Control of Golgi Morphology and Function by Sed5 t-SNARE Phosphorylation

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Previously, we demonstrated that the phosphorylation of t-SNAREs by protein kinase A (PKA) affects their ability to participate in SNARE complexes and to confer endocytosis and exocytosis in yeast. Here, we show that the presumed phosphorylation of a conserved membrane-proximal PKA consensus site (serine-317) in the Sed5 t-SNARE regulates endoplasmic reticulum (ER)-Golgi transport, as well as Golgi morphology. Sed5 is a phosphoprotein, and both alanine and aspartate substitutions in serine-317 directly affect intracellular protein trafficking. The aspartate substitution results in elaboration of the ER, defects in Golgi-ER retrograde transport, an accumulation of small transport vesicles, and the inhibition of growth of most cell types. In contrast, the alanine substitution has no deleterious effects upon transport and growth, but results in ordering of the Golgi into a structure reminiscent of mammalian apparatus. This structure seems to require the recycling of Sed5, because it was found not to occur in sec21-2 cells that are defective in retrograde transport. Thus, a cycle of Sed5 phosphorylation and dephosphorylation is required for normal t-SNARE function and may choreograph Golgi ordering and dispersal.

INTRODUCTION

The secretory pathway in eukaryotes consists of distinct membrane-bound intracellular compartments that transfer proteins and lipids in bulk from one to the other. This occurs via transport structures that undergo fusion with subsequent compartments. Because these compartments are in a constant state of flux, due to the continuous exchange of membrane and protein, obligate protein sorting and retrieval mechanisms have evolved to maintain organelle identity and integrity. Moreover, there is a specific need for recreating intact and functional organelles after mitosis, via organelle inheritance and/or biogenesis. The Golgi apparatus consists of well-defined subcompartments (cisternae) arranged in an ordered structure in higher eukaryotes, called the Golgi stack, which displays an asymmetric distribution of specific processing enzymes (reviewed in Farquhar and Palade, 1998). Cargo molecules traverse the stack via the process of cisternal maturation, which involves the retrograde flow of enzymes and transport factors back to earlier compartments while allowing the cargo to proceed (reviewed in Pelham, 1998; Pelham and Rothman, 2000). This results in steady-state distribution of the protein processing and delivery factors while allowing cargo to advance and eventually exit the Golgi via transport vesicles.

The fusion of membranes along the secretory pathway involves SNAREs, which comprise three main families of conserved membrane-associated proteins (the VAMP/syntaptoptobrevin, syntaxin, and SNAP-25/light chain families) that mediate vesicle docking and fusion (reviewed in Chen and Scheller, 2001). Conventionally, these families fall into two categories, the v-SNAREs that reside on vesicles and t-SNAREs that reside on the acceptor compartments. Both v- and t-SNAREs assemble into a four-helix bundle that bridges apposed membranes and leads to membrane fusion (reviewed in Chen and Scheller, 2001). The v-SNARE contributes one α-helix to the SNARE complex, whereas three are contributed by the t-SNAREs. Notably, every intracellular trafficking step has one syntaxin t-SNARE that serves as an essential element of the fusion complex. In yeast, the syntaxin family member, Sed5, plays an essential role in protein transport from the endoplasmic reticulum (ER) to the Golgi as well as intra-Golgi transport (Hardwick and Pelham, 1992, Nichols and Pelham, 1998). Sed5 forms functional SNARE complexes with Sec22, Bet1, and Bos1 (Sogaard et al., 1994; Parlati et al., 2002) to mediate ER–Golgi transport and with other SNAREs, such as Sft1, Ykt6, Gos1, and Vti1 to mediate intra-Golgi and endosome-Golgi transport (reviewed by Pelham, 1999). Attempts to systematically define the number of possible Sed5-containing SNARE complexes revealed that it is promiscuous and forms numerous complexes in vitro (Tsui et al., 2001); however, only two have been shown to be functional using an in vitro fusion assay (Parlati et al., 2002). Nevertheless, the large number of complexes that Sed5 forms in vitro may reflect its importance in the maintenance of Golgi structure and function.

Sed5 localizes to the cis-Golgi (Hardwick and Pelham 1992) and cycles through the ER (Wooding and Pelham, 1998). However, it is not known how Sed5 is retained at steady state in the Golgi and what physiological purpose is served by recycling. In an attempt to define its retention signal, mutations were made in the transmembrane domain (TMD), which is known to play a role in the retention of other SNAREs (Lewis et al., 2000). However, the Sed5 local-
zation signal is only partially determined by its TMD (Banfield et al., 1994), and thus an additional localization/retention mechanism exists. In addition to regulating transport to the Golgi, Sed5 and its orthologues play an important role in Golgi maintenance and structure. The loss of Sed5 function is characterized by the accumulation of small transport vesicles and an elaboration of ER membranes concomitant with a decrease in protein transport and cell viability (Hardwick and Pelham, 1992). ER expansion is indicative of a block in retrograde transport, suggesting a role for Sed5 recycling in Golgi function and structure. In particular, it may allow for the Golgi to cycle between ordered and dispersed states, the latter being important for Golgi inheritance during mitosis (Shorter and Warren, 2002).

**MATERIALS AND METHODS**

**Growth Tests**

Yeast were grown in standard amino acid-rich medium (YPD) or selective synthetic media (SC).

**Drop Tests.** Cells were grown to log phase in liquid selective medium. Next, cells were diluted to 10⁶ cells/mL, followed by five serial dilutions of 10-fold each. Aliquots of the dilutions were applied as drops onto solid media, which were then incubated at various temperatures.

**Yeast Strains**

Strains are listed in Table 1.

**Plasmids**

Constructs for SED5 Expression. SED5 was amplified by PCR using genomic DNA as a template and primers encoding Sall and SacI sites at the 5' and 3' ends, respectively. The Sall-SacI fragment was inserted in-frame and downstream to the hemagglutinin (HA) epitope encoded vector pAD54 (2×HA-HA). Next, pADHU-HASED5. SED5 is under the control of the constitutive ADH1 promoter. Point mutations were made in SED5 to create the alanine-317 and aspartate-317 substitutions, by PCR-based site-directed mutagenesis with PhaI polymerase (Stratagene, LaJolla, CA). Multicopy plasmids bearing either LEU2 or URA3 and single-copy plasmids bearing either LEU2 or TRP1, all of which express SED5, SED5S317A, or SED5S317D were created. A gene encoding green fluorescent protein (GFP) was introduced in-frame and upstream to SED5 by subcloning a Sall GFP fragment into the Sall site of the LEU2 multicopy plasmids. This created HA-GFP fusions with SED5, SED5S317A, or SED5S317D. Next, the latter being important for Golgi inheritance during mitosis (Shorter and Warren, 2002).

**Table 1. Yeast strains**

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EUROSCARF, European Saccharomyces cerevisiae archive for functional analysis.
region of the Schizosaccharomyces pombe his5+ gene and 3′ untranslated region of SED5. PCR amplified fragments were used to transform SP1 wild-type cells and were selected for on medium lacking histidine. Integration at the SED5 locus was verified by PCR. A plasmid expressing Sec22-myc-c, pWB-GalAn, was provided by H-D Schmidt (University of Gottingen, Germany).

**Metabolic Labeling In Vivo**

**Protein Phosphorylation In Vivo.** Proteins were metabolically labeled in vivo either with [32P]orthophosphate or [33P]orthophosphate (0.25 and 0.65 mCi/10 O.D.600 U, respectively; GE Healthcare, Piscataway, NJ), essentially as described previously (Marash and Gerst, 2001).

**Pulse-Chase Analysis.** Intracellular protein processing was monitored by pulse-chase analysis using [35S]methionine (GE Healthcare, as described previously (Couve et al., 1995).

**Immunoprecipitation and Subcellular Fractionation**

**Immunoprecipitation.** Immunoprecipitation from lysates was performed as described previously (Marash and Gerst, 2001). When performed in sec5-1 lysates, the cell pellet was first washed with 10 mM NaF, and both 20 mM NaF and 1 mM N-ethylmaleimide were added to the lysis buffer. For the detection of phosphorylated Sed5 in Westerns or by autoradiography, the lysis buffer was supplemented with the following phosphate inhibitors: 10 mM NaF, 20 mM NaPPi, 25 mM β-glycerophosphate, and 0.5 mM sodium vanadate. Proteins were detected in immunoblots by chemiluminescence.

**Subcellular Fractionation.** Yeast were subjected to subcellular fractionation, as described previously (Lustgarten and Gerst, 1999).

**Antibodies**

Monoclonal anti-HA antibodies (gift of M. Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) were used for both immunoprecipitation (IP) (1 μl) and detection (1:5000). Protein detection in blots was performed using monoclonal antibodies to 1.1000; Santa Cruz Biotecnology, Santa Cruz, CA) and polyclonal anti-phosphoserine (1:1000; Zymed Laboratories, South San Francisco, CA), anti-Sed5 (1:3000; gift of H. Pelham, MRC Laboratory for Molecular Biology, Cambridge, United Kingdom), anti-Bos1 (1:500; gift of C. Barlowe, Dartmouth Medical School, Hanover, NH), anti-Sec22 (1:3000; gift of S. Ferro-Novick, Yale University, New Haven, CT), anti-Vt1 (1:3000; gift of G. Fischer von Mollard, University of Gottingen); anti-Emp47 (1:3000; gift of H. Riezman, University of Geneva, Switzerland), anti-Opn1 (1:1000; Molecular Probes, Eugene, OR), and anti-Mnn1 antibodies (1:2000; gift of S. Emr, University of San Diego, San Diego, CA). IP antibodies for pulse-chase experiments included anti-Gas1 (gift of H. Riezman) and anti-CPY (gift of S. Emr).

**RESULTS**

**Sed5 Is Phosphorylated In Vivo**

PKA is involved in the regulation of the Sso and Tlg t-SNAREs, which confer exo- and endocytic transport in yeast, respectively (Marash and Gerst, 2001; Gurunathan et al., 2002). In the case of the Sso t-SNAREs, phosphorylation inhibits t-SNARE assembly and recruits a SNARE regulatory protein, Vsm1, to the autoinhibitory domain of Sso (Marash and Gerst, 2001; Marash and Gerst, 2003). Correspondingly, activation of a ceramide-activated protein phosphatase (CAPP) reduces Vsm1 binding, restores t-SNARE assembly, and confers exocytosis (Marash and Gerst, 2001; Marash and Gerst, 2003). Because phosphorylation and dephosphorylation play a significant role in t-SNARE regulation on the late secretory pathway, we examined whether ER–Golgi transport is controlled in a similar manner.

We examined whether temperature-sensitive mutations in t-SNARES functioning in the early pathway might be rescued for growth at restrictive temperatures by the exogenous addition of C2-ceramide, a CAPP activator, to the medium. We found that a temperature-sensitive mutation in SED5 (e.g., sed5-1), which encodes an essential Golgi t-SNARE of the syntaxin family, was partially rescued, whereas mutations in other SNAReS involved in ER–Golgi transport (i.e., bos1-1, bet1-1, and sec22-1) were either not affected or were inhibited (our unpublished data). Thus, Sed5 might be subject to regulation by phosphorylation.

We noticed two putative PKA phosphorylation sites present in Sed5: serine-8 and serine-317. The NH2-terminal phosphorylation site (serine-8) resides proximal to the Habc domain (Figure 1A), which has a suggested regulatory role (Yamaguchi et al., 2002). However, alignments of Sed5 orthologues revealed that only the COOH-terminal phosphorylation site (serine-317) is highly conserved from yeast to humans (Figure 1B). This site lies in the region directly downstream of the SNARE domain (residues 249–311) and proximal to the TMD (residues 325–339; Hardwick and Pelham, 1992) (Figure 1A).

To demonstrate whether Sed5 is a phosphoprotein, we first expressed a HA epitope-tagged form of Sed5 in yeast, performed in vivo labeling with [32P]orthophosphate, and immunoprecipitated the protein. We examined Sed5 phosphorylation in vivo using a number of strains (e.g., WT, sec18-1, bos1-1). In particular, we found significant incorporation of labeled phosphate into HA-Sed5 in bos1-1 cells, which bear a temperature-sensitive t-SNARE involved in ER–Golgi transport (Figure 1C). We could also demonstrate phosphorylation on serine residues using an antibody specific to phosphoserine (Figure 1D). In both experiments, a single band was observed corresponding to the molecular mass of HA-Sed5 (~42 kDa). Labeling of HA-Sed5 was
endogenous Sed5 also undergoes phosphorylation. We ex-
produced HA-Sed5 or HA-Sed5S317A (plasmids pADH-HASED5 or
317 is a bona fide phosphorylation site. Wild-type (SP1) cells over-
Immunoprecipitated proteins were detected in Westerns with anti-
Schematic of Sed5 structure. A, B, and C, /H9251
/H11011 /H9262 /H11002 /H9262
Figure 1. Sed5 is phosphorylated in bos1-1 and wild-type cells. (A) Schematic of Sed5 structure. A, B, and C, /H9251
/H11011 /H9262 /H11002 /H9262
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bos1-1
mented previously (Marash and Gerst, 2001).
which increased t-SNARE phosphorylation has been docu-
shifted to the restrictive temperatures (37°C). This increase
autoradiography (autoradiogram). Samples of the total cell extract
were detected either in westerns with anti-Sed5 antibodies or by
autoradiography (autoradiogram). Samples of the total cell extract
(TCL) are shown beneath the IP results. (D) HA-Sed5 in bos1-1 cells.
recognized by anti-phosphoserine antibodies. Same as in C, ex-
cept unlabeled bos1-1 cells were also transformed with a control
plasmid (−). Immunoprecipitated proteins were detected in West-
erns with either anti-Sed5 or anti-phosphoserine antibodies. (E) Endogenous Sed5 is phosphorylated in wild-type cells. Wild-type
(SPI) cells were grown to log phase and labeled with [32P]orthophosphate for 3 h before being either shifted to 37°C for 15 min or maintained at 26°C. Cells were lysed in the
presence of phosphatase inhibitors and HA-Sed5 was precipitated from lysates with anti-HA antibodies. Immunoprecipitated proteins were detected either in westerns with anti-Sed5 antibodies or by
autoradiography (autoradiogram). Samples of the total cell extract
results might have been observed for HA-Sed5 (Figure 1, C
and D), but its overexpression may have impeded resolution
of the two bands that correspond to the phosphorylated and
nonphosphorylated forms. The results obtained from both
bos1-1 and wild-type cells indicate that Sed5 is phosphory-
luted like the Sso and Tlg t-SNAREs (Marash and Gerst,
2001; Gurunathan et al., 2002).
An Aspartate Substitution at Position 317 of Sed5
Inhibits the Growth of Secretory Mutants
To explore the significance of Sed5 phosphorylation in vivo,
we mutated serine-317 by substitution either with alanine to
mimic the nonphosphorylated state or with aspartate to
mimic the phosphorylated state. To verify that serine-317 is
indeed phosphorylated, we immunoprecipitated HA-tagged
Sed5 proteins from wild-type cells using anti-phosphoserine
antibodies. Even though protein expression was similar, we
found a large reduction in the amount of HASed5S317A that
could be precipitated relative to HA-Sed5 (Figure 1F). This is
consistent with the idea that serine-317 undergoes phos-
phorylation in vivo.
Next, we examined the growth of yeast overexpressing
SED5 or the SED5 mutants. We found that both SED5S317A
and SED5S317D were functional in terms of conferring
growth to cells lacking the SED5 gene (Figure 2A). The loss of Sed5 function is lethal (Hardwick and Pelham, 1992), and
strains expressing a galactose-inducible form of SED5 re-
mained viable on glucose if they expressed native SED5 or either mutant (Figure 2A). Similar results were observed
with sed5-1 cells, which could be rescued at restrictive tem-
peratures (35°C) by overexpression of either mutant, al-
though SED5S317A expression was less effective in conferring
growth (Figure 2B). Interestingly, we noted that the overex-
pression of SED5 (or SED5S317D) was inhibitory to the
growth of wild-type (WT) yeast, as described previously
(Hardwick and Pelham, 1992). However, this effect was
not observed with SED5S317A.
Because neither substitution inactivates Sed5, we tested
their effects upon yeast defective in protein trafficking to and
from the Golgi (Figure 2C). We examined their effect upon
cells bearing defects in SNAREs required for ER-Golgi
transport (i.e., bos1-1, bet1-1, and sec22-2), a COPII coat com-
ponent required for anterograde transport (i.e., sec23-2), and
a t-SNARE required for retrograde Golgi-ER transport (i.e.,
ufe1-1). We found that mutants overexpressing SED5S317D
were universally inhibited for growth at the semirestrictive
temperature, whereas those expressing SED5S317A often
 grew no differently than cells expressing vector alone (Figure
2C; see bos1-1 and ufe1-1 cells). In no case was the
expression of native SED5 or the mutants able to rescue the
growth of yeast and in several cases (e.g., bos1-1 and ufe1-1 cells), both native SED5 and SED5S317D overexpression had
severe inhibitory effects at normally permissive tempera-
tures. This suggests that overexpression of the pseudophos-
phorylated form (SED5S317D) as well as native SED5, which
can undergo phosphorylation at serine-317, inhibits cell
growth.
It would seem that the nonphosphorylated and pseudophosphorylated forms of Sed5 have very different effects
upon cell growth. The effect upon ufe1-1 cells by SED5S317D
was of particular interest, because Ufe1 functions in retro-
found to increase somewhat (~30%; n = 3) in bos1-1 cells
shifted to the restrictive temperatures (37°C). This increase
may result from the block in SNARE assembly, during
which increased t-SNARE phosphorylation has been docu-
mented previously (Marash and Gerst, 2001).
Because exogenously expressed HA-Sed5 is phosphory-
lated in bos1-1 cells, it was necessary to determine whether
endogenous Sed5 also undergoes phosphorylation. We ex-
amined the phosphorylation of Sed5 expressed from its
Genomic locus in wild-type cells using [33P]orthophosphate
and immunoprecipitation with anti-Sed5 antibodies (Figure
1E). In Westerns detected with anti-Sed5, we observed two
Sed5 bands that migrated closely (~39 and 41 kDa, respect-
atively); however, autoradiography indicated that only the
higher band incorporated the radiolabel (Figure 1E). Similar
results might have been observed for HA-Sed5 (Figure 1, C
and D), but its overexpression may have impeded resolution
of the two bands that correspond to the phosphorylated and
nonphosphorylated forms. The results obtained from both
bos1-1 and wild-type cells indicate that Sed5 is phosphory-
lated like the Sso and Tlg t-SNAREs (Marash and Gerst,
2001; Gurunathan et al., 2002).
whereas the temperature-sensitive cells. Cells were grown on glucose-containing medium, diluted and plated, and grown as described in A at 26 and 35°C. The plated wild-type transformants were grown for 36 h, whereas the plated wild-type transformants were grown for 36 h, presumably due to the enhanced vesiculation of Golgi membranes (Hardwick and Pelham, 1992). In wild-type cells, however, SED5 overexpression causes Kar2 (Semenza et al., 1990) to be secreted, perhaps due to Erd2 depletion within the Golgi (Hardwick and Pelham, 1992). Because the phosphorylation state of Sed5 affects a strain (ufe1-1) known to be deficient specifically in retrograde trafficking (Figure 2C), we looked for further evidence of retrograde transport defects in WT cells overproducing Sed5 or the Sed5 mutants (Figure 3). We first examined whether cells overproducing these proteins secrete Kar2, a luminal ER resident protein secreted from yeast bearing defects in retrograde transport (Semenza et al., 1990) or retention. We found that cells overexpressing either SED5 or SED5S317D secreted considerable amounts of Kar2 onto filters (Figure 3A, left), whereas control cells or cells overexpressing SED5S317A secreted little or no Kar2. Because cell lysis might also account for this phenomenon, we examined the filters for the presence of cytosolic proteins (e.g., Sec1 and hexokinase). However, we found no evidence to suggest that cells overexpressing SED5 or SED5S317D are more labile (Weinberger and Gerst, unpublished observations). Moreover, no difference in Kar2 expression was detected in cells overexpressing SED5 or the mutants (Weinberger and Gerst, unpublished observations). Finally, wild-type yeast expressing either GFP-SED5 or GFP-SED5S317D from the SED5 locus were examined for Kar2 secretion (Figure 3A, right). Importantly, we found that cells expressing GFP-SED5S317D secreted Kar2 to a level similar to that of ufe1-1. In contrast, cells expressing GFP-SED5 (Figure 3A, right) did not secrete significant amounts of the protein. Thus, an aspartate substitution at serine-317 alone enhances Kar2 secretion. Therefore, the lack of Kar2 retention in these cells is independent of Sed5 overproduction.

Next, we followed processing of the plasma membrane GPI-anchored protein Gas1. Gas1 undergoes glycosylation upon arrival to the Golgi, which can be monitored by pulse-chase analysis and autoradiography. However, defects in retrograde transport result in an inhibition in Gas1 maturation (Sutterlin et al., 1997). We found that maturation was severely inhibited in cells overexpressing SED5 or SED5S317D (Figure 3B), and only a small amount of mature Gas1 became visible after 45 min of chase. In addition, a low-molecular-weight form (≈50 kDa) of Gas1, which may represent a cleavage product, accumulated in a similar time-dependent manner. In contrast, mature Gas1 was readily visible within 5–10 min of chase in cells overexpressing SED5S317A as well as in control cells. In these cells, no low-molecular-weight form was observed at ≤50 kDa. Thus, Gas1 maturation seems defective in cells overexpressing SED5 or SED5S317D.

The processing of the vacuolar protease carboxypeptidase Y (CPY) is used to examine anterograde transport along the secretory pathway. The p1CPY precursor is synthesized in the ER, modified in the Golgi to the larger p2CPY form, and is transported to the vacuole where it is cleaved to yield the mature form (Stevens et al., 1982). We examined CPY pro-
Figure 3. Overexpression of Sed5 or Sed5S317D inhibits Golgi–ER retrograde transport. (A) Secretion of Kar2. Left, control (ufe1-1) and bet1-1 and wild-type (SP1) cells expressing SED5, SED5S317A, and SED5S317D from multicopy plasmids (pPADH-HASED5, pADHU-HASED5S317A, and pADHU-HASED5S317D) were grown on synthetic medium at 26°C, replica-plated onto nitrocellulose filters, and grown before detection using anti-Gas1 antibodies. Right, WT (SP1) cells expressing GFP–Sed5-tagged SED5, or the mutants (Figure 3C). Sec22-α was first expressed under the control of a GAL promoter overnight and then “chased” by transferring the culture to glucose-containing medium for up to 2 h. Rapid cleavage of Sec22-α was observed in cells overproducing Sed5 or Sed5S317D and within 1 h even the full-length protein disappeared. However, in control cells and more so in cells expressing Sed5S317A, both the full-length protein and the Kex2 cleavage product were observed for up to 2 h. Quantification revealed that 12.3 and 7.2% of the initial Sec22-α signal remained after 1 h in control and SED5S317A-expressing cells, respectively. In contrast, cells overexpressing SED5 or SED5S317D showed only 0.5 and 1.1% of their initial signal. This makes it likely that Sec22-α retrieval to the ER is inhibited only in cells expressing a phosphorylatable form of Sed5. We note that Sec22-α undergoes degradation even in control cells, because its overexpression probably saturates the basal retrieval machinery (Ballensiefen et al., 1998). Thus, from three independent assays we can see severe defects in retrograde transport from the Golgi to the ER, which are inflicted upon SED5 overexpression and sustained by the phospho-mimetic form SED5S317D. In contrast, cells expressing the nonphosphorylated form of Sed5 at position 317 have no such defects.

The cells were grown to log phase on galactose-containing medium and transferred to glucose-containing medium. Aliquots were removed at the indicated times, lysed, and subjected to Western analysis. SED5 or Sed5S317D were detected using anti-HA antibodies, whereas Sed5 was detected using anti-α-Sed5 antibodies. (D) SED5 mutants form complexes with ER-Golgi SNAREs to the same extent as native Sed5. sec18-1 cells expressing HA-HASED5 (SED5), SED5S317A (S317A) or SED5S317D (S317D) from single copy plasmids (pLADH-HASED5, pLADH-HASED5S317A, and pLADH-HASED5S317D) were grown to log phase and either maintained at the permissive temperature (26°C) or shifted to the restrictive temperature (37°C) for 15 min, before processing for coimmunoprecipitation. Proteins were immunoprecipitated using anti-HA antibodies and detected in immunoblots with antibodies against Sed5, Bos1, Vt11, and Sec22. TCL, total cell lysate was detected with anti-Sed5 antibodies to demonstrate presence of HA-tagged and endogenous Sed5.
Sed5S317A and Sed5S317D Assemble into SNARE Complexes

Because the phenotype of Sed5 overexpression is abrogated by mutating serine-317 to alanine, we speculated that the mutant might be unstable and undergo degradation. This alone could prevent the defects in retrograde transport observed upon native Sed5 overexpression. To test this, we performed pulse-chase analysis with [35S]methionine and immunoprecipitated Sed5 proteins. However, we found that Sed5S317A was no less stable than native Sed5 for up to 60 min and beyond (Weinberger and Gerst, unpublished observations).

An alternative explanation for the absence of an effect by Sed5S317A on retrograde transport is an inability of the mutant to enter into SNARE complexes. To test this, we examined the ability of Sed5 and Sed5 mutants to interact with SNAREs in vivo by coimmunoprecipitation. We expressed HA-tagged Sed5 and the mutants from single-copy plasmids in sec18-1 temperature-sensitive cells, which accumulate SNARE complexes at restrictive temperatures (Sogaard et al., 1994). We followed the coprecipitation of SNAREs involved in ER–Golgi transport (i.e., Bos1, Sec22, and Bet1), intragolgi and endosomal sorting (i.e., Vti1) that are known to form complexes with Sed5 (Sogaard et al., 1994; Fischer von Mollard et al., 1997; Lupashin et al., 1997; Tsui et al., 2001; Parlati et al., 2002). We found that the ability of Sed5S317A to bind to Bos1, Sec22, Bet1, Ykt6, and Vti1 was similar to that of Sed5 and Sed5S317D (Figure 3D; Weinberger and Gerst, unpublished observations). This implies that transport defects elicited by the alanine or aspartate substitutions are probably not mediated by changes in SNARE pairing per se.

GFP-Sed5S317A Labels an Exaggerated Brefeldin A (BFA)-dissociable Compartment

Because serine-317 is proximal to the TMD of Sed5 and given that Sed5 cycles between the ER and Golgi (Wooding and Pelham, 1998), it is possible that this residue could regulate Sed5 localization. To examine this, we generated GFP-Sed5 chimeras using native Sed5 and both the alanine and aspartate substitution mutants to follow their localization in vivo by fluorescence microscopy. All three GFP-Sed5 chimeras exerted similar effects as their nonchimeric Sed5 counterparts, when expressed in sed5-1 temperature-sensitive yeast (our unpublished data). Both GFP-Sed5 and GFP-Sed5S317D had a random punctate distribution, typical of yeast Golgi markers (Figure 4A). Surprisingly, GFP-Sed5S317D was distributed differently, occurring in large aggregates that are predominantly adjacent to the bud neck in wild-type cells. This was observed using both multicity and single-copy expression plasmids as well as by genomic integration of GFP-Sed5S317A at the Sed5 locus (Figure 4A) and was confirmed by immunofluorescence studies using antibodies against HA-tagged Sed5S317A lacking the GFP fusion (Figure 4B).

Next, we verified that GFP-Sed5S317A labels Golgi membranes and is not mislocalized to other cellular compartments. We examined GFP-Sed5S317A labeling in erg6/ise1...
cells that are permeable and sensitive to BFA, a fungal inhibitor that induces disassembly of the Golgi (Vogel et al., 1993; Chardin and McCormick, 1999). Surprisingly, we were unable to transform erg6/ise1 cells with plasmids expressing GFP-SED5 or GFP-SED5S317D (our unpublished data) but were able to express GFP-SED5S317A. GFP-Sed5 S317A labeling in this strain (Figure 4C, left) was similar to that seen in WT cells (Figure 4, A and B) and could be dispersed to the ER by treatment of the cells with BFA for 15 min (Figure 4C, right). Thus, the large compartments labeled by GFP-Sed5 S317A seem to represent an exaggerated Golgi apparatus.

GFP-Sed5 S317A Colocalizes with a trans-Golgi Marker
Because Sed5 is a cis-Golgi protein (Banfield et al., 1994), we examined whether GFP-Sed5 S317A colocalizes with late Golgi markers in the enlarged Golgi compartment. We examined colocalization with a RFP-tagged form of Sec7, a trans-Golgi marker (Franzusoff et al., 1991). In the case of GFP-Sed5 and GFP-Sed5 S317D, there was little colocalization observed with RFP-Sec7; however, in cells expressing Sed5 S317A full colocalization was evident (Figure 4D). This suggested either that the cis- and trans-markers are mixed in cells expressing Sed5 S317A or that the membranes bearing these fluorescent-tagged proteins are tightly juxtaposed.

GFP-Sed5 S317D Expression Results in a Stacked Mammalian-like Golgi, Whereas GFP-Sed5 S317D Expression Results in Vesiculation and ER Elaboration
To examine the intracellular morphology of cells overproducing Sed5 and the Sed5 mutants, we used electron microscopy. We overexpressed SED5, SED5 S317D, and SED5 S317A in WT cells and performed rapid fixation using high-pressure freezing (HPF). Interestingly, the overexpression of SED5 and SED5 S317D led to cells having abundant and elaborated ER membranes distributed throughout the cytoplasm (Figure 5, B and E). In addition, clusters of small vesicles (40–50 nm) were readily apparent in cells overexpressing SED5 S317D (Figure 5E). Notably, similar clusters have been shown in cells depleted of Sed5 (Hardwick and Pelham, 1992).
More surprisingly, overexpression of SED5S317A resulted in the appearance of stacked membranes reminiscent of the Golgi observed in higher eukaryotes (Figure 5, C and D). Immunogold staining of these thin sections using antibodies against the HA-tagged protein revealed the presence of Sed5S317A within the stacks (Figure 5D). Thus, expression of both the nonphosphorylated and pseudophosphorylated forms of Sed5 has dramatic effects upon intracellular morphology in yeast.

**Golgi Stacking Does Not Change the Distribution of Golgi Markers**

To determine whether the structural changes elicited by the two forms of Sed5 alter protein distribution throughout the endomembrane system, we examined the effects of Sed5S317A and Sed5S317D expression by crude subcellular fractionation. We expressed HA-tagged forms of Sed5 and the mutants in WT cells and prepared two membrane fractions: a medium-speed pellet (P10) that is thought to contain the majority of the ER membranes and a high-speed pellet (P100) that includes the bulk of Golgi membranes. We then examined the distribution of various proteins, including Emp47, which localizes at steady state to the cis-Golgi, but recycles between the ER and Golgi (Lewis and Pelham, 1996) like Sed5 (Wooding and Pelham, 1998). We also examined the distribution of a medial-Golgi marker, Mnn1, and an ER marker, Dpm1. First, we noticed that native Sed5 localized to the P10 fraction, along with Dpm1, implying that the t-SNARE associates either with an ER-associated structure, perhaps the transitional ER, or that the cis-Golgi sedimented in this fraction (Figure 6A). Next, we found that the overproduced HA-tagged Sed5 and Sed5S317D proteins were also mainly in the P10 fraction, unlike HA-tagged Sed5S317A, which was evenly divided between the P10 and P100 fractions (Figure 6A). Emp47 and Mnn1 distributed primarily to the P10 fraction in cells overexpressing SED5 and SED5S317D, unlike in control cells or cells expressing SED5S317A, where the Golgi markers were present in both the P10 and P100 (Figure 6A). Thus, cells bearing the nonphosphorylated form of Sed5, which seem to have an ordered Golgi (Figure 5), show no alteration in the distribution of Golgi markers vis-à-vis control cells expressing SED5 at native levels. In contrast, the redistribution of Golgi markers to the P10 fraction in cells overproducing either native Sed5 or its pseudophosphorylated form implies that t-SNARE phosphorylation can alter Golgi constituency.

**The Nonphosphorylated Form of Sed5 Efficiently Incorporates into COPI Vesicles**

Because Sed5S317D overproduction inhibits retrograde transport and induces elaboration of the ER and the accumulation of vesicles, we examined its ability to enter into Golgi-derived COPI-coated vesicles thought to mediate retrograde transport. We used an in vitro Golgi budding assay (Spang and Schekman, 1998) to generate COPI vesicles in the presence of Arf1, GTP, and coatamer. COPI vesicles were generated from isolated Golgi and were fractionated by density gradient centrifugation (Figure 6B). Although Sed5S317A was efficiently incorporated into vesicles along with Emp47, both Sed5 and Sed5S317D were far less able to undergo packaging therein. This suggests that nonphosphorylated Sed5 may be better recruited into COPI vesicles and thus may undergo more efficient retrieval (either to the ER, within the Golgi, or both) in contrast to either the pseudophosphorylated or phosphorylatable forms of the t-SNARE.

![Figure 6](image.png)

- **A.** P10 and P100 fractions of cells overexpressing HA-tagged Sed5, Sed5S317A, and Sed5S317D were analyzed by SDS-PAGE and immunoblotted with anti-Sed5 and anti-Emp47 antibodies. (B) COPI budding assay. Cells were lysed in a detergent-free buffer, and the lysates were subjected to differential centrifugation. The formation of COPI-coated vesicles was induced by the addition of purified coatamer, Arf1, ATP, and GTP. Vesicles were fractionated by density gradient centrifugation, and samples from the fractions were precipitated, resolved by SDS-PAGE, and detected in blots using anti-Sed5 and anti-Emp47 antibodies. Ctrl-Golgi, a sample (30 µg) of the Golgi membranes used as the source for vesicles.
into COPI vesicles unlike Sed5 and Sed5S317D (Figure 6), it ordered Golgi (Figures 4 and 5) and is readily able to enter enlarged Golgi aggregates typically observed in cells overtures (Figure 7). Interestingly, we were unable to find the formation of GFP-Sed5S317A in retrograde Golgi-ER transport. We examined the localization of GFP-Sed5S317A in sec21-2 cells, which are defective in COPI-mediated retrograde transport at restrictive temperatures (Figure 7). Interestingly, we were unable to find the enlarged Golgi aggregates typically observed in cells overproducing GFP-Sed5S317A at any temperature (Figure 4). Instead, the labeling of GFP-Sed5S317A in sec21-2 cells (Figure 7) was similar to that observed for GFP-Sed5 in wild-type cells (Figure 4); 92% of cells (n = 100) expressing GFP-Sed5S317A did not show Golgi aggregates. In contrast, the enlarged Golgi puncta were readily observed in sec23-2 cells, which bear defects in anterograde ER-Golgi transport, at permissive temperatures, whereas labeling of the ER was observed at the restrictive temperature, as expected (Figure 7). Thus, Sed5 recycling is likely to be important for the formation of ordered Golgi structures.

Figure 7. Retrograde Golgi–ER transport is required for formation of the enlarged Golgi compartment. Yeast strains (sec21-2, sec23-2, and grh1Δ) were transformed with a plasmid overexpressing GFP-SED5S317A (pADH-HAGFPSED5S317A) and examined by confocal fluorescence microscopy. Cells were examined at a temperature permissive for growth (26°C) or were shifted to the restrictive temperature (37°C), before examination.

DISCUSSION

Because t-SNARE phosphorylation and dephosphorylation seem to play a significant role in regulation of the exo- and endocytic processes (Weinberger and Gerst, 2004), we examined whether ER–Golgi transport is controlled in a similar manner. Here, we show that an essential Golgi t-SNARE, Sed5, is a phosphoprotein and that phosphorylation controls the morphology and apparent functioning of the Golgi apparatus. Sed5 overproduction inhibits the growth of yeast (Hardwick and Pelham, 1992; Figure 2), which is recapitulated in an even stronger manner by the replacement of a conserved serine residue (serine-317) with aspartate (Figure 2, B and C). In contrast, this phenotype is abrogated by introduction of an alanine residue in place of the relevant serine (Figure 2, B and C). Thus, both phosphorylation and dephosphorylation of this residue seem to play a critical role in Sed5 functioning.

Although both the aspartate and alanine substitutions confer growth to yeast lacking a functional Sed5 (Figure 2), the aspartate-317 substitution results in a marked decrease in the growth of bos1-1, sec23-2, and ufe1-1 cells (Figure 2C). However, only ufe1-1 cells are specifically defective in Golgi–ER retrograde transport (Lewis and Pelham, 1996). Other postulated retrograde transport defects, such as Kar2 secretion, an inhibition of Gas1 processing, and the pronounced degradation of a Sec22-α-factor chimeric protein, were all observed in wild-type cells expressing SED5S317D (Figure 3). Similar results were obtained with overproduced native Sed5, which can undergo phosphorylation, but not with the nonphosphorylatable alanine substitution at position-317 (Figure 3). Importantly, these transport defects are not consistent with a general deficiency in protein export from either the ER or the Golgi. For example, Kar2 and Sec22-α-factor either reach post-Golgi compartments or are exported (Figure 3A and C). Next, GFP-Sed5 and GFP-Sed5S317D give punctate labeling that is typical of the Golgi and not the ER (Figure 4A), indicating that they can exit early compartments. Finally, CPY maturation is not blocked in cells overexpressing Sed5S317D (Weinberger and Gerst, unpublished observations). Although Gas1 maturation does seem inhibited (Figure 3B), this export defect has also been shown in cells defective in retrograde transport (Sutterlin et al., 1997). Thus, our observations implicate serine-317 phosphorylation as a potential regulator of Golgi–ER retrograde transport.

Supportive of this view is electron microscopy data showing ER elaboration and the accumulation of small clustered transport vesicles in cells expressing the aspartate-317 substitution (Figure 5E). Because a similar phenotype was observed in cells lacking Sed5 (Hardwick and Pelham, 1992), it suggests that constitutive phosphorylation of serine-317 can have an effect analogous to decreased Sed5 function in some assays. However, the overexpression of native SED5 also inhibited cell growth (Figure 2C) and induced both retrograde sorting defects and ER elaboration (Figures 3, A–C, and 5) as well as SED5S317D, but it could not induce vesicle accumulation (Figure 5). Thus, although the phospho-mi-
motic mutant is clearly functional (Figures 2A and 3D), it can exert effects unlike that of those of the native t-SNARE. Thus, the effects of SED5 and SED5^S317D overexpression are comparable but not necessarily identical. This may explain why the aspartate substitution mutant is more potent in some assays (Figures 2C, 3A, and 5). Although the mechanism for vesicle accumulation in cells expressing SED5^S317D is unclear, it suggests defects in either the fusion of Golgi-derived COPI vesicles with the ER or COPII vesicles with the Golgi. The former possibility is more likely, because defects in retrograde transport invariably block the budding of ER-derived transport vesicles and lead to the accumulation of ER membrane. More work will be required to verify the nature of these vesicles.

If the vesicles observed in SED5^S317D-expressing cells are indeed retrograde transport vesicles, why should they be less able to fuse? The answer may lie with the fact that Sed5^S317D and phosphorylatable native Sed5 inefficiently incorporate into Golgi-derived COPI vesicles in vitro (Figure 6B). Perhaps Sed5 is required for trans-SNARE pairing with ER resident SNAREs (i.e., Ufe1, Sec20, and Sec22) to form a retrograde fusion complex. Alternatively, it may be needed to chaperone cycling SNAREs (i.e., Bos1, Bet1, and Sec22) or other factors back to the ER. In either case, it would explain why Sed5 cycles between compartiments (Wooding and Pelham, 1998). Another possibility is that the phosphorylated form of Sed5 binds to Arf GTPases less well, thus decreasing the formation of retrograde transport vesicles. However, this possibility is less likely, given that such vesicles can be formed in the presence of Sed5^S317D (Figure 6B) and that in vitro binding studies with the Sed5 mutants and recombinant myristoylated Arf1 showed no difference in Arf binding (Spang, unpublished observations). Fractionation experiments suggest that the constituency of the Golgi may change upon overexpression and that a redistribution of Golgi markers to a heavier ER-enriched fraction occurs (Figure 6A). Ironically, this happens even though Sed5^S317D (or native Sed5) does not efficiently enter into COPI vesicles in vitro like Sed5^S317A (Figure 6B). Because GFP-Sed5^S317D does not yield ER-like labeling (Figure 4D), it is unlikely that it associates with ER membranes and presumably is associated with the early Golgi. More work will be required to understand the connection between altered Sed5 recruitment into COPI vesicles and the redistribution of the Golgi markers.

Our other major observation is that the nonphosphorylated form of Sed5, Sed5^S317A, results in the accumulation of an ordered Golgi reminiscent of the stacked apparatus found in higher eukaryotes (Figure 5, C and D). Although cells expressing Sed5^S317A show no defects in protein trafficking or growth (Figure 2); nonetheless, the dispersed Golgi typical of S. cerevisiae seems absent. Thus, formation of an ordered Golgi from a disordered state seems to have no debilitating effects upon the growth of yeast per se. Indeed, other yeast, such as Pichia pastoris, favor an ordered state (Glick, 1996). The differences between these yeast are thought to result from the number of sites available for COPII vesicle budding, which bud from fixed sites in P. pastoris and throughout the ER in S. cerevisiae (Rossanese et al., 1999). This may be responsible for the formation of either polarized or scattered transitional ER, respectively, which eventually coalesce to form the Golgi (Rossanese et al., 1999).

Our results imply that a lack of phosphorylation at position 317 of Sed5 induces an ordered state of the Golgi (Figure 5, C and D, and model in Figure 8). In contrast, pseudophosphorylation induces a dispersed state, if the vesicles that accumulate therein are COPI vesicles (Figure 5E and model in Figure 8). Thus, a dynamic cycle of t-SNARE phosphorylation and dephosphorylation may be necessary for the Golgi to maintain structural integrity even in S. cerevisiae. Changes in the ability of Sed5 to cycle (via phosphorylative control) can also be expected to play a role in the de novo formation of the early Golgi from the transitional ER (Rossanese et al., 2001).

How can the phosphorylation state of a SNARE influence organelle integrity so dramatically? One clue is that Sed5 acts upon multiple Golgi transport pathways, including anterograde transport to the cis-Golgi (using the Bos1, Bet1, and Sec22 SNAREs), intra-Golgi transport (using the Sft1, Got1, and Ykt6 SNAREs) as well as endosome-to-Golgi transport (using Tlg1, Vti1, and Gos1 SNAREs). Therefore, Sed5 localization must be controlled such that it maintains a steady-state distribution to the cis-Golgi (Hardwick and Pelham, 1992; Wooding and Pelham, 1998), but nevertheless can transverse the cisternae to interact productively with its other SNARE partners or to be retrieved to the ER.

We propose that Sed5 transport and retrieval through the Golgi is regulated by its phosphorylation state. The alanine substitution mutant, whose expression results in an ordered Golgi (Figures 4 and 5), and that efficiently incorporates into COPI vesicles (Figure 6B), implies that enhanced Sed5 retrieval back through the cisternae might induce stacking. This is supported by studies in sec21-2 cells, which are defective in retrograde transport, and do not allow for formation of ordered Golgi structures (Figure 7). However, we do note that Sed5^S317A colocalizes with Sec7, a trans-Golgi marker, implying a steady-state presence in the trans-Golgi (Figure 4D). This, as well, might account for the ordering phenomenon and therefore requires further study. It is noteworthy to add that stacking could also result from the alanine mutant independently of its enhanced packaging into COPI vesicles or retrograde transport. In contrast to Sed5^S317A, the aspartate substitution mutant, which is inefficiently incorporated into COPI vesicles (Figure 6B) and whose expression results in ER elaboration and vesicle ac-

![Figure 8. Model for Golgi morphology as a consequence of Sed5 phosphorylation. Sed5 in its nonphosphorylated (at position 317) state results in a stacked mammalian-like Golgi structure (Ordered Golgi). In contrast, upon phosphorylation at position 317 Sed5 induces the dispersal of Golgi markers, the accumulation of transport vesicles and ER, and inhibits cell growth (Dispersed Golgi). Thus, Golgi structure may alternate between structured and unstructured states as a consequence of t-SNARE phosphorylation.](image-url)
cumulation (Figure 5), may reside too efficiently in the cis-compartment such that it inhibits retrograde transport events and therefore cell growth (Figures 2 and 3). The mechanism for Sed5 retention is also not well understood, but previous studies suggested that it is determined by the cytoplasmic domain and not by the TMD (Banfield et al., 1994), in contrast to other SNAREs such as Snc1 (Lewis et al., 2000). Serine-317, which is adjacent to the TMD, is therefore a strong candidate for a Sed5 Golgi retention signal upon phosphorylation.

Finally, we note that stacked Golgi-like structures have been observed in other secretory mutants of S. cerevisiae; however, these are seen at temperatures where Golgi export is blocked (i.e., in sec7, sec14, and ypt13Δ ypt32-1 cells; Novick et al., 1981; Franzussof et al., 1991; Jedd et al., 1997), whereas the ordered structures observed in Sed5S317A-expressing cells occur under conditions conducive for growth (Figures 2 and 5). Thus, ordering per se has no deleterious effects although the alanine mutant is not as effective for the rescue of sed5-1 cells (Figure 2B). An ordered organization of the Golgi, as seen in higher eukaryotes, may prove useful where ER exit sites are more tightly controlled, leading to the polarized accumulation of transitional ER (and subsequent Golgi coalescence). This may be of particular importance as cell size increases, allowing for more efficient transport between cisternae as well as to target compartments. The fact that a t-SNARE mutation can bring about such morphological changes suggests that a controlled mechanism for ordering and dispersal exists. This is likely to be important for Golgi assembly and disassembly during the mitotic cell cycle in higher eukaryotes, which also involves cycles of protein phosphorylation and dephosphorylation. In particular, the functioning of several Golgi structural proteins (i.e., GM130, GRASP65, and p115) during mitosis has been shown to be modulated by phosphorylation and in a manner that correlates with Golgi fragmentation and dispersal (reviewed in Rossanese and Glick, 2001; Shorter and Warren, 2002).

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