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Inhibition of Endoplasmic Reticulum (ER)-to-Golgi Transport Induces Relocalization of Binding Protein (BiP) Within the ER to Form the BiP Bodies

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Immunofluorescence staining of yeast cells with anti-binding protein (BiP) antibodies shows uniform staining of the endoplasmic reticulum (ER). We have found that overproduction of Sec12p, an ER membrane protein, causes a change of BiP distribution within the cell. Upon induction of Sec12p by the *GAL1* promoter, the staining pattern of BiP turns into bright dots scattering in the cell, whereas the staining of Sec12p remains to be the typical ER figure. Overproduction of other ER membrane proteins, HMG-CoA reductase or Sed4 protein, does not induce such relocalization of BiP. Pulse-chase experiments and electron microscopy have revealed that the overproduction of Sec12p inhibits protein transport from the ER to the Golgi apparatus. When the transport is arrested by one of the *sec* mutations that block the ER-to-Golgi step at the restrictive temperature, the BiP staining also changes into the punctate pattern. In contrast, the *sec* mutants that block later or earlier steps of the secretory pathway do not induce such change of BiP localization. These observations indicate that relocalization of BiP is caused by the inhibition of ER-to-Golgi transport. Using immunoelectron microscopy, we have found that the punctate staining is because of the accumulation of BiP in the restricted region of the ER, which we propose to call the "BiP body." This implicates existence of ER subdomains in yeast. A vacuolar protein, proteinase A, appears to colocalize in the BiP body when the ER-to-Golgi transport is blocked, suggesting that the BiP body may have a role as the site of accumulation of cargo molecules before exit from the ER.

INTRODUCTION

The exocytic pathway of eukaryotic cells comprises a set of organelles. The endoplasmic reticulum (ER) is the first site of the biosynthetic membrane flow in the pathway. Newly synthesized proteins enter the pathway in the ER and travel in a vectorial fashion through the Golgi apparatus to their destination. As the organelle of entrance into the exocytic pathway, the ER fulfills a variety of tasks for newly synthesized proteins, such as translocation across the membrane, folding and modification, and packaging into the vesicles destined to the Golgi apparatus. In addition to functions in these secretory processes, the ER is a site of synthesis and assembly of lipids.

These diverse functions of the ER could be accomplished by the compartmentalization of the organelle. In animal cells, the presence of ER subregions has long been recognized (see Sitia and Meldolesi, 1992). The classically defined ER subcompartments are the rough ER, the smooth ER, and the nuclear envelope. A subregion of the ER specialized for vesicle formation has also been postulated (Palade, 1975; Hobman *et al.*, 1992).

In the yeast *Saccharomyces cerevisiae*, only two subcompartments of the ER have been distinguished in terms of morphology, the nuclear envelope and the peripheral ER (Preuss *et al.*, 1991). Although the recent development of electron microscopy such as the freeze-substituted fixation method (Baba *et al.*, 1989; Sun *et al.*, 1992) has enabled us to observe attachment of ri-

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bosomes to the ER membrane, clear distinction between the smooth and the rough ER is still difficult because of the high ribosome density in the yeast cytoplasm. By immunofluorescence microscopy, even the discrimination between the nuclear envelope and the peripheral ER is difficult. One needs antibodies against components of the nuclear pore complex to show the exclusive staining of the nuclear envelope (Davis and Fink, 1990; Nehrbass *et al.*, 1990). A variety of antibodies raised against yeast ER proteins, such as Kar2p (or binding protein [BiP])[1] (Rose *et al.*, 1989; Preuss *et al.*, 1991), Sec12p (Nishikawa and Nakano, 1993), Sec62p (Deshaies and Schekman, 1990), Sec63p (Feldheim *et al.*, 1992), and dolichol-phosphate-mannose synthase (Preuss *et al.*, 1991), label both the nuclear envelope and the peripheral ER. The same is true with immunoelectron microscopy (Preuss *et al.*, 1991). In all these cases, the staining of the membranes looks uniform, suggesting that these ER proteins are evenly distributed in the ER.

The yeast *SEC12* gene product, Sec12p, is a type-II integral membrane protein, which is almost exclusively localized in the ER (Nakano *et al.*, 1988; d'Enfert *et al.*, 1991a; Nishikawa and Nakano, 1993). Electron microscopy of the *sec12* mutant and in vitro ER-to-Golgi transport analyses have shown that this protein functions in the formation of the transport vesicles from the ER (Kaiser and Schekman, 1990; Rexach and Schekman, 1991; Oka and Nakano, 1994). Although Sec12p is localized to the ER in the steady state, modification of Sec12p by an early Golgi enzyme has suggested the cycling of Sec12p between the ER and the Golgi apparatus (Nakano *et al.*, 1988; d'Enfert *et al.*, 1991a; Nishikawa and Nakano, 1993). During the course of analyses of dynamic movement of Sec12p between the ER and the Golgi, we found that extreme overproduction of Sec12p induced a change of the localization of BiP (the product of the *KAR2* gene). BiP is a member of the hsp70 superfamily and is normally a soluble resident in the ER lumen. In this paper, we will show that the change of BiP localization is because of the accumulation of BiP within the restricted region of the ER, the BiP body, and that the change occurs when the protein transport from the ER to the Golgi apparatus is blocked.

[1] Abbreviations used: BFA, brefeldin A; BiP, binding protein; CPY, carboxypeptidase Y; PAGE, polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; PRA, proteinase A.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

Yeast strains used in this study are listed in Table 1. Cells were usually grown in the YPD medium containing 1% yeast extract (Difco, Detroit, MI), 2% polypeptone (Nihon Seiyaku, Tokyo, Japan), and 2% glucose. In the experiments of induction of the *GAL1* promoter, cells were grown in Wickerham's minimal medium (Wickerham, 1946) containing 2% raffinose with appropriate supplements (Rose *et al.*, 1990). In labeling experiments, sulfate salts in minimal media were replaced by chlorides.

Plasmids and DNA Manipulations

Yeast multicopy plasmids pSQ326 (*URA3*) and pYO324 (*TRP1*) have been described elsewhere (Qadota *et al.*, 1992; Ohya *et al.*, 1991). The *GAL1-SEC12* gene was constructed as described previously (d'Enfert *et al.*, 1991a) and subcloned into pSQ326 to give pSHY13-2. The *SED4* gene was subcloned from one of the ordered clones of the yeast chromosome III (Yoshikawa and Isono, 1990) (a gift from Katsumi Isono, Kobe University, Hyogo, Japan) into pBluescriptII SK$^+$ (Stratagene, La Jolla, CA). To construct the *GAL1-SED4* gene, a *Bsp*HI site was first created at the start codon of *SED4* using an oligonucleotide 5'-CTAGCATAATCATGAGTGGCAAC-3'. Using this site, the *SED4* promoter was replaced by the *GAL1* promoter (the 810-base pair *Bam*HI-*Nco* I fragment of pGCAMC1) (Sun *et al.*, 1991). The *GAL1-SED4* gene was subcloned into pSQ326 to give pSHY14-5. The *GAL1-SEC23* gene was constructed using the *Nco* I site at the start codon of the *SEC23* gene (Hicke and Schekman, 1989) and subcloned into pYO324 to give pSHY15-1. pANY2-19 (*GAL1-SAR1*) was described previously (Nakano and Muramatsu, 1989). A yeast plasmid pJR435

Table 1. Yeast strains

Strain	Genotype	Source
YPH501	*MATa/MATα ura3/ura3 lys2/lys2 ade2/ade2 trp1/trp1 his3/his3/leu2/leu2*	Sikorski and Hieter (1989)
ANY200	*MATa/MATα ura3/ura3 trp1/trp1/his3/his3 his4/his4 leu2/leu2*	This study
SEY5016	*MATα sec1-1 ura3 leu2*	S.D. Emr
MBY10-7AD	*MATa/MATα sec12-4/sec12-4 ura3/ura3 trp1/trp1 his3/his3 his4/his4 leu2/leu2*	This study
MBY3-15AD	*MATa/MATα sec13-1/sec13-1 ura3/ura3 his3/his3 his4/his4 leu2/leu2*	This study
ANS14-2C	*MATα sec14-3 ura3 his leu2*	Nakano and Muramatsu (1989)
MBY4-1AD	*MATa/MATα sec16-2/sec16-2 ura3/ura3 trp1/trp1 his3/his3 his4/his4*	This study
MBY11-1D	*MATα sec17-1 ura3 trp1 his leu2*	M. Bernstein
MBY12-6DD	*MATa/MATα sec18-1/sec18-1 ura3/ura3 trp1/trp1 his3/his3 his4/his4 leu2/leu2*	This study
MBY5-2A	*MATa sec20-1 ura3 trp1*	M. Bernstein
MBY6-4D	*MATα sec21-1 ura3 trp1 his leu2*	M. Bernstein
MBY13-2D	*MATa sec22-3 ura3 trp1*	M. Bernstein
MBY8-20CD	*MATa/MATα sec23-1/sec23-1 ura3/ura3 trp1/trp1 his3/his3 his4/his4 leu2/leu2*	This study
MBY7-5CD	*MATa/MATα sec53-6/sec53-6 ura3/ura3 trp1/trp1 his3/his3 his4/his4 leu2/leu2*	This study
TOY224	*MATα sar1::HIS3 pep4::ADE2 ura3 lys2 ade2 trp1 his3 leu2* YCp[*sar1-2 TRP1*]	T. Oka

containing the *GAL1-HMG1* gene (Wright *et al.*, 1988) was a gift from Robin Wright (University of Washington, Seattle, WA).

Escherichia coli strains SCS1 and XL1-Blue (Stratagene, La Jolla, CA) were used for the propagation of plasmids. DNA manipulations including restriction enzyme digestions, ligations, plasmid isolation, and *E. coli* transformation were carried out by standard methods (Sambrook *et al.*, 1989). Yeast transformation was performed by the quick method using lithium thiocyanate (Keszenman-Pereyra and Hieda, 1988).

Antibodies

The rabbit anti-BiP antiserum, the rabbit anti-protein disulfide isomerase (PDI) antiserum, and the affinity purified rabbit anti-HMG CoA reductase antibody used in this study were gifts from Mark D. Rose (Princeton University, Princeton, NJ), Takemitsu Mizunaga (Keisen College, Kanagawa, Japan), and Robin Wright (University of Washington, Seattle, WA), respectively. An anti-Sec12p mouse monoclonal antibody was prepared against lacZ-Sec12 hybrid protein (Nakano *et al.*, 1988) as described previously (Nishikawa *et al.*, 1990). A rabbit anti-carboxypeptidase Y (CPY) antiserum and an anti-proteinase A mouse monoclonal antibody were prepared as described (Stevens *et al.*, 1982; Nishikawa *et al.*, 1990). The fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit IgG antibody $F(ab')_2$ fragment, FITC-conjugated sheep anti-mouse IgG antibody $F(ab')_2$ fragment, and rhodamine-conjugated sheep anti-mouse IgG antibody $F(ab')_2$ fragment were from Boehringer Mannheim Yamanouchi (Tokyo, Japan). A rabbit anti-mouse IgG antibody and a mouse anti-rabbit IgG antibody were from Jackson Immunoresearch Laboratories (West Grove, PA). The colloidal gold-conjugated anti-rabbit goat antibody was from BioCell Research Laboratories (Cardiff, UK).

Immunofluorescence Microscopy

Immunofluorescent staining of yeast cells was performed as described previously (Nishikawa and Nakano, 1991) with minor modifications. Cells were fixed by adding potassium phosphate (pH 6.5) and formaldehyde to cultures at the final concentrations of 0.1 M and 5% (wt/vol), respectively. After 60 min with agitation, cells were collected by centrifugation, resuspended in 0.1 M potassium phosphate (pH 6.5) and 5% formaldehyde, and further incubated at 23°C for 60 min. The fixed cells were spheroplasted and attached to polylysine-coated multiwell slides (Flow Laboratories, Maclean, VA). For double-label immunofluorescence experiments, serial antibody incubations were performed as follows: 1) 1:500 dilution of anti-proteinase A mouse monoclonal antibody (ascite fluids), 2) 1 μg/ml rabbit anti-mouse IgG antibody, 3) 10 μg/ml mouse anti-rabbit IgG antibody, 4) 1:5000 dilution of rabbit anti-BiP antibody, 5) 1:10 dilution of FITC-conjugated sheep anti-rabbit IgG antibody $F(ab')_2$ fragment, 6) and 1:10 dilution of rhodamine-conjugated sheep anti-mouse IgG antibody $F(ab')_2$ fragment.

Electron Microscopy

Preparation of thin sections of yeast cells by the freeze-substituted fixation method was carried out as described (Sun *et al.*, 1992). For immunoelectron microscopy, cells were fixed with acetone only (without OsO_4) and embedded in the LR White resin (Polysciences, Warrington, PA). The resin was polymerized by UV irradiation at −35°C for 2 d. Immunoelectron microscopy with aldehyde-fixed yeast cells were also carried out as described by Preuss *et al.* (1991). Observations were made on a JEOL200CX electron microscope (JEOL USA, Peabody, MA) at 100 kV.

Pulse-Chase Experiments

Metabolic labeling of yeast cells with Tran^{35}S-label (ICN Biomedicals, Costa Mesa, CA) and preparation of the cell extracts were performed as described previously (Nishikawa and Nakano, 1991). Immunoprecipitation and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography were also as described previously (Nishikawa *et al.*, 1990).

RESULTS

Overproduction of Sec12p Induces a Change of Immunofluorescence Staining of BiP

The *KAR2* gene product, BiP, is an ER-resident soluble protein (Rose *et al.*, 1989). When wild-type cells are prepared for immunofluorescence microscopy using anti-BiP antibodies, one can observe staining of nuclear envelope and peripheral ER, which is typical for yeast ER proteins (Figure 1a). ER is stained uniformly, indicating that BiP resides almost everywhere in the ER. We found that overproduction of Sec12p, an integral ER membrane protein, dramatically changed the distribution of BiP. We grew yeast cells harboring a multicopy plasmid containing the *GAL1-SEC12* gene to an early log phase in a medium containing 2% raffinose

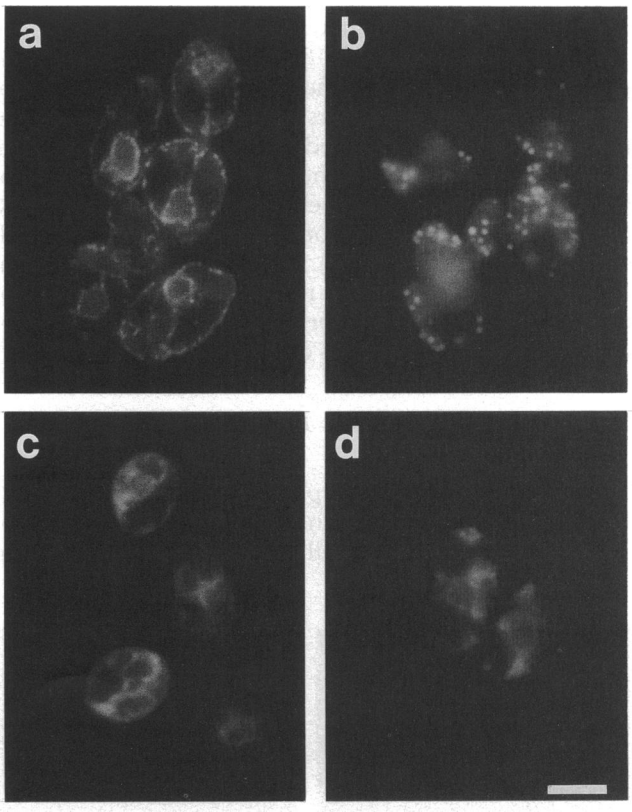

Figure 1. Overproduction of Sec12p induces relocalization of BiP. Cells of YPH501/pSQ326 (vector, a) and YPH501/pSHY13-2 (*GAL1-SEC12*, b–d) were grown at 30°C to an early log phase in minimal medium containing 2% raffinose and 0.5% casamino acids with appropriate supplements. Galactose was added to the final concentration of 3% to induce the expression of the *GAL1* promoter to overproduce Sec12p. After 3 h cells were fixed and subjected to immunofluorescence microscopy using anti-BiP (a and b), anti-Sec12p (c), and anti-PDI (d) antibodies. Bar, 2 μm.

and then induced expression of the *SEC12* transcript by the addition of galactose. Within 3 h of induction of Sec12p, the typical ER staining pattern with the anti-BiP antibody completely disappeared, and instead many bright dots emerged in the cell (Figure 1b). This was not because of the fragmentation of the ER, because antibodies to other ER proteins, such as PDI or Sec12p, stained the ER uniformly (Figure 1, c and d). In the staining of these latter two, however, we observed the proliferation of the ER membrane, as will be described later.

This change of BiP staining was not a general result of overproduction of ER membrane proteins. Overproduction of HMG-CoA reductase encoded by the *HMG1* gene was reported to induce proliferation of nuclear-associated stacked membranes, karmellae (Wright *et al.*, 1988). In Figure 2a, karmellae are visualized as HMG-CoA-reductase–positive membrane structures that partly encircle the nucleus. However, this overproduction did not result in the alteration of BiP staining (Figure 2b). Recently, DNA sequencing analysis of the yeast chromosome III revealed the presence of an open reading frame called YCR67c, which encodes a protein with a striking similarity to Sec12p (Oliver *et al.*, 1992). This gene has also been identified as *SED4*, which is one of the multicopy suppressors of the *erd2* null mutant (Hardwick *et al.*, 1992). Sed4p contains the C-terminal HDEL sequence, which should function as an ER localization signal, and is believed to reside in the ER. However, overexpression of Sed4p did not alter the BiP staining, either (Figure 2c). These results indicate that the relocalization of BiP is not because of the overproduction of ER membrane proteins in general but is specific to the overproduction of Sec12p.

Overproduction of Sec12p Inhibits ER-to-Golgi Transport

The effects of Sec12p overproduction were further analyzed. We found that enormous overexpression of Sec12p was toxic to the cell. Wild-type cells harboring a multicopy plasmid carrying the *GAL1-SEC12* construct could not grow on a galactose medium. At 3 h after the induction of the *GAL1* promoter, the amount of Sec12p was about 300-fold increased as judged by immunoblotting. As shown above, we observed proliferation of the ER membrane in these cells by immunofluorescence microscopy. We next analyzed the morphology of organelles in the growth-arrested cells by electron microscopy using the freeze-substituted fixation method. Figure 3 shows an electron micrograph of a typical cell 3 h after the induction of Sec12p by the *GAL1* promoter. Massive accumulation of the ER membrane is forming an extensive net-like structure. The tubules in the network are apparently packed together in hexagonal arrays, which look like the crystalloid ER observed in mammalian UT-1 cells overproducing HMG-CoA reductase (Orci *et al.*, 1984). Frequently, these net-like structures are in direct continuity with the nuclear envelope. When we used the conventional aldehyde fixation method, the network structure no longer persisted, and we could only observe aberrant accumulation of the ER membrane (see Figure 8A). Unlike the karmellae or the crystalloid ER, the net-like structure induced by Sec12p overproduction is probably fragile and was destroyed during the aldehyde fixation. In any case, such an extensive change of the ER structure is likely to affect the functions of the ER.

Recently, Pelham and coworkers reported that the increased gene dosage of *SEC12* by a multicopy vector slowed down the rate of ER-to-Golgi transport of a vacuolar enzyme, CPY (Hardwick *et al.*, 1992). This may well be the case in our *GAL1*-induced overproduction of Sec12p. This was confirmed by a pulse-chase analysis of CPY. Cells containing the *GAL1-SEC12* gene on a multicopy plasmid were grown to an early log phase, and the expression of the *SEC12* gene was induced by the addition of galactose. After 2.5 h, cells were pulse-labeled for 2 min with ^{35}S-labeled amino acids and chased. CPY was immunoprecipitated from the cell extracts and analyzed by SDS-PAGE and fluorography. In the Sec12p-overproducing cells, maturation of CPY

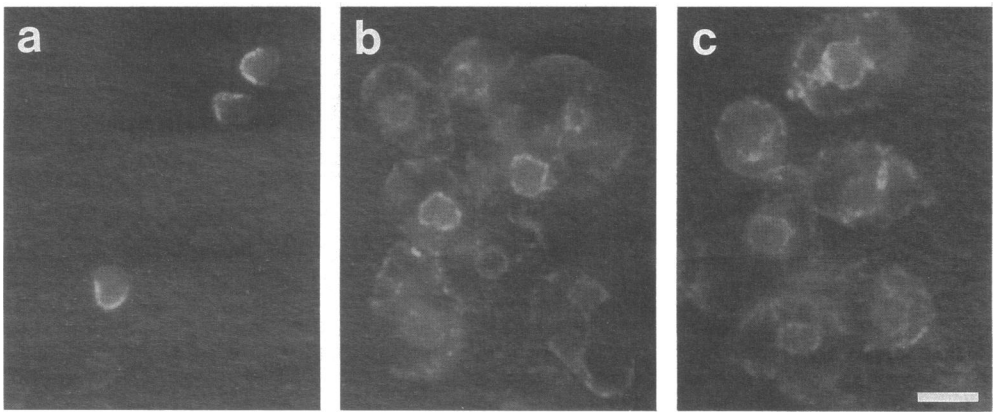

Figure 2. BiP relocalization was not induced by overproduction of other ER membrane proteins. The same experiments as in Figure 1 were done for cells of YPH501/pJR435 (*GAL1-HMG1* to overproduce HMG-CoA reductase, a and b) and YPH501/pSHY14-5 (*GAL1-SED4* to overproduce Sed4p, c). Immunofluorescence microscopy was performed using anti-HMG-CoA reductase (a) and anti-BiP (b and c) antibodies. Bar, 2 μm.

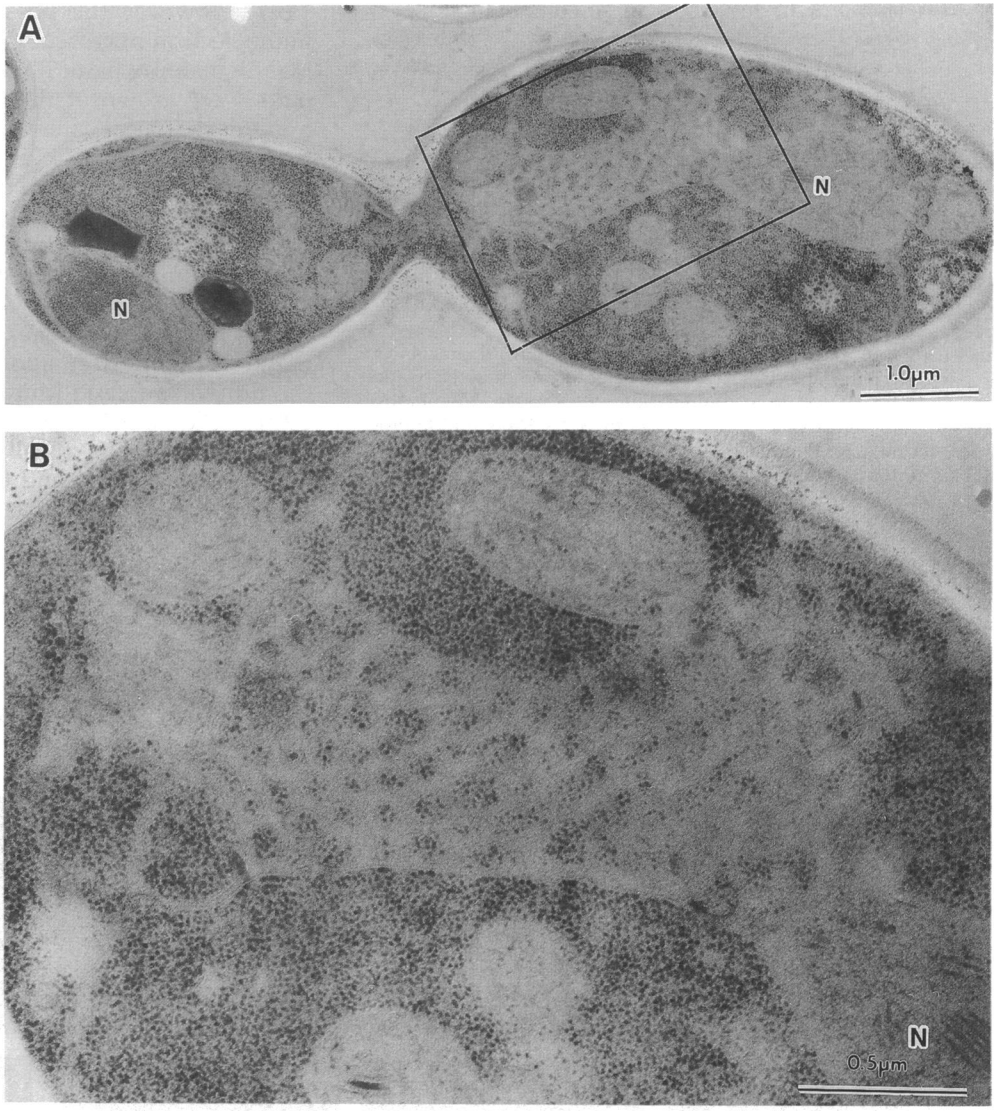

Figure 3. Electron micrographs of a cell that overproduces Sec12p by the *GAL1* promoter. Cells of YPH501/pSHY13-2 were incubated as in Figure 1 and fixed by the freeze substitution technique. (B) Magnification of the region boxed in A. N, nucleus.

was greatly inhibited (Figure 4). The majority of the CPY molecules remained as the ER form (p1) after 10 min of the chase, and the p1 form was still observed after 60 min chase. On the other hand, p1 CPY was converted to the Golgi form (p2) and further to the mature (vacuolar) form within 30 min of chase in the cells containing the vector alone. We could hardly detect the p2 form of CPY in the Sec12p-overproducing cells, suggesting that the transport from the ER to the Golgi was the rate limiting step and that the molecules that went beyond this step could reach the vacuole quickly. These results clearly indicate that overproduction of Sec12p inhibits the ER-to-Golgi transport. Overproduction of Sed4p slightly retarded the ER-to-Golgi transport of CPY; after a 10 min of chase, the amount of p1 CPY was larger than that of p2 CPY. However, the extent of the inhibition was only marginal.

Block of ER-to-Golgi Transport Induces Relocalization of BiP

From the results presented above, we hypothesized that the change of the BiP staining was induced by the block of ER-to-Golgi transport. This hypothesis was tested by immunofluorescence microscopy using various *sec* mutants. *sec* mutants grown at the permissive temperature were incubated at the restrictive temperature for 2 h, fixed, and prepared for immunofluorescence microscopy. To our surprise, the change of BiP staining was observed in all the ER-to-Golgi *sec* mutants we tested (Figure 5). For example, when ER-to-Golgi transport was blocked by the *sec18* mutation, ER staining with the anti-BiP antibody was changed to the dot pattern (Figure 5f). The staining patterns of these *sec* mutants are very similar to what we observed on the Sec12p overproducer. In most cases in Figure 5, we can count

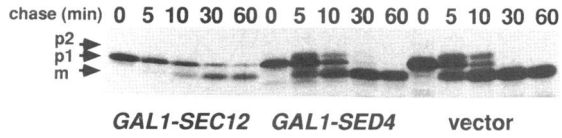

Figure 4. Overproduction of Sec12p inhibits the ER-to-Golgi transport. Cells of YPH501/pSHY13-2 (*GAL1-SEC12*), YPH501/pSHY14-5 (*GAL1-SED4*), and YPH501/pSQ326 (vector) were grown at 30°C in minimal medium containing 2% raffinose and 0.5% casamino acids. Galactose (3%) was added to induce the GAL1 promoter. After 2.5 h of incubation at 30°C, cells were collected by centrifugation, resuspended in minimal medium containing 3% galactose, and further incubated for 30 min at 30°C. Cells were pulse-labeled with Tran^{35}S-label for 2 min and chased for indicated times (min). Extracts were prepared from the labeled cells and subjected to immunoprecipitation with an anti-CPY serum in combination with Protein A-Sepharose. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. p1 and p2, ER- and Golgi-form of precursor CPY, respectively; m, mature form of CPY.

15–20 dots per cell. One may realize that the BiP staining of *sec20* appears somewhat different from others (Figure 5g). In *sec20*, BiP positive bodies were seen as a smaller number of large spots in the cytoplasm. This staining pattern might look like the staining of the Golgi apparatus (see, for example, Nishikawa and Nakano, 1991). However, this possibility was dismissed by immunoelectron microscopy as will be shown later. Normal ER staining pattern was observed when these mutants were grown at the permissive temperature. *sec* mutants that block later steps of the secretory pathway, such as *sec1* and *sec14*, did not induce such change of BiP staining at the restrictive temperature (Figure 6, a and b), indicating that the change of BiP staining was the result of the block of ER-to-Golgi transport, not of the general arrest of secretion.

Sec12p functions as a GDP-GTP exchange factor of Sar1p (Barlowe and Schekman, 1993), a small GTPase required for the formation of transport vesicles from the ER (Nakano and Muramatsu, 1989; d'Enfert *et al.*, 1991b; Oka and Nakano, 1994). Sar1p is localized on the ER membrane (Nishikawa and Nakano, 1991). Sar1p in the GTP-bound form functions in the formation of transport vesicles, and GTP hydrolysis by Sar1p takes place on the vesicles after they are released from the ER (Oka and Nakano, 1994). Another regulatory protein, Sec23p, plays a role as a GTPase-activating protein specific for Sar1p (Yoshihisa *et al.*, 1993). We found that overproduction of Sec23p also caused the relocalization of BiP (Figure 7b). Overproduction of Sar1p itself was without effect, however (Figure 7c).

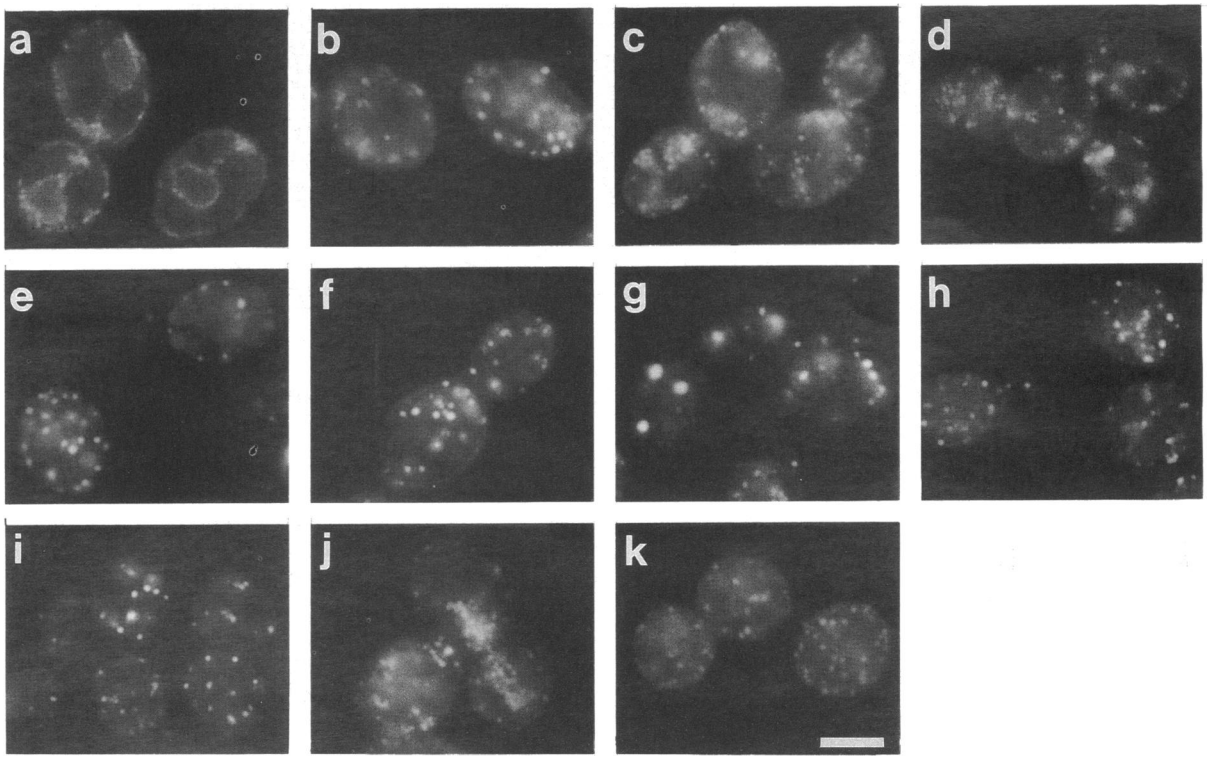

Figure 5. Block of ER-to-Golgi transport induces relocalization of BiP. Cells of wild-type (a) and mutants that block ER-to-Golgi transport at the restrictive temperature (b–k) were grown to an early log phase at 24°C in YPD medium. Cells were incubated at 37°C for 2 h and then fixed and subjected to immunofluorescence microscopy with the anti-BiP antibody. a, *SEC*$^+$; b, *sec12*; c, *sec13*; d, *sec16*; e, *sec17*; f, *sec18*; g, *sec20*; h, *sec21*; i, *sec22*; j, *sec23*; k, *sar1*. Bar, 2 μm.

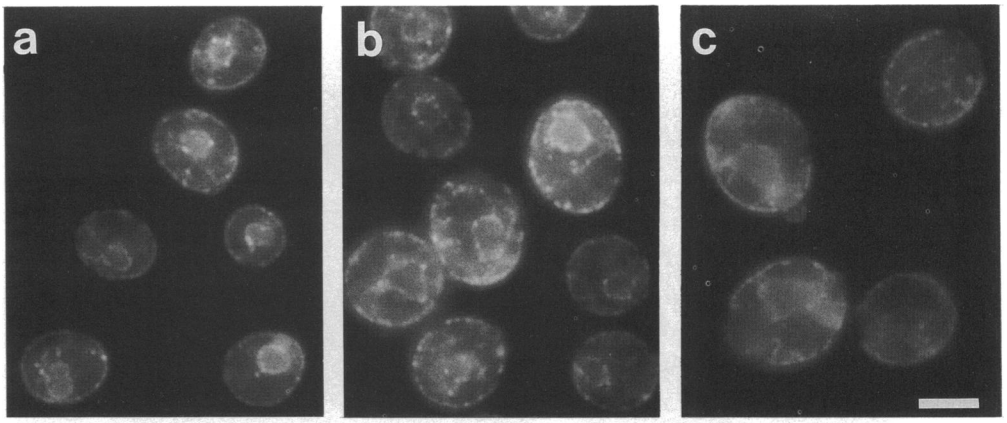

Figure 6. BiP relocalization was not induced by inhibition of other steps of the secretory pathway. Cells of *sec1* (a), *sec14* (b), and *sec53* (c) mutants were grown at 24°C and further incubated at 37°C for 2 h. Cells were fixed and subjected to immunofluorescence microscopy using the anti-BiP antibody. Bar, 2 μm.

Perhaps the overproduction of Sec12p or Sec23p would alter the ratio of Sar1p-bound guanine nucleotides, which may cause the imbalance of Sar1p cycling and thus inhibit the ER-to-Golgi transport. In contrast, the overproduction of Sar1p may not be deleterious by itself.

Accumulation of secretory proteins in the ER was reported to lead to an increased level of the *KAR2* mRNA (Normington *et al.*, 1989; Rose *et al.*, 1989). It is possible that accumulation of proteins within the ER lumen causes the induction of BiP that eventually resulted in its relocalization. The *sec53* mutant has a defect in an early step of protein glycosylation (Kepes and Schekman, 1988). In this mutant, incompletely glycosylated precursor proteins accumulate in the ER and thus the *KAR2* mRNA and its product, BiP, are highly induced (Normington *et al.*, 1989; Rose *et al.*, 1989). When this mutant was shifted to the restrictive temperature, however, no change of the BiP staining pattern was observed (Figure 6c), indicating that the BiP relocalization was not the consequence of its own induction. Defects in the vesicular traffic machinery seem to directly trigger the relocalization of BiP.

Analysis of BiP-positive Structures (BiP Bodies) by Immunoelectron Microscopy

The BiP-positive dot-like structures were further analyzed by immunoelectron microscopy. Yeast cells were fixed with aldehyde, dehydrated, and embedded in the LR White resin as described in Preuss *et al.* (1991). Thin sections were incubated with the anti-BiP antibody and then with the colloidal gold-conjugated secondary antibody. In the Sec12p-overproducing cells, remarkably dense clusters of gold particles were seen in the midst of the exaggerated ER structures (Figure 8A, arrow). The average diameter of these structures were ~200–300 nm. Such clusters of BiP staining were never observed in wild-type cells (Preuss *et al.*, 1991). Regions labeled with these gold clusters appear to represent the structure that we observed as the dot-like staining by immunofluorescence. Other compartments were not significantly labeled with the gold particles.

Similar results were obtained when ER-to-Golgi transport was blocked by *sec* mutants. Figure 8B shows the result of immunolabeling of the *sec18* mutant. In this mutant, we observed gold clusters again on the ER structure. Frequently, gold labeling was seen on the dilation of the ER or on the membrane-bounded structures emerging from the ER. This mutant accumulates small vesicles of 50 nm diameter that are believed to be the intermediate of the ER-to-Golgi traffic (Kaiser and Schekman, 1990). Neither these vesicles nor other membrane compartments were significantly labeled with the gold particles. The dense labeling with the anti-

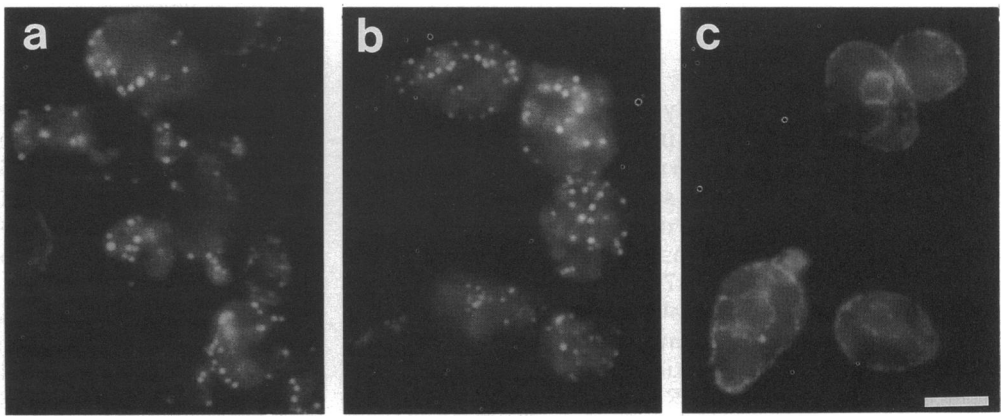

Figure 7. BiP relocalization was induced by overproduction of Sec12p and Sec23p but not Sar1p. Induction of proteins were achieved by incubating cells of YPH501/pSHY13-2 (*GAL1-SEC12*, a), YPH501/pSHY15-1 (*GAL1-SEC23*, b), and YPH501/pANY2-19 (*GAL1-SAR1*, c) in the presence of galactose as described in the legend of Figure 1. Cells were fixed and prepared for immunofluorescence microscopy using the anti-BiP antibody. Bar, 2 μm.

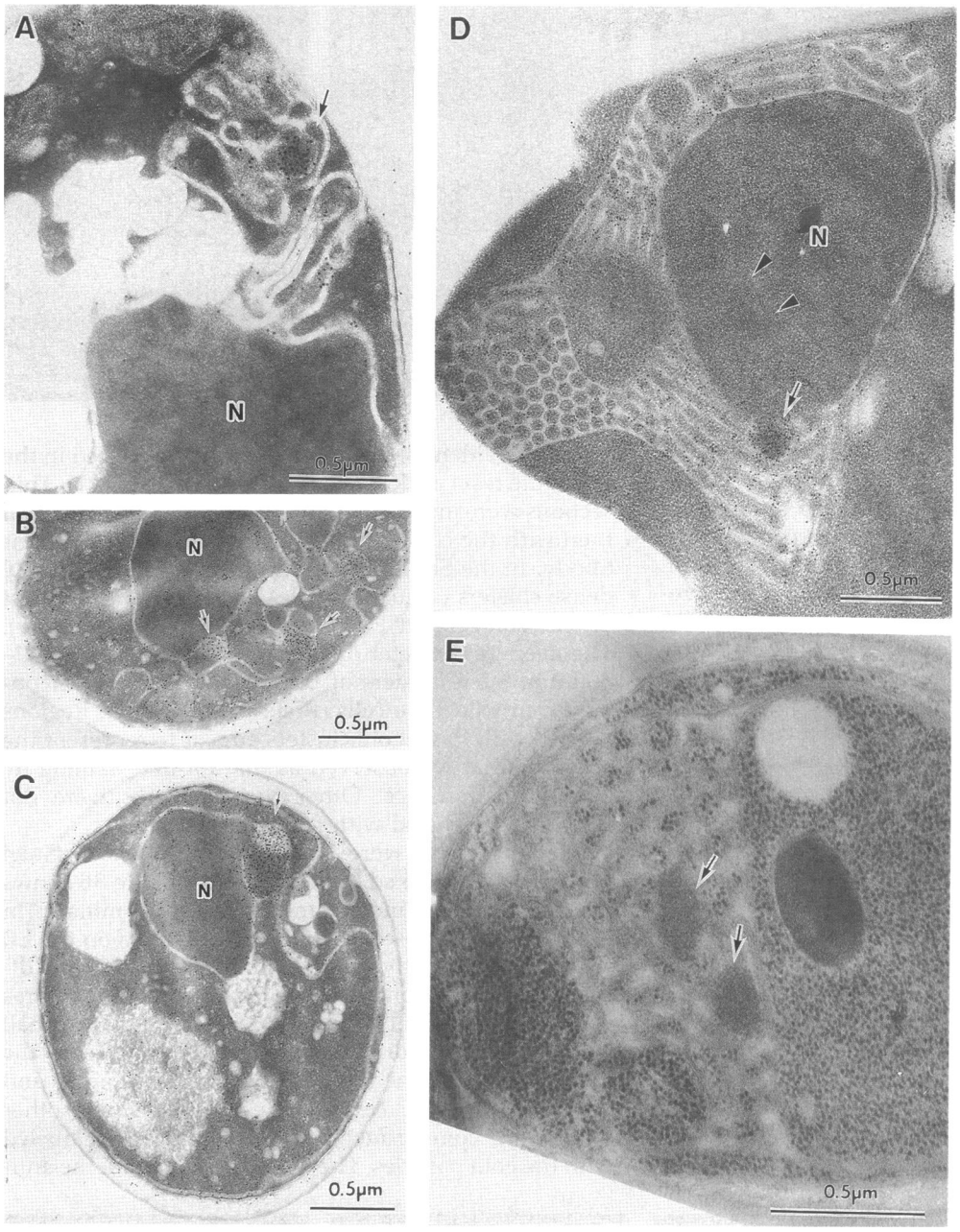

Figure 8. Electron microscopic localization of BiP in ER-accumulating cells. ER-to-Golgi transport was blocked either by overproduction of Sec12p (A, D, and E) or by incubation of *sec* mutants at the restrictive temperature (B and C) as described in Figures 1 and 6. Strains used were YPH501/pSHY13-2 (GAL1-SEC12, A, D, and E), MBY12-6DD (*sec18*, B), and MBY5-2A (*sec20*, C). Cells were fixed either with aldehyde (A–C) or by the freeze substitution technique (D), dehydrated, and embedded in the LR White resin. Thin sections were probed with the anti-BiP antibody and colloidal gold-conjugated secondary antibody. (E) Electron micrograph of a Sec12p-overproducing cell fixed by the freeze-substituted fixation method and embedded in Spurr's resin. Arrows in A–D indicate the ER regions of heavy gold labeling. Arrowheads in D note microtubules. Arrows in E point to electron dense regions within the ER lumen. N, nucleus.

BiP antibody was apparently restricted to the ER membrane. As described above, immunofluorescence staining of the *sec20* mutant with the anti-BiP antibody showed a slightly different staining pattern. A smaller number of large spots were observed in the cell, which was reminiscent of the yeast Golgi bodies as stained with antibodies against Sec7p or Kex2p (Franzusoff *et al.*, 1991; Nishikawa and Nakano, 1991; Redding *et al.*, 1991). However, the immunogold labeling was again restricted to the ER. The anti-BiP gold particles heavily labeled regions where the ER lumen dilates (Figure 8C). This structure is larger (300–500 nm) than the other cases and is very likely to represent the large spots observed in the immunofluorescence staining. All these observations indicate that the change of BiP staining by the inhibition of ER-to-Golgi transport was because of the relocalization of BiP to the restricted region within the ER.

For more precise analysis of the BiP-positive ER subregions, we tried to immunolabel sections of the Sec12p-overproducing cells that were fixed by the freeze-substitution method. For the preservation of an-

tigenicity, the osmium fixation step was omitted from the normal protocol. This technique was quite successful, and we could observe gold labeling of electron dense regions in the meshwork of the ER (Figure 8D, arrow). Such electron-dense ER regions were also observed by the normal freeze-substituted osmium-fixation method without immunogold labeling (Figure 8E). These regions were enclosed by the ER membrane and separated from the cytoplasm that was marked by ribosomes. The average size was in the range of 200 to 300 nm. We conclude that BiP is localized in a specialized subregion of the ER lumen under these conditions and would like to call this structure the BiP body.

Formation of the BiP Bodies Is Reversible

Secretory block by *sec* mutations is thermoreversible. Upon return to the permissive temperature, a large fraction of the accumulated secretory proteins are secreted to the cell surface (Novick *et al.*, 1980). We examined the fate of the BiP bodies after *sec* mutants were returned to the permissive temperature. Three *sec* mutants, *sec12*, *sec18*, and *sec20*, were once exposed to the restrictive temperature for 2 h and then further incubated at the permissive temperature. After another 2 h, cells were fixed and prepared for immunofluorescence microscopy. As shown in Figure 9, a and b, we found that the dots of the BiP bodies disappeared almost completely, and the continuous staining of the nuclear envelope and the peripheral ER reestablished in the *sec12* and *sec18* cells (compare with Figure 5, b and f.). The staining of the ER structures still looked exaggerated, suggesting that the ER-to-Golgi transport was not fully restored at this time point. The reversal of the BiP localization was also observed when the incubation at the permissive temperature was carried out in the presence of cycloheximide (Figure 9c). Although the extent of reversal was somehow affected by the drug, this would rule out the possibility that the BiP bodies were just degraded, and the reappearance of the continuous ER was due to the de novo synthesis of BiP. These results indicate that, when the block of ER-to-Golgi transport was released, the BiP molecules that had accumulated in the BiP bodies were redistributed throughout the ER. In the case of the *sec20* mutant cells, the punctate staining of the BiP bodies did not vanish completely during the 2-h incubation at the permissive temperature, perhaps because the BiP bodies of the *sec20* cells were especially magnificent (Figure 9d). However, we could clearly observe reduction of the size of the dots and signs of reappearance of the continuous ER staining (compare with Figure 5g). Taken together, we conclude that the formation of the BiP bodies is a reversible event. The BiP body is not a dead end structure but may have some physiological role in the secretory process.

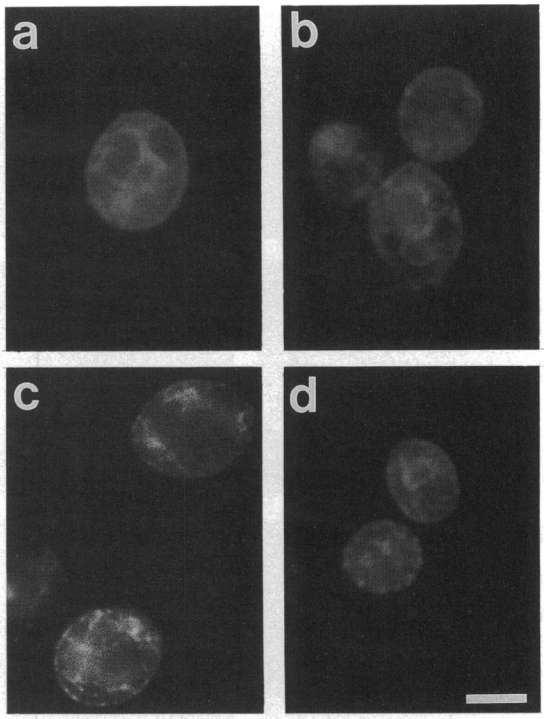

Figure 9. Formation of the BiP bodies is reversible. Cells of *sec12* (a), *sec18* (b and c), and *sec20* (d) mutants were once incubated at 37°C for 2 h and then returned to 24°C and further incubated for 2 h. In one experiment, 0.1 mg/ml cycloheximide was added at the end of the incubation at 37°C to prevent de novo synthesis of BiP when returned to 24°C (c). Cells were fixed and subjected to immunofluorescence microscopy using the anti-BiP antibody. Compare with Figure 5, b, f, and g. Bar, 2 μm.

Effects of the Block of ER-to-Golgi Transport on the Localization of PDI

To know whether the relocalization induced by ER-to-Golgi-block was specific to BiP, we examined the localization of another ER soluble protein, PDI. As described above, immunofluorescence staining of the Sec12p-overproducer with the anti-PDI antibody showed only the ER pattern (see Figure 1d). We first thought that the block of ER-to-Golgi transport had no effect on the localization of PDI. However, analyses of *sec* mutants showed that the block of ER-to-Golgi transport also induced the relocalization of PDI to some extent (Figure 10). The effects of secretory block were apparently weaker than the case of BiP though. In a set of *sec* mutants that are defective in the formation of transport vesicles (*sec12*, *sec13*, *sec16*, *sec23*, and *sar1*), staining of the exaggerated ER membranes was predominant. The dot-like staining was also observed but only occasionally. In the rest of the ER-to-Golgi *sec* mutants (*sec17*, *sec18*, *sec20*, *sec21*, and *sec22*), the dot-like staining was eminent. Identification of these dot structures with the BiP bodies would require double staining of immunofluorescence and analysis by immunoelectron

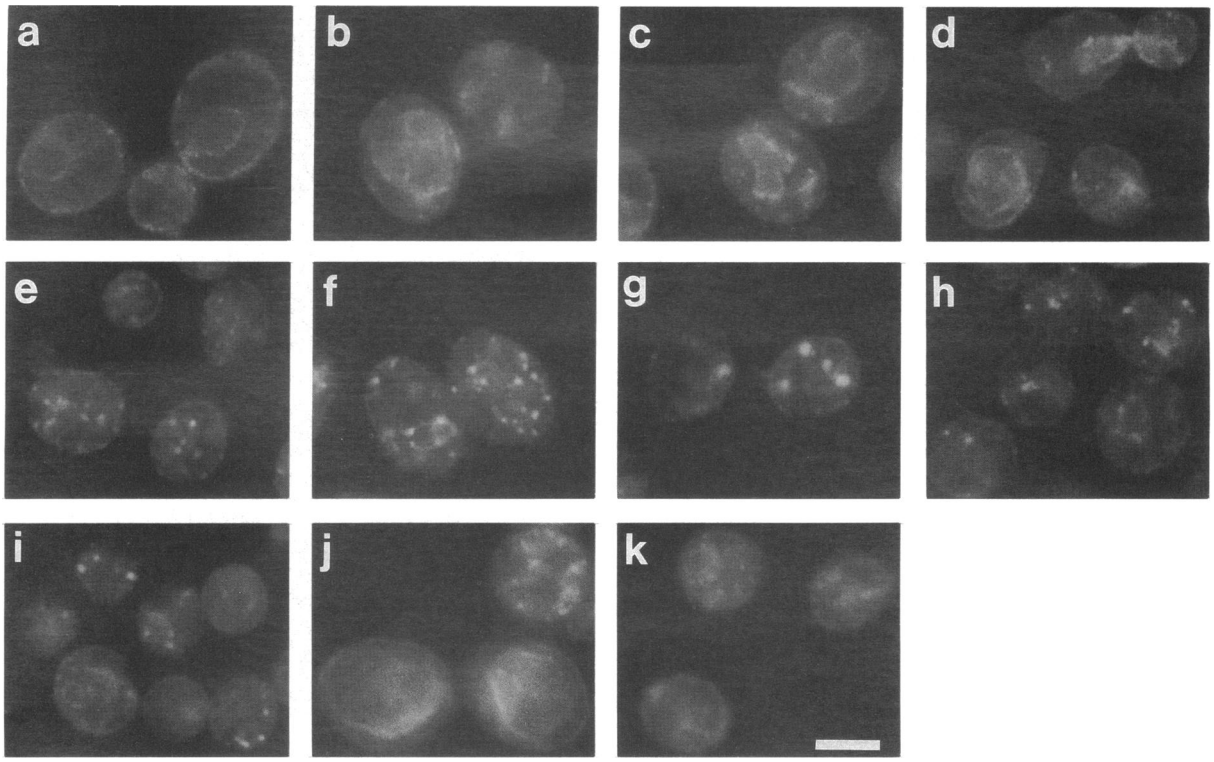

Figure 10. Effects of inhibition of ER-to-Golgi transport on the localization of PDI. Cells of wild-type (a) and ER-to-Golgi arrest mutants (b–k) were grown at 24°C in YPD medium. Log phase culture was transferred to 37°C and further incubated for 2 h. Cells were fixed and prepared for immunofluorescence microscopy with the anti-PDI antibody. a, *SEC*$^+$; b, *sec12;* c, *sec13;* d, *sec16;* e, *sec17;* f, *sec18;* g, *sec20;* h, *sec21;* i, *sec22;* j, *sec23;* k, *sar1.* Bar, 2 μm.

microscopy. However, the dot staining with the anti-PDI antibody appears always in the proximity of the ER membranes, and the pattern is quite similar to that of the BiP bodies.

Colocalization of a Cargo Protein with BiP in the BiP Bodies

To obtain a clue to understand the physiological role of the BiP bodies, we examined whether proteins that move through the secretory pathway in normal cells could colocalize in the BiP bodies or not. We performed double-label immunofluorescence microscopy using the anti-BiP antibody and several antibodies against yeast secretory and vacuolar proteins. Among the antibodies we tested, a monoclonal antibody against a vacuolar enzyme, proteinase A (PRA), worked well for the double staining experiment. Three *sec* mutants, *sec12, sec18,* and *sec20,* were incubated at the restrictive temperature for 2 h and then fixed and prepared for double-label immunofluorescence with anti-BiP and anti-PRA antibodies as described in MATERIALS AND METHODS (Figure 11). In wild-type cells, the two antibodies stained different compartments, ER and vacuoles, indicating that there is no crossreaction between these antibodies (a and b). When the *sec18* and *sec20* mutants were stained with the anti-PRA antibody, we observed clear staining of large dot-like structures (f and h), most if not all of which coincided with the BiP bodies that were stained with the anti-BiP antibody (e and g). The staining of the *sec20* cells with the anti-PRA antibody alone (i) gave a picture almost indistinguishable from that with the two antibodies (g and h), indicating that the staining of the large dots with the anti-PRA antibody was not because of the artifact of the double labeling. In the *sec12* mutant, punctate staining was not prominent as observed in the other mutants (d). This may be related to the fact that this mutant was less effective in the relocalization of PDI as described above. Even with this mutant, however, close examination of the two stainings reveals partial colocalization of PRA and BiP (c and d). At least, staining of the continuous ER by the anti-PRA antibody was not observed in any of these mutants. Thus, it appears as if the BiP body is the site where cargo molecules accumulate before exit from the ER.

DISCUSSION

Block of ER-to-Golgi Transport Induces Relocalization of BiP

In this paper, we report that the block of ER-to-Golgi transport causes dramatic relocalization of BiP. Upon

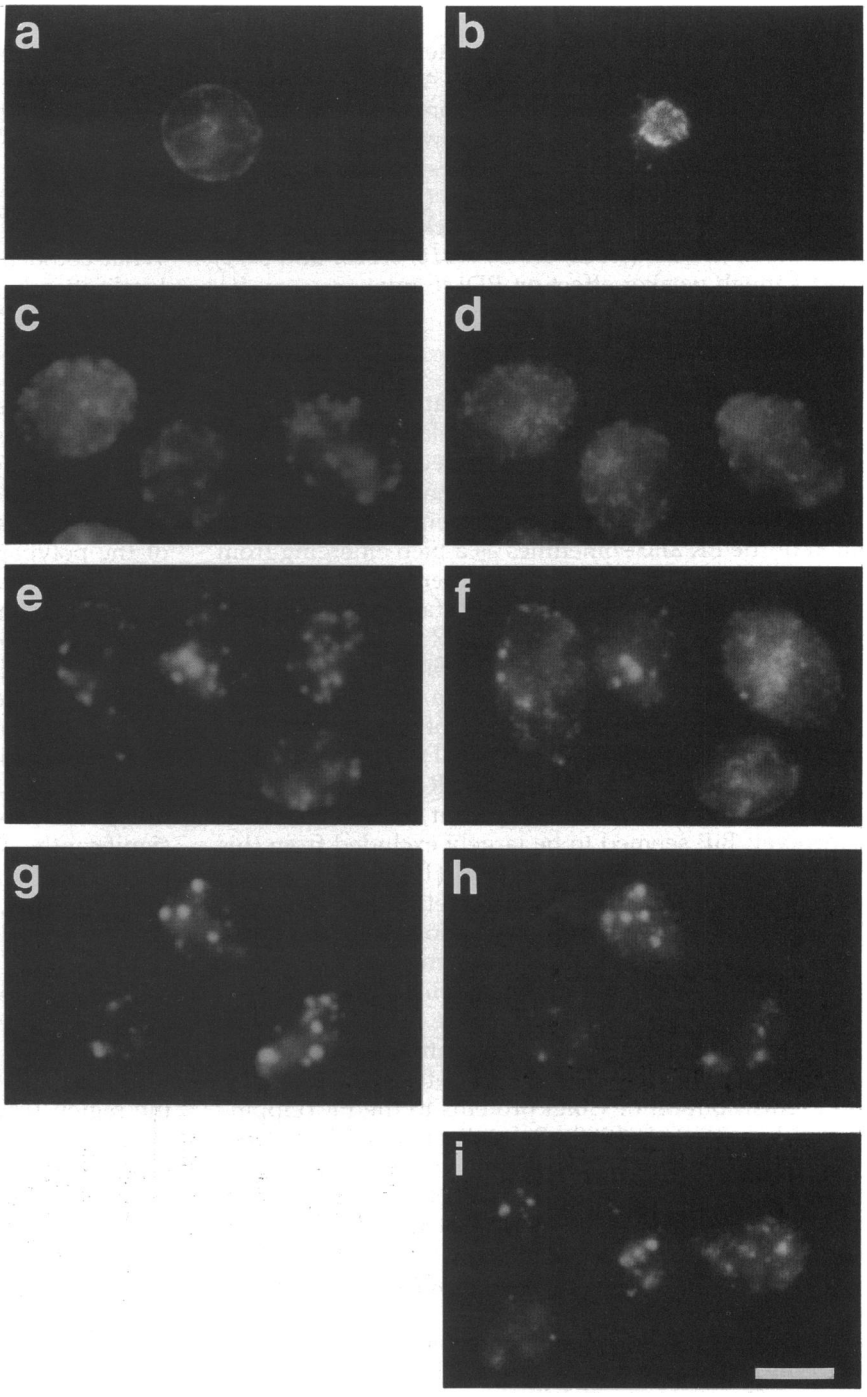

Figure 11. Colocalization of a vacuolar protein, PRA, with BiP in the BiP bodies. Cells of wild-type (a and b), *sec12* (c and d), *sec18* (e and f), and *sec20* (g–i) mutants were grown at 24°C in YPD medium. Log phase culture was transferred to 37°C and further incubated for 2 h. Cells were fixed and prepared for double-label immunofluorescence microscopy with the anti-BiP and anti-PRA antibodies (a–h). (i) Cells were stained only with the anti-PRA antibody. a, c, e, and g, fluorescein fluorescence corresponding to BiP; b, d, f, h, and i, rhodamine fluorescence corresponding to PRA. Bar, 2 μm.

onset of the transport block, BiP, a member of the hsp70 family that is ubiquitously distributed in the ER lumen in normal cells, accumulates in a distinct subregion of the ER. We first observed this phenomenon when Sec12p, an integral membrane protein in the ER, was greatly overproduced by the *GAL1* promoter. Because the overproduction of Sec12p turned out to inhibit protein transport from the ER to the Golgi, we examined various *sec* mutants and found that only the *sec* mutants that are defective in the ER-to-Golgi transport show similar relocalization of BiP. No *sec* mutants that block either later or earlier step of transport showed such a phenotype. Overproduction of other ER membrane proteins that does not lead to significant inhibition of ER-to-Golgi transport (such as HMG-CoA reductase or Sed4p) had no effect on the BiP localization. Another

ER lumenal protein, PDI, also exhibited similar relocalization in the ER, although the extent of accumulation appears lower than the case of BiP. Among *sec* mutants that block ER-to-Golgi transport, only a subset (*sec17, sec18, sec20, sec21,* and *sec22*) showed the change of PDI localization. Interestingly, these *sec* mutants are categorized as vesicle accumulators (Kaiser and Schekman, 1990). Other mutants that block formation of vesicles from the ER (*sec12, sec13, sec16, sec23,* and *sar1*) had a much weaker effect on PDI if any.

The BiP Bodies

The structure in which BiP accumulates apparently represents a part of the ER. Immunoelectron microscopy has shown that BIP is localized in electron dense bodies with the size of 200 to 500 nm. We named these structures BiP bodies. They are often seen in the dilated lumen of the ER and sometimes as a bleb emerging from the ER. A question arises on whether they bear resemblance to any known structures so far reported. In animal cells, the presence of distinct ER subcompartments has been frequently proposed (see Sitia and Meldolesi, 1992, for review). To our knowledge, however, there is no report on a specialized region of the ER in which BiP accumulates. Valetti *et al.* (1991) described Russel bodies that were formed from the ER in response to the expression of a nonsecreted mutant immunoglobulin. However, BiP seemed to be largely excluded from this structure. More recently, Orci *et al.* (1993) showed that treatment of pancreatic insulin-secreting cells with brefeldin A (BFA) resulted in the formation of "BFA bodies" that were derived from the ER cisternae. This structure concentrated nonclathrin coat proteins but was free of Sec23p. Unfortunately, the presence of BiP in the BFA bodies is unknown. In general, treatment with BFA is believed to cause inhibition of ER-to-Golgi transport and redistribution of Golgi proteins to the ER (Lippincott-Schwartz *et al.*, 1989). Relocalization of ER proteins has not been observed upon BFA treatment.

It is known that the accumulation of secretory proteins within the ER induces high level of BiP expression (Normington *et al.*, 1989; Rose *et al.*, 1989). However, our data indicate that the BiP relocalization is not because of the induction of BiP. The *sec53* cells, which accumulate underglycosylated proteins in the ER and accordingly induce BiP, are normal in the BiP distribution. One important difference between *sec53* and other ER-to-Golgi *sec* mutants is that the former probably retains an ability to secrete nonglycosylated proteins and in fact does not pile up the ER membrane (Ferro-Novick *et al.*, 1984). It appears that the complete block of vesicular traffic from the ER causes the concentration of BiP to the restricted region in the ER.

The formation of the BiP bodies is reversible. When the ER-to-Golgi transport is restored by the release of the *sec* block, the BiP bodies disappear and the localization of BiP returns to the uniform figure of the ER. In other words, the BiP body is not a dead-end structure induced by the transport block. Its formation may reflect some physiological process that is going on in normal cells.

Models of BiP Body Formation

What could the physiological meaning of the BiP relocalization be? It would be appropriate here to consider the role of BiP in the secretory pathway. As a member of the hsp70 family, BiP is believed to play a role as a molecular chaperone in the ER lumen. The basic idea is that BiP first binds to a nascent polypeptide chain during its translocation across the ER membrane, keeps the polypeptide from malfolding for some period of time (during modification and oligomerization, for example), and finally dissociates to complete the proper folding of the polypeptide. As proposed from the analyses of a mitochondrial hsp70, BiP may also fulfill a translocase function (Gambill *et al.*, 1993). In fact, some *kar2* mutations are known to cause block of ER translocation (Vogel *et al.*, 1990). What will happen to these events if the subsequent transport process is inhibited?

We can think of a few models to explain our observations. One is a random precipitation model. When the protein transport from the ER is arrested, the physiological environment in the ER lumen would change significantly. This might include alteration of pH, concentrations of ATP, Ca^{2+}, and many other ions, and so forth. Possibly, BiP cannot maintain its physicochemical properties under such a condition and may form a precipitate. Large aggregates of the BiP precipitates would eventually show up as the BiP bodies. This trivial possibility will be testable on purified BiP if the physiological change in the ER is reproduced in vitro.

The second possibility is that BiP is excluded from the region where secretory proteins wait for exit. It is established that the ER localization of BiP is achieved by the KDEL or HDEL sequence on its C-terminus (Munro and Pelham, 1987; Pelham *et al.*, 1988). Evidence is accumulating that this ER localization signal is recognized by a receptor molecule, Erd2p, that normally resides in the *cis* region of the Golgi and functions to send BiP back to the ER (Lewis *et al.*, 1990; Semenza *et al.*, 1990; Lewis and Pelham, 1992). When the K(H)DEL signal is deleted, BiP is secreted because of the lack of this recycling mechanism. However, even in the absence of the signal or in the *erd2* mutant, the rate of BiP secretion is very slow. In yeast, BiP leaves the ER with a half-life of 1.5 h without the HDEL signal (Hardwick *et al.*, 1990). Similar results have also been observed in animal cells (Munro and Pelham, 1987). Thus, there must be another mechanism that retards the exit of BiP from the ER. This mechanism might operate in the event of ER-to-Golgi transport block, that is, to keep BiP away from the accumulating secretory

proteins that are ready to exit ER. In other words, the BiP bodies may represent the site where ER resident proteins are sorted out from the bulk flow of secretion.

As mentioned above, it has been shown that BiP is required for protein translocation across the ER membrane (Vogel *et al.*, 1990; Sanders *et al.*, 1992). Genetic and biochemical analyses indicate that BiP interacts with Sec63p, an integral membrane component of the translocation machinery that contains a DnaJ-like domain (Feldheim *et al.*, 1992; Brodsky and Schekman, 1993; Scidmore *et al.*, 1993). However, BiP appears to be present in the ER lumen in a large excess over Sec63p (Brodsky and Schekman, 1993). It is unlikely that any aberrant association with the tranlocation machinery could explain the drastic relocalization of BiP.

The third model is on the contrary to the previous one. The BiP body may represent the exit site from the ER. It is conceivable that the ER-arrested proteins accumulate at the exit sites from the ER. For example, when the rubella virus E1 glycoprotein is expressed in Chinese hamster ovary cells in the absence of E2 glycoprotein, transport of E1 to the Golgi complex is arrested. E1 accumulates in a novel compartment that has been proposed to be the exit site from the ER (Hobman *et al.*, 1992). In the transport block we examined in this paper, BiP may stay complexed with accumulating secretory proteins and form an unusual structure, the BiP body.

A very important piece of information to distinguish the second and the third possibilities comes from a double staining experiment of the BiP bodies. A vacuolar enzyme PRA is known to travel the early secretory pathway to reach the vacuole. *sec* block that inhibits the ER-to-Golgi transport causes accumulation of PRA in the ER. As shown in Figure 11, the sites of PRA accumulation in the ER appear to be mostly coincident with the BiP bodies. We have been successful in double staining only with the anti-PRA antibody for some technical reasons, but if other cargo molecules also colocalize, it is quite likely that the BiP body is the site where leaving proteins accumulate. It could be either the exit site itself or a subcompartment in the ER where cargo proteins await to be packaged into the ER bud. The role of BiP in this structure may be to escort secretory proteins until they become transport-competent. Then the reason why BiP molecules do not accumulate at this site in normal cells would be that they are usually recycling between here and the bulk of the ER lumen.

Protein Sorting and Membrane Differentiation in the ER

The behavior of PDI upon inhibition of ER-to-Golgi transport is puzzling but intriguing. As described above, PDI also shows accumulation in the restricted regions of the ER, which we presume are identical to the BiP bodies. However, the accumulation of PDI is only seen in a subset of *sec* mutants, *sec17, sec18, sec20, sec21,* and *sec22.* These mutants are known to be defective in consumption of transport vesicles and accumulate them in addition to the ER membrane at the restrictive temperature. Other mutants (*sec12, sec13, sec16, sec23,* and *sar1*), as well as Sec12p overexpression that are defective in the formation of vesicles, affect only BiP's localization but not PDI's. The colocalization of PRA in the BiP bodies was also less prominent in *sec12* than in *sec18* or *sec20.* The ability of the ER membrane to form vesicles seems to influence the properties of the BiP bodies. Its meaning is unclear at the moment, but it may give us a clue to further understand the phenomenon we discovered. Possibly, vesicle-consumption mutants might accumulate in the vesicles some components that are recycling between the ER and the Golgi apparatus in normal cells. This could lead to deficiency of these components in the ER and give a more severe phenotype of the BiP body formation. Identification of such putative components would be an interesting question to be addressed in the future.

The mechanisms of ER protein localization appear to be much more complex than thought before. At least two distinct ways must be taken into account, retention and retrieval. We have started a genetic approach to identify cellular components that are required for ER membrane protein localization and found that two yeast genes are involved in the proper localization of Sec12p (Nishikawa and Nakano, 1993). We named these genes *RER1* and *RER2* for return to the ER or retention in the ER. There must be many more genes functioning in these processes. At least a set of ER proteins are presumably retained in the ER without the retrieval system. The BiP bodies could be involved in the sorting of resident proteins from leaving ones. Studies on how the BiP bodies are formed may provide important information about the mechanism of ER retention.

The BiP bodies we discovered should also represent an example of ER membrane differentiation. Like higher eukaryotic cells, yeast cells must have developed functional subdomains of the ER membrane. Further knowledge on the BiP bodies would be helpful to dissect the functions of this organelle.

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