Golgi Structure Correlates with Transitional Endoplasmic Reticulum Organization in *Pichia pastoris* and *Saccharomyces cerevisiae*

Olivia W. Rossanese, Jon Soderholm, Brooke J. Bevis, Irina B. Sears, James O'Connor, Edward K. Williamson, and Benjamin S. Glick

Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637

**Abstract.** Golgi stacks are often located near sites of "transitional ER" (tER), where COPII transport vesicles are produced. This juxtaposition may indicate that Golgi cisternae form at tER sites. To explore this idea, we examined two budding yeasts: *Pichia pastoris*, which has coherent Golgi stacks, and *Saccharomyces cerevisiae*, which has a dispersed Golgi. tER structures in the two yeasts were visualized using fusions between green fluorescent protein and COPII coat proteins. We also determined the localization of Sec12p, an ER membrane protein that initiates the COPII vesicle assembly pathway. In *P. pastoris*, Golgi stacks are adjacent to discrete tER sites that contain COPII coat proteins as well as Sec12p. This arrangement of the tER-Golgi system is independent of microtubules. In *S. cerevisiae*, COPII vesicles appear to be present throughout the cytoplasm and Sec12p is distributed throughout the ER, indicating that COPII vesicles bud from the entire ER network. We propose that *P. pastoris* has discrete tER sites and therefore generates coherent Golgi stacks, whereas *S. cerevisiae* has a delocalized tER and therefore generates a dispersed Golgi. These findings open the way for a molecular genetic analysis of tER sites.

**Key words:** apparatus, Golgi • endoplasmic reticulum • *Pichia* • *Saccharomyces* • microtubules

THE transitional ER (tER) is a specialized ER subdomain at which proteins destined for the Golgi apparatus are packaged into transport vesicles (Palade, 1975). tER sites are defined by the presence of COPII vesicles, which carry secretory cargo out of the ER (Kuehn and Schekman, 1997). Vertebrate cells contain multiple tER sites, and the COPII components Sec23p, Sec13p, and Sar1p have been localized to these sites (Orci et al., 1991; Kuge et al., 1994; Shaywitz et al., 1995; Paccaud et al., 1996; Tang et al., 1997). Although tER sites often display an elaborate architecture (Bannykh and Balch, 1997), the mechanisms that generate these structures are still mysterious.

In many cell types, ranging from algae to pancreatic acinar cells, some or all of the tER sites are directly apposed to the cis-face of Golgi stacks (Whaley, 1975; Farquhar and Palade, 1981). Such morphological data led early investigators to propose the cisternal progression model for Golgi function (Beams and Kessel, 1968; Morré, 1987). In this view, a new cis-Golgi cisterna forms by the fusion of membranes derived from the tER. The cisterna then progresses through the stack to the trans face, where it fragments into secretory vesicles. However, this model failed to explain how resident Golgi proteins maintain a polarized distribution across the stack, or why vesicles appear to bud from all Golgi cisternae. As an alternative, it was proposed that the Golgi consists of a series of stable compartments, and that transport vesicles carry the secretory cargo from one compartment to the next (Dunphy and Rothman, 1985; Farquhar, 1985; Rothman and Wieland, 1996). According to the stable compartments model, the juxtaposition of tER and Golgi structures might facilitate vesicular trafficking, but this juxtaposition would not reflect a continual formation of new Golgi cisternae.

Recently, cisternal progression has been the subject of renewed interest (Bonfanti et al., 1998). An updated model postulates that cisternal progression is coupled to retrograde vesicular transport of resident Golgi proteins (Schneff, 1993; Mironov et al., 1997; Bannykh and Balch, 1997; Love et al., 1998). This "cisternal maturation" model can account for many experimental findings, including the polarity of the Golgi stack and the existence of...
of Golgi-derived transport vesicles (Glick and M alhotra, 1998; Pelham, 1998).

Can the cisternal maturation model help explain why Golgi structure varies so dramatically between cell types (Mollenhauer and M orre, 1991)? If the Golgi is indeed an outgrowth of the tER, then some of the variation in Golgi structure may be due to differences in tER organization. To test this idea, we examined two budding yeasts that display different Golgi morphologies. Pichia pastoris has stacked Golgi organelles (Gould et al., 1992; Glick, 1996). By contrast, the Saccharomyces cerevisiae Golgi rarely shows a stacked structure, but exists primarily as individual cisternae dispersed throughout the cytoplasm (Preuss et al., 1992). Here we demonstrate that tER organization is also fundamentally different in the two yeasts. P. pastoris contains discrete tER sites. In S. cerevisiae, the entire ER network apparently functions as tER. We suggest that fixed tER sites in P. pastoris produce coherent Golgi stacks, whereas the delocalized tER in S. cerevisiae produces a dispersed Golgi. Thus, tER organization may be a key determinant of Golgi structure in budding yeasts.

Materials and Methods

Strains and Plasmids

Yeast strains and plasmids are listed in Table I. Experiments with S. cerevisiae were carried out using strain BY1034 and derivatives thereof. D BY1034 was transformed with plasmid pOH to obtain expression of Och1p-HA. Strain D BY1034-13G, in which the endogenous SEC13 gene has been replaced with SEC13-GFP, was constructed as follows. The URA3 cassette from pU CI91-URA3 (Benedetti et al., 1994) was excised with HindIII, blunted, and inserted into the SspI site of pUC19 (Yanisch-Perron et al., 1985) to create pUC19-URA3. An 1,166-bp HindIII-HindIII fragment spanning the 3′ end of SEC13 was then amplified by PCR from S. cerevisiae genomic DNA and inserted into the corresponding sites in pUC19-URA3. The resulting plasmid was mutagenized using the QuickChange kit (Stratagene Inc.) to replace the SEC13 stop codon with a SnaB1 site. The EGFP gene was excised from pEGFP-1 (Clontech) with BamHI and NotI, blunted, and inserted into this SnaB1 site. The resulting construct was linearized at the unique BstEII site and integrated into the chromosomal SEC13 gene. This “pop-in” strain was then plated on 5-fluoroorotic acid (Rothstein, 1991) to select for the “pop-out” recombinant strain D BY1034-13G. Strain D BY1034-13G, in which the endogenous SEC13 gene has been replaced with SEC13-GFP, was constructed in the same manner, beginning with the insertion into pUC19-URA3 of a 1,120-bp AflII-HindIII fragment spanning the 3′ portion of SEC13. Similar strategies were used to replace the endogenous SEC24 and SEC31 genes with EGFP fusion genes. To construct strain D BY1034-13m, in which the endogenous SEC12 gene has been replaced with SEC12-myc, a 1,685-bp HindIII-XbaI fragment spanning the 3′ portion of SEC12 was inserted into pUC19-URA3; a c-myc epitope sequence was then inserted downstream of the strong constitutive GAP promoter, pB2-OH was linearized with SalI and integrated into the his4 locus of PPY12. Strain PPY12-13G, in which the endogenous SEC13 gene has been replaced with SEC13-GFP, was constructed as follows. pSG464 (Gould et al., 1992) was digested with SnaB1 and N del, blunted, and religated to yield pUC19-A R G4. This plasmid lacks the PAR52 sequence present in pSG464, and therefore can only transform P. pastoris by integration. A 461-bp HindIII-NsiI fragment spanning the 3′ end of SEC13 was then amplified by PCR from P. pastoris genomic DNA, blunt-end inserted into pUC19-ARG4 that had been cut with HindIII and PstI. This plasmid was mutagenized to replace the SEC13 stop codon with a SnaB1 site, and the EGFP gene was inserted into this site as described above. The resulting construct was linearized at the unique Scl site and integrated into the chromosomal SEC13 gene, yielding an intact SEC13-GFP fusion gene plus a 3′ fragment of authentic SEC13. A similar strategy was used to create strain PPY12-12m: a 1,328-bp BamHI fragment spanning the 3′ end of SEC12 was inserted into pUC19-ARG4; a c-myc epitope sequence was then inserted just upstream of the HDEL sequence, and the resulting construct was linearized with XhoI for integration into the SEC12 locus. To express myc-tagged S. cerevisiae Mtn1p (Chapman and Munro, 1994) in P. pastoris, the gene encoding tagged Mtn1p was excised from pMT15B (a gift of Sean Munro) and subcloned into pOW3, a P. pastoris episomal vector that contains the GAP promoter (Gould et al., 1992; Waterham et al., 1997; O.W. Rossanese, unpublished observations). Plasmid pFB384, which encodes glutathione S-transferase fused to the NH2-terminal acidic domain of S. cerevisiae Sec7p (up to the Nael site in the coding sequence), was provided by A lex Franzusov (University of Colorado, Denver, CO).

Nocodazole Treatment

P. pastoris cultures were grown overnight at 30°C to an OD600 of ~0.15 in 1% yeast extract, 2% peptone, 2% glucose, 20 mg/liter adenine sulfate, 20 mg/liter uracil, 50 mM sodium maleate, pH 5.5. Half of a culture then received nocodazole at a final concentration of 15 μg/ml, diluted from a 10 mg/ml stock solution in water-free dimethyl sulfoxide. As a control, the other half of the culture received only dimethyl sulfoxide. Incubation was continued for up to 2.5 h, and the cells were fixed for microscopy.

Antibodies for Immunofluorescence

An n-1 HA monoclonal antibody (16B12; Berkeley Antibody Co.), anti-myc monoclonal antibody (9E10; Boehringer Mannheim Biochemicals) and anti-green fluorescent protein (GFP) monoclonal antibody (a mixture from clones 7.1 and 13.1; Boehringer M anheim Biochemicals) were used at 5 μg/ml; in cells not expressing a tagged protein, each antibody gave only a faint background signal. The anti-GFP antibody was used to supplement the endogenous GFP signal, which is diminished by the immunofluorescence procedure. Anti-Pdi1p monoclonal antibody (K M X-1; Boehringer M anheim Biochemicals) was used at 1 μg/ml. The following rabbit polyclonal antibodies were used: A nti-Pdi1p serum (a gift of Peter Walter, University of California, San Francisco, CA; originally produced by Victoria Hines, Chiron Corp., Emeryville, CA), raised against SDS-PAGE–purified S. cerevisiae Pdi1p, was used at a dilution of 1:350. A lex Franzusov generously provided an antisera raised against a fusion between β-galactosidase and the NH2-terminal portion of Sec7p (Franzusov et al., 1991); this antibody was used at a dilution of 1:500. O regon green 488-conjugated goat anti-mouse IgG and Texas red-X-conjugated goat anti-rabbit IgG (Molecular Probes, Inc.) were used at 20 μg/ml.

Controls were performed (not shown) to check the specificity of the polyclonal antibodies. In the case of anti-Pdi1p, the immunofluorescence signal could be quenched by preincubating with a protein fragment (a gift of R obert Freedman, University of K ent at Canterbury, U K) comprising the COOH-terminal 308 residues of S. cerevisiae Pdi1p. The anti-Sec7p antibody specifically recognized a glutathione S-transferase-Sec7p fusion protein on an immunoblot of an extract from Escherichia coli cells expressing this protein.

N-acetylglucosamine-containing oligosaccharides of glycoproteins were isolated from Golgi vesicles by passage of the proteins through an aminoterminal acid-extracting column. The eluted material contains a 488-conjugated goat anti-mouse IgG and Texas red-X-conjugated goat anti-rabbit IgG (Molecular Probes, Inc.) were used at 20 μg/ml.

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N-acetylglucosamine-containing oligosaccharides of glycoproteins were isolated from Golgi vesicles by passage of the proteins through an aminoterminal acid-extracting column. The eluted material contains
immunofluorescence, such antibodies primarily label early Golgi elements. Similarly, with P. pastoris, anti-α-1,6-mannose antibodies give a strong signal in Golgi and transitional ER. In several cases, we observed punctate staining with antisera raised against various COPII proteins, but this staining was actually due to contaminating anti-α-1,6-mannose antibodies. This problem can be avoided by preincubating a diluted antibody either with fixed S. cerevisiae cells or with 0.5 mg/ml purified yeast mannan (M-7504, Sigma Chemical Co.).

Electron Microscopy
Thin-section electron microscopy was performed essentially as described (Kaiser and Schekman, 1990; Gould et al., 1992). In brief, a 50-ml culture of yeast cells in rich glucose medium was grown to an OD_{600} of ~0.5. The culture was concentrated to a volume of ~5 ml with a bottom-top vacuum filter, and 40 ml of ice-cold 50 mM KPi, pH 6.8, 1 mM MgCl₂, 2% glutaraldehyde was added rapidly with swirling. A fixer fixation for 1 h on ice, the cells were washed, and then resuspended in 0.75 ml 0.1% glutaraldehyde, 0.1% glutaraldehyde, and then once more with 1 ml of the same solution. Treatment with periodate was omitted because this compound may destroy certain antibodies. The washed cells were embedded in 100 μl of low-melting-temperature agarose, which was cut into cubic-millimeter blocks and infiltrated for 2 h at room temperature in 1.7 M sucrose, 25% polyvinylpyrrolidone K15 (Fluka Chemical Corp.) in PBS, prepared according to Tokuyasu (1989). Individual agarose blocks were then mounted on copper pins, frozen in liquid nitrogen, and cryosectioned at ~80 μm. Thawed cryosections were prepared and labeled as described (Griffiths, 1993; Kärger et al., 1996), using a blocking buffer consisting of PBS, 20 mM glycine, 1% fish gelatin (Sigma Chemical Co.), and 10% fetal calf serum; the serum had been heated at 60°C for 1 h, and then centrifuged to remove complement. Dilutions were 1:10 for an affinity-purified polyclonal anti-HA antibody (a gift of Jan U. Burkhardt, University of Chicago, Chicago, IL), 1:25 for an affinity-purified polyclonal anti-GFP antibody (a gift of Judi Antebi, University of California, San Diego, San Diego, CA), and 1:50 for protein A-gold (10 nm; Goldmark Biologicals). A fixer the protein A-gold step, the sections were postfixed for 30 min in 1% glutaraldehyde in PBS, and then washed with distilled water and stained with 1.5% silicotungstic acid in 2.5% polyvinyl alcohol (MW 15,000; ICN Biomedicals Inc.). The best results were obtained using relatively thick sections and a thin layer of stain. Electron micrographs were digitized and imported into Photoshop (A dobe Systems Inc.) to adjust brightness and contrast and to compose composite images. For Figs. 3 and 7, the gold particles were darkened to improve visualization. Images were printed on an NP-1600 dye-sublimation printer (Codon, Inc.).

Immunofluorescence Microscopy
We modified previously described methods (Pringle et al., 1991) to enhance both the preservation and the visualization of intracellular structures. Improved preservation was achieved by rapidly digesting the cell wall with protease-free lyticase in sorbitol-free wash buffer, and by postfixing the cells in acetone. Improved visualization was achieved by adhering the cells directly to coverslips (Weiss et al., 1989; Helf and Stelzer, 1995) rather than to wells on a slide. A detailed protocol follows.

Fixation and Spheroplasting. A yeast culture is grown overnight in rich phase with good aeration. A 400-μl aliquot of the culture was then mixed rapidly with 400 μl of 100 mM KPi, pH 7.5, 1 mM MgCl₂, 8% formaldehyde, 0.5% glutaraldehyde. A fixer fixation for 1 h in the dark, the cells were washed twice with 1 ml PBS, and then resuspended in 50 μl PBS. For viewing by fluorescence microscopy, 1-2 μl of the resuspended cells was spotted on a slide and spread with a coverslip.
Results

Golgi Stacks in P. pastoris Are Closely Associated with tER Sites

Stacks of Golgi cisternae can be readily visualized in P. pastoris cells, with each stack containing about four cisternae (Fig. 1) (Gould et al., 1992; Glick, 1996). Previously published images of P. pastoris showed Golgi stacks near the nucleus (Gould et al., 1992; Rambourg et al., 1995; Glick, 1996). Golgi structures might form by growing out of the nuclear envelope, which constitutes a large fraction of the ER in budding yeasts (Preuss et al., 1991). Alternatively, P. pastoris Golgi stacks might be positioned near the nucleus by microtubule-dependent transport toward the centrosome, as occurs in vertebrate cells (Thyberg and Moskalewski, 1985; Kreis, 1990; Lippincott-Schwartz, 1994).

Figure 1. Thin-section electron microscopy of Golgi and ER structures in P. pastoris. (A) A representative P. pastoris cell. Golgi stacks are found adjacent to ER membranes, including both the nuclear envelope and peripheral ER elements. (B) A representative P. pastoris cell after treatment with nocodazole for 2.5 h. The nucleus has failed to divide. However, Golgi morphology is unaffected by the drug treatment. This experiment was performed with strain PPY1. Bars, 0.5 μm. G, Golgi stack; N, nucleus; ER, peripheral ER membranes; M, mitochondrion; V, vacuole.
fied immunofluorescence protocol that consistently yields marker proteins (Table I) using immunofluorescence microscopy.

We confirmed this interpretation by visualizing various organelles in P. pastoris and S. cerevisiae, but that Golgi organelles in P. pastoris are more coherent (see above). We verified this interpretation by visualizing various marker proteins (Table I) using immunofluorescence microscopy. For these experiments, we developed a modified immunofluorescence protocol that consistently yields high-quality images (see Materials and Methods).

First, thin-section electron microscopy indicates that a typical P. pastoris cell contains several distinct Golgi stacks, only some of which are located near the nucleus (Fig. 1 A). Other Golgi stacks are found next to the peripheral ER elements that underlie the plasma membrane. Second, treatment of P. pastoris cells with nocodazole does not visibly alter the structure or positioning of Golgi stacks (Fig. 1 B). A way with S. cerevisiae (Jacobs et al., 1988), nocodazole treatment of P. pastoris disrupts microtubules and inhibits nuclear division (see Fig. 4), but Golgi stacks are still observed next to the nuclear envelope and peripheral ER. In both untreated and nocodazole-treated cells, vesicular profiles are frequently seen in ER regions adjacent to the Golgi cisternae (Fig. 1) (Glick et al., 1992; Glick, 1996). These vesiculating ER regions resemble the tER sites seen in vertebrate cells (Palade, 1975; Bannykh and Balch, 1997). Hence, the morphological data suggest that Golgi stacks in P. pastoris are associated not with the centrosome, but rather with tER sites.

**Comparison of ER and Golgi Structures in the Two Yeasts by Immunostaining**

Electron microscopy indicates that general ER structure is similar in P. pastoris and S. cerevisiae, but that Golgi organelles in P. pastoris are more coherent (see above). We confirmed this interpretation by visualizing various marker proteins (Table I) using immunofluorescence microscopy. For these experiments, we developed a modified immunofluorescence protocol that consistently yields high-quality images (see Materials and Methods).

Because P. pastoris is closely related to S. cerevisiae (Higgins and Cregg, 1998), polyclonal antibodies raised against S. cerevisiae antigens often cross-react with the P. pastoris homologues. Alternatively, marker proteins were modified with epitope tags and visualized using specific monoclonal antibodies.

To visualize the general ER in P. pastoris, fixed cells were labeled with an antibody against protein disulfide isomerase (Pdi1p), a marker for the general ER (Fig. 2 D, red) (Sitia and Meldolesi, 1992; Nishikawa et al., 1994). The same cells were also incubated with Hoechst dye (Fig. 2 D, blue) to label DNA. The anti-Pdi1p antibody highlights the nuclear envelope as well as peripheral ER elements. This pattern resembles the ER distribution seen in S. cerevisiae (Rose et al., 1989; Preuss et al., 1991).

Fig. 2 A shows the S. cerevisiae Golgi. This organelle appears in immunofluorescence images as a set of punctate spots, with different Golgi markers often displaying only a partially overlapping localization (Segev et al., 1988; Franzusoff et al., 1991; Antebi and Fink, 1992; Lussier et al., 1995). In S. cerevisiae cells expressing an HA-tagged version of the early Golgi marker Och1p (Nakayama et al., 1992; Gaynor et al., 1994; Harris and Waters, 1996; Gaynor and Emr, 1997), an anti–HA monoclonal antibody (Fig. 2 A, green) reveals multiple spots of Och1p-HA labeling per cell. The same cells were also labeled with a polyclonal antibody (red) against Sec7p, a protein that is concentrated in late Golgi elements (Franzusoff et al., 1991). The two markers show no significant overlap (Fig. 2 A, merged; Table II).

Do early and late Golgi elements ever colocalize in

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**Table I. Marker Proteins, Yeast Strains, and Yeast Plasmids Used in This Study**

| Marker proteins | Yeast Strains | Yeast Plasmids |
|-----------------|--------------|---------------|
| Pdi1p           | DBY1034      | pOH           |
| Sec12p          | DBY1034-S13G | OCH1-HA URA3 CEN6 |
| Sec13p, Sec23p, Sec24p, Sec31p | DBY1034-SEC13-GFP | Harris and Waters, 1996 |
| Och1p           | DBY1034-SEC23-GFP | This study |
| Sec7p           | DBY1034-SEC24-GFP | This study |
| Sec13p, Sec23p, Sec24p, Sec31p | DBY1034-SEC24-GFP | This study |
| Och1p           | DBY1034-SEC31-GFP | This study |
| Sec7p           | DBY1034-SEC12-myc | This study |
| Sec13p, Sec23p, Sec24p, Sec31p | DBY1034-SEC12-myc | This study |
| Och1p           | DBY1034-SEC12-84 | This study |
| Sec7p           | DBY1034-SEC12-84 | This study |
| Sec13p, Sec23p, Sec24p, Sec31p | DBY1034-SEC12-84 | This study |
| Och1p           | CTY214       | ARG4 PARS2 |
| Sec7p           | CTY214       | This study |
| Sec13p, Sec23p, Sec24p, Sec31p | MATa his4 leu2 his4 ursa3 | This study |
| Och1p           | MATa his4 leu2 his4 ursa3 | This study |
| Sec7p           | MATa his4 leu2 his4 ursa3 | This study |
| Sec13p, Sec23p, Sec24p, Sec31p | MATa his4 leu2 his4 ursa3 | This study |
| Och1p           | MATa his4 leu2 his4 ursa3 | This study |
| Sec7p           | MATa his4 leu2 his4 ursa3 | This study |

*Sec7p is thought to function in both intra-Golgi and ER-to-Golgi transport (Franzusoff et al., 1991; Lupashin et al., 1996; Wolf et al., 1998). However, the immunofluorescence staining pattern of Sec7p apparently represents late Golgi elements and shows little overlap with the staining pattern of early Golgi markers (Franzusoff et al., 1991; Antebi and Fink, 1992). Vytas Bankaitis (University of Alabama, Birmingham, AL).
S. cerevisiae? In temperature-sensitive sec14 mutants of S. cerevisiae, multilamellar Golgi structures accumulate at the nonpermissive temperature (Novick et al., 1980; Rambourg et al., 1996); these structures are reminiscent of the Golgi stacks seen in higher eukaryotes. Therefore, we tested whether Och1p-HA and Sec7p colocalize in a sec14 mutant after incubation at the nonpermissive temperature of 37°C. The 37°C treatment causes redistribution of Sec7p into large clusters (Fig. 2 B, red) (Franzusoff et al., 1991). However, the staining pattern of Och1p-HA is largely unchanged in the sec14 mutant (Fig. 2 B, green), and there is still no significant overlap between Och1p-HA and Sec7p. It seems that S. cerevisiae is incapable of generating coherent Golgi stacks that contain both early and late Golgi proteins.

Fig. 2 C shows the P. pastoris Golgi. Using an integrating expression vector (Sears et al., 1998), we generated a P. pastoris strain that stably expresses HA-tagged S. cerevisiae Och1p. P. pastoris contains an α-1,6-mannosyltransferase activity like that ascribed to Och1p (Trimble et al., 1991; Nakanishi-Shindo et al., 1993), and expression of Och1p-HA in P. pastoris has no effect on growth or Golgi morphology (not shown). Och1p-HA localizes to two to six spots per P. pastoris cell (Fig. 2 C, green). A subset (~45%) of the Och1p-HA spots clearly adjoin the nucleus (see Fig. 2 C, legend). When P. pastoris cells are labeled with the antibody against S. cerevisiae Sec7p, two to six spots are again detected (Fig. 2 C, red), presumably because this antibody reacts with the P. pastoris homologue of Sec7p. By con-
Table II. Overlap between Golgi Markers as Visualized by Immunofluorescence

| Marker protein | Second marker | Spots that colocalize | Spots that fail to colocalize | Percent overlap |
|---------------|--------------|----------------------|-------------------------------|-----------------|
| Saccharomyces cerevisiae (DBY1034/pOH) | Och1p-HA | Sec7p | 50 | 611 | 7.6 |
| | Sec7p | Och1p-HA | 50 | 437 | 10.3 |
| Pichia pastoris (PPY12-OH) | Och1p-HA | Sec7p | 215 | 12 | 94.7 |
| | Sec7p | Och1p-HA | 215 | 18 | 92.3 |

Yeast cells expressing Och1p-HA were processed for double-label immunofluorescence, and overlap between Och1p-HA and Sec7p was quantified. “Colocalization” means that a spot labeled for the marker protein was also labeled for the second marker. 50 cells of each species were examined, with budded cells being counted as one. Based on the fraction of the cell area occupied by fluorescent spots, the probability that a given spot would overlap by chance with a spot representing the second marker was estimated at ~8.9% for S. cerevisiae and ~8.3% for P. pastoris. With P. pastoris, a small percentage of the fluorescent spots are labeled for only one of the two Golgi markers; it is unclear whether this phenomenon is meaningful or whether it reflects a limitation of the immunofluorescence method.

Golgi Organization in P. pastoris Is Unaffected by Microtubule Disruption or Cell Cycle Progression

P. pastoris resembles vertebrate cells in having stacked Golgi cisternae. We tested whether the two cell types are also similar with regard to Golgi dynamics. Pre-Golgi elements in vertebrate cells are transported along microtubules (Lippincott-Schwartz, 1998), but our electron microscopy data indicate that in P. pastoris, microtubules do not influence Golgi structure (see Fig. 1). This conclusion was confirmed at the immunofluorescence level. In untreated P. pastoris cells, microtubules are present and nuclei partition into daughter cells during mitosis (Fig. 4 A). Microtubule distribution shows no detectable relationship to Golgi distribution (Fig. 4 A). Nocodazole treatment depolymerizes microtubules and blocks nuclear migration, but the Golgi staining pattern is unaffected (Fig. 4 B). Moreover, the Golgi markers Sec7p and Och1p-HA still colocalize in nocodazole-treated P. pastoris cells (not shown).

To determine whether cell cycle progression alters Golgi organization in P. pastoris, as it does in vertebrate cells (Rabouille and Warren, 1997), we analyzed P. pastoris in the G1, S/G2, and M phases of the cell cycle. Immunofluorescence was used to quantify the average number of Golgi structures per cell. This number is similar in each phase of the cell cycle (Table IV). Thus, when observing P. pastoris under a variety of conditions, we consistently find that each cell contains a small number of distinct Golgi stacks.

COPII Coat Proteins Show Different Distributions in S. cerevisiae and P. pastoris

The electron microscopy data suggest that P. pastoris contains...
contains discrete tER sites, whereas S. cerevisiae lacks such sites. To explore this idea further, we compared the localizations of COPII proteins in the two yeasts. GFP was fused to the COOH terminus of S. cerevisiae Sec13p, a coat protein that is incorporated at a late stage of COPII vesicle assembly (Pryer et al., 1993; Kuehn and Schekman, 1997). Replacement of the endogenous SEC13 gene with the SEC13-GFP gene has no detectable effect on cell growth, indicating that the Sec13p-GFP fusion can perform the essential function of Sec13p (Pryer et al., 1993). When S. cerevisiae cells expressing Sec13p-GFP are viewed by fluorescence microscopy, each cell contains ~30–50 tiny spots (shown in Fig. 5A, although this pattern is difficult to photograph accurately). The spots are distributed almost evenly throughout the cytoplasm, with some cells showing an apparent concentration of spots on the nuclear envelope. We surmise that these spots represent individual COPII vesicles. A vesicle is smaller than the resolution limit of light microscopy (Lacey, 1989), so the apparent size of the spots is probably misleading, but a single COPII vesicle contains many copies of Sec13p-GFP and hence should produce a detectable fluorescence signal. GFP was also fused to three other S. cerevisiae COPII coat proteins (Kuehn and Schekman, 1997): Sec23p, Sec24p, and Sec31p. All of these fusions are functional, and they all give the same fluorescence pattern as Sec13p-GFP (Fig. 5B and data not shown), indicating that we have visualized the normal distribution of COPII vesicles. These results strongly support the notion that COPII vesicles bud from the entire ER in S. cerevisiae.

In parallel, the P. pastoris SEC13 gene was replaced with a SEC13-GFP fusion gene. The resulting fluorescence pattern is strikingly different from that seen in S. cerevisiae. Sec13p-GFP in P. pastoris is concentrated in only two to six large spots per cell (Fig. 6A). When examined by immunofluorescence microscopy, the Sec13p-GFP spots are adjacent to, but not quite overlapping, the Golgi

| Phase of cell cycle | Average number of Och1p-HA spots per cell |
|--------------------|------------------------------------------|
| G1                 | 3.8 ± 1.2                                |
| S/G2               | 4.1 ± 0.9                                |
| M                  | 3.9 ± 1.3                                |

An unsynchronized culture of P. pastoris strain PYP12-OH was processed for immunofluorescence as in Fig. 2C. Individual cells were assigned to a phase of the cell cycle based on morphology and nuclear distribution (Lew et al., 1997). 50 cells were examined for each phase of the cell cycle to determine the average number of Och1p-HA spots. The numbers listed refer to nucleated cells; buds lacking nuclei were excluded from the analysis. Standard deviations are indicated.
spots marked by the anti–Sec7p antibody (Fig. 6 B). This result suggests that Sec13p-GFP is localized to tER sites. Indeed, immunoelectron microscopy with an anti–GFP antibody revealed that Sec13p-GFP is present on tubulovesicular structures at the interface between ER membranes and Golgi stacks (Fig. 7; Table III). We conclude that COPII vesicle budding is restricted to discrete tER sites in P. pastoris.

**Sec12p Localizes to tER Sites in P. pastoris**

The earliest known player in the COPII assembly pathway is Sec12p, a membrane-bound guanine nucleotide exchange factor that recruits the small GTPase Sar1p to the ER membrane (Nakano et al., 1988; Nakano and Muramatsu, 1989; Barlowe and Schekman, 1993; Kuehn and Schekman, 1997). In previous studies of *S. cerevisiae*, Sec12p exhibited general ER staining (Nishikawa and Nakano, 1991, 1993). Our results confirm those earlier findings. *S. cerevisiae* Sec12p has traditionally been visualized in strains overexpressing this protein (Nishikawa and Nakano, 1993; Nishikawa et al., 1994). To eliminate possible ambiguities resulting from overexpression, we replaced the chromosomal *SEC12* gene with a myc-tagged version. Once again the fluorescence signal is weak, but the pattern is clearly visible. In this case, the anti–myc antibody does not give a general ER staining, but instead labels several spots per cell (Fig. 8 B). Sec12p-myc colocalizes with Pdi1p (Fig. 8 A, red) in the nuclear envelope and in peripheral ER membranes. Although Sec12p-myc sometimes shows a discontinuous staining pattern, the fluorescence signal is relatively weak, and we have observed that the ER network often appears discontinuous when it is weakly stained (not shown). Hence, the combined data suggest that Sec12p-myc is present throughout the ER in *S. cerevisiae*.

We also replaced *P. pastoris* SEC12 with a myc-tagged version. Once again the fluorescence signal is weak, but the pattern is clearly visible. In this case, the anti–myc antibody does not give a general ER staining, but instead labels several spots per cell (Fig. 8 B). Like Sec13p-GFP (see above), Sec12p-myc localizes to sites that are immediately adjacent to Sec7p-containing Golgi structures (Fig. 8 B). Thus, in *P. pastoris*, components at both early and late stages of the COPII assembly pathway are concentrated at tER sites.
Discussion

Why is the Golgi apparatus more photogenic in P. pastoris than in S. cerevisiae? These two yeasts are morphologically very similar; yet Golgi cisternae in P. pastoris are organized into stacks, whereas Golgi cisternae in S. cerevisiae are scattered throughout the cytoplasm. We propose the following hypothesis. In P. pastoris, COPII vesicles bud from fixed tER sites, and then fuse with one another to create new Golgi cisternae, which mature to yield polarized stacks (Glick and Malhotra, 1998). In S. cerevisiae, COPII vesicles bud throughout the ER, and therefore each Golgi cisterna forms at a different location (Fig. 9).

Our immunofluorescence data confirm that S. cerevisiae and P. pastoris have fundamentally different Golgi structures. A hallmark of the Golgi in S. cerevisiae is that various marker proteins often show distinct punctate distributions (Antebi and Fink, 1992; Chapman and Munro, 1994; Lussier et al., 1995). For example, the early Golgi protein Och1p-HA exhibits virtually no overlap with the late Golgi protein Sec7p. Although multilamellar Golgi structures are seen in temperature-sensitive sec7 and sec14 mutants of S. cerevisiae (Novick et al., 1980; Svodoba and Nécas, 1987; Rambourg et al., 1993, 1996), we find that early and late Golgi markers still do not colocalize in sec14 mutant cells (Fig. 2), indicating that S. cerevisiae cannot make coherent Golgi stacks. With P. pastoris, on the other hand, Och1p-HA and Sec7p overlap almost completely (Fig. 2), as expected if each Golgi stack represents an ordered set of early, middle, and late cisternae.

Does this difference in Golgi structure correlate with a difference in ER organization? The ER of both yeasts comprises the nuclear envelope plus peripheral elements. However, in P. pastoris, vesicles can often be seen budding specifically from regions of the ER adjacent to Golgi stacks (Fig. 1) (Gould et al., 1992; Glick, 1996). Such vesiculating ER regions have not been seen in S. cerevisiae (Kuehn and Schekman, 1997). These observations suggested to us that P. pastoris contains discrete tER sites, whereas S. cerevisiae does not. To test this interpretation, we used COPII coat proteins as markers for the tER (Orci...
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How is Sec13p recruited to tER sites in P. pastoris? The assembly of COPII vesicles is an ordered process that begins with the action of Sec12p (Barlowe and Schekman, 1993; Kuehn and Schekman, 1997). We compared the localization of epitope-tagged Sec12p in the two yeasts. Consistent with previous reports (Nishikawa and Nakano, 1993; Nishikawa et al., 1994), Sec12p-myc in S. cerevisiae is distributed throughout the ER. In P. pastoris, on the other hand, Sec12p-myc is concentrated at tER sites (Fig. 8). This observation suggests a working model for the organization of tER and Golgi compartments in the two yeasts. We postulate that in P. pastoris, Sec12p is anchored at tER sites by unknown partner proteins that comprise a “tER scaffold.” Because Sec12p initiates the assembly of COPII vesicles, these vesicles bud exclusively from tER sites, and successive Golgi cisternae form at fixed positions along cytoskeletal tracks. Consistent with this idea, microtubule-depolymerizing agents, entire Golgi stacks are lost when microtubules have been disrupted (B.J. Bevis, unpublished observations). The situation is quite different in S. cerevisiae—this yeast apparently lacks a tER scaffold, so Sec12p is free to diffuse throughout the ER. As a result, COPII vesicles bud from the entire ER, and successive Golgi cisternae form at different locations, yielding a dispersed organelle (Fig. 9). In this view, Golgi structure and positioning are strongly influenced by ER organization.

An important test of our model will be to alter tER organization in P. pastoris and ask whether Golgi structure is correspondingly affected. Such an experiment will reveal whether the existence of Golgi stacks in P. pastoris is due solely to the presence of fixed tER sites. The simplest view is that Golgi stacking is a kinetic phenomenon, with cisternal formation and maturation occurring too quickly for successive cisternae to diffuse away from one another. Alternatively, Golgi cisternae in P. pastoris might be held together by a cytosolic matrix (Mollenhauer and Morré, 1978; Cleton and Brown, 1992; Staeelin and Moore, 1995; Barr et al., 1997). These possibilities can be distinguished by generating P. pastoris mutants in which COPII proteins are delocalized, and then asking whether the loss of tER sites leads to Golgi dispersal. One promising approach focuses on determining whether P. pastoris Sec12p contains a tER localization signal that is recognized by specific partner proteins.

Although S. cerevisiae lacks discrete tER sites, this yeast can be used to explore the more general question of whether the Golgi is an outgrowth of the ER. Early in S-phase of the S. cerevisiae cell cycle, the small buds invariably contain both ER and Golgi structures (Segev et al., 1988; Redding et al., 1991; Preuss et al., 1991, 1992; O.W. Rossanese, unpublished observations). It is likely that the ER elements present in the emerging bud give rise to new Golgi cisternae. If so, mutants that fail to transport ER membranes into the bud should also lack Golgi cisternae in the bud. We are currently testing this prediction by characterizing S. cerevisiae mutants defective in Golgi inheritance.

A comparison of budding yeasts with other eukaryotes can indicate which aspects of the tER-Golgi system are cell type-specific. First, unlike many eukaryotes, S. cerevisiae contains neither stacked Golgi organelles nor discrete tER sites. It is unclear whether the absence of these structures in S. cerevisiae is adaptive, or whether it reflects a loss-of-function mutation during the evolution of this yeast. Second, tubular connections between Golgi stacks are present in vertebrate cells (Mironov et al., 1997), but have not been detected in P. pastoris, the fission yeast Schizosaccharomyces pombe (Chappell and Warren, 1989) or certain insect cells (Stanley et al., 1997). Third, the Golgi breaks down during mitosis in vertebrate cells (Rabouille and Warren, 1997; Acharaya et al., 1998), but not in higher plants (Drionich and Staeelin, 1997) or budding yeasts (Table IV) (Makarow, 1988).

Which aspects of the tER-Golgi system are universal? We propose that Golgi cisternae always form by the coalescence of tER-derived membranes. This relationship is particularly evident in cells that contain Golgi stacks immediately adjacent to tER sites (Whaley, 1975; Fairquhar and Palade, 1981; B Racker et al., 1996). We have now documented such an association in P. pastoris. A close apposition between tER sites and Golgi stacks is probably a general feature of cells that do not transport Golgi elements along cytoskeletal tracks. Consistent with this idea, microtubules have no influence on Golgi structure or positioning in P. pastoris (Figs. 1 and 4). However, in some cell types, nascent Golgi elements are transported away from tER sites, thereby obscuring the tER-Golgi connection. For example, in S. pombe, microtubules play a role in cisternal stacking and possibly in Golgi movement (Ayscough et al., 1993). In higher plants, Golgi structures are transported along actin filaments (Driouch and Staeelin, 1997; Boeving et al., 1998). The best-studied example of cytoskeleton-mediated Golgi movement is provided by vertebrate cells, which employ microtubules and associated motor proteins to generate a juxtanuclear Golgi ribbon (Lippincott-Schwartz, 1998; Burkhart, 1998). As a consequence, tER sites and Golgi elements normally do not colocalize in vertebrate cells. Yet recent evidence suggests that nascent Golgi structures in vertebrate cells initially coalesce at tER sites (Presley et al., 1997; Scales et al., 1997; Rowe et al., 1998); in the presence of microtubule-depolymerizing agents, entire Golgi stacks are found next to tER sites (Cole et al., 1996; Storrie et al., 1998). Thus, when microtubules have been disrupted, the tER-Golgi system in a vertebrate cell resembles the tER-Golgi system in P. pastoris. It seems that in all eukaryotes, the Golgi can be viewed as a dynamic outgrowth of the tER.
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