44λ-<i>gyrA</i>96 <i>relA</i>1</i>	Bethesda Research Laboratories, Inc. (Gaithersburg, MD)

In addition, the kinetics of protein transport in wild-type (strains X2180 and SEY6210) and *sec* mutant (strains DKY6183, SEY5188, and SF 309-2C) cells were nearly identical at 20°C (data not shown). The relative abundance and electrophoretic mobility of the different α f forms immunoprecipitated after the 20°C labeling period are shown in the first two lanes of each panel (A–C) in Fig. 2. Assuming that the modification reactions are rapid relative to protein transport, these precursor forms should mark the compartment in which the modification occurred (Balch et al., 1984) (Fig. 1). The cells were then chased at the nonpermissive temperature (37°C). In the case of wild-type cells (Fig. 2 A), the α f precursors rapidly chased to mature α f (α f) which was secreted from the cells. In contrast, further modification and processing of the ER and Golgi forms of α f were completely blocked in *sec18* mutant cells after shifting to 37°C (compare Fig. 2 B to A).

These data show that in addition to the rapid and complete block in ER to Golgi protein transport, proteins in the Golgi complex are also susceptible to the *sec18* transport block. Multiple steps in the posttranslational modification of α f could not occur in the absence of *SEC18* gene product function. In addition, most α f was retained with the cell over the 60-min chase period indicating secretion of α f also required Sec18p function. Four distinct steps in α f biosynthesis were observed to require Sec18p function: (a) Conversion of core glycosylated pro- α f to the α 1→6 mannosylated form; (b) conversion of α 1→6 modified pro- α f to the α 1→3 mannosylated form; (c) proteolytic processing of the α 1→3 modified pro- α f by Kex2 endopeptidase to the mature form (α f); and (d) secretion of α f into the media (See Fig. 1 and the legend to Fig. 2 for a description of the α f precursor forms). If these Golgi-associated reactions occurred in the same com-

partment, we would have expected these modifications to continue after the temperature shift and all of the hyperglycosylated pro- α f forms would have been converted to α f. Because this was not the case, and because Sec18p/NSF has previously been shown to function in the cytoplasm to promote the transfer of proteins from one compartment to another (Wilson et al., 1989), we suggest these processing reactions occur in separate compartments of the Golgi complex.

We considered the possibility that protein transport through the yeast Golgi complex was somehow coupled to ER to Golgi transport such that the apparent requirement for Sec18p in the Golgi complex was an indirect consequence of its requirement in protein transport from the ER. Results with the *sec23* mutant make this unlikely. In this mutant, we found that core glycosylated and the α 1→6 mannosylated form of α f were blocked from receiving further modification, but proteolytic processing of the α 1→3 mannosylated form and the secretion of α f appeared unaffected (Fig. 2 C). The data shown in Fig. 2 B and the results of others (Baker et al., 1988; Franzusoff and Schekman, 1989) indicate that α 1→6 linked mannose addition to α f does not occur in the ER, but appears to be restricted to an early Golgi compartment. Therefore, the data shown in Fig. 2 C indicate that Sec23p is required early within the yeast Golgi complex but not for steps distal to the α 1→6 linked mannose addition to pro- α f. In addition, it appears that intercompartmental protein transport events within the Golgi complex and beyond can occur efficiently in the absence of ER to Golgi transport. Therefore, the requirement for Sec18p at multiple transport steps in the secretory pathway is unlikely to be a secondary consequence of its requirement in ER to Golgi protein transport.

Figure 2. Immunoprecipitation of αf from cells pulse labeled at 20°C and then shifted to 37°C. (A) Wild-type (DKY6183 harboring the centromeric vector pTG-*SEC18*), (B) *sec18* (DKY6183), or (C) *sec23* (SF309-2C) cells were labeled at 20°C for 7-1/2 min as described in Materials and Methods. To initiate a chase at the nonpermissive temperature, an equal volume of media containing 5 mM methionine and 2 mM cysteine, prewarmed to 42°C, was added to the cultures which were then incubated at 37°C. Equal aliquots of cells were removed at the indicated time points, centrifuged briefly (20 sec) in a microfuge to separate the cells (C) from the media (M), and the chase was stopped by the addition of TCA. αf was recovered from the samples by immunoprecipitation and was subsequently eluted from the primary antibody by boiling in 1% SDS. The eluates were divided into three fractions, one fraction was saved for electrophoresis (lanes 1-6) while the other two fractions were diluted with IP buffer (Materials and Methods) and subjected to a second immunoprecipitation either with antisera specific to $\alpha 1 \rightarrow 6$ linked mannose (lanes 7-9), or specific to $\alpha 1 \rightarrow 3$ linked mannose (lanes 10-12). The $\alpha 1 \rightarrow 6$ linkage specific antisera immunoprecipitates all hyperglycosylated αf forms, including those forms that have subsequently been modified with $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 3$ mannose residues. For clarity, we have described the material that reacts with the $\alpha 1 \rightarrow 6$ but not $\alpha 1 \rightarrow 3$ linkage specific antisera as the $\alpha 1 \rightarrow 6$ mannosylated αf form. The $\alpha 1 \rightarrow 3$ linkage specific antisera only recognizes the fully hyperglycosylated form that contains $\alpha 1 \rightarrow 3$

linked mannose. The mature αf peptide is not glycosylated so the media samples were not subjected to the second immunoprecipitation. The positions of unglycosylated (20 kD), core glycosylated (26 kD), $\alpha 1 \rightarrow 6$ mannosylated (28-90 kD), $\alpha 1 \rightarrow 3$ mannosylated (~90-150 kD), and mature αf (~2 kD) forms are noted. The exposure time of lanes 7-12 was 2.5× that of lanes 1-6 in C. All other exposure times were equivalent. The hyperglycosylated material shown in lane 6 of C was not fully recovered in the second immunoprecipitation with $\alpha 1 \rightarrow 6$ linkage specific antibodies (lane 9 of C). Longer exposure of the primary immunoprecipitates (lanes 1-6), and other experiments not shown, indicate that the majority of the $\alpha 1-6$ mannosylated form is blocked from further transport in *sec23* cells at 37°C. Results with the wild-type strain isogenic to SF309-2C are not shown because the data were very similar to that shown in panel A. αf is prone to rapid degradation, particularly at 37°C; therefore, a *sec18* strain deficient in many vacuolar protease activities (DKY6183) was used to help stabilize the peptide.

Requirement for *Sec18p* and *Sec23p* in the Transport and Maturation of CPY

Consistent with in vitro observations (Wilson et al., 1989; Beckers et al., 1989; Diaz et al., 1989), our data indicate

that *Sec18p* is a general transport factor required for most, if not all, intercompartmental protein transport steps along the secretory pathway in vivo. To test whether *Sec18p* also acts in protein transport to the vacuole, we briefly labeled mutant and wild-type yeast cells at the permissive tempera-

Figure 3. Immunoprecipitation of CPY from cells pulse labeled at 20°C and then shifted to 37°C. Wild-type (SEY5188 harboring pSEY8-*SEC18*), *sec18* (SEY5188), or *sec23* (SF309-2C) cells were labeled for 10 min and then chased for 6 min at 20°C. The cultures were shifted to 37°C and aliquots of cells were removed after the indicated time and stopped by the addition of TCA. The samples were subjected to immunoprecipitation with CPY antisera as described in Materials and Methods. The position of p1, p2, and mCPY are noted.

ture (20°C) to populate prevacuolar compartments with labeled CPY precursors. The CPY precursor distribution that was present after this labeling period is shown in Fig. 3 (0 min). p1 CPY marks ER and early Golgi compartments (Franzoso and Schekman, 1989), p2 CPY is present in more distal Golgi compartment(s) and mCPY is in the vacuole (Fig. 1). Upon shifting to 37°C all of the CPY precursors efficiently chased to mCPY in wild-type cells (Fig. 3). However, in the *sec18* and *sec23* cells, the amount of p1 CPY did not change significantly over the 40-min chase period at 37°C. This indicates that these mutant *sec* gene products were rapidly thermally inactivated in this experiment. Surprisingly, p2 CPY was only partially blocked from proteolytic processing in the *sec18* cells. Approximately 70% of p2 CPY was rapidly converted to mCPY (1/2 time of 2.5 min) while the remaining 30% was stable during the entire chase

period. Both p2 CPY and mCPY in this experiment were quantitatively recovered in a second immunoprecipitation using anti- $\alpha 1 \rightarrow 3$ linkage-specific antisera indicating that all of p2 CPY had reached a compartment that contains the $\alpha 1 \rightarrow 3$ mannosyltransferase (data not shown). In *sec23* cells, the transition of p2 to mCPY went to completion even though p1 CPY was completely blocked from further modification (Fig. 3). Again, this indicates that the partial block of p2 CPY seen in the *sec18* cells was not a secondary consequence of blocking ER to Golgi transport. After shifting *sec18* cells back to the permissive temperature after 10 min at 37°C, we found that >90% of the remaining p1 and p2 CPY were converted to mCPY (data not shown). The *sec18* transport block was readily reversible; therefore, cell death or dead-end transport intermediates cannot account for the *sec18* block in p2 CPY maturation. We also found that the Golgi-modified proteinase A precursor (another vacuolar enzyme) was partially blocked in *sec18* cells at the nonpermissive temperature indicating that this phenomenon was not specific to CPY (data not shown).

Figure 4. The percentage of p2 CPY blocked from maturation in *sec18* cells depends on the age of the enzyme. Strain SEY5188 (*sec18*) was labeled for 8 min and then chased at 20°C. (A) At the time points indicated two aliquots were removed, one was immediately stopped with TCA, the other aliquot was shifted to 37°C, and incubated an additional 20 min before stopping with TCA. CPY was quantitatively recovered from each sample by immunoprecipitation and was subjected to SDS-PAGE. After autoradiography and quantitation by laser densitometry, the amount of p2 CPY present in the 37°C sample was divided by that amount in the corresponding 20°C sample. (B) The closed boxes represent the percentage of p2 CPY present in the 20°C sample blocked from maturation during the 37°C chase. The open boxes represent the percentage of p2 CPY that is converted to mCPY during the 37°C chase.

One way to explain these data is to propose that p2 CPY exists in at least two compartments in the cell. Sec18p may be required for transport of p2 CPY from the compartment in which it is formed to a later compartment, but not for transport of p2 CPY from this second compartment to the vacuole. This predicts that newly formed p2 CPY would require Sec18p function for transport to the vacuole, but as p2 CPY moved to a later compartment(s) it would become independent of Sec18p for continued transport. To test this model, an experiment was done with *sec18* cells similar to that shown in Fig. 3, except the chase times at the permissive temperature were varied from 8–28 min. This was done to generate labeled pools of p2 CPY corresponding to newly synthesized p2 CPY as well as kinetically older p2 CPY. At the time points indicated in Fig. 4 A, two equal aliquots of cells were removed. One aliquot was immediately stopped with TCA to mark the progression of CPY and the other was shifted to 37°C for 20 min and then stopped with TCA. After immunoprecipitation, electrophoresis, and autoradiography, the amount of p2 CPY in these two samples was quantitated by densitometry and compared. As an internal control, the amount of p1 CPY in each pair of samples showed that the temperature conditional block was established efficiently at each time point and that the recovery of CPY from the TCA pellets was nearly equivalent for each sample. We found that the small amount of p2 CPY formed by the first time point (new p2 CPY) was almost completely blocked from maturation upon temperature shift to 37°C. However, as the chase continued at permissive temperature, a greater percentage of p2 CPY was matured after the culture was shifted to the nonpermissive temperature. These results indicate that there were two pools of p2 CPY in these cells. One pool of newly formed p2 CPY required Sec18p for entry into the second pool. This second pool of p2 CPY, kinetically distal to the first pool, was independent of Sec18p for continued transport to the vacuole. As suggested above, it is likely that these two pools of p2 CPY represent residence in at least two compartments; one that contains the $\alpha 1 \rightarrow 3$ mannosyltransferase which is required for the formation of p2 CPY, and a later compartment that may act as a donor for transport to the vacuole.

Intercompartmental Transport to the Vacuole Is Sec18p Independent

Our data suggest that all intercompartmental protein transport events in the biosynthetic pathway, except for transport to the vacuole, require Sec18p function. This interpretation is based on the assumption that the conversion of p2 CPY to mCPY is indicative of intercompartmental transport to the vacuole. This appears to be the case for wild-type cells (Stevens et al., 1982; Mechler et al., 1987; Vida et al., 1990), but we were concerned about uncovering proteolytic processing events in *sec18* cells not normally seen in wild-type cells. We needed to show a physical difference in the compartments that contain p2 CPY and mCPY to demonstrate that an intercompartmental transport event can occur in the absence of Sec18p function. When yeast spheroplasts are diluted into a hypotonic medium, cells efficiently lyse as do their vacuoles, yet most ER and Golgi vesicles remain intact such that the soluble luminal constituents of these organelles can be sedimented with the membranes (Eakle et

al., 1988; Vida et al., 1990). If p2 CPY was being transported to the vacuole in *sec18* cells at the nonpermissive temperature then we would expect the p2 CPY present at time 0' (Fig. 3) to be in a compartment stable to hypotonic stress, but after conversion to mCPY it would be in a hypotonically fragile compartment. Yeast spheroplasts were labeled at permissive temperature as described for Fig. 3 and then chased at 37°C for 10 min. Equal aliquots of cells were taken at 0 and 10 min of chase (at 37°C) and were lysed in a hypotonic medium. The lysates were then centrifuged at 100,000 g to divide the sample into a sedimentable fraction and soluble fraction. We found that >90% of p1 and p2 CPY was recovered in the pellet fraction both before (0 min at 37°C) and after (10 min at 37°C) the temperature conditional block suggesting that little, if any, of these precursors were in the vacuole (Table II). In contrast, ~90% of the mCPY formed under these conditions was in the soluble fraction. This suggests that p2 CPY was rapidly processed to mCPY when it reached this osmotically sensitive compartment, and that maturation was not occurring in the same compartment that initially contained the Sec18p-independent pool of p2 CPY. No difference was seen between mutant or wild-type cells in this experiment (Table II). The experiment was also done with a *sec18 pep4* strain in which p2 CPY cannot be processed to mCPY even though vacuolar transport is not affected (Hemmings et al., 1981; Stevens et al., 1982; Zubenko et al., 1983). At 10 min after shifting to 37°C, ~65% of p2 CPY was in the soluble fraction as would be expected if this amount had been transferred to the vacuole. This shows that p2 CPY does not appreciably interact with the vacuolar membrane and therefore should have been released into the supernatant

Table II. Differential Sedimentation of Mature and Precursor Forms of CPY after Osmotic Lysis of *sec18* or Wild-type Spheroplasts

			Percent in pellet	Percent in supt
<i>sec18</i>	0'	p1	95	5
		p2	96	4
		m	—	—
	10'	p1	96	4
		p2	91	9
		m	8	92
SEC18	0'	p1	93	7
		p2	90	10
		m	—	—
	10'	p1	98	2
		p2	96	4
		m	10	90

Wild type (SEY5188 harboring pSEY8-SEC18) or *sec18* (SEY5188) cells were converted to spheroplasts and then labeled for 10 min and chased for 6 min at 20°C as described in Materials and Methods. The cultures were shifted to 37°C and aliquots were removed at 0 and 10 min of nonpermissive chase and diluted 10-fold in a hypo-osmotic buffer as previously described (Eakle et al., 1988). Unbroken cells were removed by centrifugation at 2,000 g for 2 min and the resulting supernatant was subjected to centrifugation at 100,000 g for 40 min. Immunoprecipitations using CPY antisera were performed on the 100,000 g pellet and supernatant fraction. A portion of the starting material was also immunoprecipitated to control for recovery from the ultracentrifuge spin. Only experiments in which >90% of the CPY was recovered were included in the quantitation. After electrophoresis and autoradiography the amount of each CPY form recovered from the fractions was quantitated by laser densitometry. The values are averaged from three experiments and expressed as the percentage of the total CPY form present in the pellet or supernatant fraction (for example, [p1 CPY in pellet/p1 CPY in pellet + p1 CPY in supernatant] × 100).

if it were in the vacuole in the experiments using the *PEP4* strain. We conclude from these experiments that p2 CPY is being transferred from an osmotically stable compartment(s) (characteristic of the Golgi vesicle) to an osmotically sensitive compartment (characteristic of the vacuole) by a Sec18/NSF independent mechanism.

Proteins Destined for the Vacuole Transit through a Compartment that Contains Kex2p

The compartmental organization of the yeast Golgi complex indicated by the above experiments raised the interesting question of where in the secretory pathway does protein sorting to the vacuole occur? Both CPY and α f require the MNN1 gene product for α 1 $\rightarrow$ 3 mannosylation suggesting that sorting occurs after this event (Ballou, 1982; data not shown). Our data indicated that a distinct compartment housing Kex2p is distal to a compartment which catalyzes α 1 $\rightarrow$ 3 linked mannose addition (Fig. 2). We reasoned that a portion, if not all, of the Sec18p independent pool of p2 CPY may be localized to the same compartment as Kex2p. It is not known if vacuolar proteins are sorted from the secretory pathway before reaching the Kex2 endopeptidase because none of the vacuolar enzymes characterized to date are known to be a substrate for this endopeptidase (Klionsky et al., 1990). We have constructed a trimeric gene that encodes a vacuolar protein that is a substrate for Kex2p by cloning a 63-bp fragment of the MF α 1 gene into a previously characterized CPY-invertase chimera (Johnson et al., 1987; see Materials and Methods). The product of this new fusion gene (C α I) contains the CPY vacuolar targeting signal at its amino terminus, separated from enzymatically active invertase at the carboxy terminus by a Kex2p cleavage site (LysArg) present in the α f insert. We isolated trimeric constructs with the α f fragment cloned in either direction (C α I, C $\bar{\alpha}$ I). While both orientations maintain the open reading frame, only the proper α f orientation encodes a Kex2 cleavage site (Fig. 5 A). If the C α I fusion protein transits through the Kex2p compartment, invertase should be cleaved free from the CPY vacuolar targeting signal. Furthermore, if this cleavage occurs before sorting and commitment of the C α I fusion protein to the vacuole then the invertase fragment released from the triple fusion should be secreted.

As previously described (Johnson et al., 1987), the parent CPY-invertase fusion is efficiently sorted to the vacuole, therefore most of the invertase activity (>95%) is retained in the cell (CYI, Fig. 5 B). The transit pathway for this fusion protein appears to be identical to intact CPY (Johnson et al., 1987). In contrast, 70% of invertase activity was secreted from cells expressing the triple fusion with the α f fragment cloned in the proper orientation (C α I). The action of Kex2 endopeptidase was required for the secretion of invertase expressed from the C α I construct as invertase is not secreted from *kex2* mutant cells expressing this construct. KEX2 cells expressing the C $\bar{\alpha}$ I construct also do not secrete invertase activity (Fig. 5 B). All of the strains expressing these constructs contain similar levels of invertase activity as determined by quantitative assays. The parent CPY-invertase fusion (CYI) is secreted by *vps* mutant yeast which exhibit pleiotropic defects in vacuolar protein sorting (Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989; Rothman and Stevens, 1986). Invertase is also secreted by

vps cells expressing the C $\bar{\alpha}$ I fusion protein. This suggests that the insertion of 20 amino acids encoded by the α f gene does not affect the vacuolar transit pathway of these fusion proteins (data not shown).

The fusion protein initially expressed from the C α I construct reacted with invertase antibodies and migrated in SDS-PAGE as a 63-kD protein after endo H treatment, consistent with the size calculated from its amino acid sequence

Figure 5. KEX2-dependent secretion of invertase from cells expressing a CPY- α f-invertase trimeric fusion protein. (A) Schematic drawing of the fusion proteins. The amino acid sequence encoded by the α f insert in the proper orientation (C α I) and in the opposite orientation (C $\bar{\alpha}$ I) are shown. The amino acid sequence of the mature α f peptide is boxed and the peptide bond that is cleaved by Kex2 endopeptidase is marked with an arrow. The hatched segment represents the first 50 amino acids of CPY which contains the signal sequence and the vacuolar targeting signal of CPY. The open box represents the full-length mature invertase segment. The positions of N-linked oligosaccharides are marked above the invertase segment. (B) Strain 6210 (KEX2, +) or XMC36-10d (KEX2, -) harboring plasmids expressing either the parent CPY-invertase fusion which lacks the α f insert (CYI) or the trimeric fusion proteins were patched onto YP fructose plates and were grown overnight. Invertase filter assays were performed as described (Klionsky et al., 1988) using filter replicas of the master plate before (Intact Cells) and after (CHCl₃ Lysed) chloroform vapor lysis of the yeast cells. Quantitative liquid invertase assays were done with the same strains grown to mid-logarithmic phase in YPD broth. The percent invertase secreted was calculated as previously described (Johnson et al., 1987). All strains expressed approximately the same level of invertase units per OD₆₀₀ of cells as determined by quantitative liquid assay.

(Fig. 6, lane 1). However, after 45 min of chase most of the protein (75%) was found in the media and the polypeptide now migrated as a 59-kD protein (lane 4). This form comigrated with endo H-treated invertase secreted from wild-type cells (data not shown). The 59-kD protein was not found in the media from spheroplasts expressing the C α I construct (lanes 5 and 6) indicating its formation required the presence of the α f Kex2 cleavage site. These data suggest that the Kex2 endopeptidase cleavage occurred at the expected site in the C α I fusion protein and only the material cleaved by Kex2p could be secreted. The intact fusion protein (63-kD form) present after the chase period was only found inside the cells (lane 3) suggesting that the material not cleaved by Kex2p was sorted to the vacuole. In this experiment the fusion proteins were expressed in a *pep4* strain because in earlier experiments we found that the proCPY portion of the fusion protein retained within the cell was unstable in *PEP4* cells (data not shown and Johnson et al., 1987). This indicates that the intact C α I fusion proteins that were retained within the cell were localized to the vacuole with the *PEP4*-dependent proteases which cleave within the pro-CPY segment of the triple fusion (Mechler et al., 1987). The data shown in Fig. 2 demonstrates that pro- α f can be blocked in the ER and early Golgi compartments for up to an hour without noticeable processing by Kex2p. In addition, when the labeled C α I fusion protein was trapped in the ER in *sec18* cells for 45 min, no Kex2p-dependent processing occurred (data not shown). Therefore, the observed results cannot be explained by cleavage of the C α I fusion protein by newly synthesized Kex2p traversing the ER or early Golgi compartments en route to the late Golgi complex. These experiments suggest that vacuolar proteins transit through the Kex2p compartment before removal from the secretory pathway, and that sorting of vacuolar proteins from secreted proteins occurs either within the Kex2p compartment or in a more distal compartment.

Figure 6. KEX2-dependent proteolytic processing of the C α I fusion protein. Strain DKY6224 harboring plasmids pC α I or pC α I were converted to spheroplasts, labeled at 30°C for 5 min, and chased for 45 min. At the indicated time points aliquots were removed, centrifuged briefly to divide the samples into an intracellular (I) and extracellular (E) fraction, and the chase was stopped by the addition of TCA. The fusion proteins were recovered from the samples by immunoprecipitation with invertase antibodies. The immunoprecipitated proteins were subsequently treated with endoglycosidase H as described (Johnson et al., 1987), and fractionated in a 10% SDS-polyacrylamide gel. The position of the intact fusion proteins is shown (C α I) as is the relative molecular weight of the gene-fusion products. The 59-kD invertase form found in the cellular fraction after the 45-min chase (lane 3) may have been retained in the periplasmic space of cells not converted to spheroplasts (8% in this experiment).

Discussion

In this work we have described a novel approach to analyzing the role of the early acting *sec* mutants in protein transport events distal to the primary block exhibited by these mutants. We have found that Sec18p and Sec23p are required for protein transport out of the ER as originally determined, but also for transport events distal to the ER (Figs. 2 and 3). Consistent with in vitro observations (Wilson et al., 1989; Beckers et al., 1989; Diaz et al., 1989), our data indicates that Sec18p/NSF performs a crucial function in most, if not all, intercompartmental protein transport events in the secretory pathway in vivo. We have demonstrated a requirement for Sec18p for the following events in α f biosynthesis: (a) Addition of α 1 $\rightarrow$ 6 linked mannose to the core glycosylated form; (b) addition of α 1 $\rightarrow$ 3 linked mannose to the α 1 $\rightarrow$ 6 mannosylated form; (c) Kex2p-dependent proteolytic processing of the α 1 $\rightarrow$ 3 mannosylated form; and (d) the secretion of m α f into the media (Fig. 2). Because Sec18p/NSF functions to catalyze the vesicle-mediated transfer of proteins from one compartment to another (Wilson et al., 1989), our results indicate that each of the events listed above require intercompartmental transfer of α f. Fig. 7 depicts this information in a schematic drawing of the yeast secretory pathway. We present evidence for three functionally distinct Golgi compartments which contain, from *cis* to *trans*; an α 1 $\rightarrow$ 6 mannosyltransferase, an α 1 $\rightarrow$ 3 mannosyltransferase, and the Kex2 endopeptidase. Sec23p has also been shown to be required both in vivo and in vitro for intercompartmental protein transport (Hicke and Schekman, 1989). We have found that both core glycosylated and α 1 $\rightarrow$ 6 mannosylated α f precursors are blocked from continued transport in *sec23* cells at the nonpermissive temperature, while pro- α f modified with α 1 $\rightarrow$ 3 linked mannose appears to be efficiently processed to m α f and secreted from the cell (Fig. 2).

Figure 7. Model for the compartmental organization of posttranslational modification and vacuolar protein sorting events in the yeast Golgi complex (see Discussion for details).

Surprisingly, Sec18p does not appear to be required for intercompartmental protein transport to the vacuole (Fig. 3). We have found that p2 CPY is present in at least two compartments in the yeast cell. Kinetic experiments have indicated that Sec18p is required for transport of p2 CPY from the compartment in which it is formed (the $\alpha 1 \rightarrow 3$ mannosyltransferase compartment) to a distal compartment. However, Sec18p is apparently not required for transport of p2 CPY from this later compartment to the vacuole (Fig. 4). All of p2 CPY is processed to mCPY in *sec23* cells at 37°C (Fig. 3). This is consistent with the results obtained using αf as the marker. Glycoproteins traversing the secretory pathway present within or beyond the $\alpha 1 \rightarrow 3$ mannosyltransferase compartment seemingly do not require Sec23p function for continued transport. This result also shows that protein export from late Golgi compartments can occur efficiently in the absence of protein import from earlier compartments. Therefore, the portion of p2 CPY blocked in *sec18* cells is not likely to be a secondary consequence of blocking earlier transport events. The difference in osmotic stability between the donor compartment containing the Sec18p-independent pool of p2 CPY and the processing compartment (the vacuole) allowed us to show that this pool of p2 CPY was in a distinct prevacuolar compartment and that CPY maturation was indicative of intercompartmental transport to the vacuole (Table II). The action of Sec18p appears to be a basic requirement for intercompartmental protein transport through the secretory pathway, yet this activity does not appear to be needed for protein transport to the vacuole. It is interesting to speculate that another protein may provide a Sec18p/NSF-like function in vacuolar protein transport, or that perhaps the cell has evolved an entirely different mechanism for intercompartmental protein transport to the vacuole. Consistent with this model, many *VPS* genes have been identified that are required for vacuolar protein transport but not for protein secretion (Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989; Rothman and Stevens, 1986).

We cannot rule out the possibility that Sec18p and Sec23p are required for all intercompartmental transport events. If this is the case, then the thermal inactivation of the *sec18-1* and *sec23-1* gene products must affect only a subset of the transport events catalyzed by these proteins. This seems unlikely, as one would have to argue for a different mechanistic requirement for these proteins at different transport steps. For example, a thermally stable domain of the Sec18-1 protein may be required for vacuolar protein transport, or perhaps this protein is protected from thermal inactivation at this step because it resides in a different protein complex. Although these explanations are possible, the simplest interpretation of our data is that Sec18p is not required for Golgi compartment to vacuole transport and Sec23p is not required for transport events distal to the $\alpha 1 \rightarrow 6$ mannosyltransferase compartment.

The diagram in Fig. 7 represents the minimal number of Golgi compartments for which we have obtained evidence. It is possible that other compartments exist. A 28-kD form of αf has been shown to be an intermediate between core glycosylated and $\alpha 1 \rightarrow 6$ linked mannose forms of αf in an in vitro ER to Golgi assay (Bacon et al., 1989). The chemical nature of this modification has not been demonstrated but it is formed within Golgi membranes and the *YPT7* gene product is required for its subsequent modification with $\alpha 1 \rightarrow 6$

linked mannose in vitro (Groesch et al., 1990). In addition, pro- αf that has been tagged at the carboxyl terminus with the yeast ER retention signal HDEL apparently receives this limited modification in the Golgi compartment and is retrieved back to the ER (Dean and Pelham, 1990). This modification has been suggested to occur in a distinct compartment that may be analogous to the salvage compartment described for mammalian cells (Warren, 1990). Although a 28-kD form of pro- αf appears to be blocked in *sec23* cells at 37°C (Fig. 2) we cannot clearly differentiate it from the more extensively modified forms migrating just above it because all of these αf forms are recognized by the $\alpha 1 \rightarrow 6$ linkage specific antisera used in this study. Thus, our results neither confirm nor deny the presence of this compartment. Yeast also contain an $\alpha 1 \rightarrow 2$ mannosyltransferase that acts on *N*-linked oligosaccharides (Ballou, 1982). Others (Franzusoff and Schekman, 1989) have suggested that this enzyme is sequestered in a compartment distinct from the $\alpha 1 \rightarrow 6$ mannosyltransferase and before the $\alpha 1 \rightarrow 3$ mannosyltransferase. This was based on the partial resistance of pro- αf , glycosylated in an in vitro ER to Golgi transport assay, to endomannanase treatment (this enzyme cleaves $\alpha 1 \rightarrow 6$ mannose linkages on *N*-linked oligosaccharides that have not been substituted with $\alpha 1 \rightarrow 2$ mannose). Yet, the apparent complete endomannanase resistance of partially modified invertase accumulated in a *sec7* mutant could argue that these mannosyltransferases are not sequestered to different compartments (Franzusoff and Schekman, 1989).

Lysosomal enzymes in mammalian cells are thought to be sorted from secreted proteins in the TGN and transported to an endosomal compartment where they dissociate from the mannose-6-phosphate receptor (Griffiths and Simons, 1986; Dahms et al., 1989). The lysosomal enzymes are subsequently transferred from the endosome to lysosomes by an unknown mechanism. It is not known if yeast vacuolar proteins transit through an endosomal compartment before arrival in the vacuole. The compartmental nature of the yeast Golgi complex suggested by our data provided us with the opportunity to ask where within the Golgi complex vacuolar proteins are sorted from secreted proteins. Both CPY and pro- αf require the MNN1 gene product for $\alpha 1 \rightarrow 3$ mannosylation suggesting that sorting occurs after this event (Ballou et al., 1990). Our data indicated that much of p2 CPY was in a compartment *trans* to the $\alpha 1 \rightarrow 3$ mannosyltransferase compartment. It seemed likely that at least a portion of this Sec18p-independent pool was in the Kex2p compartment. To test if vacuolar proteins pass through this latter compartment en route to the vacuole, we constructed a CPY- αf -invertase fusion gene to express a vacuolar protein that is a substrate for Kex2p. The efficient *KEX2*-dependent cleavage of the triple fusion protein in cells expressing this construct indicates that vacuolar proteins transit through the Kex2p compartment. Moreover, the *KEX2*-dependent secretion of invertase from these cells indicates that this cleavage event occurred before the intact fusion protein was sorted from the secretory pathway. Our data argue against this *KEX2*-dependent processing event occurring in secretory granules or in the vacuole. As previously suggested (Julius et al., 1984b), Kex2p must initially act in a late Golgi compartment before, or concomitant with, the vacuolar protein sorting event. As seen in Fig. 1, pro- αf can be blocked early in the secretory pathway in *sec18* and *sec23* cells for up to an hour without notice-

able conversion to α f. Thus, the small amount of newly synthesized Kex2p that is progressing through these early compartments is apparently inactive or at least is present in insufficient quantity to efficiently process α f. Therefore, proteolytic processing of the CPY- α f-invertase fusion protein before this protein reached the Kex2 compartment cannot account for the secretion of invertase. The small fraction of the triple fusion protein not cleaved by Kex2p may have escaped processing due either to inefficient proteolysis of this unnatural substrate or to the active sorting of this protein from the secretory pathway before Kex2p could cleave it.

Our results suggest that vacuolar proteins transit through the Kex2p compartment en route to the vacuole and that vacuolar proteins are sorted from secreted proteins in this compartment or in a more distal Golgi compartment. Because we have no evidence for a more distal Golgi compartment at this time and because α f is likely to be packaged into secretory vesicles at this step (Bresnahan et al., 1990; Fuller et al., 1989; Thomas et al., 1988; Julius et al., 1984b), we suggest that sorting occurs within the Kex2 compartment (Fig. 7). Consistent with this hypothesis, we have recently found that a subset of *vps* mutants which are defective in the sorting of vacuolar proteins also exhibit a defect in the processing of pro- α f by Kex2p (Robinson, J. S., T. R. Graham, and S. D. Emr, unpublished results). This phenotype might be expected if the *vps* mutation adversely affects a Golgi compartment in which both events occur.

In conclusion, although the details of the morphological structure of the yeast Golgi complex are not yet known, the functional organization of this organelle seems to be remarkably similar to that of the multi-cisternal Golgi complex of mammalian cells. This makes yeast an attractive model system for studying how the compartmental organization of the Golgi complex is established, how specific proteins are sorted to and maintained within each Golgi compartment, and how secreted and vacuolar proteins are transported into and out of each of these compartments.

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