Localization of Components Involved in Protein Transport and Processing through the Yeast Golgi Apparatus

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Abstract. Saccharomyces cerevisiae sec7 mutants exhibit pleiotropic deficiencies in the transit of proteins through the Golgi apparatus, and elaborate an array of Golgi apparatus-like cisternae at a restrictive growth temperature (37°C). The SEC7 gene encodes an essential high-molecular weight protein (227 kD) that is phosphorylated in vivo. In cell lysates, Sec7 protein (Sec7p) is recovered in both sedimentable and soluble fractions. A punctate immunofluorescent pattern of Sec7p-associated structures seen in SEC cells coalesces in sec14 mutant yeast that accumulate exaggerated Golgi cisternae at 37°C. Sec7p may function as a peripheral membrane protein that cycles between a soluble, cytosolic pool and a sedimentable, membrane-associated complex for its essential role in vesicular traffic through the Golgi apparatus.

The transmembrane Kex2 protease, which processes precursors of secreted peptides within the yeast secretory pathway, is also localized by indirect immunofluorescence to multiple structures in the yeast cell (Redding, K., and R. Fuller, manuscript submitted for publication). In double-immunofluorescence labeling experiments, significant colocalization of Sec7 and Kex2 proteins was found. Colocalization of the two antigens, one implicated in protein transport through the Golgi apparatus and the other in processing within a late Golgi compartment, supports the conclusion that we have visualized the yeast Golgi apparatus.

Membrane traffic and protein compartmentalization in the secretory pathway of eukaryotic cells is maintained by vesicular intermediates. The complex processes of vesicular budding from one compartment with targeting and subsequent fusion to the next compartment involve a host of cytosolic and membrane protein components. The requirement for some cellular components is reiterated at multiple steps in the pathway, such as for the N-ethylmaleimide-sensitive factor (NSF) in vesicle fusion with the target compartment (Wilson et al., 1989; Beckers et al., 1989; Diaz et al., 1989). Conversely, secretory pathway components involved in vesicle recognition and targeting to the various compartments are predicted to function at unique points in the pathway. Several approaches have been adopted to identify and to characterize proteins that facilitate these vesicular traffic processes. The biochemical reconstitution of different stages of intracellular protein transport has revealed several cellular components that participate in vesicle fusion in mammalian cells (Malhotra et al., 1988, 1989; Glick and Rothman, 1987; Block et al., 1988; Melancon et al., 1987; Weidman et al., 1989; Clary et al., 1990; Wattenberg et al., 1990).

Genetic selection in the yeast Saccharomyces cerevisiae was used to isolate a series of temperature-sensitive (sec) mutants affecting protein transport at various stages in the secretory pathway (Novick et al., 1980). Two mutations, sec7 and sec14 disrupt glycoprotein traffic from the Golgi apparatus and accumulate exaggerated Golgi cisternae at the non-permissive temperature (Novick et al., 1981). The sec7 mutation abrogates protein traffic destined for both the lysosome-like vacuoles and the cell surface (Novick et al., 1981; Stevens et al., 1982; Franzusoff and Schekman, 1989). Examination of glycoprotein intermediates accumulated in sec7 yeast at 37°C led us to propose that Sec7 protein (Sec7p) function was required at multiple steps in vesicular traffic through compartments of the Golgi apparatus (Franzusoff and Schekman, 1989). The sequence of the SEC7 gene predicts a high-molecular weight polypeptide (227 kD) with an unusual charged acidic domain near the amino terminus of the protein (Achstetter et al., 1988). In this report, we describe that Sec7p is found in both soluble and sedimentable protein pools from wild-type yeast lysates.

The Kex2 protease is a transmembrane glycoprotein that processes precursors of the α-factor and killer toxin polypeptides at pairs of basic residues during their transit of the secretory pathway (Fuller et al., 1988, 1989a, b). Accumulation of both pro-α-factor and mature α-factor species in sec7 mutant cells at the non-permissive temperature suggested that transport to the processing, possibly late, compartment of the Golgi, depends on the SEC7 gene product (Julius et
Three hybrid-fusion proteins were prepared to immunize rabbits and to verify the specificity of the resultant sera. The Bgl I-Pst I fragment from the cloned Sec7p gene (Achstetter et al., 1988) was inserted into the pUR200 vector (Ruther and Muller-Hill, 1983) to make an in-frame fusion of the bacterial lacZ and yeast SEC7 genes. The resultant plasmid pTA50 was introduced into Escherichia coli strain BMH71-18 and the transformant was induced with isopropyl-thio-β-galactoside to express a lacZ-SEC7 fusion gene product (M<sub>r</sub> ~270,000, Fig. 1 A). Extracts of the induced cells were prepared by adding 5x Laemml buffer sample buffer (Laemmli, 1970) plus 1 mM PMSF and 20 µg/ml pepstatin, and heating at 95°C 5 min. This hybrid protein was isolated from SDS gels and used for immunizing rabbits, as described by Nakano et al. (1988). Primary subcutaneous injections contained 50-100 µg of the protein in complete Freund’s adjuvant. 6-8 wk after the initial immunization, boosts of similar amounts of hybrid protein in incomplete Freund’s adjuvant were given every 2 wk. Serum obtained after the fifth boost was used in experiments for this study without further purification, except as indicated.

The same fragment of the SEC7 gene was also inserted into pATH10 (obtained from T. Koerner and A. Tragoloff, Dept. of Biological Sciences, Columbia University) to make an in-frame fusion of the bacterial trpE and yeast SEC7 genes. The resultant plasmid pTAF1-2 was introduced into E. coli strain SE10 and the transformant was induced by adding 10 µg/ml indole acetic acid to exponentially growing cells for expression of the trpE-SEC7 hybrid fusion protein (M<sub>r</sub> ~190,000, Fig. 1 A). Extracts of the induced cells were prepared as described above. E. coli SE10 cells were also transformed with the pATH10 vector alone, and lysates were prepared in the same manner.

Another hybrid construct was created by inserting the 0.7-kb Eco RI-Eco RI fragment from the 5’ end of the SEC7 gene into the pEX2 plasmid to generate a different lacZ-SEC7 gene fusion. The resultant plasmid pAP9 was introduced into E. coli strain pop2136, and the hybrid-fusion protein was induced by shifting exponentially growing cells to 42°C for 2 h, as described by Stanley et al. (1984). The hybrid fusion protein was purified from SDS gels as above, and used for immunization of rabbits (M<sub>r</sub> ~163,000, Fig. 1 A). This hybrid fusion protein showed anomalous (slower) electrophoretic mobility on SDS gels than predicted by the estimated molecular mass of the protein. This was presumably due to the high content of acidic residues in the Sec7 portion of the hybrid fusion polypeptide. Tiering against yeast Sec7p epitopes was detected 2.5 mo after primary immunization (initial plus two booster injections).

Antiserum against the LacZ-antigen fragment 1 (anti-Sec7p[large] sera) was contaminated with antibodies that gave rise to speckled Western blots, but had no effect on the immunoprecipitation of Sec7p. This appeared to be a response to acrylamide and could be removed by preincubating the serum with pieces of Laemmli stacking gel in Western blocking buffer for at least 1 h at 23°C, or overnight at 4°C.

Competition experiments were performed to verify the specificity of anti-Sec7p[large] serum. Antiserum was preincubated overnight at 4°C in PBS and 0.5% Triton X-100, with either the E. coli extract induced for trpE alone (pTA10 fusion pTAF1-2 plasmid) or extract induced for hybrid fusion trpE-SEC7 gene product (pTAF1-2 plasmid). This mixture was further incubated with extracts of radiolabeled yeast for immunoprecipitation experiments or with formaldehyde-fixed cells for indirect immunofluorescence (see below).

The IgG component of both the pre-immune and anti-Sec7p[large] serum was purified for [3H]PO₄ labeling experiments to remove any source of contamination. The serum was concentrated onto a protein A column in phosphate buffered saline at 6 ml/h. IgG was eluted from the column with 0.1 M citrate pH 3 buffer (protein fractions monitored by A₅₃₀) and the eluate was immediately titrated to neutrality with 2M Tris base. The peak protein fractions were pooled, dialyzed against 20% glycerol, 5 mM sodium azide in PBS and stored at -80°C until use.

**Fractionation and Western Immunoblotting Analysis**

Yeast were grown overnight at 24°C in YP media containing 5% glucose. Cells (200 OD₅₃₀ units) were harvested by centrifugation at 24°C, then converted to spheroplasts with 20 U lyticase per OD₅₃₀ cells for 15 min at 30°C as described previously (Scott and Schekman, 1980). Spheroplasts were washed and resuspended in 0.1 M ice-cold isotonic lysis buffer and set on ice. Acid-washed glass beads (0.3-0.5 mm diam) were added to the tube, and the suspension was agitated at half-maximal speed on a Vortex mixer (Scientific Industries, Bohemia, NY) 5 times for 10 s with 15 s intervals on ice. The lysate was diluted 1:10 in lysis buffer, centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatant fraction (LSF) was transferred to a fresh tube on ice. Aliquots of the LSS (2 OD₅₃₀ units) were diluted 10-fold into Buffer B or Buffer T plus urea, detergents or salts for treatment of the lystate (solutions are described above). The mixtures were incubated 30 min at 4°C, then centrifuged 12 or 24 min at 80 krpm at 4°C (forces
sufficient to bring down protein complexes >50 and 25S, respectively) in a T.L100 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). Proteins from the supernatant were precipitated in 9 vol of ice-cold methanol for 10 min at 4°C, then sedimented in a microcentrifuge for 5 min (12,000 g). We have found that Sec7p was not quantitatively precipitated by TCA. The precipitates were dried in a Speed-Vac (Savant Instruments, Hicksville, NY) for 5 min, then resuspended in 30 µl of 1x Laemmli sample buffer and boiled at 95°C for 5 min. The ultracentrifugation pellet fractions were resuspended in 30 µl of 1x Laemmli sample buffer and heated at 95°C for 5 min. The entire pellet and supernatant protein fractions were resolved on 7.5% SDS gels then transferred to nitrocellulose filters for Western immunoblotting analysis, as described previously (Burnette, 1981; Franzussof and Schekman, 1989).

Radiolabeling and Immunoprecipitation

The protocol for radiolabeling yeast strains with [35S]H380, or 300 µCi [35S]SO4 per OD600 cells in the same medium (Sherman et al., 1986) lacking uracil, L-isoleucine, L-glutamic acid, L-asparagine, and L-glutamic acid was overexpressed because the antibody amplification procedure needed to visualize the subcellular localization of Kex2 protein. The available Sec7p-specific and Kex2-specific sera were raised in rabbits, a method was developed that permitted dual labeling without detectable cross-reaction. The two primary antibody sera were saturated with the corresponding "linker" antibody raised in different species. The key feature of this procedure was saturation of the first rabbit primary antibody with a monovalent goat anti-rabbit Fab before addition of the second rabbit primary antibody to the fixed yeast. Bivalent goat anti-rabbit antibodies caused artifactual cross-reaction of the two primary sera (data not shown). However, bivalent mouse anti-rabbit IgG was suitable as a linker for the second primary antibody, presumably because the tertiary fluorescent-coupled rabbit antibody (anti-goat and anti-mouse Fab) fragments were not bound significantly by the mouse anti-rabbit IgG. This protocol required serial incubations with the following five antibody-antiserum mixtures (15 µl in WT buffer): (a) 1:50 dilution of anti-Sec7p antiserum, preimmune serum, or a 1:200 dilution of anti-β-tubulin; (b) 10 µg/ml Fab fragment of goat anti-rabbit IgG (H + L); (c) 1:40 dilution of affinity-purified anti-Kex2 antibody; (d) 1.5 µg/ml mouse anti-rabbit IgG (H + L); and (e) 1.5 µg/ml Texas-Red conjugated rabbit anti-goat IgG (to detect Sec7p protein) and 1.5 µg/ml FITC-conjugated rabbit anti-mouse IgG (to detect Kex2 protein). Incubations were separated by washes with 25 ml WT buffer. After the final washes, cells were stained with DAPI.

Fields of cells were observed using a Zeiss Axioskop Photomicroscope equipped for epifluorescence and photographed with Kodak Professional Ektachrome P3200/1600 slide film, pushed to ASA 1600 in development. G50-490/FT510/LP520 filters were used for observing fluorescein fluorescence, BP546/FT580/LP590 filters for Texas red fluorescence, and G655/FT395/LP420 filters for DAPI fluorescence. Separate exposures were made of each field, with each fluorescence mode and also using Nomarski optics. To assess colocalization, photographic slides of fluorescein and Texas Red fluorescence were projected onto a whiteboard, and the positions of fluorescent spots marked. Landmarks in the field were used to align the photographs precisely. The relative positions of green (fluorescein, Kex2 protein) and red (Texas Red, Sec7p or β-tubulin) spots were noted, and the Nomarski and DAPI photographs were used to assign spots to individual cells. Analysis was limited to cells that stained well for Kex2 protein (61% of total). Cell-to-cell variation in the intensity of fluorescein staining was probably due to fluctuation of the plasmid-dependent expression of Kex2 protein rather than to variability in permeabilization of cells, because Sec7p staining was relatively consistent from cell to cell. Colocalization of a green spot and a red spot was noted when their positions were indistinguishable by visual inspection. Cells (197) from 9 fields were analyzed in this way (1,135 total spots scored).

Results

Identification of the SEC7 Gene Product, Sec7p

Analysis of the subcellular distribution of the Sec7 protein (Sec7p) from yeast was facilitated by the preparation of specific antisera. Three hybrid-fusion genes were constructed to generate two different types of polyclonal antisera and to verify the specificity of the sera (Fig. 1 A, see Materials and Methods). The product of the hybrid-fusion gene lacZ-Large Fragment 1 expressed in E. coli was used to generate anti-Sec7p[large] antibodies, which showed titers against both E. coli β-galactosidase and yeast Sec7p epitopes. For most of the experiments described in this study, the antisera were used without further purification, except as indicated.

The sequence of the SEC7 gene predicts an unusual, highly acidic domain near the amino terminus of the encoded polypeptide (Achstetter et al., 1988). This 125-amino acid region is rich in serine (21%) and contains 47% acidic residues. This domain was fused in frame to the 3' end of a cro-lacZ gene under control of the bacteriophage λP8 promoter in the fusion vector pEX2 (Stanley and Luzio, 1984). E. coli transformed with this gene fusion produced a novel 163-kD polypeptide species upon heat induction (lacZ-Antigen Frag-
Figure 1. (A) Hybrid fusion gene constructs with the SEC7 gene. A restriction map of the authentic SEC7 gene and its coding region is shown at the top, with the expected size of the encoded polypeptide indicated on the right. Portions of the SEC7 gene, highlighted in black, were fused in frame to either the E. coli lacZ gene or the trpE gene for induced expression in bacteria. (B) Specific immunoprecipitation of Sec7p. Yeast cells were labeled with [35S]SO42- for 1 h at 30°C, vortexed with glass beads and 1% SDS then heated 5 min, 95°C. Anti-Sec7p[large] sera and protein A-Sepharose beads were incubated with the labeled whole cell extracts in 1 ml buffer overnight at 4°C. The precipitates were washed, boiled in Laemmli sample buffer, and resolved on 7.5% polyacrylamide SDS-gels. Lanes 1 and 2 show the immunoprecipitation of Sec7p from GPY59 or SEY2102 yeast harboring a 2µ-high-expression plasmid without or with the SEC7 gene, respectively. Lanes 3–6 verify the specificity of the anti-Sec7p[large] sera. Pre-immune serum does not precipitate the 227-kD polypeptide species (lane 3). Specific anti-Sec7p[large] sera were preincubated with no E. coli extract (lane 4), E. coli extract containing TrpE protein (lane 5), or E. coli extract containing the induced TrpE-Sec7p hybrid-fusion protein (lane 6) in 0.1 ml buffer for 4 h at 24°C. Labeled yeast extracts and protein A-Sepharose beads were added to 1 ml final volume, and incubated at 4°C. Samples were processed as above. (C) Sec7p is a phosphoprotein. GPY59 yeast were labeled with either [35S]SO42- (lanes 1–3) or [32P]PO43- (lanes 4–6). Sec7p was immunoprecipitated from whole cell extracts with anti-Sec7p[large] serum and protein A-Sepharose, and the precipitates were resolved on 7.5% SDS-gels, as described in the legend to Fig. 2 (I, lanes 3 and 6). An aliquot of total labeled proteins is shown in lanes 1 and 4 (T). Lanes 2 and 5 (NI) show precipitates from the labeled cell lysates incubated with nonimmune rabbit antiserum and protein A-Sepharose.
### Table 1. Colocalization of Sec7p and Kex2 Protein

| Antigen | Number of spots that colocalize* | Number of spots that fail to colocalize† | Colocalization % |
|---------|----------------------------------|----------------------------------------|------------------|
| Kex2p   | 577                              | 134                                    | 81               |
| Sec7p   | 577                              | 424                                    | 58               |

* Number of Kex2p spots that colocalize with Sec7p spots or Sec7p spots that colocalize with Kex2p spots.
† Number of Kex2p spots that fail to colocalize with Sec7p spots or Sec7p spots that fail to colocalize with Kex2p spots.

The specificity of the anti-Sec7p serum was tested by three criteria. First, the 227-kD species was not observed upon precipitation with preimmune sera (Fig. 1 B, lane J). Second, approximately eightfold higher levels of the high molecular weight polypeptide were immunoprecipitated from extracts of yeast harboring the SEC7 gene on a multicopy plasmid (Fig. 1 B, compare lanes 1 and 2). The third criterion for antibody specificity depended on competition for antigen recognition. A third hybrid fusion gene on the fusion vector pATH10 was constructed for these experiments. The promoter region and almost the entire coding sequence of the E. coli trpE gene was joined to the same large fragment of the SEC7 gene described above. Lysates of E. coli expressing this hybrid-fusion protein were efficient in competing for recognition of the labeled yeast protein by anti-Sec7p[large] sera (Fig. 1 B, lane 6). Extracts from bacteria induced for the production of the TrpE protein alone had no effect on the immunoprecipitation of Sec7p (Fig. 1 B, lane 5).

Biochemical fractionation of wild-type yeast extracts indicated that Sec7p exhibited the characteristics of both sedimentable and soluble proteins. Approximately 50% of Sec7p was recovered in high-speed pellet fractions of yeast lysates (see Materials and Methods). This figure varied from 40–60% in 12 separate experiments using three different strains of yeast.

![Figure 2](image-url)

**Figure 2.** Subcellular distribution of Sec7p: indirect immunofluorescence. 15-9B yeast were fixed by formaldehyde addition to 5% final concentration in the growth medium. Fixed cells were briefly spheroplasted, then attached to polyethyleneimine-coated glass slides. Cells were incubated with primary antisera (1:400 dilution) for 2 h at 24°C. FITC-coupled goat anti-rabbit antisera were added to detect the primary antibody. Preimmune primary antisera were used in A and E. B shows DAPI staining of nuclear and mitochondrial DNA in the cells shown in D. Anti-Sec7p[large] serum was preincubated in 0.1 ml buffer overnight at 4°C with E. coli extract containing TrpE protein alone (D) or containing TrpE-Sec7p hybrid-fusion protein (C) before exposure with the fixed yeast. Anti-Sec7p[acidic] serum was also used to detect Sec7p in fixed cells (F). Exposure times for photomicrographs were 2.5 (A–D), and 2 min (E and F). Images are magnified ~1,000-fold.
Figure 3. Indirect immunofluorescence of Sec7p-associated structures in sec14 yeast. HMSF 169 (sec14) yeast were grown in synthetic defined media with 2% glucose overnight at 24°C, then harvested and resuspended in the same media with 0.1% glucose. Portions of the culture were shaken for 1 h at either the permissive (24°C) or restrictive (37°C) growth temperature before formaldehyde fixation. The cells were then prepared for indirect immunofluorescence with anti-Sec7p serum as described in the legend to Fig. 2. A shows Sec7p-immunofluorescence in sec14 yeast incubated at 24°C. The pattern of Sec7p-immunofluorescence is dramatically altered in sec14 yeast shifted to the restrictive temperature (B and D). C shows the DAPI staining of the nuclear and mitochondrial DNA in the yeast cells from D. The exaggerated immunofluorescent structures seen in some of the cells of panels B and D were observed in all cells by adjusting the plane of focus.

The putative association of Sec7p with particulate subcellular structures was disrupted by incubation of the lysates with relatively low concentrations of urea (>2 M urea) causing the protein to be released into the soluble fraction. Under these conditions, carboxypeptidase Y, a soluble protease in the lumen of the vacuole was recovered in the pellet fractions. In contrast, treatment of the lysates with nonionic detergents or bile salts resulted in 40–60% Sec7p recovery in the particulate fractions, yet caused the solubilization of carboxypeptidase Y. Both Sec7p and carboxypeptidase Y were solubilized upon incubation of the lysates in 0.1 M carbonate buffer pH 11, a treatment known to release peripheral membrane proteins, but which also converts closed membrane compartments into open membrane sheets (Fujiki et al., 1982, data not shown). The sedimentation behavior of Sec7p in biochemical fractionation studies confirmed the polypep-
Posttranslational Modifications: Sec7p is a Phosphoprotein

Regulation of the distribution of Sec7p between soluble and sedimentable states could depend on post-translational modifications of the protein. The addition of long chain fatty acids has been postulated to promote the association of otherwise soluble proteins with membranes (e.g., Aderem et al., 1988). However, a canonical sequence for protein fatty acylations, palmitoylation, or myristoylation was not evident in the predicted sequence of Sec7p. Furthermore, [3H]palmitate labeling of wild-type yeast followed by immunoprecipitation of Sec7p from the lysates did not reveal a labeled polypeptide (data not shown).

An abundance of serine residues in the highly acidic domain of Sec7p suggested that the protein could be modified by phosphorylation. Yeast were grown in minimal phosphate-sulfate media, then incubated with [32P]PO43- or [35S]SO42- at 30°C for 1 h. Extracts were mixed with anti-Sec7p[large] or preimmune IgG and protein A-Sepharose beads and the immunoprecipitates were resolved on SDS-PAGE (Fig. 1 C). A single phosphorylated species was precipitated by the immune serum (Fig. 1 C, lane 6), but not by the preimmune serum (Fig. 1 C, lane 5). This species exhibited the same gel mobility as Sec7p labeled with [35S]SO42- (Fig. 1 C, compare lanes 3 and 6). Neither the site(s) of phosphorylation on Sec7p, nor the phosphoamino acid species have been determined.

Fractionation of extracts prepared from 32P- or 35S-labeled cells failed to show enrichment of newly synthesized, or phosphorylated forms of Sec7p in either the particulate or soluble fractions (data not shown). The role of phosphorylation in the function of Sec7p remains to be determined.

Sec7p Is Associated with the Yeast Golgi Apparatus

Indirect immunofluorescence of formaldehyde-fixed yeast cells was also used to examine the subcellular localization of Sec7p. Both anti-Sec7p[large] and anti-Sec7p[acidic] sera were used to detect the protein in the wild-type cells (Fig. 2). Fluorescein isothiocyanate–coupled goat anti–rabbit serum was used to recognize the primary sera and provide the fluorescent signal. Sec7p was localized in a punctate pattern consisting of multiple large and small structures (approximately four to six per focal plane, Fig. 2, D and F). Nuclei and mitochondria, seen by DAPI staining of the DNA, showed distinct and nonoverlapping distributions (Fig. 2, compare B and D). Antibodies raised against the different portions of Sec7p showed similar punctate patterns, as did cells stained with rhodamine-coupled secondary antibodies (data not shown). The cytosolic form of Sec7p detected in the biochemical fractionation experiments may be represented by the diffuse cytoplasmic fluorescence in the images.

We verified the specificity of the immunofluorescence by two criteria. First, preimmune sera failed to produce any immunofluorescent signal (Fig. 2, A and E). Second, preincubation of the anti-Sec7p[large] serum with E. coli extracts containing the TrpE–Sec7p[large] hybrid fusion protein (Fig. 1 A) effectively competed for antibody recognition of Sec7p (Fig. 2 C). Preincubation of the immune serum with extracts of E. coli induced for the production of TrpE protein alone showed no effect on the pattern of immunofluorescence (Fig. 2 D).

As discussed above, two mutations (sec7 and sec4) block protein traffic from the yeast Golgi apparatus (Novick et al., 1981; Stevens et al., 1982). Whereas morphologically identifiable stacks of Golgi cisternae are seldom observed in wild-type S. cerevisiae (Novick et al., 1981; Svoboda and Necas, 1987), sec7 and sec4 yeast accumulate exaggerated cisternae at the restrictive temperature. Correlation of the punctate pattern of Sec7p-associated structures in SEC cells with the nature of the yeast Golgi apparatus was assessed with immunofluorescence experiments in sec4 mutant yeast. The punctate pattern of Sec7p immunofluorescence in wild-type yeast was also observed in sec4 yeast grown at the permissive temperature (Fig. 3 A). When sec4 cells were incubated at 37°C for 1 h before fixation, exaggerated Sec7p-associated structures were observed in each cell scanned at multiple focal planes (Fig. 3 B).

Colocalization of Sec7p and Kex2 Protease

Indirect immunofluorescence using antibody against the yeast Kex2 protein (Kex2p) also identified a punctate pattern of fluorescence (Redding, K., and R. S. Fuller, manuscript submitted for publication). Kex2p is a membrane-bound protease that cleaves the precursor of the mating pheromone α-factor at pairs of basic residues, a reaction thought to occur in a late Golgi compartment because unprocessed intermediates as well as mature peptides are observed in sec7 yeast at the nonpermissive temperature (Julius et al., 1984; Franzusoff and Schekman, 1989). In order to evaluate the relationship between structures containing Sec7p and Kex2p, double-label immunofluorescence experiments were performed. Fig. 4 shows a typical field of Kex2p-overproducing cells [BFY125(pBM-KX22) strain] from Sec7p/Kex2p double-labeling experiments. The majority of Texas Red–labeled structures, corresponding to Sec7p (Fig. 4, A and C), and fluorescein–labeled structures, corresponding to Kex2p were coincident (Fig. 4, B and D). Additional sites labeled only by Texas Red or by fluorescein were observed in the photomicrographs (note arrows and arrowheads in Fig. 4, C and D). Photographic images of 197 cells were analyzed for colocalization of the fluorescent signals as described in Materials and Methods (Table I). Of the Sec7p signals, 58% were Kex2p positive, whereas 81% of the Kex2p signals corresponded to a Sec7p–labeled structure. At high magnification, individual Sec7p- and Kex2p-labeled signals often appeared to overlap only partially, suggesting differential organization of the two proteins within a larger extended structure.

Because both primary antibodies in the double-labeling methods were derived from rabbit antisera, two sets of control experiments were performed to ensure the fidelity of the procedure. First, the procedure was tested with anti–Kex2p serum and another rabbit antiserum that recognized morphologically distinct structures in a Kex2p-overproducing yeast strain. Anti-β-tubulin serum recognizes both intranuclear and cytoplasmic microtubules emanating from opposite faces of the spindle pole body in the nuclear envelope (Kilmartin and Adams, 1984; Pringle et al., 1986; Pilus and Solomon, 1983).
Figure 4. Double-immunofluorescence labeling of Sec7 and Kex2 proteins. Pairs of panels (A with B and C with D) represent individual fields of BFY125[pBM-KX22] cells subjected to the double-labeling protocol as described in Materials and Methods. Anti-Sec7p Ab was applied in the first incubation. (A and C) Texas Red fluorescence corresponding to Sec7 protein; (B and D) fluorescein fluorescence corresponding to Kex2 protein. Arrowheads correspond to Sec7-stained structures which do not label for Kex2 protein; the arrow corresponds to a Kex2-stained spot which does not label for Sec7 protein. Bar, 10 μm.

1986). With Texas Red-coupled antiserum marking the rabbit anti-β-tubulin serum, fluorescence was restricted to elongated structures typical of yeast microtubules (Fig. 5 C), whereas fluorescein fluorescence intended for the Kex2 protein highlighted the expected punctate pattern (Fig. 5 F). When the same protocol was applied to yeast mutants lacking Kex2 protease (BFY125(pBM743)), the fluorescein signal was absent, but Texas Red-labeled microtubules were still observed (data not shown).

A second set of controls was performed to assess possible cross-reactivity between the anti-Sec7p serum and anti-Kex2p antibody. In the Kex2p-deficient yeast strain, punctate Texas-Red fluorescence corresponding to Sec7p was observed (Fig. 5 B), but the fluorescein signal was absent (Fig. 5 E). Conversely, in a Kex2p-overproducing strain, when preimmune serum was used in place of the anti-Sec7p antiserum, no Texas Red–labeled structures were observed (Fig. 5 A), but the punctate Kex2p–fluorescein pattern was seen (Fig. 5 D). Omission of either primary antibody resulted in a fluorescent pattern corresponding to the other primary antibody (data not shown).

Discussion

We have shown by indirect immunofluorescence that the 227-kD Sec7p is associated with distinct multiple structures in the cell, probably corresponding to the yeast Golgi apparatus. The distribution of Sec7p by subcellular fractionation is divided between soluble and particulate states, and may be altered in vitro by conditions that interfere with protein–protein interactions, but not by detergent solubilization of membrane compartments. We conclude that Sec7p is a cytosolic protein that assembles into particulate complexes, presumably in proximity to the yeast Golgi compartments. Furthermore, Sec7p is phosphorylated in vivo. This posttranslational modification may play a role in the activity of Sec7p in inter-compartmental protein transport.

Nature of the Yeast Golgi Apparatus

The sec7 mutation causes accumulation of exaggerated Golgi apparatus–like cisternae and affects glycoprotein traffic through the yeast Golgi apparatus. Hence, the Sec7p-associated structures seen by indirect immunofluorescence
Figure 5. Controls for double-labeling experiments: (A and D) Control using Sec7 preimmune serum (B and E) control using a strain lacking Kex2 protein; (C and F) Control using anti-β-tubulin antiserum. Strains BFY125[pBM-KX22] (A, C, D, and F) and BFY125[pBM743] (B and E) were grown and processed as described in Materials and Methods. Pairs of panels (A and D, B and E, and C and F) represent epifluorescence of the same field of cells with different filters. A-C represent Texas Red fluorescence, corresponding to β-tubulin immunofluorescence in C and Sec7p-specific staining in A and B. D, E, and F represent fluorescein fluorescence, corresponding to staining for Kex2 protein. Cells adsorbed to multiwell slides were sequentially incubated with (a) anti-β-tubulin (C and F), anti-Sec7p antiserum (B and E), or preimmune serum (A and D); (b) Fab fragment of goat anti-rabbit IgG; (c) anti-Kex2p antibody; (d) mouse anti-rabbit IgG; and (e) a mixture of rabbit anti-goat-Texas Red and rabbit anti-mouse-fluorescein. Bar, 10 μm.

may represent either individual compartments of the Golgi apparatus, or small intact Golgi complexes. Since the detection of Sec7p is severely compromised in sec7 mutant cells, we examined Sec7p distribution in sec14 yeast that are also defective in glycoprotein traffic from the Golgi apparatus, and accumulate exaggerated membrane compartments and numerous vesicles (Novick et al., 1981). In sec14 yeast at the permissive temperature for growth, the pattern of Sec7p immunofluorescence is identical to that in SEC yeast. However, at the restrictive growth temperature, the pattern of Sec7p-associated immunofluorescent structures changes, coalescing into large fluorescent bodies. It is unlikely that alteration of the Sec7p-associated fluorescent pattern in sec14 yeast represents an artefact of the formaldehyde fixation procedures since wild-type immunofluorescent patterns were observed in the mutant cells at the permissive temperature. However, since the Sec14 protein may be involved in promoting proper membrane structure as a phosphatidylinositol transfer protein (Bankaitis et al., 1990), the observed changes in sec14 yeast may reflect aberrant membrane composition at the restrictive temperature. Furthermore, Sec7p-associated structures may represent only a subset of membrane compartments that appear by EM to be affected by the sec14 mutation. These possibilities are nevertheless consistent with the hypothesis that Sec7p-containing bodies represent part of the yeast Golgi apparatus.

Sec7 and Kex2 proteins are largely localized to either identical or closely associated structures within the yeast cell. Given the evidence for functional interaction of each protein with the Golgi apparatus, it seems likely that the sites that contain both proteins represent some part of the Golgi apparatus.

What of the sites which appear to contain one and not the other protein? We do not believe that the incomplete colocalization observed is artefactual. Although cell-to-cell variability in Kex2p-specific staining occurred, such variability was not found for Sec7p staining. Only cells that stained well for Kex2p were included in the quantitative analysis of colocalization. Differences in the efficiency of staining within different regions of a single cell also seem un-
likely to account for the observed partial colocalization. For example, Kex2-positive and negative spots within cells were found generally to be stained to the same intensity with anti-Sec7 antibody.

Sites that contain Sec7p, but not Kex2p, might represent early (cis) Golgi compartments. At the non-permissive temperature, sec7 mutants accumulate multiple forms of secretory proteins, suggesting arrest at several distinct stages of transport (Franzusoff and Schekman, 1989). The secretory protein invertase is accumulated intracellularly in sec7 mutants as both a core-glycosylated ER-type form and as intermediates that have undergone extensive addition of mannosyl residues to Asn-linked oligosaccharides. The latter modifications are characteristic of molecules that have gained access to the Golgi apparatus in yeast cells (Esmon et al., 1981; Kukuruzinska et al., 1987). However, the outer chain carbohydrate chains on glycoproteins accumulated in sec7 mutants lack terminal α-1,3-linked mannosyl residues (Franzusoff and Schekman, 1989). Ser/Thr-linked oligosaccharide in Kex2p is slowly modified during its lifetime in wild-type cells (tα ~90 min at 30°C), probably by addition of α-1,3-linked mannosyl residues, but in sec7 yeast at the nonpermissive temperature, nascent Kex2p is not modified in this way (Wilcox, C. A., and R. S. Fuller, manuscript in preparation). This is consistent with the model that Sec7p function is required for transport of cargo molecules into each successive compartment of the Golgi apparatus, with Kex2p residing in a late (trans) compartment.

In mammalian cells, the Golgi apparatus “fragments” in mitosis, presumably to ensure that the daughter cells receive a complement of the Golgi compartments for proper cellular organization (Warren, 1985; Lucoq and Warren, 1987). The whole process of mitosis, changes in the nucleus and presumably other organelles during cell division requires up to 50% of the S. cerevisiae cell cycle (Wheals, 1987). Therefore, suspending fusion of the Golgi compartments in S. cerevisiae could maintain the probability that the yeast “bud” will acquire its complement of Golgi membrane. One prediction of this model is that individual Golgi compartments in yeast cells are not organized into stacks of successive compartments that are characteristic of the Golgi apparatus in mammalian cells and Schizosaccharomyces pombe. Indeed, stacks of multiple cisternae are rarely found in thin-section EM analysis of wild-type yeast cells (Novick et al., 1981; Svoboda and Nesbitt, 1987). In contrast to the condensed perinuclear structure found in mammalian cells, the yeast Golgi apparatus appears to be a dispersed collection of structures. Sec7p-staining structures that lack Kex2p-staining may represent early Golgi compartments that are not physically associated with later compartments. Structures positively stained for Kex2 protein but not for Sec7 protein may represent the absence of Sec7 protein from a late Golgi compartment that contains Kex2 protein. Alternatively, these structures may be related to transport of Kex2 protein to its site of degradation by vacuolar proteases (Wilcox, C. A., and R. S. Fuller, manuscript in preparation).

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