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Yeast KEX2 Protease and Mannosyltransferase I are Localized to Distinct Compartments of the Secretory Pathway

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The KEX2 protease (product of the *KEX2* gene) functions late in the secretory pathway of *Saccharomyces cerevisiae* by cleaving the polypeptide chains of prepro-killer toxin and prepro-α-factor at paired basic amino acid residues. The intracellular vesicles containing KEX2 protease sedimented in density gradients to a position distinct from those containing mannosyltransferase I (product of the *MNN1* gene), a marker enzyme for the Golgi complex. The recovery of intact compartments containing these enzymes approached 80% after sedimentation. We propose that the KEX2 protease and mannosyltransferase I reside within distinct compartments.

KEY WORDS — Secretion; *Saccharomyces cerevisiae*; Golgi apparatus; protein targetting.

INTRODUCTION

Eukaryotic cells have approximately 20 distinct compartments, each of which is a membrane or a membrane-bounded aqueous space with a unique protein composition. The organelles of the secretory pathway have many compartments which coordinate the organized targetting and export of secretory proteins. For example, proteins targeted to the cell surface (either the plasma membrane or extracellular space) are synthesized with leader sequences that permit their translocation into the endoplasmic reticulum (Wickner and Lodish, 1985). Subsequently, they are glycosylated and transported to the Golgi complex where further modifications occur (Farquhar and Palade, 1981). A sorting mechanism, thought to be located in the *trans*-network of the Golgi complex, efficiently directs different proteins to either lysosomes or the cell surface (Griffiths and Simons, 1986). The proteins are then packaged into secretory vesicles which direct transport to the cell surface. Many enzymes, such as glycosidases, glycosyltransferases and endoproteinases, which modify secreted proteins, are retained within specific compartments of secretory organelles or transport vesicles (Dunphy and Rothman, 1985). The mechanisms by which these enzymes are retained in specific compartments have not yet been elucidated.

The secretory pathway in *Saccharomyces cerevisiae* is very similar to that of other eukaryotes in organization (Schekman and Novick, 1982). However, the yeast organelles involved in the process have not yet been well characterized by either biochemical or microscopic methods. The Golgi complex, for example, is difficult to visualize by electron microscopy, and at present, no marker enzymes for the yeast Golgi complex have been well characterized. Therefore, in order to understand better the Golgi complex in yeast, we sought to identify marker enzymes for this organelle.

Mannosyltransferase I is thought to reside in the Golgi complex (Schekman and Novick, 1982) based on several observations. A temperature-sensitive secretory mutant termed *sec7* accumulates flattened Golgi-like vesicles under certain conditions (Novick *et al.*, 1980, 1981). Secretory proteins in this mutant accumulate intracellularly and receive outer-chain glycosidic modifications by mannosyltransferase I (Esmon, 1986). Mannosyltransferase I catalyzes the transfer of mannose units from GDP-mannose to the outer polysaccharide chain of certain secreted mannoproteins, forming α1→3 glycosidic linkages (Nakajima and Ballou, 1975). Glycosyltransferases

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are known to reside within compartments of the Golgi complex in mammalian cells (Dunphy and Rothman, 1985).

The *KEX2* gene encodes a membrane-bound endoproteinase that is also thought to reside within the Golgi complex (Julius *et al.*, 1983; G. Ammerer, personal communication). The protease is responsible for cleaving the precursors of yeast killer toxin and α-factor (Leibowitz and Wickner, 1976; Kurjan and Herskowitz, 1982; Julius *et al.*, 1983) at paired residues of basic amino acids (lysine and/or arginine) (Julius *et al.*, 1984a). The processing of prepro-killer toxin and prepro-α-factor occurs at a kinetically late step in the secretory pathway, after these proteins have received core glycosylations in the endoplasmic reticulum (Bussey *et al.*, 1983; Julius *et al.*, 1984b). Since no significant levels of the KEX2 protease are detected at the surface of intact cells (K. Cunningham and W. Wickner, unpublished results), it is likely that the enzyme functions within the cell. Previous studies have shown that this enzyme does not reside within secretory vesicles (Holcomb, 1987b). The precise compartments in which this enzyme resides, however, have not been identified.

In this report, we employ subcellular fractionation of yeast to examine the distributions of KEX2 protease and mannosyltransferase I. We show that these enzymes are markers for different compartments which can be recovered with good yield and easily distinguished by sedimentation in density gradients.

MATERIALS AND METHODS

Reagents

QRR-MCA (Boc-Gln-Arg-Arg-7-amino-4-methyl-coumarin) was purchased from Peptides International (Osaka, Japan). GDP-[1-^{3}H]mannose (10·8 Ci/mmol) was purchased from New England Nuclear. The reduced trisaccharide rM3, used in the mannosyltransferase I assays, was prepared from cell wall mannan of bakers' yeast (Red Star variety) as described (Nakajima and Ballou, 1975). The rM3 preparation contained Manα1→2Manα1→2mannitol and approximately 20% Manα1→3Manα1→2mannitol (Stewart and Ballou, 1968). AG1-X8 resin (chloride form) from Bio-Rad Corp. was exchanged to the acetate form. Lyticase was from Sigma Chemical Company. Percoll was purchased from Pharmacia. Culture media were from Difco.

Yeast strains, extract preparation, and density centrifugation

The yeast strains used in this study are listed in Table 1. The procedures for growth, lysis, and gradient fractionation employed in this study were modified from Emr *et al.* (1984). Yeast strains were grown for 16 h at 30°C in 1 liter YEPD media (containing 1% yeast extract, 2% bacto-peptone and 2% dextrose). Cultures with an optical density (at 600 nm) between 0·5 and 1·5 were rapidly chilled to 0°C, harvested by centrifugation (2500 rpm, 10 min, 4°C), resuspended in 10 ml of 10 mM-NaN_3 at 0°C, and re-centrifuged. Washed cell pellets were suspended in 10 ml buffer A (0·1 M-Tris base, 10 mM-β-mercaptoethanol, and 10 mM-NaN_3) and incubated at 30°C for 15 min. The cells were pelleted by centrifugation and resuspended in 10 ml buffer B (1·5 M-sorbitol, 0·1 M-Tris–HCl, pH 7·5, 50 mM-β-mercaptoethanol, 10 mM-NaN_3, and 500 units of lyticase per ml). The suspension was incubated at 30°C for 1 h or until greater than 95% of the absorbance at 600 nm was lost after an aliquot was diluted 1 : 100 in water. All subsequent steps were performed at 0–4°C. The spheroplast suspension was layered over 10 ml of 1·8 M-sorbitol, 10 mM-Tris–HCl, pH 7·5, then centrifuged at 3000 × *g* for 5 min. The pellet of spheroplasts was resuspended in 0·4 ml of 1·8 M-sorbitol and then lysed osmotically by dropwise addition of 3·2 ml of cold 10 mM-Tris–HCl, pH 7·5, while vortexing. Unlysed cells and debris were removed from the supernatant by two rounds of low-speed centrifugation at 1000 × *g*, each for 15 min. The resulting supernatant (S2), containing intracellular membranes and cytosol, was fractionated on two-step self-forming Percoll gradients. Each gradient consisted of 1 ml 30% (v/v) Percoll overlayed with 8 ml 18% Percoll. Both layers contained 0·5 M-mannitol and 10 mM-Tris–HCl, pH 7·5. S2 was layered over the Percoll steps and centrifuged at 40 000 × *g* for 1–1·5 h in a JA-21 fixed-angle rotor. Fractions were stored at 0°C to preserve membrane integrity until assayed.

Enzyme assays

The KEX2 protease assay was modified from Julius *et al.*, (1984a). Reaction mixtures (200 μl) contained enzyme, 0·1 M-Tris–HCl, pH 7·5, 1 mM-$CaCl_2$, 0·5 M-mannitol, 0·1 mM-QRR-MCA substrate, and (where indicated) 1% Triton X-100. After 1 h at 30°C, the reaction was terminated by

Table 1. List of strains

Strain	Genotype	Source
X2180-1A	*MATa SUC2 mal gal2*	YGSC*
X2180-1B	*MATα SUC2 mal gal2*	YGSC
XW451-3B	*MATa mnn1* (from X2180-1B	C. Ballou
ZA396	*MATα/MATα Δkex2::LEU2 ura3-52 leu2-3 leu2-112 his4-519 suc2-Δ9 mal gal2* (homozygous diploid)	G. Ammerer

*YGSC, The Yeast Genetic Stock Center, Berkeley, CA, U.S.A.

boiling for 5 min. Samples were cooled to room temperature, then centrifuged in an Eppendorf microfuge for 10 min to remove precipitates. The fluorescent reaction product of the KEX2 protease assays (aminomethyl coumarin; MCA) was measured with an Aminco spectrofluorometer set for excitation at 380 nm and emission at 460 nm. One unit of enzyme activity is defined as the amount of enzyme required to release 1 nmol MCA per hour under these conditions.

Mannosyltransferase I was assayed by modification of the procedure of Nakajima and Ballou (1975). Briefly, the transfer of [^{3}H]mannose from GDP-1[1-^{3}H]mannose to the reduced trisaccharide acceptor (rM3) was monitored. Each 50 µl reaction contained enzyme, 50 mM-morpholinopropane sulfonic acid NaOH, pH 7·2, 10 mM-mannosyltransferase, 10 mM-rM3 (acceptor), 0·6 mM-GDP-mannose, 10^5 cpm GDP-[1-^{3}H]mannose, and (where indicated) 1% Triton X-100. After 1 h, at 30°C, 0·75 ml of 50% (v/v) Dowex 1-X8 resin (acetate form) in distilled water was added to terminate the reaction. The neutral tetrasaccharide product and other neutral reaction products were separated from the resin by centrifuging the liquid through a pinhole placed in the bottom of each 1·5 ml conical reaction tube. The radioactivity in each eluate was determined by liquid scintillation counting. Since enzymes other than mannosyltransferase I may be detected by this assay, duplicates of each sample were assayed with or without the rM3 acceptor. The acceptor-independent activity (no rM3 added) was subtracted from the total activity (rM3 added) to yield the acceptor-dependent mannosyltransferase I activity. As determined by mixing experiments and by analysis of the reaction substrates and products after completion of the assay, moderate levels of acceptor-independent activity (observed in the bottom 9 ml of the gradients) did not interfere with the accurate quantitation of authentic mannosyltransferase I activity. One unit of acceptor-dependent mannosyltransferase I activity will transfer 1 nmol of mannose to acceptor trisaccharide in 1 h under these conditions. *mnn1* mutants lack all terminal α1→3-linked mannose in mannoproteins (Raschke *et al.*, 1973) and are deficient in mannosyltransferase I activity, as assayed *in vitro* (Nakajima and Ballou, 1975). A mannosidase activity which produced free mannose from GDP-mannose was detected as the acceptor-independent activity in mannosyltransferase I assays. One unit of this activity produced 1 nmol of free mannose in 1 h under the conditions used to assay mannosyltransferase I (without rM3).

Carboxypeptidase Y was assayed as indicated (Wolf and Weiser, 1977) with the modification that 1% Triton X-100 was added to destroy compartment integrity. One unit of carboxypeptidase Y activity hydrolyzed 1 µmol of the dipeptide substrate CBZ-Phe-Leu per min under these conditions. Total protein was measured with bovine serum albumin standards (Vincent and Nadeau, 1983). Gradient density was determined by refractive index of the solution containing Percoll.

RESULTS

Fractionation of yeast secretory organelles

To obtain intact organelles, washed yeast spheroplasts obtained from mid-log cultures were lysed osmotically (as described in Materials and Methods). Unlysed cells, debris and aggregates were removed by low-speed centrifugation as determined by phase microscopy. The resulting cell-free supernatant (S2) routinely contained greater than 80% of the total KEX2 protease activity found in spheroplasts. The S2 was fractionated on self-forming

Percoll gradients, and each fraction was assayed for various enzymes in the presence or absence of detergent to permeabilize membrane barriers. KEX2 protease and mannosyltransferase I showed appreciable activity in fractions that had entered the gradient (Figure 1). In the presence of detergent, two major peaks of KEX2 protease activity (peaks *a* and *b*) could be resolved in the gradient and a third smaller peak of activity (peak *c*) which did not enter the gradient was also observed (Figure 1A). Activity in peaks *a* and *b* was highly latent since the enzyme activities were diminished greatly if detergent was omitted during the assay (open squares). This observation of latent activity would be expected if the enzyme was sequestered within a membrane barrier.

Mannosyltransferase I activity sedimented to a position in the gradient that was distinct from the positions of KEX2 protease activity (Figure 1B). The observed mannosyltransferase I activity in the peak fraction was decreased by 66% (average of five experiments) when the detergent was omitted from the assays, suggesting that this enzyme is also bounded by an impermeable membrane.

The assay of mannosyltransferase I measures the transfer of radiolabelled mannose from GDP-mannose to a neutral trisaccharide acceptor (rM3). When rM3 was omitted from the assay, hydrolysis of GDP-mannose producing free mannose could be observed. Mannosidase activity, measured in this way, sedimented as two peaks in the gradient (Figure 1C). The small peak of mannosidase activity in fraction 8 was 64% latent (average of four experiments), whereas the major peak at the top of the gradient was not latent (data not shown). The latent peak of mannosidase is presumably due to α-mannosidase found in the vacuole (Van der Wilden *et al.*, 1973) since carboxypeptidase Y, a marker enzyme for the vacuole (Stevens *et al.*, 1982), cosediments precisely with this activity (Figure 1C). Intact vacuoles banded at a different position from the compartments containing either mannosyltransferase I or KEX2 protease.

For each enzyme tested, greater than 80% of the total activities applied to the gradient (i.e. in the S2) were recovered in gradient fractions when detergent

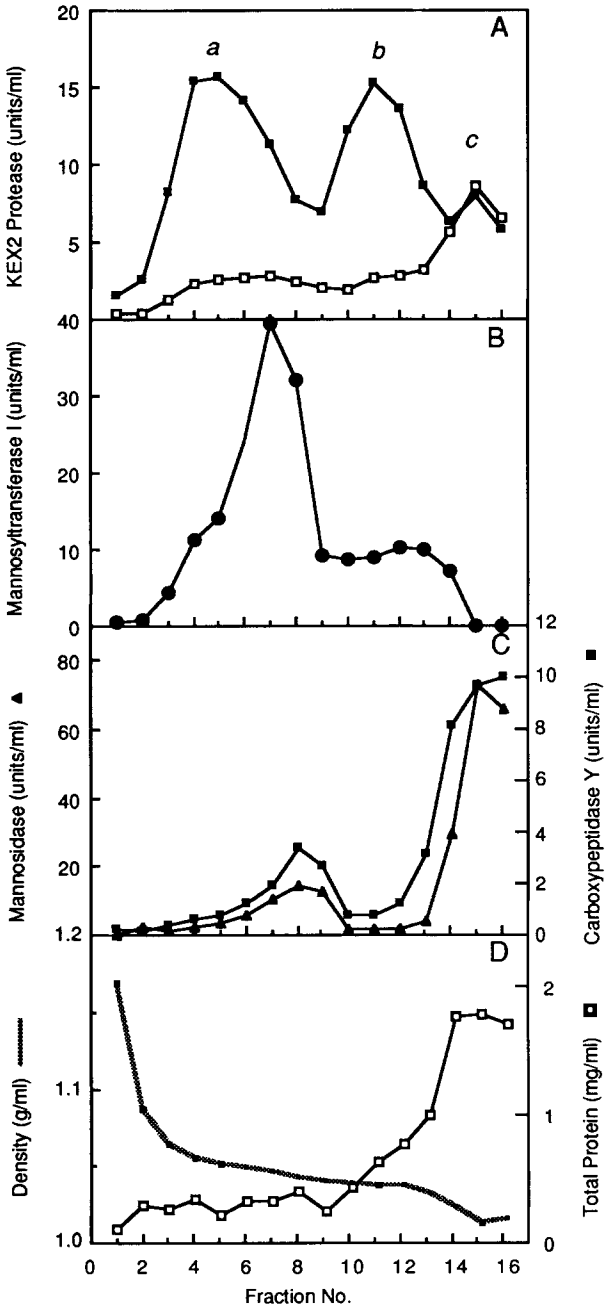

Figure 1. Fractionation of wild-type yeast organelles by density gradient centrifugation. A cell-free supernatant (S2) was prepared from a culture of X2180-1B (wild-type) as described in Materials and Methods. 1·0 ml of the S2 (3·2 mg protein/ml) was layered onto a 9 ml two-step Percoll gradient, which was then centrifuged for 1·5 h at 40 000 × *g*, 4°C. Fractions were collected from the gradient and assayed for KEX2 protease (A), mannosyltransferase I (B), and carboxypeptidase Y and mannosidase (C) either in the presence (filled symbols) or absence (open symbols) of 1% Triton X-100. Each fraction was also analyzed for total protein (open squares) and density (filled squares) (D). In panel A, the letters *a*, *b*, and *c* refer to the three major peaks of KEX2 protease activity.

Table 2. Effect of inhibitors on apparent KEX2 protease activity

Conditions	KEX2 protease activity (%) Fraction 5	Fraction 11	Fraction 15
1. No additions	100	100	100
2. EGTA added	12	9	61
3. Protease inhibitors added	105	130	20

The reactions were performed as described in Materials and Methods using fractions 5, 11 and 15 from the experiment in Figure 1. All reactions contained 1% Triton X-100 and, where indicated, either 10 mM-ethylene glycol bis(β-aminoethyl ester)-N,N,N′,N′-tetra-acetic acid (EGTA) or a mixture of protease inhibitors: phenylmethylsulfonyl fluoride (1 mM), N-tosyl-L-lysyl chloromethyl ketone (1 mM), N-tosyl-L-phenylalanyl chloromethyl ketone (1 mM), chymostatin (50 μg/ml), and bestatin (50 μg/ml). Activity is expressed as a percentage of the uninhibited control (line 1).

was included during the assays. Furthermore, mixing fractions from different regions of the gradient produced additive amounts of KEX2 protease and mannosyltransferase activities (data not shown), showing that any enzyme inhibitors or activators did not affect the analysis. The peak fractions containing KEX2 protease and mannosyltransferase I were enriched 3·1-fold and 6·1-fold, respectively, relative to the fractionation of total proteins (Figure 1D). These results show that vesicles containing the KEX2 protease and mannosyltransferase I activity can be recovered with excellent yield and distinguished by density gradient centrifugation.

The enzyme activities separated in the gradients were further analyzed to establish their authenticity. Peaks *a* and *b* (Figure 1A) were due to authentic KEX2 protease since the activity was very sensitive to EGTA and completely insensitive to a variety of common protease inhibitors (Table 2) which do not affect this enzyme but inhibit other endogenous proteases (Achstetter and Wolf, 1985; Julius *et al.*, 1984a). Furthermore, peaks *a* and *b* were not observed in gradient fractions from a *kex2* deletion mutant (ZA396) even though mannosyltransferase I was recovered normally (Figure 2). Peak *c* was not latent, was sensitive to protease inhibitors (Table 2), and was detectable in the gradients of *kex2* mutants (Figure 2), suggesting that peak *c* activity was largely due to contaminating proteases.

The mannosyltransferase I activity (Figure 1) was also found to be an accurate estimate of the authentic enzyme. Representative fractions from the gradient (fractions 7 and 15 in Figure 1) were assayed for mannosyltransferase I activity and the reaction products were analyzed by paper chromatography as described (Nakajima and Ballou, 1975). The activity from fraction 7 produced the expected

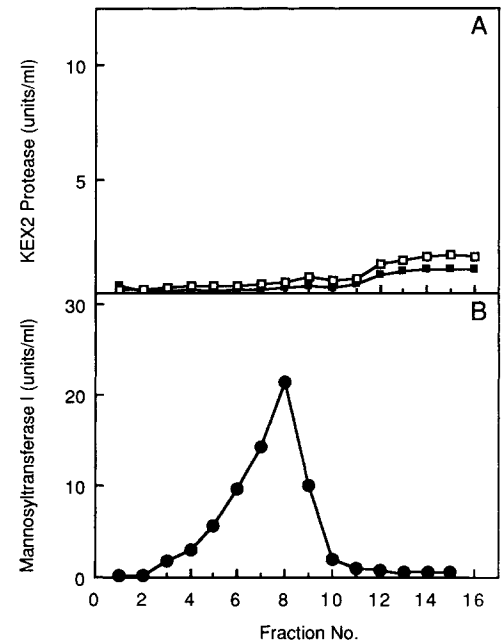

Figure 2. Fractionation of *kex2* mutant extracts. A cell-free supernatant (S2; 1·5 mg protein/ml) was prepared from a culture of ZA396 (Δ*kex2::LEU2*), fractionated on a Percoll gradient, and assayed as described in Figure 1. Assays were either in the presence (filled symbols) or in the absence (open symbols) of 1% Triton X-100.

tetrasaccharide product only when rM3 was added (Table 3) and contained little acceptor-independent mannosidase activity. In contrast, activity in fraction 15 produced only small amounts of tetrasaccharide and large amounts of free radiolabelled mannose even when rM3 acceptor was added. Fraction 15 contained a large amount of acceptor-independent mannosidase activity in comparison to

Table 3. Analysis of reaction products from assays of mannosyltransferase I

Sample	Total	Reaction products (cpm) Tetrasaccharide	Other products
Fraction 7			
+Acceptor	6240	5350	900
−Acceptor	820	<50	840
Fraction 15			
+Acceptor	4560	300	3900
−Acceptor	4810	<80	2800

The samples were from the fractionation of X2180-1B extracts shown in Figure 1. Reactions were performed as described in Materials and Methods, except that the specific activity of GDP-[^{3}H]mannose was increased to 2×10^5 cpm/reaction (7×10^9 cpm/mmol). After eluting the neutral sugars from Dowex 1-X8 resin, they were concentrated by dehydration *in vacuo* and spotted onto Whatman No. 1 paper. Descending paper chromatography of the neutral oligosaccharides (Nakajima and Ballou, 1975) was performed at room temperature in ethyl acetate : pyridine : water (5 : 3 : 2). The paper was dried *in vacuo* and sliced into strips which were analyzed by liquid scintillation counting. The detected radioactivity in each strip was corrected for background by subtracting the values of corresponding strips from a reaction that contained no enzyme.

mannosyltransferase I activity (compare Figure 1B and 1C). Gradient-fractionated extracts from an *mnn1* mutant (XW451-3B), which is deficient in mannosyltransferase I activity (Raschke *et al.*, 1973), showed very little enzyme activity in the gradient even though the KEX2 protease activity and acceptor-independent mannosidase activity sedimented to their normal positions (Figure 3). Thus, intact vesicles containing authentic KEX2 protease and mannosyltransferase I was resolved in self-forming Percoll gradients, indicating that these enzymes reside within different subcellular compartments.

KEX2 protease has previously been shown to be absent from isolated secretory vesicles by Holcomb *et al.* (1987a). KEX2 protease was well-resolved from acid phosphatase, a marker enzyme for secretory vesicles from a *sec1* mutant strain (Novick and Schekman, 1979; Holcomb, 1987b). These data strongly suggest that KEX2 protease is present in compartments which precede secretory vesicles, presumably a sub-compartment of the Golgi complex.

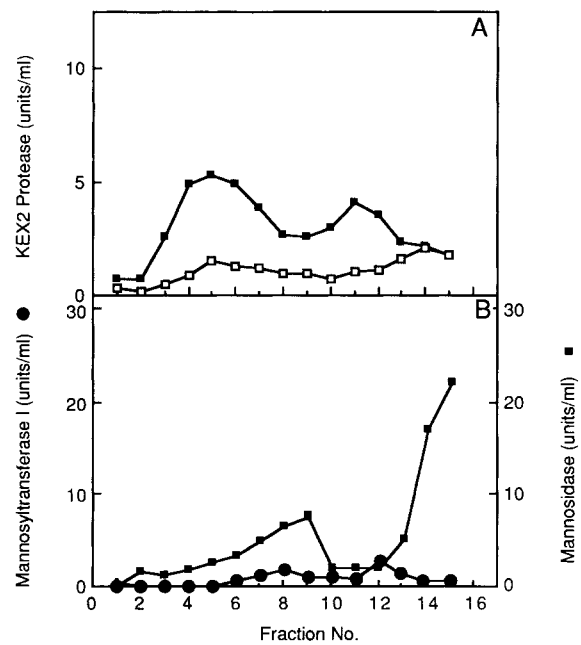

Figure 3. Fractionation of *mnn1* mutant extracts. The cell-free supernatant (S2; 1·6 mg protein/ml) was prepared from a culture of XW451-3B (*mnn1*), fractionated, and assayed as described in Figure 1. Mannosidase (filled squares) and mannosyltransferase (filled circles) activities were determined in the same assay as described in Materials and Methods.

DISCUSSION

The major organelles of the eukaryotic secretory pathway include the endoplasmic reticulum, Golgi

complex, *trans*-Golgi network, and secretory vesicles. These organelles are subcompartmentalized and are linked by various types of transport vesicles. The data presented here suggest that the yeast secretory pathway contains similar subcompartments which are distinguishable by their enzyme composition and density. In particular, the KEX2 protease and mannosyltransferase I enzymes reside within distinct subcompartments in the region of the Golgi complex or *trans*-Golgi network.

When yeast organelles are subjected to Percoll gradient sedimentation, the various compartments sediment to characteristic positions as determined by specific enzyme assays (Figure 1). The compartments containing KEX2 protease sediment to two different positions (peaks in fractions 5 and 11) and are apparently intact since much less activity is detected if detergent is omitted (Figure 1A). At present, the relationship of the two types of compartments is not understood. The compartments containing mannosyltransferase I sediment to a single major position (fraction 7 or 8) which is clearly distinct from that of KEX2 protease. Since the two enzymes do not co-sediment in these gradients and the compartments containing them are mostly intact, the two enzymes must reside within separate compartments.

What is the identity of these compartments? Several lines of evidence suggest that they are located after the endoplasmic reticulum but before the secretory vesicles in the secretory pathway. Modification of invertase by mannosyltransferase I and processing of prepro-α-factor by the KEX2 protease occur after the proteins receive core glycosylations in the endoplasmic reticulum (Bussey *et al.*, 1983; Julius *et al.*, 1984b). Mutants that are conditionally defective in transport of proteins from the endoplasmic reticulum (*sec18* mutants) are unable to deliver these proteins to the compartments where they can be modified by the KEX2 protease and mannosyltransferase I (Bostian *et al.*, 1983; Julius *et al.*, 1984a; Esmon, 1986). We find that the compartments containing mannosyltransferase I and KEX2 protease activities from *sec18* extracts are strikingly shifted in gradients to a position near the top of the gradient (unpublished data). This behaviour is consistent with the idea that their further progression was also blocked by the *sec18* phenotype.

In contrast to the *sec18* phenotype, the functions of the KEX2 protease and mannosyltransferase are not greatly affected by a *sec1* block which accumulates secretory vesicles (Julius *et al.*, 1984a,b; Esmon, 1986). The KEX2 protease does not co-purify with secretory vesicles accumulated in such a mutant (Holcomb, 1987b).

The *sec7* mutation can interfere with the action of KEX2 protease on its endogenous substrates (Bostian *et al.*, 1983; Julius *et al.*, 1984a), but still allows mannosyltransferase I to modify the carbohydrates on invertase (Esmon, 1986). The *sec7* mutant greatly reduces the transport of carboxypeptidase Y to the vacuole (Stevens *et al.*, 1982) and accumulates invertase which is still incompletely glycosylated (Esmon *et al.*, 1981). These results may suggest that mannosyltransferase I functions prior to *SEC7* action in the Golgi complex whereas sorting of vacuolar enzymes and action of the KEX2 protease occur at later steps. Our conclusion that the two enzymes reside in different compartments is consistent with this view.

The simplest interpretation of these biochemical and genetic data is that these enzymes normally reside beyond the endoplasmic reticulum but before the secretory vesicles in the secretory pathway, possibly within compartments of the Golgi complex. The exact identity of the compartments containing these enzymes, however, awaits further analysis. The studies of *sec* mutants should be interpreted with caution since the functions of most *SEC* gene products are not understood and they may have pleiotropic effects on protein transport throughout the secretory pathway.

The mammalian counterparts of the KEX2 protease and mannosyltransferase I have been localized to specific subcompartments of the Golgi complex or in post-Golgi transport vesicles. For example, glycosyltransferases of animal cells have been localized to specific subcompartments of the Golgi complex (Roth and Berger, 1982; Dunphy *et al.*, 1985). Also, maturation of pro-insulin to insulin by an enzyme in pancreatic islet B cells having similar specificity to that of the KEX2 protease has been localized *in situ* to clathrin-coated granules (Orci *et al.*, 1985). Recently, a KEX2-like protease has been implicated in the maturation of human pro-albumin (Bathurst *et al.*, 1987). These findings underscore the similarity in the organization of Golgi complexes between mammals and yeast.

Our studies show that intact compartments of the Golgi complex and other secretory organelles can be recovered in high yield with good enrichment. However, it is possible that the use of enzymatic assays may misrepresent the actual enzyme distribution in gradients if inactive forms of the enzymes are

present or if these enzymes are activated *in vitro* but regulated *in vivo*. Further studies, such as histochemical or immunocytochemical localization (Vorisek, 1986) or additional fractionation procedures (Mueller and Branton, 1984), would be needed to determine the precise identity of the compartments containing the KEX2 protease and mannosyltransferase I. Application of subsequent purification steps may yield highly enriched preparations of specific Golgi compartments, which could be used to investigate in greater detail their structures, functions, and organization.

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