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The Newly Identified Yeast *GRD* Genes Are Required for Retention of Late-Golgi Membrane Proteins

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Processing of A-ALP, a late-Golgi membrane protein constructed by fusing the cytosolic domain of dipeptidyl aminopeptidase A to the transmembrane and lumenal domains of alkaline phosphatase (ALP), serves as a convenient assay for loss of retention of late-Golgi membrane proteins in *Saccharomyces cerevisiae*. In this study, a large group of novel *grd* (for Golgi retention defective) yeast mutants, representing 18 complementation groups, were identified on the basis of their mislocalization of A-ALP to the vacuole, where it was proteolytically processed and thus became enzymatically activated. All of the *grd* mutants exhibited significant mislocalization of A-ALP, as measured by determining the kinetics of A-ALP processing and by analyzing its localization by indirect immunofluorescence microscopy. The mutants were evaluated in a variety of other phenotypic tests relevant to yeast Golgi function, including processing of the α-factor mating pheromone, sorting of the vacuolar hydrolase carboxypeptidase Y, and retention of an early-Golgi membrane protein. Mutants from three *grd* complementation groups also failed to retain an early-Golgi membrane protein, suggesting that these mutations may have more global effects on Golgi retention and function. However, the majority of the *grd* mutants appeared to be defective specifically for the retention of several late-Golgi membrane proteins. A subset of the *grd* mutants appeared defective only in retention of A-ALP and not other late-Golgi membrane proteins. The *grd* mutants define a new set of genes required for Golgi membrane protein retention in *S. cerevisiae*.

The secretory pathway is composed of many membrane-enclosed organelles, each with a unique composition of proteins and other cellular components (42). These compartments contain both transient proteins bound for other destinations and resident proteins essential for organelle function. A secretory pathway organelle retains its resident proteins while allowing high levels of nonresident proteins to pass through because there are specific retention signals on the resident proteins (23, 30, 34). For example, the retention of proteins in the lumen of the endoplasmic reticulum (ER) depends on a four-amino-acid retention signal (HDEL and KDEL in yeast and animal cells, respectively) present at the C terminus of resident ER lumenal proteins (33). Genetic approaches in *Saccharomyces cerevisiae* led to the identification of a receptor that binds the HDEL signal and is required for retention of ER lumenal proteins (22, 44). The HDEL receptor has been shown to function by retrieving ER proteins from a post-ER compartment back to the ER (21, 50). In principle, retention in a given organelle could occur by either a retrieval mechanism or a static retention mechanism or both.

Mechanisms of membrane protein retention within the Golgi apparatus appear to vary between subcompartments. In both yeast and animal cells, retention of membrane proteins in the early and medial regions of the Golgi apparatus is dependent on determinants within (and/or immediately flanking) the transmembrane domain (23). Models put forward to explain how transmembrane domains mediate retention include (i) a specific interaction of the transmembrane domain with the lipid bilayer (6, 34) and (ii) a role for transmembrane domains in oligomerization, resulting in aggregates that prevent further anterograde transport (27, 52).

In contrast, resident membrane proteins of the yeast late-Golgi compartment and the *trans*-Golgi network (TGN) of animal cells are retained via signals present in their cytosolic domains (3, 8, 29, 46, 53). In *S. cerevisiae* the three late-Golgi membrane proteins characterized to date (dipeptidyl aminopeptidase A [DPAP A], Kex1p, and Kex2p) are all single-membrane-spanning integral membrane proteins with cytosolic domains of around 100 amino acids. Aromatic residues have been shown to be involved in the retention of both DPAP A and Kex2p (30). Surprisingly, retention-defective forms of membrane proteins of the yeast late-Golgi compartment were found to be transported directly to the vacuole (40, 53). Site-directed mutagenesis experiments identified a sequence, FXFXD, in the cytosolic domain of DPAP A that is essential for its late-Golgi retention; the two phenylalanine residues were most important for the function of this sequence (29). Similarly, Kex2p contains a critical aromatic residue (Tyr-713) in its cytosolic domain that is absolutely required for its retention in the late-Golgi compartment (53). The DPAP A and Kex2p retention signals are similar to a general motif for sorting into clathrin-coated pits of animal cells (51). This type of sorting motif is also found in the mannose 6-phosphate receptor, an integral membrane protein that cycles between the TGN, prelysosome, and plasma membrane (4). Since clathrin coats have been shown to be involved in receptor mediated transport at both the plasma membrane and the TGN, it is conceivable that yeast late-Golgi retention signals may function by interaction with clathrin coats.

While amino acid motifs necessary for retention of membrane proteins in the late-Golgi apparatus have been well characterized, little is known about the proteins that recognize these signals and effect the actual retention. At present, two

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference
pSN55	*STE13-PHO8* gene fusion in pRS316	29
pSN246	*STE13-PHO8* gene fusion in pRS315	This study
pSN269	*pep8*Δ::*LEU2* in Bluescript KS$^+$	This study
pSN273	*pep4*Δ::*LEU2* in Bluescript KS$^+$	This study
pSN274	Construct for expression of maltose-binding protein–ALP fusion protein antigen	This study
pSN275	Construct for expression of glutathione *S*-transferase–ALP fusion protein antigen in pGEX-5X-3	This study
pSN280	*MNT1-PHO8* gene fusion in pRS314	This study
pKJH2	*vps27*Δ::*LEU2* in pUC19	37
pSL1572	*ren1*::*LEU2* in Bluescript KS$^+$	11
pTS18	*PEP4* gene in YCp50	1

genes have been reported as being necessary for late-Golgi retention of both DPAP A and Kex2p: the clathrin heavy-chain gene, *CHC1* (43), and *VPS1*, which encodes a 80-kDa GTPase (28, 54). More recently, Graham et al. (14) have shown that clathrin heavy chain is also required for retention of Mnn1p, a type II membrane protein localized to a medial-Golgi compartment by a mechanism independent of its cytosolic domain. These results indicate that Chc1p plays a general role in Golgi retention that is not limited to membrane proteins with cytosolic retention signals. Both Chc1p and Vps1p have either mammalian homologs or closely related proteins that are involved in vesicle formation, namely, clathrin heavy chain (32) and dynamin (7, 10), respectively. Mutations in *vps1* were shown to cause both a late-Golgi membrane protein and a vacuolar membrane protein to be initially mislocalized to the plasma membrane before being transported to the vacuole via the endocytic pathway (28). Likewise, a temperature-sensitive *chc1* mutant mislocalized both Kex2p and DPAP A to the plasma membrane (43). These results are consistent with a model in which late-Golgi membrane proteins are transported to the prevacuolar compartment and are subsequently retrieved to the Golgi apparatus.

While the study of Chc1p and Vps1p has shed some light on the mechanism of Golgi membrane protein retention, several features of the retention mechanism remain elusive. For example, the receptor that recognizes the cytosolically oriented Golgi retention signal has not been identified, and it is not known whether this recognition takes place at the late-Golgi or post-Golgi compartment. Here we report the development of a genetic screen for yeast mutants that fail to properly localize late-Golgi membrane proteins. Characterization of the large group of *grd* (Golgi retention deficient) mutants should further our understanding of the machinery and mechanism of Golgi protein retention.

MATERIALS AND METHODS

Plasmids, strains, and materials. The plasmids used in this study are listed in Table 1. pSN269 is pBluescript KS$^+$ (Stratagene, La Jolla, Calif.) with a 2.0-kbp *Xho*I-*Xba*I PCR-generated insert consisting of the entire *PEP8* gene (2) in which the *PEP8* open reading frame was precisely replaced with the *LEU2* gene. pSN273 was constructed by subcloning the 3.0-kbp *Sac*I-*Xho*I fragment from pTS17 (1) into the *Sac*I-*Xho*I sites of pBluescript KS$^+$. To construct plasmids pSN274 and pSN275, the 2-kbp *Xho*I-*Sal*I fragment from the *PHO8* gene was subcloned into the *Sal*I sites of pMAL-c2 + stop (New England Biolabs Inc., Beverly, Mass.) and pGEX-5X-3 (Pharmacia Biotech, Uppsala, Sweden), respectively. pSN280 was constructed by three-way ligating the 0.5-kbp *Kpn*I-*Sac*I fragment and the 2.8-kbp *Sac*I-*Pst*I fragment (both from pRCMMMP [6]) into the *Kpn*I-*Pst*I sites of pRS314 (45), a *CEN*-based vector containing the *TRP1* gene. Plasmid pSN246 was constructed by subcloning the *Sac*I-*Eco*RV fragment, containing the *STE13-PHO8* fusion, into the *Sac*I-*Sma*I sites of pRS315 (a *LEU2*-based centromere [*CEN*] plasmid [45]).

The yeast strains used in this study are indicated in Table 2. Strains SNY41 and SNY60 were constructed by transforming strain SNY36-9B with pSN269 DNA digested with *Xba*I and *Xho*I and pSL1572 DNA digested with *Bam*HI-*Sac*I, respectively. In both cases, gene disruptions were selected by growth in the absence of leucine. NBY56 was constructed by transforming strain SNY36-9A with a PCR-generated fragment consisting of the *TRP1* gene flanked on the 5′ and 3′ sides with 30 bp from the 5′ and 3′ ends, respectively, of the *VPS36* open reading frame. The resulting Trp$^+$ transformants were checked for the *vps36*Δ::*TRP1* construct by PCR analysis.

The following reagents were obtained from the indicated sources: [^{35}S] Express label was from New England Nuclear (Boston, Mass.), oxalyticase was from Enzogenetics (Corvallis, Oreg.), Fast Red salt, naphthol AS phosphate, and α-naphthyl phosphate were from Sigma Chemical Co. (St. Louis, Mo.), and all secondary and fluorochrome-conjugated antibodies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, Pa.).

The *grd* mutant screen and ALP activity assays. The *MAT*α parental strain SNY17 carrying pSN55 was mutagenized by both ethyl methanesulfonate (EMS) and UV irradiation. *MAT***a** parental strains SNY18 and SNY36-9A were mutagenized by UV irradiation and EMS, respectively. Mutagenesis using EMS was carried out as described by Lawrence (20). Cells that had been mutagenized with EMS to 80% killing were plated onto SD-Ura plates at a density of 1,000 colonies per plate and screened by using the ALP activity assay (below). For UV mutagenesis, cells were plated onto SD-Ura at a density of 3,000 viable cells per plate. They were then immediately placed into a UV cross-linking apparatus (Stratagene) with the lids removed and subjected to UV irradiation (27,500 μJ/cm^2). The plates were kept in the dark at 30°C for 12 h after irradiation and then incubated for an additional 2 to 3 days before screening. This level of UV light exposure typically resulted in 60 to 70% killing.

The assay used for measuring ALP activity of yeast colonies on plates was essentially as described by Chapman and Munro (6). Briefly, a Whatman no. 1 filter was saturated with 1 ml of the reaction mixture (0.5 M Tris [pH 9.0], 25 mM $MgSO_4$, 1% Triton X-100, 10 mg of Fast Red salt per ml, 1 mg of naphthol as phosphate per ml) and overlaid onto an SD-Ura plate containing approximately 1,000 yeast colonies. The rate at which the yeast colonies turn red is proportional to the amount of ALP activity (6); therefore, mutant colonies exhibiting elevated levels of ALP activity were identified as deep red colonies in a background of

TABLE 2. Yeast strains used in this study

Strain	Genotype	Reference
SNY17	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 lys2-801 suc2-*Δ*9 pho8*Δ*::LEU2*	29
SNY18	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 ade2-101 suc2-*Δ*9 pho8*Δ*::ADE2*	29
SNY36-9A	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*901 suc2-*Δ*9 pho8*Δ*::ADE2*	29
SNY36-9B	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*901 suc2-*Δ*9 pho8*Δ*::ADE2 lys2-801*	29
SNY38	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*901 suc2-*Δ*9 pho8*Δ*::ADE2 lys2-801 vps1*Δ*::LEU2*	29
SNY41	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*901 suc2-*Δ*9 pho8*Δ*::ADE2 lys2-801 pep8*Δ*::LEU2*	This study
SNY60	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*901 suc2-*Δ*9 pho8*Δ*::ADE2 lys2-801 ren1*::*LEU2*	This study
AHY14	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*901 suc2-*Δ*9 pho8*Δ*::ADE2 lys2-801 vps27*Δ*::LEU2*	This study
NBY57	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 lys2-801 suc2-*Δ*9 pho8*Δ*::LEU2 vps36*Δ*::TRP1*	This study
NBY56	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*901 pho8*Δ*::ADE2 vps36*Δ*::TRP1*	This study
SNY71	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 ade2-101 suc2-*Δ*9 pho8*Δ*::ADE2 pep4*Δ*::LEU2*	This study
SNY86-2D	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 ade2-101 suc2-*Δ*9 pho8*Δ*::ADE2 pep4*Δ*::LEU2 pep8*Δ*::LEU2*	This study
SNY83-3B	*MAT***a** *ura3-52 leu2-3,112 his3-*Δ*200 trp1-901 lys-801 suc2-*Δ*9 pho8*Δ*::ADE2 gem3-45*	This study

pink colonies. With this assay, a total of 50,000 EMS-mutagenized colonies and 50,000 UV-mutagenized colonies from the SNY17 background, 50,000 EMS-mutagenized colonies from the SNY36-9A background, and 50,000 UV-mutagenized colonies from the SNY18 background were screened (i.e., 200,000 colonies in total).

A liquid assay to quantify the relative amounts of ALP activity in yeast strains was performed on whole cells with α-naphthyl phosphate as the substrate, using a method similar to those previously published (26, 48). Cells at an optical density at 600 of 1 were pelleted and washed once with 1 ml of assay buffer (250 mM Tris, 10 mM $MgSO_4$, 10 μM $ZnSO_4$ [pH 9.0]). The cells were then resuspended in 1 ml of 4% ethanol–1% toluene and vortexed for 10 min. After centrifugation, the cells were resuspended in 1 ml of assay buffer, and 100 μl of assay buffer containing 55 mM α-naphthyl phosphate was added. The reaction was allowed to proceed at 30°C for 20 min. The reaction was stopped by the addition of 1 ml of 2 M glycine [pH 11]. The cells were then pelleted, and the α-naphthyl product of the reaction in the supernatant was detected by measuring the fluorescence at 472 nm when excited at 345 nm. The reaction time and amount of cells used were within the linear range for these parameters. A unit of activity was defined as the production of 1 μmol of product per min.

Antibodies and immunoblot analysis. Antibodies were generated against a fusion protein consisting of the lumenal domain (amino acids 155 to 487) of ALP fused to the C terminus of glutathione *S*-transferase. This fusion protein was produced in *Escherichia coli* via plasmid pSN275, purified, and injected into rabbits. The resulting rabbit serum was affinity purified (41) by using an *E. coli*-expressed fusion protein consisting of the same region of ALP fused to the C terminus of the maltose-binding protein (plasmid pSN276). In addition, the fusion protein produced in *E. coli* from pSN275 was injected into mice to generate anti-ALP monoclonal antibodies by using standard techniques (25). Monoclonal antibody 1D3A10 was generated and used for these studies.

The steady-state extent of processing of the DPAP A-ALP (A-ALP) fusion protein was determined by a Western blotting (immunoblotting) procedure (49) of protein extracts separated on sodium dodecyl sulfate (SDS)–6% polyacrylamide gels. A 1:1,000 dilution of affinity-purified rabbit anti-ALP antibody was used, followed by incubation with a horseradish peroxidase conjugated anti-rabbit secondary antibody and detection by chemiluminescence (Amersham Life Science Inc., Arlington Heights, Ill.).

The extent of pro-α-factor secretion was determined by replica plating yeast patches onto YEPD plates (1% yeast extract, 1% peptone, 2% dextrose), overlaying the plates with nitrocellulose, and incubating the plates for 16 h at 30°C. The nitrocellulose filter was then rinsed under a stream of distilled water to remove yeast cells. After blocking in BLOTTO (18), the blots were incubated with a 1:5,000 dilution of rabbit anti-α-factor serum and subjected to chemiluminescence detection as described above.

Radiolabeling and immunoprecipitation. The procedure for [^{35}S]Met labeling of yeast cultures, cell lysis, and immunoprecipitation of A-ALP has been described in detail previously (28). Immunoprecipitation of carboxy peptidase Y (CPY) from extracellular and intracellular fractions after a 10-min pulse and 45-min chase was performed as described previously (36). The percentage of A-ALP processed at each time point and the relative amounts of CPY in the intracellular and extracellular fractions were quantified by scanning SDS-polyacrylamide gels with an AMBIS Radioanalytic Imaging System (AMBIS Inc., San Diego, Calif.).

Immunofluorescence microscopy. The procedures for preparation of fixed spheroplasted yeast cells, attachment to microscope slides, and costaining of the A-ALP fusion protein and the 60-kDa subunit of the vacuolar proton-translocating ATPase (V-ATPase) by using an anti-ALP polyclonal antibody and anti-60 kDa monoclonal antibody 13D11-B2 (Molecular Probes Inc., Eugene, Oreg.) were as previously described (28, 41). For costaining of Vps10p and A-ALP, fixed spheroplasts attached to slides were incubated with the following solutions followed by extensive washing with 5 mg of bovine serum albumin (BSA) per ml in phosphate-buffered saline (PBS) after each step: (i) PBS-BSA containing a 1:10 dilution of mouse anti-ALP monoclonal antibody 1D3-A10 (Molecular Probes) and a 1:300 dilution of affinity-purified rabbit anti-Vps10p polyclonal antibody (35), (ii) a 1:500 dilution of both a rat anti-mouse immunoglobulin G (IgG; heavy and light chains [H+L]) and a biotin-conjugated goat anti-rabbit IgG (H+L), and (iii) a 1:500 dilution of both Texas red-conjugated streptavidin and mouse anti-rat IgG (H+L) conjugated to fluorescein isothiocyanate (FITC).

RESULTS

A screen for late-Golgi retention-defective mutants. Retention-defective forms of membrane proteins of the yeast late-Golgi compartment are transported directly to the vacuole (40, 53). In *vps1* mutants, Golgi membrane proteins are initially mislocalized to the plasma membrane before also being transported to the vacuole as their final destination (7). Unlike *vps1* mutants, clathrin heavy-chain yeast mutants (*chc1*) accumulate late-Golgi membrane proteins on the cell surface rather than the vacuolar membrane (31, 43), probably because of a decreased rate of endocytosis.

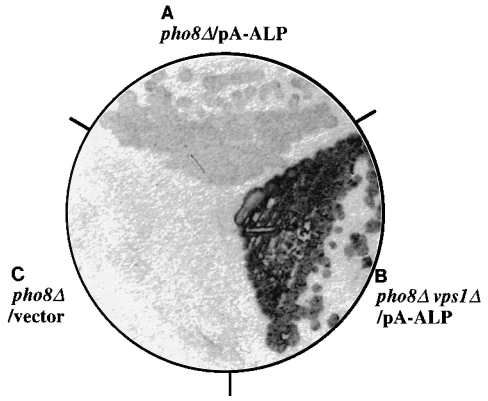

FIG. 1. Assay for measuring A-ALP activity of yeast strains on plates. Strains: A (*pho8*Δ/pA-ALP), wild type (SNY17) expressing A-ALP (via pSN55 [*CEN* A-ALP]); B (*pho8*Δ*vps1*/pA-ALP), *vps1* mutant (SNY38) expressing A-ALP (via pSN55); and C (*pho8*Δ/vector), wild type (SNY17) carrying a plasmid with no insert (pRS316). The assay was performed by overlaying a filter paper saturated with ALP assay solution (see Materials and Methods) onto the yeast strains, subsequently removing it, and photographing the plates after the reaction had proceeded.

To design a screen for mutants that fail to retain late-Golgi membrane proteins, we assumed that late-Golgi membrane proteins in such mutants would be mislocalized to the vacuolar membrane. Vacuolar delivery of the fusion protein A-ALP, a late-Golgi membrane protein consisting of the cytosolic domain of DPAP A fused to the transmembrane and lumenal domains of ALP, results in removal of its C-terminal propeptide (~3 kDa) by vacuolar proteases and causes A-ALP to become an active ALP enzyme (29). Therefore, retention-defective mutant cells expressing A-ALP but not endogenous ALP (*pho8*Δ) are predicted to exhibit elevated ALP activity in the colorimetric assay developed by Chapman and Munro (6). Strains in which the *PHO8* gene (encoding ALP) has been deleted were used to reduce background ALP activity. In the ALP assay, a dramatic increase in A-ALP specific activity was observed for a *vps1* mutant strain (Fig. 1B) compared with wild-type cells (Fig. 1A). The assay is highly specific for A-ALP activity, as demonstrated by the lack of detectable activity in a strain not expressing the A-ALP fusion (Fig. 1C).

Yeast cells expressing A-ALP were mutagenized, and approximately 100,000 colonies each from *MAT*α and *MAT***a** backgrounds were screened for elevated levels of ALP activity. Mutants exhibiting high ALP activity (*grd* mutants) were rescreened on plates, and A-ALP processing was directly determined by Western blot analysis (data not shown). Complementation analysis was carried out on the 178 recessive *grd* mutants by mating the *MAT*α mutants with the *MAT***a** mutants and assaying the resulting diploids for ALP activity. Because many of the *grd* mutants secreted CPY (see below), they were also tested for complementation against the *vps* mutants, which were previously identified on the basis of CPY secretion (for reviews, see references 17 and 38). Genetic analysis indicated that the *grd* mutants could be assigned to 18 complementation groups (Table 3).

Golgi retention phenotypes of the *grd* mutants. To measure quantitatively a defect in retention of late-Golgi membrane proteins, the kinetics of A-ALP processing was determined for a representative of each complementation group. Immunoprecipitation of A-ALP from [^{35}S]Met-labeled wild-type yeast

TABLE 3. Summary of *grd* complementation groups obtained

Group	No. of isolates		Fails to complement:
	a	α	
grd1	1	1	*vps1*
grd2	1	1	
grd3	2	1	
grd4	1	1	
grd5	1	2	
grd6	0	2	*vps26/pep8*
grd7	1	1	*vps2/ren1*
grd8	6	1	*vps15*
grd9	1	1	*vps35*
grd10	1	2	
grd11	1	0	*vps27*
grd12	1	0	*vps36*
grd13	0	1	*vps4*
grd14	1	1	
grd15	3	2	
grd16	4	2	
grd17	3	2	
grd18	4	2	

cells demonstrated that little if any A-ALP was proteolytically processed within a 180-min chase period (Fig. 2 and reference 29). In contrast, 50% of A-ALP expressed in strains containing the *grd2-1*, *grd5-1*, or *grd16-1* mutation was processed from the precursor to the mature form in 110, 60, or 100 min, respectively (Fig. 2). The processing half-times for the other *grd* mutants are summarized in Table 4 and ranged from 60 min for some of the strongest mutants (*grd1*, *grd5-1*, *grd7*, *grd9-1*, *grd10-2*, *grd11-1*, *grd12*Δ, and *grd13-1* mutants) to 180 min for the weakest (*grd8-1* mutant). Interestingly, the A-ALP processing rate in many of the mutants is similar to the rate at which retention-defective mutant forms of A-ALP lacking the aromatic retention signal are processed (60 to 70 min; [29]).

To address whether the processing of A-ALP by the *grd* mutants reflected delivery to the vacuole, A-ALP was localized in the mutants by indirect immunofluorescence microscopy. Figure 3 shows an experiment in which wild-type, *grd3*, and *grd6* cells were costained for the A-ALP fusion protein and Vma2p, the 60-kDa subunit of V-ATPase. In wild-type cells, A-ALP exhibited a punctate staining pattern typical of yeast late-Golgi membrane proteins (Fig. 3B) (29, 39). In contrast, much of the A-ALP protein in the *grd3* and *grd6* mutants (Fig. 3E and H) was localized to the vacuolar membrane, as shown by comparison of its staining pattern with that in the corresponding Nomarski (Fig. 3D and G) and V-ATPase (Fig. 3F and I) images. A-ALP and Vma2p also colocalized in the other *grd* mutants, and in every case a significant portion of A-ALP was detected on the vacuolar membrane (data not shown). Taken together, these results demonstrate that the A-ALP processing phenotype of the *grd* mutants is indeed due to a lack of retention in the late-Golgi compartment and subsequent delivery to the vacuole.

The Kex2p endoprotease is a type I membrane protein that is localized to the late-Golgi apparatus, where it performs the initial processing event to convert the pro-α-factor pheromone to mature α-factor (13). A loss of Kex2p Golgi retention has been shown to result in secretion of unprocessed pro-α-factor rather than the mature form (12), and thus secretion of pro-α-factor serves as an assay for Kex2p retention. Kex2p, like DPAP A, is retained in the Golgi compartment via an aromatic amino acid-based retention signal present in its cytosolic domain (53). To determine whether the *grd* mutants correctly localize Kex2p, a colony immunoblot blot assay was used to detect secretion of unprocessed α-factor (Table 4). Whereas the wild-type strain SNY17 did not secrete detectable amounts of pro-α-factor, all of the *grd* mutants with the exception of *grd10*, *grd15*, *grd16*, and *grd17* mutants secreted significant amounts of unprocessed pheromone. These data indicate that most of the *grd* mutants are defective for Kex2p as well as A-ALP retention and thus fail to retain multiple late-Golgi membrane proteins.

Overlap of late-Golgi retention and vacuolar protein-sorting functions. The vacuolar hydrolase CPY is known to be sorted in a late-Golgi compartment (15) as a result of its binding to Vps10p, the membrane-bound CPY-sorting receptor (9, 24). The cellular itinerary of Vps10p appears to include the late-Golgi apparatus, where it recognizes CPY. Recently, the cytosolic domain of this integral membrane protein has been demonstrated to contain sequences critical for its localization to the Golgi complex and recycling back from the prevacuolar compartment (5, 9, 35). Mutations in these Vps10p cytosolic domain sequences result in the rapid transport of Vps10p to the vacuole. Because recycling of Vps10p back from the prevacuolar compartment is required for CPY sorting, failure to recycle Vps10p results in CPY secretion.

Since the sorting of CPY occurs in the late-Golgi compartment and is mediated by the membrane protein Vps10p, the *grd* mutants were checked for secretion of CPY by quantifying the amounts of extracellular and intracellular CPY in radiolabeled yeast cultures. Whereas wild-type yeast cells secrete very little CPY (5%), many *grd* mutants secreted significant amounts of the newly synthesized CPY (Fig. 4). As CPY passes through the Golgi apparatus, it is processed from the p1CPY to the p2CPY precursor form and is further modified to the mature form upon transport to the vacuole (47). The observation that CPY was secreted from the *grd* mutants as the Golgi-modified precursor form (Fig. 4) indicates that the CPY was transported directly from the Golgi apparatus to the plasma membrane and did not first encounter a compartment containing activated vacuolar proteases.

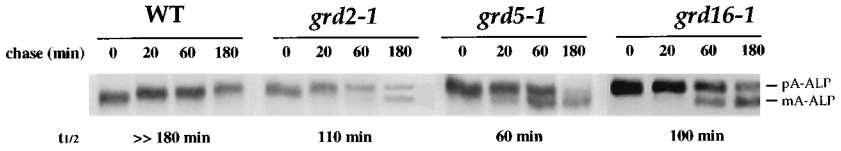

FIG. 2. Kinetics of A-ALP processing in wild-type and *grd* mutant strains. Wild-type (SNY17) and *grd2-1* (SNY47-7D), *grd5-1* (SNY50-9C), and *grd16-1* (SNY17-14) mutant strains carrying pSN55 (*CEN* A-ALP) were labeled with [^{35}S]Met for 10 min and then chased for the indicated times by adding 50 μg of methionine and cysteine per ml. At the indicated times, the cells were spheroplasted and lysed, and proteins from extracts were immunoprecipitated with a polyclonal antibody specific for ALP and then subjected to SDS-polyacrylamide gel electrophoresis and fluorography to assess the conversion of pro-A-ALP (pA-ALP) to mature A-ALP (mA-ALP). The processing half-times shown at the bottom were calculated by measuring the relative amounts of pro-A-ALP and mature A-ALP at each time point and performing linear regression analysis.

TABLE 4. Summary of *grd* mutant phenotypes[a]

Strain[b]	*grd* genotype	A-ALP processing $t_{1/2}$ (min)	% CPY secretion	Pro-α-factor secretion	Mnt1-ALP retention[c]
SNY17	*GRD*	>>180	<5	−	+
SNY38	*grd1*Δ/*vps1*Δ	60	85	+	+
SNY47-7B	*grd2-1*	110	72	+	+
NBY50-7C	*grd3-2*	120	70	+	+
SNY18-97	*grd4-2*	100	22	+	−
SNY50-8D	*grd5-1*	60	49	+	+
SNY41	*grd6*/*pep8*Δ	70	80	+	+
SNY60	*grd7*/*ren1*Δ	60	30	+	+
SNY51-5B	*grd8-1*	180	49	+	+
SNY52-11B	*grd9-1*	60	85	+	+
SNY36-9AN48	*grd10-2*	60	<10	−	−
SNY36-9AN57	*grd11-1*	60	36	+	+
NBY56	*grd12*Δ/*vps36*Δ	60	39	+	+
SNY17-N79	*grd13-1*	60	45	+	+
SNY17-51B	*grd14-1*	100	35	+	+
SNY36-9AN27	*grd15-1*	90	<10	−	+
SNY17-228	*grd16-1*	100	<10	−	+
SNY17-N15	*grd17-1*	ND	<10	−	+
SNY17-36	*grd18-1*	120	<10	+	−

[a] The kinetics of A-ALP processing, percent CPY secretion, and Mnt1p-ALP retention were determined as described in the legends to Fig. 2, 4, and 5, respectively. Secretion of pro-α-factor was scored by a colony blot procedure as described in Materials and Methods. ND, not determined.

[b] In some cases (*grd1 grd6*, *grd7*, and *grd12*), gene disruptions of the corresponding *vps* or *pep* mutants were substituted for the original *grd* mutant isolate for these phenotypic analyses. In cases where *MAT***a** strains are listed, a *MAT*α strain carrying the same *grd* allele was substituted to assess pro-α-factor secretion. Exceptions are *grd10* and *grd15*, in which cases *MAT*α strains carrying the *grd10-1* and *grd15-2* alleles were used for the pro-α-factor secretion analysis.

[c] Data taken from Fig. 5. +, normal retention of Mnt1p-ALP; −, defective retention.

A summary of the CPY secretion defects of the *grd* mutants is presented in Table 4. The majority of *grd* mutants were found to secrete significant amounts of CPY, exceptions being *grd10*, *grd15*, *grd16*, *grd17*, and *grd18* mutants. Given that most *grd* mutants secreted CPY, complementation analysis was performed between the *grd* mutants and the *vps* mutants, which were isolated as defective for CPY sorting (17, 38). Eight of the 18 *grd* complementation groups were found to overlap with *vps* mutant groups, including *vps1* (Table 3).

The identification of *vps1* in the *grd* screen was reassuring since previous work demonstrated that *VPS1* is required for retention of both DPAP A and Kex2p (28, 54). Similarly, the identification of several class E *vps* mutants in the *grd* screen (*vps2*, *vps4*, *vps27*, and *vps36* mutants) was anticipated since class E *vps* mutants accumulate late-Golgi membrane proteins in an exaggerated prevacuolar compartment (35, 37). Previous studies have shown that the prevacuolar compartment that accumulates in class E *vps* mutants contains activated vacuolar proteases (35). The 13 *vps* mutants belonging to class E (*vps2*, *vps4*, *vps20*, *vps22*, *vps23*, *vps24*, *vps25*, *vps27*, *vps28*, *vps31*, *vps32*, *vps36*, and *vps37* mutants) were tested for A-ALP processing by Western blot analysis, and all of these class E *vps* mutants (with the exception of *vps31* mutants) failed to retain A-ALP in the Golgi apparatus (data not shown).

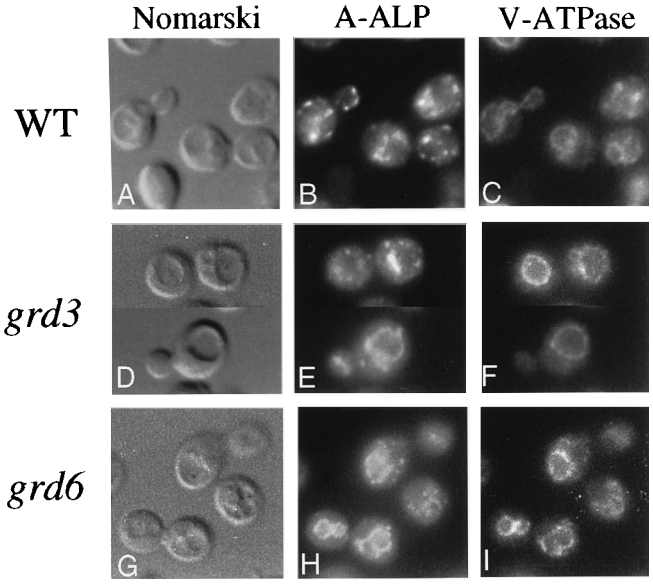

FIG. 3. A-ALP is localized to the vacuolar membrane in the *grd* mutants. Wild-type (WT; SNY71; A to C), *grd3-2* (SNY85-4A; D to F), and *grd6-1* (SNY86-2D; G to I), cells carrying plasmid pSN55 (*CEN* A-ALP) were fixed, spheroplasted, and costained with a rabbit antibody against A-ALP (B, E, and H) and a mouse antibody against the 60-kDa subunit of V-ATPase (C, F, and I). The cells were viewed by Nomarski optics (A, D, and G) and by epifluorescence through filter sets specific for fluorescein (B, E, and H) and Texas red (C, F, and I).

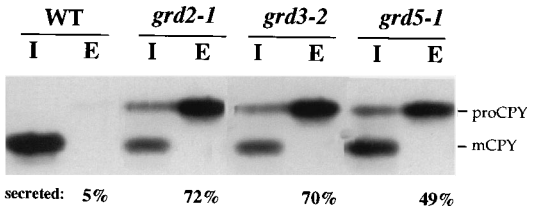

FIG. 4. A subset of the *grd* mutants are defective for sorting of CPY to the vacuole. Wild-type (WT; SNY17), *grd2-1* (SNY47-7D), *grd3-2* (NBY50-7C), and *grd5-1* (SNY50-9C) strains were labeled with [^{35}S]Met for 10 min and then chased for 45 min by adding 50 μg of methionine and cysteine per ml. The cultures were then divided into intracellular (I) and extracellular (E) fractions that were subjected to immunoprecipitation with an anti-CPY polyclonal antibody. The washed immunoprecipitates were then run on SDS-polyacrylamide gels and subjected to fluorography. The positions of precursor (proCPY) and mature (mCPY) forms of CPY are indicated. The percentage of CPY found in the extracellular fraction is indicated below each E lane.

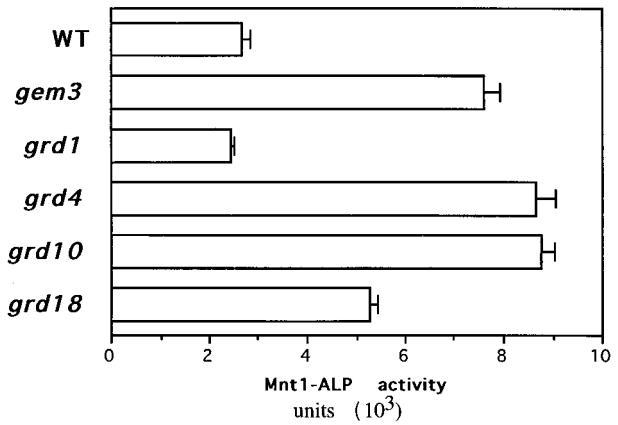

FIG. 5. The *grd4*, *grd10*, and *grd18* mutants are defective for retention of the Mnt1-ALP fusion protein. Wild-type (WT; SNY17), *gem3-45* (SNY83-3B), *grd1*Δ *vps1*Δ (SNY38), *grd4-2* (SNY18-97), *grd10-1* (SNY17-34), and *grd18-1* (SNY17-36) strains carrying the Mnt1-ALP plasmid (pSN280) were assayed for Mnt1-ALP activity by using α-naphthyl phosphate as a substrate (see Materials and Methods). The average and standard deviation of three independent measurements for each strain are shown.

Retention of an early-Golgi membrane protein is not affected in most *grd* mutants. Clearly, the *grd* mutants exhibit defects in retention of late-Golgi membrane proteins; however, it was possible that membrane protein retention at earlier-Golgi compartments were also affected. The specificity of the *grd* mutants for the retention of late-Golgi membrane proteins was investigated by determining their abilities to retain a model early-Golgi membrane protein, using a procedure developed by Chapman and Munro (6). This protein, Mnt1-ALP, consisted of the ALP lumenal domain fused to the C terminus of Mnt1p, a type II early-Golgi membrane protein. As is the case with A-ALP, a loss of Mnt1p-ALP Golgi retention results in mislocalization to the vacuolar membrane, where the protein is processed and thereby activated (6). Three mutant complementation groups (*gem1*, *gem2*, and *gem3*) that fail to retain Mnt1-ALP have been identified (6).

The retention of Mnt1-ALP produced in the *grd* mutants was determined by performing a fluorometric enzymatic assay on permeabilized cells, using α-naphthyl phosphate as the substrate. This substrate was highly specific for the Mnt1-ALP fusion since the activity was reduced more than 20-fold if the fusion was not expressed (data not shown). Under the conditions of the assay, the *gem3* mutant, as a positive control, consistently exhibited Mnt1-ALP activity approximately threefold greater than that in wild-type cells expressing the fusion protein. Whereas most *grd* mutants did not exhibit elevated ALP activity when expressing Mnt1-ALP (Fig. 5, *grd1*), representative alleles from a small subset of the *grd* complementation groups (*grd4*, *grd10*, and *grd18*) exhibited elevated levels of Mnt1-ALP activity. The *grd4* and *grd10* mutants appeared to be particularly defective for retention of Mnt1-ALP. These data indicate that most of the *grd* mutants are specifically defective for the retention of late-Golgi membrane proteins.

A subset of the *grd* mutations specifically perturb A-ALP retention. Most of the *grd* mutations appear to perturb multiple processes important for late-Golgi function, including retention of A-ALP and Kex2p as well as the recycling of the CPY-sorting receptor Vps10p. However, mutants from three of the *grd* complementation groups (*grd15*, *grd16*, and *grd17*) exhibited only defects in retention of A-ALP. The lack of CPY secretion in mutants from these three groups suggests that localization of the CPY receptor, Vps10p, is unaffected, whereas A-ALP is mislocalized to the vacuole. To address whether localization of A-ALP and Vps10p is uncoupled in such mutants, the two proteins were detected simultaneously by indirect immunofluorescence microscopy. In wild-type yeast cells, A-ALP (Fig. 6B) and Vps10p (Fig. 6C) exhibited extensive colocalization, consistent with Vps10p being predominantly localized to the late-Golgi compartment (5, 9, 24, 35). In contrast, when double labeling was performed in a *grd16* mutant, A-ALP was localized to the vacuolar membrane (Fig. 6E), whereas Vps10p exhibited a punctate distribution (Fig. 6F) similar to that seen in wild-type cells (5, 9, 35). In *grd* mutants that secrete CPY, such as *vps1 grd1* mutants, both A-ALP and Vps10p are mislocalized to the vacuole (reference 28 and data not shown). These data suggest that certain aspects of the retention machinery for A-ALP (DPAP A) are distinct from those responsible for proper localization of Vps10p.

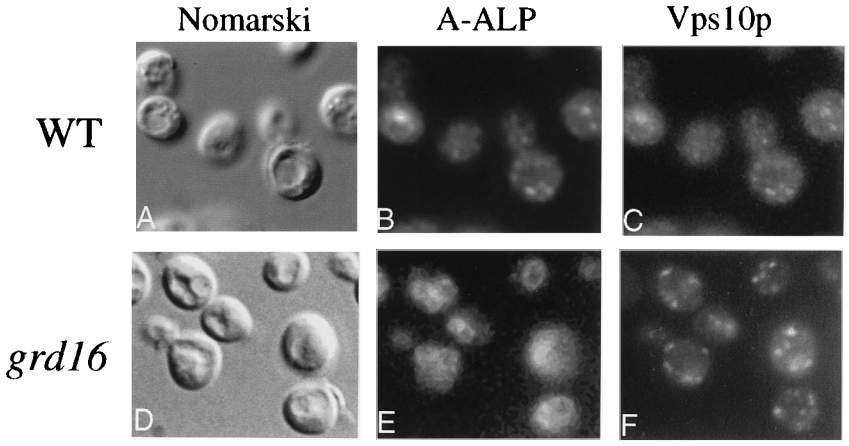

FIG. 6. The *grd16-1* mutation affects A-ALP but not Vps10p localization. Wild-type (WT; SNY17) and *grd16-1* (SNY17-N4) strains each expressing A-ALP (via pSN55 [*CEN* A-ALP) are shown. Each strain was fixed, spheroplasted, and costained with a mouse antibody against A-ALP (B and E) and a rabbit antibody against Vps10p (C and F). The cells were viewed by Nomarski optics (A and D) and by epifluorescence through filter sets specific for fluorescein (B and E) and Texas red (C and F).

DISCUSSION

In this report, we describe a genetic screen for yeast mutants defective in Golgi membrane protein retention that is based on an assay for the localization of a Golgi membrane protein, A-ALP. The *grd* mutants isolated in this screen fell into 18 complementation groups. Mutants in 15 of the 18 *grd* complementation groups did not exhibit defects in retention of an early-Golgi membrane protein, Mnt1-ALP, and thus are likely to be specifically required for retention of late-Golgi proteins. Most of the *grd* mutants exhibited defects in retention of another late-Golgi membrane protein, Kex2p, and in recycling of the CPY-sorting receptor, Vps10p, which results in secretion of CPY. In contrast, a small subset of the mutants are specific for the retention of A-ALP (DPAP A), since the retention defect was limited to this Golgi protein.

The observation that most of the *grd* mutants retained an early-Golgi membrane protein is consistent with previous results from both yeast and animal cells that underscores the differences between retention in the late-Golgi compartment (or TGN) and retention occurring in earlier-Golgi subcompartments (reviewed in reference 23). Retention signals present in the yeast late-Golgi membrane proteins DPAP A, Kex1p, and Kex2p are found in the cytosolic domain (8, 29, 53), whereas the retention of the early-Golgi membrane protein, Mnt1p, is mediated by its transmembrane domain (6). The medial-Golgi membrane protein, Mnn1p, contains retention information in both the transmembrane and lumenal domains, while the cytosolic domain is expendable for retention (16). Clathrin heavy chain has been shown to be required for transmembrane domain-mediated Mnn1p retention (14, 16) and is essential for retention of late-Golgi membrane proteins (31, 43). We have identified three yeast genes (*GRD4*, *GRD10*, and *GRD18*) that are required for both A-ALP and Mnt1-ALP retention. These results indicate that transmembrane-mediated and cytosolic domain-mediated retention mechanisms may not be completely independent of one another. The observation that mutations in *GRD4*, *GRD10*, and *GRD18* cause defects in separate Golgi compartments suggests that these genes may be required for overall Golgi organization. It is unlikely that any of the *grd* mutants have drastic defects in general Golgi functions since Golgi-specific carbohydrate processing (47) of CPY (both secreted and vacuolar compartment-localized CPY) is normal in all of the mutants. However, given our limited knowledge of yeast late-Golgi functions, we cannot rule out the possibility that some of the *GRD* genes are required for late-Golgi assembly or maintenance.

The significant overlap in genes required for Golgi retention and CPY sorting found in this study indicates that multiple late-Golgi membrane proteins follow the same post-Golgi trafficking steps as the CPY receptor Vps10p. By analogy with the mannose 6-phosphate receptor of animal cells (19), Vps10p binds CPY in the late-Golgi compartment and enters vesicles bound for a prevacuolar compartment. Upon reaching the prevacuolar compartment, CPY dissociates from Vps10p and is eventually delivered to the vacuole, whereas Vps10p recycles back to the Golgi complex for another round of sorting (5, 9, 35).

Several class E *vps* mutants were isolated as *grd* mutants, consistent with previous reports indicating that A-ALP, Kex2p, and Vps10p are all localized to the prevacuolar compartment that accumulates in class E *vps* mutants such as *vps27* mutants (5, 35, 37). In fact, mutants from 12 of the class E *vps* complementation groups failed to retain A-ALP. Experiments carried out with a *vps27-ts* mutant have demonstrated that the recycling of Vps10p from the prevacuole back to the Golgi complex is rapidly blocked upon Vps27p inactivation, leading to its accumulation in the prevacuole (35). These observations, along with our discovery that 12 of the class E *vps* genes are required for the retention of late-Golgi membrane proteins, provide substantial evidence in support of the model that late-Golgi membrane proteins are continually retrieved from the prevacuolar compartment.

Interestingly, mutations in the *VPS1* gene, which is required for Golgi-to-prevacuole vesicular traffic, have dramatic effects on retention of late-Golgi membrane proteins. In *vps1* mutant cells, both Golgi and vacuolar membrane proteins are initially mislocalized to the plasma membrane and are then delivered to the vacuole via the endocytic pathway (28). These data indicate that late-Golgi membrane proteins are not properly retained in the absence of a functional anterograde Golgi-to-prevacuole pathway.

Although the mechanism of localization of Golgi membrane proteins such as DPAP A appears to be quite similar to that of Vps10p, the identification of mutants (*grd10*, *grd15*, *grd16*, *grd17*, and *grd18*) that affect A-ALP but not Vps10p indicates that the processes are not strictly coupled. In contrast to enzymes such as DPAP A, which act only in the late-Golgi compartment, cycling of Vps10p between the Golgi apparatus and the prevacuolar compartment is key to its role as the CPY-sorting receptor (5, 9). Therefore, it is not surprising that the machinery for localization of the two types of Golgi proteins would not overlap completely. It is quite possible that a static retention mechanism unique to DPAP A operates to reduce the rate of DPAP A departure from the late-Golgi compartment (29) and thereby keep most of the DPAP A in the Golgi apparatus, where it is required for processing of pro-α-factor (13).

Surprisingly, mutants from four of the *grd* complementation groups (*grd10*, *grd15*, *grd16*, and *grd17*) were found to not only sort CPY normally but also retain Kex2p efficiently on the basis of a lack of pro-α-factor secretion. It is possible that subtle effects on Kex2p retention that are not reflected by examining pheromone precursor processing occur in these mutants. Nevertheless, these data argue that differences exist between the mechanisms for retention of Kex2p and DPAP A. Both proteins are known to be retained by aromatic amino acid retention signals, although very little similarity can be seen at the primary sequence level (30). The best evidence that the two proteins may be recognized by the same retention signal receptor is that overproduction of wild-type Kex2p, but not the retention-defective form lacking the tyrosine residue, significantly reduced the efficiency of the retention of A-ALP (29). Whereas this experiment suggested at least overlap in the sorting apparatus for the two proteins, the identification of *GRD* genes that are required only for DPAP A retention suggests that components of this apparatus, and perhaps the retention signal receptor itself, may be different. It is also interesting that the half-time of Kex2p turnover in the vacuole is 80 min (53), compared with >>180 min for A-ALP processing. Therefore, it is also possible that DPAP A-specific retention machinery prevents DPAP A departure from the Golgi apparatus, thereby causing it to be more effectively retained than Kex2p. Experiments are under way to test whether the A-ALP-specific *grd* mutants possess the properties expected of a receptor for the aromatic amino acid-based retention signal.

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