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Regulation of COPII vesicle formation and protein sorting by the *BST* genes in *Saccharomyces cerevisiae*

by

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Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy
at the
Massachusetts Institute of Technology
June 1998

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ABSTRACT

Protein transport through the secretory pathway of eukaryotic cells consists of a series of vesicle budding, targeting, and fusion steps that deliver protein cargo from one membrane-bounded organelle to the next. Proper sorting of secretory proteins requires that cargo molecules be segregated from resident proteins as transport vesicles form. While significant progress had been made in discovering the mechanisms of protein transport, less is known about how proteins are sorted. The characterization of the *BST* genes and their role in protein sorting and COPII vesicle formation from the endoplasmic reticulum of the yeast *Saccharomyces cerevisiae* is the subject of this doctoral thesis.

The *BST* genes (*BST1, EMP24/BST2,* and *ERV25/BST3*) were isolated as bypass suppressors of mutations in the essential COPII gene *SEC13*. *bst* mutations also partially suppress the temperature-sensitivity of all other COPII *sec* mutants but are unable to suppress deletions in these genes. The suppression is specific, since *bst* mutations exacerbate the temperature-sensitivity of other ER to Golgi *sec* mutants. *In vitro* experiments demonstrate that Sec13p is not absolutely required for the budding of COPII-coated vesicles and suggest a role for Sec13p in stabilizing the coat. This stabilizing function is essential when *BST* genes are active (the wild-type situation), indicating that *BST* protein functions may destabilize the coat.

bst mutants also show defects in discriminating between cargo proteins and resident proteins during sorting into COPII vesicles: a subset of secretory proteins are transported with reduced kinetics in *bst* mutants, and resident proteins leak more rapidly from the ER in these strains. The transport of GPI-anchored proteins is particularly sensitive to mutations in *BST* genes. *BST1* encodes a resident ER membrane glycoprotein with a motif characteristic of lipases. *EMP24/BST2* and *ERV25/BST3* encode ER membrane proteins of the p24 family and are major constituents of COPII-coated vesicles.

These data suggest a model in which the *BST* proteins directly affect protein sorting into COPII vesicles by altering the lipid environment in the vicinity of forming vesicles and, as a consequence, indirectly influence the assembly of the COPII coat.

Thesis Supervisor: Chris A. Kaiser,
Title: Associate Professor of Biology

to my parents, Dr. Jon D. and Mary B. Erickson,
and to my wife Monicia Elrod-Erickson

ACKNOWLEDGDMENTS

A number of people have helped me in various ways during my tenure as a graduate student and I would like to take this opportunity to thank them.

First, I would like to thank my advisor Chris Kaiser for his guidance. In particular, he has helped me develop the skills necessary to think about and present my work in (what I hope is) a clear and concise fashion, a gift for which I shall remain eternally grateful.

I wish to thank the members of my thesis committee (Charlie Barlowe, Gerry Fink, Monty Krieger and Frank Solomon) for their interest, time, helpful advice and support.

Every member of the Kaiser lab, past and present, deserves thanks for helping create and maintain a working environment that was both fruitful and enjoyable. Thanks to Steve Bickel, Esther Chen, Elizabeth Chitouras, Michelle Crotwell, John Cuazzo, Peter Espenshade, Alison Frand, Pam Gannon, Ruth Gimeno, Masa Hirata, Elizabeth Hong, Bill Payne, Rachna Ram, Kevin Roberg, Neil Rowley and David Shaywitz.

I would like to thank my classmates, especially those members of the class with whom I've continued to interact: Peter Espenshade and David Shaywitz as labmates as well as classmates; Mark (and Leila) Borowsky, Christopher Cilley, Sandy Gilbert, Adam Grancell, Elizabeth Hong and Ann-Marie White for all the shared adventures; Mark Metzstein and Gillian Stanfield for gatherings of the usual suspects; and Alok Shrivastava for encouraging my philosophical side.

Thanks to my parents, sister, brother and other close friends for encouragement and support. You are the people that matter most to me.

Thanks most of all to my wife Monicia Elrod-Erickson for being my classmate, my friend and confidant, for sharing in all the daily joys and frustrations of the last six years.

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Secretory protein maturation, sorting, and transport at the endoplasmic reticulum

Overview

Most eukaryotic cells contain multiple membrane-bounded organelles, each of which houses a unique assortment of proteins required to carry out its specialized cellular function. A complete understanding of the biogenesis and functions of each organelle, as well as a complete description of the mechanisms by which proteins are transported from their site of synthesis in the cytosol to their final address within these organelles, are major goals in cell biology.

The sorting and transport of most organellar proteins occurs via the secretory pathway. Proteins destined for secretion or for residence in the plasma membrane, lysosome (the yeast vacuole), or endosomal compartments are first translocated into the endoplasmic reticulum (ER) and then transported sequentially through the *cis*-, *medial*-, and *trans*-Golgi before being sent to their final destination (Palade, 1975). Each transport step is accomplished by the packaging of proteins into vesicles that bud from the donor membrane and then fuse with the target organelle, thus delivering their contents (reviewed in Rothman and Orci, 1992; Kaiser et al., 1997). Estimates from the yeast *Saccharomyces cerevisiae*, for which a list of all potential cellular proteins exists, suggest that 10% - 20% of yeast proteins transit at least some portion of the secretory pathway and are therefore dependent upon its function for their localization (Kaiser et al., 1997). The overall pathway and many of the proteins required for its function are highly conserved among eukaryotes (Novick et al., 1981; Clary et al., 1990; d'Enfert et al., 1992; Bennett and Scheller, 1993). In some cases the homologous proteins from different species have been shown to be functionally interchangeable (Griff et al., 1992; Shaywitz et al., 1995), suggesting that the underlying mechanisms of protein transport are also similar among eukaryotes.

As the entry point to the secretory pathway, the ER is the site of several unique and essential functions. Proteins are first translocated from the cytosol into the lumen or

membrane of the ER. Once within the ER, proteins must assume their functional conformation. This folding and maturation process is aided by luminal chaperones and can involve a variety of modifications, including signal sequence cleavage, attachment of N-linked and/or O-linked carbohydrates, disulfide bond formation (and rearrangement), and attachment of a glycosylphosphatidylinositol (GPI) anchor. Additionally, some proteins must oligomerize or form multi-protein complexes before becoming fully functional and competent for transport. Finally, mature proteins are sorted away from resident ER proteins and packaged into transport vesicles destined for the *cis*-Golgi.

To ensure the proper localization of proteins -- and thus the unique identity of each organelle and the viability of the organism -- proteins must be both transported and sorted properly. There are two processes required for the correct sorting of secretory proteins: first the appropriate cargo must be packaged into transport vesicles, and then these vesicles must be fused to the correct target membrane. The latter process will not be discussed in this introduction. A thorough discussion of protein sorting in the context of ER vesicle formation will be conducted in a later section of this introduction.

A growing body of evidence suggests that the maturation of secretory proteins, their sorting into transport vesicles, and the mechanism of vesicle formation at the ER are coupled. Acquiring a better understanding of these processes and how they are connected was the goal of the experiments described in this thesis. Therefore, a careful review of what is known about secretory protein maturation, sorting, transport vesicle formation, and related topics forms the remainder of this introduction. Because the experiments described in this thesis were done in the budding yeast *Saccharomyces cerevisiae*, this review will emphasize what is known in this organism. However, information from other systems will be included to strengthen arguments made in yeast, to highlight differences between systems, or to introduce ideas that currently have no counterpart in yeast.

Protein maturation in the ER

Regardless of whether a secretory protein is co-translationally or post-translationally translocated into the ER, it enters as an unfolded polypeptide that must go through a number of steps before it can be packaged into transport vesicles and moved through the secretory pathway. Attributes such as conformation, presence of attached carbohydrates (or GPI-anchors), and oligomeric state not only influence the activity of a protein but are also critical determinants for its intracellular transport.

Signal peptidase and the requirement for signal sequence cleavage in protein transport

In both yeast and mammals a multiprotein, membrane-associated endopeptidase (termed signal peptidase) is situated within the lumen of the ER and is responsible for the proteolytic removal of the amino-terminal signal sequence from secreted proteins. The yeast signal peptidase complex contains four polypeptides of 11, 17, 18, and 21 kDa. Two of these proteins, Sec11p (17 kDa) and Spc3p (21 kDa), have been shown to be essential for both signal peptidase activity and cell viability (Bohni et al., 1988; Fang et al., 1997). The remaining two yeast subunits, Spc1p (11 kDa) and Spc2p(18 kDa), are dispensable for both growth and enzyme activity but appear to increase the efficiency of the enzyme (Fang et al., 1996; Mullins et al., 1996). The mammalian complex contains five proteins related to those found in yeast, with two homologs of Sec11p (Shelness and Blobel, 1990). Signal peptidase from hen oviduct contains only two subunits, homologs of Sec11p and Spc3p, further demonstrating that these two components are sufficient for catalytic activity (Baker and Lively, 1987; Shelness et al., 1988; Newsome et al., 1992).

Eukaryotic signal sequences generally consist of a short region at the amino-terminus of a protein that contains one or more positively charged residues followed by a hydrophobic region of 7-15 residues and a polar region of 3-7 amino acids (von Heijne, 1990). Comparison of a large number of known cleavage sites within eukaryotic signal

sequences shows that signal peptidase cleaves within the polar region of the signal sequence and requires small neutral or polar amino acids at positions -1 and -3 from the cleavage site (von Heijne, 1983).

The removal of the signal sequence is an essential process in the maturation of secretory proteins. Signal sequence cleavage is obviously necessary to release soluble secretory proteins from the membrane. Interestingly, the removal of the signal peptide is not required for the proper folding of all secretory proteins, although it may be in some instances. An allele of the yeast *SUC2* (invertase) gene that alters the signal peptidase cleavage site -- and drastically reduces cleavage of the signal sequence -- produces a functional enzyme (Schauer et al., 1985). However, this mutant invertase is significantly delayed in its transport from the ER. This delay appears to be a general effect of failure to remove the signal sequence, because similar transport defects were observed for many invertase mutants with uncleaved signal sequences that were generated by randomizing the amino acid sequence of the signal peptide (Kaiser et al., 1987). In addition, the *SEC11* component of signal peptidase was first identified and classified as a gene required for ER to Golgi transport in yeast (Novick et al., 1981). The transport defect caused by an uncleaved signal sequence is not at all understood, but could be the result of the uncleaved signal peptide resembling an unfolded region more than a transmembrane domain, making it subject to the retention mechanisms that prevent the transport of unfolded proteins. Thus, signal sequence cleavage is an essential prerequisite for the efficient transport of secretory proteins from the ER.

Addition of N- and O-linked carbohydrates and their influence on secretory protein transport

Two additional enzymatic activities act on nascent polypeptides emerging from the translocation channel into the lumen of the ER: 1) oligosaccharyltransferase (OTase)

transfers $GlcNAc_2Man_9Glc_3$ from Dol-PP to the amide group of the asparagine side chain in the tripeptide motif Asn-X-Ser/Thr (Lehle and Bause, 1984), and 2) protein:O-mannosyltransferase (PMTase) transfers a mannose from Dol-P-Man to serine and threonine residues (Tanner and Lehle, 1987). Proteins required for the biosynthesis of the oligosaccharide precursors also affect the glycosylation of secretory proteins (reviewed in Orlean, 1997) but will not be discussed here. A role for N-linked oligosaccharides in protein folding and multimerization, and therefore secretion competency, is now well established. The role of O-linked sugars on proteins is less clear and will be discussed first.

The addition of mannose residues to serine or threonine side chains of secretory proteins in the yeast ER is carried out by one of seven protein:O-mannosyltransferases encoded by the genes *PMT1-7* (reviewed in Orlean, 1997). The PMTases are thought to have different substrate specificities and therefore only partially overlapping function. This idea is supported by two sets of results: 1) the O-mannosylation of chitinase is greatly reduced in a *pmt1Δ pmt2Δ* strain, but is normal in a *pmt4Δ* mutant, whereas the O-mannosylation of Gas1p is affected only in a *pmt4Δ* mutant, and 2) the deletion of any single *PMT* gene is not lethal, but strains containing deletions of three or more of the genes are inviabile (Gentzche and Tanner, 1996). This inviability clearly demonstrates that the process of O-mannosylation as a whole is essential for yeast cells, but so far only a single yeast protein (the bud site selection protein Axl2p) has been observed to require its O-linked sugars for proper function. In the absence of O-glycosylation Axl2p is mislocalized and degraded (Sylvia Sanders, personal communication). Most O-mannosylated proteins in yeast are structural components of the cell wall or are involved in its formation. The proposed functions for O-mannosylation are to confer an extended or "stiff" conformation to the heavily modified portions of cell wall proteins, and to protect or stabilize the proteins (Jentoft, 1990; Stratford, 1994). In the case of rat pancreatic bile salt-dependent lipase (BSDL), the addition of O-linked sugars is thought

to stabilize the protein by masking PEST sequences that otherwise target BSDL for rapid degradation, thereby allowing the protein to be secreted (Bruneau et al., 1997). Thus, O-linked oligosaccharides can be important for the proper localization and stability of secretory proteins.

The addition of N-linked carbohydrates to yeast secretory proteins requires the products of eight genes: *WBP1, SWP1, OST1, OST2, OST3, OST4, OST5,* and *STT3*. Only five of these genes are essential for viability, but mutations in any of them reduce the number of oligosaccharide chains transferred to proteins in vivo and cause decreased OTase activity in vitro (reviewed in Orlean, 1997). Each of the encoded proteins is present in equimolar amounts in a purified complex (Karaoglu et al., 1997), although catalytically active subcomplexes as small as four subunits have also been described (Knauer and Lehle, 1994; Pathak et al., 1995). An intriguing role has been proposed for the nonessential *OST3* gene in positioning the OTase complex for N-glycosylation of proteins that use the signal recognition particle (SRP)-dependent translocation pathway. This idea is based on the findings that *ost3* disruptants show a biased underglycosylation of membrane proteins that use the SRP-dependent pathway as compared to soluble secretory proteins (e.g. carboxypeptidase Y) that use the SRP-independent pathway (Karaoglu et al., 1995). Thus, while only five of the eight OTase subunits are essential, and catalytic activity may only require four or fewer subunits, each of the proteins in the complex may play important roles in vivo to ensure proper N-glycosylation of secretory proteins.

The mammalian OTase complex consists of four known subunits: ribophorin I (homolog of Ost1p), ribophorin II (homolog of Swp1p), OST48 (homolog of Wbp1p) and DAD1 (homolog of Ost2p) (Kelleher and Gilmore, 1997). Multiple subunit binding interactions have been described (Fu et al., 1997), but, as with the yeast enzyme, little is known about the function of any of the subunits within the complex.

N-glycosylation is an essential process. The addition of N-linked sugars to secretory proteins is thought to help prevent misfolding and aggregation, to aid in multimerization, and, as a result of these functions, to promote the secretion competence of secretory proteins in the ER. Mutations in the *WBP1* subunit of OTase have been shown to exacerbate the temperature sensitivity of conditional alleles of *KAR2*, which encodes an ER chaperone (Te Heesen and Aebi, 1994). Thus, defects in chaperone-mediated folding of secretory proteins are more extreme when there are lower levels of N-glycosylation. Similarly, the addition of tunicamycin (an inhibitor of N-glycosylation) to yeast cultures causes a rapid induction of the unfolded protein response, leading to increased levels of ER chaperones, presumably in response to the folding problems that occur in the absence of N-glycosylation (Shamu et al., 1994). In general, the sugar moieties are thought to help keep the unfolded protein soluble and prevent aggregation, but they may also directly aid in the folding process itself. Studies of glycopeptides from proteins of known three-dimensional structure show that the modified peptides adopt a different profile of conformations (often more compact and more closely resembling the conformation they have in the folded protein) than do the corresponding unmodified peptides (Imperiali and Rickert, 1995). Thus, in addition to preventing aggregation, N-oligosaccharides may promote nucleation events that assist in protein folding. These properties of N-glycosylation are important for increasing the secretion competency of proteins. Numerous proteins have been observed to have reduced rates of transport in the absence of N-glycosylation, including invertase, carboxypeptidase Y(CPY), Prc1p, pro-α-factor, and Pho5p (Trimble et al., 1983; Caplan et al., 1991; Riederer and Hinnen, 1991; Winther et al., 1991).

N-linked oligosaccharides are also thought to be important for the multimerization of several secretory proteins, including invertase, Mel1p, and Pho3p (Chu et al., 1983; Esmon et al., 1984; Schulke and Schmid, 1988). Invertase produced in the presence of tunicamycin is not only delayed in its transport, but dimers, rather than the usual

octamers, are formed (Esmon et al., 1987). Exactly how the presence of N-linked sugars promotes or stabilizes multimers of these proteins, and why multimerization leads to more rapid secretion, are as yet unanswered questions.

N-glycosylation of secretory proteins in mammalian cells serves many of the same functions as in yeast. In addition, a "quality control" system has been described that uses the N-linked oligosaccharides as part of the mechanism that keeps partially folded or misfolded proteins in the ER until they adopt their native conformation (Hammond and Helenius, 1995). The mechanism involves the constant deglucosylation and reglucosylation of the core oligosaccharide ($GlcNAc_2Man_9Glc_3$). The enzyme responsible for reglucosylation is only active on denatured substrates, and the resulting mono-glucosylated (unfolded) glycoprotein is bound by the membrane-associated chaperone calnexin and is retained in the ER for further attempts at folding (Hebert et al., 1996). Once the protein is folded, it is no longer reglucosylated and can exit the ER. The *CNE1* gene encodes the yeast homolog of calnexin and has been implicated in the retention of incorrectly folded proteins in the ER (Parlati et al., 1995), but since the reglucosylation activity has not yet been detected in *S. cerevisiae*, the extent to which a similar mechanism functions in yeast is currently unknown.

Protein folding in the ER: the role of folding factors in the transport of secretory proteins

As alluded to in the previous section, a secretory protein in the ER must become fully folded to be efficiently transported. Folding is aided by luminal chaperones and by factors involved in disulfide bond formation/rearrangement. Kar2p is the essential yeast homolog of the mammalian immunoglobulin binding protein (BiP) and is probably the major chaperone in the lumen of the ER (Normington et al., 1989; Rose et al., 1989). BiP has been shown to associate with newly synthesized proteins, denatured protein aggregates, unassembled subunits of multi-protein complexes, and ER retained proteins

(Jamsa et al., 1995; Beggah et al., 1996; Oda et al., 1996; Kuznetsov et al., 1997; Zhang et al., 1997). The best evidence for Kar2p playing an active role in folding and transport in yeast comes from experiments using conditional *kar2* mutants. In order to bypass the requirement for Kar2p in translocation, mutant strains are treated with DTT at permissive temperatures to delay the folding and transport of CPY (which requires intramolecular disulfide bond formation to fold). The cells are then shifted to nonpermissive temperatures and washed to remove the DTT. In *kar2* mutant strains CPY remains in the ER and is found in large aggregates that also contain the mutant Kar2p, whereas in wild-type strains CPY transport is rapidly restored (Simons et al., 1995). These results show clearly that Kar2p is necessary to restore secretion competency to reduced CPY and suggest that Kar2p normally acts as a chaperone in the maturation of ER proteins.

Another key aspect of protein folding in the ER is the formation of native disulfide bonds. As described above, carboxypeptidase Y is a good example of a yeast protein whose folding -- and therefore transport from the ER -- is dependent upon disulfide bond formation. Two essential activities are required for the proper formation of disulfide bonds in ER protein folding: 1) maintenance of an oxidizing environment in the ER, which is necessary for the formation of disulfides, and 2) the catalyzed rearrangement of nonnative disulfides. The *ERO1* gene of yeast encodes an essential ER glycoprotein involved in maintaining the oxidizing potential of the ER. The levels of Ero1p appear to determine the oxidizing capacity of cells, since deletion of *ERO1* causes hypersensitivity to reducing agents like DTT, while overexpression of the gene confers resistance to DTT. In addition, CPY transport is abolished in conditional *ero1* mutants at nonpermissive temperatures because of a failure to form disulfide bonds (Frand and Kaiser, 1998). Homologs of Ero1p are found in other fungi, insects, plants, and animals, suggesting that the mechanism of ER oxidation is conserved among all eukaryotes.

The best characterized factor known to catalyze the rearrangement of disulfide bonds is protein disulfide isomerase (PDI). PDI can bind peptides and catalyze the *in vitro*

refolding of reduced substrates like ribonuclease A or bovine pancreatic trypsin inhibitor (BPTI) under oxidizing conditions (LaMantia et al., 1991; Lyles and Gilbert, 1991). The yeast PDI (encoded by the *PDI1* gene) shows both sequence and functional homology to mammalian PDI (LaMantia et al., 1991; Scherens et al., 1991). *PDI1* is an essential gene, and depletion of Pdi1p from yeast cells by regulated repression of the gene causes CPY to accumulate in the ER (Tachibana and Stevens, 1992). The essential function of PDI is catalyzing the rearrangement of nonnative disulfide bonds, since mutations that render the protein inactive for dithiol oxidation but do not affect its ability to catalyze reshuffling do not affect cell viability (LaMantia and Lennarz, 1993; Laboissiere et al., 1995). There are two other yeast genes known to encode proteins with protein disulfide isomerase activity. Both *EUG1* and *MPD1* show sequence homology to *PDI1* and can suppress the lethality of a *PDI1* deletion when they are overexpressed (Tachibana and Stevens, 1992; Tachikawa et al., 1995). However, the rate of CPY transport under these conditions is slower than in wild-type cells, indicating that each of these proteins is likely to perform slightly different functions or have different substrate specificities.

The addition of GPI-anchors and their effect on protein transport

Another modification that occurs within the ER and is found on a subset of secretory proteins is the addition of a GPI-anchor. GPI-anchored proteins are found in all eukaryotes and serve diverse cellular functions, including cell adhesion, transmembrane signalling, and cell wall synthesis (Lisanti et al., 1990; Klis, 1994). Proteins destined for GPI-anchor attachment contain a C-terminal signal similar in structure to the N-terminal signal sequence (Yan et al., 1998). The glycolipid anchor is preassembled in the ER membrane starting from phosphotidylinositol and is attached *en mass* through a transamidation reaction that displaces the C-terminal signal peptide (Maxwell et al., 1995). Seven mutants (*sec53, sec59, dpm1, gpi1, gpi2, gpi3/spt14,* and *gpi7*) have been

isolated in yeast that are known to disrupt the assembly of the glycolipid anchor precusor, thus affecting the modification and transport of GPI-proteins. These genes will not be discussed further in this introduction, but are reviewed in Orleans, 1997. Two yeast genes, *GAA1* and *GPI8*, are known to be essential for the transamidation reaction that attaches the glycolipid anchor to proteins. Conditional mutations in either of these genes cause a defect in anchor attachment and an accumulation of the complete GPI-anchor precursor at restrictive temperatures (Hamburger et al., 1995; Benghezal et al., 1996). After attachment, the fatty acid portion of the anchor generally undergoes one of three types of remodeling: 1) the diacylglycerol portion of the anchor can be remodeled in the ER to ceramide; 2) the diacylglycerol can be remodeled in the ER to a more hydrophobic diacylglycerol, containing a long-chain (C26:0) fatty acid in the *sn*-2 position of glycerol; or 3) the diacylglycerol can be remodeled in the Golgi to a more polar ceramide (Sipos et al., 1997). The role of remodeling is not yet understood, but it may be important for partitioning GPI-anchored proteins into sphingolipid rich domains within the membrane, and may therefore have a role in the sorting of these proteins (see below).

The attachment of the GPI-anchor is necessary for the efficient sorting and transport of GPI-proteins. In yeast, blocking attachment of the anchor to the model GPI-protein Gas1p causes a defect in the transport of the protein from the ER to Golgi, whereas removal of the C-terminal signal for anchor attachment causes the truncated protein to be secreted into the medium (Nuoffer et al., 1991; Hamburger et al., 1995). Interestingly, such a block can be imposed not only by using conditional *gpi8* or *gaa1* mutants or by altering the GPI attachment site on a protein, but can also be achieved by starving inositol auxotrophs for inositol. Apparently, the biosynthesis of the GPI precursor is particularly sensitive to low levels of inositol (Doering and Schekman, 1996).

In polarized epithelial cells, GPI-anchors are thought to be involved in the sorting of the proteins that contain them in the *trans*-Golgi. GPI-anchored proteins and glycosphingolipids are preferentially sorted to the apical surface (Simons and van Meer,

1988; Lisanti et al., 1990). In addition, converting a basolaterally targeted membrane protein into a GPI-anchored form redirects this protein to the apical surface in MDCK cells (Brown et al., 1989). The GPI-proteins and sphingolipids are thought to associate in the Golgi, forming microdomains or "rafts", which are then recruited into vesicles bound for the apical surface (Simons and Wandinger-Ness, 1990). Evidence supporting such a model comes from the observation that detergent insoluble complexes form in the Golgi that contain both GPI-anchored proteins and glycosphingolipids but lack basolaterally sorted proteins (Brown and Rose, 1992).

The transport of GPI-proteins from the yeast ER may also involve association with sphingolipids. Gas1p transport is blocked in cells treated with myriocin, a drug that blocks ceramide synthesis in yeast (Horvath et al., 1994). Similarly, mutant strains lacking sphingolipids (but viable because of suppressor mutations that allow production of glycerophospholipids capable of mimicking sphingolipid structures), also show defects in the transport of GPI-anchored proteins (Skrzypek et al., 1997). In both cases anchor attachment to Gas1p is normal, but remodelling does not occur. Since the anchor normally found on mature Gas1p does not contain ceramide (Frankhauser et al., 1993), these results suggest that Gas1p may need to cluster with other GPI-anchored proteins in sphingolipid enriched subdomains of the ER membrane in order to be efficiently packaged into transport vesicles. Remodelling of the anchor may also be important in this process.

Assembly of multi-protein complexes as a prerequisite for transport

Some secretory proteins are not secretion competent until they multimerize or are incorporated into a multi-protein complex. In mammalian cells, influenza hemagglutinin and vesicular stomatitis virus glycoprotein, for example, must trimerize before leaving the ER. Similarly, multi-subunit membrane protein complexes (e.g. T-cell receptor

complexes, immunoglobulins, and class I major histocompatability complexes) are usually assembled in the ER and require complete assembly of the complex to exit the ER (reviewed in Rose and Doms, 1988). In some cases assembly into multimeric complexes involves disulfide bond formation. The unassembled subunits of these complexes therefore have exposed thiols that can cause retention of the proteins by making reversible disulfide bonds with resident ER folding factors (Isidoro et al., 1996; Reddy et al., 1996). This quality control mechanism thereby ensures that only subunits that have assembled into complexes are capable of leaving the ER.

In yeast, the iron permease complex and the vacuolar ATPase (V-ATPase) complex require assembly in the ER for transport (Bauerle et al., 1993; Stearman et al., 1996). In the case of the V-ATPase, at least three proteins (Vma12p, Vma21p, and Vma22p) are required for the stable assembly (and thus transport) of the complex, but are not themselves part of the V-ATPase (Hill and Stevens, 1995). The existence of the *VMA* genes suggests that there may be other cofactors involved in the assembly of multiprotein complexes or the maturation of membrane proteins in the ER. The SHR3 gene product may act analogously as a cofactor for the folding/maturation of amino acid permeases (Ljungdahl et al., 1992). Alternatively, Shr3p may act to sort permeases into ER-derived transport vesicles and will be discussed in that context in the next section.

Protein sorting into transport vesicles at the ER

Secretory proteins that have been modified, folded, and assembled into their mature, secretion competent form in the ER must next be sorted into transport vesicles if they are to be moved forward through the secretory pathway. A key aspect of sorting is the segregation of secretory (cargo) molecules from resident proteins, such that cargo is efficiently transported to the Golgi while resident proteins remain in the ER. Two models have dominated the thinking about how proteins are sorted and trafficked through the

secretory pathway. In the first, the pathway behaves like a river with a strong current. Cargo proteins are passively carried forward into transport vesicles. Resident proteins must be actively kept out of vesicles to resist the flow and remain within an organelle, and (later in the pathway) cargo must be actively sorted into a different set or class of vesicles to change course. The other model likens the pathway to a series of connected trains. If cargo proteins are to be moved forward to the next stop/compartment, they must be actively packaged into transport vesicles headed in the direction of their destination. Resident proteins are not packaged and remain behind. The emerging picture of sorting at the ER has elements of both models, with data suggesting active mechanisms during vesicle formation for both the concentration of at least some cargo proteins and the exclusion of resident proteins. These data will be presented below.

Sorting functions in other parts of the secretory pathway: models for sorting at the ER

A number of sorting mechanisms that have been described for other steps in the secretory pathway may provide insight and help direct investigations into the sorting mechanisms at the ER. The current view of cargo sorting into clathrin-coated endocytic vesicles in mammalian cells has been highly influential in shaping ideas about sorting in the ER. In this system, cargo is selected by the assembling coat, which forms an affinity matrix that partitions receptors and receptor bound molecules into the budding vesicle (coated pit) according to their affinity for the coat (reviewed in Pearse and Robinson, 1990). The very different environments found in the lumen of the ER and the extracellular space, however, make it likely that sorting in the ER will have novel components, even if some elements of the mechanisms turn out to be similar.

Another idea from work on sorting at the cell surface and the *trans*-Golgi that has been adopted (and may apply) to the ER is the notion of receptors for sorting soluble cargo molecules. At the cell surface, soluble factors interact with membrane receptors

and are subsequently clustered in coated pits and internalized (Pearse and Robinson, 1990). Similarly, binding of the mannose-6-phosphate/insulin-like growth factor 2 receptor (LGF2/MPR) to lysosomal enzymes in the *trans*-Golgi is required to direct the transport of the enzymes to the late endosome (Griffiths et al., 1988). In yeast, the Vps10 protein plays an analogous role in the targeting and transport of CPY and misfolded proteins from the Golgi to the vacuole (Marcusson et al., 1994; Hong et al., 1996). The p24 family of transmembrane proteins has been proposed to act as receptors for soluble cargo in the earlier steps of the secretory pathway, but direct evidence of such a function is still lacking. This family of proteins will be discussed in more detail later in this section because of its relevance to the data presented in this thesis.

A further idea that may prove useful in thinking about sorting at the ER is the notion that the lipids in the membrane may play a role in the sorting of membrane proteins. The role of sphingolipids in the sorting of GPI-linked proteins was discussed previously in the section on GPI-anchor addition. More recent results implicate the rare, highly hydrophobic, acidic phospholipid lysobisphosphatidic acid (LBPA) -- found in the internal membranes of multilamellar late endosomes -- in the trafficking of LGF2/MPR (Kornfeld, 1992; Kobayashi et al., 1998). The importance of the membrane in the sorting of transmembrane proteins is further implicated by the growing number of recent reports in both yeast and mammals of domain swapping experiments that show transmembrane domains are often both necessary and sufficient to determine localization of a protein to the ER or other organelles (Raynor and Pelham, 1997; Yang et al., 1997; Cocquerel et al., 1998). Determining which, if any, of the mechanisms discussed in this section have counterparts in the ER and whether novel sorting functions exist are key questions in the analysis of protein transport between the ER and Golgi.

Evidence for active concentration of cargo molecules into transport vesicles at the ER

A large body of evidence is accumulating that clearly shows at least some secretory proteins are actively packaged and concentrated into ER-derived transport vesicles. Quantitative immuno-electron microscopy studies in mammalian cells show that some soluble and integral membrane proteins are concentrated approximately 5-fold within regions of vesicle budding in the ER (Mizuno and Singer, 1993; Balch et al., 1994). Similarly in yeast, comparing the composition of vesicles produced *in vitro* with that of the ER microsomes from which they are derived shows that cargo molecules are enriched 5 to 10-fold in vesicles (Rexach et al., 1994). Transport vesicles can also be formed *in vitro* using either of two types of vesicle coats (COPI or COPII) to drive vesicle budding from the ER/nuclear envelope of isolated yeast nuclei. In this system, the soluble secretory protein α-factor is selectively packaged into COPII vesicles but not COPI vesicles, demonstrating that vesicle budding is not sufficient to capture this cargo molecule (Bednarek et al., 1995). Further *in vivo* evidence in yeast comes from the examination of mutants that show defects in the transport of only a subset of secretory proteins. The *bst1*, *emp24/bst2*, and *erv25/bst3* mutants transport most secretory proteins (e.g., CPY, α-factor, and acid phosphatase) at normal rates, but show a significant delay in the transport of some cargo, including invertase, and Gas1p and other GPI-linked proteins (Schimmöller et al., 1995; Elrod-Erickson and Kaiser, 1996 [Chapter 2]; Belden and Barlowe, 1996; and Chapter 4 of this thesis). The invertase in these mutants is active, glycosylated, and forms multimers at normal rates, indicating that the defect is in the packaging of the protein into vesicles (Schimmöller et al., 1995). Similarly, mutations in *SHR3* cause selective defects in the transport of amino acid permeases and in their incorporation into *in vitro* generated COPII vesicles (Ljungdahl et al., 1992; Kuehn et al., 1996). However, in this case a role in the folding of the permeases is much harder to rule out (as mentioned above). Certain alleles of *SEC21* can also cause

selective defects in transport from the ER to Golgi (Gaynor and Emr, 1997). Finally, recent *in vitro* work implicates COPII coat subunits in the selective packaging of subsets of cargo molecules into vesicles (Campbell and Schekman, 1997; Kuehn et al., 1998; see the review of the Sec23/24 complex below for a more thorough discussion of these results). Collectively these data make a strong case for the active sorting of cargo molecules into budding vesicles at the ER.

Retention mechanisms in sorting at the ER

Active mechanisms involved in the retention of immature secretory proteins in the ER are well documented and involve interactions with resident ER proteins (see the discussion of quality control mechanisms in the sections above on N-linked glycosylation and assembly of multiprotein complexes). The mechanisms that in turn act to retain resident proteins have been more difficult to uncover, but some evidence suggests that such mechanisms do exist. Short peptide sequences on resident ER proteins act as retrieval signals, directing their capture in the Golgi and return transport of the small fraction of these molecules that escape the ER (reviewed in Pelham, 1995). These signals are not sufficient, however, to explain retention, because resident proteins from which the signal has been removed are still largely retained in the ER (Nilsson et al., 1989; Hardwick et al., 1990). As discussed in the previous paragraph, transport vesicles produced *in vitro* lack resident proteins and are enriched in cargo. However, this data is also consistent with an absence of active sorting of resident proteins into vesicles. The best evidence suggesting that retention is an active process comes from experiments described in this thesis on yeast mutants that cause defects in the retention of resident ER proteins in a manner that is independent of known retrieval pathways. Deletion of *BST1, EMP24/BST2*, or *ERV25/BST3* not only slows the transport of a subset of secretory proteins (see above), but also increases the leakage of resident and retained proteins from

the ER (Elrod-Erickson and Kaiser, 1996 [Chapter 2 of this thesis]). The affect of *bst* mutants on both forward transport and retention suggests that the *BST* proteins are part of a mechanism that allows discrimination between cargo molecules and resident proteins during sorting. The function of the *BST* proteins also influences the requirements for vesicle assembly itself, indicating that sorting processes and the coat assembly that drives vesicle budding are coupled. These ideas will be elaborated in the following chapters.

The p24 family of proteins and their role in protein sorting

The *EMP24/BST2* and *ERV25/BST3* genes described in this thesis encode members of the p24 protein family. All members of this family are predicted based on their primary sequence to be type I membrane proteins of approximately 24 kDa with the bulk of the protein in the lumen and a short cytosolic C-terminus (Stamnes et al., 1995). The region of highest homology among p24 proteins begins within the luminal domain near the transmembrane segment and extends through the membrane to include the short cytosolic tail. On the luminal side of the membrane is a region containing heptad repeats of hydrophobic amino acids that are characteristic of coiled-coil structures and that may mediate association of family members (Stamnes et al., 1995; Belden and Barlowe, 1996; Dominguez et al., 1998). The function of p24 proteins has yet to be demonstrated, but these proteins are currently of great interest because they are major constituents of both COPI and COPII vesicles (Stamnes et al., 1995; Belden and Barlowe, 1996). (It should be noted, however, that the yeast p24 proteins that have been characterized to date are nonessential.) The cytosolic tail of p24 proteins governs their binding to vesicle coat proteins and therefore their incorporation into transport vesicles (Fielder et al., 1996; Sohn et al., 1996; Dominguez et al., 1998). As mentioned above, deleting Emp24p/Bst2p and/or Erv25p/Bst3p (two of the yeast members of the p24 family) slows the transport of a subset of secretory proteins from the ER. This observation, and the fact that these

proteins are major components of transport vesicles, has led to the idea that p24 proteins may act as receptors for soluble secretory proteins, but so far no interaction has been observed between the p24 proteins and the cargo they are thought to concentrate (Schimmöller et al., 1995; Belden and Barlowe, 1996). The data presented in this thesis, along with the observations outlined above, appear to support a different view of the role of the p24 proteins in protein transport. A model summarizing this view will be put forward at the end of this chapter.

COPII vesicle formation at the ER

The budding of transport vesicles from the ER is a regulated process driven by the assembly of a proteinacious coat (termed COPII) on the cytosolic surface of the membrane. Seven key proteins (Sec12p, Sec13p, Sec16p, Sec23p, Sec24p, Sec31p, and Sar1p) are known to be essential for vesicle formation in yeast, and all but Sec12p are components of the vesicle coat (reviewed in Kaiser et al., 1997). *SEC12, SEC13, SEC16,* and *SEC23* were among the 23 genes identified in the original screen for *sec* mutants (Novick et al., 1980). Examination of the maturation of marker proteins in these mutants and careful genetic and morphological analyses demonstrated that each was involved in the formation of transport vesicles from the ER (Novick et al., 1981; Kaiser and Schekman, 1990). Sec24p and Sec31p were identified because they copurify with Sec23p and Sec13p, respectively, and these two complexes were subsequently shown to be essential for vesicle budding (Hicke et al., 1992; Pryer et al., 1993; Salama et al., 1993). Sar1p was identified as a high copy suppressor of mutations in *SEC12* and *SEC16* and was later also shown to be essential for vesicle formation (Nakano and Muramatsu, 1989; Oka et al., 1991; Barlowe et al., 1993). The development of an *in vitro* assay (Baker et al., 1988; Ruohola et al., 1988) that reconsitutes vesicle budding from ER membranes has allowed further dissection of the role of each of these proteins.

The GTPase cycle of Sar1p regulates assembly and disassembly of the COPII coat

The assembly and disassembly of the COPII coat is thought to be regulated by the GTPase cycle of Sar1p. *SAR1* is essential for ER to Golgi transport and encodes a small GTP-binding protein related to ARF, which regulates the assembly of the coatamer (COPI) coat of Golgi-derived vesicles (Oka et al., 1991; Barlowe et al., 1993). Sar1p is required for vesicle formation *in vitro* and is a component of the COPII coat (Barlowe et al., 1994). In the reconstituted budding reaction, Sar1p is required in its GTP-bound form, but hydrolysis of GTP is not required during budding (Oka et al., 1991; Rexach and Schekman, 1991; Barlowe et al., 1993). However, GTP hydrolysis by Sar1p is required for the vesicle fusion step of the complete ER to Golgi transport reaction (Barlowe et al., 1994; Oka and Nakano, 1994). The current model is that Sar1p in its GTP-bound form stimulates assembly of the COPII coat, and the later hydrolysis of GTP by Sar1p triggers disassembly of the coat, a prerequisite for vesicle fusion.

Sec12p regulates the assembly of the COPII vesicle coat

Sec12p has a unique function in the budding of transport vesicles. It is a type II integral ER membrane protein and the only known essential protein in vesicle budding that is not incorporated into the vesicle (Nakano et al., 1988; Barlowe et al., 1994). The cytosolic N-terminus of the protein stimulates guanine nucleotide exchange by Sar1p, and thereby recruits Sar1p to the membrane (d'Enfert et al., 1991a; d'Enfert et al., 1991b; Barlowe and Schekman, 1993). Sec12p can bind to the C-terminus of Sec16p *in vitro* and this binding interaction may function to localize the exchange activity of Sec12p to sites of vesicle assembly (P. Espenshade, Ph.D. dissertation, 1997). Thus, the role of Sec12p may be -- through its localization and effect on Sar1p -- to restrict the initiation of COPII coat assembly to the ER membrane.

Sec16p may nucleate assembly of the COPII coat on the ER membrane

Sec16p is a large (240 kDa), hydrophilic, multidomain protein essential for vesicle formation *in vivo* (Kaiser and Schekman, 1990; Espenshade et al., 1995). The protein is tightly associated with the cytosolic face of the ER membrane and is incorporated into transport vesicles produced *in vitro* (Espenshade et al., 1995). The inability to extract Sec16p from the ER by urea washing may explain why this protein is not considered one of the essential cytosolic components required for vesicle budding from ER membranes *in vitro* (Espenshade et al., 1995). *SEC16* exhibits genetic interactions with each of the five other COPII genes, and the protein can physically interact with Sec12p, Sec23p, Sec24p, and Sec31p (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Espenshade et al., 1995; Gimeno et al., 1996; Shaywitz et al., 1997). These collective data suggest that Sec16p on the membrane may localize or nucleate COPII coat assembly, possibly as a scaffold around which the remaining coat components assemble.

The Sec23p/Sec24p complex performs multiple functions in COPII vesicle formation

The Sec23p/Sec24p complex is thought to serve both structural and catalytic functions in the formation of COPII-coated vesicles. The complex can be purified from yeast as a 300-400 kDa species of unknown stoichiometry that is a major component of the COPII coat and is required for its formation (Hicke et al., 1992; Barlowe et al., 1994). Sec23p and Sec24p can bind independently to adjacent but different sites on Sec16p (Gimeno et al., 1996). Sec23p can also function as a GTPase activating protein (GAP) for Sar1p, and this activity is not influenced by Sec24p (Yoshihisa et al., 1993). Finally, recent exciting results show that the Sec23p/Sec24p complex, along with Sar1p in its GTP bound state, can form complexes with cargo molecules in the ER membrane (Kuehn et al., 1998). These results have been interpreted to suggest that such interactions direct cargo sorting

into the budding vesicle. However, it should be noted that none of the reported experiments rule out the possibility that cargo molecules are concentrated by some other means to sites of vesicle budding and that the Sec23p/Sec24p-Sar1p(GTP) complex is itself recruited to these sites by its affinity for cargo or other vesicle components. Nonetheless, these results do make the model of protein sorting into budding ER vesicles via an affinity for the coat -- either directly (for membrane proteins) or indirectly through membrane protein receptors (for soluble cargo) -- an attractive possibility.

The Sec13p/Sec31p complex performs at least a structural function in the COPII coat

The Sec13p/Sec31p complex has the least understood function in vesicle formation. The complex can be purified from yeast as an approximately 700 kDa complex of unknown stiochiometry that, like the Sec23p/Sec24p complex, is a major component of the COPII coat and is required for its formation (Pryer et al., 1993; Salama et al., 1993; Barlowe et al., 1994). Both *SEC13* and *SEC31* are essential genes that show genetic interactions with the other COPII genes (Kaiser and Schekman, 1990; A. Frand and C. Kaiser, unpublished observations). Sec13p is composed almost entirely of six WD40 repeats, a motif specifying a β-propeller structure (Sondek et al., 1996), and appears to bind to Sec31p via the WD40-containing domain of Sec31p. Through separate regions of the protein, Sec31p can also bind to Sec16p, Sec23p, and Sec24p (Shaywitz et al., 1997). Besides such binding data, little is known about the function of this complex. These two proteins may simply play a structural role as part of the coat. In fact, data presented in this thesis suggests that Sec13p is not absolutely essential for the budding of COPII vesicles and may only be required to stabilize the coat at high temperatures or other conditions where the structural integrity of the coat is critical.

Other data suggests that Sec13p may have additional functions. Sec13p has recently been shown to also be involved in sorting and transport at the *trans*-Golgi of two

coordinately regulated permeases, Gap1p (the general amino acid permease) and Put4p (the proline permease). Sec13p may function at the Golgi, directly affecting the sorting and transport of these proteins, or Sec13p may be specifically required for the transport of a cofactor of these permeases that is necessary for their proper localization (Roberg et al., 1997). Furthermore, Sec13p (but not Sec31p) has been found in a subcomplex of the nuclear pore along with a Sec13p homolog, Seh1p (Siniossoglou et al., 1996). However, *sec13* mutants show no defects in nuclear pore function, whereas *seh1* mutants do appear to affect pore function. Thus, Sec13p may simply be structurally similar enough to its nuclear pore homolog that it can be integrated into this subcomplex of nucleoporins. Nonetheless, these data illustrate the need for further clarification and examination of the functions of Sec13p.

Summary

Numerous processing and sorting steps are required to prepare secretory proteins for efficient transport from the ER to the Golgi. In many cases, the modification of a protein is essential for its proper sorting and transport. Furthermore, recent results suggest that sorting is coupled to the assembly of the vesicle coat that drives vesicle formation at the ER. The following chapters of this thesis describe the isolation and characterization of the *BST* genes of *Saccharomyces cerevisiae*. These analyses allow several general conclusions to be drawn that are relevant to many of the topics discussed in this introduction. These conclusions will be summarized here, while leaving their justification for later chapters.

- Sec13p is not absolutely required for COPII vesicle formation, but is essential under conditions where coat stability is challenged and is important for sorting/transport late in the secretory pathway.

- The *BST1, EMP24/BST2,* and *ERV25/BST3* genes encode nonessential proteins whose functions restrict COPII-coated vesicle formation from the ER.
- The functions of the *BST* proteins enable efficient discrimination between cargo molecules and resident ER (or ER retained) proteins during sorting into COPII-coated vesicles.

In addition to these conclusions, several further observations are important for thinking about the role of the *BST* genes in protein sorting and transport.

- Removing *BST* gene functions allows COPII vesicle formation both in the absence of Sec13p and when other COPII coat proteins are crippled by conditional mutations.
- Emp24p/Bst2p and Erv25p/Bst3p are ER membrane proteins that form a complex and are incorporated into COPII vesicles (Belden and Barlowe, 1996), whereas Bst1p is a permanent resident of the ER membrane and is excluded from vesicles.
- The sorting of GPI-anchored proteins appears to be particularly sensitive to mutations in *BST* genes (especially mutations in *BST1*).
- Mutations in the lipase motif of Bst1p (which is highly conserved between Bst1p and its *C. elegans* homolog) disrupt function, suggesting a possible role for Bst1p in lipid modification.

Model for the function of the BST proteins in protein sorting and vesicle formation

A model can be derived that incorporates all of the above general conclusions and observations, as well as additional data described in this introduction. This model is presented now in order to provide a framework for the interpretation of the results described in the remainder of this thesis. The function of the *BST* proteins in this model

is to modify the lipid composition of the membrane in regions of vesicle assembly, which then facilitates the sorting of resident proteins from cargo molecules.

Vesicle formation is triggered by the recruitment and assembly of the COPII vesicle coat proteins on the ER membrane. Integral membrane components of the vesicle, including v-SNARES (required for fusion of the vesicle) and p24 proteins (Emp24p/Bst2p and Erv25p/Bst3p) are recruited to sites of coat assembly -- or recruit the coat to regions of the membrane where they reside -- by the binding of their cytosolically exposed domains to components of the vesicle coat. A putative complex containing Bst1p, Emp24p/Bst2p and Erv25p/Bst3p is thereby positioned at sites of vesicle assembly. The interaction of the complex with coat components releases Bst1p, which can then locally modify lipids in membrane. This local change in lipid composition facilitates the partitioning of membrane protein cargo into the region of vesicle budding and of resident membrane proteins (including Bst1p itself) out of these regions. Interactions between cargo proteins and coat proteins may also play a role in this process. Soluble cargo is concentrated by binding interactions with sorting receptors (potentially a further function of the p24 proteins) or membrane protein cargo, and resident proteins are excluded through interactions with resident membrane proteins. A further consequence of the local change in lipid composition and subsequent sorting is an increased requirement for a stable coat.

Thus, in the absence of any of the *BST* proteins the localized change in lipid composition does not occur. Sorting is less efficient, resulting in a decreased rate of transport for cargo molecules sensitive to the change in lipid composition and an increased rate of transport of resident proteins. Without the local change in lipid composition there is less need for stability in the coat, allowing COPII vesicles to form in the absence of Sec13p or at higher temperatures in strains with conditional mutations in other COPII coat proteins.

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Chapter Two:

Genes that control the fidelity of ER to Golgi transport identified as suppressors of vesicle budding mutations

PREFACE

This chapter has been published in Molecular Biology of the Cell as:

Elrod-Erickson, M. J. and C. A. Kaiser (1996). Genes that control the fidelity of endoplasmic reticulum to Golgi transport identified as suppressors of vesicle budding mutations. Mol. Biol. of the Cell *7*, 1043-1058.

ABSTRACT

While convergent evidence suggests that proteins destined for export from the endoplasmic reticulum (ER) are separated from resident ER proteins and are concentrated into transport vesicles, the proteins that regulate this process have remained largely unknown. In a screen for suppressors of mutations in the essential COPII gene *SEC13*, we identified three genes (*BST1*, *BST2/EMP24* and *BST3*) that negatively regulate COPII vesicle formation, preventing the production of vesicles with defective or missing subunits. Mutations in these genes slow the secretion of some secretory proteins and cause the resident ER proteins Kar2p and Pdi1p to leak more rapidly from the ER, indicating that these genes are also required for proper discrimination between resident ER proteins and Golgi-bound cargo molecules. The *BST1* and *BST2/EMP24* genes code for integral membrane proteins that reside predominantly in the ER. Our data suggest that the *BST* gene products represent a novel class of ER proteins that link the regulation of vesicle coat assembly to cargo sorting.

INTRODUCTION

Protein transport through the secretory pathway of eukaryotic cells consists of a series of vesicle budding, targeting and fusion steps that move protein cargo from one membrane-bounded organelle to the next (Palade, 1975). These processes have been studied mainly by biochemical dissection of the cytosolic components required for mammalian and yeast cell-free transport assays and by the analysis of yeast mutants that completely block the flow of cargo through the secretory pathway (reviewed in Pryer *et al.*, 1992; Rothman and Orci, 1992). Such approaches have identified many of the essential structural components of vesicles, but much less is known about how cargo molecules are segregated into vesicles. Cargo that is to be carried forward must be packaged into vesicles while permanent residents of an organelle must, to at least some degree, be kept out of the transport vesicles in order to maintain the integrity of individual organelles (reviewed in Pfeffer and Rothman, 1987).

The first known sorting step occurs as proteins exit the endoplasmic reticulum (ER). Short peptide sequences carried by resident ER proteins act as signals for the retrieval to the ER of the small fraction of these proteins that have escaped to the Golgi (reviewed in Pelham, 1995). However, when the known retrieval signals are removed from ER resident proteins, secretion to the cell surface is still much slower than for actual secretory proteins, implying that additional retention mechanisms exist (Nilsson *et al.*, 1989; Hardwick *et al.*, 1990). Additionally, the concentration of at least some secretory proteins into regions of vesicle budding (Mizuno and Singer, 1993; Balch *et al.*, 1994) and the selective packaging of α-factor into COPII-coated but not COPI-coated vesicles that bud from the ER (Bednarek *et al.*, 1995) argue that sorting functions may act not only to restrict the progression of resident proteins but also to concentrate some cargo molecules into vesicles.

Cargo selectivity and the fidelity of vesicle assembly have been difficult to study since biochemical assays for these processes have not yet been developed. Furthermore, informative mutations that reduce the fidelity of transport vesicle function have not yet been isolated, presumably because the phenotype of such mutants would be too subtle to have been recognized in screens for secretory mutants. An opportunity to identify genes that control the accuracy of vesicle formation presented itself when we found that recessive mutations in three genes can efficiently bypass the secretion block that results from null mutations of the essential vesicle coat protein gene *SEC13*. We reasoned that a negative regulatory mechanism may prevent the completion of defective vesicles, resulting in the block in vesicle transport exhibited by *sec13* mutants. The bypass suppressors could then represent mutations that inactivate this vesicle quality control system, allowing vesicles to form without Sec13p.

Bypass suppressors of *sec13Δ* were unexpected since Sec13p is one of the seven key proteins (Sec12p, Sec13p, Sec16p, Sec23p, Sec24p, Sec31p and Sar1p) required for budding of transport vesicles from the ER in *Saccharomyces cerevisiae* (Nakano *et al.*, 1988; Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Hicke *et al.*, 1992; Pryer *et al.*, 1993; Salama *et al.*, 1993; Espenshade *et al.*, 1995). For six of these proteins, involvement in vesicle formation has been demonstrated for ER to Golgi transport reconstituted *in vitro* (d'Enfert *et al.*, 1991; Oka *et al.*, 1991; Hicke *et al.*, 1992; Salama *et al.*, 1993), and all but Sec12p are found as components of the protein coat of vesicles produced *in vitro* (Barlowe *et al.*, 1994; Espenshade *et al.*, 1995). The elements of this coat are collectively known as COPII, and the assembly of this structure is thought to drive vesicle formation (Barlowe *et al.*, 1994). An analogous structure exists in mammalian cells, since homologs of Sec13p and Sec23p are localized to the transitional ER of mammalian cells (Orci *et al.,* 1991; Shaywitz *et al.,* 1995). These results demonstrate the central and evolutionarily conserved role played by Sec13p in the process of vesicle formation.

This report describes the suppressors of *sec13Δ* mutations which lie in three genes called *BST* for bypass of *SEC13*. The *BST1* gene was cloned and encodes a membrane protein situated in the ER. The *BST2* gene was also cloned and found to be identical to *EMP24,* whose product was previously shown to be an ER protein and a membrane component of COPII vesicles (Schimmöller *et al.*, 1995). In addition to bypassing the need for Sec13p in ER to Golgi transport, the *bst* mutations were found to increase the rate by which resident ER proteins (Kar2p and Pdi1p) and retained proteins (s11-invertase) leave the ER and to decrease the rate by which the secretory protein invertase is transported from the ER. These genes therefore define a new type of ER function that specifies both the fidelity of cargo sorting and the cytosolic protein requirements for vesicle assembly. A simple explanation for the phenotypes of the *bst* mutants is that the processes of vesicle assembly and cargo loading are coupled. The loss of a quality control mechanism for vesicle assembly could allow inappropriate or premature formation of vesicles that have not yet properly segregated cargo away from ER resident proteins.

MATERIALS AND METHODS

Media and Yeast Strains

Growth and maintenance of strains, preparation of standard media (Difco, Detroit, MI), crosses and other genetic manipulations were performed as described in Kaiser *et al.*, 1994. *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. The *sec13Δ1* allele is a deletion of the entire coding sequence of *SEC13* (R. Gimeno, this laboratory) and the *sec13Δ2::LEU2* allele is a deletion/disruption as described in Pryer *et al.*, 1993. The *SUC2-s11* allele is an Ala to Ile substitution at position -1 of the signal peptidase cleavage site that blocks peptidase cleavage (Bohni *et al.*, 1987). In strain construction, the *suc2Δ* allele was scored by invertase assay and the *KAR2ΔHDEL* allele was followed by the dominant Kar2p secretion phenotype it confers. *bst* alleles were scored by assaying suppression of *sec13Δ* and Kar2p secretion. The former trait was often examined by testing our ability to generate viable transformants of a strain with the *SEC13* knockout construct pCK1316 (Pryer *et al.*, 1993). This assay was used in addition to crosses to *bst sec13Δ* because the low viability (between 50 and 70%) of *bst sec13Δ* ascospores often rendered test-crosses alone inconclusive. Isogenic *KAR2ΔHDEL, bst1Δ KAR2ΔHDEL* and *bst2Δ KAR2ΔHDEL* strains were constructed from CKY190 by disruption of the *BST* genes using the plasmids pME1165 and pME262 respectively. Isogenic *suc2Δ* and *bst1Δ suc2Δ* strains were similarly constructed from CKY343.

Plasmids and DNA Manipulations

DNA manipulations were performed as described in Sambrook *et al.*, 1989. pRS306-2μ is an episomal derivative of pRS306 (Sikorski and Hieter, 1989; Miller and Fink, unpublished). pCK1390 is a *GAL1*-promoted *SEC13* plasmid as described in Shaywitz *et al.*, 1995. pCK1391 is *SEC13* in pRS306-2μ (C. Kaiser, unpublished). pEHB29 is *SUC2-s11* in pRS316 (Sikorski and Hieter, 1989; E. Hong, unpublished). pME11 is a

YCp50 library (Rose *et al.*, 1987) plasmid containing *BST1*. pME1113 was constructed by recircularization of SalI digested pME11. pME1101 was constructed by ligating the 3.8 kb ClaI – KpnI fragment from pME11 into the ClaI – KpnI sites of pRS316. pME1120 and pME1121 are two plasmids from a series of nested deletions generated from pME1101 according to the method of Henikoff, 1984. These plasmids have deletions of approximately 700 bp and 900 bp respectively (see Figure 5A). pME1108 is the 3.5 kb ClaI - XhoI fragment of pME11 in pRS316. pME1165 was constructed by ligating the 1.3 kb SpeI – XhoI fragment of pME1101 into the SpeI – XhoI sites of pRS305 (Sikorski and Hieter, 1989). pME1170 was constructed in two steps. First, a NotI site was inserted at a position between codons 86 and 88 of *BST1* by site directed mutagenesis according to Kunkel *et al.*, 1987. The sequence of the oligonucleotide used was 5'-GAT GGA AAC ATA TAG ATG CGG CCG CTT TCA CAC TGA GGA GCA TCT GC-3'. A 117 bp NotI fragment from pGTEPI (Tyers *et al.*, 1993) containing three tandem repeats of the hemagglutinin (HA) epitope (Kolodziej and Young, 1991) was then ligated into the newly created NotI site to give pME1170. pME21 is a YCp50 library (Rose *et al.*, 1987) plasmid containing *BST2/EMP24*. pME253 was constructed by ligating the 3.7 kb HindIII fragment of pME21 into the HindIII site of pRS316. pME262 was constructed by first ligating the 0.5 kb EcoRI – SacI fragment from pME253 into the EcoRI – SacI sites of pRS306 to generate pME260. The 1.4 kb EcoRI – SalI fragment from pME253 was then ligated into the EcoRI – SalI sites of pME260.

Isolation and Complementation Testing of bst Mutants

One hundred colonies of CKY321 were cultured for 3 days in liquid YEP (2% galactose). For each culture, $8x10^6$ viable cells were plated onto an SC (2% glucose) 5-FOA plate at 24°C. After 3 days of growth, a single 5-FOA resistant colony from each plate was chosen for further analysis.

Initially, complementation tests were done by crossing a *bst sec13Δ* mutant isolate to several other outcrossed *bst sec13Δ* mutant strains of the opposite mating type and with complementary auxotrophies. Mating mixtures were grown on rich medium (YPD) overnight, then replica plated to minimal medium (SD) selective for the growth of diploids. Growth of the *sec13Δ/sec13Δ bst/bst* diploids indicated failure of the recessive *bst* mutations to complement. Once initial testing indicated three complementation groups, test strains representing each group (CKY325, CKY326 or CKY327) were used to classify the remaining mutants.

Protein Gels, Immunoblotting and Quantitation

Protein extracts were prepared from 1-5 OD_{600} units of cells by boiling and lysis with glass beads in 30µl of sample buffer (80 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 10% bromophenol blue). Proteins from the culture medium were precipitated by adding 100% TCA to a final concentration of 10% and incubating at 0°C for 30 min. The precipitates were pelleted in a microfuge, washed with 100% acetone (-20°C for 30 min) and resuspended in sample buffer. Samples were heated at 95°C for 3 min before being resolved by SDS-PAGE. Proteins were transferred to nitrocellulose in a semi-dry transfer apparatus (Owl Scientific Plastics, Inc., Cambridge, MA) at 500 mV for 45 min. Blots were blocked and then incubated for 1 h with primary antibody in TBS-T (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20), 2% milk at the following dilutions: affinity purified rabbit anti-Sec13p antibody was used at 1:1,000 dilution, affinity purified rabbit anti-KAR2p antibody (a kind gift from M. Rose, Princeton University) was used at 1:10,000 dilution, anti-Pdi1p antibody (a kind gift from C. Shamu) was used at 1:2,000 dilution and anti-HA antibody (12cA5 ascitic fluid; BABCO, Richmond, CA) was used at 1:1,000 dilution. Blots were then washed three times for 10 min each in TBS-T, incubated with a 1:10,000 dilution of either goat anti-rabbit IgG-HRP or sheep anti-mouse IgG-HRP (both Amersham Corp., Arlington Heights, IL) in TBS-T, 1% BSA

for 1 h, washed 3 times for 10 min each in TBS-T, developed for chemiluminescence using the ECL system (Amersham) and exposed to film.

For quantitation of Western blots, a serial dilution of sample was first immunoblotted as described above and quantitated on an LKB 2202 Ultroscan laser densitometer (LKB, Bromma, Sweden) to determine the range in which a linear response was observed. Working within this range, samples were loaded in duplicate and all values reported are the average of the duplicate samples scanned three times each.

An immunoblotting assay was used to score Kar2p secretion for the cloning of *BST1* and *BST2/EMP24* (Figure 4A). Colonies on solid medium were covered with a wetted nitrocellulose filter (S&S BA85 0.45μm circles, Keene, NH) and grown for 16-24 hours (YPD) or 40-48 hours (synthetic medium). Cells were washed from the filter with 10mM Tris-HCl (pH 7.5), 0.5M NaCl and the filters were processed by immunoblotting as described above.

Radiolabeling and Immunoprecipitation

Radiolabeling and immunoprecipitations were carried out essentially as described in Gimeno et al., 1995. The IP buffer used was 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100. 1μl of anti-CPY or 2μl of anti-invertase antibody was used per OD_{600} unit of labeled cell extract. For the reimmunoprecipitation of s11-invertase with anti-α1,6 antibody, the precipitated primary immune complexes were disrupted by boiling in 30μl of sample buffer (as above). 20μl were removed and diluted in 1ml of IP buffer. 0.5μl of anti-α1,6 antibody was then used to reimmunoprecipitate the α1,6 modified protein. 10μl of each sample was separated by SDS-PAGE. Gels were soaked in 1 M sodium salicylate for 30 min before being dried and exposed to a phosphorimaging screen. Images were analyzed using a 445si PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Cloning of BST1 and BST2/EMP24

BST1 and *BST2* were cloned by complementation of the Kar2p secretion phenotype. Plasmids were rescued and shown to complement both the Kar2p secretion phenotype of the corresponding *bst* mutant strain and the suppression of *sec13Δ* mutations. The latter was tested in transformation experiments where our inability to generate viable transformants of the corresponding *sec13Δ bst* mutant strain was scored as complementation of the suppression phenotype. Controls for these experiments included showing that the *sec13Δ bst* mutant strain could be transformed with a control plasmid, and that the clone being tested could transform a control strain.

Once clones were obtained, they were subcloned into pRS316 (Sikorski and Hieter, 1989), and the minimal complementing region was identified using the transformation assay described above. For *BST1,* a series of nested deletions were generated from both directions as described in Henikoff, 1984. These clones were used to sequence both strands by the dideoxy method following the Sequenase protocol (USB, Cleveland, OH). Sequence generated in this manner was used to search both DNA and protein databases for homologies. The *BST1* gene sequence is found on Chromosome VI as YFL025C (The Saccharomyces Genomic Information Resource [http://genome-www.stanford.edu/]). For *BST2,* sequence analysis identified the complementing region as the previously identified *EMP24* locus (Schimmöller *et al.*, 1995).

Bst1p Localization

To analyze the subcellular distribution of Bst1p, cells (CKY10) expressing either (HA) epitope tagged *BST1* (pME1170) or untagged *BST1* (pME1101) were examined by Western analysis using anti-HA antibody, which detected a single diffuse band that was only present in strains containing the epitope tagged *BST1*. This protein migrated with an apparent molecular mass of 142-146 kDa (Figure 5). Cell fractionation was carried out on these strains as described in Espenshade *et al.*, 1995. EndoH$_f$ treatments were performed

by diluting 3.5 fold into 20mM sodium citrate (pH 5.5), adding 8 units of EndoH$_f$ (New England Biolabs, Beverly, MA) and incubating at 37°C for 3 hours. Indirect immunofluorescence of Bst1p-HA was carried out essentially as described in Espenshade *et al.*, 1995. A yeast strain (CKY10) expressing *BST1-HA* (pME1170) was grown selectively and then transferred to YPD for 2 hours before fixation. The fixed cells were washed once in 0.1% SDS for 5 minutes prior to application to the slide in order to enhance the detection of the protein. Bst1p-HA was visualized using a 1:200 dilution of anti-HA antibody (12cA5 ascitic fluid; BABCO, Richmond, CA) and a 1:200 dilution of fluorescein-conjugated sheep anti-mouse secondary antibody (Amersham). Kar2p was visualized using a 1:5,000 dilution of affinity purified anti-Kar2p antibody (a kind gift from M. Rose, Princeton University) and a 1:200 dilution of rhodamine-conjugated goat anti-rabbit secondary antibody (Boehringer Mannheim, Indianapolis, IN). For the double-label immunofluorescence experiments an additional blue excitation filter (Zeiss, #467974) was used to eliminate crossover fluorescence.

Genetic Interaction between bst and sec Mutations

To minimize the possible variation due to genetic background, *sec13Δ bst* mutant isolates were outcrossed and the *bst* mutant spore clones were sequentially backcrossed four times to either CKY8, CKY10, CKY13 or CKY14. These strains are members of a set of isogenic strains derived from S288C (D. Botstein, Stanford). In the final backcrosses, the Kar2p secretion and suppression phenotyes cosegregated 2:2 in the tetrads analyzed. Two such *bst* mutant strains (CKY330 and CKY332) were then crossed to the *sec* mutant strains listed in Table 1. Tetrads were tested for growth at 24, 28, 30, 33, 36 and 38°C. Genetic interactions (either partial suppression or synthetic enhancement of the temperature sensitivity) were observed in crosses where 2:2, 3:1 and 4:0 ascospore viability patterns were seen at temperatures above or below the "cutoff temperature" for a given *sec* mutant. The suppression or synthetic effects (seen when in combination with a

sec mutation) completely cosegregated with the Kar2p secretion phenotype of the *bst* mutation in the cross, as assayed by the immunoblotting filter assay described above. In crosses where the *sec* mutant involved also secretes Kar2p (*sec17* and *sec18*; Semenza *et al.*, 1990), only NPD tetrads were considered. In representative tetrads the segregation of the *bst* mutation in the cross was then confirmed by scoring the *sec13Δ* suppression phenotype. Isogenic *sec* and *sec bst* strains were constructed by transforming the *sec bst* double mutant strains from Table 1 with the corresponding *BST* gene on a CEN plasmid (pME1101 or pME253) or with a CEN vector (pRS316) control plasmid. The growth of these isogenic pairs of strains was compared on synthetic complete medium lacking uracil.

Invertase Assays

Invertase assays were performed on either whole cells (external) or cell lysates (total) prepared in the following manner. Exponentially growing cultures in either YPD or SD + casamino acids (2% glucose) were pelleted and resuspended at a density of 1 OD_{600} unit/ml in medium with 0.1% glucose. Cells were then incubated for 2 hours. 1 OD_{600} unit of cells was removed to an equal volume of 20 mM Tris (pH 7.5), 20 mM NaN_3 at 0°C, washed twice in ice cold 10 mM Tris (pH 7.5) and resuspended in either 1 ml of 10 mM Tris (pH 7.5) for assaying external invertase or 30 µl of 10 mM Tris (pH 7.5), 1% Triton for assaying total invertase activity. For assay of total activity, the cells were lysed by vigorous vortexing with glass beads for 3-5 min and then diluted to 1 ml in 10 mM Tris (pH 7.5). Invertase was assayed as described in Gascón *et al.*, 1968 in duplicate for each strain tested.

RESULTS

Isolation and Complementation Testing of bst sec13Δ Mutant Strains

SEC13 is an essential gene, since the conditional allele *sec13-1* is inviable above 30°C and spores bearing a deletion of the gene (*sec13Δ*) never germinate on rich medium (Pryer *et al.*, 1993). To examine the phenotype of cells lacking Sec13p, we constructed a strain with *sec13Δ* on the chromosome and a plasmid-borne copy of *SEC13* under *GAL1* promoter control. Under conditions where Sec13p expression is repressed by growth on glucose, this strain does not grow, yet rare revertant colonies arise on glucose plates. To explore the genetic basis of this reversion, a collection of revertants was isolated as follows. The strain CKY321 has a chromosomal deletion of the *SEC13* coding sequence and was therefore dependent on the *GAL1*-promoted *SEC13-URA3*-containing plasmid pCK1390 for viability. (This strain was designed to minimize the likelihood of reversion by plasmid integration or gene conversion: the plasmid carries a centromere and only shares homology with the chromosome in the 3' region of the *SEC13* locus.) Cultures of CKY321 were plated on medium containing 5-FOA to select for cells that had lost the *SEC13-URA3* plasmid. Robust colonies arose on these plates after three days of growth at 24°C at an average frequency of $2x10^{-6}$ colonies per viable cell. After longer incubations (up to seven days), additional colonies arose at a 10-fold higher frequency. These additional colonies comprise a second class of suppressors, weaker than those discussed in this report, that are currently under investigation. After three days of growth, 100 independent revertants were chosen for further analysis.

Preliminary experiments suggested that most of the revertants contained unlinked suppressor mutations that segregated as single genes on backcrossing. To distinguish suppressor containing strains from other possible types of revertants, a number of independent tests were performed. Transformation with *URA3*-containing plasmids restored 98 of the isolates to Ura^+, indicating that in these cases 5-FOA resistance was

probably due to plasmid loss, as expected. The two isolates that remained Ura$^-$ in this test were not considered further since they still contained Sec13p detected by Western blotting and probably resulted from *ura1* mutation. Southern analysis on representatives of the 98 isolates confirmed that the deletion at the *SEC13* locus was still intact. Finally, cell extracts from representative isolates did not contain Sec13p by Western blotting. Figure 1A shows the absence of Sec13p in three such representatives that were subsequently identified as isolates from the three major complementation groups (see below). Together these findings show that reversion had occurred because of suppressor mutations that allowed growth in the absence of Sec13p. We therefore called these *bst* mutants (for **b**ypass of *s*ec **t**hirteen).

To determine the number of genes represented by this collection of mutants, *bst sec13Δ* strains that were appropriate for complementation testing were constructed by backcrossing. The test strains were mated to the 98 isolates and diploids were selected by use of complementary auxotrophic mutations. For all 98 isolates, the resulting diploids were found to be inviable for many of the test crosses. This showed that the suppressor mutations were recessive and could be organized into complementation groups as follows. In crosses where the parental *bst* mutations are in different genes, complementation in the diploid should prevent suppression of the homozygous *sec13Δ* mutation, giving an inviable diploid; viable diploids should be recovered in crosses where both suppressor mutations are in the same gene. This analysis allowed the mutants to be organized into three major complementation groups, designated *BST1* (52 isolates), *BST2* (24 isolates) and *BST3* (19 isolates). The remaining three isolates grew poorly and mated inefficiently, and therefore gave ambiguous results in the complementation test. An example of complementation tests among the three groups is shown in Figure 1B.

ER to Golgi Transport is Restored in bst sec13Δ Mutants

The strains with suppressed *sec13Δ* grow almost as well as wild-type: the doubling times in rich medium at 24°C for *bst1 sec13Δ*, *bst2 sec13Δ*, and *bst3 sec13Δ* are 170 min, 165 min, and 155 min, as compared to 150 min for wild-type. The ability of *bst sec13Δ* strains to grow well indicated a functional secretory pathway. To examine secretion more closely, the maturation of the vacuolar protease carboxypeptidase Y (CPY) was followed as a marker for early events in the secretory pathway. CPY is found in three distinct forms, P1, P2 and mature, that represent progression to the ER, Golgi and vacuole, respectively (Stevens *et al.*, 1982). In the *bst sec13Δ* strains CPY was targeted and transported normally at 24°C (Figure 2A), although somewhat more slowly than in the wild-type control (Figure 2C).

The transport of secreted invertase in *bst sec13Δ* strains was also examined. These strains showed normal levels of extracellular invertase activity, indicating that there was no block to invertase secretion. A more detailed examination of the kinetics of invertase secretion was performed by pulse-chase analysis. As with CPY, secreted invertase matured normally in *bst sec13Δ* strains at 24°C, although more slowly than in the wild-type control (Figures 2B and 2C); this kinetic defect was more pronounced in *bst2 sec13Δ* and *bst3 sec13Δ* mutants than in *bst1 sec13Δ*. The acquisition by invertase of extensive outer-chain addition at later times showed that transport through the Golgi occurred normally in the mutant strains. Thus, the bypass that occurs in *bst sec13Δ* strains does not completely circumvent any of the normal transport steps to the cell surface or vacuole.

ER to Golgi Transport in bst Mutant Strains

In order to understand better the role of the *BST* genes in the secretory process, the *bst* mutations were introduced into an otherwise wild-type genetic background. To construct these strains, *bst* mutant segregation was followed in crosses to wild-type by subsequent

test crosses to *bst sec13Δ* strains and by transformation experiments to test whether individual segregants could be transformed with an integrating plasmid that knocks out the *SEC13* gene. The growth characteristics and secretion phenotypes of these *bst* mutant strains were then analyzed. The *bst1, bst2* and *bst3* strains (CKY330, CKY332 and CKY334, respectively) grew as well as wild-type at all temperatures. All of the parental *bst sec13Δ* strains were unable to grow at temperatures above 30°C. This temperature sensitivity can be ascribed to a temperature dependence of the bypass process since the *bst* mutations themselves do not cause temperature sensitivity.

The transport of CPY and invertase in *bst* strains was analyzed by pulse-chase experiments at 30°C. Invertase matured normally in the *bst1* strain, but showed a significant delay in transport in *bst2* and *bst3* mutants (Figures 3B and 3C). CPY matured normally in all three mutants (Figures 3A and 3C). Thus, *bst2* and *bst3* mutations slowed invertase transport from the ER, but otherwise the *bst* mutations did not have a marked effect on secretory protein transport.

Cloning and Analysis of BST1

We also examined the effect of *bst* mutations on the retention of the resident ER protein Kar2p. The test was conducted by growing strains in contact with a nitrocellulose filter and then probing the filters with antibody to detect Kar2p released into the extracellular space. Both *bst sec13Δ* and *bst* strains showed a dramatic increase in the amount of Kar2p detectable by this assay compared to the wild-type control (Figure 4A). In tests of diploids, Kar2p secretion was recessive for *bst1*, *bst2*, and *bst3* mutations and therefore provided a plate assay for cloning the genes by complementation.

The *BST1* gene was cloned by transforming the *bst1-1* strain CKY331 with a genomic library in YCp50 and screening 6,000 transformants for those that no longer secreted excess Kar2p. Three independent isolates of the same library clone were identified, and the minimum complementing region within this plasmid was determined

by subcloning (Figure 5A). The 3.8 kb ClaI – KpnI fragment of pME1101 was shown to be sufficient to complement both the Kar2p secretion (see Figure 4A) and *sec13Δ* suppression phenotypes of *bst1* mutant strains (our unpublished results). The latter property was tested in transformation experiments where complementation of *bst1* was scored by the failure of a plasmid carrying the complementing gene to form viable transformants of a *bst1 sec13Δ* strain. The nucleotide sequence at the ClaI site was found to lie within the *STE2* locus, which is adjacent to the complementing region of the clone (Figure 5A). This observation places the *BST1* locus on the left arm of Chromosome VI.

The 3.3 kb of pME1101 adjacent to the *STE2* locus was sequenced and contains a single open reading frame predicted to encode a protein of 118 kDa. Analysis of the hydrophobicity of this sequence, using the algorithm of Kyte and Doolittle (1982), predicts multiple potential membrane spanning domains in the C-terminus and a single transmembrane domain near the N-terminus. Additional features are 14 potential N-linked glycosylation sites throughout the protein sequence and a double-arginine motif at the fourth and fifth amino acids from the N-terminus. These aspects of the sequence are diagrammed in Figure 5B.

To confirm that this open reading frame corresponds to the *BST1* gene, a disruption of the gene was generated by subcloning the 1 kb SpeI – XhoI fragment that lies within the coding sequence into the *LEU2*-marked integrating vector pRS305 (Sikorski and Hieter, 1989). The resulting plasmid, pME1165, was linearized within the insert at the PstI site and integrated into the wild-type diploid CKY348 by transformation. Integration results in two truncated copies of the gene separated by plasmid sequences, and neither truncated copy should be functional according to the complementation behavior of subclones. The resulting heterozygous diploid was sporulated and all tetrads examined had four viable ascospores (n=30), demonstrating that *BST1* is not essential. In each tetrad the *LEU2* marker segregated 2:2 and showed complete linkage to excess Kar2p secretion as determined by the immunoblotting filter assay. The Leu^+ spore clones also

exhibited the ability to suppress *sec13Δ* both in crosses to *bst1 sec13Δ* strains and in transformation experiments where the *SEC13* gene was disrupted directly. Finally, in crosses between the Leu^+ spore clones and *bst1* mutant strains, complete linkage was demonstrated by 4:0 segregation of the Kar2p secretion phenotype (n=36). We therefore refer to this gene as *BST1*. This analysis also demonstrated that the alleles of *bst1* isolated as suppressors of *sec13Δ* have the same phenotype as the disrupted allele.

Bst1p is an Integral Membrane Glycoprotein that Resides in the ER

The predicted amino acid sequence of Bst1p indicated an integral membrane glycoprotein. To examine the protein, a 30 amino acid epitope was inserted near the N-terminus of the protein (see Figure 5) to produce a tagged version of the gene, *BST1*-HA. A centromere plasmid carrying *BST1*-HA (pME1170) complemented all of the phenotypes of *bst1* mutant strains, showing that insertion of the epitope did not disrupt function. Cells expressing *BST1*-HA were converted to spheroplasts, lysed gently, and fractionated by differential centrifugation. Bst1p-HA detected by immunoblotting was found exclusively in the 500***g*** and 10,000***g*** pellets, suggesting membrane association (Figure 6A). Cytosolic invertase in these samples was found almost entirely in the 150,000***g*** supernatant, demonstrating that the cells were efficiently lysed. After cell lysates were incubated in 1% Triton X-100 and centrifuged at 150,000***g***, much of the Bst1p-HA became soluble (Figure 6B), consistent with the behavior expected of an integral membrane protein. Treatment of cell lysates with 0.5 M NaCl, 2.5 M urea or carbonate buffer, pH 11.5, did not solubilize Bst1p (our unpublished results).

To determine whether Bst1p is a glycoprotein, cell extracts were treated with $EndoH_f$ to remove N-linked oligosaccharide chains. Treatment with $EndoH_f$ resulted in an increase in the mobility of Bst1p-HA consistent with the removal of six or seven core oligosaccharide chains from the protein (Figure 6B). This result demonstrated that Bst1p is a glycoprotein and indicated that the large domain of the protein between the single N-

terminal and multiple C-terminal transmembrane sequences resides in the lumen of the ER (diagrammed in Figure 5B).

The demonstration that Bst1p is an integral membrane glycoprotein limited its subcellular distribution to the organelles of the secretory pathway. Additionally, the predicted sequence of the protein shows an N-terminal double arginine motif at positions 4 and 5, which has been shown to be an ER retention signal for type II integral membrane proteins in mammalian cells (Schutze *et al.*, 1994). To determine whether Bst1p-HA progresses beyond the ER, we examined the extent of glycosylation of the protein in a *sec18-1* mutant blocked for ER to Golgi transport. Western blots of extracts from this strain grown either under permissive conditions or shifted to nonpermissive temperatures for 2 hours showed no detectable difference in the amount or mobility of the Bst1p-HA. Most of the protein, therefore, is not subject to post-ER glycosylation and presumably resides in the ER (Figure 6B). As a control to demonstrate the efficacy of the *sec18* block, CPY was shown to remain in the P1 form in the *sec18* strain at the restrictive temperature.

The localization of Bst1p was further examined by indirect immunofluorescence microscopy. In order to enhance the detection of the protein in cells expressing *BST1-HA* (from the centromere plasmid pME1170), fixed cells were treated with SDS in a manner similar to that described for the optimal detection of Sec62p (Deshaies and Schekman, 1990). Cells treated in this manner and stained with anti-HA antibody showed a continuous band of concentrated staining surrounding the DAPI-stained nucleus. This staining pattern is characteristic of ER proteins (Rose et al., 1989; Deshaies and Schekman, 1990), and was not seen in cells expressing untagged *BST1*. In double staining experiments the Bst1p staining was coincident with that of the luminal ER protein Kar2p (Figure 7), consistent with an ER localization for Bst1p.

BST2 is Identical to EMP24

The *BST2* gene was cloned using the same strategy as that used to isolate *BST1*. Approximately 10,000 YCp50 library transformants of a *bst2-1* mutant were screened to find two overlapping clones that were shown to complement both the Kar2p secretion and *sec13Δ* suppression phenotypes of *bst2* mutant strains. Subclones were tested to find the minimal complementing region, and the sequence of this region showed it to be the *EMP24* locus. *EMP24* encodes a 24 kDa integral membrane protein that resides in the ER and in COPII vesicles that bud from the ER (Schimmöller *et al.*, 1995). A mammalian homolog of EMP24 was found to be enriched in COPI vesicles (Stamnes *et al.*, 1995). Disruption of *EMP24* was shown to decrease the transport of invertase and Gas1p from the ER, consistent with the transport defect of the *bst2* mutants described here (Schimmöller *et al.*, 1995).

To confirm that *BST2* and *EMP24* are identical, a disruption of the *EMP24* coding sequence was constructed by replacing an internal SacI–SalI fragment of *EMP24* with the *URA3*-marked integrative plasmid pRS306 in the wild-type diploid CKY348. Sporulation of the resulting diploid gave four viable spores in which the *URA3* marker segregated 2:2 (n=29), confirming that *EMP24* is a nonessential gene. The disrupted allele was shown to confer the Kar2p secretion and *sec13Δ* suppression phenotypes of *bst2* mutant strains and to be linked to the isolated *bst2* mutations (n=40), as described above for *BST1*. The *bst2* null alleles behaved identically to the *bst2* mutations isolated as suppressors of *sec13Δ* .

bst Mutations Cause Leakage of the Resident ER Proteins Kar2p and Pdi1p from the ER

The most striking phenotype of *bst* mutants is their defect in the retention of Kar2p. To investigate the nature and magnitude of this defect, we assayed the quantity of Kar2p released from cells by TCA precipitation of the growth medium and quantitation of

Kar2p by Western blotting. Comparison of the amount of Kar2p in the medium to the amount in the corresponding cell extracts gave the fraction of Kar2p secreted. Both *bst sec13Δ* and *bst* mutant strains secreted about 17% of the total Kar2p in 3 hr after transfer to fresh culture medium, whereas the wild-type strain secreted less than 1% of the total Kar2p (Figure 4B). Probing the medium for the presence of CPY (with anti-CPY antibody) showed that the *bst sec13Δ* and *bst* mutant strains had the same low levels of CPY in the medium as the wild type control (P2 form only), ruling out the possibility that the Kar2p in the medium was from cell lysis (our unpublished results).

Excessive secretion of Kar2p into the culture medium is a hallmark of *erd* mutants, which are defective in the retrieval from the Golgi of resident ER proteins that, like Kar2p, have the motif HDEL at their C-terminus (Hardwick *et al.*, 1990; Semenza *et al.*, 1990). Mutants of *erd2* secrete as much Kar2p into the medium as do strains bearing an allele of *KAR2* that lacks the HDEL retention signal (*KAR2ΔHDEL*) (Semenza *et al.*, 1990). In both cases, the rate of Kar2p secretion is slow compared to the rate of secretion for actual secretory proteins such as invertase. The slow secretion of Kar2p$^{\Delta HDEL}$ implies that mechanisms independent of HDEL-dependent recycling contribute to retention of Kar2p in the ER.

The amount of Kar2p secreted by *bst* mutants was equivalent to that secreted by strains expressing the *KAR2ΔHDEL* allele (Figure 4B), suggesting that the *bst* mutations might affect the HDEL-dependent retrieval of Kar2p. Alternatively, the mutations could affect an HDEL-independent retention mechanism (*i.e.* the rate at which Kar2p exits the ER). To distinguish between these possibilities, we examined the extent of Kar2p$^{\Delta HDEL}$ secretion in *bst KAR2ΔHDEL* double mutant strains to determine whether the effect of *bst* mutations on Kar2p secretion depended on HDEL. Removal of HDEL from Kar2p should completely eliminate the *ERD2*-dependent retrieval of this protein; if a *bst* mutation affects a retention mechanism that does not depend on HDEL, then a *bst KAR2ΔHDEL* double mutant should secrete more Kar2p$^{\Delta HDEL}$ than should either single

mutant alone. Quantitative Western blotting of Kar2p$^{\Delta HDEL}$ from both the culture medium and cell extracts revealed that while all the mutants maintained wild-type levels of internal Kar2p (through increased production of the protein, presumably as a result of the unfolded protein response), the *bst1Δ KAR2ΔHDEL* and *bst3-2 KAR2ΔHDEL* mutants consistently secreted approximately twice as much Kar2p$^{\Delta HDEL}$ into the culture medium (in a fixed amount of time) as did the single mutant strains (Figure 4C), suggesting that *bst1* and *bst3* mutations affect a retention mechanism that is independent of the HDEL-dependent retrieval pathway.

The similarity of the phenotypes of *bst1, bst2* and *bst3* mutants suggested these genes might perform similar functions. To address the degree to which the functions of the *BST* genes overlap, the effect of double mutants on the extent of Kar2p secretion was examined. Neither the *bst1Δ bst2Δ* nor *bst2Δ bst3-2* double mutants showed a significant increase in Kar2p secretion over that of the single mutants, indicating that loss of *BST2* does not exacerbate the defect already present in either *bst1* or *bst3* mutants. In contrast, the *bst1Δ bst3-2* double mutant secreted three to four times more Kar2p into the culture medium than did either single mutant (Figure 4C). The additive effects of these mutations show that even when Bst3p is absent the *BST1* gene product performs a function that contributes to the retention of Kar2p.

The effect of *bst1* and *bst3* mutations on the retention of Kar2p$^{\Delta HDEL}$ suggested that the *bst* mutations might affect the escape of other resident proteins from the ER. We therefore examined the retention of the luminal resident ER protein Pdi1p in *bst* mutants by TCA precipitation of the culture medium and quantitative Western analysis as was done for Kar2p. All three *bst* mutants secreted approximately 50% of the total Pdi1p into the culture medium during a 3 hour incubation , whereas the wild-type strain secreted no detectable Pdi1p (Figure 8). Pdi1p, like Kar2p, contains a C-terminal HDEL retrieval signal, and removal of this sequence results in the secretion of a large fraction of the protein (LaMantia and Lennarz, 1993 and Figure 8). Because such a large fraction of the

Pdi1p that lacks the HDEL was secreted from the cells, the double mutant test that we applied to Kar2p was not feasible. The secretion of both Kar2p and Pdi1p in *bst* mutants indicates a general defect in the retention of ER resident proteins.

bst Mutations also Cause Leakage of Retained Proteins from the ER

As an independent test for a defect in the proper retention of proteins in the ER, we examined fate of invertase that is retained in the ER because of the presence of an uncleaved signal sequence. The *SUC2-s11* allele of invertase is an Ala to Ile substitution at the signal peptide cleavage site that blocks cleavage by signal peptidase and dramatically slows the exit of s11-invertase from the ER, presumably because the protein has the qualities of a misfolded protein and is recognized by the ER quality control system (Bohni *et al.*, 1987). s11-invertase is retained in the ER in an enzymatically active state, and the small fraction that is secreted acquires carbohydrate modifications that allow the progression of the protein through the secretory pathway to be monitored. The effect of *bst* mutations on the secretion of s11-invertase was examined by assaying the fraction of active invertase at the cell surface. The ratio of external to total invertase activity two hours after induction of *SUC2-s11* showed that a *bst1::LEU2* mutant secreted 1.7 times and a *bst3-2* mutant secreted 1.4 times the amount of s11-invertase as did the wild-type control (Table 2). The *bst2-1* mutant did not significantly increase secretion of s11-invertase. These data were consistent with the effects of *bst* mutations on retention of Kar2p$^{\Delta HDEL}$ and demonstrated the generality of the retention defect of *bst1* and *bst3* mutants.

The rate of s11-invertase transport from the ER to the Golgi was measured directly in *bst1::LEU2, bst2-1, bst3-2* and *BST*$^{+}$ strains. Pulse-chase experiments were performed by shifting cultures to low-glucose medium for 30 min to induce the expression of s11-invertase, labeling the cells for 20 min with [^{35}S]-methionine and then chasing with unlabeled methionine. s11-invertase was first immunoprecipitated with anti-invertase

antibody, and then a portion of each sample was re-immunoprecipitated with antibodies directed against the Golgi-specific modification α1,6 mannose in order to determine the fraction of the protein that had reached the Golgi. The rate at which s11-invertase acquired α1,6 mannose modifications in the *bst1::LEU2* mutant was approximately twice that of the isogenic *BST*$^+$ control (Figure 9). Thus, an increase in the rate of exit from the ER accounts for the increased secretion of s11-invertase in the *bst1* mutant. Similarly, for the *bst3-2* mutant the rate was approximately 1.5 times that of wild-type, while that of the *bst2-1* mutant was indistinguishable from wild-type. Because the rate of transport for wild-type invertase from the ER in *bst2* and *bst3* mutants is 3 fold slower than it is in a wild-type strain (Figure 3B and 3C), the rates observed for s11-invertase transport in the *bst2* and *bst3* mutants probably understate the effect these mutants have on the escape of s11-invertase from the ER.

Genetic Interactions Between bst Mutants and sec Mutants

The bypass of the cellular requirement for Sec13p by *bst* mutations suggested that these mutations might also bypass the requirement for other *SEC* genes. We tested the ability of the *bst* mutations to suppress null alleles of other *SEC* genes in crosses segregating both a *bst* mutation and a null *SEC* gene allele. Null alleles of *SEC12*, *SEC23* and *SEC31* were tested and none were suppressed by *bst1*, *bst2* or *bst3* mutations. In addition, selections for suppressors similar to the one used to isolate the *bst* mutations were tried with null alleles of *SEC12*, *SEC23* and *SEC16* and all failed to yield extragenic suppressor mutations (R. Gimeno and P. Espenshade, personal communications). Thus, *bst* mutations do not bypass the need for other essential ER vesicle proteins.

Suppression of temperature-sensitive alleles of *SEC13* was also examined. A *sec13-1* strain was inviable at temperatures above 30°C, whereas isogenic *bst1 sec13-1* and *bst2 sec13-1* double mutants grew at temperatures up to 36°C (Figure 10). Thus, *bst* mutants can partially suppress *sec13-1*, and the inability to suppress at temperatures above 36°C

was presumably due to the failure of suppression at high temperatures that was observed for suppression of *sec13Δ*.

The ability to detect suppression of temperature-sensitive alleles allowed us to extend our evaluation of suppression to other *SEC* genes that are involved in ER to Golgi transport. The *bst1* and *bst2* mutant strains CKY330 and CKY332 were crossed to each of the *sec* mutant strains listed in Table 1. Tests of the temperature sensitivity of double-mutant segregants showed that both *bst* mutations could suppress to at least some degree the temperature sensitivity of all of the COPII vesicle formation mutants (*sec12, sec13, sec16* and *sec23*). In contrast, an exacerbation of the temperature sensitivity of vesicle fusion mutants (*sec17* and *sec18*) and COPI mutants (*sec21* and *sec27*) occurred when these mutations were combined with either *bst1* or *bst2* mutations.

To avoid possible effects of genetic background in tetrad analysis, *sec bst* strains were transformed with either the corresponding *BST* gene on a centromere plasmid or with a control plasmid. The growth of these isogenic pairs at different temperatures confirmed the suppression and synthetic interactions observed by tetrad analysis (Figure 10). Similar results were also seen with the *bst3* mutation in crosses to representative *sec* mutants, but a more rigorous confirmation of these results using truly isogenic strains must await cloning of the *BST3* gene. CPY pulse-chase experiments on isogenic pairs of *sec* and *sec bst* mutant strains confirmed that the temperature sensitive growth was always accompanied by a corresponding ER to Golgi transport defect (our unpublished results). Thus, the *bst* mutations cause ER to Golgi transport to becomes less dependent on the COPII vesicle formation genes and more dependent on vesicle fusion genes and COPI genes.

DISCUSSION

In this report, we describe the identification of three genes, *BST1*, *BST2/EMP24* and *BST3*, that when mutated suppress the lethal secretion defect caused by deletion of the *SEC13* gene. Sec13p is known to be essential for formation of transport vesicles both *in vivo*, where *sec13* mutations cause a defect in ER vesicle formation (Kaiser and Schekman, 1990) and *in vitro*, where Sec13p is one of the five cytosolic COPII proteins that are necessary and sufficient for vesicle formation from ER membranes that have been depleted of peripheral proteins (Kaiser and Schekman, 1990; Pryer *et al.*, 1993; Salama *et al.*, 1993). It was therefore surprising to find that the cellular requirement for Sec13p could be bypassed by second-site mutations. A key observation concerning the mechanism of suppression is that the suppressor mutations are genetically recessive. We show that null alleles of *BST1* and *BST2/EMP24* are suppressors. Because loss of *BST* gene function gives suppression, we deduce that the *BST* proteins act negatively to prevent transport from the ER to the Golgi. Furthermore, Sec13p is apparently needed to overcome the transport block caused by the *BST* gene products, perhaps in a late step in vesicle assembly. Thus the function of the *BST* genes in vesicular transport is formally like the inhibitory checkpoints that block the progression of the mitotic cell cycle in response to the incomplete assembly of the mitotic spindle or the incomplete replication of chromosomes.

There are two prominent possibilities for processes that could be inhibited by the *BST* gene products (diagrammed in Figure 10). One possibility is that the *BST* gene products directly block completion of incorrectly assembled COPII vesicles. Thus, when Sec13p is absent, the observed secretory block would result from the action of the *BST* proteins. In addition, under semi-permissive conditions mutations in other COPII genes would also bring about a *BST* dependent block, since loss of *BST* gene function renders less restrictive mutations in all COPII genes tested. The purpose of such a negative

regulatory mechanism could be to improve the fidelity of vesicle assembly by preventing the pinching off of vesicles that have not been completely assembled.

An alternative possibility is that the *BST* proteins perform an organizational function to maintain a distinct subcompartment within the ER from which vesicle formation can take place. Disruption of this organization by a *bst* mutation might then either allow COPII-driven vesicle formation in the absence of Sec13p or give access to a new pathway out of the ER, perhaps utilizing COPI rather than COPII coat proteins (Bednarek *et al.*, 1995). A predicted consequence of the opening of a COPII-independent process for transport from the ER is that complete elimination of other COPII proteins would also be bypassed by *bst* mutations. Because *bst* mutations do not bypass null alleles of the COPII genes *SEC12*, *SEC31* or *SEC23*, complete bypass of the COPII step is unlikely. We can not rule out, however, the possibility that the COPII proteins other than *SEC13* are required for an essential process, in addition to ER to Golgi transport, that cannot be suppressed by *bst* mutations.

Cargo Segregation in bst Mutant Strains

When *bst* mutations are placed in a wild-type strain background, the most pronounced effect of the mutations is to cause secretion of the resident ER proteins Kar2p and Pdi1p into the extracellular space. It was by complementation of the recessive Kar2p secretion trait that we cloned the *BST1* and *BST2* genes. For *bst1* and *bst3* mutations, an increase in the rate of Kar2p secretion, even when HDEL-dependent retrieval of Kar2p from the Golgi has been inactivated, indicates that the increased Kar2p secretion is the result of an increased flux of Kar2p out of the ER. Further, invertase that in a wild-type background is retained because of an uncleaved signal sequence more readily escapes the ER in *bst1* and *bst3* mutants. Thus, these mutants cause the ER to exhibit a general reduction in the ability to retain proteins, and this property suggests that these genes may have general utility for overriding the normal inhibitions for the secretion of misfolded

proteins. The magnitude of these effects can be considerable: Kar2p secretion for all three mutants is the same as that for a derivative of Kar2p lacking the C-terminal HDEL retrieval sequence and roughly half of the total Pdi1p is secreted from these mutants in 3 hours of growth. In *bst1 bst3* double mutants the Kar2p secretion phenotype is even more pronounced: about 35% of the total Kar2p is secreted into the medium during 3 hours of growth, which is the most pronounced, non-lethal ER retention defect yet documented.

Not only do *bst* mutations increase the leakage of resident proteins from the ER, but they also decrease the rate of transport of a subset of secretory cargo. This effect can be seen in the selective effects of *bst2/emp24* and *bst3* mutations on the rate of invertase transport shown here and previously reported for *bst2/emp24* mutant strains (Schimmöller *et al.*, 1995). Taken together, the effect of disruption of the *BST* genes on the sorting of luminal proteins is generally to decrease the rate of secretion of proteins that are normally secreted (invertase) and to increase the rate of export of proteins that are normally retained in the ER (Kar2p, Pdi1p and invertase with an uncleaved signal sequence). Thus, loss of *BST* gene function reduces the capacity of the ER to discriminate between secreted and retained proteins, thereby causing secretion in *bst* mutants to resemble a condition known as bulk flow where cargo leaves the ER at a rate corresponding to its concentration in the vicinity of the forming vesicle.

The existence of mutations with the properties of the *bst* mutations suggests a general model for how cargo sorting could be coupled to vesicle coat assembly. The prevailing view of how cargo is selected by vesicles is that the vesicle coat forms an affinity matrix, and that a given type of cargo molecule is partitioned into the budding vesicle according to its affinity for the coat (in the case of membrane proteins) or for coat associated receptors (in the case of luminal proteins). To accomplish this partitioning, the coat must exist for a time in a partially assembled state similar to the clathrin-coated pits that are thought to be the precursors of clathrin-coated endocytic vesicles (reviewed in Pearse and

Robinson, 1990). The negative effect of *BST* proteins on the formation of transport vesicles could simply provide a delay in the pinching off of vesicles that would give time for sorting to take place. In the absence of the restriction imposed by the *BST* checkpoint, vesicles could form prematurely before cargo sorting is completed. The cargo content of the vesicles thus formed would reflect reduced discrimination between resident proteins and secretory proteins. This putative *BST* checkpoint on vesicle completion may also be imposed in response to incomplete or improper assembly of the vesicle coat, so that in the absence of *BST* quality control crippled but functional vesicles could form when the coat structure is compromised by mutation of COPII proteins.

Molecular analysis of the *BST1* and *BST2/EMP24* genes show that these proteins reside primarily in the ER membrane, a location consistent with their proposed role in regulating the fidelity of vesicle assembly and cargo loading. The double-arginine motif at the N-terminus in the predicted sequence of Bst1p may function to govern the retrieval of this protein in a manner similar to that observed for KKXX-containing membrane proteins (Gaynor *et al.*, 1994; Schutze *et al.*, 1994).

It is also noteworthy that Bst2p/Emp24p is a member of a family of proteins that have been found in COPII-coated vesicles (Schimmöller *et al.*, 1995) and COPI-coated vesicles (Stamnes *et al.*, 1995). An appreciation of the reduced rate of invertase and Gas1p transport in *emp24* mutants led to the suggestion that these proteins are cargo receptors for a subset of proteins (Schimmöller *et al.*, 1995). However, this suggestion does not explain the effects of these mutations on Kar2p release from the ER and suppression of *sec13* mutations that we describe here.

Interaction Between BST Genes and Other SEC Genes.

While the *bst* mutations fully or partially suppress mutations in COPII genes, they exacerbate mutations in COPI genes and vesicle fusion genes. These interactions can be explained in light of the proposed participation of *BST* genes in ensuring the fidelity of

vesicle assembly. The exacerbation of the temperature sensitive defects of *sec17-1* and *sec18-1* mutants may reflect structural defects in the vesicles formed in the absence of *BST* function: if proteins required for stability or for the targeting and fusion of the vesicles (V-SNARES) are not assembled as efficiently into the budding vesicles, this could cause the enhancement of the defect seen in *sec17-1* and *sec18-1* mutants. Additionally, the greatly increased flux of Kar2p and possibly other resident proteins from the ER in *bst* mutants may produce an unusually great load on the systems that retrieve ER proteins from the Golgi. The COPI vesicle proteins Sec21p, Sec27p and Ret1p are required for the retrieval of type I ER membrane proteins bearing the retention signal KKXX at their cytosolic C-termini (Letourner *et al.*, 1994). If COPI coated vesicles are needed to retrieve from the Golgi a variety of ER-resident proteins, then the increased load on the retrieval system in *bst* mutants may be lethal when combined with the COPI *sec* mutants that reduce the capacity of the retrieval system.

Relationship to Other Genes Involved in the Retention of Resident ER Proteins

A number of genes have been reported to influence the retention and retrieval of resident ER proteins in yeast. The *rer* mutants were identified in screens for mutants that mislocalize hybrid transmembrane proteins normally retained in the ER (Nishikawa and Nakano, 1993; Boehm *et al.*, 1994). The defect in *rer1* and *rer3* mutants is for transmembrane proteins only, since both show normal retention of the soluble Kar2p (Boehm *et al.*, 1994). The *rer2* mutant is defective in the retention of Kar2p and may, like the *bst* mutants, perform a more general retention function (Nishikawa and Nakano, 1993). A more general retention defect has been observed for strains in which the *CNE1* gene has been deleted (Parlati *et al.*, 1995), but secretion of Kar2p was not examined for this mutant, nor was the capacity to suppress COPII mutations tested, so the relationship of *CNE1* to the *bst* mutants is not known.

The isolation of the *bst* mutants has given us a new insight into how cargo sorting could be related to the assembly of vesicle coats. The key questions now are how the *BST* proteins operate at a molecular level: how they negatively regulate vesicle transport, and what the inputs are that trigger this regulation. The isolation of the *BST* genes and their products provides a way to address these questions by studying the structure of the *BST* proteins, their possible association with one another, and their proximity to proteins of the vesicle coat and to cargo molecules.

ACKNOWLEDGEMENTS

We thank the members of the Kaiser lab for providing an interactive and stimulating environment in which to conduct this work and for technical assistance. We are especially grateful to Monicia Elrod-Erickson, P. Espenshade, R. Gimeno and D. Shaywitz for their comments on this manuscript. We thank G. Fink, E. Hong, R. Gimeno, R. Schekman and M. Rose for sharing strains and reagents and C. Shamu for suggesting experiments on Pdi1p and providing the necessary reagents. This work was supported by the Searle Scholars program (to C.K.) and a predoctoral fellowship from the National Science Foundation to M.J. Elrod-Erickson. C.A. Kaiser is a Lucille P. Markey Scholar, and this work was funded in part by a grant from the Lucille P. Markey Charitable Trust.

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Table 1. Strain List

Strain	Genotype	Source
CKY8	*MATα ura3-52 leu2-3,112*	Kaiser lab collection
CKY10	*MATa ura3-52 leu2-3,112*	Kaiser lab collection
CKY13	*MATα his4-619 lys2-801*	Kaiser lab collection
CKY14	*MATa his4-619 lys2-801*	Kaiser lab collection
CKY40	*MATa sec12-4 ura3-52*	Kaiser lab collection
CKY46	*MATa sec13-1 ura3-52 his4-619*	Kaiser lab collection
CKY53	*MATa sec16-1 ura3-52 leu2-3,112*	Kaiser lab collection
CKY55	*MATa sec17-1 ura3-52 his4-619*	Kaiser lab collection
CKY59	*MATa sec18-1 ura3-52 his4-619*	Kaiser lab collection
CKY69	*MATa sec21-1 ura3-52 his4-619*	Kaiser lab collection
CKY79	*MATa sec23-1 ura3-52 leu2-3,112*	Kaiser lab collection
CKY100	*MATa sec27-1 ura3-52 leu2-3,112*	Kaiser lab collection
CKY190	*MATα KAR2ΔHDEL ura3 leu2 his4 suc2*	M. Rose (Princeton)
CKY321	*MATα sec13Δ1 ade2 ade3 ura3-52 leu2-3,112* (pCK1390)	This study
CKY322	*MATα sec13Δ1 bst1-2 ade2 ade3 ura3-52 leu2-3,112*	This study
CKY323	*MATα sec13Δ1 bst2-2 ade2 ade3 ura3-52 leu2-3,112*	This study
CKY324	*MATα sec13Δ1 bst3-2 ade2 ade3 ura3-52 leu2-3,112*	This study
CKY325	*MATa sec13Δ2::LEU2 bst1-1 ura3-52 leu2-3,112 his4-619*	This study
CKY326	*MATa sec13Δ2::LEU2 bst2-1 ura3-52 leu2-3,112 his4-619*	This study
CKY327	*MATa sec13Δ2::LEU2 bst3-1 ura3-52 leu2-3,112 his4-619*	This study
CKY330	*MATα bst1-1 ura3-52 leu2-3,112*	This study
CKY331	*MATa bst1::LEU2 ura3-52 leu2-3,112*	This study
CKY332	*MATα bst2-1 ura3-52 leu2-3,112*	This study
CKY333	*MATa bst2::URA3 ura3-52 leu2-3,112*	This study
CKY334	*MATα bst3-2 ura3-52 leu2-3112 ade2 ade3*	This study
CKY335	*MATα bst1::LEU2 KAR2ΔHDEL ura3 leu2 his4 suc2*	This study
CKY336	*MATα bst2::URA3 KAR2ΔHDEL ura3 leu2 his4 suc2*	This study
CKY337	*MATα bst3-2 KAR2ΔHDEL ura3 leu2 his4*	This study
CKY339	*MATa bst1::LEU2 bst2::URA3 ura3-52 leu2-3,112*	This study
CKY340	*MATα bst1::LEU2 bst3-2 ura3-52 leu2-3,112 ade2 ade3*	This study
CKY341	*MATα bst2::URA3 bst3-2 ura3-52 leu2-3,112 ade2 ade3*	This study
CKY343	*MATa suc2Δ9 ura3-52 leu2-3,112* (pEHB29)	This study
CKY344	*MATa bst1::LEU2 suc2Δ9 ura3-52 leu2-3,112* (pEHB29)	This study
CKY345	*MATa bst2-1 suc2Δ9 ura3-52 leu2-3,112* (pEHB29)	This study
CKY346	*MATα bst3-2 suc2Δ9 ura3-52* (pEHB29)	This study
CKY348	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112*	This study
CKY349	*MATα bst1-1 sec12-1 ura3-52 leu2-3,112*	This study
CKY352	*MATa bst2-1 sec12-1 ura3-52*	This study
CKY355	*MATa bst1-1 sec13-1 ura3-52 leu2-3,112*	This study
CKY358	*MATα bst2-1 sec13-1 ura3-52 leu2-3,112*	This study
CKY361	*MATa bst1-1 sec16-1 ura3-52 leu2-3,112*	This study
CKY364	*MATa bst2-1 sec16-1 ura3-52 leu2-3,112*	This study
CKY367	*MATa bst1-1 sec17-1 ura3-52 leu2-3,112*	This study
CKY370	*MATa bst2-1 sec17-1 ura3-52 leu2-3,112*	This study
CKY373	*MATa bst1-1 sec18-1 ura3-52 leu2-3,112*	This study
CKY376	*MATα bst2-1 sec18-1 ura3-52 his4-619*	This study
CKY379	*MATa bst1-1 sec21-1 ura3-52 his4-619*	This study
CKY382	*MATa bst2-1 sec21-1 ura3-52 leu2-3,,12*	This study
CKY385	*MATα bst1-1 sec23-1 ura3-52 leu2-3,112*	This study
CKY388	*MATα bst2-1 sec23-1 ura3-52 leu2-3,112*	This study
CKY391	*MATα bst1-1 sec27-1 ura3-52 leu2-3,112*	This study
CKY394	*MATa bst2-1 sec27-1 ura3-52 leu2-3,112*	This study
CKY395	*MATa ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 pdi1::TRP1 PDIΔHDEL*	C. Shamu (UCSF)

Table 2. Secretion of s11-invertase in *bst* mutants

Strain	Relevant genotype	External activity *
CKY343	*suc2Δ [suc2-s11]*	13.9 ± 0.5%
CKY344	*bst1::LEU2 suc2Δ [suc2-s11]*	24.0 ± 1.0%
CKY345	*bst2::URA3 suc2Δ [suc2-s11]*	15.8 ± 0.2%
CKY346	*bst3-2 suc2Δ [suc2-s11]*	19.3 ± 0.6%

* Intact cells and cell lysates were assayed to determine the fraction of total invertase activity that was extracellular. Three determinations on each of two independent transformants were averaged and standard deviations are shown.

Figure 1. Analysis of *sec13Δ* revertants.

(**A**) A wild-type strain CKY10, carrying either the empty vector pRS306-2μ (lane1) or the *SEC13*-containing high copy plasmid pCK1391 (lane2), and three *sec13Δ* revertant strains (CKY322, CKY323 and CKY324) carrying pRS306-2μ (lanes 3-5), were grown in selective medium at 24˚C. Sec13p was detected by immunoblotting using affinity purified anti-Sec13p antibody. (**B**) Complementation analysis of *bst sec13Δ* mutant isolates. Strains of the indicated genotype (CKY322, CKY323, CKY324, CKY 325, CKY326 and CKY327) were patch mated, and diploids were selected.

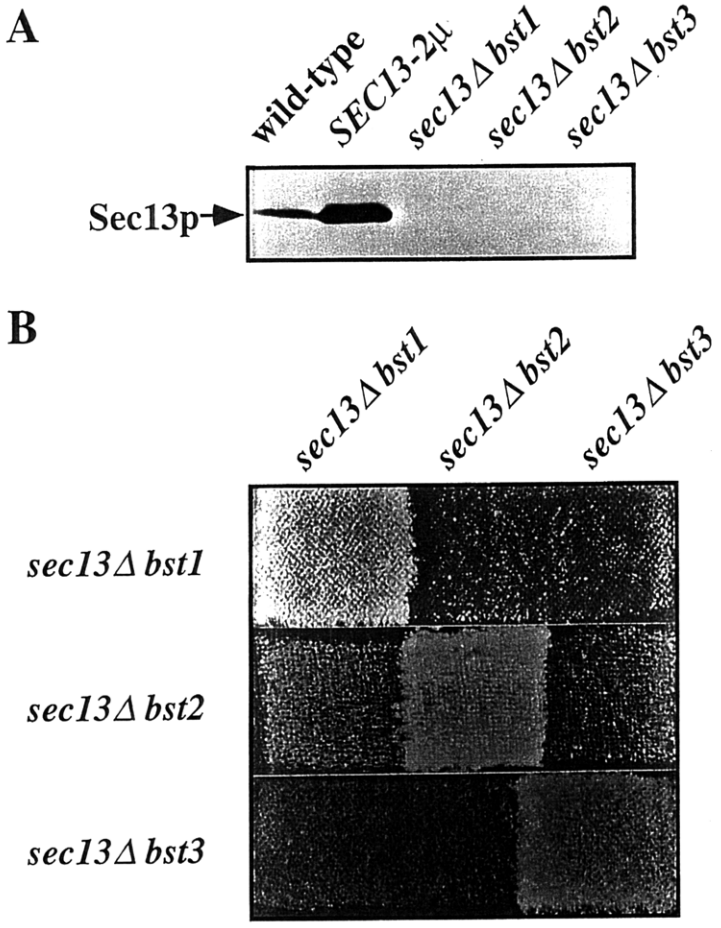

A
wild-type
SEC13-2μ
sec13Δ bst1
sec13Δ bst2
sec13Δ bst3
Sec13p
B
sec13Δ bst1
sec13Δ bst2
sec13Δ bst3
sec13Δ bst1
sec13Δ bst2
sec13Δ bst3

Figure 2. ER to Golgi transport in *bst sec13Δ* mutant strains.

A wild-type strain (CKY10) and three *bst sec13Δ* mutant strains (CKY322, CKY323 and CKY324) were grown in supplemented minimal medium at 24°C. (**A**) Cultures were pulse-labeled with ^{35}S trans-label for 5 min and chased for the times indicated. CPY was immunoprecipitated from the labeled extracts, resolved by SDS-PAGE and imaged on a PhosphorImager (for quantitation) and by autoradiagrapy. (**B**) Cultures were shifted to supplemented minimal medium containing 0.1% glucose to induce the expression of invertase, labeled and chased as in (A). Invertase was immunoprecipitated from the labeled extracts and treated as in (A). (**C**) The individual forms of CPY and invertase were quantitated and expressed as a percentage of the total counts for each timepoint. Shown graphically are the average values for the appearance of the mature, fully processed form of each protein from two experiments, which varied by less than 10%.

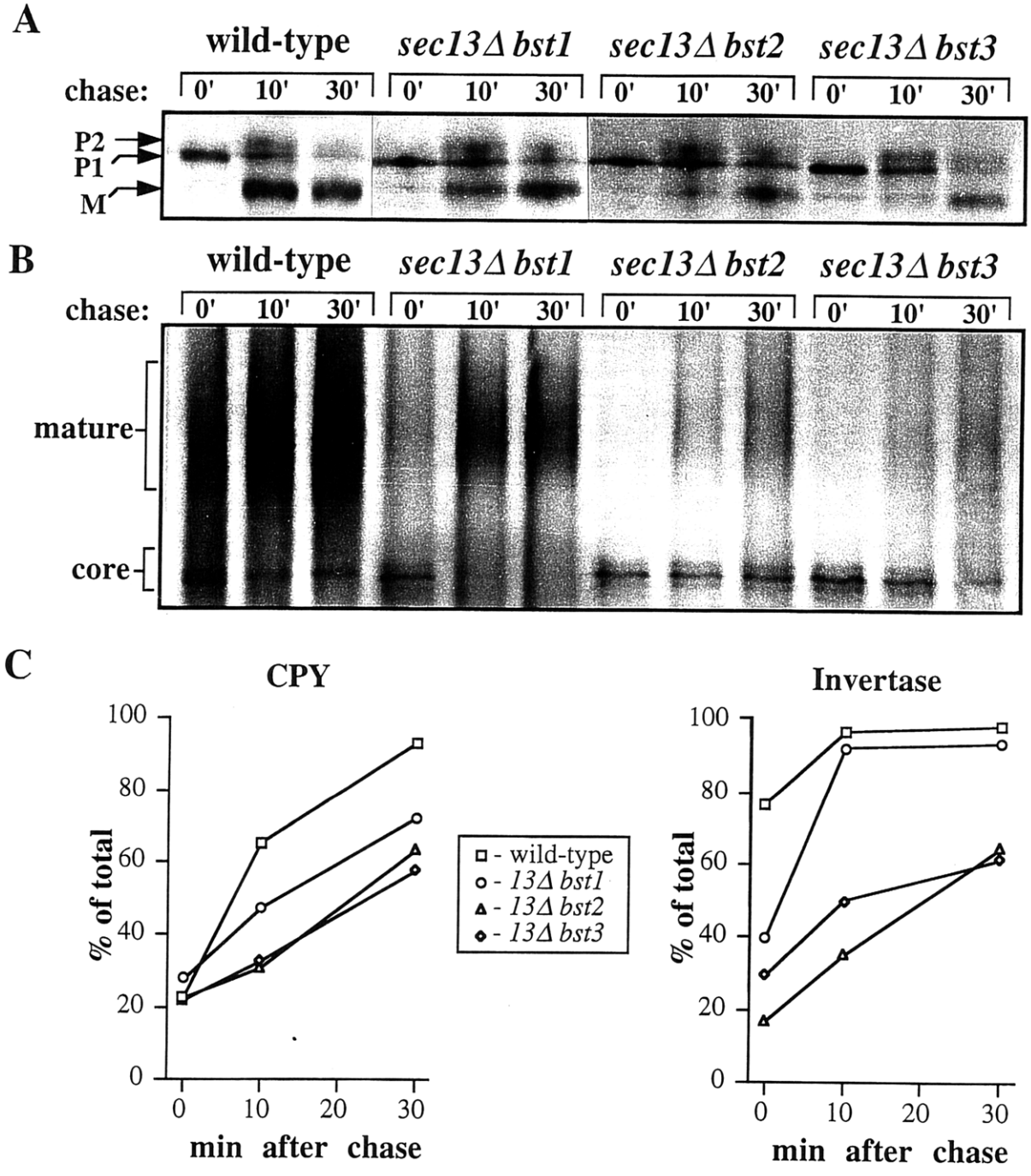

A
wild-type
sec13Δ bst1
sec13Δ bst2
sec13Δ bst3
chase:
0'
10'
30'
P2
P1
M
B
wild-type
sec13Δ bst1
sec13Δ bst2
sec13Δ bst3
chase:
0'
10'
30'
mature
core
C
CPY
Invertase
% of total
100
80
60
40
20
0
0
10
20
30
min after chase
□ - wild-type
○ - 13Δ bst1
△ - 13Δ bst2
◇ - 13Δ bst3

Figure 3. ER to Golgi transport in *bst* mutant strains.

A wild-type strain (CKY10) and three *bst* mutant strains (CKY330, CKY332 and CKY334) grown in supplemented minimal medium at 30°C were shifted to medium with 0.1% glucose to induce the expression of invertase, pulse-labeled with ^{35}S trans-label for 5 min, and chased for the times indicated. (**A**) Immunoprecipitated CPY. (**B**) Immunoprecipitated invertase. (**C**) The individual forms of CPY and invertase were quantitated and expressed as a percentage of the total counts for each timepoint. Shown graphically are the average values for the appearance of the mature, fully processed form of each protein from two experiments, which varied by less than 10%.

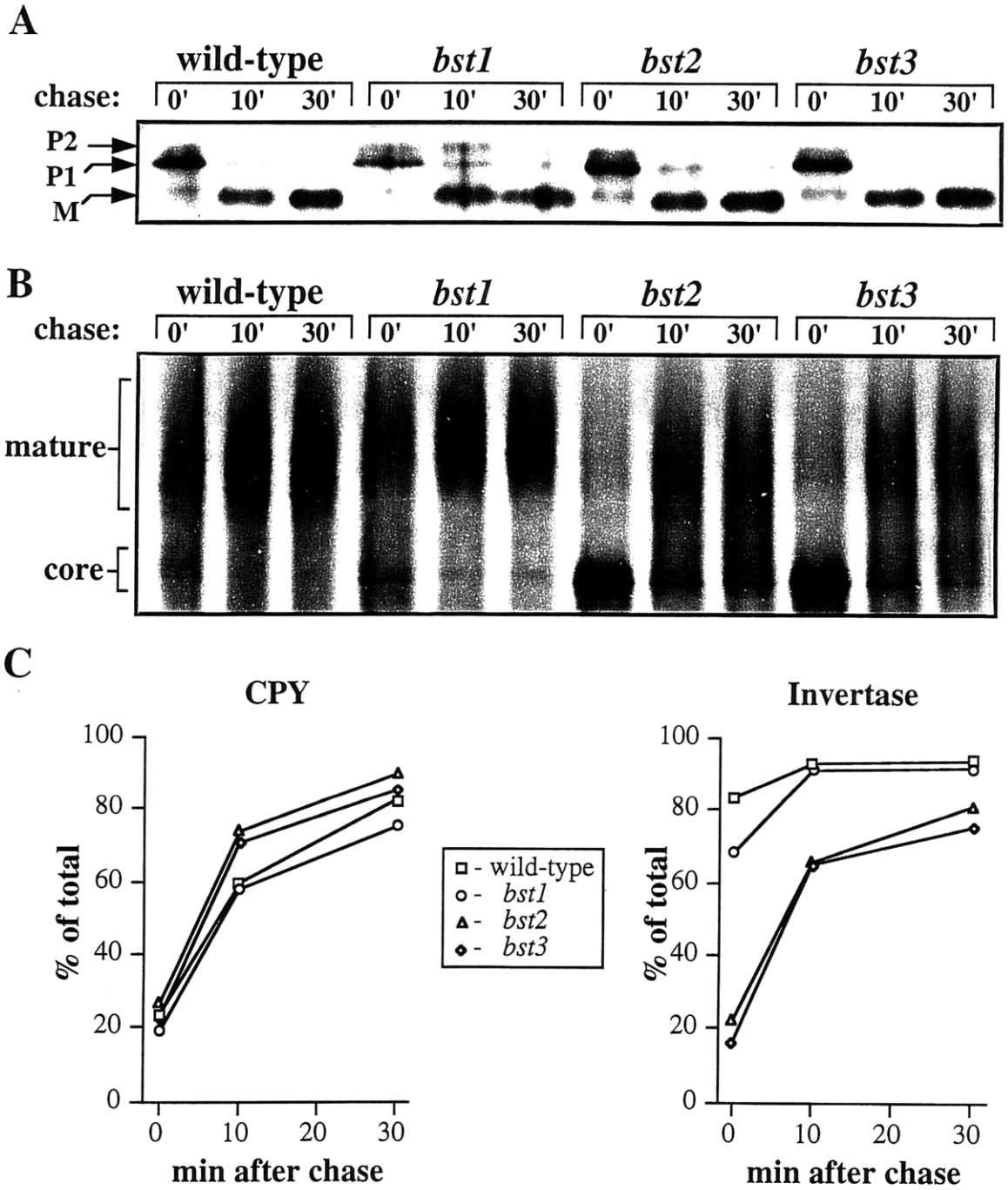

A
wild-type
bst1
bst2
bst3
chase:
0'
10'
30'
P2
P1
M
B
mature
core
C
CPY
Invertase
% of total
min after chase
wild-type
bst1
bst2
bst3

Figure 4. *bst* mutations cause defects in Kar2p retention that are independent of the HDEL retrieval pathway.

Strains of the indicated genotype used were CKY10, CKY190, CKY322 - CKY324, CKY330 - CKY337 and CKY339 - CKY341. (**A**) A wild-type strain, a *KAR2ΔHDEL* strain, three *bst sec13Δ* strains and three *bst* strains carrying the empty vector pRS316, a *bst1* strain carrying the *BST1*-containing plasmid pME1101 and a *bst2* strain carrying the *BST2*-containing plasmid pME253 were spotted onto selective medium, covered with a nitrocellulose filter and grown for 48 h at 24°C. Kar2p secreted onto the filter was detected by immunoblotting using anti-Kar2p Ab. (**B**) Exponentially growing cultures were washed, suspended in fresh medium, incubated for 3 h at 24°C and split into cell and medium samples. Extracts from 0.5 OD_{600} units of cells and medium samples from 2.5 OD_{600} unit equivalents were resolved by SDS-PAGE. Kar2p was detected by Western analysis with anti-Kar2p Ab. (**C**) Samples from experiments as described in (B) were quantitated by densitometry. The value for the *KAR2ΔHDEL* control in each experiment was the baseline for comparison and shown are the average values from three experiments for the relative amounts of Kar2p detected in the culture medium. Error bars represent one standard deviation. The inlay is a Western blot showing a sample of the data used to generate these values for the correspondingly numbered strain in the graphic below.

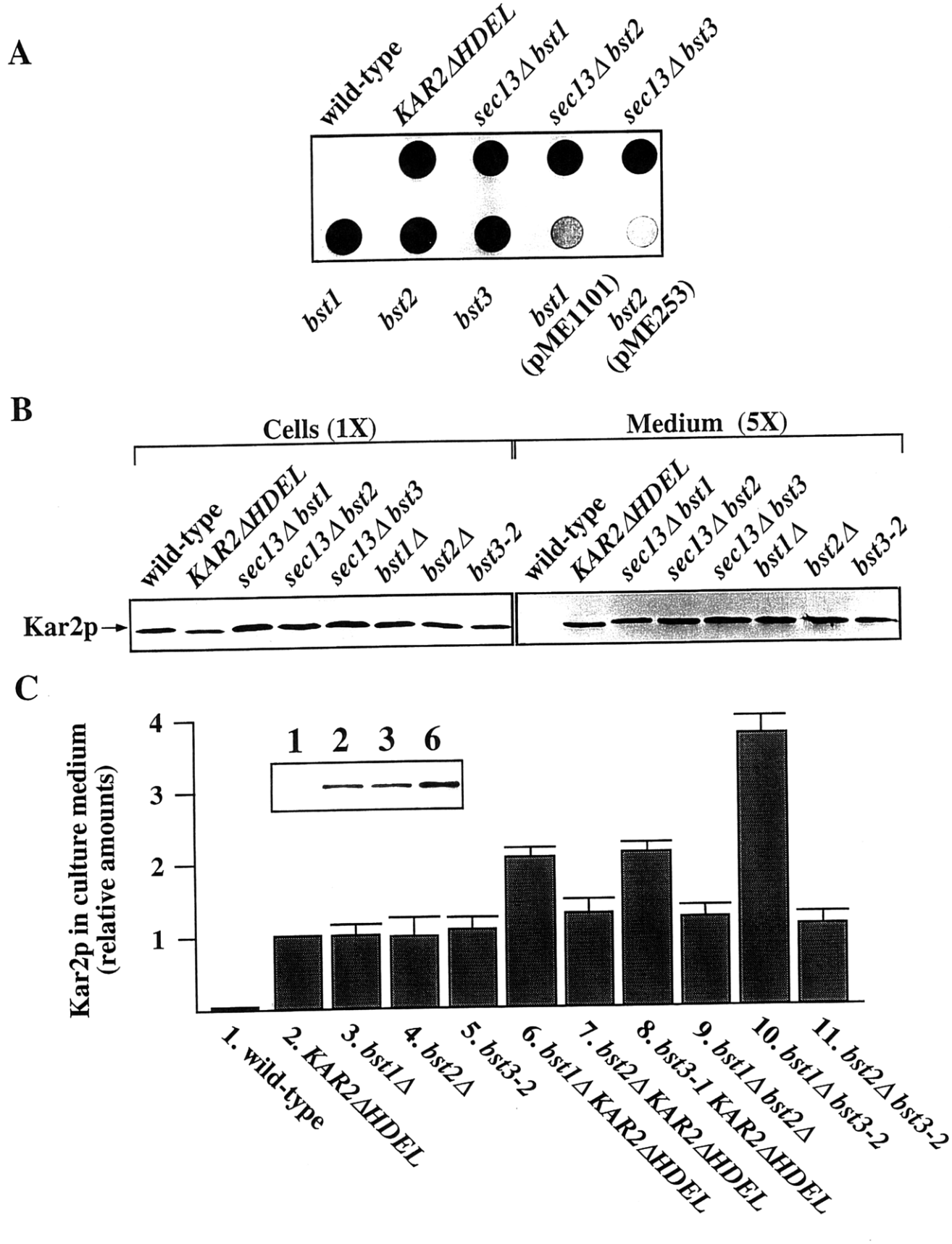

A
wild-type
KAR2ΔHDEL
sec13Δbst1
sec13Δbst2
sec13Δbst3
bst1
bst2
bst3
bst1 (pME1101)
bst2 (pME253)
B
Cells (1X)
Medium (5X)
wild-type
KAR2ΔHDEL
sec13Δbst1
sec13Δbst2
sec13Δbst3
bst1Δ
bst2Δ
bst3-2
Kar2p
C
Kar2p in culture medium (relative amounts)
1 2 3 6
1. wild-type
2. KAR2ΔHDEL
3. bst1Δ
4. bst2Δ
5. bst3-2
6. bst1Δ KAR2ΔHDEL
7. bst2Δ KAR2ΔHDEL
8. bst3-1 KAR2ΔHDEL
9. bst1Δ bst2Δ
10. bst1Δ bst3-2
11. bst2Δ bst3-2

Figure 5. Physical map of *BST1*

(**A**) The plasmid subclones of the *BST1* locus are shown along with their ability to complement the *bst1-1* mutation. The large solid arrow represents the predicted open reading frame. (**B**) Schematic representation of Bst1p. The shaded regions represent potential membrane spanning regions, and (*) represents the location of potential N-linked glycosylation sites. The first five amino acids are shown to highlight the double arginine motif. The location of the epitope tag in *BST1-HA* is indicated. The predicted topology shown is based on the number of glycosylation sites that are apparently utilized (see text).

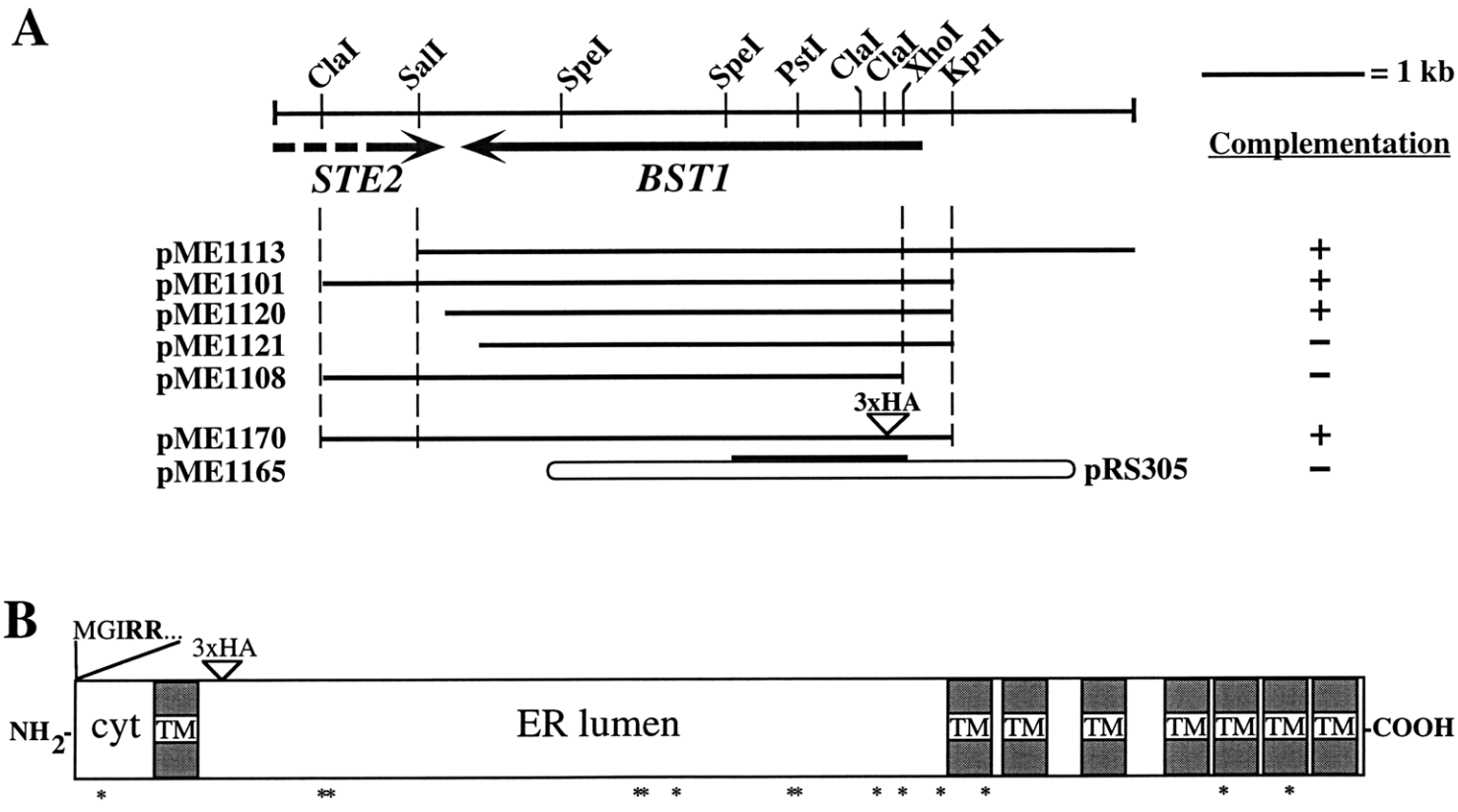

A
ClaI
SalI
SpeI
SpeI
PstI
ClaI
ClaI
XhoI
KpnI
= 1 kb
Complementation
STE2
BST1
pME1113
pME1101
pME1120
pME1121
pME1108
3xHA
pME1170
pME1165
pRS305
+
+
+
−
−
+
−
B
MGIRR...
3xHA
NH2-
cyt
TM
ER lumen
TM
TM
TM
TM
TM
TM
TM
-COOH

Figure 6. Bst1p is an integral membrane glycoprotein.

(**A**) A cell lysate of a wild-type strain (CKY10) expressing Bst1p-HA from a CEN plasmid (pME1170) was subjected to a series of centrifugation steps resulting in 500***g***, 10,000***g*** and 150,000***g*** pellets (P) and a 150,000***g*** supernatant (S). An equal number of cell equivalents were loaded in each lane. (**B**) Lanes 1 and 2: cell lysates of a wild-type strain (CKY10) carrying the *BST1-HA*-containing plasmid pME1170 (as in A) or the *BST1*-containing plasmid pME1101 respectively. Lanes 3 - 6: a cell lysate from the *BST1-HA*- containing strain in (A) was incubated in 1% Triton at 4˚C for 1 h and centrifuged at 150,000***g*** to give pellet (P) and supernatant (S) fractions (Lanes 3 and 4), or was treated (or mock treated) with endoH_f (Lanes 5 and 6). Lanes 7 and 8: cell lysates from a *sec18-1* mutant strain (CKY59) containing *BST1-HA* (pME1170) grown continuously at 24˚C or shifted to 37˚C for 2 h. An equal number of cell equivalents was used for each treatment. Bst1p-HA was detected by SDS-PAGE and Western analysis with the 12cA5 monoclonal antibody.

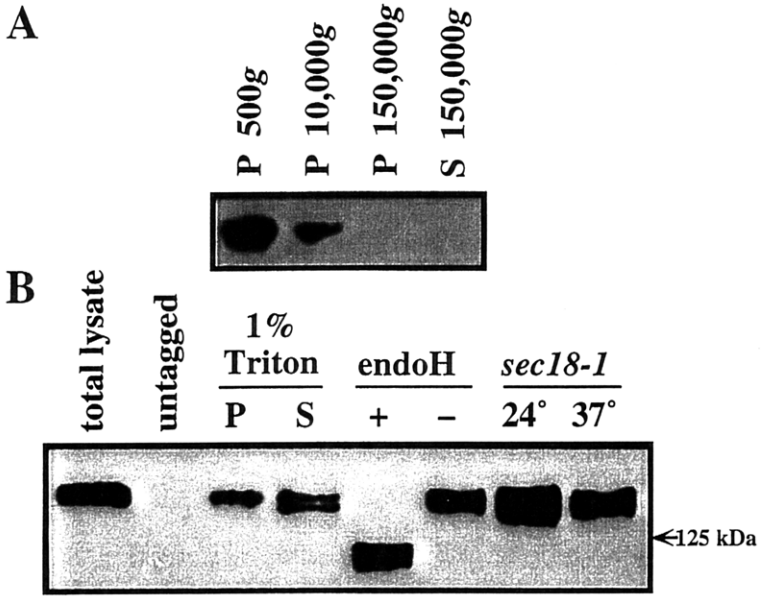

A
P 500g
P 10,000g
P 150,000g
S 150,000g
B
total lysate
untagged
1% Triton
P
S
endoH
+
–
sec18-1
24°
37°
125 kDa

Figure 7. Bst1p colocalizes with the luminal ER protein Kar2p. Cells (CKY10) expressing *BST1-HA* from the centromere plasmid pME1170 were fixed, spheroplasted, washed with 0.1% SDS and processed for double-label indirect immunofluorescence. (A) Indirect immunofluorescence of Bst1p-HA . (B) Indirect immunofluorescence of Kar2p. (C) DAPI stained nuclear DNA. (D) Cell bodies visualized using Nomarski optics. All panels are 1250X magnification.

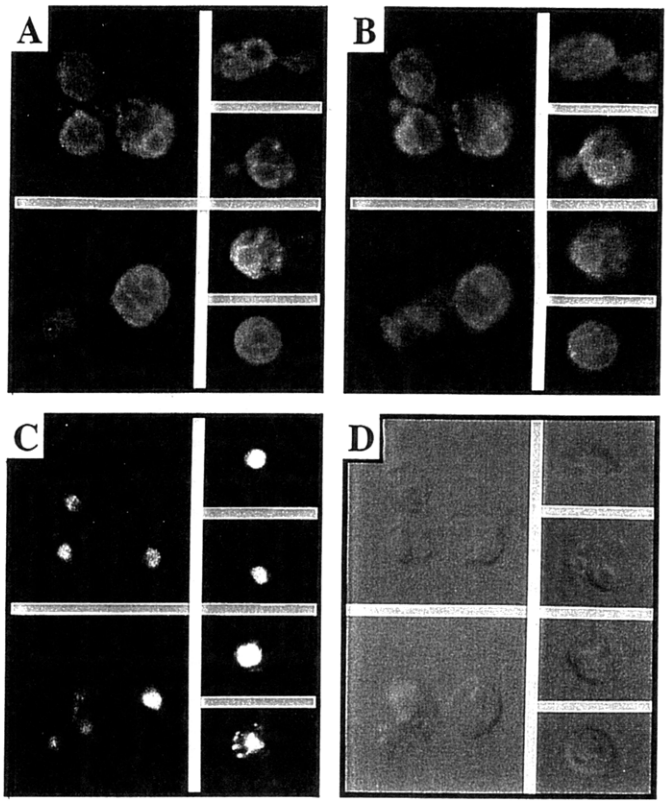

A
B
C
D

Figure 8. *bst* mutants secrete the luminal ER protein Pdi1p. Samples from a wild-type strain (CKY10), a *PDIΔHDEL* strain (CKY395), *bst1-1, bst2-1* and *bst3-2* mutant strains (CKY330, CKY332 and CKY334) were made as described for Figure 4B. Pdi1p was detected by Western analysis with anti-Pdi1p antibody. (*) represents the glycosylated form of Pdi1p that is secreted from cells.

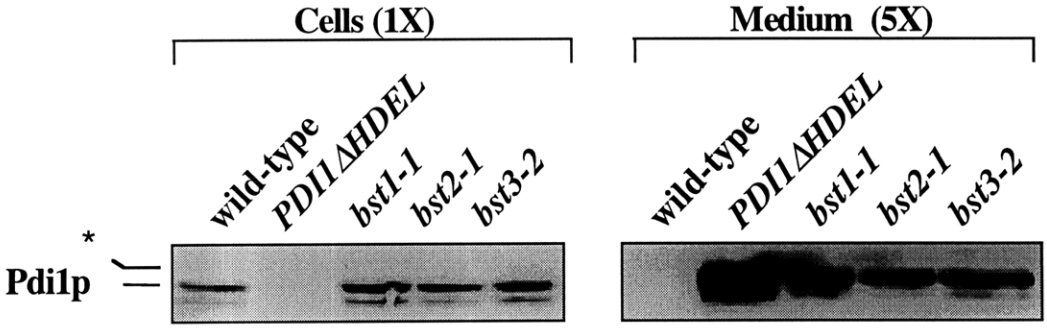

Cells (1X)
Medium (5X)
wild-type
PDI1ΔHDEL
bst1-1
bst2-1
bst3-2
wild-type
PDI1ΔHDEL
bst1-1
bst2-1
bst3-2
*
Pdi1p

Figure 9. *bst* mutants increase the rate at which s11-invertase leaks from the ER. Isogenic *suc2Δ* (CKY343) and *bst1Δ suc2Δ* (CKY344) strains, a *bst2-1 suc2Δ* (CKY345) and a *bst3-2 suc2Δ* (CKY346) expressing s11-invertase from a plasmid (pEHB29) were grown in supplemented minimal medium at 30°C, pulse-labeled with ^{35}S trans-label for 20 min and chased for the times indicated. s11-invertase was immunoprecipitated and then boiled in SDS to disrupt the immune complexes, and a fraction was reimmunoprecipitated with α1,6-specific Ab. Samples were treated with endoH_f, resolved by SDS-PAGE, visualized and quantitated on a PhosphorImager. Shown is the average value from three experiments for the fraction of the total s11-invertase that was recovered in the α1,6 precipitation. Error bars represent one standard deviation.

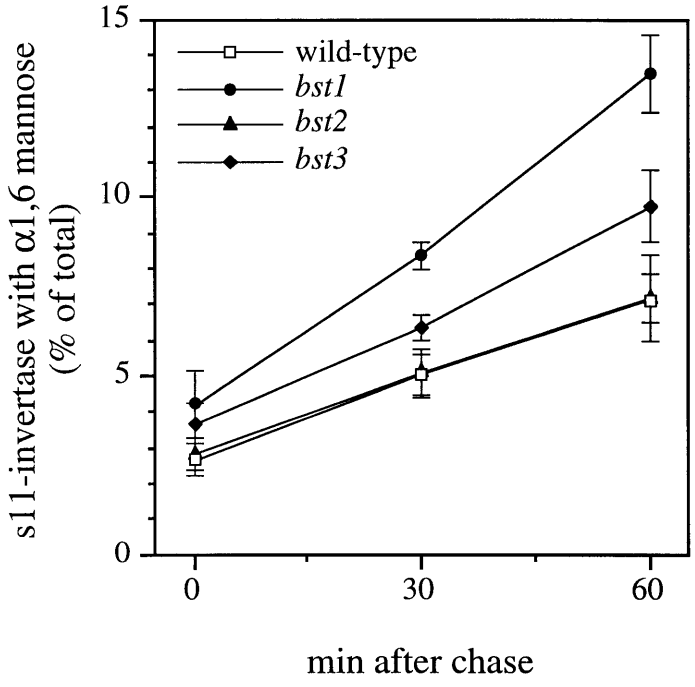

15
10
5
0
0
30
60
wild-type
bst1
bst2
bst3
s11-invertase with α1,6 mannose
(% of total)
min after chase

Figure 10. Genetic interactions between *bst* mutants and *sec* mutants.
Each of the *bst sec* double mutant strains listed in Table 1 carrying either the vector pRS316 or the corresponding *BST* gene-containing plasmid (pME1101 or pME253) was assayed in streakouts on selective medium for growth at temperatures over the range indicated. Shown is a graphic summary of the data.

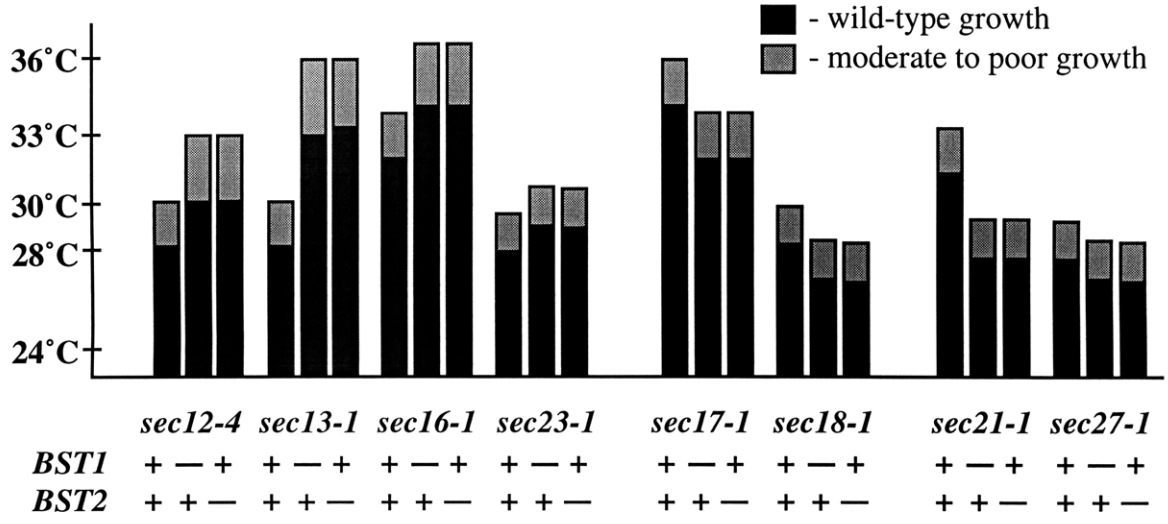
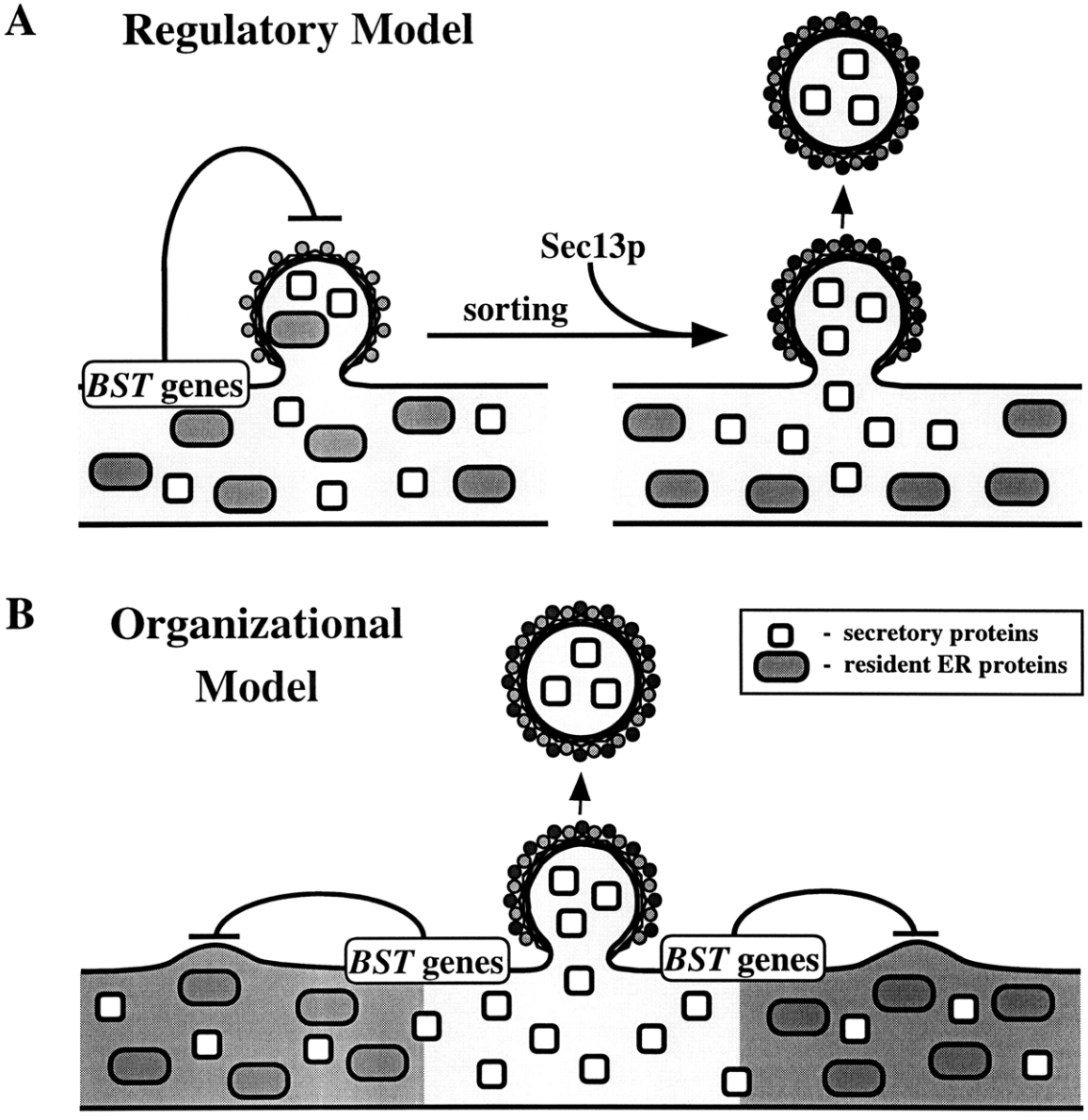

- wild-type growth
- moderate to poor growth
36°C
33°C
30°C
28°C
24°C
sec12-4 sec13-1 sec16-1 sec23-1 sec17-1 sec18-1 sec21-1 sec27-1
BST1 + — + + — + + — + + — + + — + + — + + — + + — +
BST2 + + — + + — + + — + + — + + — + + — + + — + + —

Figure 11. Models for the function of the *BST* genes.

(**A**) Regulatory model in which the *BST* genes inhibit the completion of transport vesicle formation, allowing cargo sorting to occur. (**B**) Organizational model in which the *BST* genes maintain distinct subcompartments in the ER from which vesicle formation occurs. Resident proteins are excluded from these regions and vesicle budding is inhibited outside these regions.


A
Regulatory Model
Sec13p
sorting
BST genes
B
Organizational
Model
- secretory proteins
- resident ER proteins
BST genes
BST genes

Chapter Two - Appendix

Further characterization of the *BST* genes

PREFACE

Since the publication of Chapter 2, a few additional pieces of data have been generated that fit best as part of that body of work and are therefore included here as an appendix to Chapter 2. These results include an extension of the genetic analysis to incorporate the COPII mutants *sec31* and *sec24*, the recent appearance in the database of a *C. elegans* homolog of *BST1*, and the identification of *BST3* as *ERV25* (in collaboration with C. Barlowe, Dartmouth).

MATERIALS AND METHODS

Genetic interactions between bst mutants and sec mutants

Crosses and tetrad analysis were conducted as described in Chapter 2. The strains used were CKY496 (MATα ura3-52 leu2-3,112 sec24), CKY507 (MATα ura3-52 sec31-1), CKY331, CKY333 (both described in Chapter 2), and MEY529 (MATa ura3-52 leu2-3,112 bst3-2).

Determination of identity between BST3 and ERV25

The *erv25Δ* strain CBY114 (MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 erv25::HIS3; from C. Barlowe, Dartmouth) was crossed to both wild-type (CKY10) and *bst3-2* (MEY529 - see above) mutant strains. Diploids from these crosses were analyzed for Kar2p secretion using the immunoblotting filter assay (see Methods from Chapter 2) to test for complementation. Diploids were then sporulated and the resulting tetrads were also tested for Kar2p secretion by this assay to assess linkage.

RESULTS AND DISCUSSION

bst *mutations partially suppress the temperature sensitivity of* sec24-1 *and* sec31-1

bst mutations can partially suppress temperature-sensitivity of *sec12-4, sec13-1, sec16-1,* and *sec23-1* mutant strains (as described in Chapter 2). The recent isolation of temperature-sensitive alleles of the two remaining COPII genes, *SEC24* and *SEC31* (A. Frand and C. Kaiser, unpublished), allowed the analysis of suppression by *bst* mutations to be extended to include these genes. The *bst1, bst2*, and *bst3* mutant strains CKY330, CKY332, and MEY529 were crossed to the *sec24-1* and *sec31-1* mutant strains CKY496 and CKY507, and diploids were isolated. Diploids were then sporulated and segregants from the resulting tetrads were analyzed for temperature-sensitivity and Kar2p secretion to determine their genotype. The results of these tests showed that all three *bst* mutations could partially suppress the temperature-sensitivity of both *sec24-1* and *sec31-1* mutant strains. Although *bst* mutations partially suppress conditional mutations in all the *SEC* genes involved in COPII vesicle formation, the suppression is nonetheless specific since *bst* mutations exacerbate the temperature-sensitivity of *sec* mutants involved in vesicle fusion or COPI vesicle formation. A summary of all genetic interactions between *bst* mutants and ER to Golgi *sec* mutants is shown in Figure 1 of this appendix. These results further demonstrate that deleting *BST* gene function removes a restriction to COPII vesicle formation.

BST1 and a C. elegans homolog share a motif characteristic of triglyceride lipases

Since the publication of Chapter 2, a *C. elegans* ORF (T19B10.8) predicted to encode a protein with significant homology to Bst1p has been identified(Figure 2A). Both proteins have a similar overall structure, consisting of a large luminal domain at the N-terminus and multiple membrane spanning regions at the C-terminus (Figure 2B). In addition, both proteins contain a motif characteristic of the active site of triglyceride lipases that is

situated in the presumptive luminal domain where the two proteins show the highest degree of homology (~30% identity and 62% similarity; Figures 2B, and 2C). The consensus pattern for this motif is [LIV]-X-[LIVFY]-[LIVMST]-G-[HYWV]-**S**-X-G-[GSTAC] (Prosite PS00120; LIPASE_SER; Figure 2C). The highlighted serine has been shown in characterized lipases to participate, with an aspartic acid and a histidine, in a charge relay system similar to that of serine proteases (Blow, 1990). This motif is also found in prokaryotic lipases and in lecithin-cholesterol acyltransferase, which catalyzes the transfer of fatty acids between phosphatidylcholine and cholesterol (McLean et al., 1986). Thus, the motif appears to be suggestive of esterase activity capable of removing (or transferring) fatty acids from triglycerides or, in some cases, phospholipids. The relevance of this motif in Bst1p will be addressed in Chapter 5.

BST3 is identical to ERV25

Erv25p is a member of the p24 protein family and a major component of COPII-coated vesicles. The protein forms a complex with Emp24p/Bst2p, and *erv25Δ* mutants show selective transport defects identical to those of an *emp24/bst2* mutant (Belden and Barlowe, 1996). In addition, *erv25Δ* mutants show defects in Kar2p retention that are identical to those described for *bst1, emp24/bst2,* and *bst3* mutants (C. Barlowe, personal communication). Because of the similarity in phenotypes between *erv25* and *bst3* mutants, as well as the association of Erv25p with Emp24p/Bst2p, we (in collaboration with C. Barlowe, Dartmouth) tested directly whether *ERV25* and *BST3* are identical. The *erv25Δ* strain CBY114 was crossed to both wild-type (CKY10) and *bst3-2* mutant (MEY529) strains and the resulting diploids were tested for Kar2p secretion. The *erv25Δ* and *bst3-2* mutations failed to complement, as the diploid from this cross still secreted excess Kar2p, whereas the diploid from the control cross showed normal low levels of Kar2p secretion. Diploids from each cross were then sporulated and the resulting tetrads were examined for Kar2p secretion. In the cross between *erv25Δ* and *bst3-2*, complete

linkage of these mutations was demonstrated by 4:0 segregation of the Kar2p secretion phenotype (n=11), whereas the control cross showed the expected 2:2 segregation of this phenotype (n=10). On the basis of the linkage and noncomplementation of *erv25* and *bst3* mutations, we conclude that *ERV25* and *BST3* are identical.

The identity of *ERV25* and *BST3* places two of the *BST* proteins as major constituents of COPII-coated vesicles. This observation, along with the specificity of bypass suppression shown by *bst* mutations, suggests that the model in which the *BST* proteins regulate or influence COPII-coated vesicle formation (rather than an alternative pathway from the ER to the Golgi) is more likely. Further, and more direct, experiments aimed at distinguishing between these two possibilities are the subject of Chapter 3.

REFERENCES

Belden, W. J. and C. Barlowe (1996). Erv25p, a component of COPII-coated vesicles, forms a complex with Emp24p that is required for efficient endoplasmic reticulum to Golgi transport. J. Biol. Chem. *271*, 26939-26946.

Blow, D. (1990). Enzymology. More of the catalytic triad. Nature *343*, 694-695.

McLean, J., C. Fielding, D. Drayna, H. Dieplinger, B. Baer, W. Kohr, W. Henzel and R. Lawn (1986). Cloning and expression of human lecithin-cholesterol acyltransferase cDNA. Proc. Natl. Acad. Sci. USA *83*, 2335-2339.

Figure 1. Summary of genetic interactions between *bst* mutants and *sec* mutants. A graphic representation of growth comparisons over the indicated temperature range is shown. The data for *BST1* and *BST2* involve comparison of isogenic single and double mutants as described in Figure 10 of Chapter 2. The data for *BST3* are comparisons of several *bst3 sec* double mutants with the *sec* mutant parent and *sec* mutant segregants in the cross.

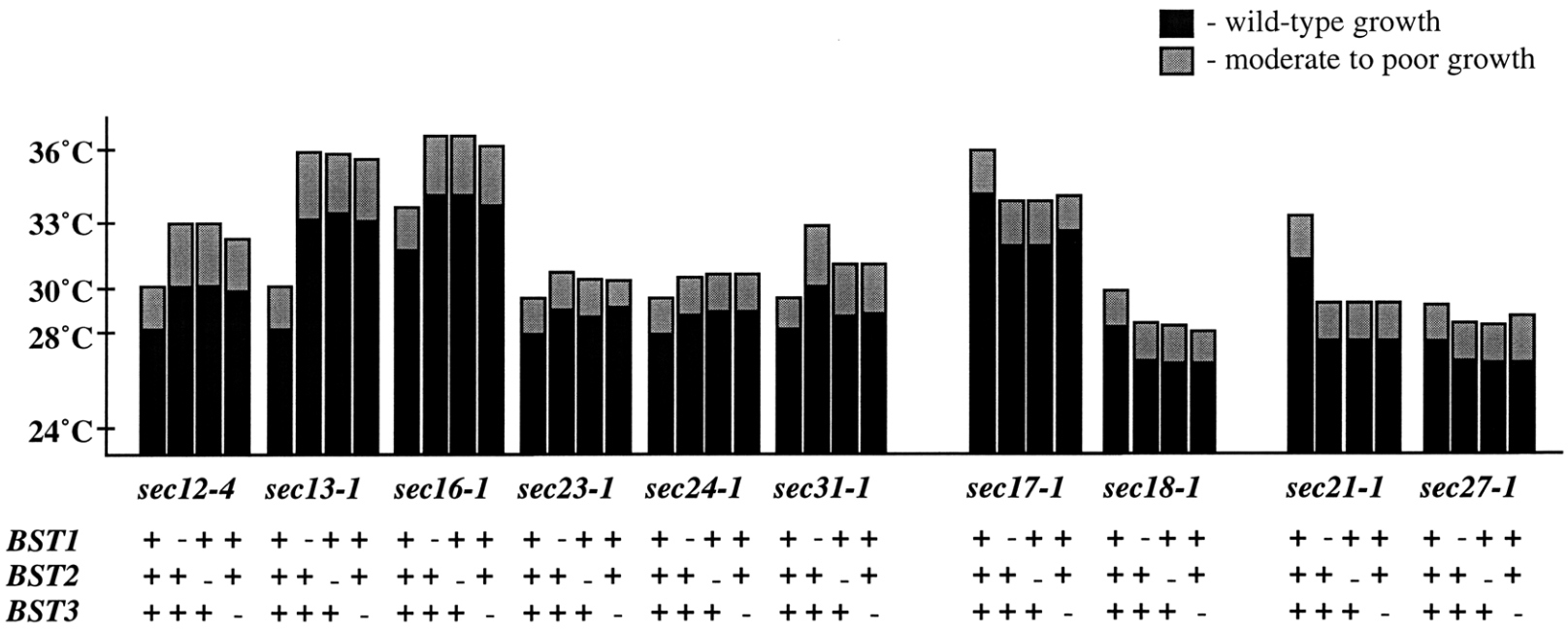
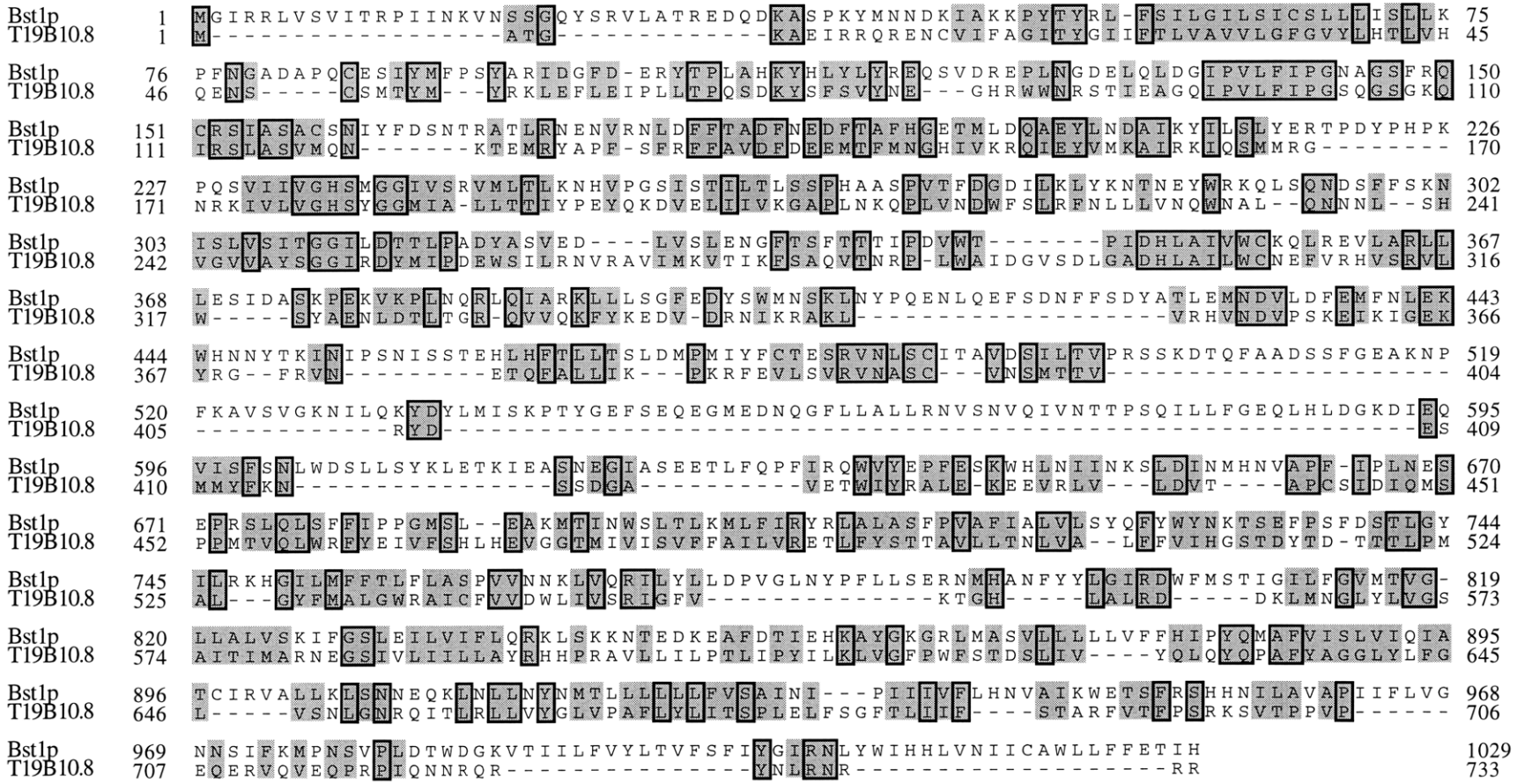
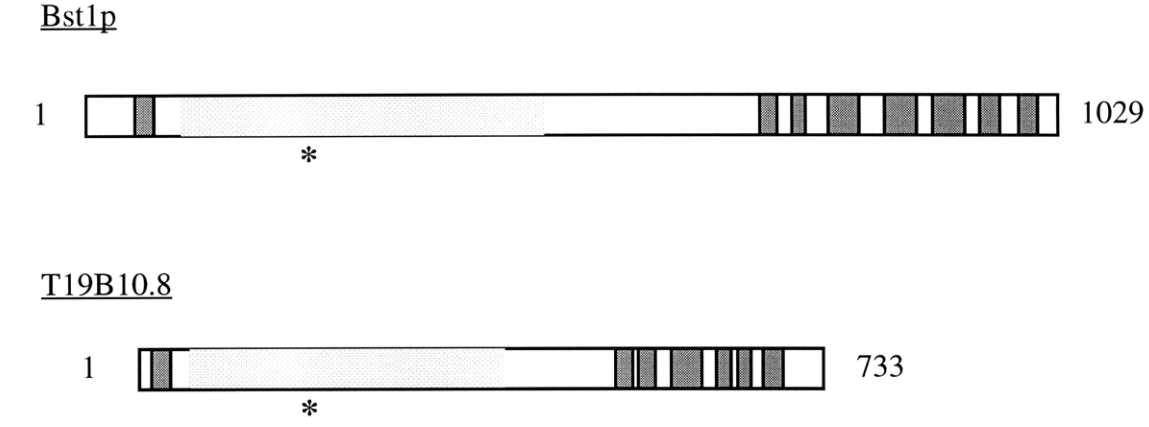

- wild-type growth
- moderate to poor growth
36°C
33°C
30°C
28°C
24°C
sec12-4
sec13-1
sec16-1
sec23-1
sec24-1
sec31-1
sec17-1
sec18-1
sec21-1
sec27-1
BST1 + - + + + - + + + - + + + - + + + - + + + - + + + - + + + - + + + - + +
BST2 + + - + + + - + + + - + + + - + + + - + + + - + + + - + + + - + + + - +
BST3 + + + - + + + - + + + - + + + - + + + - + + + - + + + - + + + - + + + -

Figure 2. Comparisons of Bst1p and a *C. elegans* homolog. (A) Comparison of the protein sequences of Bst1p and the *C. elegans* ORF T19B10.8. Boxed regions indicate identical amino acids, and shaded regions indicate similar amino acids. Amino acids 48 to 404 of T19B10.8 and 78 to 498 of Bst1p (the majority of the luminal domains of each protein) share 30% identical and 62% similar amino acids. (B) Schematic diagram comparing the overall structure of both proteins. Dark shaded regions represent potential membrane spanning segments, and the light shaded areas represent the region of highest homology. (*) indicates the location of the lipase motif in each protein. (C) Comparison of the lipase motifs of Bst1p and T19B10.8 with the consensus sequence.

A



B



C

	V I L	X	Y F V I L	T S M V I L	G	V W Y H	**S**	X	G	C A T S G
Bst1p:	V	I	I	V	G	H	**S**	M	G	G
T19B10.8:	I	V	L	V	G	H	**S**	Y	G	G

Chapter Three:

COPII vesicle budding *in vitro* in the absence of Sec13p

Evidence supporting a role for the *BST* proteins in regulating COPII vesicle formation

ABSTRACT

The budding of transport vesicles from the endoplasmic reticulum (ER) is driven by the assembly of a proteinacious coat (termed COPII) on the cytosolic surface of the membrane. *SEC13* is an essential gene required for this process and encodes a major component of the COPII coat. Mutations in any one of three genes (*BST1, EMP24/BST2,* or *ERV25/BST3*), however, enable growth and ER to Golgi transport in the absence of Sec13p. This report describes experiments investigating whether the transport observed in *sec13Δ bst* mutants occurs via COPII vesicles (lacking Sec13p) or via an alternative transport pathway. Examination of *bst* and *sec13Δ bst* mutants by electron microscopy shows that secretory organelles in these mutants have essentially normal morphology. *In vitro* experiments demonstrate that the budding of COPII vesicle markers from microsomal membranes does not require Sec13p, suggesting that COPII vesicles can form without Sec13p and that this is the likely mechanism of transport in *sec13Δ bst* strains. In addition, using the *in vitro* budding assay, we demonstrate that Bst1p (unlike Emp24p/Bst2p and Erv25p/Bst3p) is not incorporated into transport vesicles. The relevance of these results to models for the function of the *BST* genes is discussed.

INTRODUCTION

The movement of secretory proteins from the endoplasmic reticulum (ER) to the Golgi is mediated by transport vesicles that are formed by the assembly of cytosolic coat proteins on the ER membrane (Schekman and Orci, 1996). In the yeast *Saccharomyces cerevisiae*, six proteins (Sec16p, Sar1p, Sec13p, Sec23p, Sec24p, and Sec31p) are known to be components of the COPII coat (Barlowe et al., 1994; Espenshade et al., 1995). The current view of the function of each of these proteins was reviewed in Chapter 1.

SEC13 is an essential gene required for ER vesicle formation *in vivo*. Conditional mutants exhibit complete blocks in the transport of marker proteins from the ER and accumulate ER membranes at restrictive temperatures (Kaiser and Schekman, 1990), and spores carrying deletions of *SEC13* fail to germinate on rich medium (Pryer et al., 1993). In addition, *sec13* mutants show genetic interactions with each of the five other vesicle formation *sec* mutants (Kaiser and Schekman, 1990).

The importance of Sec13p in COPII vesicle formation has also been examined in cell-free assays that reconstitute vesicle budding using microsomal membranes and cytosol (or purified cytosolic proteins). Antibodies to Sec13p block vesicle release in these assays, and this block can be overcome by the addition of purified Sec13p/Sec31p complex (Salama et al., 1993). In addition, the Sec13p/Sec31p complex is one of the three purified factors (Sar1p and the Sec23p/Sec24p complex being the others) that are both necessary and sufficient to satisfy the cytosolic protein requirement in an assay using urea washed membranes (Salama et al., 1993; Barlowe et al., 1994). Cytosol from conditional *sec13* mutants is not, however, defective *in vitro*, although this could be due to the fact that the assay itself is temperature sensitive and the temperatures required to see a defect with *sec13* cytosol may be above those at which the assay functions (Pryer et al., 1993). These results demonstrate that the Sec13p/Sec31p complex is essential for COPII vesicle budding *in vitro*, but fail to address directly the requirement for Sec13p.

The essential nature of Sec13p in COPII vesicle formation (suggested by the results outlined above) is challenged by the observation that mutations in any one of three genes (*BST1, EMP24/BST2,* or *ERV25/BST3*) enable growth and ER to Golgi transport in the absence of Sec13p (Elrod-Erickson and Kaiser, 1996). This bypass of the requirement for Sec13p could be explained by either of two models. In the first model, the *BST* proteins impose a restriction on COPII vesicle formation, and Sec13p is required to overcome this restriction. Eliminating the *BST* restriction (by mutation) allows COPII vesicles to form without Sec13p. In the second model, the *BST* proteins inhibit an alternative pathway for transport between the ER and Golgi that is revealed by mutations in *BST* genes. In this model, Sec13p would be essential for COPII vesicle formation.

Several lines of evidence suggest that the first model is correct. The inability of *bst* mutations to suppress the deletion of other COPII genes (Elrod-Erickson and Kaiser, 1996) makes the complete bypass of COPII-mediated transport unlikely. In addition, the finding that Emp24p/Bst2p and Erv25p/Bst3p are major constituents of COPII-coated vesicles (Schimmöller et al., 1995; Belden and Barlowe, 1996) is also more consistent with a role for these proteins in the assembly of COPII vesicles. Here we describe further experiments that show COPII vesicles can form *in vitro* without Sec13p. Collectively, these data argue strongly that the *BST* proteins restrict the formation of COPII-coated vesicles and help focus future experiments on the nature of this restriction and why it exists.

MATERIALS AND METHODS

Electron microscopy

The yeast strains CKY10, CKY325, CKY326, CKY330 and CKY332 were used for this study (see Table 1, Chapter 1 for their complete genotype). Cultures (100 ml) grown exponentially in YPD at 25°C for 18 hrs were collected at an OD_{600} of 0.5, concentrated to a volume of 5 ml, and fixed by the addition of fresh glutaraldehyde to a final concentration of 2%. Cells were immediately pelleted and washed twice with 2 ml of dH_2O. The cells were then incubated for 4 hours at 4°C in 1 ml fresh 4% $KMnO_4$ with shaking, washed 5 times with dH_2O, and moved to fresh tubes where they were incubated in 2% uranyl acetate for 18 hrs at 4°C in the dark. The cells were again washed 5 times with dH_2O and dehydrated in a graded series of ethanol washes : 70% ethanol for 3 min, 80% ethanol for 3 min, 95% ethanol for 5 min, and 100% ethanol (fresh bottle) 4 x 5 min. The fixed, dehydrated cells were embedded in Spurr's resin and sectioned to a thickness of 70-90nm. Sections were stained with a 1:5 dilution of Reynolds' lead citrate (Reynolds, 1963) for 2.5 min to enhance membrane profiles and were visualized on a JOEL 1200CX electron microscope at 80 kV.

In vitro assay for vesicle formation

Cytosol was prepared from cultures grown in YPD 5% glucose to an OD_{600} of 4.0. Cells were harvested, washed twice in WB (20 mM HEPES-KOH, pH 6.8, 150 mM KOAc, 5 mM MgOAc, 250 mM sorbitol), and lysed by vortexing with glass beads in 2 ml per L starting culture of RB (WB containing 1 mM DTT, 1 mM ATP, 0.5 mM PMSF, 0.7µg/ml leupeptin and 0.5 µg/ml pepstatin A). The lysate was collected and the beads were washed 4x with 2 ml per L starting culture RB. Lysate and washes were mixed and cleared of all insoluble material by centrifugation for 10 min /4°C /32,000 rpm in a TLA100.3 rotor, and then for 1 h /4°C /100,000 rpm in a TLA100.3 rotor (Beckman). The soluble protein concentration was generally ~10 mg/ml by Lowry assay using

lysozyme as a standard. The cytosol was aliquoted, frozen in liquid nitrogen, and stored at -80°C until needed.

Membranes for *in vitro* assays were prepared from cultures grown in YPD 4% glucose to an OD_{600} of 2.0. Cells were harvested, incubated in 10 ml per L starting culture of 100 mM Tris-SO_4, pH 9.4, 100 mM β-ME for 10 min, and spheroplasted using 40 U/ OD cells of bacterially expressed lyticase in SB (10 mM Tris-HCl, pH 7.5, 0.7 M sorbitol, 1.5% (w/v) Bactopeptone, 0.75% (w/v) yeast extract, 0.5% glucose). Spheroplasts were collected after being spun through a sucrose/Ficoll cushion (20 mM HEPES-KOH, pH 7.4, 0.8 M sucrose, 1.5% (w/v) Ficoll 400) and then gently lysed with glass beads in lysis buffer (20 mM HEPES-KOH, pH 7.4, 0.1 M sorbitol, 50 mM KOAc, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.7μg/ml leupeptin, 0.5μg/ml pepstatin A). The lysate was collected and the beads were washed 4x with 2 ml per L starting culture lysis buffer. The lysate and washes were combined and cleared of unlysed cells by centrifugation at 500***g*** /4°C /2 min. Membranes from the cleared lysate were then collected by pelleting at 13,000***g*** /4°C /2 min in a microfuge. These membranes were resuspended in lysis buffer, and 0.5 ml aliquots were layered on top of sucrose step gradients (2.25 ml 1.2 M sucrose in lysis buffer carefully layered on top of 1.5 M sucrose in lysis buffer in 5 ml Beckman ultraclear ultracentrifuge tubes). Gradients were centrifuged at 32,500 rpm /4°C /1 hr in an SW 50 Ti rotor (Beckman). The microsomal membranes banded at the interface of the sucrose steps and were collected, diluted 5-fold in RB, pelleted at 13,000***g*** /4°C / 2 min in a microfuge, and washed 2x with RB. The membranes were then resuspended in ~300μl of RB per 1000 OD units of cells, frozen in aliquots in liquid nitrogen, and stored at -80°C. The protein concentration was generally ~2 mg/ml by modified lowry assay (DC protein assay kit, BioRad) using lysozyme as a standard.

Reactions were performed using 50μg of microsomal membranes and 1 mg of cytosol in a final volume of 500μl. The reactions also contained (final conc.) 1 mM ATP,

40 mM creatine phosphate (CP; disodium salt, Sigma), 0.2 mg/ml creatine phosphokinase (CPK; type 1 from rabbit muscle, Sigma), 1 mM GDP-mannose (type 1 sodium salt, Sigma), 0.1 mM GTP, and 1 mM PMSF in RB. Reaction mixtures were assembled on ice, with membranes being added last, and then the reactions were incubated for 1 hr at 20°C. Control reactions were as follows: (4°C) - was kept on ice during 1 hr incubation, (+ apy) - 10U/ml apyrase (grade VII, Sigma) was substituted for ATP, CP and CPK, and (- cyt) - RB was substituted for cytosol. After incubation, reactions were placed on ice for 5 min, the donor membranes were pelleted at 13,000***g*** /4°C /2 min, and vesicles were collected from the supernatants by centrifugation at 150,000***g*** /4°C /30 min in a TLA100.3 rotor. The vesicle fractions, as well as 2.5µg and 5µg of microsomal membranes, were analyzed by SDS-PAGE and Western blotting using antibodies against the vesicle marker Sec22p (1:1000 dilution), the resident ER marker Sec61p (1:1000 dilution) and the COPII marker Sec16p (1:1000 dilution). Western blots were developed using the ECL system (Amersham) and quantitated using an LKB 2202 Ultroscan laser densitometer (LKB, Bromma, Sweden).

RESULTS

Examination of sec13Δ bst and bst mutant strains by electron microscopy

sec13Δ bst mutant strains grow almost as well as wild-type strains at 25°C and show normal (although slightly delayed) transport of secretory proteins (Elrod-Erickson and Kaiser, 1996). To characterize these mutants more fully we examined the morphology of the secretory organelles in both *sec13Δ bst* and *bst* mutant strains by electron microscopy. The strains CKY10 (wild-type), CKY325 (*sec13Δ bst1*), CKY326 (*sec13Δ bst2*), CKY330 (*bst1*), and CKY332 (*bst2*) were fixed and stained with permanganate to aid in the visualization of membranes as described in Kaiser and Schekman (1990). Wild-type cells show ER membrane around the nucleus (the nuclear envelope) and at the extreme periphery of the cell, just under the plasma membrane (Figure 1A). Both *sec13Δ bst* mutant strains show a steady-state accumulation of ER membrane within the interior of the cell that is similar to the phenotype observed for vesicle formation *sec* mutants that have been held at restrictive temperatures for ~30 min (Kaiser and Schekman, 1990; Figures 1B and 1C). This accumulation of ER was observed in greater than 90% of all cells and is consistent with the delay in transport of secretory proteins observed in these mutants. *sec13Δ bst* mutant strains did not show any other abnormal morphologies. The *bst* mutant strains were virtually indistinguishable from the wild-type control (Figures 1D and 1E). Thus *sec13Δ bst* mutants show a steady state accumulation of ER membrane that is consistent with slower kinetics of transport out of the ER, but are otherwise morphologically normal, and *bst* mutant strains show no aberrant morphologies.

Vesicle budding in vitro does not require Sec13p

Depletion and antibody block experiments, as well as the reconstitution of vesicle budding using purified cytosolic factors and washed membranes, have established the Sec13p/Sec31p complex as an essential component in the formation of COPII-coated

vesicles *in vitro* (Pryer et al., 1993; Salama et al., 1993; Barlowe et al., 1994). Because *bst* mutations can bypass the cellular requirement for Sec13p *in vivo*, we wondered whether Sec13p itself was absolutely required for COPII vesicle formation. We therefore used an *in vitro* vesicle budding assay (basically as described in Wuestehube and Schekman, 1992) to assess the requirement for Sec13p.

The assay uses ER-enriched membrane preparations and cytosol, and is outlined in Figure 2. Basically, an easily sedimentable microsomal membrane fraction (donor membranes) is mixed with cytosol, an ATP regeneration system, and GTP at 20°C for 1 hour. The donor membranes are then removed by a medium speed (13,000***g***) spin, leaving any vesicles that have been produced in the supernatant. The vesicles can then be collected from this supernatant by pelleting at high speed (150,000***g***). The reaction is known to require an energy source (is inhibited by apyrase), cytosol, and incubation temperatures of 20 - 25°C (is inhibited at 4°C; Wuestehube and Schekman, 1992).

Using this assay we compared the efficiency of vesicle budding when both membranes and cytosol were prepared either from a wild-type strain (CKY10) or from a *sec13Δ bst1* mutant strain (CKY325). The use of a *sec13Δ bst1* mutant as a source of both membranes and cytosol ensured that these reactions were completely free of Sec13p. As can be seen in Figure 3, the reactions using *sec13Δ bst1* components were indistinguishable from those using wild-type components. Release of the vesicle marker, Sec22p, and the COPII marker, Sec16p, from the donor membranes into the vesicle fraction was equally efficient in both cases and was dependent on an energy source, the addition of cytosol, and incubation at 20°C. Furthermore, the resident ER marker protein, Sec61p, was not efficiently released from the donor membranes, demonstrating that the reactions measure vesicle production (not fragmentation of the donor membranes) and faithfully segregate resident proteins from vesicle proteins. These results demonstrate that Sec13p is not required for the budding of COPII vesicle markers in the context of *bst* mutant membranes.

We also examined vesicle budding from wild-type membranes using either wild-type (CKY10) or *sec13Δ* cytosol (prepared from the *sec13Δ bst1* mutant strain CKY25) to address whether Sec13p is required in this context. As shown in Figure 4A, both cytosols were able to promote vesicle budding from wild-type membranes to a similar extent. Vesicle production required the addition of cytosol, indicating that the membranes do not contain sufficient amounts of COPII proteins to effectively drive vesicle budding. We could not, however, rule out the possibility that there was sufficient Sec13p on the membranes to allow budding after the addition of cytosol. We therefore urea washed wild-type membranes to remove peripheral proteins, and compared the efficacy of wild-type and *sec13Δ* cytosol to promote vesicle budding from the washed membranes. The overall efficiency of vesicle budding decreased with increasing stringency of the urea wash, but under no conditions did we detect a difference between *sec13Δ* and wild-type cytosols in their ability to promote vesicle budding from these membranes (Figure 4B). We therefore conclude that Sec13p is not required for COPII vesicle formation *in vitro*.

Bst1p is not packaged into transport vesicles produced in vitro

Both Emp24p/Bst2p and Erv25p/Bst3p have been shown to be components of COPII-coated vesicles (Schimmöller et al., 1995; Belden and Barlowe, 1996). In order to address whether Bst1p is also incorporated into transport vesicles, we examined the release of Bst1p-HA from donor membranes (prepared from the wild-type strain CKY10 expressing Bst1p-HA from the centromere-based plasmid pME1170 - see Chapter 2) in the *in vitro* budding assay. Donor membranes and vesicle fractions were examined by SDS-PAGE and immunoblotting, using antibodies against both Sec22p and the HA epitope. The efficient release of the Sec22p marker demonstrated the production of vesicles. Bst1p-HA, however, was not detected in the vesicle fractions at levels above background, indicating Bst1p-HA is not incorporated into COPII-coated vesicles and is instead a permanent resident of the ER membrane (Figure 5).

DISCUSSION

The identification of the *BST* genes as bypass suppressors of *sec13Δ* raised a number of questions about the role of Sec13p in COPII-coated vesicle formation and the function of the *BST* genes in ER to Golgi transport. The experiments described here allow us to address some of these questions. We have demonstrated that Sec13p is not absolutely required for COPII vesicle formation *in vitro*, by showing that COPII vesicle markers are efficiently released from donor membranes in cell-free assays using *sec13Δ bst* components. Additional data suggests that Sec13p is not required *in vitro* even in the context of budding from wild-type membranes. Thus, explanations for the bypass of Sec13p do not require hypotheses of a novel transport mechanism between the ER and Golgi, since COPII vesicles are capable of forming without Sec13p. The morphological examination of *sec13Δ bst* and *bst* mutant strains by electron microscopy also provides no evidence of novel structures or aberrant morphologies that might suggest a novel transport pathway. The simplest explanation for these results is that the *BST* genes impose a restriction on COPII vesicle formation and that Sec13p is required to overcome this restriction. In the absence of the restriction, Sec13p is no longer strictly required to transport proteins via COPII vesicles. We have also provided evidence that Bst1p is not a component of COPII vesicles, an observation that distinguishes it from Emp24p/Bst2p and Erv25p/Bst3p and helps to further refine models for the function of the *BST* proteins.

COPII vesicle formation at the ER

Sec13p is a component of the COPII coat whose assembly on the ER membrane drives the formation of COPII vesicles. The results presented here indicate that Sec13p is not strictly required for vesicle formation in cell free assays, and those of Chapter 2 show that Sec13p is dispensable *in vivo* if cells have a mutation in any one of the three *BST* genes.

How can these results be reconciled with the body of evidence suggesting that Sec13p plays a central and essential role in COPII vesicle formation?

One possible unifying idea is that Sec13p is required for maximal stability of the COPII coat; thus, Sec13p, while important for vesicle coat assembly, would only be essential under conditions where stability is critical. This could explain why the bypass of *SEC13* is temperature dependent. If the absence of *BST* function stabilizes the coat (or makes coat stability less important), then Sec13p would not be essential at lower temperatures but would still be essential at higher temperatures where coat stability is perhaps more critical. The requirement for Sec13p in stabilizing the coat at high temperatures might also account for the apparent lack of a requirement for Sec13p *in vitro*, because our cell-free assays are carried out at 20°C. (Direct tests of this idea are not possible, however, because the assay itself is extremely inefficient at temperatures above 30°C.) The inviability of *sec13Δ BST*$^{+}$ strains could be explained if the normal function of the *BST* gene products destabilizes the coat during vesicle formation (see below).

Previous results demonstrating a requirement for the Sec13p/Sec31p complex *in vitro* can now be understood to imply an essential requirement for Sec31p. Our use of *sec13Δ bst* mutants as the source of membranes and cytosol for *in vitro* budding reactions not only ensured that the reactions were completely free of Sec13p, but also allowed us to address the question of the requirement for Sec13p without having to devise a means of purifying functional Sec31p away from Sec13p, a difficulty that is likely to be responsible for this question having never been directly addressed.

Functions of the BST proteins in COPII vesicle formation

Several lines of evidence, some of which are described in this chapter, strongly suggest a role for the *BST* proteins during COPII vesicle formation. The inability of *bst* mutations to suppress deletions in other COPII genes (Chapter 2) indicates that the suppression of

sec13Δ is probably not due to a complete bypass of COPII vesicle-mediated transport. The results presented in this chapter show that COPII vesicles can indeed form without Sec13p, strengthening the idea that *bst* mutations allow the budding of COPII vesicles in the absence of Sec13p. Furthermore, the fact that two of the *BST* proteins (Emp24p/Bst2p and Erv25p/Bst3p) are themselves components of COPII vesicles (Schimmöller et al., 1995; Belden and Barlowe, 1996) argues that these proteins have a function in COPII-coated vesicle formation. What, then, is the nature of this function?

The phenotypes of *bst* mutant strains indicate that the function of the *BST* proteins affects both protein sorting and the assembly of the COPII coat. Either the regulation of vesicle coat assembly by *BST* proteins allows efficient sorting to occur, or the *BST* sorting function alters conditions for coat assembly such that there is a strict requirement for a fully functional coat.

As suggested in Chapter 2, the *BST* proteins could, by restricting the completion of vesicle assembly, keep the vesicle coat in a partially assembled state long enough to allow sorting to occur . In this model, Sec13p would be required to progress beyond the *BST* restriction. However, there are several problems with this model. *sec13Δ bst* mutants have a slightly decreased rate of transport for all secretory proteins, suggesting a decreased rate of vesicle release (Chapter 2). These mutants nonetheless show sorting defects (leak resident proteins). If all the *BST* proteins do is provide sufficient time for sorting to occur, one might expect the *sec13Δ bst* mutants to sort efficiently. In addition, explaining rates of transport becomes difficult (although not insurmountable) in a model where maximal rates are achieved by slowing vesicle release in order to increase the concentration of a subset of cargo proteins. (Why aren't most proteins transported faster in *bst* mutants than in wild-type if a restriction to vesicle release is removed?) Furthermore, if Sec13p has a special role in overcoming the *BST* regulation, and can therefore be bypassed by removing this regulation, why do *bst* mutations also partially suppress mutations in all the other COPII subunits?

Most of these problems are overcome by proposing that cells monitor both sorting and vesicle assembly such that vesicle production is restricted by the *BST* proteins when sorting is incomplete or when the coat is compromised by mutation. This *BST* "checkpoint" would therefore ensure efficient sorting and vesicle assembly in a manner similar to the checkpoints in the cell cycle that ensure efficient segregation of chromosomes during mitosis. According to this model, one might expect to be able to generate dominant mutations in *BST* genes that block or slow vesicle formation. We explored this possibility extensively and were unable to find such mutants, but our inability to generate dominant mutants in no way serves as a test of the model. Thus, the checkpoint analogy still proves a plausible model for the function of the *BST* proteins if they act primarily as regulators of vesicle assembly.

Alternatively, if we adopt the idea from the previous section that Sec13p is only required under conditions where coat stability is critical, the *BST* proteins could instead perform a sorting function that indirectly affects coat assembly by changing the physiology of the forming vesicle in a way that makes coat stability important. Thus, when the *BST* sorting function is active, Sec13p would be required. In the absence of the sorting function, Sec13p would not be required, except at high temperatures where coat stability is again important. This idea is appealing because it easily explains why *bst* mutations partially suppress temperature sensitive mutations in all COPII subunits (Chapter 2 and its appendix); decreasing the requirement for a stable coat (by removing the *BST* sorting function) would make coat assembly easier, even with mutant components. This model also explains why the rate of transport of most proteins in *bst* mutants is identical to that seen in wild-type strains. Vesicles form at similar rates with or without the *BST* sorting function; it is only the requirement for stability in the coat (and the sorting of some proteins) that is affected by *BST* protein activity. How a sorting function might increase the need for stability in the vesicle coat is now the obvious question and will be discussed in detail in Chapter 5.

ACKNOWLEDGEMENTS

We would like to thank Peter Espenshade and Bill Payne for technical advice and all the members of the Kaiser Lab and Monicia Elrod-Erickson for helpful discussions during the course of this work.

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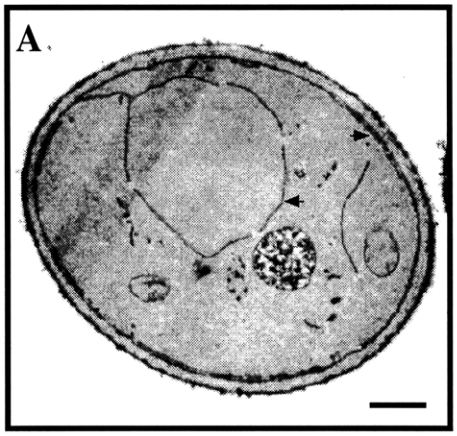
Figure 1. Analysis of *sec13Δ bst* and *bst* mutant strains by electron microscopy. Representative micrographs are shown for (A) the wild-type strain CKY10, (B) the *sec13Δ bst1-1* strain CKY325, (C) the *sec13Δ bst2-1* strain CKY326, (D) the *bst1-1* strain CKY330, and (D) the *bst2-1* strain CKY332. All strains were grown at 25˚C and processed as described in the Materials and Methods. Arrows indicate ER membranes. The size bars are 500nm.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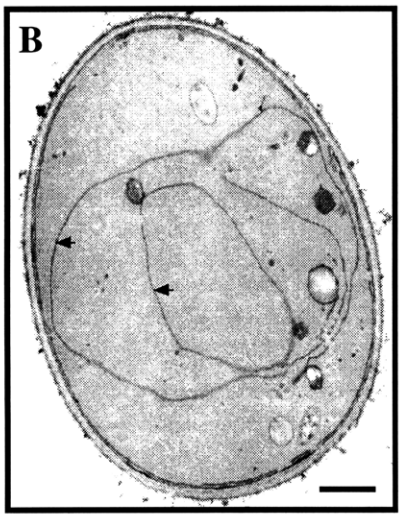
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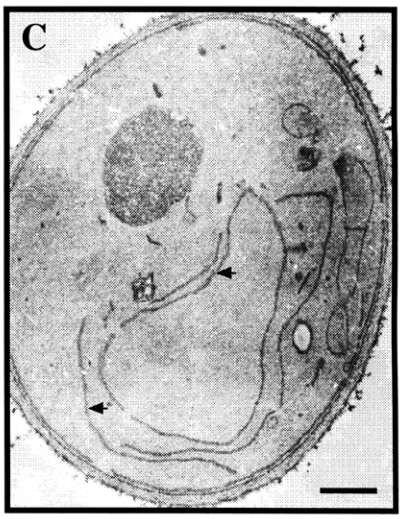
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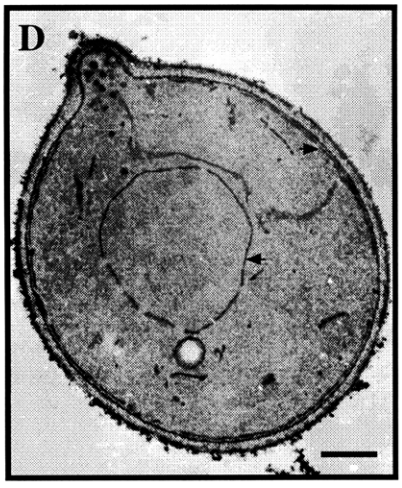
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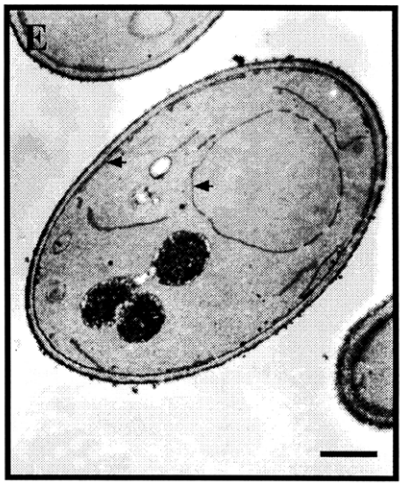
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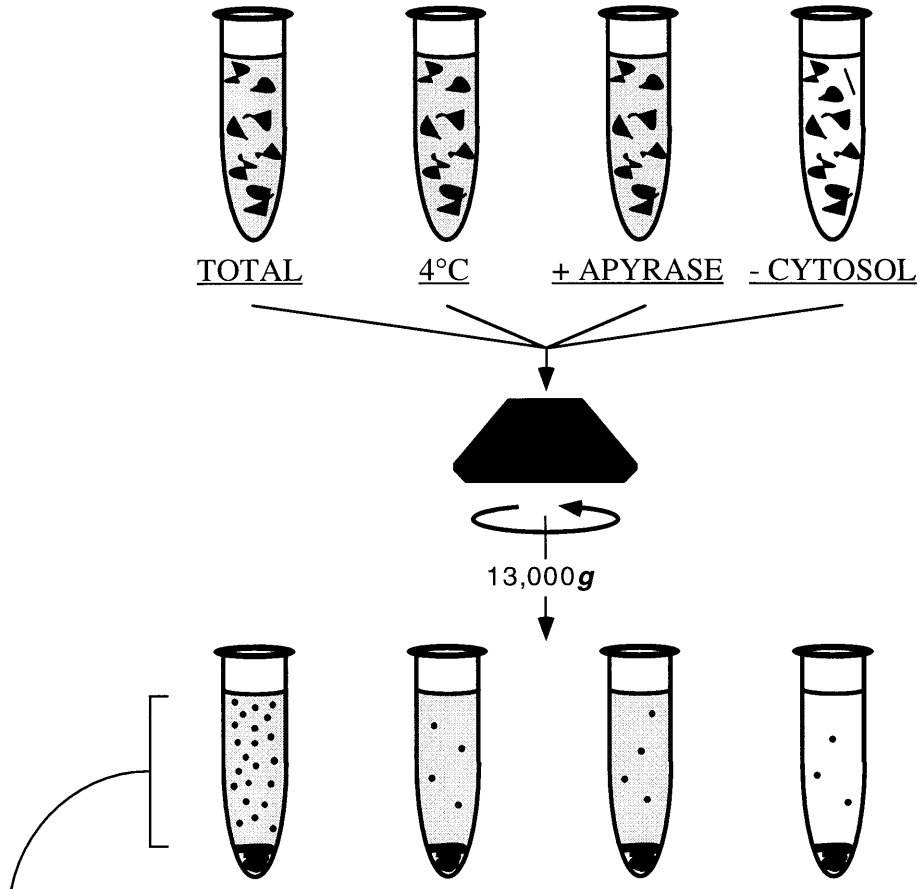
Figure 2. Schematic diagram outlining the *in vitro* assay for vesicle budding. ER-enriched microsomal membranes are mixed with cytosol, an ATP regeneration system and GTP at 20°C for 60 minutes as described in the Materials and Methods. Microsomal membranes are then pelleted by centrifugation at 13,000***g***. Vesicles remain in the supernatant and are collected by pelleting in a subsequent centrifugation step at 150,000***g***. Samples are prepared and analyzed by SDS-PAGE and Western blotting using antibodies to vesicle markers.

ER microsomes + cytosol + ATP regeneration system + GTP
20°C for 60 min



collect supernatants, pellet at 150,000*g* to collect vesicles, and assay by quantitative Western for the presence of vesicle markers

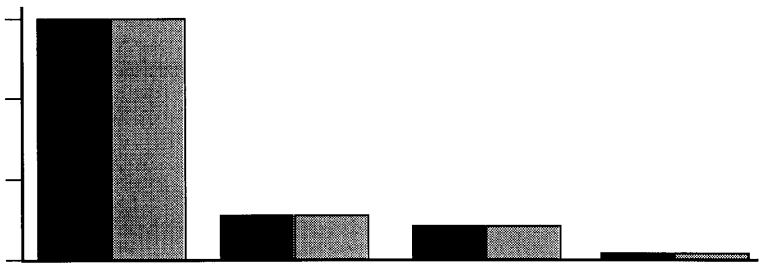


Figure 3. Sec13p is not required for COPII vesicle budding *in vitro*. Vesicle budding reactions were performed using either wild-type membranes and cytosol or *sec13Δ bst1* membranes and cytosol as described in the Materials and Methods. Vesicle fractions were analyzed by immunoblotting and quantitated densitometrically. Percent release was calculated by comparing the amount of marker proteins detected in vesicle fractions with the amount detected in the original donor membranes. Shown are the percentage of three marker proteins released from donor membranes in total and control reactions.

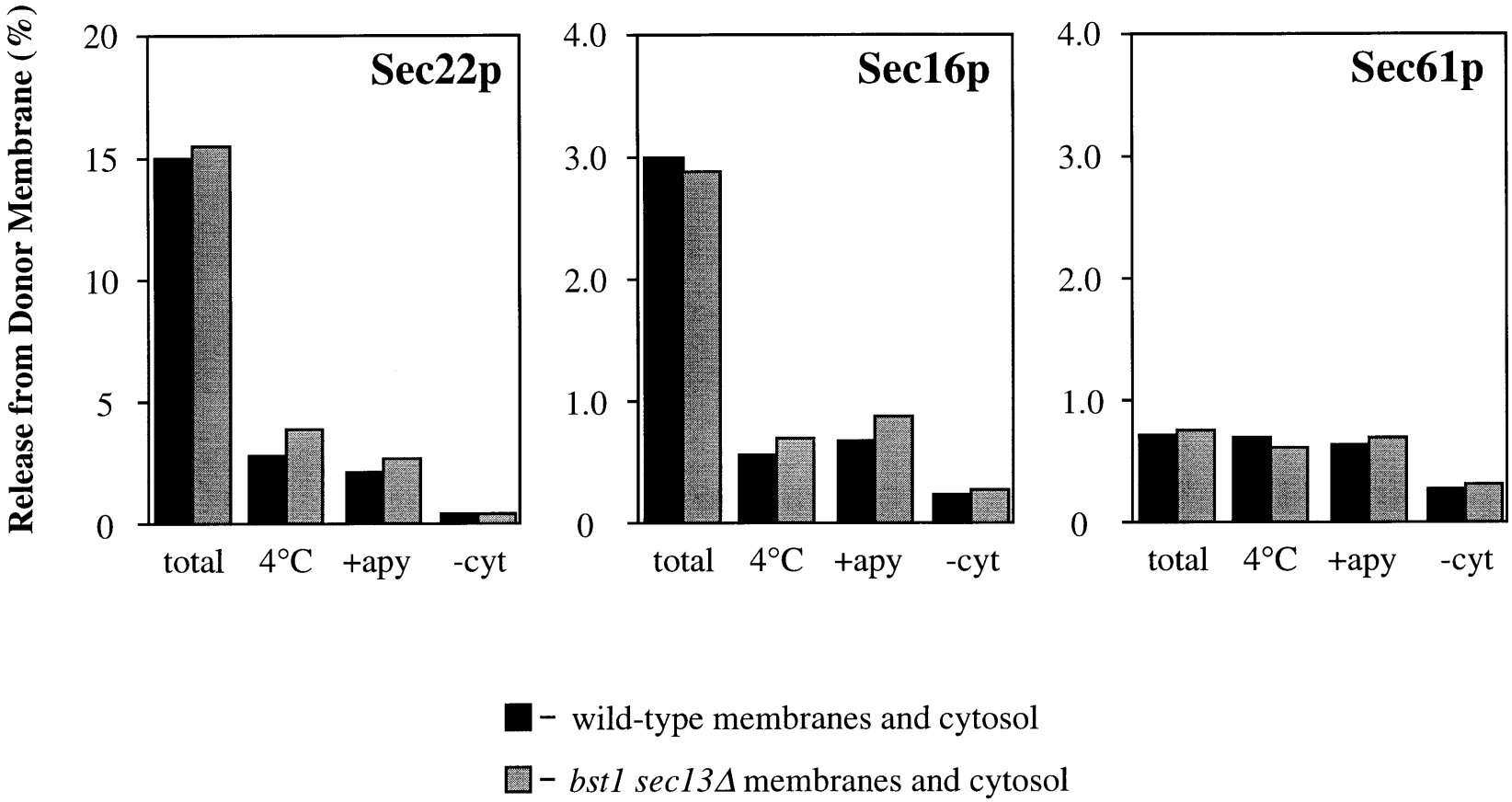

Release from Donor Membrane (%)
Sec22p
Sec16p
Sec61p
20
15
10
5
0
4.0
3.0
2.0
1.0
0
total
4°C
+apy
-cyt
– wild-type membranes and cytosol
– bst1 sec13Δ membranes and cytosol

Figure 4. Sec13p is not required for vesicle budding from wild-type membranes. Vesicle budding reactions were performed using wild-type membranes and either wild-type or *sec13Δ bst1* mutant cytosol as described in the Materials and Methods. Vesicle fractions were analyzed as described for Figure 3. (A) The percentage of Sec22p released into the vesicle fraction in total and control reactions. (B) The percentage of Sec22p released from the washed membranes in total budding reactions using either wild-type or *sec13Δ bst1* mutant cytosols.

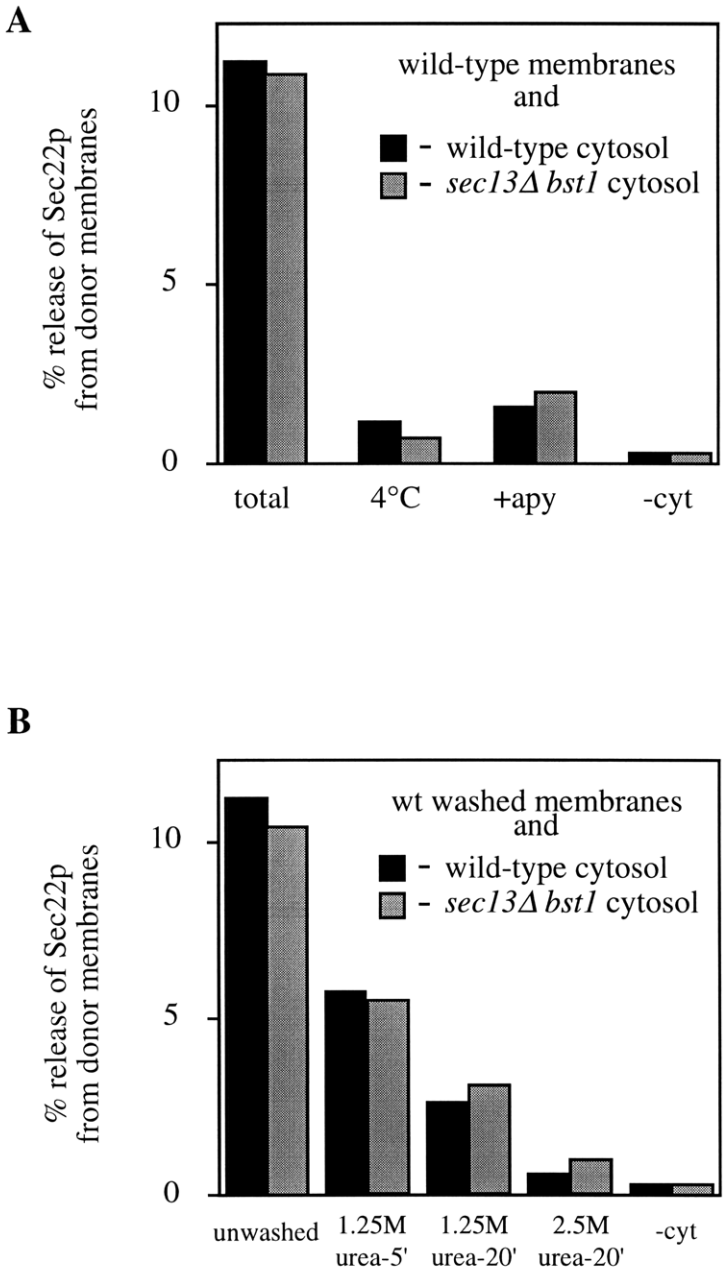
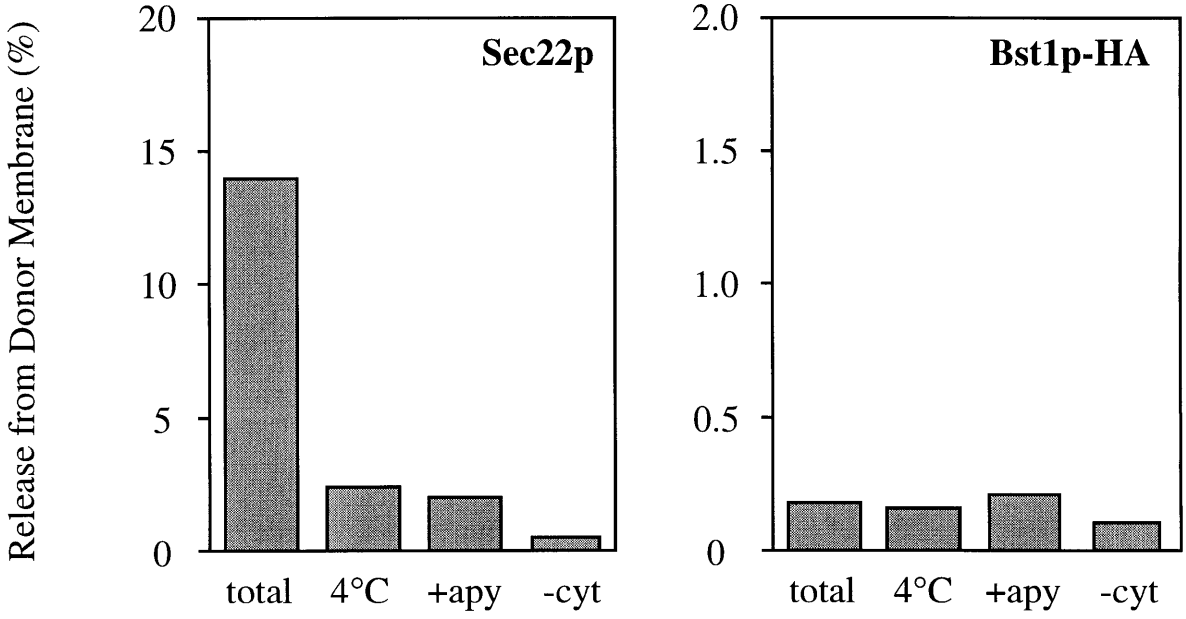

A
% release of Sec22p
from donor membranes
10
5
0
wild-type membranes
and
- wild-type cytosol
- sec13Δ bst1 cytosol
total
4°C
+apy
-cyt
B
% release of Sec22p
from donor membranes
10
5
0
wt washed membranes
and
- wild-type cytosol
- sec13Δ bst1 cytosol
unwashed
1.25M
urea-5'
1.25M
urea-20'
2.5M
urea-20'
-cyt

Figure 5. Bst1p-HA is not incorporated into vesicles produced *in vitro*. Vesicle budding reactions were performed using wild-type membranes (from a strain expressing Bst1p-HA) and wild-type cytosol as described in the Materials and Methods. Vesicle fractions were analyzed as described for Figure 3 and shown are the values for the percent release of the vesicle marker Sec22p and Bst1p-HA in total and control reactions.


Release from Donor Membrane (%)
20
15
10
5
0
Sec22p
total
4°C
+apy
-cyt
2.0
1.5
1.0
0.5
0
Bst1p-HA
total
4°C
+apy
-cyt

Chapter Four:

Characterization of *ICWP* as a reporter for secretory defects

Evidence that the *BST* genes are important for efficient transport of GPI-anchored proteins

ABSTRACT

As the study of the secretory pathway moves from the characterization of essential components to the identification of factors required for efficiency and fidelity within the pathway, the use of sensitive markers for perturbations in protein transport will become increasingly important. In this report we describe the characterization of an inner cell wall protein (Icwp) as an extremely sensitive reporter of secretory defects. Icwp is a GPI-anchored membrane protein that is rapidly transported through the secretory pathway. At the cell surface the protein is cross-linked to the glucan layer of the cell wall, making it completely insoluble and immunologically undetectable unless the wall is digested with glucanase. In wild-type cells, the steady state amounts of intracellular (detectable) protein are extremely small. In mutant strains with perturbations in protein transport, the protein accumulates intracellularly and is easily detected, thus providing a simple and sensitive assay for identifying mutants with secretory defects. Icwp accumulation identifies all *sec* mutants as well as additional mutants that show only subtle kinetic defects when examined using current secretory marker proteins, demonstrating the sensitivity of this assay. In addition, the steady state accumulation of Icwp and Gas1p (both GPI-anchored proteins) observed in *bst* mutant strains suggests a role for the *BST* genes in the efficient transport of GPI-anchored proteins.

INTRODUCTION

The yeast cell wall is composed of glucans, mannoproteins, and chitin in a layered structure. The inner layer is composed of β-1,3 and β-1,6 glucans, small amounts of chitin, and mannoproteins and is responsible for the mechanical strength of the cell wall and for maintaining its shape. The outer layer is composed of mannoproteins and determines the porosity of the wall, as well as the surface properties of the cell, such as charge, flocculence, and sexual agglutinability (reviewed in Fleet, 1991).

Mannoproteins of the cell wall can be divided into three groups according to the methods that are capable of extracting them: sodium dodecyl sulfate (SDS)-extractable mannoproteins, reducing agent-extractable mannoproteins, and glucanase-extractable mannoproteins (Valentin et al., 1984). The mechanism by which glucanase-extractable mannoproteins are incorporated into the cell wall is thought to require that the proteins be GPI-anchored during their transport to the plasma membrane. Once at the cell surface, the mannoproteins (along with some of the sugar residues) are removed from the lipid portion of their anchors and covalently attached to the cell wall glucans in a transglycosylation reaction (De Nobel and Lipke, 1994). The incorporation into the glucan layer of the cell wall renders such proteins completely insoluble and undetectable by normal immunological means (Valentin et al., 1984).

Here we report the characterization of Icwp, a glucanase-extractable mannoprotein of the inner cell wall (Moukadiri et al., 1997), as a new marker protein that provides a simple but sensitive assay for detecting perturbations in secretory pathway function. Fortuitous antibodies present in one of our polyclonal antisera recognize several protein species from cell extracts of mutants with defects in protein transport, but not from cell extracts of wild-type strains. One of these proteins was purified from yeast, sequenced, and found to be Icwp. The detection of Icwp is dependent on its intracellular accumulation, apparently because once it reaches the cell surface the protein is covalently

attached to the glucan layer of the cell wall, rendering it insoluble and undetectable. Icwp transport provides a particularly sensitive assay because the steady state amount of intracellular (detectable) protein is very small. Only by causing the levels of intracellular protein to increase can Icwp be easily detected. Thus, any mutation or treatment of cells that blocks or sufficiently slows the transport of the protein through the secretory pathway shows detectable levels of Icwp. All *sec* mutants, as well as additional mutants with more subtle transport defects, show accumulation of detectable amounts of Icwp, demonstrating the utility of the assay.

We also show that Gas1p transport is defective in *bst1*-1 mutant strains. The fact that Icwp and Gas1p (also GPI-anchored) are two of the three proteins known to be transported inefficiently in *bst* mutant strains suggests that GPI-anchored proteins are particularly sensitive to defects in the *BST* sorting function. The implications of this idea are discussed briefly here and developed further in Chapter 5.

MATERIALS AND METHODS

General techniques

Growth and maintenance of strains, preparation of standard media (Difco, Detroit, MI), crosses and other genetic manipulations were performed as described in Kaiser *et al.*, 1994. *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. DNA manipulations were performed as described in Sambrook *et al.*, 1989. pME702 was generated by PCR amplification of the YRL391w-A ORF plus approximately 500 nucleotides of flanking DNA both upstream and downstream of the gene using the primers: 5 '- CGGGATCCGTGGGAGTCTACACGGGCACGAG - 3 ' and 5 '- GGACTAGTGAATTACGGTTAAAGGATCACCCC - 3 '. The underlined sequences engineered a BamHI site upstream of the gene and a SpeI site downstream of the gene. This fragment was then cloned into the yeast episomal plasmid pRS306-2μ (Miller and Fink, see Chapter 2 for details) by replacing the BamHI - SpeI fragment of the polylinker.

Characterization of BAP137 antibodies

Immunoblotting with the polyclonal anti-invertase antiserum 137 unexpectedly revealed the presence of several prominent protein species in *sec* mutant extracts. Analysis of *sec suc2Δ* double mutants indicated that the bands were not encoded by the *SUC2* (invertase) gene. Using the *bst1 ire1Δ* strain RRY315, the cross-reacting antibodies were blot-affinity purified as described (Tang, 1993) and called BAP137 antibodies. BAP137 antibodies were unable to detect invertase on immunoblots, further demonstrating that these antibodies recognize an epitope (or epitopes) unrelated to invertase. All four species observed on blots from extracts of RRY315 were detected by BAP137 antibodies regardless of which band was used for the blot-affinity purification. (S. McGuire et al., UROP thesis 1995).

Protein gels and immunoblotting

Protein extracts were prepared from 1-5 OD_{600} units of cells by boiling and disruption with glass beads in 30µl of sample buffer (80 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 10% bromophenol blue). Extracts were diluted in sample buffer and heated at 95°C for 3 min before being resolved by SDS-PAGE. Proteins were transferred to nitrocellulose in a semi-dry transfer apparatus (Owl Scientific Plastics, Inc., Cambridge, MA) at 500 mV for 45 min. Blots were blocked and then incubated for 1 h with primary antibody in TBS-T (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20), 2% milk at the following dilutions: BAP137 antibodies were used at 1:500, and anti-Gas1p antibody (a kind gift from H. Riezman) was used at 1:1,000 dilution. Blots were then washed three times for 10 min each in TBS-T, incubated with a 1:10,000 dilution of donkey anti-rabbit IgG-HRP (Amersham Corp., Arlington Heights, IL) in TBS-T, 1% BSA for 1 h, washed 3 times for 10 min each in TBS-T, developed for chemiluminescence using the ECL system (Amersham) and exposed to film.

Purification of Icwp

The purification of Icwp was as follows. Cultures (13.5L) of RRY315 were grown in YPD 4% glucose to an OD_{600} of 2.5, harvested, washed and resuspended in 50 ml of 100 mM Tris-SO_4, pH 9.4, 100 mM β-ME for 10 min at room temperature. The cells were then spheroplasted using 40 U/OD of bacterially expressed lyticase in 150 ml SB (10 mM Tris-HCl, pH 7.5, 0.7 M sorbitol, 1.5% (w/v) Bactopeptone, 0.75% (w/v) yeast extract, 0.5% glucose). Spheroplasts were spun through an equal volume of sucrose/Ficoll cushion buffer (20 mM Tris-Cl, pH 8.0, 0.8 M sucrose, 1.5% (w/v) Ficoll 400) and then vigorously lysed with glass beads in lysis buffer P (20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM PMSF) on ice. The lysate was collected and the beads were washed 4x with 10 ml of ice cold lysis buffer P . The lysate and washes were combined and cleared of unlysed cells by centrifugation at 500***g*** /4˚C /2 min in a clinical centrifuge.

Membranes from the cleared lysate were then collected by pelleting at 13,000*g* /4°C /5 min in an SS34 rotor (DuPont-Sorvall), washed in cold lysis buffer P, resuspended in lysis buffer P containing 2.5 M urea and placed on ice for 30 min. The ratio of buffer to membranes from this point on was kept at approximately 2:1 (vol:vol of pelleted membranes). The urea washed membranes were washed 2x in 20 mM Tris-Cl, pH 8.0, 10 mM NaCl and solubilized in 2 volumes of 20 mM Tris-Cl, pH 8.0, 10 mM NaCl, 0.1% Triton X-100 on ice for one hour. The insoluble material was removed by centrifugation at 200,000*g* /4°C /1 hr in a SW41Ti rotor (Beckman) and the soluble material was collected. The soluble protein was then subjected to a slow freeze/thaw (placed at -20°C overnight and thawed on ice), cleared of precipitated material by centrifugation at 200,000*g*, boiled for 5 min, and again cleared of precipitate by centrifugation at 200,000*g* /4°C / 1 hr in a SW41Ti rotor. The soluble protein at this stage was passed over a MonoQ column (Pharmacia) and eluted with a NaCl gradient. Fractions were collected and analyzed by SDS-PAGE and immunoblotting using BAP137 antibodies. Icwp eluted at approximately 150mM NaCl in a fraction with only a few minor contaminants (see Figure 4). This fraction was concentrated by TCA precipitation and run on an 8% polyacrylamide SDS gel. The gel was Coomassie stained and the ~75 kDa band was excised and submitted for automated sequencing at the MIT Biopolymers laboratory.

RESULTS

Immunoblotting with BAP137 antibodies provides a simple and sensitive assay for identifying mutants or treatments that perturb secretory pathway function

BAP137 antibodies were identified (as described in the Materials and Methods) because they detect (on immunoblots) a protein species in extracts from *bst* mutant strains but not from wild-type strains (Figure 1A). *bst* mutants have been shown to have subtle and selective defects in protein transport (Chapter 2). To determine whether the presence of the band detected with BAP137 antibodies was due to defects in protein transport (and whether there was any specificity with respect to where in the pathway the block occurs), we examined all of the *sec* mutant strains listed in Table 1. Cell extracts from cultures that were either grown continuously at the permissive temperature of 24°C, or grown at 24°C and then shifted for one hour to the restrictive temperature of 37°C, were analyzed by SDS-PAGE and immunoblotting, using BAP137 antibodies. One to four species were detected in every *sec* mutant examined (the results from representative *sec* mutants are shown in Figure 1B). For most of the mutants, the bands were observed exclusively in the 37°C sample. The species of approximately 75 kDa was observed in every mutant, although it sometimes appeared smeary and ran at almost 100 kD (as in *sec12*, Figure 1), and additional bands appeared in some mutants (examined more carefully below). The presence of BAP137-specific bands on these blots appeared to be due to the block in protein transport induced at high temperatures in *sec* mutants, since no bands were observed at 24° or 37°C in extracts from either wild-type strains (Figure 1B) or randomly chosen temperature sensitive mutants that have no *sec* defect (data not shown). For some of the mutants (e.g. *sec4-8*, Figure 1B), less intense bands were present in the 24°C samples, suggesting that these mutants show subtle defects in transport even at the permissive temperature. Immunoblotting with BAP137 antibodies therefore appeared to

be a sensitive and specific assay for identifying strains with defects in protein transport at any step in the pathway between the ER and the cell surface.

To determine how sensitive this assay is compared to others currently employed, we examined three additional mutants that show very subtle or selective defects in protein transport. The deletion of *SED4* in an S288C strain background causes a modest kinetic defect in protein transport between the ER and Golgi: CPY maturation is slowed approximately 1.6-fold in a *sed4Δ* strain compared to a wild-type strain (Gimeno et al., 1995). This defect requires a careful pulse-chase analysis to be detected. Similarly, *ISS1* encodes a nonessential homolog of *SEC24* that shows genetic interactions with a subset of ER to Golgi *SEC* genes, suggesting it may be important for efficient transport, but an *iss1Δ* strain has no detectable defect in the transport of commonly used marker proteins (R. Gimeno, PhD dissertation, 1996). Finally, *LST1* encodes another *SEC24* homolog and shows genetic interactions with the COPII vesicle formation *SEC* genes. *lst1Δ* mutants exhibit a kinetic defect in the transport of the plasma membrane ATPase from the ER at 37°C , but show no detectable defect in the transport of invertase or CPY at this temperature (K. Roberg, PhD dissertation, 1997). We therefore examined extracts from *sed4Δ*, *iss1Δ*, and *lst1Δ* strains by immunoblotting using BAP137 antibodies as described above. All three mutants showed BAP137-detectable species at 37°C, indicating that this assay is extremely sensitive (Figure 1C).

The presence of multiple species on immunoblots probed with BAP137 antibodies correlated with two factors: 1) the step in the pathway where the mutant is defective, and 2) the length of time the mutant was kept at the restrictive temperature. Mutants affecting early steps in protein transport were more likely to show multiple species than mutants defective in later transport steps (compare the early acting mutants *sec18-1* and *sec21-1* with the late acting mutant *sec4-8* in Figure 1B). In addition, the longer a mutant spent at restrictive temperatures, the more likely it was to show multiple bands. Figure 2A shows a kinetic analysis of the appearance of BAP137 detectable species in *sec18-1* and *sec1-1*

mutant strains. Cultures grown at 24°C were filtered and resuspended in prewarmed media at 37°C to effect a rapid shift in incubation temperature. Samples taken at intervals after the shift were analyzed by SDS-PAGE and immunoblotting with BAP137 antibodies. In both mutants, p75 is the first detectable species to appear and additional bands become visible after longer incubation. (Figure 2A; *sec1-1* does eventually show small amounts of p105, p120, and p160 after longer incubation times than those shown here). Interestingly, a lag of approximately 20 minutes was observed in the *sec1-1* mutant before detectable increases in p75 were visible. A similar lag was observed for another late acting mutant, *sec6-4* (data not shown). Since kinetic experiments of this kind have only been done for *sec18-1, sec1-1* and *sec6-4*, it is not known whether this lag is a characteristic of all late acting *sec* mutants or is simply an indication that the block in transport in the *sec1* and *sec6* mutants is not as rapid as in *sec18-1*.

We also examined whether perturbing protein transport in a wild-type strain by prolonged exposure to tunicamycin or DTT was sufficient to give rise to BAP137-reactive species in the immunoblotting assay. Tunicamycin or DTT was added to exponentially growing cultures of the wild-type strain CKY10 to give a final concentration of 5μg/ml or 5mM, respectively. Samples removed at intervals over a two hour period were analyzed by SDS-PAGE and immunoblotting with BAP137 antibodies. As seen in Figure 2B, after a lag of approximately one hour, BAP137-reactive species were detectable in samples from cultures treated with either compound, and the intensity and number of bands increased with time. Thus, perturbing protein transport either by mutation or by chemically treating cells causes the appearance of BAP137-detectable proteins on Western blots.

Purification and identification of p75 as Icwp

To clarify many questions about the nature of the protein(s) recognized by BAP137 antibodies, we purified the ~75 kDa species (p75) from a *bst1-1 ire1Δ* strain and

sequenced the protein. This double mutant showed higher steady state levels of the protein by Western blot than did *bst* single mutants (Figure 1A) and had comparable amounts to that seen with *sec* mutants shifted to restrictive temperatures for 1.5 hours. Cell fractionation studies showed that p75 (as well as the other BAP137-reactive species) behaves like an integral membrane protein: the protein was readily solubilized by treating extracts with detergent, but was not solubilized by treating with 0.5 M NaCl, 2.5 M urea, or carbonate buffer at pH 11.5 (Figure 3). Additional fractionation and immunofluoresence experiments suggest that the protein(s) localizes to the ER in *bst1* mutant strains (data not shown). We therefore spheroplasted approximately 45,000 OD_{600} units of cells, lysed them with glass beads, and collected 13,000***g*** membranes. The membranes were washed with urea and solubilized in 0.1% Triton X-100. The Triton soluble material was then taken through several denaturation steps (as described in the Materials and Methods) that caused 80 - 90% of the soluble protein to precipitate. The material that remained soluble was then passed over an anion-exchange column. p75 bound to the column and eluted at approximately 150 mM NaCl in a fraction with two or three minor contaminants of much different size (see Figure 4). The proteins in this fraction were then separated by SDS-PAGE. p75 (approximately 30 μg) was cut out of the gel and digested with Lys C, and the resulting peptides were HPLC purified for automated sequencing (Biopolymers laboratory, MIT).

The sequence of two such peptides (the bold sequences in Figure 5) mapped into an open reading frame (YLR391w-A) that encodes a 238 amino acid protein recently described as a mannoprotein of the inner cell wall called Icwp (Moukadiri et al., 1997). The protein sequence shows potential signals for translocation, for GPI-anchor attachment, and for addition of a single N-linked carbohydrate (Figure 5). In addition, 42% of the amino acids are either serine or threonine and results reported by Moukadiri et al. suggest that the protein is highly O-glycosylated. We find that the protein binds the lectin concanavalin A even after treatment with endo H (which removes N-linked

carbohydrates), confirming that the protein is an O-linked mannoprotein (data not shown).

To demonstrate that Icwp is indeed the protein (or one of the proteins) recognized by BAP137 antibodies, we cloned the gene into a high copy plasmid and introduced this construct (pME702) into both wild-type and *bst1-1* mutant strains by transformation. Extracts from these strains and from controls transformed with the vector alone were analyzed by SDS-PAGE and immunoblotting with BAP-137 antibodies. As seen in Figure 6, overexpression of Icwp from pME702 significantly increased the amount of the BAP137-reactive band of ~75 kDa, but not any of the other species, suggesting that this gene does indeed encode one of several proteins recognized by BAP137 antibodies.

bst1 mutants have defects in the transport of Gas1p

All three *bst* mutants show Kar2p and Pdi1p retention defects and inefficient transport of Icwp. *emp24/bst2* and *erv25/bst3* mutants have also been shown to have kinetic defects in the transport of invertase and Gas1p, but not α-factor, CPY, or acid phosphatase (Schimmöller et al., 1995; Belden and Barlowe, 1996; and Chapter 2). *bst1* mutants, on the other hand, do not show an appreciable defect in invertase transport (Elrod-Erickson and Kaiser, 1996 - Chapter 2; Gas1p was not examined). Because Icwp and Gas1p are both GPI-anchored proteins, we reasoned that the transport of GPI-anchored proteins may be particularly sensitive to loss of *BST* gene function. To determine if Gas1p transport is affected in *bst1* mutants as it is in *emp24/bst2* and *erv25/bst3* mutants, we examined the steady state distribution of the protein in *bst1-1* mutant strains. Gas1p is produced in the ER as a 105 kDa protein with both N- an O-linked carbohydrates. These oligosaccharides are extended as the protein moves through the Golgi, giving rise to the 125 kDa mature form of Gas1p (Nuoffer et al., 1991). In wild-type cells the vast majority of the protein is found in the mature form. As seen in Figure 7, a significant fraction of the protein is found in the ER form in a *bst1-1* mutant strain, demonstrating that Gas1p is

inefficiently transported in this mutant. Thus, the only known secretory proteins whose transport is impaired in *bst1-1* mutant strains are Gas1p and Icwp, both of which are GPI-anchored proteins.

DISCUSSION

As the analysis of protein transport through the secretory pathway becomes more detailed, it will become increasingly important to use a variety of marker proteins to assess the role of new functions. To date, most of the tests of secretory pathway function in the yeast *Saccharomyces cerevisiae* have utilized three soluble proteins -- the secreted pheromone α-factor, the secreted enzyme invertase, and the vacuolar protease carboxypeptidase Y (CPY) -- as markers for secretory protein transport. As soluble proteins, these markers represent a minor class of secretory cargo molecules, the majority of which are integral membrane proteins (Kaiser et al., 1997). Major differences in the transport of membrane proteins and soluble proteins are known, indicating that the use of soluble proteins as markers for protein transport is insufficient. For instance, transport to the cell surface appears to be the default pathway for soluble proteins, whereas transport to the vacuole seems to be the default pathway for membrane proteins in yeast (Nothwehr and Stevens, 1994). Thus, while the current marker proteins have proven sufficient for the identification and characterization of much of the basic machinery of the secretory pathway, additional markers may prove to be crucial for the discovery and examination of novel functions. In addition, increasingly sensitive assays for secretory defects are likely to be needed for the investigation of factors involved in efficiency or fidelity functions within the pathway, because mutants with defects in such processes may have too subtle a phenotype for easy detection using current assays.

Another argument for increasing the number of proteins used to assess transport through the secretory pathway is the fact that different classes or families of proteins appear to have different requirements for efficient transport. For example, amino acid permeases require Shr3p for efficient transport (Ljungdahl et al., 1992; Kuehn et al., 1996), and GPI-anchored proteins seem to have special lipid requirements for efficient transport from the ER in yeast (Horvath et al., 1994; Skrzypek et al., 1997). Unless the

transport of a number of different marker proteins is assessed, it will be difficult to generate an accurate picture of the role of a given protein in secretory protein transport.

In this report we describe an extremely sensitive assay for detecting perturbations in secretory pathway function. Western analysis of whole cell extracts using BAP137 antibodies detects the intracellular form of Icwp, a glucanase-extractable mannoprotein of the inner cell wall. Because this protein is rapidly incorporated into the wall, where it is insoluble and undetectable, the steady-state detectable fraction of Icwp is very small (wild-type in Figure 1). Mutants or treatments that perturb the transport of the protein to the cell surface can therefore be identified because they show a dramatic increase in the amount of detectable Icwp (Figures 1 and 2). We have demonstrated that this assay identifies *sec* mutants that block at all transport steps between the ER and cell surface (Figure 1), and that it is sensitive enough to reveal very subtle defects that are difficult to detect using other common transport assays. Thus, Icwp should prove to be a valuable tool for further exploration of the details of secretory pathway function.

Implications for the function of the BST genes

bst mutants have an obvious defect in the transport of Icwp. The steady-state levels of detectable Icwp in *bst* mutant strains (at all temperatures) is comparable to the amount that accumulates in a *sec18-1* mutant that has been held at restrictive temperatures for 20-30 minutes (our unpublished observations). This defect is clearly selective, because the transport of CPY (and most other secretory proteins) is completely normal in these mutants (Elrod-Erickson and Kaiser, 1996 - Chapter 2). Gas1p transport is also defective in *bst* mutant strains (Schimoller et al., 1995; Belden and Barlowe, 1996; and Figure 7). Thus, it appears that the transport of GPI-anchored proteins in general may be particularly sensitive to loss of *BST* gene function. Several factors are already known to be important for the transport of GPI-anchored proteins (as discussed in Chapter 1). Mutants that affect the assembly or attachment of the GPI-anchor precursor cause the

accumulation of unanchored proteins in the ER (reviewed in Orleans, 1997). *bst* mutants are unlikely to affect either of these processes, since the Icwp that accumulates in (and was purified from) a *bst1* mutant contains an intact anchor based on its Triton X-114 partitioning behavior (our unpublished observations). The transport of GPI-anchored proteins from the ER is also dependent upon sphingolipid biosynthesis in a manner that is not entirely understood (Horvath et al., 1994; Skrzypek et al., 1997). Although it is unlikely that *bst* mutations affect the biosynthesis of sphingolipids, it is possible that the *BST* proteins could locally affect the lipids in the ER membrane, since Bst1p contains a motif characteristic of lipases and acyltransferases (Chapter 2 - Appendix). Although clearly speculative, a function for the *BST* proteins in locally altering the lipid composition of the membrane could help explain how these mutant show selective defects in the transport of secretory proteins and defects in the retention of resident ER proteins. The modification of lipids in the vicinity of a forming vesicle could be important in helping to partition some proteins into the vesicle and resident proteins out of the vesicle. If the lipid composition of the membrane (or the consequences of proper sorting) also influenced the assembly or stability of the vesicle coat, such a function for the *BST* proteins could also explain the effects of *bst* mutations on COPII vesicle formation. (These ideas are elaborated in the next chapter.) We now have the tools in hand that should allow us to test this model.

ACKNOWLEDGEMENTS

We would like to thank Monicia Elrod-Erickson for technical advice on protein purification, the Pabo Lab for the use of their FPLC and MonoQ column, and the members of the Kaiser lab for helpful discussions during the course of this work. We also thank R. Ram, and H. Riezman for gifts of strains and antibodies.

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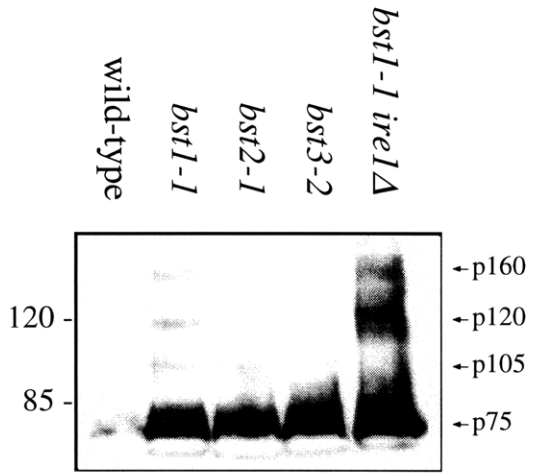
Table 1. Strain List

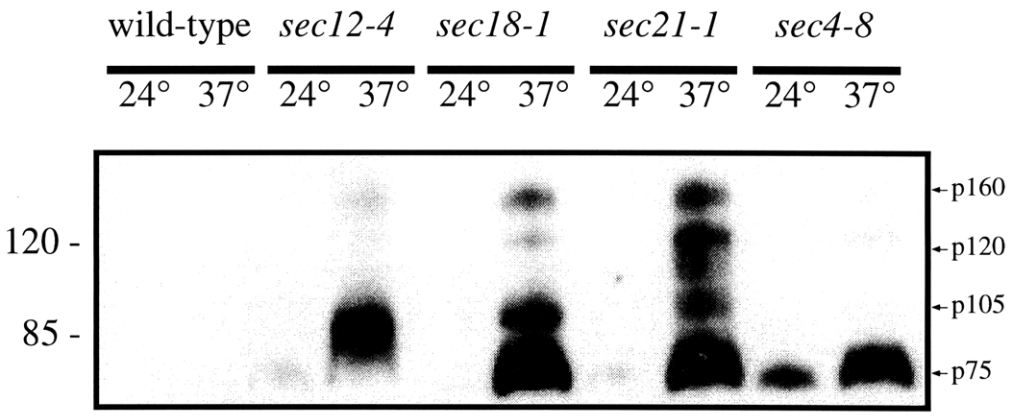
Strain	Genotype	Source
CKY10	*MATa ura3-52 leu2-3,112*	Kaiser lab collection
CKY40	*MATa sec12-4 ura3-52*	Kaiser lab collection
CKY46	*MATa sec13-1 ura3-52 his4-619*	Kaiser lab collection
CKY53	*MATa sec16-1 ura3-52 leu2-3,112*	Kaiser lab collection
CKY55	*MATa sec17-1 ura3-52 his4-619*	Kaiser lab collection
CKY59	*MATa sec18-1 ura3-52 his4-619*	Kaiser lab collection
CKY62	*MATα sec19-1 ura3-52 his4-619*	Kaiser lab collection
CKY64	*MATα sec20-1 ura3-52 his4-619*	Kaiser lab collection
CKY69	*MATa sec21-1 ura3-52 his4-619*	Kaiser lab collection
CKY70	*MATα sec22-3 ura3-52 his4-619*	Kaiser lab collection
CKY79	*MATa sec23-1 ura3-52 leu2-3,112*	Kaiser lab collection
CKY100	*MATa sec27-1 ura3-52 leu2-3,112*	Kaiser lab collection
CKY162	*MATa sec1-1 ura3-52*	Kaiser lab collection
CKY164	*MATa sec6-4 ura3-52*	Kaiser lab collection
CKY165	*MATa sec10-2 ura3-52*	Kaiser lab collection
CKY166	*MATa sec15-1 ura3-52*	Kaiser lab collection
CKY169	*MATa sec4-8 ura3-52*	Kaiser lab collection
CKY170	*MATa sec8-9 ura3-52*	Kaiser lab collection
CKY251	*MATα sed4-Δ1 ura3-52 leu2-3,112*	Kaiser lab collection
CKY330	*MATα bst1-1 ura3-52 leu2-3,112*	Kaiser lab collection
CKY332	*MATα bst2-1 ura3-52 leu2-3,112*	Kaiser lab collection
CKY334	*MATα bst3-2 ura3-52 leu2-3112 ade2 ade3*	Kaiser lab collection
CKY499	*MATα iss1-Δ1::TRP1 ura3-52 leu2-Δ1 his3-d200 ade2-101 trp1-d63*	Kaiser lab collection
CKY536	*MATa lst1::LEU2 leu2-3,112*	Kaiser lab collection
RRY315	*MATa bst1-1 ire1Δ::URA3 ura3-52 leu2-3,112*	R. Ram, Kaiser lab

Figure 1. Immunoblotting with BAP137 antibodies identifies mutants with defects in protein transport. (A) Extracts from wild-type (CKY10), *bst1-1* (CKY330), *bst2-1* (CKY332), *bst3-2* (CKY334), and *bst1-1 ire1Δ* (RRY315) strains grown at 30°C. (B) Extracts from wild-type (CKY10), *sec12-4* (CKY40), *sec18-1* (CKY59), *sec21-1* (CKY69), and *sec4-8* (CKY169) strains grown at 24°C, or at 24°C and then shifted to 37°C for 1.5 hours. (C) Extracts from *sec18-1* (CKY59), *iss1Δ* (CKY499), *sed4Δ* (CKY251), and *lst1Δ* (CKY536) strains grown at 24°C, or at 24°C and then shifted to 37°C for 1.5 hours.

A


wild-type
bst1-1
bst2-1
bst3-2
bst1-1 ire1Δ
120 -
85 -
←p160
←p120
←p105
←p75

B


wild-type
sec12-4
sec18-1
sec21-1
sec4-8
24° 37° 24° 37° 24° 37° 24° 37° 24° 37°
120 -
85 -
←p160
←p120
←p105
←p75

C

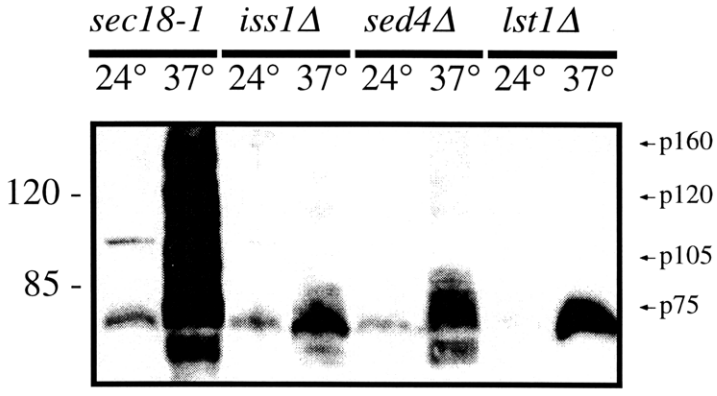
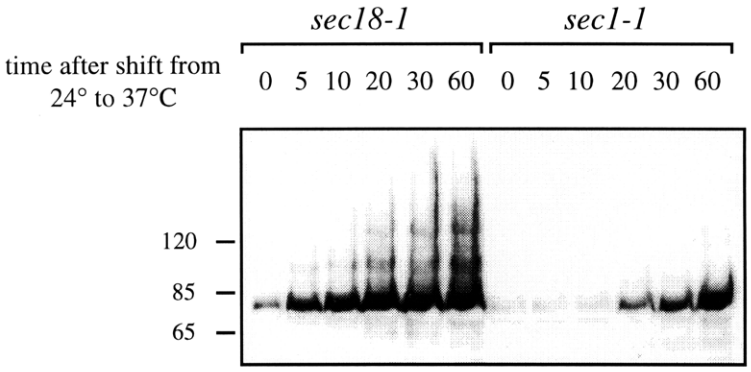

sec18-1
iss1Δ
sed4Δ
lst1Δ
24° 37° 24° 37° 24° 37° 24° 37°
120 -
85 -
←p160
←p120
←p105
←p75

Figure 2. Time course experiments. (A) Multiple BAP137-reactive species accumulate upon prolonged incubation at restrictive temperatures. Extracts from *sec18-1* (CKY59) and *sec1-1* (CKY162) were prepared at the indicated times after shifting the growth temperature form 24° to 37°C and analyzed by immunoblotting with BAP137 antibodies. (B) Extracts from the wild-type strain CKY10 were prepared at the indicated times after addition of either tunicamycin or DTT and analyzed by immunoblotting with BAP137 antibodies.

A


sec18-1
sec1-1
time after shift from 24° to 37°C
0 5 10 20 30 60 0 5 10 20 30 60
120
85
65

B

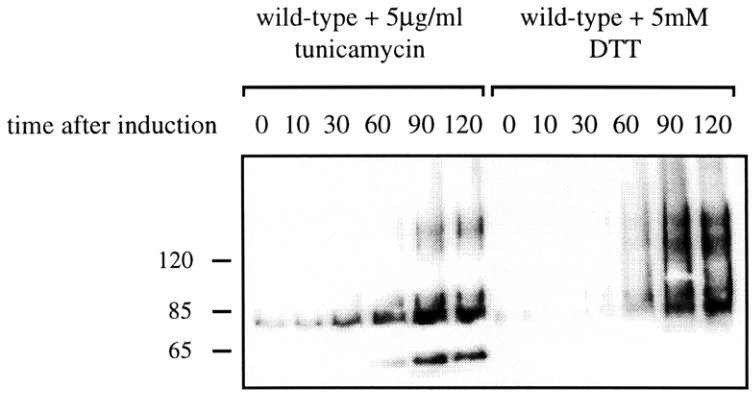
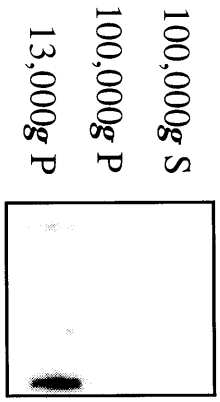

wild-type + 5µg/ml tunicamycin
wild-type + 5mM DTT
time after induction
0 10 30 60 90 120 0 10 30 60 90 120
120
85
65

Figure 3. Cell fractionation of BAP137-reactive material. Fractionation was carried out as described (Espenshade et al., 1995). (A) Differential centrifugation of cell lysates from the *bst1-1* mutant strain CKY330. (B) Cell lysates were incubated on ice in the presence of 0.5M NaCl, 2.5M urea, carbonate buffer at pH 11, or 1% Triton for one hour and then separated into pellet (P) and supernatant (S) fractions by centrifugation at 150,000***g***.

A


13,000g P
100,000g P
100,000g S

B

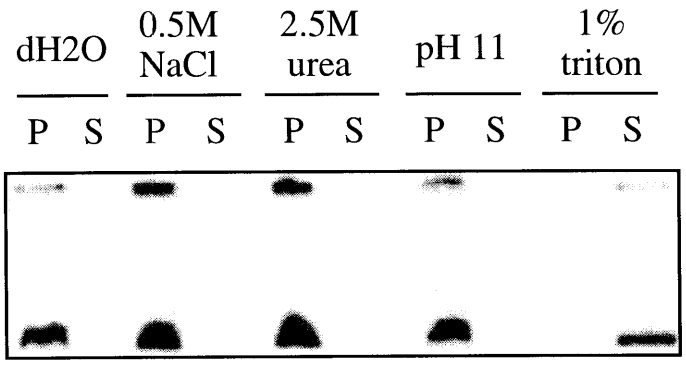
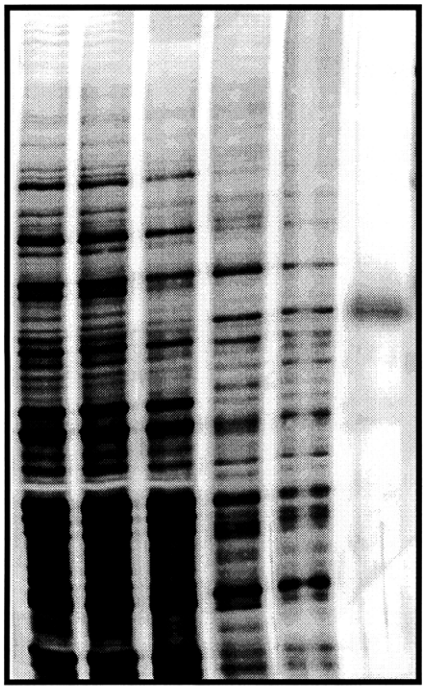

dH2O
0.5M NaCl
2.5M urea
pH 11
1% triton
P S P S P S P S P S

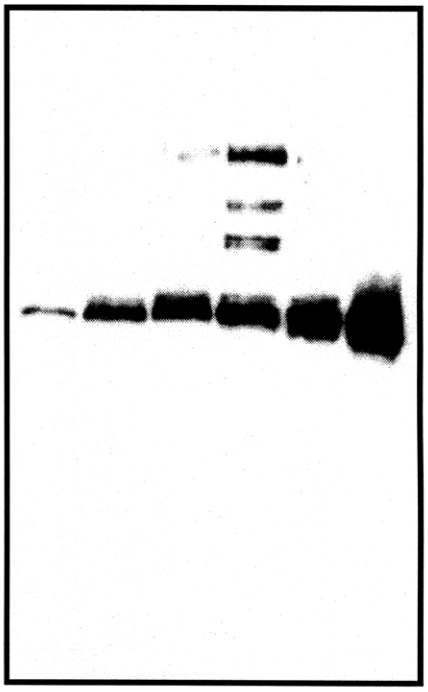
Figure 4. p75/Icwp purification. Fractions from various steps in the purification were analyzed by silver staining (A) and immunoblotting with BAP137 antibodies (B). Lanes are as follows: 1) whole cell lysate, 2) spheroplast lysate, 3) 13,000*g* membranes, 4) Triton soluble material from 13,000*g* membranes, 5) loaded on MonoQ column, 6) pure Icwp from 150 mM NaCl fraction. Lanes 1-4 of the silver stained gel are at 1/8 the concentration of the equivalent lanes from the Western blot.

A



silver stained gel

B



Western blot

Figure 5. Analysis of the amino acid sequence of Icwp. (A) The predicted sequence of Icwp. In bold are the peptide sequences from purified p75 that identified the protein as Icwp. The open arrow marks the putative signal sequence cleavage site. The shaded arrow marks the putative site for GPI-anchor attachment. (*) indicates the single potential N-linked glycosylation site in the protein. (B) Hydropathy plot (Kyte - Doolittle algorithm, with a window of 11 amino acids) showing the hydrophobic N- and C-terminal regions that are the putative signals for translocation and GPI-anchor attachment, respectively.

A

```
                                    ⇩
1   MRATTLLSSVVSLALLSKEVLATPPACLLACVAQVGKSSSTCDSLNQVTC   50
                                                 *
51  YCEHENSAVKKCLDSICPNNDADAAYSAFKSSCSEQNASLGDSSSSASSS  100

101 ASSSSKASSSTKASSSSASSSTKASSSSASSSTKASSSSAAPSSSKASST  150

151 ESSSSSSSSTKAPSSEESSSTYVSSSKQASSTSEAHSSSAASSTVSQETV  200

201 SSALPTSTAVISTFSEGSGNVLEAGKSVFIAAVAAMLI               238
                    ⇧
```

B

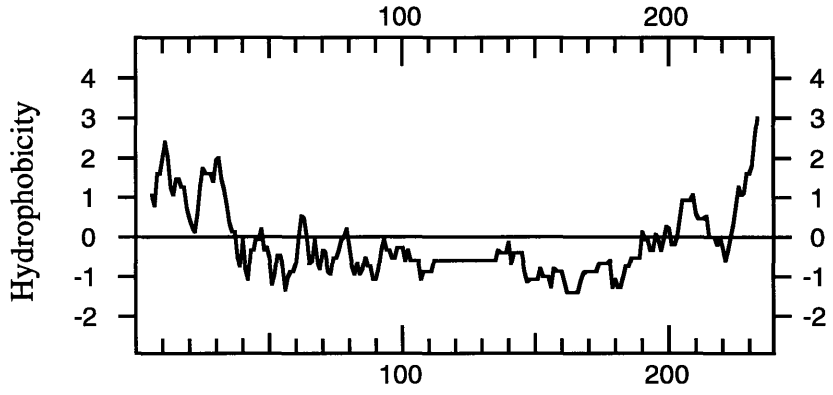


Figure 6. *ICWP* encodes p75. Extracts from cultures of wild-type (CKY10) and *bst1-1* (CKY330) strains containing either the empty vector pRS306-2μ or pME702 (2μ-*ICWP*) were analyzed by immunoblotting with BAP137 antibodies.

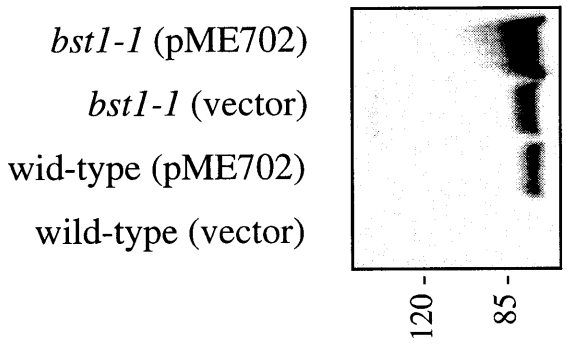
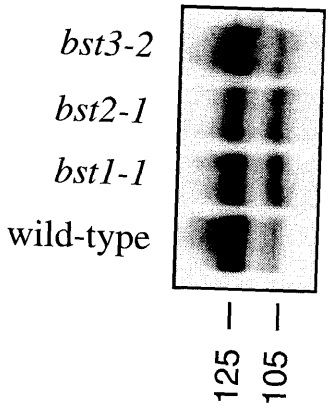

bst1-1 (pME702)
bst1-1 (vector)
wid-type (pME702)
wild-type (vector)
120 -
85 -

Figure 7. *bst1-1* is defective in the transport of Gas1p. Extracts from cultures of wild-type (CKY10), *bst1-1* (CKY330), *bst2-1*(CKY332), and *bst3-2* (CKY334) mutant strains grown at 30°C were analyzed by immunoblotting with anti-Gas1p antibodies.


bst3-2
bst2-1
bst1-1
wild-type
125
105

Chapter Five:

Future directions for the analysis of the role of the *BST* proteins in cargo sorting and COPII vesicle formation

Summary and model

The focus of the work described in this thesis has been to characterize the role of the *BST* genes in COPII vesicle formation and protein sorting. The isolation of the *BST* genes as bypass suppressors of *SEC13* (Chapter 2) and the demonstration that COPII vesicle budding *in vitro* does not require Sec13p (Chapter 3), indicate that Sec13p is not absolutely required for COPII vesicle formation. Sec13p is, however, essential *in vivo* at temperatures above 30°C and at lower temperatures when the *BST* genes are functional (Chapter 2), suggesting that Sec13p may be necessary for maximal stability of the COPII veisle coat and that both higher temperatures and the activity of the *BST* proteins destabilize the coat.

Sec13p has also been implicated in protein transport between the Golgi and the plasma membrane (Roberg et al., 1997). *sec13Δ bst* mutants grow almost as well as wild-type strains at 25°C, suggesting either that the *bst* mutations also bypass the function of Sec13p involved in transport later in the pathway or that this function is not essential for vegetative growth. The latter model may be correct. Only about 50% of *sec13Δ bst* mutant spores germinate, and suppressed null strains rapidly lose viability if allowed to grow to saturation (our unpublished observations). *bst* mutant strains show neither of these defects, suggesting that Sec13p is necessary for the significant "remodeling" of cell surface proteins that occurs as cells enter and exit stationary phase and that the *bst* mutations do not affect this function of Sec13p.

The *BST* proteins are also clearly important for efficient discrimination between cargo molecules and resident ER (or ER retained) proteins during sorting into COPII-coated vesicles. *bst* mutations slow the transport of a subset of secretory proteins: the transport of Gas1p and Icwp is affected in *bst1* mutants (Chapters 2 and 4), whereas the transport of Gas1p, Icwp, and invertase is affected in *emp24/bst2* and *erv25/bst3* mutants (Chapter 2; Schimmöller et al., 1995; Belden and Barlowe, 1996; and Chapter 4). The

fact that Gas1p and Icwp are both GPI-anchored proteins suggests that the *BST* proteins are particularly important for the efficient transport of GPI-proteins. All three *bst* mutants also show defects in the retention of resident ER and ER retained proteins (Kar2p, Pdi1p, an s11-invertase; Chapter 2). The selective transport defects of *emp24/bst2* and *erv25/bst3* mutants and the observation that Emp24p/Bst2p and Erv25p/Bst3p are major components of COPII vesicles (Schimmöller et al., 1995; Belden and Barlowe, 1996) have led to the idea that these two proteins (or perhaps all members of the p24 protein family) act as sorting receptors. However, the fact that *bst* mutants also show defects in retention of resident proteins and the observation that *bst1* mutants show nearly identical sorting defects even though Bst1p is not a component of COPII vesicles suggest that the *BST* proteins must play a more general role in protein sorting during COPII vesicle formation.

Bst1p and its *C. elegans* homolog both have a motif that is characteristic of lipases. Two observations make this fact potentially important. First, changing the active site serine in this motif to alanine disrupts *BST1* function as judged by the fact that the mutant protein expressed from a plasmid no longer complements the *sec13Δ* suppression or the Kar2p retention defect of a *bst1-1* mutant strain (our unpublished observations). Second, the transport of GPI-linked proteins appears to be particularly sensitive to mutations in *BST* genes, and it is clear that lipids can influence the transport of GPI-anchored proteins.

A model incorporating all of the above general conclusions and observations can be generated. The function of the *BST* proteins in this model is to modify the lipid composition of the membrane in regions of vesicle assembly, which then facilitates the sorting of resident proteins from cargo molecules and indirectly influences the assembly of the COPII vesicle coat.

The recruitment and assembly of the COPII vesicle coat proteins on the ER membrane drives the formation of transport vesicles. Integral membrane components of the vesicle, including Emp24p/Bst2p and Erv25p/Bst3p, are recruited to sites of coat

assembly -- or recruit the coat to regions of the membrane where they reside -- through the interaction of their cytosolically exposed domains with vesicle coat proteins. This interaction thereby localizes a complex containing Bst1p, Emp24p/Bst2p and Erv25p/Bst3p to sites of vesicle assembly. As coat assembly begins, Bst1p is released from the *BST* complex and can then locally modify membrane lipids. This local change in lipid composition helps to partition membrane protein cargo into the region of vesicle budding and resident membrane proteins out of these regions. Soluble cargo is concentrated through interactions with sorting receptors or membrane protein cargo, and resident proteins are excluded through interactions with resident membrane proteins. The local change in lipid composition and subsequent sorting also increases the requirement for stability in the assembling vesicle coat.

Thus, in the absence of any of the *BST* proteins the localized change in lipid composition does not occur. Sorting is less efficient, resulting in a decreased rate of transport for cargo molecules sensitive to the change in lipid composition and an increased rate of transport of resident proteins. However, there is less need for stability in the coat, allowing COPII vesicles to form in the absence of Sec13p or at higher temperatures in strains with conditional mutations in other COPII coat proteins.

Future directions

The work described in this thesis has allowed the model outlined above to be generated. The task at hand is to test the various aspects of this model so that it can be refined. Some aspects of the model are clearly more speculative than others and should therefore be addressed first. The remainder of this chapter will be spent providing suggestions for how to test the model.

The most important aspect of the model to address is whether Bst1p can indeed act to modify lipids in the membrane. The fact that a serine to alanine change in the conserved active site of the lipase motif in *BST1* appears to disrupt function is promising, but a

direct demonstration of lipolytic activity would significantly strengthen arguments for this part of our model. We have constructed (or are constructing) various GST-BST1 fusion constructs, containing either the wild-type *BST1* gene or the serine to alanine mutant, that should allow the purification of Bst1p for use in assaying lipolytic activity. A variety of assays have been described, some of which may be general enough to prove useful. The use of the serine to alanine mutant protein will enable us to correlate *in vitro* activity and *in vivo* function.

An additional approach to addressing the role of lipids in protein sorting and COPII vesicle formation would be to alter the lipid composition in cells by feeding them excess lipids of various types or by using lipid biosynthesis mutants. Protein sorting could be easily assessed using the Kar2p secretion assay described in Chapter 2. The growth of *sec13Δ bst* mutant strains could be used to assess the effect on COPII vesicle formation. Interestingly, one of the weak suppressors of *sec13Δ* alluded to in Chapter 2 was identified as an *ada5* mutant. In addition to a number of other phenotypes, *ada5* mutants are inositol auxotrophs due to a failure to transcribe the *INO1* gene. The ability of *ino1* mutants to suppress *SEC13* is currently being investigated. Furthermore, acidic phospholipids have been recently implicated in the binding of the COPII coat to ER membranes *in vitro* (Matsuoka et al., 1998), supporting the idea that the lipid environment influences coat assembly.

Another prediction of the model is that Bst1p should be localized in the vicinity of budding vesicles and should physically interact with Emp24p/Bst2p and Erv25p/Bst3p. While electron microscopy of intact cells rarely captures a budding vesicle in *Saccharomyces cerevisiae*, the analysis of *in vitro* vesicle budding from isolated nuclei by immuno-electron microscopy has been used to demonstrate that COPII proteins localize to budding vesicles (Bednarek et al., 1995). A similar approach could be used to address whether Bst1p is concentrated in the vicinity of budding vesicles in the ER membrane. Two-hybrid experiments using the luminal domains of the *BST* proteins and/or co-

immunoprecipitation experiments could be used to address whether Bst1p physically interacts with either Emp24p/Bst2p or Erv25p/Bst3p.

The notion that *bst* mutations affect the transport of GPI-anchored proteins could be strengthened by demonstrating that the transport of additional GPI-anchored proteins is affected by *bst* mutations. One general approach to addressing this issue involves the specific radiolabelling of GPI-anchored proteins using [H^3] myo-inositol. After the separation of proteins by SDS-PAGE, the amount of label in proteins of different sizes is determined. Most GPI-anchored proteins are highly glycosylated species of very large apparent molecular weight. Blocking the transport of GPI-proteins from the ER (using a *sec* mutant) causes the bulk of the radiolabeled protein to shift from high molecular weight fractions to lower molecular weight fractions (Horvath et al., 1994). This type of analysis could reveal whether the bulk of GPI-proteins are delayed in the ER in *bst* mutant strains. In addition, the transport of GPI-anchored proteins is particularly sensitive to inositol starvation in an *ino1Δ* background (Doering and Schekman, 1996). Preliminary results suggest that *bst1 ino1* mutants are more sensitive to inositol starvation than *ino1* single mutants. *bst1* mutants also appear to be more sensitive to the cell wall destabilizing compound calcofluor white. Both of these observations are consistent with a role for Bst1p in the transport of GPI-anchored proteins.

These are just some of the interesting possibilities for the further investigation of the role the *BST* proteins play in secretory protein transport and COPII vesicle formation.

ACKNOWLEDGMENTS

I would like to thank Monicia Elrod-Erickson for comments on drafts of this thesis.

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