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A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole

Jennifer J.Vowels and Gregory S.Payne[1]

Department of Biological Chemistry, UCLA School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA

[1]Corresponding author
e-mail: gpayne@biochem.medsch.ucla.edu

Transport of yeast alkaline phosphatase (ALP) to the vacuole depends on the clathrin adaptor-like complex AP-3, but does not depend on proteins necessary for transport through pre-vacuolar endosomes. We have identified ALP sequences that direct sorting into the AP-3-dependent pathway using chimeric proteins containing residues from the ALP cytoplasmic domain fused to sequences from a Golgi-localized membrane protein, guanosine diphosphatase (GDPase). The full-length ALP cytoplasmic domain, or ALP amino acids 1–16 separated from the transmembrane domain by a spacer, directed GDPase chimeric proteins from the Golgi complex to the vacuole via the AP-3 pathway. Mutation of residues Leu13 and Val14 within the ALP cytoplasmic domain prevented AP-3-dependent vacuolar transport of both chimeric proteins and full-length ALP. This Leucine–Valine (LV)-based sorting signal targeted chimeric proteins and native ALP to the vacuole in cells lacking clathrin function. These results identify an LV-based sorting signal in the ALP cytoplasmic domain that directs transport into a clathrin-independent, AP-3-dependent pathway to the vacuole. The similarity of the ALP sorting signal to mammalian dileucine sorting motifs, and the evolutionary conservation of AP-3 subunits, suggests that dileucine-like signals constitute a core element for AP-3-dependent transport to lysosomal compartments in all eukaryotic cells.

Keywords: ALP sorting signal/AP-3 pathway/dileucine-like signal/vacuolar transport

Introduction

The secretory pathway in eukaryotic cells serves as a major protein traffic route to different subcellular compartments including the endoplasmic reticulum (ER), the Golgi complex, the plasma membrane and lysosomes. Sorting of proteins to these various destinations is thought to occur by incorporation of selected cargo into distinct coated vesicles which mediate transport between compartments (Schekman and Orci, 1996). Selectivity relies on short sequences in the cytoplasmic domains of cargo membrane proteins, referred to as sorting signals, that specify sequestration into budding vesicles by interaction with vesicle coat components. Deciphering the sorting signals for particular coated vesicle transport pathways is therefore central to understanding sorting in the secretory pathway.

Within the secretory pathway, selected membrane proteins are sorted from the *trans*-Golgi network (TGN) through the endosomal pathway to the major hydrolytic compartment, mammalian lysosomes or yeast vacuoles (Kornfeld and Mellman, 1989; Stack *et al.*, 1995). In mammalian cells, this sorting process can be directed by tyrosine-based or dileucine-based sorting signals (Sandoval and Bakke, 1994; Mellman, 1996). The tyrosine-based signals are generally present as YXXZ, where X is any amino acid and Z represents a bulky hydrophobic amino acid. Dileucine-based signals consist of an invariant leucine in the first position and a hydrophobic residue, L, I, V or M, in a more tolerant second position (Sandoval and Bakke, 1994). One mechanism for sorting of membrane proteins bearing tyrosine-based sorting signals involves incorporation into nascent clathrin-coated vesicles budding from the TGN (Mellman, 1996; Marks *et al.*, 1997). Association of these proteins with clathrin coats is proposed to occur by an interaction between the sorting signal and clathrin adaptor-1 (AP-1) complexes (Mellman, 1996; Marks *et al.*, 1997), heterotetrameric assemblies of two large (γ and β1), one medium (μ1) and one small (σ1) subunit (Kirchhausen, 1993; Robinson, 1994; Kirchhausen *et al.*, 1997; Marks *et al.*, 1997). AP-1 will also bind to clathrin and stimulate coat assembly, thereby coupling cargo recruitment to coated vesicle formation (Robinson, 1994; Kirchhausen *et al.*, 1997). A similar AP-1-mediated mechanism has been proposed for sorting of membrane proteins containing dileucine sorting signals (Sandoval and Bakke, 1994). Both tyrosine and dileucine signals will also interact with another adaptor complex, AP-2, present in plasma membrane clathrin coats, thus allowing for endocytosis of membrane proteins containing these signals (Kirchhausen *et al.*, 1997; Marks *et al.*, 1997). A recently discovered AP complex, AP-3, is related to AP-1 and AP-2 but does not appear to be associated with clathrin-coated vesicles (Newman *et al.*, 1995; Simpson *et al.*, 1996, 1997; Dell'Angelica *et al.*, 1997b). Although AP-3 localizes to the TGN and more peripheral structures (Simpson *et al.*, 1996, 1997; Dell'Angelica *et al.*, 1997a), a sorting role for mammalian AP-3 has not been established.

In the yeast *Saccharomyces cerevisiae*, two genetically distinct intracellular pathways for routing proteins from the Golgi complex to the vacuole have been defined by mutants defective in vacuolar protein sorting (*vps* mutants). Elucidation of the first pathway resulted from analysis of a soluble vacuolar protein, carboxypeptidase Y (CPY) (Stack *et al.*, 1995; Bryant and Stevens, 1997). The CPY pathway commences in a TGN-like compartment where CPY, bound to its receptor Vps10p, is directed by vesicular traffic to an intermediate endosome en route to the vacuole. The CPY transport pathway is blocked specifically by

mutations in proteins such as the endosomal t-SNARE Pep12p, or the Sec1 homolog Vps45p, both of which are involved in vesicle targeting to endosomes (Cowles *et al.*, 1994; Piper *et al.*, 1994; Becherer *et al.*, 1996). Genetic studies have also provided evidence that clathrin is involved in CPY and Vps10p transport from the TGN (Seeger and Payne, 1992b). Although subunits homologous to mammalian AP-1 have been identified in yeast, their role is unclear since deletions of genes encoding AP-1 subunits do not cause CPY missorting (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). Most endosome-targeted and vacuolar membrane proteins studied to date follow the clathrin- and *PEP12*-dependent pathway from the TGN. However, attempts to define signals mediating sorting into the pathway have not been successful, and it has been proposed that the pathway functions as a default route for untargeted membrane proteins (Nothwehr and Stevens, 1994).

The second biosynthetic pathway from the Golgi to the vacuole was uncovered by characterization of the type II vacuolar membrane protein alkaline phosphatase (ALP) (Klionsky and Emr, 1989, 1990; Cowles *et al.*, 1997b; Piper *et al.*, 1997). Like CPY, ALP reaches the vacuole from the Golgi complex by an intracellular route that does not include transport to the cell surface followed by endocytosis (Klionsky and Emr, 1989; Nothwehr *et al.*, 1995). However, ALP transport is unaffected by mutations which block CPY transport from the TGN to endosomes (*pep12* or *vps45*), or which block CPY and endocytic traffic from endosomes to vacuoles (*vps4* and *vps27*), indicating that ALP bypasses the CPY pathway through endosomes (Babst *et al.*, 1997; Cowles *et al.*, 1997b; Piper *et al.*, 1997). Recently, it was found that the 'ALP pathway' is dependent on a yeast AP complex homologous to mammalian AP-3 (Cowles *et al.*, 1997a; Stepp *et al.*, 1997). Sorting of ALP into the *PEP12*-independent, AP-3-dependent pathway depends on sequences within the N-terminal cytoplasmic domain, but a clear definition of the sorting signal has been hampered by conflicting results (Cowles *et al.*, 1997b; Piper *et al.*, 1997). Here we use chimeric proteins and site-directed mutagenesis to identify a dileucine-related leucine–valine (LV) sequence in the ALP cytoplasmic domain that serves as a major determinant for sorting into the AP-3 pathway. Additionally, we show that this LV-mediated vacuolar transport is unaltered upon inactivation of clathrin heavy chain, providing functional evidence that the AP-3 pathway is independent of clathrin. These results suggest that dileucine-like motifs can act as targeting signals for a clathrin-independent AP-3 pathway to vacuoles.

Results

A clathrin-independent mechanism for ALP transport to the vacuole

A role for clathrin in the CPY sorting pathway was revealed by analyzing strains carrying a temperature-sensitive allele of the clathrin heavy chain gene (*chc1-ts*) (Seeger and Payne, 1992b). Shifting *chc1-ts* cells to a non-permissive temperature of 37°C blocks sorting of CPY and other soluble vacuolar proteins, as well as a mutant form of the TGN membrane protein Kex2p that mislocalizes to the vacuole in wild-type cells (Seeger and Payne, 1992b; Redding *et al.*, 1996). In contrast, clathrin inactivation does not impede delivery of ALP to the vacuole, suggesting that clathrin is not required for transport through the AP-3-mediated vacuolar pathway (Seeger and Payne, 1992a,b). However, an alternative explanation has arisen from studies of cells carrying a disruption of the *VPS1* gene (*vps1*Δ). In *vps1*Δ cells, ALP is delivered to the vacuole with near wild-type kinetics even though the mutation causes aberrant routing of ALP to the vacuole by way of the plasma membrane (Nothwehr *et al.*, 1995). Such misrouting to the vacuole via the cell surface in *chc1-ts* cells could obscure a role for clathrin in the AP-3-mediated transport of ALP.

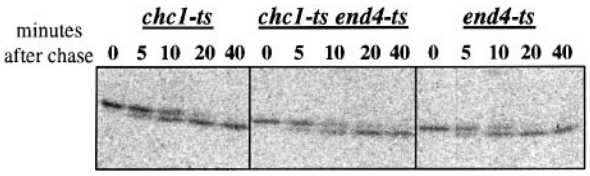

Fig. 1. Effect of *chc1-ts* and *end4-ts* on ALP transport to the vacuole. GPY418 (*chc1-ts*), GPY1526 (*chc1-ts end4-1*) and GPY1508 (*end4-1*) were incubated at the restrictive temperature of 37°C for 15 min to impose the *chc1-ts* and *end4-ts* blocks. Cells were then labeled with [^{35}S]methionine and cysteine for 5 min, followed by addition of unlabeled amino acids (chase), and harvested at the indicated chase times. ALP was immunoprecipitated and analyzed by SDS–PAGE. The upper and lower bands in each gel represent precursor and mature ALP, respectively (see text for details).

To address the possibility that loss of clathrin function results in routing of ALP to the vacuole by way of the plasma membrane, we analyzed *chc1-ts* cells also carrying the temperature-sensitive *end4-1* mutation (Raths *et al.*, 1993). The endocytic block imposed by *end4-1* at the non-permissive temperature has been shown to prevent vacuolar delivery of ALP from the cell surface (as in *vps1*Δ cells), but has no effect on intracellular sorting of ALP (Nothwehr *et al.*, 1995). A pulse–chase immunoprecipitation regimen was used to follow ALP maturation in the *chc1-ts end4-1* double mutant and congenic strains carrying either mutation alone. Strains were shifted to a non-permissive temperature for 15 min to allow both *chc1-ts* and *end4-1* to institute the transport blocks. Subsequently, cells were labeled for 5 min with [^{35}S]methionine and cysteine (pulse), and labeling was quenched by the addition of unlabeled amino acids (chase). Cells were harvested at designated time periods for lysis and immunoprecipitation of ALP, and immunoprecipitates were subjected to SDS–PAGE (Figure 1). In wild-type cells, ALP is synthesized as an inactive 74 kDa precursor form which is matured upon delivery to the vacuole by proteolytic cleavage near the C-terminus to produce the active 72 kDa enzyme (Klionsky and Emr, 1989). Thus, conversion of the 74 kDa precursor to the 72 kDa mature form during the chase period is a convenient indicator of the kinetics of ALP delivery to the vacuole. Figure 1 shows that the 74 kDa precursor form of ALP was matured to 72 kDa with wild-type kinetics in all three test strains. In contrast, analysis of CPY maturation indicated that the CPY sorting pathway was effectively blocked in *chc1-ts*-containing strains (data not shown). Thus, like certain *vps* mutations, *chc1-ts* can be used to distinguish between the ALP and CPY pathways to the vacuole. These results indicate that ALP is not misrouted to the cell surface after

clathrin inactivation and provide further evidence that ALP is directed to the vacuole through a clathrin-independent pathway.

A novel strategy to identify sorting signals in vacuolar membrane proteins

The sorting signal(s) within membrane proteins dictates the particular pathway which the protein will transit to reach its resident organelle. If a sorting signal is absent or defective, the protein can enter a default pathway. For localization-defective membrane proteins of the secretory pathway in yeast, the vacuole is the default destination (Nothwehr and Stevens, 1994). For example, mutation of the localization signals in ER and Golgi membrane proteins leads to transport from the Golgi complex to the vacuole without passage through the plasma membrane (Roberts *et al.*, 1992; Wilcox *et al.*, 1992; Gaynor *et al.*, 1994). This apparent default pathway is blocked in *chc1-ts* cells and in *vps* mutants with defects in export from endosomes, suggesting that default transport follows the endosomal CPY pathway to the vacuole (Redding *et al.*, 1996; Brickner and Fuller, 1997). The vacuolar destination of membrane proteins lacking sorting signals confounds approaches to identify vacuolar sorting signals since, even in the absence of such signals, the proteins would be expected to reach the vacuole by default.

To circumvent the potential problem of default vacuolar transport in characterizing sorting signals in vacuolar membrane proteins, we applied a strategy intended to change the default destination of proteins carrying defective vacuolar sorting signals. For this purpose, chimeric proteins were created in which the cytoplasmic domain of a given vacuolar membrane protein was joined to the transmembrane and lumenal domains of guanosine diphosphatase (GDPase), a resident Golgi membrane protein with the same type II topology as the vacuolar membrane proteins (Abeijon *et al.*, 1993). The GDPase transmembrane and lumenal domains are sufficient to specify Golgi residence; deletion of the cytoplasmic domain or replacement with sequences lacking sorting signals still leads to proper Golgi localization (Vowels and Payne, 1998). We reasoned that cytoplasmic vacuolar sorting signals appended to the GDPase transmembrane and lumenal domains might override the Golgi localization information and direct the chimeric proteins to the vacuole. If so, mutation of the vacuolar sorting signal would allow the GDPase localization information to take effect and alter the destination from the vacuole to the Golgi complex.

Vacuolar localization of chimeric proteins containing the cytoplasmic domains of ALP or DPAP B

To test our strategy, the entire cytoplasmic domains of ALP (amino acids 1–33) or dipeptidyl aminopeptidase B (DPAP B; amino acids 1–29) were fused to the GDPase transmembrane and lumenal domains. DPAP B is a type II vacuolar membrane protein localized to the vacuole via the CPY pathway (Roberts *et al.*, 1989; Piper *et al.*, 1997). ALP–GDPase and DPAP B–GDPase chimeric proteins are referred to as AGG and DGG, respectively, where each letter represents a topological domain of the protein of origin. As described in detail below, localization of each chimeric protein was compared with localization of GDPase (Golgi) and ALP (vacuole) using several different criteria: (i) protein stability and glycosylation status; (ii) steady-state intracellular location; and (iii) Golgi-specific GDPase function.

GDPase and ALP display clear differences in protein stability and glycosylation status which can be monitored conveniently by pulse–chase immunoprecipitation to distinguish between Golgi complex and vacuole localization (Vowels and Payne, 1998). These differences, which provide sensitive kinetic measures of localization, are illustrated in Figure 2A. GDPase (GGG) is a relatively stable protein (half-life $\geqslant$120 min) that undergoes a progressive size increase due to extension of the oligosaccharide side chains during residence in the Golgi complex (Vowels and Payne, 1998). After a 10 min labeling with [^{35}S]methionine and cysteine (0 min chase), a prominent 67 kDa doublet, as well as a 65 kDa minor form, of GDPase was apparent. The lower component of the 67 kDa doublet is the ER form of GDPase which is converted into the upper form upon transport to the Golgi complex where the oligosaccharide side chain extension occurs (Vowels and Payne, 1998). The minor 65 kDa species, which always parallels the larger form, probably represents a form of GDPase with fewer oligosaccharide side chains (Vowels and Payne, 1998). At later chase times (Figure 2A, 15 min and longer chase times), the 67 kDa doublet coalesces into a single band, and both this band and the minor GDPase form display incremental size increases and slow declines in intensity over time. Since GDPase is retained in the Golgi complex efficiently and only slowly leaks to the vacuole (Vowels and Payne, 1998), there was little difference in the results of a pulse–chase immunoprecipitation of GDPase from *pep4*Δ cells which are deficient for vacuolar proteases (Figure 2A, GGG, *pep4*Δ panel) (Jones, 1991). The same protocol revealed that transport of ALP to the vacuole was sufficiently rapid to generate both precursor and mature forms by the 0 min chase time (Figure 2A, AAA, *PEP4* panel). Maturation was complete by 15 min of chase and was entirely dependent on Pep4p (Figure 2A, AAA, *PEP4* and *pep4*Δ panels). There was no incremental size increase in ALP, either in *PEP4* or *pep4*Δ strains, since transport to the vacuole sequesters ALP from the action of secretory pathway mannosyltransferases.

When AGG and DGG were analyzed by pulse–chase immunoprecipitation, both proteins displayed the major and minor forms attributable to the heterogeneous number of oligosaccharide side chains attached to the GDPase lumenal domain (Figure 2A, AGG and DGG panels). Both major and minor forms of AGG and DGG disappeared by the 60 min chase point in *PEP4* cells but persisted in *pep4*Δ cells, suggesting that the proteins were degraded in the vacuole. Consistent with delivery to the vacuole, neither AGG nor DGG underwent slow incremental increases in glycosylation. From these findings, it appears that fusion of the cytoplasmic domain of either ALP or DPAP B to the transmembrane and lumenal domains of GDPase can target the chimeric proteins to the vacuole.

To provide additional evidence that AGG is transported to the vacuole, the steady-state localization of AGG was examined by indirect immunofluorescence using cells carrying the *pep4*Δ mutation to prevent degradation of vacuolar AGG (Figure 2B). For comparison, GDPase and

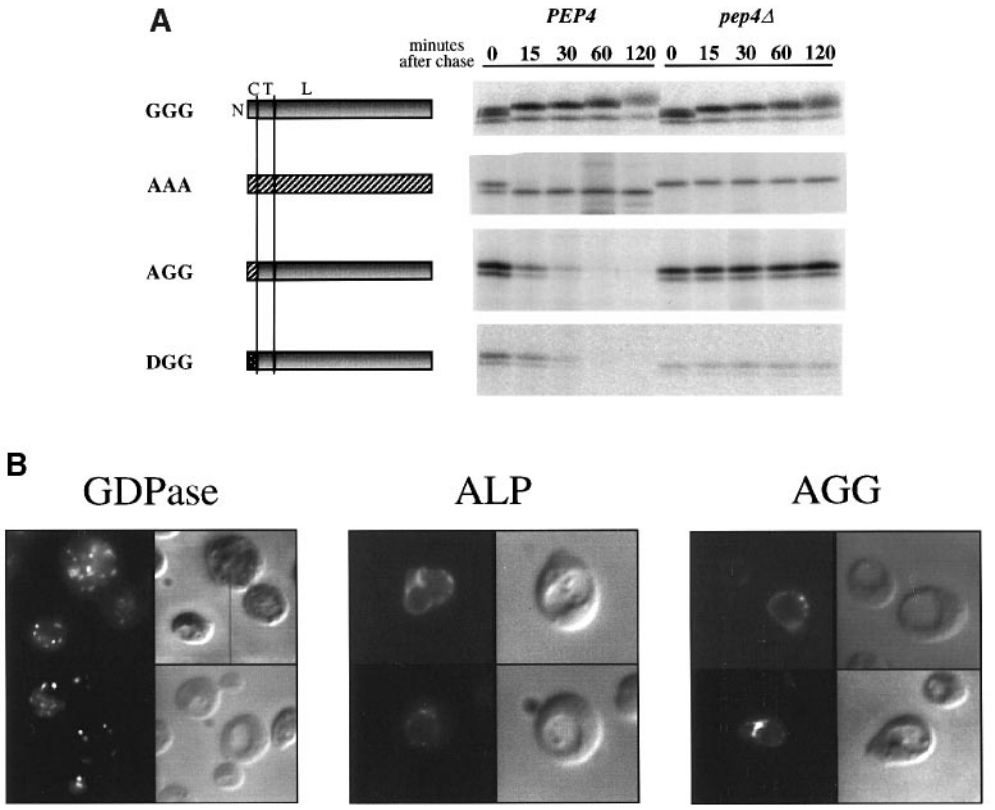

Fig. 2. (**A**) Analysis of AGG and DGG transport to the vacuole by pulse–chase immunoprecipitation. Protein designations: each letter corresponds to a domain in the chimeric protein, and represents the origin of that domain; G refers to GDPase, A refers to ALP and D refers to DPAP B. The domain structure of each protein is drawn with GDPase domains shaded, ALP domains diagonally hatched and DPAP B domains dotted. Above the GGG diagram, C = cytoplasmic domain, T = transmembrane domain and L = lumenal domain. Vertical lines represent the membrane. Proteins GGG, AGG and DGG were analyzed in strains G2-11 (*gda1*Δ *PEP4*) and GPY1452 (*gda1*Δ *pep4*Δ). Labeling (10 min) and chase (indicated times) were performed at 30°C, and proteins were precipitated with GDPase antibodies. AAA was analyzed in the same manner in DKY6280 (*pho8*Δ *PEP4*) and GPY1250 (*pho8*Δ *pep4*Δ), and the protein was precipitated with antibodies to ALP. (**B**) Representative immunofluorescence staining patterns of GDPase, ALP and AGG. In each panel, immunofluorescent images appear on the left, Nomarski optics images are on the right. GDPase and AGG were expressed in GPY1452 (*gda1*Δ *pep4*Δ), and cells were incubated with antibody to GDPase. ALP was expressed in GPY1250 (*pho8*Δ *pep4*Δ), and cells were incubated with affinity-purified antibody to the lumenal domain of ALP.

ALP were also monitored. AGG, like ALP, was distributed in a pattern that coincided with the vacuole membrane as visualized by differential interference optics (Figure 2B, AGG and ALP panels). This pattern was easily distinguished from the punctate staining of GDPase, typical of Golgi-localized proteins (Figure 2B, GDPase panel). Low expression levels of DGG thwarted attempts at immunofluorescence localization. However, the concordance of immunofluorescence and pulse–chase results for both ALP and AGG supports the reliability of the conclusions derived from pulse–chase analyses. Thus, the steady-state vacuolar localization of AGG shown by immunofluorescence provides corroborative evidence that AGG is transported to the vacuole efficiently.

As another means to assess Golgi complex versus vacuolar localization, chimeric proteins containing the enzymatically active GDPase lumenal domain were tested for the ability to reverse Golgi-specific glycosylation defects in cells lacking the endogenous GDPase gene (*GDA1*) (Abeijon *et al.*, 1993; Vowels and Payne, 1998). Such defects can be detected easily by monitoring the glycosylation status of CPY (Abeijon *et al.*, 1993). In wild-type cells, the 67 kDa core-glycosylated precursor form of CPY (p1 form) is elaborated by Golgi-resident mannosyltransferases to produce the 69 kDa p2 form

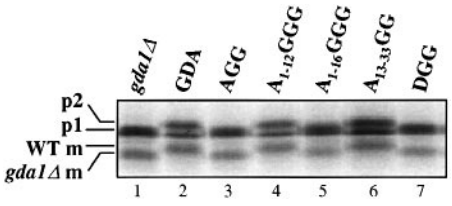

Fig. 3. Complementation of the *gda1*Δ CPY glycosylation defect by GDPase chimeric proteins. G2-11 cells containing either no plasmid (lane 1) or plasmids expressing either wild-type GDPase or GDPase chimeric proteins as indicated (lanes 2–7) were labeled for 5 min, chased with unlabeled amino acids for 5 min, and subjected to immunoprecipitation with CPY antibodies. The immature forms of CPY (p1 and p2), and mature forms of CPY from wild-type (WTm) or the *gda1*Δ mutant (*gda1*Δm) are as indicated on the left.

(Stevens *et al.*, 1982). Proteolytic maturation of p2 CPY in the vacuole yields the active 61 kDa protein (Stevens *et al.*, 1982). All CPY forms were detected by immunoprecipitation after a 5 min labeling followed by a 5 min chase (Figure 3, lane 2). The Golgi-specific glycosylation defects in *gda1*Δ cells were manifested by the absence of the p2 form and the correspondingly smaller (59 kDa) mature form of CPY (Figure 3, lane 1; Abeijon *et al.*, 1993). As shown in Figure 3, lanes 3 and 7, expression of AGG or DGG in *gda1*Δ cells did not restore wild-type

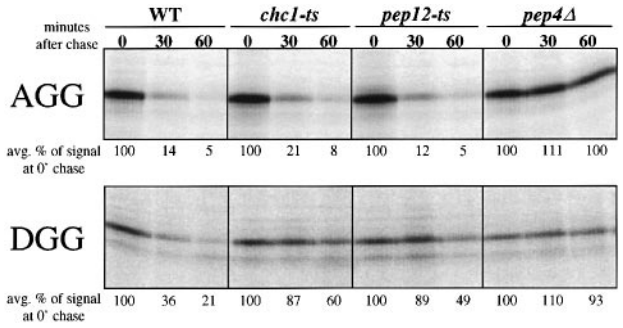

Fig. 4. Effect of *chc1-ts* and *pep12-ts* on AGG and DGG transport to the vacuole. Congenic strains SEY6210 (WT), GPY982 (*chc1-ts*), CBY9/5C (*pep12-ts*) and TVY1 (*pep4*Δ) expressing either AGG or DGG were incubated at the restrictive temperature of 37°C for 5 min prior to labeling for 10 min. After the addition of unlabeled amino acids, cells were harvested at the indicated chase times, and chimeric proteins were immunoprecipitated with antibodies to GDPase. Before analysis by SDS–PAGE, immunoprecipitated proteins were treated with EndoH to remove N-linked glycosyl residues. The faint band migrating below AGG and DGG is the endogenous GDPase. AGG and DGG band intensities were measured using ImageQuaNT™ software by Molecular Dynamics (Sunnyvale, CA). Values shown below each lane are the percentage of the 0 min signal that remains at each chase time, and represent the average of two experiments.

glycosylation of CPY. These data argue that neither protein is present in the Golgi complex long enough to complement the *gda1*Δ defect, as expected if the proteins are delivered to the vacuole efficiently.

By the multiple criteria presented in this section, both AGG and DGG are transported to the vacuole efficiently. Since the GDPase transmembrane and lumenal domains present in both proteins contain sorting information sufficient for Golgi localization, the results indicate that the cytoplasmic domains of both ALP and DPAP B are able to override the GDPase Golgi localization signals and direct transport to the vacuole.

Distinct vacuolar transport pathways for AGG and DGG

ALP and DPAP B have been shown to follow distinct pathways to the vacuole (Piper *et al.*, 1997). To examine whether the AGG and DGG chimeric proteins are also sorted into separate pathways, we analyzed AGG and DGG transport to the vacuole in *chc1-ts* and *pep12-ts* strains. Wild-type and mutant cells expressing either AGG or DGG were shifted to 37°C for 5 min to impose the CPY pathway block in mutant cells, and then the strains were subjected to pulse–chase immunoprecipitation analysis. As shown in Figure 4, the rate of *PEP4*-dependent degradation of AGG was the same in wild-type and mutant strains; by the 30 min chase point ~80% or more of AGG was degraded. In contrast, DGG was substantially stabilized by the *chc1-ts* and *pep12-ts* mutations. This was particularly evident at the 30 min chase time where >60% degradation of DGG occurred in wild-type cells compared with little if any degradation of DGG in the mutant cells. Analysis of CPY transport in each of the strains indicated that the temperature shift blocked CPY sorting in the mutant strains as expected (data not shown). These observations argue that AGG and DGG reach the vacuole by two different pathways. AGG is sorted into a clathrin- and *PEP12*-independent pathway whereas DGG follows the clathrin- and *PEP12*-dependent CPY pathway.

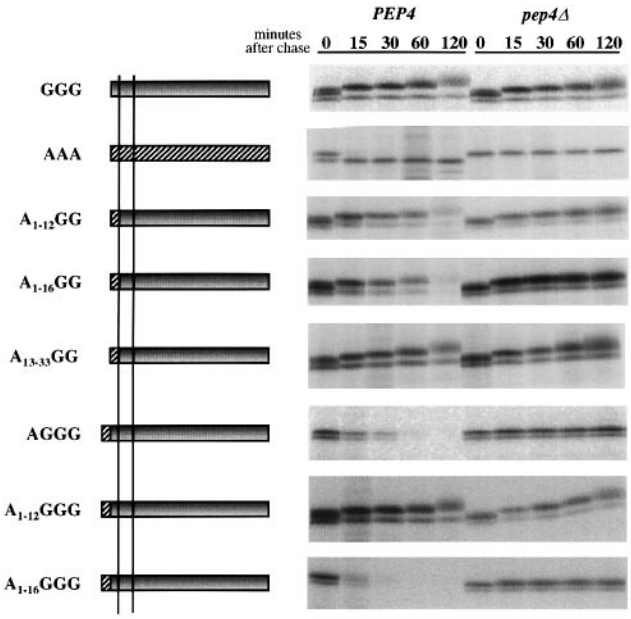

Fig. 5. Characterization of the ALP sorting signal by pulse–chase immunoprecipitation of ALP–GDPase chimeric proteins. Proteins are named and represented as described in Figure 2A. GGG and AAA immunoprecipitations, shown in Figure 2A, have been reproduced in this figure for convenient comparison with the chimeric proteins. All other proteins were expressed in congenic strains G2-11 (*gda1*Δ *PEP4*) and GPY1452 (*gda1*Δ *pep4*Δ). Labeling and immunoprecipitation of GDPase chimeras were as described in Figure 2A. The 33 amino acids of the ALP cytoplasmic domain are shown for reference.

An AP-3 pathway sorting signal in the N-terminal 16 amino acids of ALP

A series of deletions were introduced into the 33 amino acid ALP cytoplasmic domain of AGG to define the sequences necessary for sorting to the vacuole. Based on the reasoning described above, mutations affecting the vacuolar sorting signal in AGG were expected to uncover the Golgi localization signal, thus shifting localization from the vacuole to the Golgi complex. Chimeric proteins containing ALP residues 1–12 ($A_{1–12}$GG), 1–16 ($A_{1–16}$GG) or 13–33 ($A_{13–33}$GG) were analyzed by pulse–chase immunoprecipitation. All three proteins displayed the relative stability and incremental size increase diagnostic of Golgi localization, although $A_{1–16}$ GG was mostly degraded by the 120 min chase point in *PEP4* cells (Figure 5). In addition, each protein complemented the CPY glycosylation defect when expressed in *gda1*Δ cells, as diagnosed by the presence of p2 CPY and a mature form of 61 kDa (Table I; $A_{13–33}$GG is shown in Figure 3, lane 6). These data show that alterations of the ALP cytoplasmic sequences in AGG can dramatically slow transport to the vacuole and increase residence in the Golgi complex, thereby validating the strategy of using chimeric constructs containing Golgi localization signals. We conclude that the ALP sorting signal is perturbed in each chimeric protein (perhaps less so in $A_{1–16}$GG).

Table I. Summary of chimeric protein phenotypes

Construct	Stability	Glycosylation	Complements *gda1*Δ	Localization (vacuole or Golgi)
GGG	+	+	+	G
AAA	–	–	NA	V
AGG	–	–	–	V
DGG	–	–	–	V
$A_{1–12}$GG	+	+	+	G
$A_{1–16}$GG	+/–	+	+	G
$A_{13–33}$GG	+	+	+	G
AGGG	–	–	–	V
$A_{1–12}$GGG	+	+	+	G
$A_{1–16}$GGG	–	–	–	V
A_{AA}GGG	+	+	+	G
$GA_{13–33}$GG	+	+	ND	G

Constructs are named as described in Figure 2A. For stability, '+' denotes protein stability within the 2 h period of the experiment, and '+/–' or '–' represents decreased or no stability, respectively. For glycosylation, '+' indicates Golgi-specific glycosylation of the chimeric protein and '–' indicates lack of protein glycosylation. In the Complements *gda1*Δ column, '+' indicates that the protein rescues the CPY glycosylation defect of a *gda1*Δ mutant and '–' indicates that the protein fails to rescue this mutant phenotype. NA means not applicable, and ND indicates that the experiment has not been done. Localization to the vacuole (V) or Golgi complex (G) is inferred on the basis of the localization assay results.

In the $A_{1–12}$GG and $A_{1–16}$GG proteins, deletion of ALP cytoplasmic sequences moved the N-terminal ALP sequences adjacent to the GDPase transmembrane domain (TMD). We considered the possibility that this abnormal juxtaposition with the membrane might prevent recognition of a vacuolar sorting signal present in the N-terminal ALP sequences. To test this possibility, ALP residues 1–12 and 1–16 were fused to the N-terminus of GDPase so that the nine amino acid GDPase cytoplasmic tail separated the ALP sequences from the membrane ($A_{1–12}$GGG and $A_{1–16}$GGG). The GDPase sequences bear no resemblance to the sequences following amino acids 12 or 16 in the native ALP cytoplasmic domain. As a control, the complete ALP cytoplasmic domain was also joined to full-length GDPase (AGGG). Like AGG, AGGG was delivered rapidly to the vacuole where it was degraded (Figure 5). The ability of the ALP cytoplasmic domain to direct GDPase to the vacuole prevented AGGG from complementing the *gda1*Δ glycosylation defect (Table I). $A_{1–12}$GGG also mimicked its shorter version, $A_{1–12}$GG; $A_{1–12}$GGG was stable, progressively glycosylated and complemented *gda1*Δ (Figure 5; Figure 3, lane 4; Table I), signifying Golgi complex localization. In contrast, $A_{1–16}$GGG was strikingly different from $A_{1–16}$GG. Addition of the nine amino acid GDPase cytoplasmic domain between the ALP sequences and the TMD resulted in a protein that was degraded rapidly in the vacuole and failed to complement *gda1*Δ (Figure 5; Figure 3, lane 5; Table I). The results with $A_{1–16}$GGG identify a signal in the first 16 amino acids of ALP which acts as a vacuolar sorting signal if sufficiently separated from the membrane. Furthermore, the inability of ALP amino acids 1–12 to direct $A_{1–12}$GGG to the vacuole identifies ALP amino acids 13–16 as at least part of the vacuolar targeting determinant.

To establish that $A_{1–16}$GGG follows the AP-3-dependent ALP pathway to the vacuole, the pathway was blocked by disruption of the gene encoding the AP-3 β-subunit (*APL6*). Elimination of any AP-3 subunit greatly slows transport of ALP to the vacuole and leads to accumulation of precursor ALP, but has no effect on proteins transported via the CPY pathway (Cowles *et al.*, 1997a; Stepp *et al.*, 1997). AGGG, $A_{1–16}$GGG and DGG vacuole-dependent turnover was compared in wild-type and congenic *apl6*Δ strains by pulse–chase immunoprecipitation (Figure 6). To facilitate quantitation of the protein signal, chimeric proteins were subjected to endoglycosidase H (EndoH) treatment prior to SDS–PAGE, thus removing any N-linked glycosyl residues. Vacuole-dependent degradation of the chimeric proteins in wild-type cells proceeded rapidly, with the majority degraded by the 30 min chase point. Similarly, in cells lacking AP-3 function, DGG degradation occurred with near wild-type kinetics, consistent with transport of this protein through the clathrin-dependent pathway (Figure 6). In comparison, little turnover of the AGGG and $A_{1–16}$GGG chimeric proteins occurred in the *apl6*Δ mutants by the 30 min chase point, and at least 65% of the starting material was still present after 60 min. This severe retardation of vacuole-dependent turnover provides evidence that transport of $A_{1–16}$GGG to the vacuole, like that of full-length ALP, requires AP-3. Thus, the first 16 amino acids of ALP contain a signal that is sufficient for sorting into the AP-3-dependent pathway.

A critical LV sequence in the ALP sorting signal

A comparison of $A_{1–12}$GGG (Golgi) and $A_{1–16}$GGG (vacuole) localization indicates that the amino acids 13–16 of ALP, LVPG, are important for vacuolar targeting. Based on work in animal cells that implicates dileucine motifs in lysosomal membrane protein sorting (Sandoval and Bakke, 1994), we assessed the role of the LV pair at positions 13 and 14 in ALP transport. These residues were converted to alanines by site-directed mutagenesis of AGGG to generate A_{AA}GGG. Transport of A_{AA}GGG to the vacuole was virtually abolished by this mutation (Figure 7). Without a vacuolar targeting signal to override the Golgi localization information present in the GDPase sequences, A_{AA}GGG was localized to the Golgi complex as demonstrated by the stability and progressive size increase of this chimera compared with AGGG (Figure 7). Also, unlike AGGG, A_{AA}GGG complemented *gda1*Δ (Table I). From these results, the LV pair at position 13–14 of the ALP cytoplasmic domain appears to be an important component of the vacuolar targeting signal in the chimeric protein.

The role of Leu13Val14 in vacuolar sorting was assessed similarly by changing the two residues to alanines in native ALP (ALP_{AA}). Pulse–chase immunoprecipitation revealed that mutation of Leu13Val14 in ALP substantially slowed, but did not prevent, delivery to the vacuole as monitored by proteolytic maturation (Figure 8). Wild-type ALP was processed completely by the 15 min chase point (Figure 8, lane 2) while ~50% of ALP_{AA} still remained in precursor form at the 30 min chase point (Figure 8, lane 11). To test whether the residual maturation of ALP_{AA} was due to rerouting through the CPY pathway, maturation of wild-type and mutant ALP was measured in *pep12-ts* cells shifted to 37°C for 5 min to block traffic through the endosomal pathway. Imposition of the *pep12* block had no effect on wild-type ALP (Figure 8, lanes 5–8), but

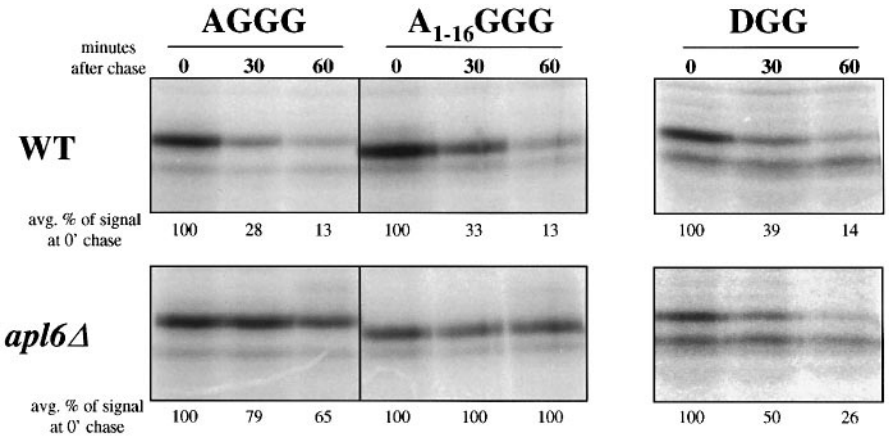

Fig. 6. Effect of *apl6*Δ on transport of AGGG, $A_{1–16}$GGG and DGG to the vacuole. Congenic strains LRB770 (WT) or LRB771 (*apl6*Δ) expressing either AGGG, $A_{1–16}$GGG or DGG were subject to pulse–chase immunoprecipitation and EndoH treatment as described for Figures 2A and 4. The percentage of protein signal at each time point was determined as described in Figure 4. The lower, stable band in each lane is endogenous GDPase.

effectively prevented maturation of ALP_{AA} (Figure 8, lanes 13–16). The sensitivity of ALP_{AA} maturation to *pep12-ts* argues that mutation of Leu13Val14 prevents sorting into the AP-3-dependent pathway and leads to slow progression of the mutant protein through the endosomal 'default' route to the vacuole. These results demonstrate that the LV pair is an important component of the sorting signal that directs ALP into the AP-3 pathway to the vacuole.

Discussion

We have described a strategy to identify vacuolar localization signals that utilizes chimeric membrane proteins whose potential default destination has been altered from the vacuole to the Golgi complex by the presence of Golgi localization information. Our strategy stemmed from the finding that, when present together in a chimeric protein, a vacuolar targeting signal in the ALP cytoplasmic domain can override Golgi localization determinants in GDPase transmembrane and lumenal domains. This hierarchical relationship between sorting signals offered a means to define the ALP vacuolar sorting determinant through mutations which eliminate vacuolar sorting, thereby uncovering the GDPase localization signal and shifting localization from the vacuole to the Golgi complex. Circumventing the problem of default vacuolar transport of membrane proteins allowed characterization of the ALP sorting signal in wild-type cells, without the use of mutations in vacuolar transport pathways that previously were necessary to monitor sorting into the ALP pathway (Cowles *et al.*, 1997b; Piper *et al.*, 1997). Our analysis demonstrates that the N-terminal 16 amino acids of the ALP cytoplasmic domain are sufficient to redirect a GDPase-containing chimeric protein from the Golgi complex to the vacuole. Within the ALP cytoplasmic sequence, the L and V residues at positions 13 and 14 are critical for vacuolar targeting of chimeric proteins and full-length ALP to the vacuole through a clathrin-independent, AP-3-dependent pathway.

Additional features of the LV-based sorting signal are revealed by variations in the efficiency of vacuolar targeting among different chimeric proteins. First, the position of the LV sequence in the cytoplasmic domain is important.

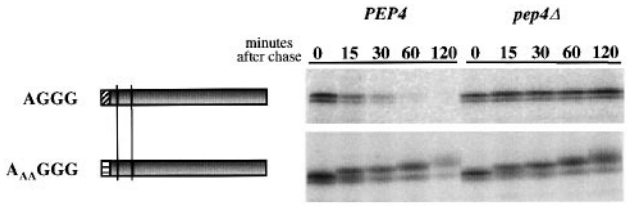

Fig. 7. Effect of mutating ALP cytoplasmic domain residues Leu13Val14 on vacuolar transport of AGGG. Protein names, diagrams, immunoprecipitation procedures and protein analysis are as described in Figure 2A, except that the mutant ALP cytoplasmic domain is shown as a striped box in the diagrammatic representation of the protein. The AGGG figure has been reproduced here for ease of comparison with A_{AA}GGG.

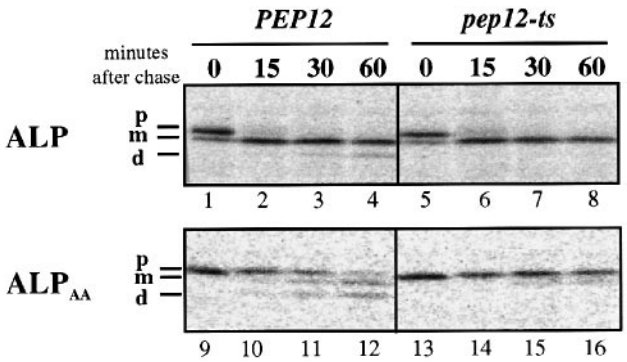

Fig. 8. Effect of mutating ALP cytoplasmic domain residues Leu13Val14 on vacuolar transport of full-length ALP in wild-type (*PEP12*) and *pep12-ts* cells. Congenic strains SEY6210 (*PEP12*; lanes 1–4) and CBY9/5C (*pep12-ts*; lanes 5–8) expressing native ALP, or congenic strains DKY6280 (*pho8*Δ; lanes 9–12) and GPY1697 (*pep12-ts pho8*Δ; lanes 13–16) expressing ALP_{AA} were incubated for 5 min at the restrictive temperature of 37°C to impose the *pep12-ts* transport block. Analysis was as described in Figure 2A. Proteins were immunoprecipitated with antibodies to ALP. ALP precursor (p) and mature (m) forms are indicated, as is a degradation product (d) of ALP.

Little vacuolar targeting occurred when the sorting signal was positioned six amino acids from the TMD in $A_{1–16}$ GG, but efficient vacuolar targeting occurred when the signal was spaced 13 amino acids from the TMD by the GDPase cytoplasmic domain in $A_{1–16}$GGG. This difference suggests that effective presentation of the LV signal requires some separation from the membrane. Such

separation is achieved in native ALP where the LV sequence is located 20 residues from the TMD. Secondly, consideration of the properties of the $A_{13–33}GG$ chimera indicates that the LV pair is not sufficient for vacuolar localization since this chimera contains LVPG at residues 2–5 but is localized to the Golgi complex efficiently. Adding the nine amino acid GDPase cytoplasmic domain to the N-terminus of $A_{13–33}GG$ to create $GA_{13–33}GG$, and thereby positioning the LV sequence further from the N-terminus of the protein, also did not result in vacuolar targeting (data not shown). These results imply that the vacuolar targeting signal in ALP consists of sequences including, and N-terminal to, the LV residues. The acidic glutamate residue four amino acids N-terminal to the leucine is noteworthy. Studies of dileucine targeting signals in mammalian proteins have shown a requirement in some cases for acidic residues 3–5 amino acids preceding the first leucine (Motta *et al.*, 1995; Pond *et al.*, 1995; Dietrich *et al.*, 1997). Additional mutagenesis studies of ALP–GDPase chimeric proteins will allow assessment of the importance of the nearby acidic residue as well as other flanking residues. In addition to ALP, the only other known cargo for the AP-3-dependent pathway is the vacuolar t-SNARE Vam3p (Cowles *et al.*, 1997b; Piper *et al.*, 1997). Vam3p contains four potential targeting signals consisting of dileucine-related sequences preceded by an acidic residue 3–4 amino acids before the first leucine, and none are proximal to membrane sequences.

Two prior studies have addressed the ALP vacuolar targeting signals and reported different conclusions. Both studies employed fusions between ALP and vacuolar membrane proteins that are delivered to the vacuole via the CPY pathway, and distinguished the two vacuolar transport pathways with mutants defective in transport through the CPY pathway. Cowles *et al.* (1997b) generated chimeric proteins containing domains from ALP and from the vacuolar membrane protein carboxypeptidase S (CPS). In agreement with our results, the first 16 amino acids of ALP were found to be sufficient for sorting to the ALP pathway. Similarly, Piper *et al.* (1997) reported that the ALP cytoplasmic domain joined to DPAP B is sufficient to target the chimeric protein into the ALP pathway. However, additional studies by this group suggested that the first 16 amino acids of ALP are not absolutely necessary for vacuolar targeting (Piper *et al.*, 1997). A deletion mutant of full-length ALP lacking amino acids 2–21, and thus Leu13Val14, was still delivered, albeit slowly, to the vacuole when the CPY pathway was blocked. This result suggests the presence of a targeting signal for the ALP pathway located between residues 22 and 33 of ALP. Our analysis did not detect vacuolar targeting of $A_{13–33}GG$, which indicates that if a vacuolar targeting signal is located between amino acids 22 and 33, it is not sufficiently strong to override the GDPase Golgi localization signals. Furthermore, we observed that mutation of the LV to alanines in full-length ALP resulted in slowed maturation that was almost entirely dependent on a functional CPY transport pathway. Thus, in the context of the full-length cytoplasmic domain, sequences other than the LV pair are ineffective in targeting transport into the ALP pathway. The basis for these discrepancies is not clear; however, the results can be reconciled by a model in which the LV sequence constitutes the primary vacuolar targeting signal in ALP. Removal of this signal by deletion of amino acids 2–21 could expose a weak, otherwise cryptic targeting signal between residues 22 and 33.

LV-based sorting into the ALP pathway relies on AP-3. From this finding, it is tempting to speculate that the LV signal mediates sorting by interacting directly with AP-3 on nascent transport vesicles budding from the Golgi complex, in the same way that tyrosine- and dileucine-based signals are thought to be recognized by AP-1 or AP-2 to direct cargo incorporation into clathrin-coated vesicles in mammalian cells (Kirchhausen *et al.*, 1997; Marks *et al.*, 1997). However, preliminary attempts to detect interaction of the ALP signal with AP-3 subunits using the yeast two-hybrid assay have been unsuccessful (J.J.Vowels, unpublished results; C.Cowles, G.Odorizzi and S.Emr, personal communication). Thus, it remains to be determined whether the LV signal interacts with AP-3 directly, or whether it functions through interaction with additional sorting factors.

The LV-based sorting signal in ALP resembles mammalian dileucine and dileucine-like signals which act in endocytosis and sorting from the TGN to endosomes and lysosomes (Sandoval and Bakke, 1994; Mellman, 1996). These dileucine motifs have been proposed to direct incorporation into clathrin-coated vesicles at the TGN in mammalian cells (Sandoval and Bakke, 1994), an idea which contrasts with the clathrin-independent vacuolar sorting mediated by the ALP LV signal in yeast. Although compelling evidence indicates that dileucine-containing mammalian proteins enter clathrin-coated pits at the plasma membrane (Miettinen *et al.*, 1989), and dileucine motifs bind AP-2 adaptor complexes *in vitro* (Amigorena *et al.*, 1992; Heilker *et al.*, 1996; Dietrich *et al.*, 1997), dileucine-directed sorting into clathrin-coated vesicles at the TGN has only been inferred by analogy with endocytosis (Sandoval and Bakke, 1994), and from the ability of dileucine motifs to bind AP-1 complexes *in vitro* (Amigorena *et al.*, 1992; Heilker *et al.*, 1996; Dietrich *et al.*, 1997). In most cases, functional or morphological evidence for association of dileucine-containing proteins with clathrin-coated membranes at the TGN is lacking. Notable exceptions are the mannose-6-phosphate receptors (M6PRs) (Kornfeld and Mellman, 1989), but recent results raise the possibility that a casein kinase II phosphorylation site, not the dileucine sorting signal, is responsible for interaction of the cation-dependent M6PR with AP-1 (Mauxion *et al.*, 1996). Based on the ability of the LV signal in ALP to direct sorting into a clathrin-independent AP-3 pathway in yeast, and the evolutionary conservation of AP-3 subunits, we propose that dileucine signals can function as sorting signals for an analogous AP-3-dependent pathway to lysosomal compartments in mammalian cells. Like the yeast ALP pathway, the mammalian AP-3 pathway is likely to be independent of clathrin since AP-3 does not co-purify with clathrin-coated vesicles and does not co-localize with TGN clathrin coats (Newman *et al.*, 1995; Simpson *et al.*, 1996, 1997; Dell'Angelica *et al.*, 1997b). In this model, preference of dileucine signals for AP-3 versus AP-1 at the Golgi complex could be a function of the affinity of the sorting signal for a particular AP complex, which could be determined, at least in part, by sequences flanking the signal. A similar model has already been put forward for tyrosine-based

Table II. Yeast strains used in this study

Strains	Genotype	Reference
G2-11	*MATα gda1::LEU2 ade2-101(oc) his3-Δ200 leu2-Δ1 lys2-801(am) trp1-Δ1 ura3-52*	Abeijon *et al.* (1993)
GPY1452	*MATα gda1::LEU2 ade2-101(oc) his3-Δ200 leu2-Δ1 lys2-801(am) trp1-Δ1 ura3-52 pep4Δ*	Vowels and Payne (1998)
GPY1100	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1*	Payne and Schekman (1989)
GPY418	GPY1100; *chc1-521*	Tan *et al.* (1993)
GPY1305	GPY1100; *end4-1::URA3*	this study
GPY1306	GPY418; *end4-1::URA3*	this study
GPY1508	GPY1305; *end4-1*	this study
GPY1526	GPY1306; *end4-1*	this study
DKY6280	*MATa pho8::TRP1 ade2-101 his3-Δ200 leu2-3, 112 suc2Δ9 trp1-Δ901 ura3-52*	Klionsky and Emr (1989)
GPY1250	*MATa pho8::TRP1 ade2-101 his3-Δ200 leu2-3, 112 suc2Δ9 trp1-Δ901 ura3-52 pep4::LEU2*	Vowels and Payne (1998)
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.* (1988)
GPY982	SEY6210; *chc1-521*	this study
CBY9/5C	*MATa pep12-ts ade2-101 leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	gift of C.Burd, UCSD
TVY1	SEY6210; *pep4Δ::LEU2*	gift of S.Emr, UCSD
LRB770	*MATa bar1 trp1 leu2 his3 ura3*	gift of L.Robinson, LSU
LRB771	*MATa bar1 trp1 leu2 his3 ura3 apl6::LEU2*	gift of L.Robinson, LSU
GPY1697	CBY9/5C; *pho8Δ::LEU2*	this study

binding to AP-1 and AP-2 (Marks *et al.*, 1997). Common interaction of different dileucine signals with AP-2 at the plasma membrane would then allow endocytic retrieval of proteins which escape sorting in the TGN, thereby conferring additional fidelity to the localization of proteins to the lysosomal compartment.

Materials and methods

Media and strains

YPD medium contains 1% Bacto-yeast extract, 2% Bactopeptone without amino acids (Difco Laboratories, Inc., Detroit, MI) and 2% dextrose. SD medium contains 0.67% yeast nitrogen base without amino acids (Difco) and 2% dextrose. Supplemented SD is SD containing 40 μg/ml adenine, 30 μg/ml leucine and lysine, and 20 μg/ml histidine, uracil and tryptophan (Sigma Chemical Co., St. Louis, MO). SD CAA is supplemented SD containing 5 mg/ml vitamin assay casamino acid mix (Difco). SD CAA-ura is SD CAA lacking uracil. SDYE is SD with 0.2% yeast extract. Cell densities in liquid culture were measured in a 1 cm plastic cuvette in a Beckman Instruments DU-62 spectrophotometer. One OD_{500} unit is equivalent to 2.3×10^7 cells/ml.

The genotypes of yeast strains used in this study are listed in Table II.

One-step gene replacement (Rothstein, 1991) was used to substitute the wild-type *END4* gene in GPY1100 and GPY418 with the *end4-1* allele present on the *URA3* integrating vector, pSN261 (gift of Howard Riezman, University of Basel), thus creating GPY1305 and GPY1306, respectively. Since all plasmids transformed into these strains utilized uracil-based selection, GPY1305 and GPY1306 were converted to *ura3* by growth on 5-fluoro-orotic acid. Conversion of *URA3* to *ura3* in GPY1305 and GPY1306 generated GPY1508 and GPY1526, respectively. The presence of the *end4-1* allele in GPY1508 was confirmed by the temperature-sensitive growth conferred by this allele (Raths *et al.*, 1993). The *end4-1* allele in the *chc1-ts end4-1* double mutant strain, GPY1526, was confirmed by complementation analysis.

To create GPY982, *chc1-521* was integrated into SEY6210 as described in Tan *et al.* (1993). To generate the GPY1697 strain, the pGP10 plasmid containing *pho8::LEU2* (gift of Tom Stevens, University of Oregon) was used to disrupt *PHO8* in the CBY9/5C strain by the gene replacement method of Rothstein (1991). An alkaline phosphatase overlay assay, adapted from Chapman and Munro (1994) by Nothwehr *et al.* (1996), was used to confirm loss of *PHO8* activity in GPY1697. Ten mg/ml Fast Red Dye (Sigma #F8764) and 1 mg/ml naphthol as phosphate (Sigma #N9252) were mixed into an agar solution (23% melted 1.5% agar, 500 mM Tris pH 9.0, 5 mM $MgSO_4$ and 1% Triton X-100) equilibrated to 60°C. This mixture was poured carefully over fresh patches of cells grown on minimal media plates. *PHO8* strains turn red, while *pho8* mutants remain white.

Transformations into yeast were performed by the lithium acetate procedure (Ito *et al.*, 1983).

Plasmid constructions

DNA manipulations were carried out essentially as described in Sambrook *et al.* (1989). Constructions of pRS *GDA1* (containing the *GDA1* gene encoding GDPase) and pRS *PHO8* (containing the *PHO8* gene encoding ALP) are described in Vowels and Payne (1998). The numbering system for *GDA1* DNA sequences is as follows: base pairs 1–132, promoter sequences; 133–159, cytoplasmic sequences; 160–204, TMD sequences; and 205–1689, lumenal sequences. The numbering system for *PHO8* DNA sequences is as follows: base pairs 1–99, cytoplasmic sequences; 100–177, TMD sequences; 178–1702, lumenal sequences.

The polymerase chain reaction (PCR) (Saiki *et al.*, 1988) was used in all plasmid constructions. PCR fragments were sequenced to confirm proper fragment orientation and lack of sequence error. Sequencing was performed either by the dideoxy chain termination procedure (Sanger *et al.*, 1977) using the Sequenase enzyme (United States Biochemical Corp., Cleveland, OH), or performed in the UCLA DNA Sequencing Facility using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and AmpliTaq® DNA polymerase from Perkin Elmer (Foster City, CA). All constructs are subcloned into the pRS316 vector (Sikorski and Hieter, 1989), and have *GDA1* promoter sequences, except pRS *PHO8* which has native *PHO8* promoter sequences. The details of construction of each chimeric protein are given below, and a partial peptide sequence for each construct is shown in Table III.

AGG and DGG. Step 1: *PHO8* sequences 1–100 from pRS *PHO8* and *DPP2* sequences 1–87 from plasmid pGP6 (gift of Tom Stevens, University of Oregon) were amplified by PCR using appropriate 5′ and 3′ primers into which *Nde*I restriction sites were engineered. Step 2: each PCR fragment was inserted into the *Nde*I site of pBKS $GG_{205–465}$ (Vowels and Payne, 1998), resulting in pBKS $AGG_{205–465}$ and pBKS $DGG_{205–465}$. Step 3: the *Hin*dIII–*Bsp*EI fragment from pBKS $AGG_{205–465}$, or the *Eco*RV–*Bsp*EI fragment from pBKS $DGG_{205–465}$ was ligated into pRS *GDA1* digested with *Kpn*I (filled in) and *Bsp*EI to generate full-length pRS AGG and pRS DGG.

$A_{1–12}GG$, $A_{1–16}GG$ and $A_{13–33}GG$. *PHO8* sequences 1–36 ($A_{1–12}GG$), 1–48 ($A_{1–16}GG$) or 37–100 ($A_{13–33}GG$) were amplified using appropriate 5′ and 3′ primers containing *Nde*I sites. In $A_{1–16}GG$, an additional arginine residue was added after base pair 48 just before the transmembrane sequences to ensure proper positioning of the chimeric protein in the membrane. The resulting PCR fragments were used to generate the full-length constructs as described in steps 2 and 3 above.

AGGG, $A_{1–12}GGG$ and $A_{1–16}GGG$. *GDA1* sequences 1–332 were PCR amplified using a 5′ primer containing an *Nde*I site engineered such that the ATG of the *Nde*I sequence coincides with the initiating methionine. The *Nde*I–*Bsp*EI PCR fragment was ligated into pBKS $GG_{205–465}$ to generate Nde-$GGG_{205–465}$. *Nde*I fragments from pBKS $AGG_{205–465}$, pBKS $A_{1–12}GG_{205–465}$ and pBKS $A_{1–16}GG_{205–465}$ containing the relevant segments of the ALP cytoplasmic tail were inserted into the *Nde*I site of Nde-$GGG_{205–465}$ (thus generating pBKS $AGGG_{205–465}$, pBKS $A_{1–12}GGG_{205–465}$ and pBKS $A_{1–16}GGG_{205–465}$, respectively). pRS AGGG, $A_{1–12}GGG$ and $A_{1–16}GGG$ chimeric genes were generated as described in step 3 above.

Table III. Partial amino acid sequence of ALP–GDPase chimeric constructs

Construct	Cytoplasmic tail sequence	Transmembrane domain sequence	Partial lumenal domain sequence
GGG	MAPIFRNYR	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND
AAA	MMTHTLPSEQTRLVPGSDSSSRPKKRRISKRSK	IIVSTVVCIGLLLVLVQLAFPSSFAL	RSASHKKKN
AGG	MMTHTLPSEQTRLVPGSDSSSRPKKRRISKRSK**HMR**	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND
DGG	*MEGGEEEVERIPDELFDTKKKHLLDKLIR***HMR**	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND
A_{1-12}GG	MMTHTLPSEQTR**HMR**	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND
A_{1-16}GG	MMTHTLPSEQTRLVPG**RHMR**	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND
A_{13-33}GG	MLVPGSDSSSRPKKRRISKRSK**HMR**	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND
AGGG	MMTHTLPSEQTRLVPGSDSSSRPKKRRISKRSK**HM** APIFRNYR	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND
A_{1-12}GGG	MMTHTLPSEQTR**HM**APIFRNYR	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND
A_{1-16}GGG	MMTHTLPSEQTRLVPG**RHM**APIFRNYR	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND
A_{AA}GGG	MMTHTLPSEQTR***AA***PGSDSSSRPKKRRISKRSK**HM**APIFRNYR	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND
ALP_{AA}	MMTHTLPSEQTR***AA***PGSDSSSRPKKRRISKRSK	IIVSTVVCIGLLLVLVQLAFPSSFAL	RSASHKKKN
GA_{13-33}GG	MAPIFRNYRLVPGSDSSSRPKKRRISKRSK**HMR**	FAIGAFAVIMLILLI	KTSSIGPPSIARTVTPNASIPKTPEDISILPVND

Amino acid sequences from GDPase are shown in regular font. Amino acids from ALP are underlined. Italicized amino acids come from DPAP B. Bold letters are amino acids encoded by restriction sites that were introduced during construction of the protein, or that were introduced purposely to ensure proper positioning of the protein in the membrane. The alanines in bold italic font indicate residues mutated by site-directed mutagenesis.

$A_{AA}GG$ and $A_{AA}GGG$. Amino acids Leu13 and Val14 in the cytoplasmic tail of ALP were changed to alanines in the pBKS $AGG_{205–465}$ plasmid using site-directed mutagenesis (McClary *et al.*, 1989). The resulting plasmid, pBKS $A_{AA}GG_{205–465}$, was cleaved with *Hin*dIII and *Bsp*EI, and the mutant ALP sequences were ligated into pRS *GDA1* to generate $A_{AA}GG$. To construct $A_{AA}GGG$, an *Xho*I fragment from pBKS $A_{AA}GG_{205–465}$ was inserted into pBKS $AGGG_{205–465}$. pRS $A_{AA}GGG$ was generated from pBKS $A_{AA}GGG_{205–465}$ as described in step 3 above.

$GA_{13–33}GG$. Two PCR fragments were generated independently: the first contained *GDA1* residues 133–160 fused to *PHO8* sequences 37–48, the second includes *GDA1* sequences 149–160 fused to *PHO8* base pairs 37–100. In a third PCR, the two PCR fragments were mixed, and because they contain homologous *GDA1* and *PHO8* regions, they generated a third PCR fragment containing *GDA1* sequences 133–160 fused to *PHO8* 37–100 residues. This product was ligated into the *Nde*I site of pBKS $AGG_{205–465}$. The resulting plasmid, pBKS $GA_{13–33}GG_{205–465}$, was subjected to step 3 to generate the full-length construct.

$ALP_{LV–AA}$. pBKS *PHO8* [2.3 kb *Eco*RV–*Sal*I *PHO8* fragment in pBKS (Stratagene, La Jolla, CA)] was used as the template for single strand oligonucleotide mutagenesis (McClary *et al.*, 1989) to mutate Leu13 and Val14 in the ALP cytoplasmic tail to alanines. An *Nhe*I fragment from pBKS $ALP_{LV–AA}$ was reinserted into full-length pRS *PHO8* to generate $ALP_{LV–AA}$.

Metabolic labeling and immunoprecipitation

Metabolic labeling and immunoprecipitation were performed as previously described (Seeger and Payne, 1992a; Vowels and Payne, 1998). Unless noted otherwise, temperature-sensitive strains were incubated at the restrictive temperature for 5 min prior to the addition of radioactive [^{35}S]methionine and cysteine, and then labeled for 10 min. Antibodies to GDPase were a gift of P.Berninsone and C.Hirschberg (Berninsone *et al*., 1995). Antibodies to ALP are described in Seeger and Payne (1992a). Antibodies to CPY were a gift from Scott Emr.

Treatment of immunoprecipitates with EndoH is described in Vowels and Payne (1998). Deglycosylated proteins were subject to SDS–PAGE, and proteins were analyzed by PhosphorImage analysis using a Molecular Dynamics PhosphorImager 445-SI (Sunnyvale, CA). To determine the percentage of signal at each time point, the volume of signal in each protein band was quantitated using the ImageQuaNT™ program by Molecular Dynamics. No background correction was applied since background signal was minimal. The numerical results shown are the average signal from at least two experiments.

Immunofluorescence

Indirect immunofluorescence was performed as described in Vowels and Payne (1998). Affinity-purified ALP antibodies were the generous gift of J.Shaw.

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