Requirement for Neo1p in Retrograde Transport from the Golgi Complex to the Endoplasmic Reticulum

Zhaolin Hua and Todd R. Graham*

Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee 37235-1634

Submitted July 2, 2003; Revised July 31, 2003; Accepted August 1, 2003

Monitoring Editor: Benjamin Glick

Neo1p from *Saccharomyces cerevisiae* is an essential P-type ATPase and potential aminophospholipid translocase (flippase) in the Drs2p family. We have previously implicated Drs2p in protein transport steps in the late secretory pathway requiring ADP-ribosylation factor (ARF) and clathrin. Here, we present evidence that epitope-tagged Neo1p localizes to the endoplasmic reticulum (ER) and Golgi complex and is required for a retrograde transport pathway between these organelles. Using conditional alleles of *NEO1*, we find that loss of Neo1p function causes cargo-specific defects in anterograde protein transport early in the secretory pathway and perturbs glycosylation in the Golgi complex. Rer1-GFP, a protein that cycles between the ER and Golgi complex in COPI and COPII vesicles, is mislocalized to the vacuole in *neo1-ts* at the nonpermissive temperature. These phenotypes suggest that the anterograde protein transport defect is a secondary consequence of a defect in a COPI-dependent retrograde pathway. We propose that loss of lipid asymmetry in the *cis* Golgi perturbs retrograde protein transport to the ER.

INTRODUCTION

Yeast *Saccharomyces cerevisiae* contains 16 P-type ATPases that can be phylogenetically grouped into five subfamilies: heavy metal ion transporters, Na⁺ transporters, Ca²⁺ transporters, H⁺ transporters, P4 ATPases with unknown substrate, and potential aminophospholipid translocases (APTs; Catty *et al.*, 1997). The APT subfamily contains five genes, *DRS2*, *DNF1*, *DNF2*, *DNF3*, and *NEO1*, that share 40–83% amino acid sequence similarity with each other (Catty *et al.*, 1997; Hua *et al.*, 2002). APTs, or flippases, couple ATP hydrolysis to the translocation of phosphatidylserine (PS) and/or phosphatidylethanolamine (PE) from the external leaflet of a membrane bilayer to the cytosolic leaflet and appear to be responsible for concentrating PS and PE in the cytosolic leaflet of biological membranes (Balasubramanian and Schroit, 2003). This activity has been primarily defined in the plasma membrane of blood cells (Seigneuret and Devaux, 1984; Auland *et al.*, 1994) and in bovine chromaffin granule membranes (Zachowski *et al.*, 1989). The latter APT appears to be equivalent to ATPase II, which is 47% identical to Drs2p from yeast (Tang *et al.*, 1996). The human genome encodes multiple members of the APT subfamily including genes implicated in genetic disorders. FIC1 mutations cause a defect in bile secretion and ATP10C is in an imprinted region of chromosome 18 linked to Angelman syndrome and potentially autism (Thompson and Jansen, 2000; Herzog *et al.*, 2001; Meguro *et al.*, 2001). In addition, the mouse ATP10C gene is linked to an increased body fat phenotype (Dhar *et al.*, 2000).

The localization and cellular requirements for the yeast APTs are starting to be defined. Drs2p and Dnf3p localize to late Golgi and perhaps endosomal membranes, whereas

Dnf1p and Dnf2p localize primarily to the plasma membrane (Chen *et al.*, 1999; Hua *et al.*, 2002; Pomorski *et al.*, 2003). Deletion of all four of these genes is lethal in yeast, indicating that *DRS2* and the *DNFs* form an essential gene group (Hua *et al.*, 2002). Strains harboring disruptions of *DNF1,2* or *DRS2* exhibit a defect in lipid translocation across the plasma membrane and expose more PE on the external leaflet than wild-type cells (Tang *et al.*, 1996; Gomes *et al.*, 2000; Pomorski *et al.*, 2003). These mutant phenotypes are consistent with the proposed translocase activity for the Drs2/Neo1 family ATPases, although this conclusion has been controversial (Siegmund *et al.*, 1998; Marx *et al.*, 1999) and direct evidence that these proteins catalyze lipid translocation is still lacking.

Drs2 and Dnf proteins are also required for protein trafficking between the Golgi complex, plasma membrane, and endosomal/vacuolar system (Chen *et al.*, 1999; Gall *et al.*, 2002; Hua *et al.*, 2002; Pomorski *et al.*, 2003). *DRS2* interacts genetically with clathrin and ARF, and *drs2Δ* exhibits defects in late Golgi function that are similar to clathrin mutants (Chen *et al.*, 1999). In addition, *drs2* mutants also exhibit a defect in generating a specific class of exocytic vesicles carrying invertase and acid phosphatase (Gall *et al.*, 2002). These vesicles require clathrin for their formation and at least a portion of them are clathrin-coated. The *drs2Δ dnf1Δ* double mutant exhibits a significantly stronger defect in the alkaline phosphatase and carboxypeptidase Y (CPY) vacuolar transport pathways compared with the single mutants. This suggests that Drs2p and Dnf1p are functionally redundant in their ability to support vacuolar protein transport, whereas only Drs2p can support the generation of exocytic vesicles. Moreover, the *dnf1Δ dnf2Δ dnf3Δ* triple deletion mutant exhibits a defect in an endosome to TGN recycling pathway traveled by a v-SNARE protein (Hua *et al.*, 2002). ARF, clathrin, and/or adaptins (AP-1, AP-3 and GGAs) have been implicated in each of these pathways that require Drs2/Dnf proteins for normal function.

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-07-0463. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-07-0463.

* Corresponding author. E-mail address: tr.graham@vanderbilt.edu.

Among the five potential APTs in yeast, *NEO1* is unique in that deletion of this gene alone is lethal (Prezant *et al.*, 1996), and so none of the other APT subfamily members can perform the essential function of Neo1p. In addition, overexpression of *NEO1* cannot suppress the *drs2Δ* cold-sensitive growth defect, nor can overexpression of *DRS2* rescue *neo1Δ* lethality (Hua *et al.*, 2002). *NEO1* was first identified in a screen for genes that confer resistance to the aminoglycoside neomycin upon overexpression (Prezant *et al.*, 1996). Neo1p overexpression did not confer resistance to other drugs tested including ethidium bromide, cycloheximide, and chloramphenicol, indicating that Neo1p is not a multidrug resistance protein. The Neo1p ATPase activity is required to support cell viability and to confer neomycin resistance (Prezant *et al.*, 1996). It is not known if Neo1p can directly pump neomycin out of the cell or if it confers the drug resistance through another mechanism. Nothing is known about the essential cellular functions of Neo1p.

Because Drs2p and Dnf proteins are involved in protein transport in the late secretory and endosomal pathways, we hypothesized that Neo1p might also be required for protein transport in the secretory pathway. To test this possibility, conditional alleles of *neo1* were generated and their effect on protein transport was determined. We found that Neo1p is indeed required for efficient protein transport in the early secretory pathway and Golgi-dependent glycosylation. Protein transport between the ER and Golgi is mediated by small transport vesicles, with COPII-coated vesicles carrying cargo in the anterograde direction and COPI-coated vesicles carrying cargo in the retrograde direction (Kirchhausen, 2000). Temperature-sensitive mutations in COPII subunits (e.g., *sec12* or *sec23*) cause a block in the ER-to-Golgi transport and the accumulation of all cargo proteins in the ER at the nonpermissive temperature (Barlowe, 2002). COPI mutations (e.g., *sec21*) perturb retrograde transport and can also cause a defect in ER-to-Golgi transport, although not all proteins are subject to this block. For example, Hsp150 and invertase are secreted efficiently from *sec21* cells, whereas carboxypeptidase Y (CPY) and pro- α -factor are blocked in the ER (Gaynor and Emr, 1997). Mutations in early Golgi ARF GAPs (Glo3 and Gcs1) also perturb CPY transport and invertase glycosylation but not invertase secretion (Poon *et al.*, 1999). Rer1p can be used to distinguish a primary defect in anterograde or retrograde transport, because this protein is trapped in the ER in COPII mutants but mislocalized to the vacuole in COPI mutants (Sato *et al.*, 2001). The *neo1* mutants also exhibit cargo-selective defects in anterograde protein transport and mislocalize Rer1p to the vacuole. These phenotypes suggest that Neo1p is required for COPI-dependent retrograde transport from the Golgi to the ER.

MATERIALS AND METHODS

Media, Strains, and Plasmids

Yeast strains were grown in standard rich medium (YP) or synthetic minimal (SM) media containing required supplements, with 2% glucose (YPD) or 2% galactose as indicated. YPD sorbitol plates were made by autoclaving YPD agar supplemented with the indicated amount of sorbitol. YPD, pH 8.0, plates were made by adding 10 mM Tris base to YPD and adjusting to pH 8.0 before autoclaving. Neomycin, cation, ethanol, calcofluor white (CW), and NaF plates were made by adding the indicated concentration of the chemicals after autoclaving. Sporulation was done by growing cells in YP 2% KOAc presporulation media to 1.0 OD/ml before shifting to SPM media (0.3% KOAc, 0.02% raffinose, and 0.25 $\times$ supplements) for 3–7 d.

Yeast strains used in this study are listed in Table 1. The yeast knockout strain collection was originally purchased from Research Genetics, which is now Resgen, Invitrogen Corporation (Carlsbad, CA). ZHY9075E was generated by sporulation of BY4743 *neo1Δ* pBM743-NEO1 (Hua *et al.*, 2002).

ZHY219RR and all ZHY628 strains were generated by replacing pBM743-NEO1 in ZHY9075E by either p413-NEO1 (carrying wild-type *NEO1*) or p413-*neo1-1* through 6 (carrying *neo1 ts* alleles).

The plasmid p413-NEO1PM was generated by replacing the ADH promoter from p413-ADH with the PCR-amplified *NEO1* promoter region (518 bp) on a *SacI/XbaI* fragment. The *NEO1* coding sequence from pYGW1-NEO1 (Hua *et al.*, 2002) was cloned into the *EcoRI/Sall* site of p413-NEO1PM to generate p413-NEO1. Sequencing of p413-NEO1 revealed two nucleotide changes compared with published sequence and causes two amino acid changes (V186M and T558A). This plasmid fully rescues the *neo1Δ* lethality phenotype. Other plasmids used in this study are pSKY5/RER1-0 (a cent-based URA3 plasmid carrying Rer1-GFP; Sato *et al.*, 2001), pOH-URA3 (a 2- μ m plasmid with OCH1-HA; Harris and Waters, 1996), and pRS426-MNN1 (Graham *et al.*, 1994).

Isolation of *neo1-ts* Alleles

Random PCR mutagenesis (with 3.5 mM MgCl₂, 0.5 mM MnCl₂, 0.45 mM dATP, 0.72 mM dCTP, 0.16 mM dGTP, and 1.12 mM dTTP in the PCR reaction for 35 cycles) was used to generate mutations in the *NEO1* gene. Initial attempts at mutagenesis using higher concentrations of dNTPs and fewer cycle numbers failed to yield a *neo1-ts* allele. Primers used for PCR were 5'-TTTGTGCCAACCCCTATTATGAA, and 5'-GCATTGTTTATTCATGTGTAGA. Plasmid p413-NEO1 was gapped using *NcoI* and *StuI* and was cotransformed with the mutagenized PCR products into ZHY9075E. The resulting transformants (~10,000) were then replicated onto 5-fluoroorotic acid (5-FOA) plates to select against the original wild-type *NEO1* plasmid (pBM743-NEO1 harboring URA3). Approximately 1000 colonies grew on the 5-FOA plates, which were then tested for growth at 37°C. Six colonies exhibited a tight *ts* growth defect at 37°C. Plasmids were rescued from these six strains and named p413-*neo1-1* to p413-*neo1-6*. The following mutations are found in the six *neo1 ts* alleles: *neo1-1*, D356V, I381V, A420V, E528D, T529P, Q538L, D568V, T604N, Q727R, R805K, E807K, T1043S; *neo1-2*, H346L, A431V, V457I, L465I, P491L, S502C, S542C, P581L, S618T, K672E, L719W, D725V, I732N, L752S, Q812R, A854V; *neo1-3*, H279Q, T337I, A370V, A375S, V402A, V430L, N437I, R496H, M569K; *neo1-4*, M234I, S297T, C319S, N366I, E528K, R565H, E624D, V674A, D716E, S729N, M872K; *neo1-5*, A370V, P491Q, S542R, G698S, S908T, A940V; *neo1-6*, C380Y, F661S, C855S, A951T, Y957N, V986I, H993L, T1000S.

Integration of *neo1-ts* Alleles into the Yeast Genome

To facilitate genetic crosses of *neo1-ts* mutants with other mutants, *neo1-ts* alleles were integrated into the *NEO1* locus. The integrating *neo1-ts* plasmids (pZH1125-15B and pZH1125-34A for *neo1-1* and *neo1-2*, respectively) were made by first inserting the *SacI/Sall* fragment from p413-*neo1-1* or p413-*neo1-2* into pRS303, and then a 600-bp PCR-amplified KanMX fragment was inserted into the *Apal/XhoI* site of the resulting plasmid. pZH1125-15B and pZH1125-34A were then cut with *NruI*, a unique restriction enzyme site located within the KanMX fragment, and then transformed into BY4743 *neo1Δ::KanMX*, to generate the BY4743 *neo1Δ::neo1-1* (or *neo1-2*) *HIS3* strain. These strains were then subjected to sporulation, and the progeny were tested for *ts* growth to identify the integrated *neo1-ts* mutants (ZHY129-15B28A and ZHY129-34A2A). To integrate *neo1 ts* alleles into the SEY6210 background, a PCR-amplified KanMX-NEO1 knockout module (http://www.sequence.sanford.edu/group/yeast_deletion_project/deletions3.html) was first transformed into a SEY6210.5 diploid (Robinson *et al.*, 1988). Then the *neo1 ts* integration plasmids pZH1125-15B and pZH1125-34A were transformed into the resulting strain. The transformants were then subjected to sporulation and tested for a *ts* growth phenotype to identify *neo1-ts* integrated mutants (All ZHY124 strains are in SEY6210 background).

Genetic Analysis of *neo1 ts* Mutants

Genetic crosses between *neo1-ts* (ZHY129-15B28A and ZHY129-34A2C) and *arf1Δ*, *glo3Δ*, *gcs1Δ*, *gda1Δ*, *mmn9Δ*, *dnf1Δ*, *dnf2Δ*, and *dnf2Δ* strains were done in the BY4741/BY4742 strain background. Genetic crosses between *neo1-ts* (ZHY124-15B1B, ZHY124-15B1C, ZHY124-34A2A, and ZHY124-34A2B) and *sec21-1*, *ret1-1*, *sec18-1*, *sec23-1*, *sec1-1*, *sec12-1*, *sec14-1*, and *sec7-4* were done in the SEY6210 strain background. Diploids from the above crosses were subjected to sporulation and tetrad dissection, and the double mutants were collected if available.

Immunological and Imaging Methods

Cell labeling, immunoprecipitation (Gaynor and Emr, 1997), and immunoblotting (Chen *et al.*, 1999) were performed as described previously. Anti- α -factor (Graham and Emr, 1991) and anti-carboxypeptidase Y (CPY) serum (Klionsky *et al.*, 1988) were used for immunoprecipitation. The 9E10 mouse monoclonal c-Myc antibody (Oncogene Research Products, Darmstadt, Germany) (1:100), polyclonal affinity-purified rabbit anti-HA antibody (Zymed Laboratories, South San Francisco, CA; 1:100), and anti-Mnn1p antibody (Graham *et al.*, 1994; 1:50) were used for immunofluorescence microscopy. Immunofluorescence and green fluorescent protein (GFP) fluorescence were

Table 1. Yeast strains used in this study

Strain	Genotype	Source
BY4742	<i>MATα his3 leu2 ura3 lys2</i>	Invitrogen
BY4743 <i>neo1Δ</i>	<i>MATα/his3/his3 leu2/leu2 ura3/ura3 lys2/LYS2 met15/MET15 neo1Δ::KanMX/NEO1</i>	Invitrogen
ZHYNEO1-MYC	<i>MATα his3 leu2 ura3 lys2 NEO1::13XMYC</i>	Hua <i>et al.</i> (2002)
ZHY9075E	<i>MATα his3 leu2 ura3 neo1Δ pBM743-NEO1</i>	This study
ZHY219RR	<i>MATα his3 leu2 ura3 neo1Δ p413-NEO1</i>	This study
ZHY628-15B	<i>MATα his3 leu2 ura3 neo1Δ p413-neo1-1</i>	This study
ZHY628-34A	<i>MATα his3 leu2 ura3 neo1Δ p413-neo1-2</i>	This study
ZHY628-12F	<i>MATα his3 leu2 ura3 neo1Δ p413-neo1-3</i>	This study
ZHY628-22D	<i>MATα his3 leu2 ura3 neo1Δ p413-neo1-4</i>	This study
ZHY628-26H	<i>MATα his3 leu2 ura3 neo1Δ p413-neo1-5</i>	This study
ZHY628-36A	<i>MATα his3 leu2 ura3 neo1Δ p413-neo1-6</i>	This study
ZHY129-15B28A	<i>MATα his3 leu2 ura3 lys2 neo1-1::HIS3-KanMX</i>	This study
ZHY129-34A2C	<i>MATα his3 leu2 ura3 lys2 neo1-2::HIS3-KanMX</i>	This study
ZHY124-15B1B	<i>MATα his3 leu2 ura3 ade2 trp1 suc2 neo1-1::HIS3-KanMX</i>	This study
ZHY124-15B1C	<i>MATα his3 leu2 ura3 ade2 trp1 suc2 neo1-1::HIS3-KanMX</i>	This study
ZHY124-34A2A	<i>MATα his3 leu2 ura3 ade2 trp1 suc2 neo1-2::HIS3-KanMX</i>	This study
ZHY124-34A2B	<i>MATα his3 leu2 ura3 ade2 trp1 suc2 neo1-2::HIS3-KanMX</i>	This study
BY4741 <i>arf1Δ</i>	<i>MATα his3 leu2 ura3 met15 arf1Δ</i>	Invitrogen
BY4741 <i>glo3Δ</i>	<i>MATα his3 leu2 ura3 met15 glo3Δ</i>	Invitrogen
BY4741 <i>gcs1Δ</i>	<i>MATα his3 leu2 ura3 met15 gcs1Δ</i>	Invitrogen
BY4741 <i>gda1Δ</i>	<i>MATα his3 leu2 ura3 met15 gda1Δ</i>	Invitrogen
BY4741 <i>mn9Δ</i>	<i>MATα his3 leu2 ura3 met15 mn9Δ</i>	Invitrogen
BY4741 <i>dnf1Δ</i>	<i>MATα his3 leu2 ura3 met15 dnf1Δ</i>	Invitrogen
BY4741 <i>dnf2Δ</i>	<i>MATα his3 leu2 ura3 met15 dnf2Δ</i>	Invitrogen
ZHY615D1C	<i>MATα his3 leu2 ura3 lys2 drs2Δ</i>	Hua <i>et al.</i> (2002)
EGY1211-6B	<i>MATα leu2 ura3 his3 trp1 suc2 sec21-1</i>	Gaynor <i>et al.</i> (1998)
EGY101-16D	<i>MATα leu2 ura3 his3 trp1 suc2 ret1-1</i>	Gaynor <i>et al.</i> (1998)
TBY102	<i>MATα ura3 leu2 his3 trp1 lys2 suc2 sec18-1</i>	Brigance <i>et al.</i> (2000)
TBY103	<i>MATα leu2 ura3 his3 trp1 sec23-1</i>	Chen <i>et al.</i> (1999)
TGY144	<i>MATα leu2 ura3 his3 trp1 lys2 sec1-1</i>	Chen <i>et al.</i> (1999)
TBY120	<i>MATα leu2 ura3 his3 trp1 lys2 sec12-4</i>	This study
CTY1-1A	<i>MATα leu2 his3 lys2 sec14-1</i>	Bankaitis <i>et al.</i> (1989)
AFM69-1A	<i>MATα leu2 ura3 his3 sec7-4</i>	Franzusoff and Schekman (1989)

observed using an Axioplan microscope (Carl Zeiss, Thornwood, NY), and fluorescent images were processed using MetaMorph 4.5 software (Universal Imaging, Downingtown, PA). Samples for electron microscopy were prepared as described previously (Rieder *et al.*, 1996) and sections (50–60 nm) were viewed on a CM12 electron microscope (Philips, Eindhoven, Netherlands).

RESULTS

Neo1-myc Localizes to the ER and Golgi Complex

To better understand the essential function of Neo1p, we first examined the intracellular localization of this protein by immunofluorescence. A 13-myc epitope tag was integrated into the genome at the 3' end of *NEO1*. This strain grew as well as wild-type, indicating that Neo1-myc was fully functional. Immunofluorescence localization of myc-tagged Neo1p suggested a role for this protein in the early secretory pathway. Neo1-Myc was found in a continuous membrane structure that surrounds the nucleus and extends to the periphery of the cell body (Figure 1A), which is typical for ER localization in yeast cells. Neo1-Myc was also found in punctate structures where it partially colocalized with both the early Golgi marker Och1p (Figure 1B) and the late Golgi marker Mnn1p (Figure 1C). Therefore, Neo1-Myc localizes to both ER and Golgi membranes. This conclusion is consistent with the distribution of Neo1-HA in subcellular fractions examined by the Holthuis group, although they did not observe ER staining by immunofluorescence (Pomorski *et al.*, 2003). Thus, it is possible that the c-myc epitope tag induced an artificially high concentration of Neo1-myc in the ER of our strain.

Neo1p Depletion Causes Defects in Protein Transport and Golgi Glycosylation

A conditional allele of *NEO1* was produced by cloning this gene under transcriptional control of the *GAL1* promoter (Hua *et al.*, 2002) so that its expression could be controlled by carbon source (Johnston and Davis, 1984). The *neo1 Δ GAL::NEO1* strain grew well on inducing galactose media, but did not form visible colonies on glucose media where Neo1p expression was repressed. After initial growth in the presence of galactose, ~18 h were required for cell growth to slow down in YP glucose medium and ~24 h in synthetic minimal glucose medium. This represented ~10 generations for both media, which is typical for proteins that do not turn over rapidly.

To determine if Neo1p is involved in protein trafficking, cells depleted for Neo1p were pulse-labeled and chased to examine the kinetics of pro- α -factor processing and transport (Figure 2). The yeast α -factor mating pheromone is synthesized in the ER as a high-molecular-weight precursor and is further modified in the Golgi complex to produce the heterogeneously glycosylated pro- α -factor form that migrates as a high-molecular-mass smear by SDS-PAGE. This precursor is processed in the TGN through a series of proteolytic events, initiated by Kex2p, to produce the mature α -factor peptide, which is secreted (Fuller *et al.*, 1988).

The *neo1 Δ GAL::NEO1* strain was grown in SM galactose and shifted to SM glucose for 24 h to deplete Neo1p. Cells were then labeled with [³⁵S]methionine/cysteine for 15 min and chased for the time points indicated. In cells maintained

Figure 1. Neo1-Myc localizes to the ER and Golgi complex. (A) Strain ZHYNEO1-MYC was labeled with a mouse monoclonal anti-myc antibody. (B) The Neo1-Myc strain (ZHYNEO1-MYC) expressing HA-tagged *OCH1* (from pOH-URA3) was labeled with rabbit anti-HA antibody and mouse anti-myc to detect Och1-HA and Neo1-Myc. (C) Strain ZHYNEO1-MYC overexpressing Mnn1p (pRS426 MNN1) was stained with affinity-purified polyclonal rabbit anti-Mnn1p and mouse anti-myc. DNA was stained with DAPI to identify the position of the nucleus in all samples. For the overlay images, Neo1-Myc localization is shown in red, Och1-HA or Mnn1p is in green, and DNA is in blue.

in galactose, pro- α -factor was processed to the mature form very quickly (Figure 2, galactose), indicating that protein transport from the ER through the Golgi complex was efficient. However, for cells grown in glucose media, both the ER and Golgi forms of pro- α -factor were present through-

out the chase. In addition, the Golgi pro- α -factor forms appeared to be substantially underglycosylated because most of this precursor migrated just above the ER form (Figure 2, glucose). This result indicates that depletion of Neo1p causes defects in protein transport from the ER to the Golgi and potentially through the Golgi complex. Depletion of Neo1p also appeared to perturb Golgi-dependent glycosylation.

Figure 2. Neo1p depletion causes protein transport and glycosylation defects. ZHY9075E (*neo1 Δ GAL::NEO1*) was grown in galactose medium and subcultured in media containing either galactose or glucose media for 24 h. Galactose induces high-level expression of Neo1p and glucose shuts off expression from the *GAL::NEO1* construct. Cells were labeled with [35 S]methionine/cysteine for 15 min and then chased for the times indicated. α -Factor was recovered from each sample by immunoprecipitation and subjected to SDS-PAGE.

Isolation of *neo1-ts* Mutants

To facilitate studies of its essential function, we screened for temperature sensitive (*ts*) alleles of *NEO1*. The middle three fourths of the *NEO1* gene, including the ATPase domain and six flanking transmembrane domains, was targeted for mutagenesis (Figure 3 and MATERIALS AND METHODS). Among nearly 10,000 transformants screened, we found six colonies that grew well at 27°C but exhibited a tight *ts* growth defect. Three mutants could not form visible colonies at 34°C, and three could grow at 34°C but not 37°C (Figure 4, YPD). All six mutant alleles have been sequenced and each mutant contains from 6 to 16 missense mutations, with 10 mutations on average, spanning the targeted 810 amino acids (Figure 3). It does not appear that the P-type ATPase conserved motifs were more mutated than the other regions. Instead, it seems that the mutations clustered at the predicted transmembrane motifs, especially in transmembrane domain 3 (TMD3) where five of the six alleles carried mutations. Other clustered mutations included four residues that were mutated in two different alleles, and five sites where adjacent amino acids were mutated in the same or different alleles. The *neo1-5* allele only contains six mutations, and three of them overlap with mutations from other alleles, suggesting that mutations of A370, P491, and S542 likely contribute to the *ts* phenotype (Figure 3). Another interesting phenomenon is that the mutations tend to cluster even within one allele. For example, five of eight mutations

Figure 3. Mutations present in the six *neo1-ts* alleles. The *NEO1* wild-type sequence from amino acid 234–1043 subjected to mutagenesis is shown with corresponding residue numbers on top. Amino acid substitutions found in the six *neo1 ts* alleles are aligned underneath the corresponding wild-type residue. The superscript for each amino acid substitution indicates the *neo1* allele number from which it is derived (*neo1-1* to *neo1-6*). P-type ATPase conserved motifs are marked with asterisks. Predicted transmembrane domains three through seven are labeled as TMD3–7.

from *neo1-6* reside within 50 of the total of 810 amino acids targeted. Eight of nine mutations in *neo1-3* reside within the first third of this region (Figure 3). This phenomenon might reflect a destabilization of local secondary structures in the *ts* mutant proteins.

The *neo1-ts* mutants we isolated can still divide a few times after shifting to the nonpermissive temperature. The

neo1-ts mutants formed microcolonies containing a few to several dozens of cells when grown at 37°C and this varied depending on the allele. In addition, the growth rate of *neo1-ts* mutants in liquid media continued unperturbed for ~6 h when cells were shifted from 27 to 37°C. These data indicate that either all of the *neo1-ts* alleles are slowly inactivated after temperature shift, that they never lose complete

Figure 4. The growth defect of *neo1-ts* mutants is suppressed by osmotic support or nonfermentable carbon sources. Serial dilutions of the wild-type strain (BY4742) or *neo1Δ* carrying indicated alleles (expressing from plasmids) were spotted onto YPD plates, YPD supplemented with 1 or 1.5 M sorbitol, or YP with 3% glycerol as the carbon source. Plates were incubated at the indicated temperatures for two or three days before photographing.

Table 2. Growth profile of six *neo1-ts* mutants

	WT	<i>neo1-1</i>	<i>neo1-2</i>	<i>neo1-3</i>	<i>neo1-4</i>	<i>neo1-5</i>	<i>neo1-6</i>	<i>neo1Δ</i>
27°C Glucose	+++	+++	+++	+++	+++	+++	+++	—
34°C Glucose	+++	—	++	—	—	++	+	—
37°C Glucose	+++	—	—	—	—	—	—	—
27°C Galactose	+++	+++	+++	+++	+++	+++	+++	—
34°C Galactose	+++	+	+++	+	+	+++	++	—
37°C Galactose	+++	—	+	—	—	+	—	—
37°C 1 M sorbitol	+++	+	+++	++	++	+++	++	—
37°C 1.5 M sorbitol	+++	+++	+++	+++	+++	+++	+++	—
37°C 1 M NaCl	+++	+	+++	+	+	+++	++	—
27°C pH 8.0	+++	+++	+++	+++	+++	+++	+++	nd
500 μg/ml neomycin	+++	—	—	+	—	—	+++	nd
200 mM Ca ²⁺	+++	+++	+++	+++	+++	+++	+++	nd
500 mM Mg ²⁺	+++	+++	+++	+++	+++	+++	+++	nd
5 mM Zn ²⁺	+++	++	++	++	++	++	+++	nd
5 mM Mn ²⁺	+++	+++	++	+++	+++	+++	+++	nd
100 μM Co ²⁺	+++	+++	+++	+++	+++	+++	+++	nd
34°C Glycerol	+++	+++	+++	+++	+++	+++	+++	—
37°C Glycerol	++	++	+++	++	++	+++	++	—
34°C Ethanol	++	+	++	+	+	++	+	—
37°C Ethanol	+	+	++	+	+	++	+	—
20 μg/ml CW	+++	—	—	—	—	—	—	nd
60 mM NaF	+++	++	+	++	++	+++	+++	nd

Growth was examined on indicated plates at 27°C other than indicated temperature. Symbols indicate relative growth rate from wild-type (+++) to no growth (—). nd, not determined.

function at 37°C, or that cells can divide a few times in the absence of Neo1p function.

The *ts* Growth Defect of *neo1-ts* Mutants Is Suppressed by High Osmotic Support

The glycosylation defect observed by depleting Neo1p suggested that cell wall biosynthesis might be perturbed in *neo1* mutants. Mutants that grow poorly because of a defective cell wall can be remedied by sorbitol or other osmotic support and all six *neo1-ts* mutants can be rescued by osmotic support at the nonpermissive temperature. The growth of most mutants improved significantly on 1 M sorbitol, and they grew nearly as well as wild-type on 1.5 M sorbitol (Figure 4 and Table 2). In addition, 1 M NaCl also suppressed the *neo1 ts* growth defect (Table 2). The yeast cell wall consists of glucans, mannans, and chitin (Klis *et al.*, 2002). Many cell wall biosynthesis mutants are hypersensitive to the chitin-binding compound calcofluor white (CW). The *neo1-ts* mutants were also hypersensitive to CW (Table 2), further suggesting a cell wall defect.

Interestingly, nonfermentable carbon sources like glycerol or ethanol also supported *neo1-ts* growth at the nonpermissive temperature (Figure 4 and Table 2), and even galactose partially supported *neo1-ts* growth (Table 2). One possible explanation for these results is that cells grow more slowly on the nonglucose carbon source, and this might allow for more time to assemble a functional cell wall. However, it is also possible that in nonglucose carbon source media, other pathways are activated to suppress the *neo1-ts* mutant phenotype. It is noteworthy that neither the high osmotic support, nor the nonglucose carbon sources were able to suppress *neo1Δ* lethality (Table 2), indicating that Neo1p is not solely involved in cell wall synthesis. This also indicates that despite the heavy mutagenesis, all of the *neo1-ts* alleles retain some function at 37°C; otherwise these mutants would fail to grow on sorbitol.

Because *NEO1* confers resistance to neomycin when overexpressed, we tested the *neo1-ts* mutants for neomycin hypersensitivity. As predicted, most *neo1-ts* mutants are hypersensitive to neomycin (Table 2). Interestingly, *neo1-6* is not nearly as sensitive to neomycin as the other mutants. This suggests that the different *neo1 ts* alleles might affect different functions of Neo1p. Neo1p is homologous to Drs2p, and *drs2* has been shown to be hypersensitive to Mn²⁺, Ca²⁺, and other cations. Therefore, the *neo1-ts* mutants were also tested on the plates containing different cations at permissive temperature. The *neo1-ts* mutants are not significantly hypersensitive to any cation tested (Table 2). However, a few *neo1 ts* alleles did exhibit an increased sensitivity to NaF, an inhibitor of P-type ATPases (Table 2). Hypersensitivity to calcofluor white, neomycin, and NaF at the permissive growth temperature indicates that all of the *neo1-ts* alleles have partially lost function at 27°C. Consistent with this, we find that the *neo1-ts* cells grown at permissive temperature are enlarged and a small percentage of the cells are also elongated (Figure 5, A and B). This distinctive phenotype is not significantly aggravated when cells are shifted to the nonpermissive temperature. Interestingly, growth in 1.5 M sorbitol appeared to have partially suppressed the increased width of the cell, but severely exacerbated the hyperelongated morphology (Figure 5, A and B).

Protein Transport and Glycosylation Defects Exhibited by *neo1-ts*

Strains harboring a strong (*neo1-1*) or weak (*neo1-2*) *ts* allele of *NEO1* were examined for defects in protein transport and modification at the permissive and nonpermissive temperatures. Cells growing at 27°C, or shifted to 37°C for 1 or 3 h, were subjected to pulse-chase analysis of CPY and α -factor transport. CPY is synthesized in the ER as the p1 precursor form and is modified on N-linked oligosaccharides by Golgi mannosyltransferases to form the p2 precursor. p2 CPY is

Figure 5. *neo1-ts* cells are large. (A) Wild-type (BY4742) and *neo1-1* (ZHY628-15B) strains were grown at 27°C in YPD media or YPD media supplemented with 1.5 M sorbitol and imaged. (B) Fifty cells of wild-type and *neo1-1* strains grown at 27°C in YPD media or YPD sorbitol media were measured for length and width. A Student's *t* test indicated that the mutant cell sizes are significantly different from the wild-type ($p < 0.0001$).

sorted from secreted proteins in the late Golgi and is transported through a prevacuolar endosome to the vacuole where it is processed to the mature form (mCPY; Stevens *et al.*, 1982; Vida *et al.*, 1993).

At 27°C, the kinetics of transport and modification of CPY and α -factor in the *neo1* mutants was similar to wild-type cells, but a defect became apparent after incubation at 37°C (Figure 6). In wild-type cells preincubated at 37°C for 1 h, about half of the labeled CPY was in the p2 form at the beginning of the chase period (0 min), and most of the protein was converted to the mature form 10 min later. However, most of CPY was still in the ER form at 0 min in the *neo1* mutants and the p2 form of CPY was underglycosylated, causing a faster migration in the gel so that it could not be clearly separated from the p1 form (Figure 6). For the "tighter" *neo1-1* mutant, only half of CPY was converted to the mature form in 10 min. The transport and glycosylation defects became more apparent for both mutants after 3 h at 37°C.

After 1 h of incubation at the nonpermissive temperature, the transport and modification of pro- α -factor (immunoprecipitated from the same cell extracts as CPY) was surprisingly normal (Figure 6). A defect became apparent after a 3-h shift to the nonpermissive temperature in the *neo1* mutants, with more ER form accumulating and less mature form present at the beginning of the chase. The fully glyco-

sylated form of pro- α -factor, which can be seen in the 37°C 1-h panel (Figure 6, lanes 9 and 11), was replaced by underglycosylated precursor in the 3 h *neo1-ts* samples (Figure 6, lanes 15 and 17). These results are consistent with what was observed in the Neo1p depletion experiments and suggest that Neo1p is required for efficient protein transport from the ER to the Golgi and for synthesis of the outer chain on N-linked oligosaccharides in the Golgi complex.

neo1-ts Exhibits COPI Mutant Phenotypes

Proteins are transported from the ER to the Golgi complex in COPII-coated transport vesicles. Temperature-sensitive COPII mutants (e.g., *sec12* or *sec23*) exhibit a block in the ER-to-Golgi transport of all cargo proteins tested (Hicke and Schekman, 1989; Barlowe and Schekman, 1993). The differential effect of *neo1-ts* on CPY and α -factor transport noted above is more consistent with a defect in Golgi-to-ER retrograde transport mediated by COPI. COPI mutants are known to exhibit cargo-specific defects in anterograde transport that is presumably a secondary effect of perturbing the recycling of cargo receptors (Gaynor and Emr, 1997).

To test if *neo1* mutants exhibit a cargo-specific defect in protein transport, general secretion was examined in the *neo1 ts* mutants (Figure 7). Cells maintained at 27°C or shifted to 37°C for 2 or 3 h were labeled with [³⁵S]methionine/cysteine. Cells and medium were separated by centrifugation, and proteins secreted into the medium were precipitated with TCA and subjected to SDS-PAGE. At 27°C, the general secretion of proteins into the media was about the same between the wild-type strains (WT and *NEO1*) and *neo1* mutants. When cells were shifted to 37°C for 2 h, Hsp150, the top band on the gel migrating at about 150 kDa, was still secreted from both *neo1* mutants, whereas lower molecular weight proteins were missing (Figure 7, 2 h). This pattern of secreted proteins is similar to the COPI mutant phenotype, in which only a subset of secreted proteins were affected at the nonpermissive temperature (Gaynor and Emr, 1997). When cells were shift to 37°C for 3 h, Hsp150 secretion also decreased in *neo1-1* (Figure 7, 3 h), although *neo1-2* still exhibited the cargo-specific defect in secretion. We also noticed that Hsp150 from *neo1 ts* mutants labeled at the nonpermissive temperature migrated slightly faster in gels than that from the wild-type cells. This suggests that Golgi O-glycosylation is partially defective in *neo1*, because Hsp150 is extensively O-glycosylated with no N-linked oligosaccharides.

Rer1p is a protein that continuously cycles between the early Golgi complex and the ER, and is a sensitive marker to distinguish whether a mutant exhibits a primary defect in anterograde or retrograde transport (Sato *et al.*, 2001). Sato *et al.* (2001) found that Rer1-GFP is primarily localized to the Golgi in wild-type cells, but is trapped in the ER in COPII mutants. To test if Neo1p is involved in anterograde or retrograde transport between the ER and Golgi complex, Rer1-GFP was expressed in *neo1-ts* cells. At the permissive temperature, Rer1-GFP was mainly localized in punctate structures in both wild-type and *neo1-1* cells, although a slightly more diffuse localization was noted for the *neo1-1* cells (Figure 8, 27°C). After cells were shifted to nonpermissive temperature for 2 h, a significant amount of Rer1-GFP localized to vacuoles in *neo1-1* cells (Figure 8, 37°C). Wild-type cells also exhibited more Rer1-GFP in the vacuole at 37°C, but not to the extent observed in *neo1-1* cells. No ER accumulation of Rer1-GFP was found in the *neo1-1* mutant. This result suggests that *neo1* does not directly perturb the COPII-depen-

Figure 6. *neo1-ts* exhibits Golgi glycosylation and protein transport defects at the nonpermissive temperature. Wild-type (BY4742) and two *neo1-ts* strains (ZHY628–15B and ZHY628–34A) were grown at 27°C, and then shifted to 37°C for 1 or 3 h. Cells were then labeled with [³⁵S]methionine/cysteine for 6 min, and chased for 0 and 10 min. CPY and α-factor were immunoprecipitated from cells collected at each chase time and subjected to SDS-PAGE.

dent anterograde pathway but does perturb a COPI-dependent retrograde transport pathway. Consistent with these results, we also find that *neo1-ts* cells secrete more Kar2p (an

ER resident protein bearing the HDEL retrieval signal) than wild-type yeast (our unpublished results).

Mutations affecting the same pathway will often show genetic interactions and so double mutants were constructed by crossing *neo1-1* with several different mutants with de-

Figure 7. General secretion defect in *neo1-ts*. Wild-type (BY4742) and *neo1Δ* harboring either *NEO1* (ZHY219RR), *neo1-1* (ZHY628–15B), or *neo1-2* (ZHY628–34A) were grown at 27°C, and part of each culture was shifted to 37°C for 2 or 3 h. Cells were then labeled with [³⁵S]methionine/cysteine for 10 min and then chased for 30 min. Cells and media were separated by centrifugation, and the proteins in the media were precipitated with TCA and subjected to SDS-PAGE.

Figure 8. Rer1-GFP is mislocalized to the vacuole of *neo1-1* cells. Wild-type (BY4742) and *neo1-1* (ZHY628–15B) harboring an Rer1-GFP plasmid were grown at 27°C, and half of each culture was shifted to 37°C for 2 h. Fluorescent images were captured and representative cells are shown. Cells with large vacuoles were chosen to better distinguish vacuolar fluorescence from the Golgi signal. No ER localization was observed in any of the cells.

Figure 9. Genetic interactions between *neo1-1* and *drs2* or COPI mutations. (A) Summary of double mutant phenotypes. Strains harboring the alleles indicated were crossed with integrated *neo1-1* or *neo1-2* mutant, sporulated, and subjected to tetrad dissection. Double mutants harboring *neo1-1* and the alleles listed in the viable column were recovered at the expected frequency and showed a ts growth profile comparable to one of the two parents. In contrast, most *neo1-1 drs2Δ* or *neo1-2 drs2Δ* double mutants were inviable, and the *neo1-1 sec21-1* and *neo1-1 ret1-1* strains were more temperature sensitive than either parent (Lethal or sick column). (B) Synthetic lethality between *neo1-2* and *drs2Δ*. Dissected tetrads from the *neo1-2/drs2Δ* cross are shown and the missing colonies in rows labeled "T" (tetratype) were predicted to be double mutants. "P" is a parental ditype with two pairs of single mutant progeny. (C) Growth profile of *neo1-1 sec21-1* compared with both parental strains at 30°C, a semipermissive temperature for *neo1-1*.

fects in protein transport or glycosylation (Figure 9A). The viable double mutants were then tested for growth at several different temperatures. No interaction was observed between *neo1-1* and the COPII alleles (*sec12-4*, *sec23-1*), but the *neo1* COPI (*sec21-1*, *ret1-1*) double mutants grew slower on the tetrad dissection plates and showed an increase in temperature sensitivity relative to the single mutants (Figure 9C and our unpublished results). The strongest genetic interaction was observed between *neo1-1* and *drs2Δ*. Most of the double mutants produced from this cross failed to grow (Figure 9B), and the few that did survive grew extremely slowly. Although the *drs2 neo1* synthetic lethality may be caused by the combined defects in clathrin and COPI pathways, it more likely reflects a common biochemical function for Neo1p and Drs2p in the Golgi complex. These proteins are 30% identical in amino acid sequence and are both in the APT family of P-type ATPases. No significant genetic interaction was observed between *neo1-1* and *arf1Δ*, ARF-GAPs (*gcs1Δ*, *glo3Δ*), or an ARF-GEF (*sec7-4*), which was surprising considering that both *drs2 arf1* and *drs2 neo1-ts* are synthetically lethal. In addition, no genetic interaction was observed with mutations that perturb later steps in protein transport (*sec14-3*, *sec1-1*) or glycosylation (*gda1Δ*, *mn9Δ*), or deletions of other APT family members (*dnf1Δ*, *dnf2Δ*; Figure 9A). Thus, although the genetic interaction found between *neo1-1* and COPI alleles was modest, it was specific and supported a role for Neo1p in COPI function.

The anterograde protein transport defect appeared more severe for cells depleted for Neo1p compared with the experiments using *neo1-ts* mutants. We suspected that the different temperatures used to assay protein transport might have contributed to these differences. Therefore, we labeled Neo1-depleted cells at 37°C to examine CPY and α -factor

Figure 10. Neo1p depletion causes a more severe protein transport defect at lower temperature. Wild-type and *neo1 GAL::NEO1* strains were grown in galactose and then shifted to glucose media for 24 h at 30°C. For labeling at 37°C, half of the cultures cells were shifted to 37°C for the last 2.5 h before labeling. Cells were then labeled with [³⁵S]methionine/cysteine for 8 min and chased for 0, 8, and 16 min. CPY and α -factor was immunoprecipitated from cells collected at each chase time and subjected to SDS-PAGE.

transport at the higher temperature. CPY transport was approximately twofold faster for both wild-type and Neo1p-depleted cells at 37°C (compared with 30°C) but the transport defect was still apparent in the depleted cells (Figure 10, CPY). In contrast to CPY, the protein transport defect was significantly alleviated at 37°C for pro- α -factor (Figure 10, 37°C). More ER form of pro- α -factor was present at the beginning of the chase (time 0) in the Neo1-depleted cells than in wild-type, but this quickly chased to the mature form. These results are very similar to those obtained with the *neo1-ts* strains at 37°C and suggest that there is a greater demand for Neo1p function in recycling cargo receptors at lower temperatures.

The *neo1-1* Mutant Accumulates Abnormal Membrane Structures

Because the *neo1* mutants exhibited significant defects in the secretory pathway, we examined these cells by electron microscopy (EM) to see if any organelle membrane or intermediate transport vesicles accumulate in this mutant. The appearance of *neo1-1* cells grown at 27 and 37°C were quite different (Figure 11). At the permissive temperature, the *neo1-1* mutant accumulated small vesicles (40.9 ± 9.2 nm in diameter) and lipid droplets (Figure 11, A and B) compared with wild-type cells. Vacuoles were also fragmented, which was confirmed by staining living cells with FM4-64 or CDC-FDA (our unpublished results). The fragmented vacuole phenotype was not completely penetrant, and cells with normal vacuoles could easily be found (see Figure 8). When *neo1* was shifted to the nonpermissive temperature for 3 h, the number of small vesicles was substantially reduced, and several kinds of abnormal membrane structures were found to accumulate. Some cell sections contained multiple vacuole-like structures, although they did not stain darkly as do

Figure 11. *neo1-ts* cells accumulate abnormal membrane structures. (A and B) *neo1-1* (ZHY628-15B) was grown at the permissive temperature of 27°C and processed for electron microscopy. (C) Wild-type cells (BY4742) grown at 37°C for 3 h. Wild-type cells grown at 27°C were indistinguishable and are not shown. (D–F) *neo1-1* grown at 37°C for 3 h. Arrows indicate small vesicles in A and B, poorly stained fragmented vacuoles in D, abnormal Golgi membranes in E, and enlarged ER membrane in F.

vacuoles in wild-type cells (Figure 11D, arrows). Other sections showed an accumulation of abnormal membrane structures that are likely enlarged Golgi cisternae (Figure 11E, arrows), and continuously looped membrane sheets, which are likely an expanded ER (Figure 11F, arrow). The accumulation of different abnormal membrane structures in *neo1* indicates a defect in membrane trafficking through the secretory pathway.

DISCUSSION

Neo1p is an integral membrane P-type ATPase and potential APT. Other members of the yeast APT family (Drs2p and Dnf1, 2, and 3) have been implicated in protein transport in the late secretory and endosomal/vacuolar pathways. In this study, we present several lines of evidence implicating Neo1p in protein transport and modification in the early secretory pathway. 1) Neo1-myc localizes to both ER and Golgi compartments. 2) Neo1p depletion at 30°C causes a strong defect in CPY and pro- α -factor transport from the ER to the Golgi and potentially through the Golgi complex. 3) Golgi-dependent modification of N-linked oligosaccharides on CPY and pro- α -factor is perturbed upon Neo1p depletion. 4) The *neo1-ts* mutations cause these same protein transport and glycosylation defects at the nonpermissive temperature, albeit less severely, and seem to preferentially perturb CPY exit from the ER. 5) The *neo1-ts* mutants exhibit a block in the secretion of a subset of proteins, whereas others, such as Hsp150, are secreted efficiently. 6) Rer1-GFP, a protein that continuously cycles between the ER and Golgi, is mislocalized to the vacuole in *neo1-ts*. 7) The *neo1-ts* mutants accumulate small vesicles at the permissive temperature and accumulate ER and Golgi membranes at the nonpermissive temperature.

The *neo1* mutants exhibit several phenotypes in common with COPI mutants. These include a cargo-specific defect in secretion, aberrant glycosylation of cargo in the Golgi, and mislocalization of Rer1-GFP to the vacuole. Rer1p is an integral membrane protein required for retrograde transport of other membrane proteins to the ER. It has a C-terminal dilysine-like motif that binds COPI and links other cargo (Sec12p or Sec71p) to COPI via transmembrane domain interactions (Sato *et al.*, 1997, 2001). Thus, Rer1p appears to be a cargo receptor for proteins retrieved from the Golgi to the ER in COPI vesicles and must continuously shuttle between the ER and Golgi to perform this task. COPII mutants accumulate Rer1-GFP in the ER and COPI mutants mislocalize Rer1-GFP to the vacuole (Sato *et al.*, 2001). The mislocalization of Rer1-GFP to the vacuole of *neo1-ts* suggests that these cells have a primary defect in retrograde rather than anterograde transport. In addition, the *sec21* (γ -COPI) mutant perturbs anterograde transport of a subset of secreted proteins and the spectrum of affected and unaffected proteins is similar in the *neo1* mutants (Gaynor and Emr, 1997). This is likely caused by a failure to recycle cargo receptors needed for anterograde transport of some proteins.

Although all COPI mutants appear to exhibit a defect in retrograde transport, not all display a defect in anterograde protein transport (Cosson *et al.*, 1996; Duden *et al.*, 1998). Thus, the modest anterograde transport defect observed in *neo1-ts* at 37°C does not indicate that the retrograde defect is marginal. In fact, the Neo1p requirement for anterograde transport appears less critical at higher temperatures because depletion of Neo1p at 30°C produced a stronger pro- α -factor block than depletion at 37°C. Moreover, it is clear that none of the *neo1-ts* alleles cause a complete loss of

function at the nonpermissive temperature. The *ts* growth defect of all of the *neo1-ts* mutants can be suppressed by osmotic support, but this treatment does not allow *neo1 Δ* cells to grow at any temperature tested. Therefore, the protein trafficking defects observed in *neo1-ts* cells was caused by a partial loss of Neo1p function. Neo1p depletion caused a stronger defect in anterograde protein transport and even these experiments may not represent a complete loss of function phenotype. These cells were labeled at a time of depletion where a growth defect first became apparent, rather than when growth stopped. This was done in an attempt to define the immediate consequences of diminished Neo1p function. Likewise, *neo1-ts* cells were examined 1–3 h after temperature shift instead of the 6 h required to completely inhibit growth.

Surprisingly, *neo1-ts* cells accumulated a large number of small vesicles (30–50 nm) at the permissive temperature, where it is clear from the cell morphology and hypersensitivity to various chemicals that Neo1p is partially defective. This same vesicle accumulation phenotype is observed when *sec21* is incubated at a semipermissive temperature (Rambourg *et al.*, 1994). These vesicles are likely in transit between the ER and Golgi, but whether they are COPI or COPII vesicles is not known. Most of these vesicles disappear when *neo1-ts* is shifted to 37°C and ER and Golgi membranes accumulate. COPI mutants also accumulate ER at the nonpermissive temperature but the Golgi accumulation appears to be unique to *neo1*. This difference may reflect the hypomorphic nature of the *neo1-ts* alleles or that *neo1* perturbs additional transport steps through, or from, the Golgi complex. However, it is important to note that *neo1* does not appear to perturb COPII function and at least some proteins (Hsp150) are secreted normally. In addition, the CPY that escapes the ER-to-Golgi block in *neo1* is sorted to the vacuole. Thus, a number of transport steps are occurring normally in *neo1* and the defect appears rather specific to the Golgi-to-ER retrograde pathway.

Both COPI and *neo1* mutations perturb glycosylation in the Golgi complex. For *neo1-ts* strains, the glycosylation defect appears to be the primary cause of the 37°C growth defect. This growth defect is sorbitol-remedial and the *neo1-ts* mutants are hypersensitive to calcofluor white. These phenotypes suggest an abnormal cell wall and are consistent with the Golgi-dependent glycosylation defects observed for CPY and α -factor. How *neo1* mutants perturb the function of Golgi glycosyltransferases is not known. However, localization of an α -1,6-mannosyltransferase (Och1p) that initiates outer chain elongation is perturbed in *sec21*. Rather than being mislocalized to the vacuole, Och1p changes from a punctate distribution by immunofluorescence to a dispersed granular appearance that might reflect residence in small vesicles or Golgi fragments (Gaynor and Emr, 1997). In addition, two different α -1,6-mannosyltransferase complexes containing Mnn9p, which elongate the outer chain on N-linked oligosaccharides, have been shown to cycle between the ER and Golgi complex. Like Rer1p, these proteins are mislocalized to the vacuole when retrograde transport is perturbed (Todorow *et al.*, 2000). Therefore, it is possible that a reduced ability to form COPI vesicles in *neo1* causes the glycosylation defect by mislocalizing α -1,6-mannosyltransferases. The fact that *neo1 Δ* lethality cannot be rescued by osmotic support indicates that Neo1p has an essential function independent of its role in glycosylation and cell wall synthesis. This essential Neo1p function appears to be in COPI-dependent Golgi-to-ER retrograde protein transport, an essential process in yeast.

Figure 12. Model for the role of Neo1p in retrograde transport. (A) In wild-type cells, Neo1p probably establishes phospholipid asymmetry in early Golgi membranes (exhibited as light and dark shades of the bilayer membrane), and COPI vesicles form normally. (B) When Neo1p is not functional, lipid asymmetry is lost and the efficiency of COPI vesicle formation is greatly reduced.

How do potential APTs influence protein transport in the secretory pathway? Drs2p appears to play a direct role in forming secretory vesicles from Golgi membranes because inactivation of Drs2 or clathrin ts proteins causes a rapid loss of a specific class of exocytic vesicles (Gall *et al.*, 2002). Drs2p also interacts directly with Gea2p, an ARF guanine nucleotide exchange factor, physically coupling Drs2p to vesicle budding machinery (Chantalat *et al.*, 2004). The data presented in this work implicate Neo1p in COPI vesicle budding from early Golgi membranes, but whether this is a direct or indirect requirement remains to be determined. The onset of defects in transport is rather slow after shifting the *neo1-ts* mutants to the nonpermissive temperature. This could be explained by the hypomorphic nature of the *neo1-ts* alleles, but it could also indicate an indirect requirement for Neo1p in COPI vesicle formation. One model to explain the requirement for Neo1p is that an asymmetric distribution of phospholipids in the *cis*-Golgi is required for optimal COPI vesicle formation. ER and Golgi membranes in mammalian cells have been suggested to be asymmetric (Bollen and Higgins, 1980; Higgins, 1984). Neo1p might establish this phospholipid asymmetry, but it slowly decays after shifting the *neo1-ts* mutants to the nonpermissive temperature. As the membrane becomes more symmetric, the ability of ARF and COPI to form retrograde vesicles would diminish (Figure 12). It is also possible that Neo1p plays a more direct role in this process by interacting with components of the vesicle budding machinery, or by facilitating outward bending of the membrane during vesicle formation.

We have recently found that late Golgi membranes purified from a *drs2-ts* mutant display a ts defect in translocating NBD-PS from the luminal to the cytosolic leaflet. The *drs2* mutants also expose more PS on the outer leaflet of the plasma membrane than do wild-type cells (our unpublished

results). These data strongly support the proposed APT activity for Drs2p. It is possible that both Neo1p and Drs2p control lipid asymmetry in the Golgi complex, with Neo1p acting in the *cis* compartments and Drs2p in the *trans*. In this case, loss of both proteins would severely perturb the trans-bilayer lipid distribution in the entire Golgi complex, perhaps explaining the synthetic lethality between *drs2Δ* and *neo1-1*.

COPI and clathrin-coated vesicles have been generated from synthetic liposomes using only cytosolic coat components (Spang and Schekman, 1998; Takei *et al.*, 1998), suggesting that coat components alone are sufficient to drive vesicle formation without contribution from membrane proteins. However, these studies could not determine if vesicle formation occurred in vitro with the same rate and efficacy required in vivo. We have found that Neo1p, an essential P-type ATPase and integral membrane protein, is required for efficient Golgi-to-ER protein transport. All five members of the Drs2/Neo1 family proteins have now been implicated in protein trafficking at different steps in the secretory or endocytic pathways (Chen *et al.*, 1999; Gall *et al.*, 2002; Hua *et al.*, 2002; Pomorski *et al.*, 2003). The Drs2/Neo1 proteins appear to be APTs (Tang *et al.*, 1996; Gomes *et al.*, 2000; Pomorski *et al.*, 2003), suggesting that the regulation of membrane phospholipid asymmetry plays an important role in the formation of coated transport vesicles.

ACKNOWLEDGMENTS

We thank Ken Sato, Akihiko Nakano, Gerald Waters, and Scott Emr for plasmids, strains, and antibodies. We also thank Sophie Chen from the Graham laboratory for help in strain and media preparation during the course of these experiments. This work was supported by National Institutes of Health Grant GM-62367 (to T.R.G.).

REFERENCES

- Auland, M.E., Roufogalis, B.D., Devaux, P.F., and Zachowski, A. (1994). Reconstitution of ATP-dependent aminophospholipid translocation in proteoliposomes. *Proc. Natl. Acad. Sci. USA* 91, 10938–10942.
- Balasubramanian, K., and Schroit, A.J. (2003). Aminophospholipid asymmetry: a matter of life and death. *Annu. Rev. Physiol.* 65, 701–734.
- Bankaitis, V.A., Malehorn, D.E., Emr, S.D., and Greene, R. (1989). The *Saccharomyces cerevisiae* SEC14 gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. *J. Cell Biol.* 108, 1271–1281.
- Barlowe, C. (2002). COPII-dependent transport from the endoplasmic reticulum. *Curr. Opin. Cell Biol.* 14, 417–422.
- Barlowe, C., and Schekman, R. (1993). SEC12 encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. *Nature* 365, 347–349.
- Bollen, I.C., and Higgins, J.A. (1980). Phospholipid asymmetry in rough- and smooth-endoplasmic-reticulum membranes of untreated and phenobarbital-treated rat liver. *Biochem. J.* 189, 475–480.
- Brigance, W.T., Barlowe, C., and Graham, T.R. (2000). Organization of the yeast Golgi complex into at least four functionally distinct compartments. *Mol. Biol. Cell* 11, 171–182.
- Catty, P., de Kerchove d'Exaerde, A., and Goffeau, A. (1997). The complete inventory of the yeast *Saccharomyces cerevisiae* P-type transport ATPases. *FEBS Lett.* 409, 325–332.
- Chantalat, S., Park, S.-K., Hua, Z., Liu, K., Gobin, R., Peyroche, A., Rambourg, A., Graham, T.R., and Jackson, C.L. (2004). The Arf activator Gea2p and the P-type ATPase Drs2p interact at the Golgi in *Saccharomyces cerevisiae*. *J. Cell Sci.* (in press).
- Chen, C.Y., Ingram, M.F., Rosal, P.H., and Graham, T.R. (1999). Role for Drs2p, a P-type ATPase and potential aminophospholipid translocase, in yeast late Golgi function. *J. Cell Biol.* 147, 1223–1236.

- Cosson, P., Demolliere, C., Hennecke, S., Duden, R., and Letourneur, F. (1996). Delta- and zeta-COP, two coatomer subunits homologous to clathrin-associated proteins, are involved in ER retrieval. *EMBO J.* 15, 1792–1798.
- Dhar, M., Webb, L.S., Smith, L., Hauser, L., Johnson, D., and West, D.B. (2000). A novel ATPase on mouse chromosome 7 is a candidate gene for increased body fat. *Physiol. Genomics* 4, 93–100.
- Duden, R., Kajikawa, L., Wuestehube, L., and Schekman, R. (1998). epsilon-COP is a structural component of coatomer that functions to stabilize alpha-COP. *EMBO J.* 17, 985–995.
- Franzusoff, A., and Schekman, R. (1989). Functional compartments of the yeast Golgi apparatus are defined by the sec7 mutation. *EMBO J.* 8, 2695–2702.
- Fuller, R.S., Sterne, R.E., and Thorner, J. (1988). Enzymes required for yeast prohormone processing. *Annu. Rev. Physiol.* 50, 345–362.
- Gall, W.E., Geething, N.C., Hua, Z., Ingram, M.F., Liu, K., Chen, S.I., and Graham, T.R. (2002). Drs2p-dependent formation of exocytic clathrin-coated vesicles in vivo. *Curr. Biol.* 12, 1623–1627.
- Gaynor, E.C., Chen, C.Y., Emr, S.D., and Graham, T.R. (1998). ARF is required for maintenance of yeast Golgi and endosome structure and function. *Mol. Biol. Cell* 9, 653–670.
- Gaynor, E.C., and Emr, S.D. (1997). COPI-independent anterograde transport: cargo-selective ER to Golgi protein transport in yeast COPI mutants. *J. Cell Biol.* 136, 789–802.
- Gomes, E., Jakobsen, M.K., Axelsen, K.B., Geisler, M., and Palmgren, M.G. (2000). Chilling tolerance in Arabidopsis involves ALA1, a member of a new family of putative aminophospholipid translocases. *Plant Cell* 12, 2441–2454.
- Graham, T.R., and Emr, S.D. (1991). Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast sec18 (NSF) mutant. *J. Cell Biol.* 114, 207–218.
- Graham, T.R., Seeger, M., Payne, G.S., MacKay, V.L., and Emr, S.D. (1994). Clathrin-dependent localization of alpha 1, 3 mannosyltransferase to the Golgi complex of *Saccharomyces cerevisiae*. *J. Cell Biol.* 127, 667–678.
- Harris, S.L., and Waters, M.G. (1996). Localization of a yeast early Golgi mannosyltransferase, Och1p, involves retrograde transport. *J. Cell Biol.* 132, 985–998.
- Herzing, L.B., Kim, S.J., Cook, E.H., Jr., and Ledbetter, D.H. (2001). The human aminophospholipid-transporting ATPase gene ATP10C maps adjacent to UBE3A and exhibits similar imprinted expression. *Am. J. Hum. Genet.* 68, 1501–1505.
- Hicke, L., and Schekman, R. (1989). Yeast Sec23p acts in the cytoplasm to promote protein transport from the endoplasmic reticulum to the Golgi complex in vivo and in vitro. *EMBO J.* 8, 1677–1684.
- Higgins, J.A. (1984). The transverse distribution of phospholipids in the membranes of Golgi subfractions of rat hepatocytes. *Biochem. J.* 219, 261–272.
- Hua, Z., Fatheddin, P., and Graham, T.R. (2002). An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. *Mol. Biol. Cell* 13, 3162–3177.
- Johnston, M., and Davis, R.W. (1984). Sequences that regulate the divergent GAL1-GAL10 promoter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 4, 1440–1448.
- Kirchhausen, T. (2000). Three ways to make a vesicle. *Nat. Rev. Mol. Cell. Biol.* 1, 187–198.
- Klionsky, D.J., Banta, L.M., and Emr, S.D. (1988). Intracellular sorting and processing of a yeast vacuolar hydrolase: proteinase A propeptide contains vacuolar targeting information. *Mol. Cell. Biol.* 8, 2105–2116.
- Klis, F.M., Mol, P., Hellingwerf, K., and Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 26, 239–256.
- Marx, U., Polakowski, T., Pomorski, T., Lang, C., Nelson, H., Nelson, N., and Herrmann, A. (1999). Rapid transbilayer movement of fluorescent phospholipid analogues in the plasma membrane of endocytosis-deficient yeast cells does not require the Drs2 protein. *Eur. J. Biochem* 263, 254–263.
- Meguro, M., Kashiwagi, A., Mitsuya, K., Nakao, M., Kondo, I., Saitoh, S., and Oshimura, M. (2001). A novel maternally expressed gene, ATP10C, encodes a putative aminophospholipid translocase associated with Angelman syndrome. *Nat. Genet.* 28, 19–20.
- Pomorski, T., Lombardi, R., Riezman, H., Devaux, P.F., Van Meer, G., and Holthuis, J.C. (2003). Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. *Mol. Biol. Cell* 14, 1240–1254.
- Poon, P.P., Cassel, D., Spang, A., Rotman, M., Pick, E., Singer, R.A., and Johnston, G.C. (1999). Retrograde transport from the yeast Golgi is mediated by two ARF GAP proteins with overlapping function. *EMBO J.* 18, 555–564.
- Prezant, T.R., Chaltrow, W.E., Jr., and Fischel-Ghodsian, N. (1996). Identification of an overexpressed yeast gene which prevents aminoglycoside toxicity. *Microbiology* 142, 3407–3414.
- Rambourg, A., Clermont, Y., Jackson, C.L., and Kepes, F. (1994). Ultrastructural modifications of vesicular and Golgi elements in the *Saccharomyces cerevisiae* sec21 mutant at permissive and non-permissive temperatures. *Anat. Rec.* 240, 32–41.
- Rieder, S.E., Banta, L.M., Kohrer, K., McCaffery, J.M., and Emr, S.D. (1996). Multilamellar endosome-like compartment accumulates in the yeast vps28 vacuolar protein sorting mutant. *Mol. Biol. Cell* 7, 985–999.
- Robinson, J.S., Klionsky, D.J., Banta, L.M., and Emr, S.D. (1988). Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* 8, 4936–4948.
- Sato, K., Sato, M., and Nakano, A. (1997). Rer1p as common machinery for the endoplasmic reticulum localization of membrane proteins. *Proc. Natl. Acad. Sci. USA* 94, 9693–9698.
- Sato, K., Sato, M., and Nakano, A. (2001). Rer1p, a retrieval receptor for endoplasmic reticulum membrane proteins, is dynamically localized to the Golgi apparatus by coatomer. *J. Cell Biol.* 152, 935–944.
- Seigneuret, M., and Devaux, P.F. (1984). ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proc. Natl. Acad. Sci. USA* 81, 3751–3755.
- Siegmund, A., Grant, A., Angeletti, C., Malone, L., Nichols, J.W., and Rudolph, H.K. (1998). Loss of Drs2p does not abolish transfer of fluorescence-labeled phospholipids across the plasma membrane of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 273, 34399–34405.
- Spang, A., and Schekman, R. (1998). Reconstitution of retrograde transport from the Golgi to the ER in vitro. *J. Cell Biol.* 143, 589–599.
- Stevens, T., Esmon, B., and Schekman, R. (1982). Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell* 30, 439–448.
- Takei, K., Haucke, V., Slepnev, V., Farsad, K., Salazar, M., Chen, H., and De Camilli, P. (1998). Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes. *Cell* 94, 131–141.
- Tang, X., Halleck, M.S., Schlegel, R.A., and Williamson, P. (1996). A subfamily of P-type ATPases with aminophospholipid transporting activity [published erratum appears in *Science* 1996 Dec 6;274(5293):1597]. *Science* 272, 1495–1497.
- Thompson, R., and Jansen, P.L. (2000). Genetic defects in hepatocanalicular transport. *Semin. Liver Dis.* 20, 365–372.
- Todorow, Z., Spang, A., Carmack, E., Yates, J., and Schekman, R. (2000). Active recycling of yeast Golgi mannosyltransferase complexes through the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* 97, 13643–13648.
- Vida, T.A., Huyer, G., and Emr, S.D. (1993). Yeast vacuolar proenzymes are sorted in the late Golgi complex and transported to the vacuole via a pre-vacuolar endosome-like compartment. *J. Cell Biol.* 121, 1245–1256.
- Zachowski, A., Henry, J.P., and Devaux, P.F. (1989). Control of transmembrane lipid asymmetry in chromaffin granules by an ATP-dependent protein. *Nature* 340, 75–76.