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Structure of the Yeast Endoplasmic Reticulum: Localization of ER proteins using Immunofluorescence and Immunoelectron Microscopy

DAPHNE PREUSS[1,*], JON MULHOLLAND[1,2], CHRIS A. KAISER[3], PETER ORLEAN[4,†], CHARLES ALBRIGHT[4], MARK D. ROSE[5], PHILLIPS W. ROBBINS[4] AND DAVID BOTSTEIN[1,2,‡]

[1]*Department of Genetics, Stanford University Medical Center, S-337, Stanford, CA 94305 U.S.A.*
[2]*Genentech, Inc., 450 Point San Bruno Blvd, S. San Francisco, CA 94080, U.S.A.*
[3]*Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.*
[4]*Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.*
[5]*Department of Biology, Lewis Thomas Laboratory, Princeton University, Princeton, NJ 08544, U.S.A.*

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The endoplasmic reticulum (ER) and other secretory compartments of *Saccharomyces cerevisiae* have biochemical functions that closely parallel those described in higher eukaryotic cells, yet the morphology of the yeast organelles is quite distinct. In order to associate ER functions with the corresponding cellular structures, we localized several proteins, each of which is expected to be associated with the ER on the basis of enzymatic activity, biological function, or oligosaccharide content. These marker proteins were visualized by immunofluorescence or immunoelectron microscopy, allowing definition of the *S. cerevisiae* ER structure, both in intact cells and at the ultrastructural level. Each marker protein was most abundant within the membranes that envelop the nucleus and several were also found in extensions of the ER that frequently juxtapose the plasma membrane. Double-labeling experiments were entirely consistent with the idea that the marker proteins reside within the same compartment. This analysis has permitted, for the first time, a detailed characterization of the ER morphology as yeast cells proceed through their growth and division cycles.

KEY WORDS — Yeast; *Saccharomyces cerevisae*; endoplasmic reticulum; ultrastructure; cell cycle.

INTRODUCTION

The endoplasmic reticulum (ER) consists of an extensive membranous network that appears to originate at the nuclear envelope and emanates through the cytoplasm (Porter *et al.*, 1945; Palade, 1956). The secretion process is initiated as proteins enter this organelle. Morphologically, the ER is a particularly prominent feature of cells that specialize in protein export. Observations of living cells indicate that this structure is not static, but that ER tubules undergo frequent branching and sliding movements (Lee and Chen, 1988).

Secreted proteins are first targeted to the ER by an amino-terminal signal peptide. Upon entry into the ER lumen, the signal peptide is removed, and, in at least some cases, the mature protein assumes its final, folded conformation for the first time. Those proteins that do not fold properly or fail to form correct multimeric structures are often associated with an abundant ER binding protein known as BiP (Haas and Wabl, 1983). This protein is thought to recognize and bind to specific domains of misfolded proteins or of protein subunits that do not form proper multimers, and may also serve to catalyse the folding or assembly of normal protein structures (for reviews, see Lodish, 1988; Rose and Doms, 1988; Rothman, 1989; Verner and Schatz, 1988).

In addition to its function in the secretion process, the ER is the site at which the synthesis of lipids and the assembly of lipid bilayers are initiated (reviewed in Fawcett, 1981). The ER also contains biochemical machinery devoted to the assembly of dolichol-linked oligosaccharides and to the transfer of oligosaccharide units to certain asparagine

*Present address: Department of Biochemistry, Beckman Center, B400, Stanford University, Stanford, CA 94305, U.S.A.
†Present address: Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, U.S.A.
‡To whom correspondence should be addressed. Telephone: (415) 723-3488; Telefax: (415) 723-7016.

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residues of secreted proteins (reviewed in Kornfeld and Kornfeld, 1985).

Genetic and biochemical analyses of protein secretion in *Saccharomyces cerevisiae* have established that this process parallels that found in other eukaryotic cells (Novick *et al.*, 1980, 1981; reviewed in Schekman, 1985). In fact, several components of the yeast secretory apparatus can substitute for their mammalian counterparts (for example, see Segev *et al.*, 1988; Haubruck *et al.*, 1989; Normington *et al.*, 1989; Wilson *et al.*, 1989; Clary *et al.*, 1990). Yeast proteins that play an essential role in the translocation of proteins across the ER membrane and in signal peptide cleavage have been identified (reviewed in Deshaies *et al.*, 1989) and the synthesis of core oligosaccharides proceeds by identical mechanisms in yeast and higher cells (Tanner and Lehle, 1987). *S. cerevisiae* contains a homolog of BiP, and mutations within the *KAR2* gene that encodes this protein have provided enlightening evidence that BiP plays a crucial role in the earliest stages of protein translocation (Rose *et al.*, 1989; Vogel *et al.*, 1990; Normington *et al.*, 1989).

Electron micrographs of yeast cells, like micrographs of other eukaryotic cells not specialized for secretion, typically depict few ER tubules. Nonetheless, ER structures have been observed in yeast near the nuclear membrane or along the inner surface of the plasma membrane, and connections between the nuclear envelope and peripheral tubules have been described (Matile *et al.*, 1969; van Rijn *et al.*, 1975). Such structures are dramatically altered in mutant yeast strains that are deficient in the transport of proteins from the ER to the Golgi apparatus, as judged by the accumulation of core glycosylated intermediates (Novick *et al.*, 1980; Esmon *et al.*, 1981; Novick *et al.*, 1981). These secretory (*sec*) mutant strains accumulate an extensive network of membranous tubules that wind through the cytoplasm and often juxtapose the plasma membrane. In contrast, strains that express elevated levels of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), an integral component of the ER membrane, do not have alterations in peripheral membrane structures, but instead accumulate stacks of membranes closely associated with the nuclear envelope (Wright *et al.*, 1988; Wright and Rine, 1989).

To identify unambiguously the compartments in wild-type yeast cells that perform the biochemical functions characteristic of the ER, we employed immunofluorescent and immunoelectron microscopy to localize several yeast proteins that are residents of this organelle. Initially, the subcellular location of dolichol-phosphate-mannose (Dol-P-Man) synthase, a membrane-bound ER enzyme that catalyses the formation of Dol-P-Man from Dol-P and GDP-mannose, was investigated. Dol-P-Man serves as a mannose donor for the synthesis of N-linked, core oligosaccharides, and is also required for the formation of glycosyl phosphatidylinositol membrane anchors and for O-mannosylation of proteins (Sharma *et al.*, 1974; Kornfeld and Kornfeld, 1985; Tanner and Lehle, 1987; Orlean, 1990). Immunofluorescence microscopy has been used to localize the yeast BiP homolog, the Kar2 protein, to the perinuclear region and to filamentous structures within the cytoplasm (Rose *et al.*, 1989). We compared the location of Dol-P-Man synthase to that of the Kar2 protein, and also determined the location of slowly secreted, mutant forms of invertase that receive only core oligosaccharide modifications, suggestive of their accumulation within the ER. Using each of these marker proteins, we examined the fine structure of the ER throughout the yeast cell cycle.

MATERIALS AND METHODS

Yeast strains and culture medium

Yeast strains are listed in Table 1. DBY2449 and DBY5408 were used for immunoelectron microscopy, and immunofluorescence microscopy was performed with DBY5408 (for invertase localization), and, for localization of Dol-P-Man synthase, a diploid strain (formed by crossing DBY1315 to DBY2068) was used. Localization of the Suc2-s1 protein was performed with strain DBY2449 carrying the mutant invertase gene on plasmid pRB58 (Schauer *et al.*, 1985). The *sec18* allele is in DBY2356 (obtained from R. Schekman), and wild-type invertase was introduced into this strain on plasmid pRB420 (Kaiser and Botstein, 1986).

Yeast media were prepared as described by Sherman *et al.* (1986). Minimal medium supplemented with vitamin assay casamino acids (Difco) at 0·2% was used to grow auxotrophic uracil strains containing episomes. Carbon sources were 5% glucose (for repression of invertase synthesis) or 2% lactate adjusted to pH 5·5 with potassium hydroxide (for elimination of glucose repression).

Table 1. Yeast strains

Strain	Genotype	Source
DBY954	*MATa lys2-802 suc2-533*	M. Carlson
DBY955	*MATa lys2-802 suc2-538*	M. Carlson
DBY1034	*MATa his4-539 lys2-801 ura3-52 SUC2*	D. Botstein
DBY1315	*MATα leu2-3 leu2-112 lys2-801 ura3-52*	D. Botstein
DBY2068	*MATa his4-619 leu2-3 leu2-112 ura3-52*	D. Botstein
DBY2356	*MATα leu2-3 leu2-112 ura3-52 suc2-Δ9 sec18-1*	R. Schekman
DBY2449	*MATα ade2-101 ura3-52 suc2-Δ9*	D. Botstein
DBY2617	*MATa his4-539 lys2-801 ura3-52 suc2-438*	C. Kaiser
DBY5408	*MATα ade2-101 ura3-52 suc2-Δ9 (URA3 suc2-533)*	D. Botstein
DBY5410	*MATα ade2-101-ura3-52 suc2-Δ9 (URA3 suc2-s1)*	R. Schekman

Preparation and purification of antibodies

Purified invertase was obtained from the yeast strain DBY2617, which produces non-glycosylated invertase that accumulates within the cytoplasm (Kaiser and Botstein, 1986). Purification procedures were based on the method of Goldstein and Lampen (1975), and 10 mg of purified invertase were obtained (D. Preuss, PhD Thesis, Massachusetts Institute of Technology, Cambridge, 1990). The purified, lyophilized protein was suspended at a final concentration of 300 μg/ml in 150 mM-NaCl, 50 mM-potassium phosphate buffer (pH 7·5) with 0·1% SDS, and was denatured by heating to 100°C for 5 min. Freund's adjuvant was added, and 100 μg of invertase were injected into guinea pigs. After two additional immunizations, a highly specific, concentrated serum was obtained from each of the guinea pigs.

Antiserum to Dol-P-Man synthase was produced by immunizing rabbits with a *DPM1-trpE* fusion protein that included amino acids 10–191 of the Dpm1 protein (C.F. Albright, manuscript in preparation). The immune and preimmune serum were extensively purified first by adsorption to a column containing the fusion protein. Antibodies to the trpE portion of the fusion protein were then dramatically reduced by adsorption to a column containing the trpE protein. The resulting immune serum bound tightly to the Dpm1 protein as judged by immunoblotting and immunoprecipitation. The minor proteins that were detected by these procedures reacted with the purified immune and preimmune sera and formed weak complexes with these purified antibodies.

Preparation of antibodies to the KAR2 protein has been described previously (Rose *et al.*, 1989), and the rabbit anti-β-galactosidase antibodies were from Organon Teknika (Durham, NC).

Construction of SUC2-lacZ *gene fusions*

The high copy-number plasmid pRB400, which carries the *SUC2* gene, was cleaved and digested with *Bal*31 to produce carboxy-terminal deletions of the *SUC2* gene (Kaiser and Botstein, 1986, 1990; C.A. Kaiser, PhD Thesis, Massachusetts Institute of Technology, Cambridge, 1987). The *SUC2* promoter and residual coding sequences from the digested DNA were then inserted into a high copy-number plasmid adjacent to a copy of the *Escherichia coli lacZ* gene that lacks all 5′ noncoding sequences and the first eight amino acid codons of β-galactosidase. This gave rise to a hybrid gene consisting of the regulatory sequences and amino-terminal coding sequences of the *SUC2* gene fused to the β-galactosidase coding sequence. Yeast transformants that expressed the *SUC2* and *lacZ* gene sequences fused in-frame were identified by their blue color when plated onto medium containing the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Restriction enzyme mapping of the resulting plasmids identified fusion 806 as the shortest fusion that produced active enzyme. The junctions of fusions 895, 804 and 807 were all in the signal sequence region, but none had the proper reading frame. The reading frame was restored in these plasmids by filling in the cohesive ends of a restriction site at the fusion juction, thus generating fusions 895f, 804f and 807f. The DNA sequences of each of the fusions was determined by chain-termination sequencing (Sanger *et al.*, 1977). The fusions 895f, 804f, 807f and 806 respectively contained the first 5, 19, 25 and

38 amino acids of the *SUC2* coding sequence fused to β-galactosidase.

Denaturing gels and immunoblots

Yeast cells were grown in YEP medium supplemented with 5% glucose, and the synthesis of invertase or invertase-β-galactosidase hybrids was induced by shifting exponential-phase cultures to YEP medium with 0·1% glucose for 2–3 h. Tunicamycin (Sigma) was added at 10 μg/ml during induction to inhibit glycosylation (Kuo and Lampen, 1974). Strains were grown at 30°C, except for the *sec18* strains, which were maintained at 20°C (permissive) or induced at 37°C (restrictive). 2×10^7 cells were collected and washed once with 25 mM-Tris–hydrochloride (pH 7·5), 10 mM-sodium azide, heated to 95°C for 3 min and then lysed in 20 μl of denaturing sample buffer [80 mMTris–HCl (pH 6·8), 2% SDS, 100 mM-DTT, 10% glycerol, 0·1% bromophenol blue] by vortexing with glass beads. Samples treated with endo-β-N-acetylglucosaminidase H (endo H) were diluted with 60 μl of 20 mM-sodium citrate (pH 5·5) and incubated for 12 h at 37°C in the presence of endo H at 3 μg/ml. Prior to electrophoresis, 30–80 μl of denaturing sample buffer was added, and each lysate was heated to 95°C for 3 min. Proteins were fractionated on a 7·5% gel according to the method of Laemmli (1970), and then transferred to nitrocellulose filters. Immunoblotting was performed as described previously (Kaiser and Botstein, 1986) with guinea pig anti-invertase antibodies at a 1 : 5000 dilution, or with rabbit anti-β-galactosidase antibodies (Organon Teknika) at a dilution of 1 : 50.

Protease sensitivity of invertase

An exponential culture of 2×10^9 yeast cells was shifted to medium containing 0·1% glucose for 2 h to induce invertase synthesis. Cells were harvested and washed in 25 mM-Tris–HCl (pH 7·5), 10 mM-NaN_3, and spheroplasts were formed by incubation in 3·5 ml of 1 M-sorbitol, 25 mM-Tris–HCl, 5 mM-$MgCl_2$, 40 mM-2-mercaptoethanol and 10 mg of Zymolyase 100 000 (Seikagaku Kogyo, Tokyo) for 1 h at 30°C. Spheroplasts were washed in 1·4 M-sorbitol, 25 mM-Tris–HCl (pH 7·5), and then suspended in 3·5 ml of cold 400 mM-sorbitol, 100 mM-NaCl, 10 mM-Tris–HCl (pH 7·5), 5 mM-$MgCl_2$. The spheroplasts were lysed by dounce homogenization at 4°C, and lysis was confirmed by light microscopy. Two aliquots of 200 μl of lysed spheroplasts were removed and 10 μl of either 1·4 M-sorbitol or 10% Triton X-100 were added. A 1 mg/ml stock of trypsin was added to a final concentration of 50 μg/ml and timed proteolysis reactions were performed on ice. Reactions were terminated by removing 40 μl aliquots into 4 μl of soybean trypsin inhibitor (Boehringer Mannheim Biochemicals) at a final concentration of 100 μg/ml for 5 min at 37°C. Prior to electrophoresis, 40 μl of denaturing sample buffer were added, and samples were heated to 95°C for 3 min.

Immunofluorescence microscopy

Yeast cells with high copy-number plasmids encoding either the *DPM1* gene (Orlean *et al.*, 1988), the *suc2-533* allele, or the *SUC-lacZ* fusions were grown in minimal medium supplemented with casamino acids, and for the latter strains, the *SUC2* promoter was derepressed by shifting the cells to minimal medium containing either 2% lactate or 0·1% glucose. Cells were fixed as described (Kaiser and Botstein, 1990; C.F. Albright, PhD Thesis, Massachusetts Institute of Technology, 1989) or by adding an equal volume of 10% formaldehyde (Polysciences) for 2 h at 26°C. Fixed cells were washed three times in 1·4 M-sorbitol, 50 mM-Tris–HCl (pH 7·5), and cell walls were removed as described by Adams and Pringle (1984) and Kilmartin and Adams (1984). Multi-well glass slides were treated by applying 15 μl of 1 mg/ml poly-L-lysine, which was removed by aspiration after 10 min. The treated wells were hydrated by incubation in water for 20–30 min, and then the spheroplasts were added to each well and allowed to stand for 5 min. For invertase and Dol-P-Man synthase labelling, dehydration was performed in methanol at −20°C for 5 min, followed by acetone at −20°C for 30 s. After air drying, the slide was rehydrated with TSA buffer [100 mM-NaCl, 20 mM-Tris–HCl (pH 7·5), 100 μg/ml bovine serum albumin (BSA)]. Invertase, β-galactosidase and tubulin antisera were diluted into TSA buffer; Dol-P-Man synthase antibodies were diluted into phosphate-buffered saline containing 1 mg/ml BSA. Antibody incubations were 45 min each, followed by three washes in buffer. Guinea pig anti-invertase serum was diluted 1 : 5000, as was fluorescein-conjugated goat anti-guinea pig serum (Organon Teknika). To visualize β-galactosidase, monoclonal anti-β-galactosidase was applied at a 1 : 50 dilution, followed by rabbit anti-mouse IgG, and fluorescein-conjugated goat anti-rabbit IgG at 1 : 500 (Organon Teknika). Rat

anti-tubulin serum (Accurate Chemical Co., No. YOL1/34) was diluted 1 : 250, and rhodamine-conjugated goat anti-rat serum was diluted 1 : 800. Purified Dol-P-Man synthase antibodies were applied overnight at a 1 : 15 dilution, and fluorescein-conjugated anti-rabbit antibodies (Kirkegaard and Perry Laboratories) at a 1 : 300 dilution were subsequently added. DAPI (4′, 6-diamidine 2-phenyl-indole) was added at 1 μg/ml in TSA buffer for a 5 min incubation, and then mounting solution [1 mg/ml *p*-phenylenediamine, 90% glycerol, 0·85% NaCl, 66 mM-Na_3PO_4 (pH 9·0)] and a cover slip were placed on the slide. The cells were visualized with a standard fluorescence photomicroscope (Zeiss).

Electron microscopy

The yeast strains DBY2449 and DBY5408 were grown in minimal glucose medium and then shifted briefly to minimal medium containing 2% lactate in order to relieve catabolite repression. Fixation and embeding were performed as described by Wright *et al.*, (1988) and Wright and Rine (1989), except that sorbitol was added to a final concentration of 1·25 M during fixation. Dehydration was performed by washing the cells in 30% ethanol, followed by 50%, 70% and 95% ethanol, and finally, by three incubations in 100% ethanol. The dehydrated cells were infiltrated with LR White resin (Polysciences) by incubation for 1 h in resin diluted three-fold with ethanol, followed by 1 h and then overnight incubations in a 1 : 1 mixture of resin and ethanol. Finally, two 1h incubations in 100% resin with a 15 min degassing at 20 psi were performed. Embeding was carried out by transferring the cell pellets into gelatin capsules containing fresh 100% resin, and polymerization was done at 47°C for 2 days. Sections measuring approximately 90 nm (as determined by thin section interference color) were cut using a diamond knife and mounted on 300-mesh nickel grids.

Anti-invertase antibodies were extensively adsorbed against an extract from DBY2449, and incubations with cell sections were performed with antibodies diluted 1 : 1000 in PBST (140 mM-NaCl, 3 mM-KCl, 8 mM-Na_2HPO_4, 1·5 mM-KH_2PO_4, 0·05% Tween 20) containing 2% ovalbumin. Antibodies to the KAR2 protein were diluted 1 : 350, and the affinity-purified anti-*DPM1* antibodies were diluted 1 : 5 in PBST with 2% ovalbumin. Gold-conjugated secondary antibodies were diluted as suggested by the manufacturer (BioCell, Cardiff, U.K.). All incubations were performed for 45 min at 25°C, and the grids were washed extensively with PBST, followed with water prior to staining with 2% aqueous uranyl acetate for 4 min, and then 0·4% lead citrate for 30 s. Observations were made on a Phillips EM300 scope at 80 kV.

RESULTS

Localization of Dol-P-Man synthase by immunofluorescence microscopy

The first ER marker protein that we localized was Dol-P-Man synthase. This protein has a single membrane spanning domain at the carboxy-terminus (Orlean *et al.*, 1988) and a cytoplasmic orientation (C.F. Albright and P. Orlean, unpublished observations). Dol-P-Man synthase is a strong candidate for a marker of the yeast ER since Dol-P-Man synthase activity cofractionates with ER markers in cell fractionation experiments with yeast cells (Marriot and Tanner, 1979) and mammalian cells (Czichi and Lennarz, 1977). The *S. cerevisiae DPM1* gene that encodes this enzyme is essential for the growth of yeast cells (Orlean *et al.*, 1988) and is functionally identical to its mammalian counterpart, since it can restore both N-linked glycosylation and glycosyl-phophatidylinositol anchoring to mammalian cells lacking Dol-P-Man synthase activity (Beck *et al.*, 1990; De Gasperi *et al.*, 1990). Moreover, when the yeast protein is expressed in mammalian cell lines, it resides within the ER, as indicated by immunofluorescence microscopy (Beck *et al.*, 1990).

Dol-P-Man synthase was localized in yeast cells using an affinity-purified anti-Dpm1 serum and indirect immunofluorescence microscopy (Figure 1). The nuclear DNA in these cells was simultaneously identified by DAPI staining. Labeled cells showed prominent staining of the region surrounding the nuclear DNA and of filamentous extensions that might emanate from the nucleus. In cells with multiple copies of the *DPM1* gene, the staining pattern was identical, but dramatically intensified (Figure 1). The relative increase in the immunofluorescent signal observed in these cells was consistent with the changes in Dpm1 protein levels as judged by immunoblotting and enzyme assays (not shown). No staining was observed with the pre-immune serum, further indicating that the affinity-purified antibodies specifically recognized the Dpm1 protein.

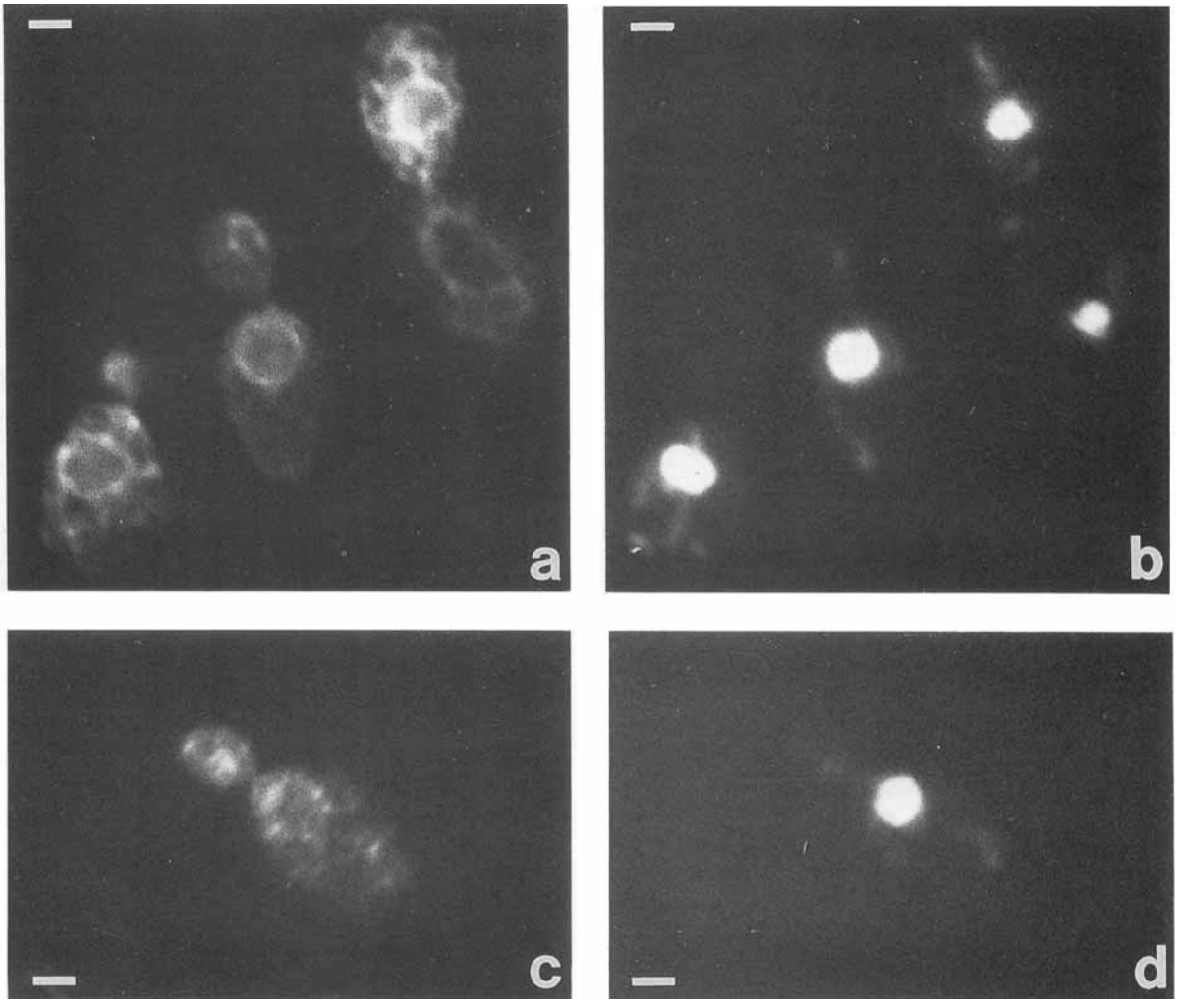


Figure 1. Immunofluorescent localization of Dol-P-Man synthase. Fixed yeast cells were stained with antibodies to the *DPM1* gene product (a and c) and with DAPI, which labels the nuclear DNA (b and d). The cells in panels (a) and (b) have a high copy-number plasmid encoding the *DPM1* gene; and in (c) and (d) have only the chromosomal copy of the gene. Bars indicate 1 μm.

Identification of mutant forms of invertase that accumulate within the secretory pathway

To provide additional markers for the yeast ER, we identified mutations within the secreted protein invertase that result in its accumulation within the secretory pathway. Because the abundance of this protein can be controlled, it is an ideal candidate for these studies, and, as described below, mutant forms of invertase that reside within the ER were characterized.

Wild-type invertase, the product of the yeast *SUC2* gene, is transported rapidly through the secretory pathway, and is modified by the addition of core oligosaccharides in the ER, followed by outer-chain glycosylation in subsequent secretory compartments (Esmon *et al.*, 1981). Because these changes dramatically alter its molecular weight, invertase is a convenient and commonly used indicator of the function of the yeast secretory pathway. Though secretion of this enzyme is not essential for cell viability, external invertase is required for the growth of yeast cells on sucrose medium. This growth assay can be used to identify yeast strains that are defective in invertase secretion, and several strains that fail to grow on sucrose medium due to

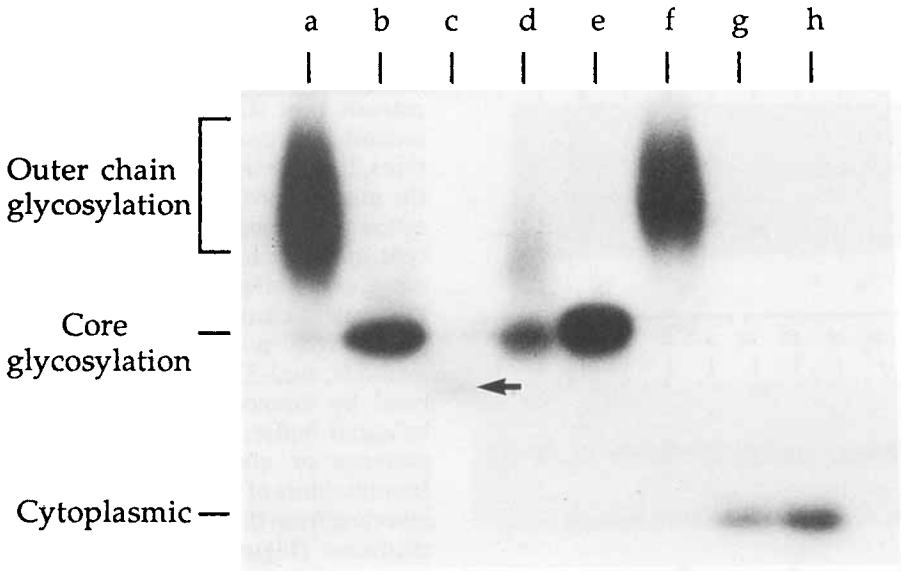


Figure 2. Identification of invertase mutants that are incompletely glycosylated. Invertase synthesis was induced by shifting exponential cultures to medium with low levels of glucose, and cell extracts were electrophoresed on a denaturing gel and immunoblotted with anti-invertase antibodies. Equivalent amounts of protein were loaded in each lane. Gel lanes are labeled as follows: (a) wild-type cells (DBY1034); (b) a *suc2-533* strain (DBY954); (c) a *suc2-538* strain (DBY955); (d) DBY2449 encoding *suc2-s1* on a low copy-number plasmid; (e) and (f) DBY2356 (*sec18-1*) encoding the wild-type *SUC2* gene on a low copy-number plasmid grown at the restrictive (e) and permissive (f) temperatures; (g and h) invertase synthesis was induced in medium containing tunicamycin using a wild-type culture (DBY1034; g) and a *suc2-533* culture (DBY954; h). The positions of cytoplasmic invertase (no oligosaccharide chains) and invertase with core oligosaccharides or heterogeneous, outer-chain modifications are indicated. Invertase from the *suc2-538* strain is present in relatively low abundance, and the position of this band is indicated (arrow).

mutations within the *SUC2* gene have been previously described (Carlson *et al.*, 1981). The invertase produced in these strains was analysed by separating whole-cell extracts on denaturing gels, followed by immunoblotting with anti-invertase antiserum. In this manner, we identified two mutant proteins (encoded by the *suc2-533* and *suc2-538* alleles) that have no enzymatic activity and are incompletely glycosylated, indicating that they are delayed at least in their transport through the secretory pathway.

Unlike wild-type invertase, the inactive *suc2-533* and *suc2-538* proteins lack the heterogeneous oligosaccharide modifications indicative of transport beyond the ER, and instead have the mobility expected of invertase modified by only core oligosaccharides (Figure 2). The mobility of these proteins was compared to that of invertase synthesized in a strain carrying a *sec18* mutation; a deficiency that, at the restrictive temperature, prevents protein transport beyond the ER (Novick *et al.*, 1981). As shown in Figure 2, the *suc2-533* protein comigrates with the core glycosylated invertase from a *sec18* strain, and the *suc2-538* strain produces low levels of protein that migrates faster than core glycosylated invertase. Incubation of these strains in the presence of tunicamycin, a drug that inhibits asparagine-linked glycosylation, indicated that the *suc2-533* protein consists of a full-length polypeptide chain (Figure 2), although it appears that the *suc2-538* protein might be truncated (data not shown). These results indicate that the aberrant migration of the *suc2-533* protein in the absence of tunicamycin is due to its lack of outer-chain carbohydrates.

Another mutant form of invertase that accumulates within the secretory pathway has been described previously (Schauer *et al.*, 1985). This protein, which is encoded by the *suc2-s1* allele, has an alteration at the signal cleavage site, and produces invertase that has a high specific activity and is associated with the ER membrane. While most of the *suc2-s1* protein has only core oligosaccharide chains, approximately one-third of the *suc2-s1* invertase has some outer-chain glycosylation

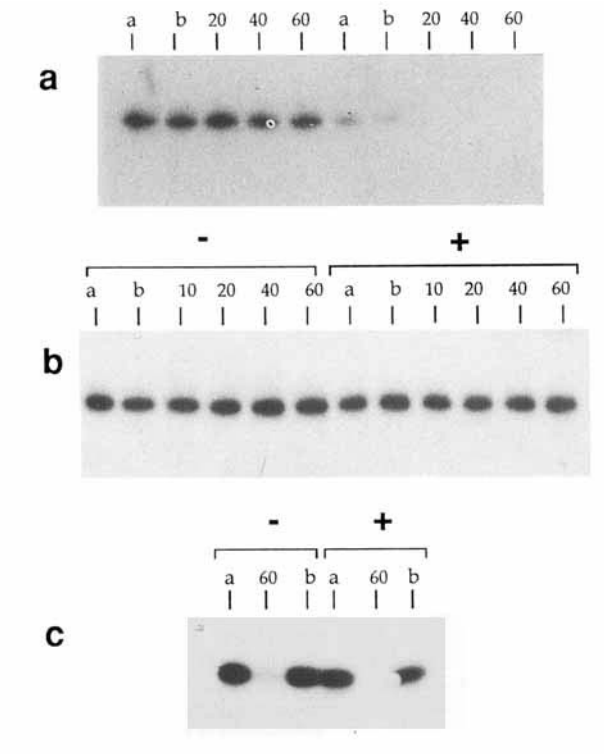


Figure 3. Sensitivity of invertase to protease treatment. Yeast cells were shifted to medium with low levels of glucose to induce invertase synthesis, and spheroplasts from a *suc2-533* strain (DBY5408; a and c), and from a *suc2-s1* strain (DBY5410; b) were prepared. The spheroplasts were homogenized and treated with trypsin in the presence (+) or absence (−) of 0·5% Triton X-100, and proteins were subsequently resolved on a denaturing gel and immunoblotted with anti-invertase antibodies (a and b) or with antibodies to the YPT1 protein (c). Lanes are as follows: (a) no trypsin added; (b) samples treated with the trypsin inhibitor prior to addition of trypsin; numbers above lanes indicate the duration of the trypsin incubation in minutes. As expected, the active invertase encoded by the *suc2-s1* strain (b) is stable under these conditions, and the cytoplasmic YPT1 protein is degraded both in the presence or absence of detergents (c).

(Figure 2). In contrast, the secretion defect in the *suc2-533* and *suc2-538* strains is much more severe. Fully glycosylated invertase was never detected in these strains, even after increasing invertase levels 20-fold (not shown).

Mutant proteins that accumulate within the ER of mammalian cells are often improperly folded (Carrell and Travis, 1985; Gething *et al.*, 1986). Initial experiments with the *suc2-533* and *suc2-538* proteins indicated that they might also be incorrectly folded. First, these proteins have no detectable enzymatic activity. In addition, the non-glycosylated, cytoplasmic invertase that normally constitutes 1% of the invertase found in wild-type strains is notably absent from *suc2-533* and *suc2-538* extracts (not shown), further suggesting that the mutant invertase proteins have altered conformations. In order to probe directly the conformation of the inactive proteins encoded by these strains, we tested their sensitivity to protease digestion. Wild-type invertase is highly resistant to protease treatment, as are other active forms of invertase, such as the protein encoded by the *suc2-s1* allele. Spheroplasts from a wild-type strain and from the *suc2-533*, *suc2-538* and *suc2-s1* mutant strains were lysed by dounce homogenization in osmotically balanced buffer, and treated with proteases in the presence or absence of a non-ionic detergent. Immunoblots of the extracts demonstrated that the invertase from the *suc2-s1* strain was resistant to this treatment (Figure 3), indicating that ER localization and incomplete oligosaccharide modification, *per se*, do not result in protease sensitivity. However, invertase from the *suc2-533* or *suc2-538* strains was resistant to proteolytic digestion only in the absence of detergents. When detergents were added, the mutant invertase proteins were degraded rapidly (Figure 3), indicating that these proteins fail to obtain stable, folded conformations and that they reside within a membranous structure.

As a control for the above experiment, we confirmed that protease digestion of cytoplasmic proteins occurred both in the presence and absence of detergents. Cell lysates were also immunoblotted with antibodies to the YPT1 protein (Segev *et al.*, 1988, and, as shown in Figure 3, the YPT1 protein was degraded regardless of whether detergents were added. Finally, to substantiate that the *suc2-533* protein resides within a membranous compartment, and to exclude the possibility that detergents alter the conformation of the mutant protein and thus make it accessible to protease digestion, spheroplasts were vigorously lysed with glass beads, and the extracts were exposed to proteases. As expected, the proteins encoded by the *suc2-s1* or the wild-type allele were resistant to protease digestion, while the *suc2-533* protease was rapidly degraded (not shown).

Localization of mutant invertase proteins that accumulate within the ER

The observation that the improperly folded *suc2-533* protein receives only core oligosaccharides indicates that this mutant protein resides within the ER. To characterize this organelle further, the

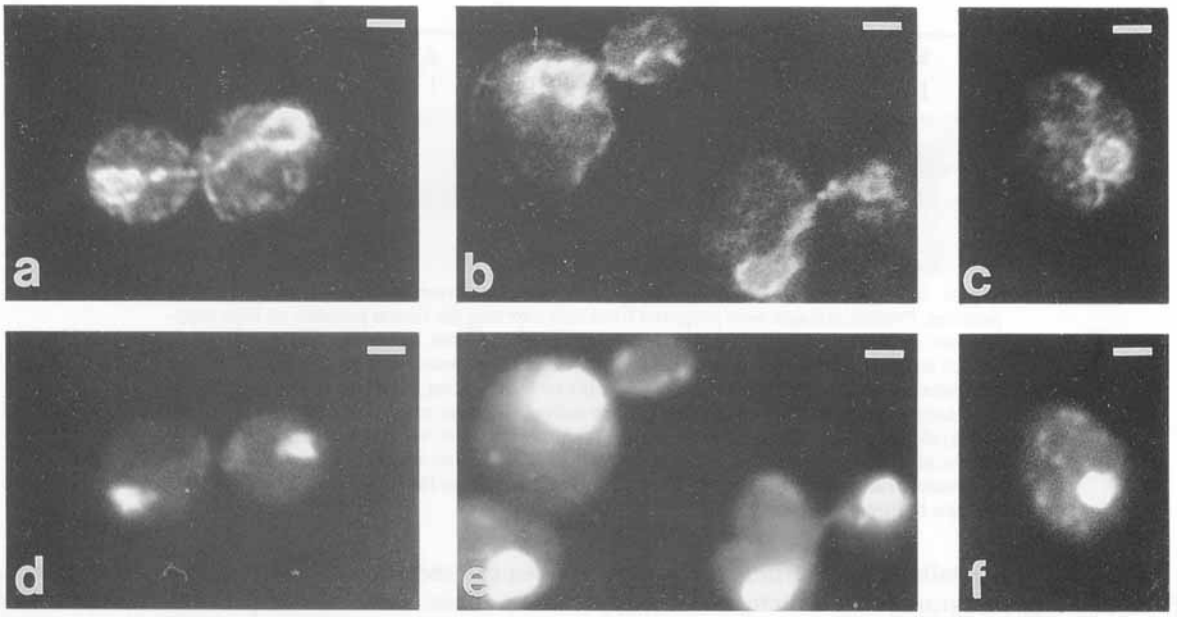


Figure 4. Immunofluorescent localization of improperly folded invertase. Invertase synthesis was induced by shifting cells encoding the *suc2-533* gene on a high copy-number plasmid to medium containing lactate as a carbon source. Cells were fixed and labeled with anti-invertase antibodies followed by fluorescein-conjugated secondary antibodies (a–c) and with DAPI (d–f). Similar, though less intense patterns were observed with strains carrying a single copy-number plasmid, and in *suc2-538* strains. No labeling of *suc2*Δ strains was detected. Bars indicate 1 μm.

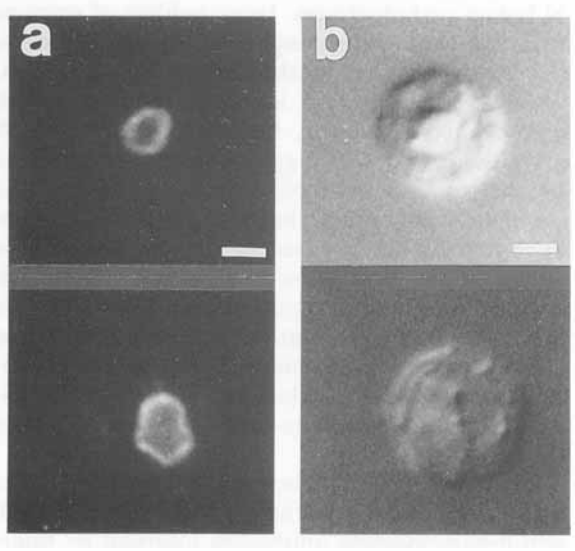


Figure 5. Immunofluorescent localization of the s1 mutant form of invertase. DBY2449 cells expressing the *suc2-s1* mutation were fixed and labeled with anti-invertase antibodies (a) and with DAPI fluorescence in combination with Nomarski optics (b). Bars indicate 1 μm.

abundant, mutant invertase was localized using indirect immunofluorescence.

Rapid induction of invertase was achieved by shifting cell cultures from glucose medium to lactate-containing medium, thus permitting observation of the invertase protein in a population of growing and dividing cells. Antibodies that specifically recognize invertase were used to label *suc2-533* cells (Figure 4), or *suc2*Δ controls. Intense immunofluorescent staining of the perinuclear region was clearly visible in every cell that expressed the mutant protein, while no staining was observed when the antibodies were applied to cells with a deletion of the invertase gene (not shown). The prominent perinuclear staining of the *suc2-533* cells permitted analysis of the invertase distribution throughout the nuclear division cycle. In mitotic cells, the perinuclear invertase staining extended between the mother and daughter nuclei, even when segregation of the nuclear DNA was complete (Figure 4). This localization pattern suggests that the mutant invertase is closely associated with the yeast nuclear envelope, which remains intact during the mitotic cycle and enters daughter cells during nuclear division (Matile *et al.*, 1969).

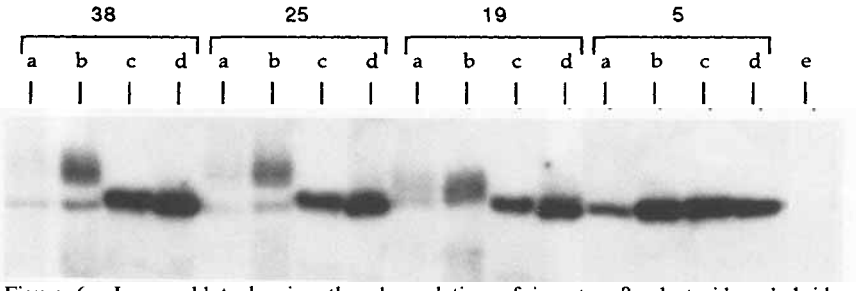


Figure 6. Immunoblot showing the glycosylation of invertase-β-galactosidase hybrid proteins. Protein extracts were prepared from cells carrying the fusion proteins on high copy-number plasmids, and cells were grown in either 5% glucose (invertase repressed) (a), or shifted to 0·1% glucose (invertase induced) (b, c and d). Proteins without glycosylation were produced by treating extracts with endo H (c) or by inducing invertase in the presence of tunicamycin (d). The portion of the invertase-coding sequence present in each hybrid is indicated (38, 25, 19 or 5 amino acids). Lane (e) represents a strain without a plasmid. Molecular weight markers indicated that the fusions with 38 or 25 amino acids have three to four core oligosaccharide chains (measuring 2000 daltons each) and that the fusion with 19 amino acids has two to three core chains.

In addition to staining the perinuclear region, faint staining of cytoplasmic structures was also observed in the mutant cells. This staining was often punctate, and filamentous structures, reminiscent of ER tubules, were occasionally observed near the plasma membrane (Figure 4). These structures entered growing buds before nuclear division, suggesting that the ER-associated material enters the daughter cell at an early stage in bud growth.

The anti-invertase staining pattern of the *suc2-533* strain was compared to that observed in a strain expressing the ER-localized *suc2-s1* protein. The *suc2-s1* cells exhibited intense staining at the nuclear perimeter that was similar to that observed in the other mutant strains (Figure 5). However, because these cells also express a stable, cytoplasmic form of invertase, intense background immunofluorescence was detected. Although this only rarely obscured the perinuclear staining, it prohibited observation of peripheral compartments.

Subcellular localization of invertase-β-galactosidase hybrid proteins

The presence of the invertase signal sequence at the amino-terminus of the *E. coli* β-galactosidase protein targets the resulting fusion protein to the yeast secretory pathway (Emr *et al.*, 1984). In the process of characterizing the invertase signal sequence, several additional invertase-β-galactosidase hybrids that contain smaller portions of the *SUC2* gene were constructed (Kaiser and Botstein, 1990). Those fusion proteins that are targeted to the secretory pathway remain associated with ER, presumably because β-glactosidase does not readily cross cell membranes (for example, see Lee *et al.*, 1989). Thus, these hybrid proteins have provided additional markers for the yeast ER.

To identify those fusion proteins that are targeted to the secretory pathway, each of the hybrids was examined for the presence of oligosaccharide modifications. Glycosylation of a given fusion protein could be inferred by a decrease in the relative molecular weight brought about by either exposing cells to the glycosylation inhibitor tunicamycin, or by treatment of cell extracts with endo H to remove N-linked carbohydrates. Immunoblots of extracts from strains expressing the hybrid proteins (Figure 6) indicate that the shortest fusion protein, which contains only 5 invertase residues, is not glycosylated. However, fusions of 19, 25 or 38 invertase residues to β-galactosidase are modified by two to four core oligosaccharide chains and completely lack outer-chain modifications (Figure 6). Some of the oligosaccharide chains must be added to the β-galactosidase polypeptide (which encodes six potential glycosylation sites) since the first potential glycosylation site in the invertase coding sequence is at residue 23. These results indicate that as few as 19 amino acids of the *SUC2* signal sequence are sufficient to target β-galactosidase to the ER.

The subcellular location of the fusion proteins was determined by staining fixed yeast cells with anti-β-galactosidase antibodies followed by fluorescein-conjugated secondary antibodies. Immunofluoresence microscopy of strains expressing the glycosylated hybrids revealed a striking ring of fluorescence at the perimeter of the DAPI-staining

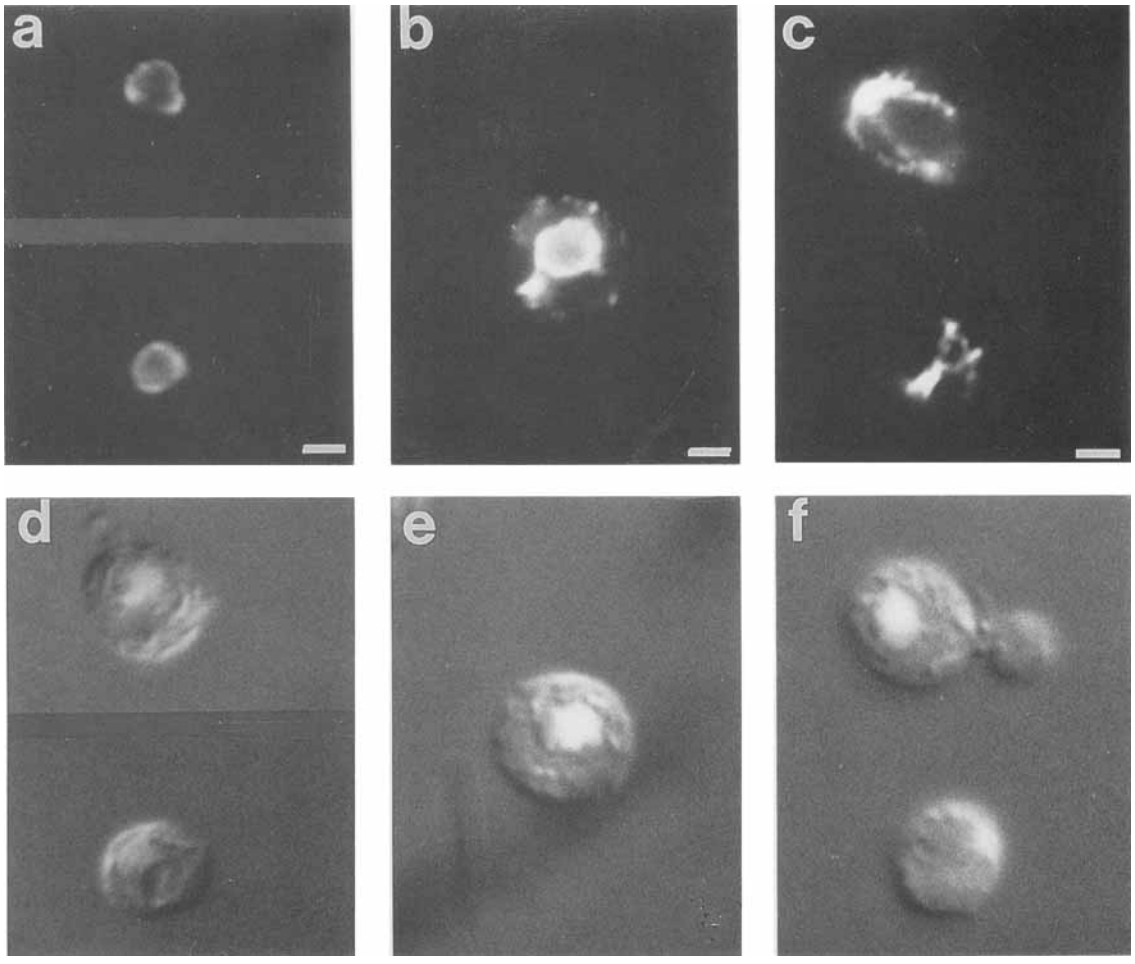


Figure 7. Immunofluorescent localization of the invertase-β-galactosidase hybrid protein that contains 19 invertase residues. Cells were stained with monoclonal antibodies to β-galactosidase followed by fluorescein-conjugated secondary antibodies (a–c); the same cells with DAPI staining and viewed with Nomarski optics (d–f). DBY2449 cells expressing the fusion protein (a). The same fusion was expressed in *sec18* cells (DBY2356) grown at the permissive temperature (b); and at the restrictive temperature (c). Bars indicate 1 μm.

nuclear DNA (Figure 7). Nuclear association was also evident in dividing cells where fluorescent staining formed a continuous sheath around the nuclei in both mother and bud and was drawn to a thin filament through the bud neck (not shown). Fusions longer than 19 amino acids also exhibited fluorescent staining throughout the cell body. Presumably, these constructs resulted in expression of substantial levels of a cytoplasmic hybrid protein (not shown).

We also tested the effect of the *sec18* mutation, a defect known to perturb the structure of the ER, on the localization of the hybrid proteins. When *sec18-1* mutant cells are grown at the non-permissive temperature, they accumulate secretory proteins within enormously amplified ER structures that are readily visualized using electron microscopy (Esmon *et al.*, 1981; Novick *et al.*, 1981). The distribution of the hybrid protein with 19 invertase amino acids was affected by the *sec18-1* mutation (Figure 7). At the permissive temperature, staining was predominantly at the nuclear perimeter, while at the restrictive temperature abundant filaments of fluorescent staining were distributed at the cell periphery. The changes in the distribution of the hybrid β-galactosidase brought about by the *sec18-1* mutation further support the interpretation that the hybrid proteins are localized within the ER.

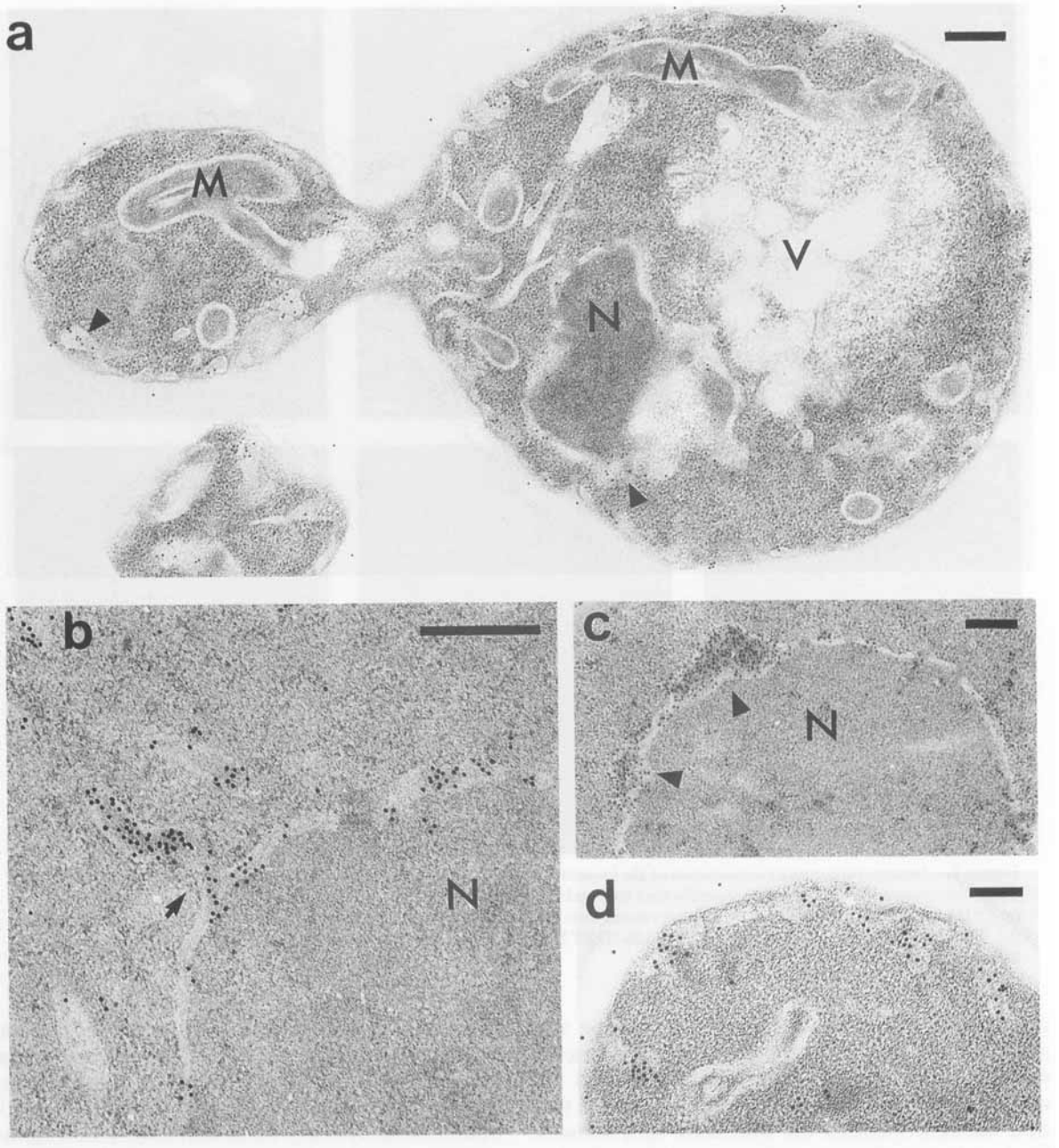


Figure 8. Immunolocalization of improperly folded invertase from a *suc2-533* strain. Cells were grown as described in Figure 4. A representative, budding cell is depicted in (a); arrows note gold particles within the lumen of the nuclear envelope and at the cell periphery. A connection between the nuclear envelope and ER tubules was occasionally observed (b, arrow), as were distensions of the nuclear envelope (c, arrows). A high magnification view of peripheral ER membranes is shown in (d). N, nucleus; V, vacuole; M, mitochondrion; bars indicate 0·25 μm.

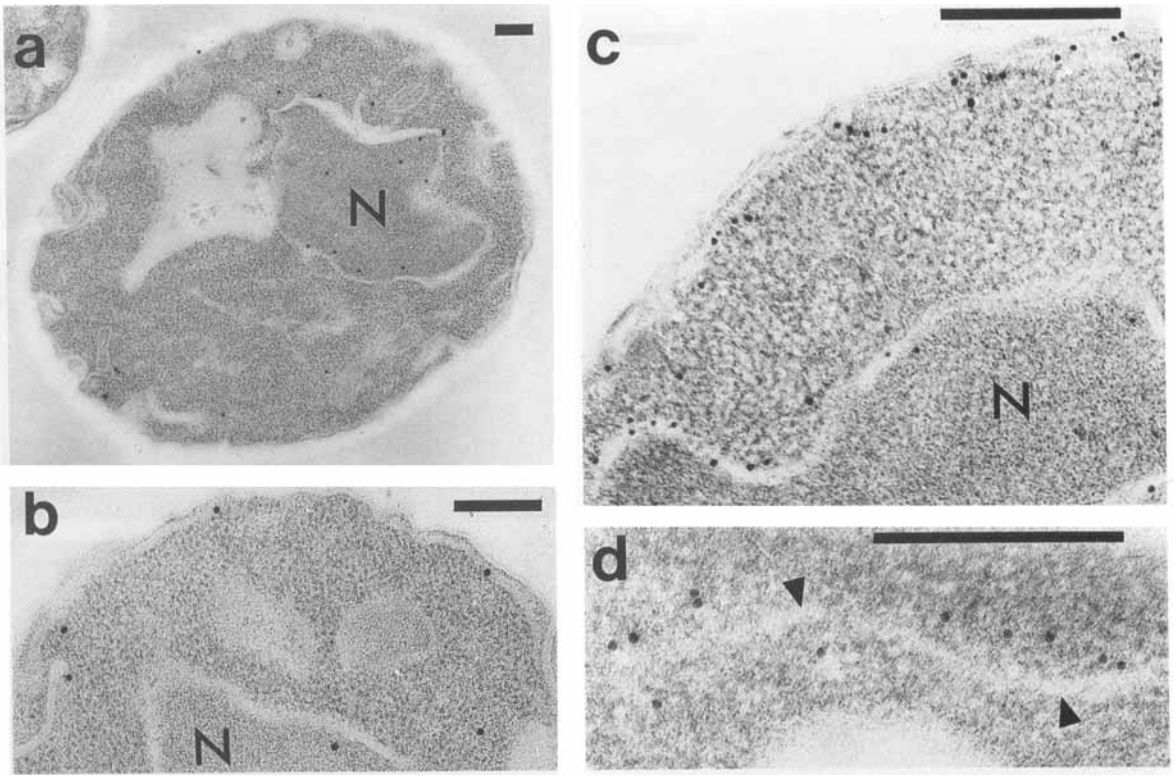


Figure 9. Immunolocalization of Dol-P-Man synthase in yeast strains encoding *suc2-533* invertase (a and b), or in a *SUC2* strain with multiple copies of the *DPM1* gene (c and d). Arrows in (d) note the position of an ER tubule. N, nucleus; bars indicate 0·25 μm. Similar patterns were observed in cultures grown in glucose- or lactate-containing medium, and in strains with a deletion of the *SUC2* gene.

Localization of the ER marker proteins using immunoelectron microscopy

Each of the proteins described above is a resident of the ER, as judged by its enzymatic function or by its oligosaccharide content. Furthermore, the similarities in the immunofluorescent staining patterns suggest that these proteins are contained within the same cellular compartment. Each protein was clearly located at the nuclear perimeter, and several were also observed in filamentous structures within the cytoplasm. To improve resolution of these structures and to examine the morphology of the ER in detail, this analysis was extended by localizing the marker proteins with immunoelectron microscopy.

Yeast cells encoding the misfolded *suc2-533* protein were prepared as described for indirect immunofluorescence, and localization was performed using purified invertase antibodies followed by gold-conjugated secondary antibodies. These antibodies were specific for invertase proteins since no labeling was detected in a *suc2* deletion strain that was grown and fixed in parallel (not shown).

In general, the morphology of the *suc2-533* cells was normal. As expected, the mutant invertase proteins were concentrated at the perimeter of the nucleus, and the distribution of these proteins within the nuclear envelope was fairly uniform (Figure 8). Extensions of the nuclear envelope into the cytoplasm that contained the mutant protein were occasionally observed. In rare cases, the lumen of the nuclear envelope was enlarged and heavily labeled with invertase antibodies (Figure 8c). Similar distensions of these membranes were not observed in the *suc2* deletion strain, and therefore they might be a consequence of expression of the improperly folded mutant proteins.

A large fraction of the mutant invertase protein was also contained within compartments in the cytoplasm (Figure 8d). These compartments were apparently enclosed by a lipid bilayer, and were

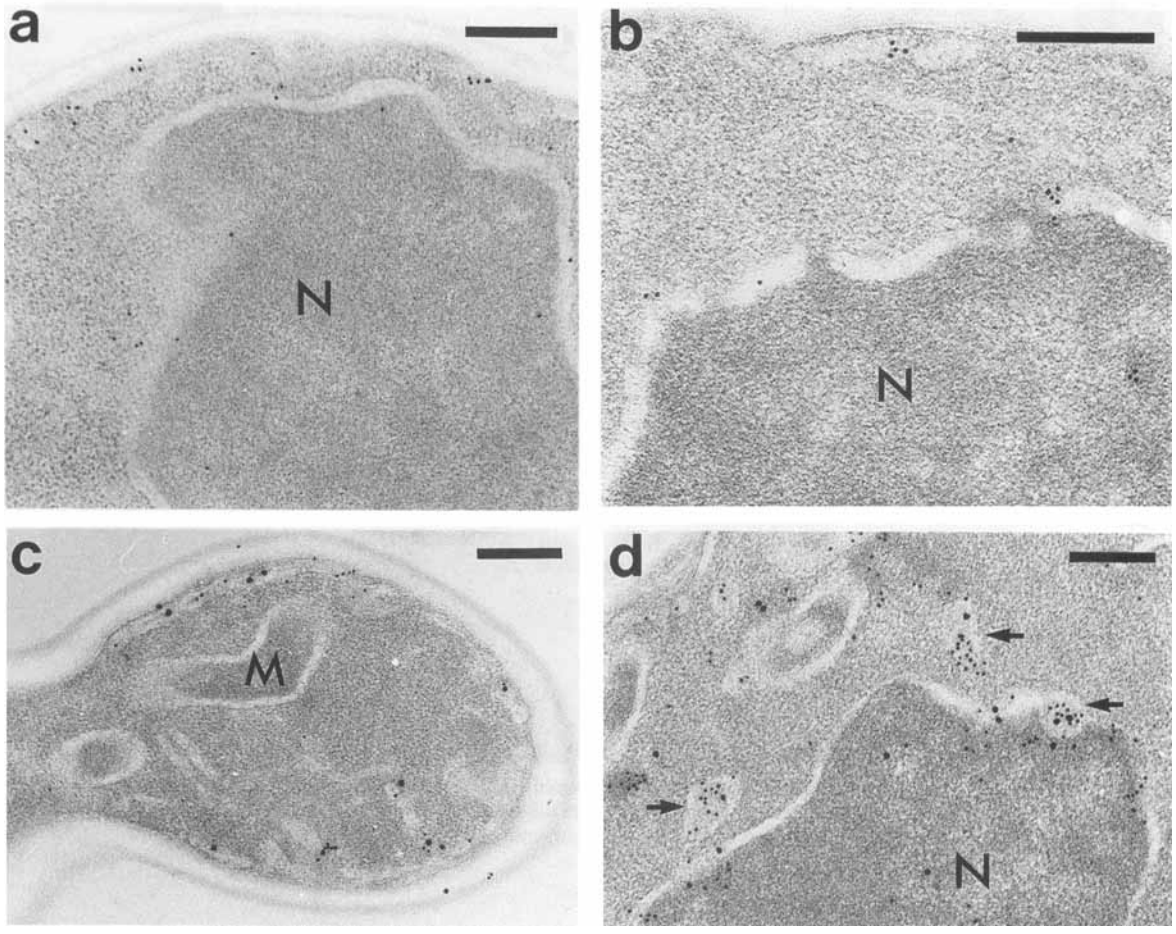


Figure 10. Immunolocalization of the Kar2 protein in DBY5408 (*suc2-533*; a) and in DBY2449 (*suc2*Δ; b) and colocalization of the Kar2 and *suc2-533* proteins in DBY5408 (c and d). In the latter case, 20 nm gold particles were used to secondarily label the Kar2 protein, and 10 nm particles were used to label invertase. In (d), arrows note compartments that clearly contain large and small gold particles. On occasion, the anti-Kar2 antibodies also labeled the nucleus itself, but this staining is probably non-specific since it was not reproduced in every cell. N, nucleus; M, mitochrondrion; bars indicate 0·25 μm.

typically adjacent to the plasma membrane. Though some of the labeled structures were longitudinal sections of ER tubules, most appeared to be cross-sections of tubules or discrete vesicles.

Because mutant invertase proteins are not normal constituents of the ER, we compared the location of these proteins to that of Dol-P-Man synthase. Sections from *suc2-533*, *suc2*Δ and *SUC2* strains were labeled with affinity-purified antibodies to the *DPM1* gene product. Although only a few grains of gold were found in each cell, the labeling was highly specific. The Dol-P-Man synthase antibodies were localized exclusively to the nuclear envelope and to membranes within the cytoplasm (Figure 9a and b). As in the immunofluorescence experiments, no labeling was observed when the sections were treated with the preimmune serum (not shown). Similar structures were labeled, regardless of the *suc2* genotype of the cells, and these staining patterns were intensified in cells with multiple copies of the *DPM1* gene (Figure 9c and d).

The gold-conjugated antibodies that reacted with the anti-*DPM1* antibodies were rarely found within the lumenal space of the ER membranes (Figure 9d); roughly 75% of the labeling (128/170 gold particles) was more than 10 nm from the ER surface. In contrast, only 14% of the invertase labeling (24/169 gold particles) was removed from the ER lumen. These observations are in agreement with biochemical studies that indicate that the Dol-P-

Man synthase polypeptide is located primarily on the extralumenal face of the ER membrane (C.F. Albright and P. Orlean, unpublished observations).

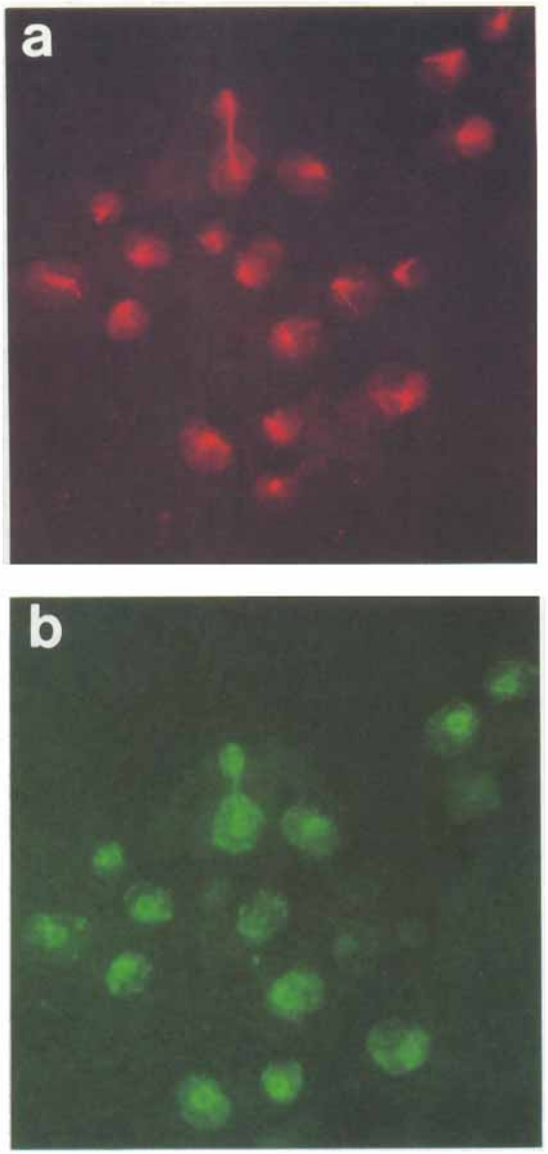


Figure 11. Immunofluorescent staining of the mitotic spindle and of the ER structures containing the suc2-533 protein. Yeast cells (DBY5408) were labeled with antibodies to tubulin, and secondarily with rhodamine-conjugated antibodies (a); and with antibodies to invertase, followed by fluorescein-conjugated secondary antibodies (b). Cells with elongated spindles also have extended perinuclear membranes between the mother and daughter nuclei. Bars indicate 1 μm.

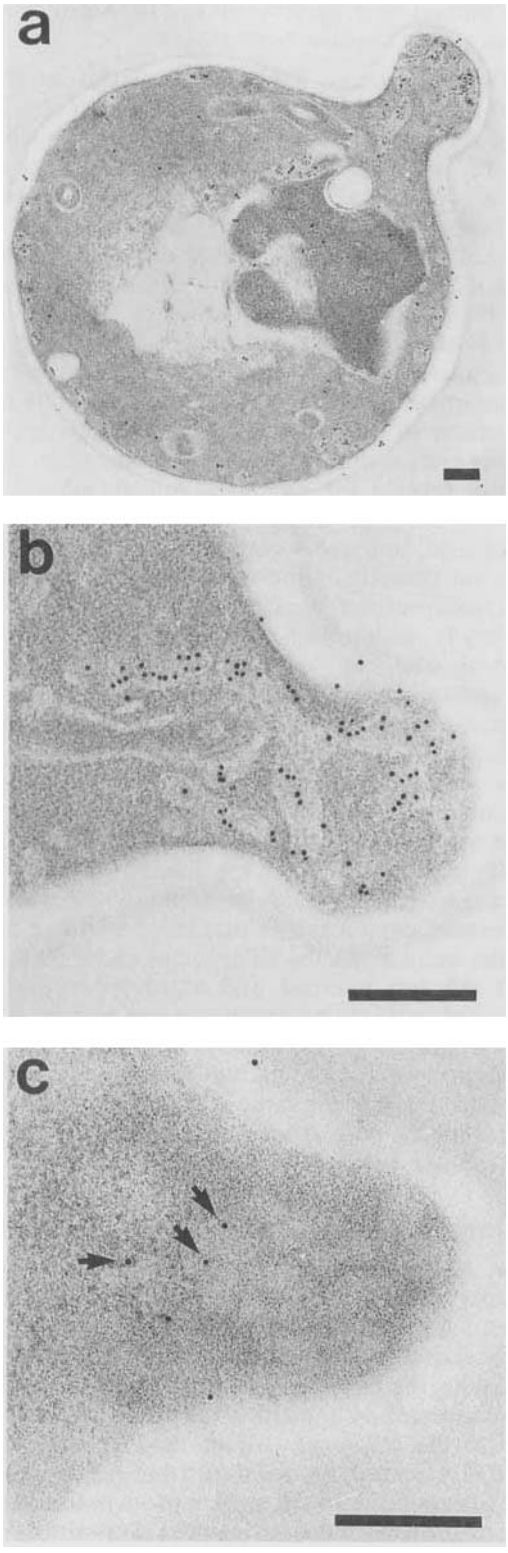
The mutant invertase proteins and the KAR2 protein reside within the same compartment

The *S. cerevisiae KAR2* gene encodes an abundant protein that, like its mammalian homolog BiP, probably resides within the lumen of the ER (Rose *et al.*, 1989; Normington *et al.*, 1989). Localization of the Kar2 protein by immunofluorescence microscopy has previously demonstrated that it is present at the nuclear perimeter and within filamentous structures extending into cytoplasm (Rose *et al.*, 1989). To determine whether the Kar2 protein, Dol-P-Man synthase and the misfolded invertase proteins are indeed associated with the same cellular compartment, we examined the Kar2-containing structures in cells expressing the *suc2-533* protein and in cells with a deletion of the *suc2* gene. Antibodies specific for the Kar2 protein labeled the nuclear envelope as well as compartments in the cytoplasm, and the location of the Kar2 protein was not affected by the presence of the misfolded invertase proteins (Figures 10a and 10b).

Finally, to establish conclusively that the Kar2 protein and the *suc2-533* protein are indeed contained within the same membranous structures, sections from the *suc2-533* strain were doubly stained with antibodies to each of these proteins. The antibodies were labeled differentially with secondary antibodies: the anti-invertase was reacted with a 10 nm gold-conjugated anti-guinea pig antibody, and the anti-Kar2 was labeled with a 20 nm gold-conjugated anti-rabbit antibody. Control experiments demonstrated that none of these antibodies has unexpected affinities for each other, and that the anti-invertase and anti-Kar2 antibodies react only with their respective target proteins (data not shown). Figures 10c and 10d show that the Kar2 protein and the mutant invertase are clearly contained within the same compartment. They are in proximity, both at the nuclear perimeter, and in ER tubules within the cytoplasm.

Distribution of the ER throughout the yeast cell cycle

In cells undergoing nuclear division, the perinuclear membranes that contain the ER proteins extend between the mother and daughter nuclei, even after the DNA has divided (Figures 1 and 4). Likewise, the DAPI-stained portions of the nucleus are connected by a microtubule-based spindle until late in the cell cycle (Adams and Pringle, 1984; Huffaker *et al.*, 1988; Kilmartin and Adams, 1984). We have utilized the ER marker proteins to examine the relationship between spindle disassembly and



division of the nuclear envelope. The *suc2-533* cells were doubly labeled with antibodies to invertase and to tubulin, and the anti-invertase was reacted with a fluorescein-conjugated secondary antibody, while the anti-tubulin was secondarily labeled with rhodamine. As demonstrated by control experiments, each of the antibodies was highly specific; no unexpected cross-reactivity was observed (not shown). A sample of 100 cells with invertase staining that extended between the dividing nuclei was examined. In every case, anti-tubulin staining of the spindle structure was also observed (Figure 11). Likewise, of cells that exhibited elongated spindles and prominent invertase staining, the invertase was always visible between the dividing nuclei. Thus, spindle disassembly and division of the perinuclear membranes occur within a short time in the cell cycle.

To discern whether buds might acquire peripheral ER structures at an earlier point in the cell cycle, we followed the marker proteins in cells with newly formed buds. Immunofluorescent labeling of cells expressing the misfolded invertase proteins indicated that these proteins enter buds well before the nuclear DNA (Figure 4). We have used immunoelectron microscopy to examine this process in more detail. As shown in Figure 12, early buds can be labeled with anti-invertase or anti-Kar2 antibodies. The labeled compartments often resemble ER tubules and vesicular structures can also be observed. These compartments enter growing buds well before other organelles such as the vacuole or mitochondrion. Thus, daughter cells acquire components of the ER early in the growth cycle, and the nuclear-associated ER components subsequently enter the buds during nuclear division.

DISCUSSION

Localization of the ER marker proteins

By using indirect immunofluorescence we demonstrated that Dol-P-Man synthase, two mutant forms of invertase, and an invertase-β-galactosidase fusion protein are concentrated both at the nuclear

Figure 12. Antibodies to the ER proteins label yeast daughter cells at an early stage in bud growth. DBY5408 cells (*suc2-533*) doubly labeled with antibodies to the Kar2 and invertase proteins (a); DBY5408 cells labeled with invertase antibodies (b); DBY2449 (*suc2Δ*) cells labeled with antibodies to the Kar2 protein (c). Arrows note gold particles within secretory vesicles. Bars indicate 0·25 μm.

perimeter and within filamentous ER tubules. A similar immunofluorescent staining pattern was also obtained when yeast cells were stained with antibodies to the Kar2 protein (Rose *et al.*, 1989). Moreover, we employed immunoelectron microscopy to confirm that the misfolded invertase proteins and the Kar2 protein reside within the lumen of these organelles and that Dol-P-Man synthase is associated with the extralumenal face of ER membranes. These studies have permitted analysis of the ultrastructure of the ER throughout the yeast cell cycle.

A large fraction of the ER proteins are associated with the nuclear envelope

Striking immunofluorescent staining of the perinuclear membranes was observed when antibodies to each of the marker proteins were applied to yeast cells. The outer nuclear envelope of mammalian cells contains ribosomes and is continuous with the ER (see Fawcett, 1981, for a review), and freeze substitution electron microscopy has demonstrated that the yeast nuclear envelope is also studded with ribosomes (Baba and Osumi, 1987). Yeast HMG-CoA reductase, an integral component of the ER membrane, may be preferentially inserted into the nuclear envelope, since cells that express high levels of this protein accumulate aberrant stacks of membranes around the nucleus, but not in the periphery of the cell (Wright *et al.*, 1988; Wright and Rine, 1989). None of the proteins we examined exhibited preferential localization within the nuclear envelope. Nonetheless, the appearance of each of the ER proteins in the perinuclear membranes clearly indicates that the yeast nuclear envelope contains all of the components required for targeting of proteins to the secretory pathway, and presumably for processing and transporting these proteins to subsequent secretory organelles.

Unlike the mammalian organelles, the yeast nuclear envelope (Matile *et al.*, 1969) and ER (Figures 1 and 4) remain intact during mitosis, and can thus form a continuous network throughout the cell cycle. Though the yeast nuclear envelope clearly contains some unique proteins, such as those that form nuclear pore complexes (Davis and Fink, 1990; Hurt, 1988), this organelle may, in fact, participate in a number of ER functions. Genetic selections have been utilized to identify yeast strains with deficiencies in the translocation of secretory proteins into the ER (Deshaies and Schekman, 1987; Rothblatt *et al.*, 1989; reviewed in Deshaies *et al.*, 1989), or with defects in the import of proteins into the nucleus (Sadler *et al.*, 1989). In at least one case, these studies have identified the same genetic locus, known as *SEC63* or *NUP1* (Sadler *et al.*, 1989), and have thus linked the functions of the nuclear envelope with those of the ER. In addition, mutations within the *KAR2* gene can impair the translocation of proteins into the ER lumen, but also can reduce the efficiency of nuclear fusion following yeast conjugation (Polaina and Conde, 1982; Vogel *et al.*, 1990). Though the latter phenotype may be an indirect consequence of the *KAR2* mutation, these observations provide additional evidence that the functions of the nuclear envelope are linked to those of the ER.

The ER proteins also reside within a network of membranous compartments

Although the peripheral ER tubules were not easily visualized with light microscopy, extensive membrane structures were revealed by employing immunoelectron microscopy. The appearance of the yeast ER network differs from the highly organized stacks of ER cisternae that are generally observed in mammalian cells that are specialized for secretion (Palade, 1956; Fawcett, 1981). The yeast tubules are not abundant near the nuclear envelope (though occasional connections were detected, Figures 4 and 8), and appear to be highly convoluted, since long sections of tubules are rarely observed. A vesiculated and disorganized ER, that nonetheless consists of an interconnected reticulum, has been observed in some mammalian cells (Palade, 1956). To demonstrate conclusively that the yeast ER is a single, interconnected organelle, and that all of the structures that are labeled by the marker antibodies are a part of this network, reconstruction of serial cell sections coupled with immunolabeling may be required. We are presently developing methods that might permit such experiments.

Most of the peripheral ER tubules were found adjacent to the plasma membrane. Though this association was observed with immunofluorescent labeling (Figure 4), its extent was only clarified when the cellular ultrastructure was examined. The intimate association of the ER membranes with membranes at the cellular periphery suggests that these bilayers, or their associated proteins, might interact. Since other membranous organelles, such as the mitochondria and vacuoles, are scattered throughout the cytoplasm, the association of the ER and plasma membrane may be a specific one.

ER membranes that lack associated ribosomes ('smooth' ER) have been observed in many cell types, and the synthesis and assembly of some complexes, such as those found in lipoproteins, require both smooth and rough ER compartments (Jones *et al.*, 1967). Connections between the smooth and rough ER have been described, and the smooth ER is often concentrated near the plasma membrane (Palade, 1956; Fawcett, 1981). The abundance of free ribosomes in the yeast cytoplasm makes observation of membrane-bound ribosomes difficult. However, freeze-substitution techniques have clearly demonstrated that the yeast ER membranes that juxtapose the plasma membrane have associated ribosomes (Baba and Osumi, 1987). Therefore, there is presently no evidence that the function of these peripheral ER membranes differs from the ER that is associated with the nucleus.

Intracellular distribution of the ER proteins

As described above, each of the ER marker proteins was present both in the perinuclear and peripheral ER. In order to estimate the relative abundance of ER proteins in these membranous compartments, we quantitated the antibody labeling of these structures in several cell sections labeled for immunoelectron microscopy. Using these data, and an estimate of relative nuclear and cellular volumes, about 30% of each ER marker protein was found to reside within the perinuclear membranes. Further, because these membranes occupy a relatively small volume, the concentration of the marker proteins in the vicinity of the nucleus is about four times higher than in the rest of the cell. Thus, when intact cells were examined with immunofluorescence microscopy, the overwhelming fluorescent signal arose from the marker proteins contained within the nuclear envelope.

As with purification of mammalian membranes, standard techniques used to purify yeast ER membranes typically discard nuclei (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Goud *et al.*, 1988). However, the results presented here indicate that this practice removes a substantial portion of the yeast ER and, presumably, of the ER-associated proteins.

The ER enters yeast buds at an early point in the cell cycle

Secreted proteins are exported from yeast cells primarily on the surface of growing buds (Field and Schekman, 1980), and electron micrographs of daughter cells often reveal extensive membranous structures (Matile *et al.*, 1969). The micrographs presented here indicate that many of the organelles that are present in growing buds contain the mutant invertase and Kar2 proteins (Figure 12). The labeled structures often resemble the abundant ER tubules found within the mother cell. However, we cannot conclude that every compartment that contains the marker proteins indeed represents the ER since a small fraction of the marker proteins may leave the ER and enter ER-derived vesicles or the Golgi apparatus.

Newly initiated buds are filled with secretory vesicles measuring 80–100 nm (Matile *et al.*, 1969; Byers, 1981), and the ER marker proteins were found within some of the vesicles in the vicinity of these small buds (Figure 12c). In buds that are slightly larger, the ER structures, like those in the mother cell, become associated with the plasma membrane of the daughter cell. Thus, growing buds obtain the ER proteins, and perhaps functional ER membranes, at an early point in the cell cycle. The appearance of the Ypt1 protein (Segev *et al.*, 1988) and of the Kex2 protein (a marker of the yeast Golgi apparatus; Redding *et al.*, 1991) in emerging buds further suggests that daughter cells have a functioning secretory pathway from an early stage in their growth.

The mechanism that targets the ER and other membranous organelles to growing buds is as yet undetermined. However, the availability of marker proteins and immunolocalization techniques, such as those described here, provides the means for addressing this fundamental question in yeast cell growth. In particular, a variety of mutant yeast strains can be investigated to determine whether the mutations directly affect the morphology of the ER.

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