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

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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)[1] 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 (Schnepf, 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

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1. *Abbreviations used in this paper:* DIC, differential interference contrast; GFP, green fluorescent protein; HA, hemagglutinin epitope; tER, transitional ER.

of Golgi-derived transport vesicles (Glick and Malhotra, 1998; Pelham, 1998).

Can the cisternal maturation model help explain why Golgi structure varies so dramatically between cell types (Mollenhauer and Morré, 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 DBY1034 and derivatives thereof. DBY1034 was transformed with plasmid pOH to obtain expression of Och1p-HA. Strain DBY1034-S13G, in which the endogenous *SEC13* gene has been replaced with *SEC13-GFP*, was constructed as follows. The *URA3* cassette from pUC1318-URA3 (Bénédetti 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 HincII-HindIII fragment spanning the 3′ portion 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 QuikChange kit (Stratagene Inc.) to replace the *SEC13* stop codon with a SnaBI site. The *EGFP* gene was excised from pEGFP-1 (Clontech) with BamHI and NotI, blunted, and inserted into this SnaBI 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 DBY1034-S13G. Strain DBY1034-S23G, in which the endogenous *SEC23* gene has been replaced with *SEC23-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 *SEC23*. Similar strategies were used to replace the endogenous *SEC24* and *SEC31* genes with *EGFP* fusion genes. To construct strain DBY1034-S12m, in which the endogenous *SEC12* gene has been replaced with *SEC12-myc*, a 1,685-bp HincII-XbaI fragment spanning the 3′ portion of *SEC12* was inserted into pUC19-URA3; a c-myc epitope sequence was then inserted before the stop codon using the QuikChange kit, and the resulting construct was linearized with SspI for "pop-in" integration into the *SEC12* locus.

Experiments with *P. pastoris* were carried out using the prototrophic wild-type strain PPY1 or the isogenic *his4 arg4* auxotroph PPY12 and derivatives thereof (Table I). General methods for growth and transformation of *P. pastoris* have been described elsewhere (Sears et al., 1998). Strain PPY12-OH, which expresses Och1p-HA, was constructed as follows. A modified gene encoding tagged Och1p (Chapman and Munro, 1994) was excised from plasmid pOCHFT (a gift of Sean Munro, Medical Research Council, Cambridge, UK) by digesting at an upstream HindIII site (sequence including the start codon: AAGCTTAGAGATCATG), blunting, and digesting at an XbaI site immediately after the stop codon. This fragment was subcloned into pIB2 (Sears et al., 1998) that had been digested with SmaI and SpeI. The resulting plasmid was digested with BstEII and PstI, and the corresponding BstEII-PstI fragment from pOH was inserted to create pIB2-OH, in which a gene encoding triple-hemagglutinin epitope (HA)–tagged Och1p is downstream of the strong constitutive *GAP* promoter. pIB2-OH was linearized with SalI and integrated into the *his4* locus of PPY12. Strain PPY12-S13G, in which the endogenous *SEC13* gene has been replaced with *SEC13-GFP*, was constructed as follows. pSG464 (Gould et al., 1992) was digested with SnaBI and NdeI, blunted, and religated to yield pUC19-ARG4. This plasmid lacks the *PARS2* 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 and inserted into pUC19-ARG4 that had been cut with HindIII and PstI. This plasmid was mutagenized to replace the *SEC13* stop codon with a SnaBI site, and the *EGFP* gene was inserted into this site as described above. The resulting construct was linearized at the unique MscI 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-S12m: 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* Mnt1p (Chapman and Munro, 1994) in *P. pastoris*, the gene encoding tagged Mnt1p was excised from pYMT1BS (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 pAFB584, which encodes glutathione *S*-transferase fused to the NH_2-terminal acidic domain of *S. cerevisiae* Sec7p (up to the NaeI site in the coding sequence), was provided by Alex Franzusoff (University of Colorado, Denver, CO).

Nocodazole Treatment

P. pastoris cultures were grown overnight at 30°C to an OD_{600} 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

Anti–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 Mannheim 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–β-tubulin monoclonal antibody (KMX-1; Boehringer Mannheim Biochemicals) was used at 1 μg/ml. The following rabbit polyclonal antibodies were used. Anti–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. Alex Franzusoff generously provided an antiserum raised against a fusion between β-galactosidase and the NH_2-terminal portion of Sec7p (Franzusoff et al., 1991); this antibody was used at a dilution of 1:500. Oregon 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 Robert Freedman, University of Kent at Canterbury, UK) 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.

Many rabbit antisera contain traces of reactivity against α-1,6-mannose linkages, and because *S. cerevisiae* and *P. pastoris* both add α-1,6-mannose residues to glycoproteins transiting through the Golgi (Orlean, 1997; Trimble et al., 1991), these contaminating antibodies give a spurious labeling of Golgi structures (not shown). With *S. cerevisiae*, anti–α-1,6-mannose antibodies label Golgi cisternae in immunoelectron microscopy experiments (Preuss et al., 1992); and when used at very high dilutions for

immunofluorescence, such antibodies primarily label early Golgi elements. Similarly, with *P. pastoris*, anti–α-1,6-mannose antibodies give a strong and relatively specific Golgi labeling. 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 bottle-top vacuum filter, and 40 ml of ice-cold 50 mM KP_i, pH 6.8, 1 mM $MgCl_2$, 2% glutaraldehyde was added rapidly with swirling. After fixation for 1 h on ice, the cells were washed repeatedly, and then resuspended in 0.75 ml 4% $KMnO_4$ and mixed for 1 h at room temperature. The cells were washed, and then resuspended in 0.75 ml 2% uranyl acetate and mixed for 1 h at room temperature. Finally, the cells were embedded in Spurrs resin; 50 ml of yeast culture yielded enough cells for three BEEM capsules. The resin was polymerized for 2 d at 68°C. Sections were stained with uranyl acetate and lead citrate, and viewed on an electron microscope (100 CXII; JEOL U.S.A. Inc.).

For immunoelectron microscopy, we used a modification of previously published methods (Kärgel et al., 1996; Rieder et al., 1996). A 50-ml log-phase culture was concentrated to a volume of <5 ml (see above) and fixed by adding 40 ml of 50 mM KP_i, pH 6.8, 1 mM $MgCl_2$, 4% formaldehyde, 0.1% glutaraldehyde (aldehydes were EM grade from Ted Pella, Redding, CA). After 1 h at room temperature, the cells were washed twice with 20 ml PBS, pH 7.4, containing 0.5% 2-mercaptoethanol, 0.5 mM *o*-phenanthroline, 0.25 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and then once more with 1 ml of the same solution. Treatment with periodate was omitted because this compound may destroy certain antigens by reacting with amino acid side chains (Clamp and Hough, 1965). 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°C. Thawed cryosections were prepared and labeled as described (Griffiths, 1993; Kärgel 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 Burkhardt, University of Chicago, Chicago, IL), 1:25 for an affinity-purified polyclonal anti–GFP antibody (a gift of Charles Zuker, University of California, San Diego, San Diego, CA), and 1:50 for protein A-gold (10 nm; Goldmark Biologicals). After 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 (Adobe Systems Inc.) to adjust brightness and contrast and to create 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 (Codonics).

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; Hell 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 or selective medium with good aeration to an OD_{600} of 0.25–1.0. 8 ml of this culture is pipetted onto a 150-ml bottle-top filter, and the liquid is removed by vacuum. The cells are immediately resuspended in 5 ml of freshly prepared 50 mM KP_i, pH 6.5, 1 mM $MgCl_2$, 4% formaldehyde (EM grade; Ted Pella). After fixation for 2 h at room temperature in a 15-ml tube (Falcon Labware), the cells were centrifuged for 3 min at 1,000 *g* (2,000 rpm) in a tabletop centrifuge, and the supernatant is aspirated completely with a Pasteur pipet. The cells are resuspended in 5 ml of freshly prepared wash buffer (100 mM KP_i, pH 7.5, 1 mM $MgCl_2$, 0.25 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin), and then centrifuged again as above. Finally, the cells are resuspended in wash buffer to an OD_{600} of 10. To 100 μl of concentrated cell suspension is added 0.6 μl of 2-mercaptoethanol followed by 20 μl of recombinant yeast lytic enzyme (20,000 U/ml; ICN Biomedicals Inc.). After mixing end-over-end for 15–30 min at room temperature, the spheroplasts are centrifuged for 2 min at 400 *g* (2,000 rpm) in a microfuge, gently resuspended in 100 μl wash buffer, and then centrifuged and resuspended once again.

Adhesion and Permeabilization. To create "wells" on a coverslip, a suitable piece is cut from the adhesive backing of an express courier document pouch, holes are made with a rotating-head hole punch, and the perforated plastic is attached to the coverslip. 10 μl of 0.1% polylysine (P-1524; Sigma Chemical Co.) is added to each well, and then removed by vacuum aspiration. Each well is washed three times with water, and 10 μl of spheroplast suspension is added. After 3 min, excess liquid is blotted off with a cotton swab, and the dried coverslip is immersed in 40 ml of acetone precooled to −20°C in a 50-ml tube (Falcon Labware). (This organic solvent fixation step is important for preserving tER and Golgi structure. For some antigens methanol works better, but in most cases acetone is superior.) After 5 min, the coverslip is removed, inverted onto a paper towel to blot off excess solvent, and allowed to dry.

Antibody Incubations. Each well is precoated for 30 min with a drop of PBS-Block (PBS, pH 7.4, 1% dried milk, 0.1% bovine serum albumin, 0.1% octyl glucoside). 10 μl of primary antibody mixture in PBS block is then added, and the coverslip is incubated in a humid chamber for 1 h. Each well is washed eight times with PBS block, and 10 μl of secondary antibody mixture in PBS block is added. 2 μg/ml 33258 (Hoechst AG) is included in the secondary antibody solution to stain DNA. After a 30-min incubation in the dark, each well is washed eight times with PBS block. The liquid is aspirated completely after the final wash. Each well then receives 5 μl of phenylenediamine-containing mounting medium (Pringle et al., 1991). The coverslip is inverted onto a slide and sealed with nail polish.

Microscopy. Samples were viewed on an Axioplan microscope equipped with a 100× Plan-Apo 1.4 NA objective (Carl Zeiss, Inc.) and with band-pass filters for visualizing Hoechst dye, fluorescein/Oregon green, and Texas red. Images were captured with a Photometrics CCD camera using the Openlab software package (Improvision). For each field of cells, multiple images were recorded from focal planes spaced 0.2 μM apart; three to four of these images were deblurred using a simple deconvolution algorithm, and then combined to form a single projected image. Where indicated, the same cells were also photographed in differential interference contrast (DIC) mode at a single focal plane. Photoshop was used for colorization, adjustments of brightness and contrast, and creation of composite and merged images. Manipulations with the Openlab and Photoshop programs improved the quality of the images, but did not significantly alter their information content. Fluorescence and DIC images were printed on a Stylus Photo color inkjet printer (Epson Electronics America Inc.).

To quantify the overlap between Och1p-HA and Sec7p, digital images were examined with Photoshop. Cells that showed clear staining with both antibodies were used for quantitation. A spot of one color was scored as overlapping with a spot of the second color if the center of the first spot was contained within the second spot. To estimate the percentage of Golgi structures that are perinuclear in *P. pastoris*, the perinuclear and peripheral Och1p-HA spots were counted in 50 cells that showed faint nuclear envelope staining (see Fig. 2 C). To estimate the average percentage of the cell area occupied by fluorescent spots of a given marker protein, 25 cells stained to reveal Och1p-HA and Sec7p were analyzed using NIH Image (http://rsb.info.nih.gov/nih-image/): the total areas of the Och1p-HA and Sec7p spots were summed and divided by twice the total areas of the cells.

Visualization of GFP Fusion Proteins in Intact Cells

Yeast cells expressing a GFP-labeled COPII protein were grown to log phase with good aeration. A 400-μl aliquot of the culture was then mixed rapidly with 400 μl of 100 mM KP_i, pH 6.5, 2 mM $MgCl_2$, 8% formaldehyde, 0.5% glutaraldehyde. After 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 were 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,

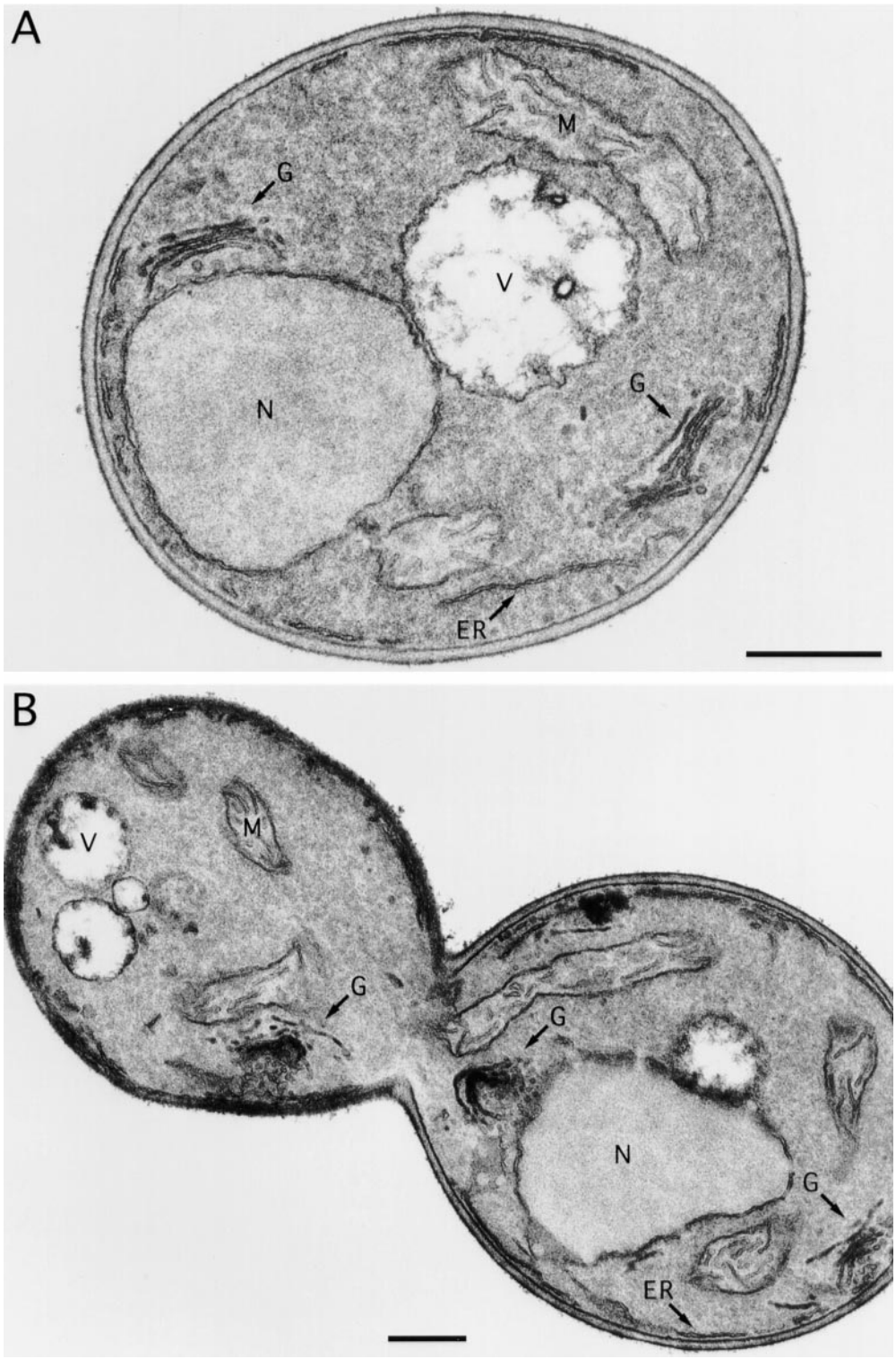

Figure 1. Thin-section electron microscopy of Golgi and ER structures in *P. pastoris.* (A) A representative *P. pastoris* cell. Golgi stacks are found adjacent to ER membranes, including both the nuclear envelope and peripheral ER elements. (B) A representative *P. pastoris* cell after treatment with 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.

1998). Such a role for microtubules is excluded by the following two observations.

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). As 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) (Gould 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

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

Marker proteins		
Pdi1p	Lumenal ER protein. General ER marker.	
Sec12p	ER membrane protein. Initiates the COPII assembly pathway.	
Sec13p, Sec23p, Sec24p, Sec31p	COPII coat proteins. Incorporated at a late stage of coat formation.	
Och1p	Golgi membrane protein. Early Golgi marker.	
Sec7p	Peripherally associated Golgi protein. Late Golgi marker.*	
S. cerevisiae strains and plasmids		
Strains		
DBY1034	*MATa his4 lys2 ura3*	Segev and Botstein, 1987
DBY1034-S13G	DBY1034 *SEC13-GFP*	This study
DBY1034-S23G	DBY1034 *SEC23-GFP*	This study
DBY1034-S24G	DBY1034 *SEC24-GFP*	This study
DBY1034-S31G	DBY1034 *SEC31-GFP*	This study
DBY1034-S12m	DBY1034 *SEC12-myc*	This study
CTY214	*MATa ura3 ade2 leu2 his4 sec14-1^{ts}*	Vytas Bankaitis‡
Plasmids		
pOH	OCH1-HA URA3 CEN6	Harris and Waters, 1996
P. pastoris strains and plasmids		
Strains		
PPY1	Prototrophic	Gould et al., 1992
PPY12	*his4 arg4*	Gould et al., 1992
PPY12-OH	PPY12 OCH1-HA HIS4	This study
PPY12-S13G	PPY12 SEC13-GFP ARG4	This study
PPY12-S12m	PPY12 SEC12-*myc* ARG4	This study
Plasmids		
pOW3-FLMNT1	MNT1-*myc* ARG4 PARS2	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).

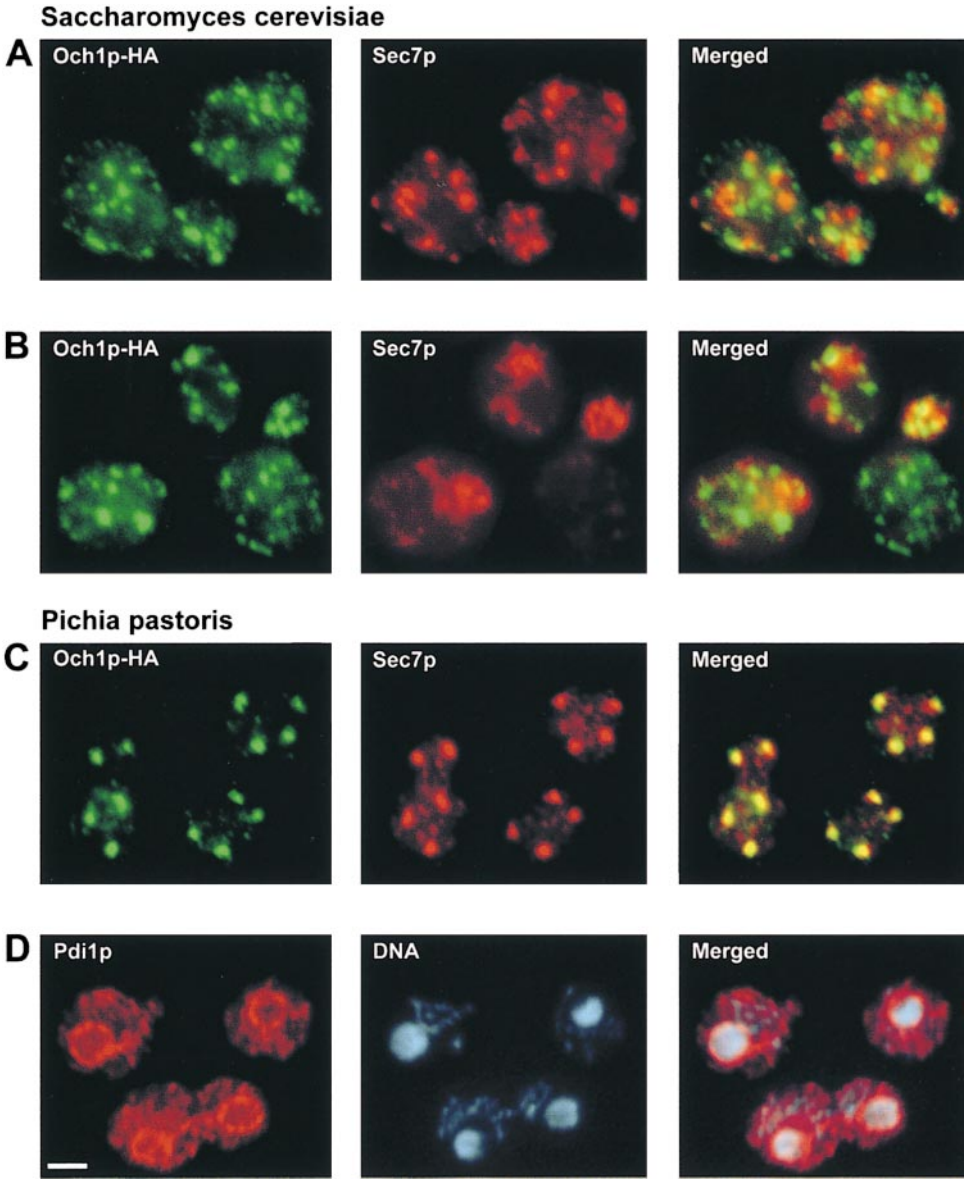

Figure 2. Immunofluorescence staining of Golgi and ER structures. (A) Golgi labeling in *S. cerevisiae*. Fixed cells of strain DBY1034/pOH were incubated with a monoclonal anti–HA antibody to label Och1p-HA, followed by Oregon green–conjugated anti–mouse antibody (green). The same cells were also incubated with a polyclonal antibody against Sec7p, followed by Texas red–conjugated anti–rabbit antibody (red). The merged image shows very little overlap between the two Golgi markers. (B) Golgi labeling in a *sec14* mutant of *S. cerevisiae*. Strain CTY214 was grown at the permissive temperature of 23°C, and then shifted to the nonpermissive temperature of 37°C for 1 h before fixation. Antibody labeling was as in A. After the temperature shift, Sec7p-containing structures reorganize into large clusters, but the staining pattern of Och1p-HA is not significantly altered. Surprisingly, as illustrated by the cell on the right, budded *sec14* cells often partition the Sec7p clusters into the bud. (C) Golgi labeling in *P. pastoris*. Fixed cells of strain PPY12-OH were incubated with the same primary and secondary antibodies as in A. Och1p-HA staining overlaps strongly with Sec7p staining, as shown in the merged image. As in some strains of *S. cerevisiae* (O.W. Rossanese, unpublished observations), Och1p-HA often gives a faint staining of the general ER in *P. pastoris*; for example, the cell on the lower left shows weak nuclear envelope staining, which reveals that two of the three labeled Golgi spots adjoin the nucleus. (D) General ER labeling in *P. pastoris*. Fixed cells of strain PPY12 were incubated with a polyclonal antibody against *S. cerevisiae* Pdi1p, followed by Texas red–conjugated anti–rabbit antibody (red). The same cells were also incubated with Hoechst dye to stain DNA (blue). As shown in the merged image, Pdi1p is present in the nuclear envelope and peripheral ER structures. Bar, 2 μm.

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). As predicted from electron microscopy, 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.

trast to the situation in *S. cerevisiae*, Och1p-HA and Sec7p exhibit nearly quantitative overlap in *P. pastoris* (Fig. 2 C, Merged; Table II). Similarly, when a tagged version of the *S. cerevisiae* medial-Golgi protein Kre2p/Mnt1p (Chapman and Munro, 1994; Lussier et al., 1995) is expressed in *P. pastoris*, it colocalizes with Sec7p (not shown). Colocalization at the light microscopy level indicates that two markers are either in the same compartment or in closely apposed structures. These data support the conclusion that Golgi organelles are more coherent in *P. pastoris* than in *S. cerevisiae*.

To confirm that the structures visualized in *P. pastoris* by immunofluorescence are indeed Golgi membranes, we performed immunoelectron microscopy on thawed cryosections. Using a polyclonal anti–HA antibody to detect Och1p-HA, we see a specific labeling of Golgi stacks (Fig. 3; Table III).

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.

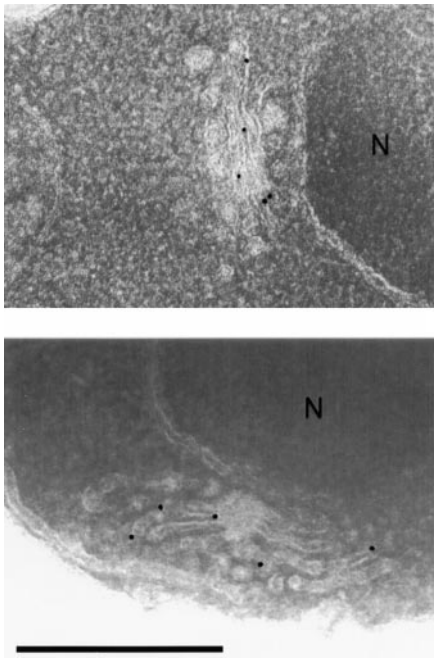

Figure 3. Immunoelectron microscopy of a Golgi marker in *P. pastoris*. *P. pastoris* cells of strain PPY12-OH were fixed and cryosectioned, and then incubated with a polyclonal anti–HA antibody followed by protein A-gold to detect Och1p-HA. Gold particles are consistently observed over Golgi stacks. In cells of the parental PPY12 strain, which does not express Och1p-HA, no specific labeling is seen with the anti–HA antibody (not shown). Bar, 0.5 μm. N, nuclei.

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

The electron microscopy data suggest that *P. pastoris* con-

Table III. Quantitation of Immunoelectron Microscopy Data for P. pastoris

Compartment	Total number of gold particles	Average number of gold particles
		per μm²
Och1p-HA labeling		
General ER	0	0.0
Transitional ER	2	1.6
Golgi	28	16.3
Sec13p-GFP labeling		
General ER	6	1.3
Transitional ER	63	47.0
Golgi	3	1.4

Data are from the experiments described in Figs. 3 (for Och1p-HA) and 7 (for Sec13p-GFP). In each case, 20 cells that showed good membrane preservation were photographed without regard to the labeling pattern. Stereology was then performed using the method of Griffiths (1993). The anti–HA and anti–GFP antibodies both gave negligible labeling of nuclei, vacuoles, and mitochondria.

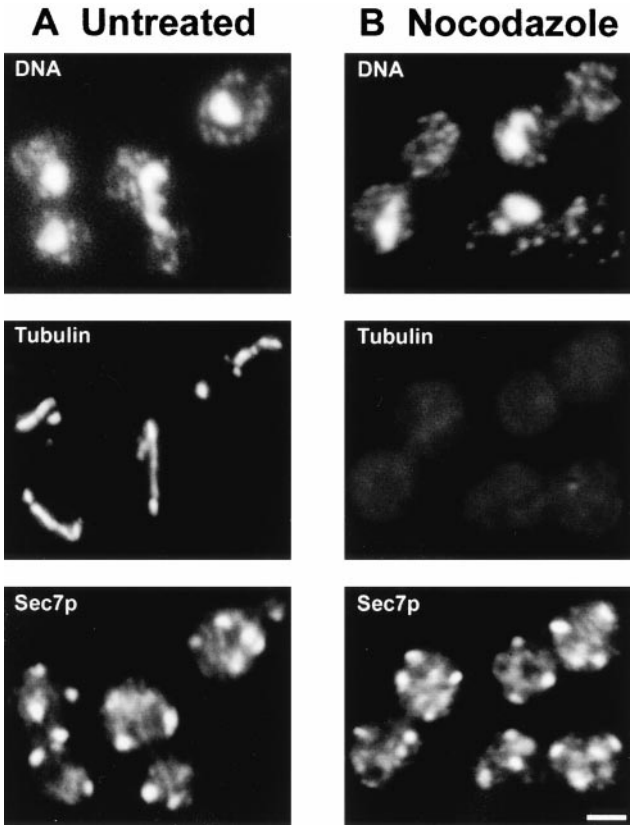

Figure 4. Immunofluorescence analysis of nocodazole-treated *P. pastoris* cells. (A) DNA, tubulin, and Sec7p distributions in untreated *P. pastoris* cells. Fixed cells of strain PPY12 were incubated with Hoechst dye to stain DNA. Microtubules were visualized with a monoclonal anti–tubulin antibody followed by Oregon green–conjugated anti–mouse antibody. The Golgi marker Sec7p was visualized as in Fig. 2. (B) DNA, tubulin, and Sec7p distributions in *P. pastoris* cells treated with nocodazole for 2.5 h. Fixed cells were labeled as in A. As previously described for *S. cerevisiae* (Jacobs et al., 1988), microtubules are virtually undetectable within 1 h after nocodazole addition (not shown). Bar, 2 μm.

tains 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. 5 A, 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. 5 B 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. 6 A). When examined by immunofluorescence microscopy, the Sec13p-GFP spots are adjacent to, but not quite overlapping, the Golgi

Table IV. The P. pastoris Golgi Retains Its Organization Throughout the Cell Cycle

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 PPY12-OH was processed for immunofluorescence as in Fig. 2 C. 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.

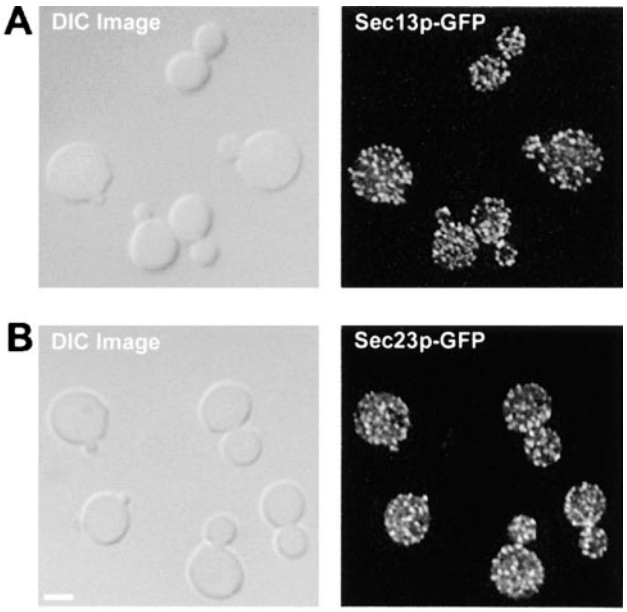

Figure 5. Visualization of GFP-labeled COPII coat proteins in intact *S. cerevisiae* cells. (A) Sec13p-GFP fluorescence in *S. cerevisiae*. Cells of strain DBY1034-S13G were fixed and viewed directly. The same cells were imaged in DIC and fluorescence modes. (B) Sec23p-GFP fluorescence in *S. cerevisiae* strain DBY1034-S23G. The experiment was performed as in A. Bar, 2 μm.

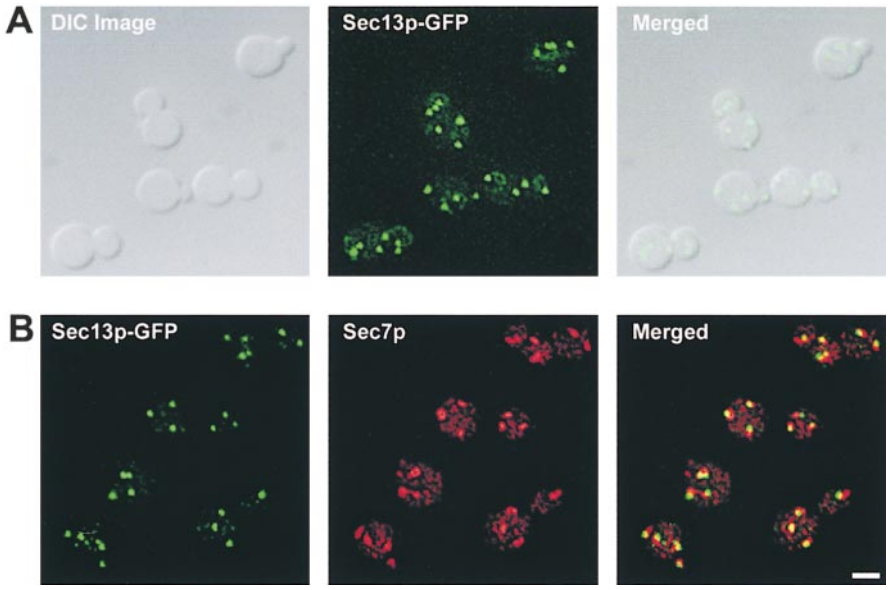

Figure 6. Visualization of Sec13p-GFP in *P. pastoris.* (A) Sec13p-GFP fluorescence in intact cells. *P. pastoris* cells of strain PPY12-S13G were fixed and viewed directly. DIC and fluorescence images were collected separately, and then combined to generate the merged image. (B) Immunofluorescence localization of Sec13p-GFP and Sec7p. Fixed *P. pastoris* cells of strain PPY12-S13G were incubated with a monoclonal anti–GFP antibody followed by Oregon green–conjugated anti–mouse antibody (green). The same cells were also stained for Sec7p (red) as in Fig. 2. The merged image shows that Sec13-GFP– and Sec7p-containing structures are closely apposed, but distinct. Bar, 2 μm.

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.*

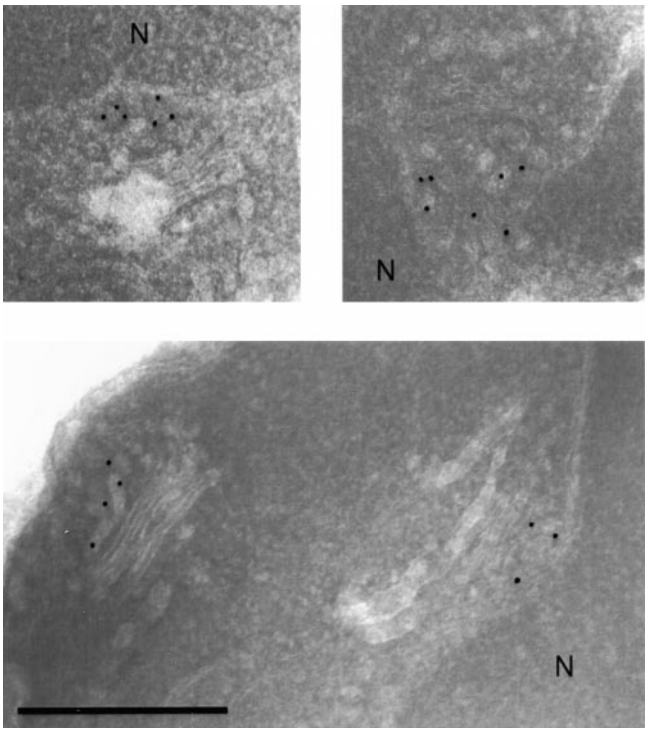

Figure 7. Immunoelectron microscopy of a tER marker in *P. pastoris. P. pastoris* cells of strain PPY12-S13G were fixed and cryosectioned. The localization of Sec13p-GFP was determined by incubating with a polyclonal anti–GFP antibody followed by protein A-gold. Gold particles are consistently observed over tER regions, which are often associated with indentations of the nuclear envelope. In cells of the parental PPY12 strain, which expresses wild-type Sec13p, no specific labeling is seen with the anti–GFP antibody (not shown). Bar, 0.5 μm. N, nuclei.

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. The resulting strain grows like the wild-type (not shown), implying that Sec12p-myc can functionally replace the essential wild-type protein (Nakano et al., 1988). Sec12p-myc (Fig. 8 A, green) 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, green). 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.

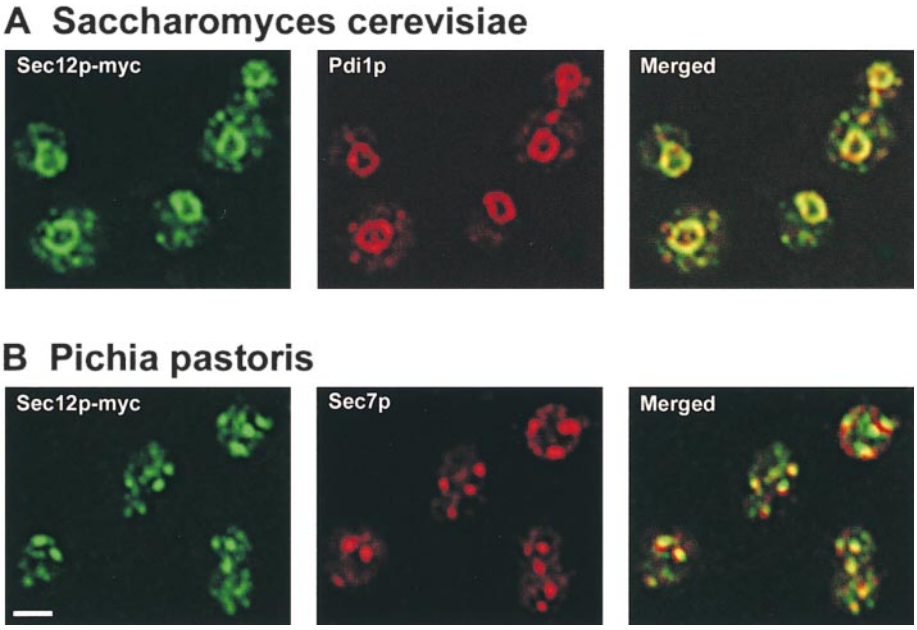

Figure 8. Immunofluorescence localization of tagged Sec12p in the two yeasts. (A) Colocalization of Sec12p-myc with Pdi1p in *S. cerevisiae*. Fixed cells of strain DBY1034-S12m were incubated with a monoclonal anti–myc antibody followed by Oregon green–conjugated anti–mouse antibody to visualize Sec12p-myc (green). The same cells were also stained for Pdi1p (red) as in Fig. 2. As shown in the merged image, the staining patterns for the two proteins largely overlap. (B) Localization of Sec12p-myc in *P. pastoris*. Fixed cells of strain PPY12-S12m were stained as in A to visualize Sec12p-myc (green); Sec7p (red) was visualized as in Fig. 2. The merged image shows that the two staining patterns are closely apposed, but distinct. Bar, 2 μm.

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 Necas, 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

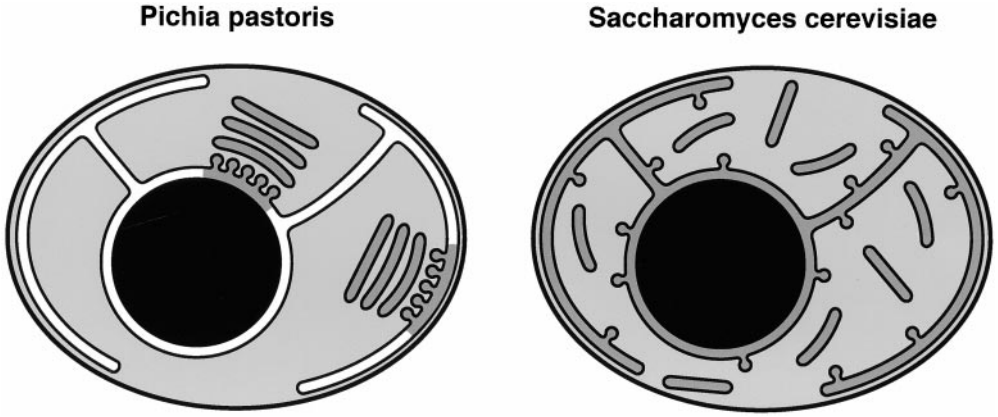

Figure 9. Summary and interpretation of the experimental data. *P. pastoris* contains discrete tER sites that produce coherent Golgi stacks. In *S. cerevisiae*, COPII vesicles bud throughout the ER network, resulting in a dispersed Golgi. The shaded portions of the ER are the regions that can function as tER. See text for details.

et al., 1991; Kuge et al., 1994; Shaywitz et al., 1995; Paccaud et al., 1996; Tang et al., 1997). With *S. cerevisiae*, fusing GFP to Sec13p or other COPII coat proteins revealed many small fluorescent spots that probably represent individual COPII vesicles (Fig. 5). These spots are found throughout the cytoplasm, consistent with the notion that COPII vesicles bud at random from the entire ER. By contrast, a Sec13p-GFP fusion in *P. pastoris* localizes to a small number of discrete regions (Fig. 6). These regions are immediately adjacent to Golgi stacks, and they contain tubulovesicular membranes (Fig. 7). Hence, we propose that Sec13p-GFP marks a compartment in *P. pastoris* that is analogous to the tER sites described previously in vertebrate cells (Palade, 1975; Bannykh and Balch, 1997).

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 locations to generate polarized stacks. This model assumes that tER sites are relatively stable entities, and, indeed, our studies of Sec13p-GFP dynamics in *P. pastoris* confirm that tER sites are long lived and slow moving (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 tER 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; Cluett and Brown, 1992; Staehelin 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; Acharya et al., 1998), but not in higher plants (Driouich and Staehelin, 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; Farquhar and Palade, 1981; Bracker 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 (Driouich and Staehelin, 1997; Boevink 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; Burkhardt, 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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