Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting

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Yeast endosomes, like those in animal cells, invaginate their membranes to form internal vesicles. The resulting multivesicular bodies fuse with the vacuole, the lysosome equivalent, delivering the internal vesicles for degradation. We have partially purified internal vesicles and analysed their content. Besides the known component carboxypeptidase S (Cps1p), we identified a polyphosphatase (Phm5p), a presumptive haem oxygenase (Ylr205p/Hmx1p) and a protein of unknown function (Yjl151p/Sna3p). All are membrane proteins, and appear to be cargo molecules rather than part of the vesicle-forming machinery. We show that both Phm5p and Cps1p are ubiquitylated, and that in a *doa4* mutant, which has reduced levels of free ubiquitin, Cps1p, Phm5p and Hmx1p are mis-sorted to the vacuolar membrane. Mutation of Lys 6 in the cytoplasmic tail of Phm5p disrupts its sorting, but sorting is restored, even in *doa4* cells, by the biosynthetic addition of a single ubiquitin chain. In contrast, Sna3p enters internal vesicles in a ubiquitin-independent manner. Thus, ubiquitin acts as a signal for the partitioning of some, but not all, membrane proteins into invaginating endosomal vesicles.

Keywords: endosome/multivesicular body/ubiquitin/vacuole/yeast

Introduction

The endosomes of both yeast and mammalian cells undergo a process of membrane segregation that is crucial to their function. Regions of the limiting endosomal membrane invaginate to form internal vesicles and the resulting structure is known as a multivesicular body. Membrane proteins that enter the internal vesicles are sequestered from the cytoplasm, thus terminating any interactions they may have with signal transduction pathways. In yeast, the internalization process occurs mainly in late endosomes and seems to be irreversible; mature multivesicular bodies fuse with the vacuole, releasing the internal vesicles into the vacuolar interior where they are destroyed by hydrolytic enzymes (Odorizzi et al., 1998; Lemmon and Traub, 2000). Proteins that do not enter the internal vesicles are either removed by conventional vesicular transport prior to fusion with the vacuole, or become constituents of the delimiting vacuolar membrane.

The sorting process that delivers some membrane proteins to the internal membranes for destruction whilst others remain outside is not well understood. Most of the proteins known to reach the vacuole interior are plasma membrane proteins which undergo regulated turnover, such as the pheromone receptors and some permeases (Berkower et al., 1994; Volland et al., 1994; Egner et al., 1995; Lai et al., 1995; Hicke and Riezman, 1996; Medintz et al., 1996; Beck et al., 1999; Springael et al., 1999), but one hydrolytic enzyme, carboxypeptidase S (Cps1p), is also known to follow this route (Odorizzi et al., 1998). Once inside the vacuole, Cps1p is processed to release a soluble form of the active enzyme. We have recently shown that sorting of membrane proteins into the internal vesicles can be influenced by the nature of their transmembrane domains, the insertion of a potentially charged residue being sufficient to cause the internalization and destruction of Pep12p, a SNARE protein that normally remains on the endosomal surface (Reggiori et al., 2000).

To gain further insight into the sorting process, we have partially purified the vesicles that accumulate inside the vacuoles of a hydrolase-deficient yeast strain, identified proteins that are in them, and studied the targeting of these components. We detected three new proteins that follow the same pathway as Cps1p: a polyphosphatase (Phm5p), a protein with homology to haem oxygenases (Hmx1p) and a possible proton transporter (Sna3p). All are membrane proteins and appear to represent cargo molecules rather than components of the sorting machinery itself.

In animal cells, the fate of internalized epidermal growth factor (EGF) receptors has been shown to be controlled by ubiquitylation, which prevents their recycling to the surface and instead promotes their degradation in lysosomes (Levkowitz et al., 1998). Similarly, transport of the interleukin-2 receptor from early to late endosomes is ubiquitin dependent (Rocca et al., 2001). Turnover of plasma membrane proteins in yeast is also known to be regulated by ubiquitylation. Conjugation of ubiquitin, a 76 residue polypeptide, to the cytosolic regions of a variety of proteins stimulates their endocytosis (Hicke, 2001). Ubiquitin has also been implicated in the regulated sorting of tryptophan permease and of general amino acid permease from the *trans*-Golgi network to endosomes (Beck et al., 1999; Helliwell et al., 2001), and recent reports have suggested a possible involvement of ubiquitylation in vacuolar degradation of the **a**-factor transporter Ste6p and other membrane proteins (Amerik et al., 2000; Losko et al., 2001).

We show here that ubiquitylation of Phm5p is both necessary and sufficient for its incorporation into the internal vesicles of multivesicular bodies. Ubiquitin is also involved in the targeting of Cps1p and Hmx1p to these vesicles. However, Sna3p follows the same pathway in a
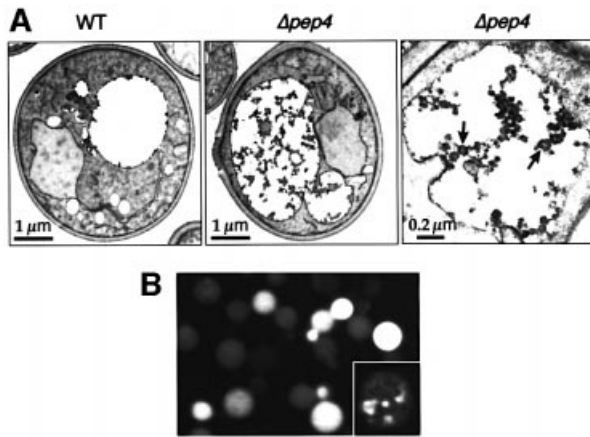
Fig. 1. Vesicles accumulate inside protease-deficient vacuoles. (**A**) EM sections of wild-type (WT) and *Δpep4* yeast. Vacuoles appear as large white areas in these permanganate-fixed cells, which are empty in the wild-type cells but contain aggregates with vesicular profiles in the mutant (arrows). (**B**) Isolated vacuoles purified from a *Δpep4* strain expressing a GFP-tagged mutant form of Pep12p show internal fluorescence. The inset shows an enlarged single confocal scan of a vacuole, revealing punctate structures within it.

Fig. 2. Isolation of internal vesicles from vacuoles. (**A**) The fractionation procedure described in the text and Materials and methods was carried out on *pep4* cells expressing GFP–PEP3D. Immunoblotting was used to follow the distribution of proteins in total cell extract (total), purified vacuoles, the P15 pellet, the S500 supernatant and the P500 pellet. The P500 and S500 samples were overloaded as indicated; the total extract was substantially underloaded since only a few percent of the vacuoles were recovered in the initial purification step. Intact GFP–PEP3D is under-represented in the total vacuole fraction due to proteolysis during sample preparation (data not shown). Markers are alkaline phosphatase (ALP, vacuolar membrane marker), carboxypeptidase Y (CPY, vacuole lumen), phosphoglycerate kinase (Pgk1p, cytosolic), dolicholphosphomannose synthase (Dpm1p, ER) and the syntaxin Sso2p (plasma membrane). (**B**) Coomassie Blue-stained gel of the S500 and P500 fractions from a scaled-up preparation. Molecular weight markers are indicated on the left (kDa). Proteins in the P500 sample were identified by tryptic digestion and mass spectrometry. These were often present in multiple bands, presumably due to proteolysis, and these are indicated by the linked lines. Asterisks mark bands containing the GFP–PEP3D marker, which also contaminated all three of the Lap4p bands. Numbered bands were identified as follows: 1, Fas1p (fatty acyl CoA synthase); 2, Ena2p (plasma membrane Na⁺-ATPase); 3, Pma1p (plasma membrane H⁺-ATPase); 4, a mixture of Tef2p (translation elongation factor) and Eno2p (enolase); 5, Adh1p (alcohol dehydrogenase); 6, Tdh3p; 7, Tdh2p (glyceraldehyde-3-P dehydrogenases). Phm5p was not identified from this gel, but the relevant part of a silver-stained gel from which it was obtained is shown on the right.

ubiquitin-independent manner. Thus, ubiquitylation marks some, but not all, membrane proteins for sorting to the vacuolar interior.

Results

Multivesicular body purification

It has been shown that vesicles derived from multi-vesicular bodies accumulate inside the vacuole in *Δvma4* cells. Vma4p is a subunit of the vacuolar H⁺-ATPase and in its absence vacuoles are not properly acidified, leading to a reduction of vacuolar hydrolase activity and a consequent stabilization of the internal vesicles (Morano and Klionsky, 1994; Odorizzi *et al.*, 1998; Wurmser and Emr, 1998). *Δpep4* cells also have a reduced vacuolar protease activity and similarly accumulate 40- to 50-nm vesicles inside the vacuole (Figure 1A; Baba *et al.*, 1997). Some of these structures are derived from the cytoplasm to vacuole targeting (Cvt) pathway (Baba *et al.*, 1997), a pathway akin to autophagy in which the cytosolic protein aminopeptidase I (API) is wrapped in membranes and delivered directly to the vacuole (Kim and Klionsky, 2000), but the remaining vesicles should be derived from multivesicular endosomes. In agreement with this, trans-membrane proteins internalized into multivesicular bodies are not degraded in this *Δpep4* strain (Reggiori *et al.*, 2000).

To mark endosome-derived vesicles, the *Δpep4* strain was transformed with a green fluorescent protein (GFP)-tagged version of Pep12p with an aspartate in the third position of the transmembrane domain (TMD) (GFP–PEP3D). This construct is transported to late endosomes, enters internal vesicles, and is delivered to the vacuolar lumen (Reggiori *et al.*, 2000). Figure 1B shows vacuoles purified from this strain (Haas, 1995), which on visual inspection could be seen to contain numerous rapidly moving internal fluorescent structures.
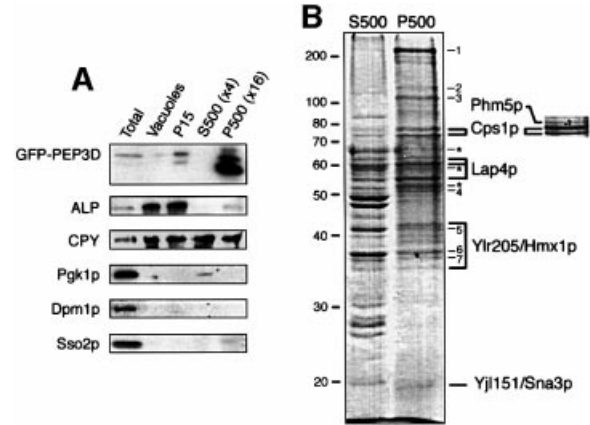
Their motion created a homogeneous blur in confocal images obtained with multiple laser scans, but single-scan pictures revealed structures within this (Figure 1B, inset).

To identify proteins in these internal vesicles, we developed a procedure to enrich for them. Vacuoles were purified, collected by sedimentation, then osmotically lysed and the outer vacuole membranes pelleted at 15 000 *g* (P15). Soluble vacuolar proteases and internal vesicles were partially released into the supernatant, which was then centrifuged at 500 000 *g* to separate the small vesicles (P500) from soluble proteins (S500). GFP–PEP3D was strongly enriched in the P500 pellet, whereas the soluble vacuolar protease carboxypeptidase Y (CPY) and the vacuole membrane protein alkaline phosphatase (ALP) were not (Figure 2A). Other membranes such as the endoplasmic reticulum (ER), marked by Dpm1p, and the plasma membrane, marked by Sso2p, were largely removed during initial purification of the vacuoles, as was the cytosolic enzyme Pgk1p (Figure 2A).

Identification of proteins in internal vesicles

The purification procedure was scaled up, and the proteins in the various fractions separated by SDS–PAGE
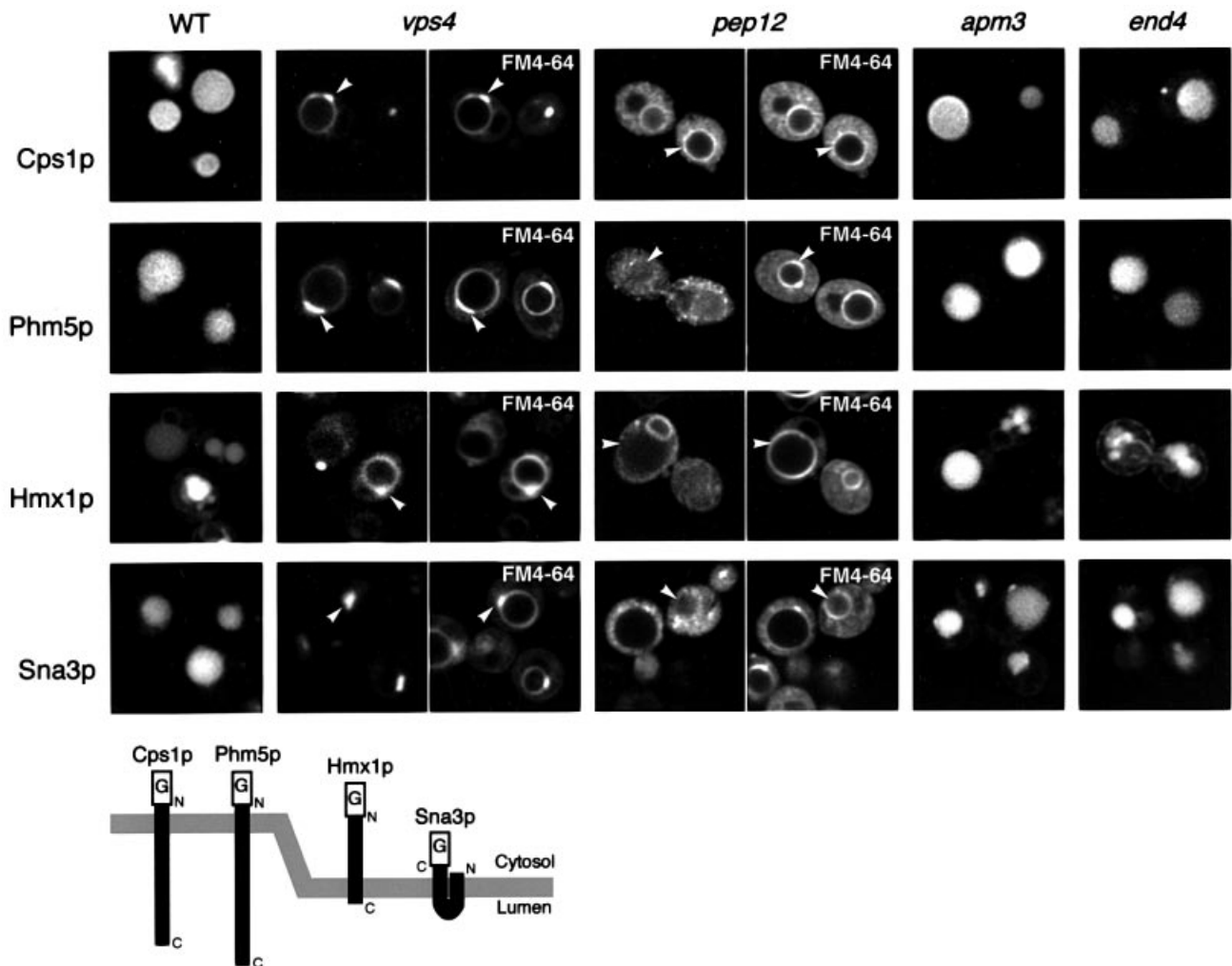
Fig. 3. Delivery of proteins to the vacuole interior occurs via endosomes. GFP-tagged versions of the four proteins identified in internal vesicles were expressed in the indicated deletion mutant strains. The *vps4* and *pep12* cells were double labelled with the dye FM4-64 to reveal vacuoles and endosomal compartments. The diagram at the bottom indicates the position of the GFP tag (G) and the topology of the proteins in the membrane, as predicted by the TMHMM program (http://www.cbs.dtu.dk/services).

(Figure 2B). Bands enriched in the P500 fraction were identified by mass spectrometry of their tryptic peptides. In addition to the Pep12 chimera used as a marker, five other proteins were identified that might be in internal vesicles: Cps1p, Phm5p, Lap4p, Ylr205p and Yjl151p (Figure 3). Phm5p was found in only one preparation, perhaps because its synthesis depends on growth conditions (see below), but the others were each found at least twice. A number of other proteins from the P500 fraction were also identified, and found to be abundant cytosolic or plasma membrane proteins (see legend to Figure 2 for details). It is not clear whether these are simply contaminants, or whether some are partially present within the vacuole, but we did not investigate them further.

Two of the enriched proteins were expected components of internal vesicles. Cps1p, a single transmembrane protein with a short cytoplasmic N-terminus and a large luminal C-terminus, was previously known to reach the vacuole in the vesicles delivered from endosomes (Odorizzi *et al.*, 1998). Aminopeptidase I (Lap4p) is a soluble protease transported from the cytosol to the

vacuole via the Cvt pathway and, as mentioned above, accumulates in internal vacuolar vesicles in a *Δpep4* strain (Baba *et al.*, 1997).

The other three proteins are all predicted to be integral membrane proteins. Phm5p is a polyphosphate endo-phosphatase. Yeast cells accumulate large amounts of polyphosphate chains in the vacuole which are used as a reservoir for phosphate (Urech *et al.*, 1978). Phm5p is required to mobilize phosphate from these chains and its transcription is induced when extracellular phosphate levels fall (Ogawa *et al.*, 2000). Phm5p has a single TMD and is predicted to have a topology similar to that of Cps1p.

Ylr205p is a C-tail anchored protein with a high similarity to members of the family of haem oxygenases; on the basis of this similarity, we propose the name *HMX1* for the corresponding gene. These oxygenases cleave the haem ring to form biliverdin and carbon monoxide, and are found in both eukaryotes and photosynthetic bacteria. Despite extensive studies of these proteins in animal cells, no precise subcellular localization has been determined.

However, human monocytes phagocytose the malarial pigment haemozoin, a polymer of haem rings, and this compound seems to be degraded by haem oxygenase in the lysosome (Schwarzer *et al.*, 1999). The catalytic domain of Hmx1p is predicted to be exposed initially to the cytosol, but would end up in the vacuolar lumen; where it performs its function is unclear.

Yjl151p is a very small protein with two TMDs. It has three homologues in yeast and several in fungi, *Caenorhabditis elegans* and plants. One of the yeast homologues, Pmp3p/Sna1p, has been shown to be localized to the plasma membrane and to be involved in salt and charge homeostasis. It has been hypothesized to form a proton channel that limits the polarization of the plasma membrane, thus reducing the uptake of sodium ions (Navarre and Goffeau, 2000). Because of its similarity to *SNA1*, we propose the name *SNA3* for *YJL151C*, and for the other two homologues *SNA2* (*YDR525W-A*) and *SNA4* (*YDL123W*), the numerical order reflecting the intracellular locations of the gene products (see below).

The newly identified proteins are delivered to the vacuole lumen through late endosomes

We tagged Phm5p, Hmx1p, Sna3p and Cps1p with GFP and expressed them in a wild-type strain (Figure 3, WT). All constructs showed a clear staining of the vacuole lumen. Since each was tagged on the predicted cytosolic portion of the protein, the luminal fluorescence suggests that they entered internal vesicles, as expected from their fractionation behaviour. Part of the GFP–Hmx1p remained in the ER, as indicated by fluorescence of the nuclear envelope. This may reflect a normal dual location, but we cannot exclude the possibility that the presence of the GFP tag slows the exit of Hmx1p from the ER.

Formation of multivesicular bodies is an endosomal function and seems to occur primarily in late endosomes in yeast. The process is blocked in class E vacuolar protein sorting mutants such as *vps4* (Odorizzi *et al.*, 1998; Reggiori *et al.*, 2000). In *vps4* cells, abnormal enlarged prevacuolar compartments accumulate (Babst *et al.*, 1997), which can be visualized using the endocytic tracer dye FM4-64 (Vida and Emr, 1995). As shown in Figure 3, all our chimeras were trapped in these structures in *vps4* cells, indicating that they normally pass through late endosomes.

Because one *vps4* allele is known to affect autophagy (Shirahama *et al.*, 1997), and thus conceivably other processes such as the Cvt pathway, we also expressed the chimeras in a *Δpep12* mutant. Removal of Pep12p does not affect the Cvt pathway (Abeliovich *et al.*, 1999), but prevents fusion of vesicles with the late endosome (Becherer *et al.*, 1996). In the *Δpep12* strain, Cps1p and the three newly identified proteins accumulated in very small structures, probably vesicles, without reaching the vacuole lumen (Figure 3); this was less clear for Hmx1p, due to its tendency to accumulate in the ER. Some of the GFP–Cps1p did reach the vacuole in *Δpep12* cells, but it remained on the outer membrane. This may reflect some transport by the direct route from the Golgi to the vacuole, mediated by the AP-3 coat protein, which bypasses late endosomes and hence the invagination process that forms internal vesicles (Cowles *et al.*, 1997; Stepp *et al.*, 1997). This route is blocked in a *Δapm3* mutant, which lacks one
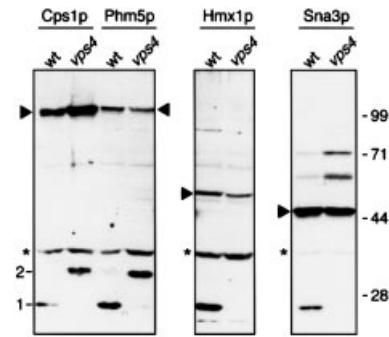

Fig. 4. Analysis of GFP chimeras. Western blots of total extracts from cells expressing the GFP-tagged versions of the indicated proteins were probed with anti-GFP antibodies. Triangles indicate full-length chimeras; asterisks mark a yeast protein that cross-reacts with the anti-GFP antibody. Bands at position 1 correspond to free GFP, those at position 2 contain GFP linked to the cytoplasmic tail and TMD of Cps1p or Phm5p. Molecular weight markers (kDa) are indicated on the right. The nature of the bands migrating more slowly than GFP–Sna3p is unknown; they may represent conformational isomers or protein complexes.

of the AP-3 subunits, but as expected the *apm3* mutation did not prevent any of the GFP chimeras from reaching the vacuole lumen when the endosomal pathway was intact (Figure 3).

These results confirm that Cps1p, Phm5p, Hmx1p and Sna3p all normally reach the vacuole from late endosomes, and that passage through endosomes is essential for their delivery to the vacuole interior. Their localization was also largely unaffected in the endocytosis-deficient mutant *end4*, indicating that they pass directly from the Golgi to endosomes rather than via the plasma membrane (Figure 3).

To confirm the predicted membrane orientation of the proteins, we analysed the GFP-tagged versions by immunoblotting extracts of wild-type and *vps4* cells with anti-GFP antibodies. In wild-type cells, entry into the vacuole should eventually expose the GFP tag to vacuolar proteases, whereas in *vps4* cells, which cannot form multivesicular bodies, it should remain exposed to the cytosol. Figure 4 shows that partial cleavage of all of the proteins occurred in wild-type cells to yield free GFP (band 1). In *vps4* cells, this band was not observed with any chimera, confirming that the GFP was not exposed to proteases. However, both Cps1p and Phm5p showed a GFP product that was 4 kDa larger (band 2), corresponding to cleavage immediately beyond the transmembrane domain. Such cleavage, mediated by proteases within the prevacuolar compartment or vacuoles, is a known feature of Cps1p biogenesis (Spormann *et al.*, 1992). Hmx1p and Sna3p did not show cleavage in *vps4* cells, consistent with their minimal luminal domains. Thus, this analysis fully supports the predicted structures of the proteins.

Since Sna3p is a member of a family of proteins in yeast, we investigated whether any other members of this family shared its location. Alignment of the four sequences shows that they are highly similar in the transmembrane domains, but diverge at the C-terminus (Figure 5A). GFP tagging confirmed the plasma membrane location of Sna1p and revealed a punctate pattern for Sna2p (not shown). However, Sna4p was on the outer vacuolar membrane (Figure 5B), raising the question of
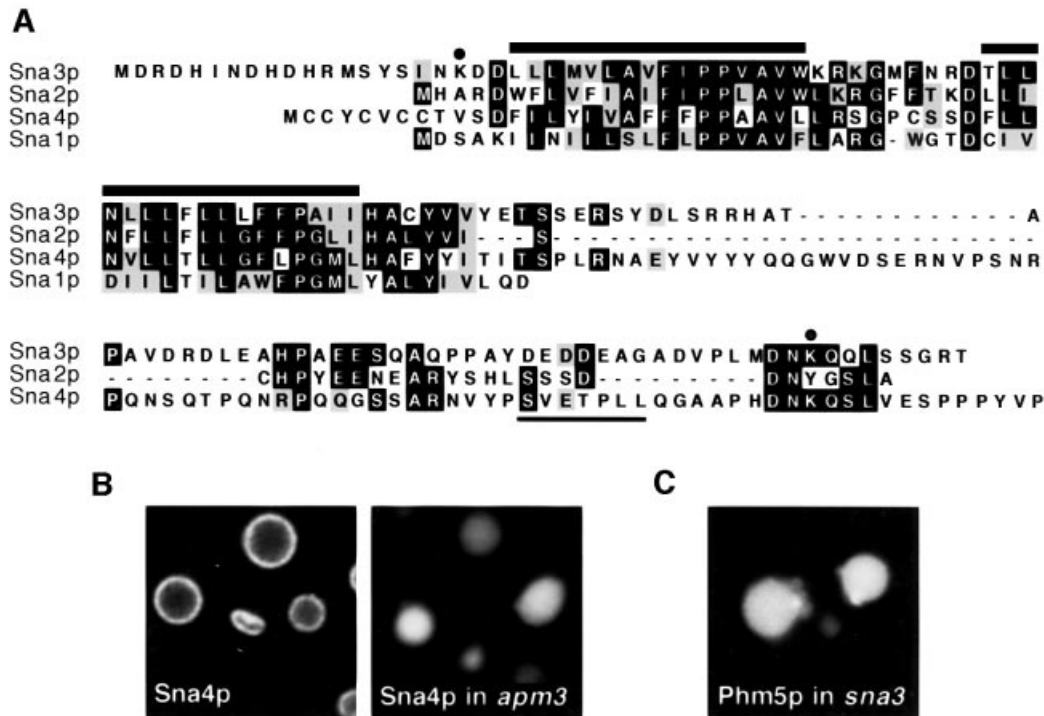
Fig. 5. The Sna family of proteins in yeast. (**A**) Alignment of the Sna3p sequence with the other three, with identities and similarities indicated by black and grey backgrounds. Thick bars over the sequences indicate the TMD. Dots indicate cytoplasmic lysines in Sna3p; the acidic di-leucine motif in Sna4p is underlined. (**B**) GFP-tagged Sna4p is on the vacuolar membrane in wild-type cells, but inside the vacuoles in Δapm3 cells. (**C**) GFP–Phm5p shows its normal intra-vacuolar pattern in a Δsna3 mutant.

how it escapes the fate of its relative, Sna3p. One possibility is that it bypasses late endosomes by following the AP-3 pathway directly to vacuoles (Odorizzi *et al.*, 1998; Reggiori *et al.*, 2000), and indeed it contains an acidic di-leucine motif (Figure 5A), which is a likely AP-3 sorting signal (Darsow *et al.*, 1998). We tested this hypothesis by expressing GFP–Sna4p in a Δapm3 mutant. In these cells, Sna4p is forced to pass through late endosomes to reach the vacuole, and like Sna3p is found in the lumen (Figure 5B). Hence, the ability to enter multivesicular bodies is not unique to Sna3p, but may be a common property of these proteins, perhaps mediated by their similar transmembrane segments.

We also investigated whether Sna3p is itself required for the formation of multivesicular bodies, but found that delivery of Phm5p to the vacuole interior was normal in a Δsna3 strain (Figure 5C). We conclude that the three new proteins we have identified are cargo molecules that are sorted into the interior of multivesicular bodies. Although we cannot rule out a redundant role, we have no evidence that they are part of the machinery for the formation of these structures. Nevertheless, they provide tools to investigate the basis of this endosomal sorting step.

Ubiquitylation affects internalization in multivesicular bodies

A role for ubiquitin in endosomal sorting has been suggested recently (Amerik *et al.*, 2000; Losko *et al.*, 2001). These studies used a deletion strain lacking Doa4p, a ubiquitin-specific protease that removes ubiquitin from protein conjugates (Papa and Hochstrasser, 1993). Doa4p

is important in the recycling of ubiquitin and a loss of its function results in a depletion of free ubiquitin, thus interfering with all ubiquitin-dependent processes (Swaminathan *et al.*, 1999). When GFP-tagged versions of Cps1p, Phm5p and Hmx1p were expressed in the Δdoa4 strain, all three proteins were found predominantly on the vacuolar membrane, rather than inside the vacuole, indicating that their efficient internalization was dependent on ubiquitin (Figure 6).

Ubiquitin could have either a direct effect, marking proteins for internalization, or a more indirect effect on the formation of multivesicular bodies in general. As a first step to distinguish these possibilities, we investigated whether the proteins themselves were ubiquitylated. We focused on Cps1p and Phm5p, because they were delivered to the vacuole more efficiently than Hmx1p. These proteins were each tagged with protein A, with a tobacco etch virus (TEV) protease cleavage site at the junction to allow subsequent removal of the protein A. The constructs were introduced into a Δdoa4 strain that expressed myc-tagged ubiquitin from the *CUP1* promoter, thus allowing ubiquitylated proteins to be detected by their acquisition of the myc epitope (Ellison and Hochstrasser, 1991). Expression of the tagged ubiquitin was induced by adding copper to the medium, the cells lysed, and the protein A chimeras isolated using an IgG–Sepharose column. They were released from the Sepharose beads by cleavage with TEV protease, and the presence of myc-ubiquitin tested by immunoblotting. As shown in Figure 7, both Cps1p and Phm5p yielded several myc-containing bands, their mobilities indicating that multiple ubiquitins
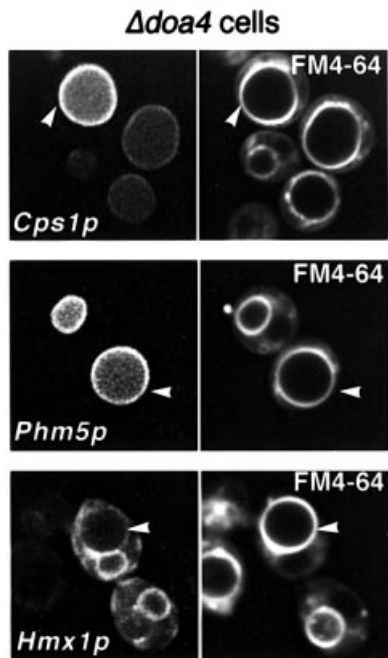
Fig. 6. Delivery to the vacuole interior is inhibited in *Δdoa4* cells. GFP-tagged Cps1p, Phm5p and Hmx1p were expressed in *Δdoa4* cells, which were also labelled with FM4-64. GFP fluorescence is more prominent on the vacuolar membrane than inside the vacuoles, in contrast to wild-type cells (see Figure 3). The brightest rings visible with GFP–Hmx1p correspond to the nuclear envelope.

Fig. 7. Ubiquitylation of Cps1p and Phm5p. (**A**) *Δdoa4* cells expressing myc-tagged ubiquitin and either protein A-tagged Cps1p, or only endogenous Cps1p, were processed in parallel. Total extracts, and protein purified by binding to IgG–Sepharose followed by cleavage with TEV protease to release Cps1p from the protein A tag, were immunoblotted with anti-protein A (PA) or anti-myc. Note that protein A is absent from the purified material. Cps1p migrates as a doublet, due to variable glycosylation (Spormann *et al.*, 1992). The faint bands above the protein A chimera are likely to be ubiquitylated forms, but these are under-represented in total cell extracts due to de-ubiquitylation during sample preparation. (**B**) As (A), but cells expressed protein A-tagged Phm5p. The lower protein A-containing band results from processing close to the transmembrane domain (see Figure 4). In addition, some more random degradation was detectable, which accounts for the smaller myc-tagged proteins in the purified fraction. In both panels, the asterisks indicate the approximate mobility expected for a mono-ubiquitylated protein.

could be added in each case. These modified bands were hard to see in total cell extracts, because it proved difficult to inhibit the numerous de-ubiquitylating enzymes during sample preparation.

Ubiquitin chains are added to lysine residues, so if ubiquitylation is necessary for internalization of the proteins in late endosomes, mutation of the relevant lysines should prevent delivery to the vacuole interior. The cytoplasmic domain of Phm5p contains four lysines (Figure 8). Mutation of Lys6 to either glycine (not shown) or arginine caused the protein to accumulate on the vacuole membrane, just as it did in the *Δdoa4* strain (Figure 8). This indicates that Lys6 is an important site of ubiquitylation. However, an effect of mutating this lysine was apparent in only ~70% of the cells, whereas changing all four lysines to arginine gave vacuolar membrane fluorescence in all the cells (data not shown). Thus, one or more of the other, membrane-proximal lysines is also likely to be a substrate for ubiquitylation. Lysines present in GFP were evidently not able to substitute for those in the Phm5p tail.

To confirm that the lysine requirement was solely for ubiquitylation, we added ubiquitin to the Lys6 mutant, by incorporating its sequence at the N-terminus of the GFP tag. The ubiquitin sequence was modified at the junction with GFP to prevent its removal (Gly76 to Val; see Weissman, 2001). In addition, lysines 29, 48 and 63, which are the known sites for the addition of further ubiquitins in yeast (Arnason and Ellison, 1994), were replaced with arginines. These modifications ensure that a single ubiquitin is present on the construct. As shown in Figure 8, the addition of ubiquitin was sufficient to restore
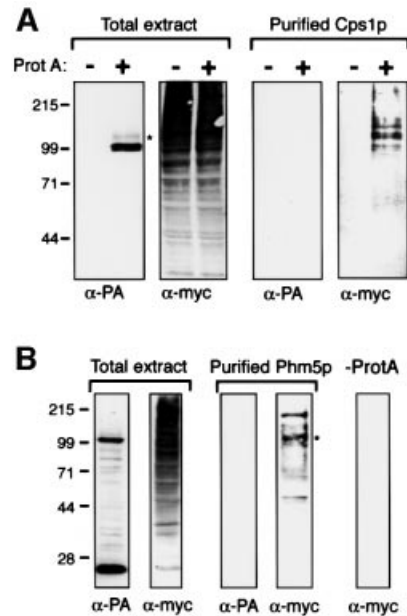
delivery of the Phm5p K6R mutant to the vacuole interior, in all cells. Furthermore, delivery was restored even in *Δdoa4* cells, indicating that the sorting defect in these cells is due to the lack of ubiquitylation of Phm5p, rather than to any general effect on the sorting machinery.

From these results we conclude that Phm5p is sorted into internal vesicles in late endosomes by a mechanism that requires it to be ubiquitylated. The same is likely to be true for Cps1p and Hmx1p, since both are affected in a *doa4* strain, and we have shown that Cps1p is also ubiquitylated. Although both Cps1p and Phm5p can receive several ubiquitins, at least in the *doa4* strain, a single ubiquitin is a sufficient signal for this sorting process.

Sna3p is internalized into multivesicular bodies without being ubiquitylated

In contrast to the other proteins studied, we found that Sna3p reached the inside of the vacuole even in *Δdoa4* cells. One possibility was that overexpression of this protein might induce vigorous invagination of endosomal membranes and make internalization signal independent. To check this, we co-expressed GFP–Sna3p with yellow fluorescent protein (YFP)–Phm5p. In wild-type cells, both were present inside vacuoles; in *Δdoa4* cells, Sna3p was inside while Phm5p was on the outer membrane of the

GFP-Phm5p

GFP-Phm5p(K6R)

Ub-GFP-Phm5p(K6R)

$$\overset{6}{\text{MVVVGKSEVRNVSMSRP}}\textbf{KKK}...$$
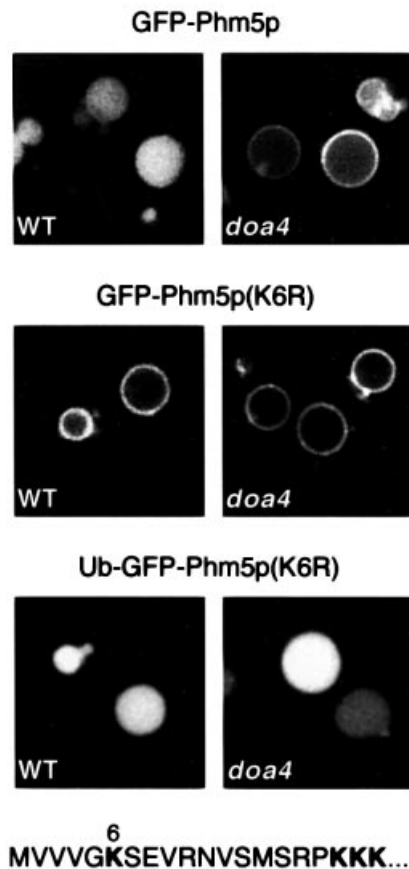
Fig. 8. Sorting of Phm5p is blocked by mutation of Lys6, but restored by the addition of ubiquitin. GFP–Phm5p, the K6R mutant, and the mutant with ubiquitin added to the N-terminus of the GFP were expressed in both wild-type cells (WT) and the Δdoa4 mutant. The sequence of the Phm5p cytoplasmic tail is shown at the bottom.

A

B

Sna3p(K19R, K125R)

Fig. 9. Ubiquitylation of Sna3p is not required for it to enter the vacuole. (**A**) Double labelling with YFP–Phm5p and GFP–Sna3p in wild-type (WT) and Δdoa4 cells. (**B**) GFP–Sna3p with both cytoplasmic lysines changed to arginines expressed in wild-type cells and in a Δsna3 mutant.

very same vacuoles (Figure 9A). Thus, Sna3p is specific-ally internalized in a manner that appears independent of ubiquitylation.

To confirm this, we changed the two lysine residues (19 and 125) in the predicted cytoplasmic portions of Sna3p to arginines, thus making ubiquitylation of these residues impossible. As shown in Figure 9B, this had no effect on the sorting of the protein. The same result was obtained in a Δsna3 strain, eliminating the possibility that sorting of the mutant Sna3p was dependent on oligomerization with wild-type protein. An analogous observation was that Sna4p truncated at position 108, which lacks lysines altogether, also reached internal vesicles (data not shown). We conclude that Sna3p (as well as Sna4p) has features that allow its selective inclusion in invaginating vesicles, and that this process does not require ubiquitylation of either Sna3p itself or of any other component that is sensitive to the effects of the *doa4* mutation.

Discussion

Components of multivesicular bodies

Our analysis of the vesicles that accumulate within the vacuole of a protease-deficient yeast strain identified four proteins that are specifically enriched: Cps1p, Phm5p,
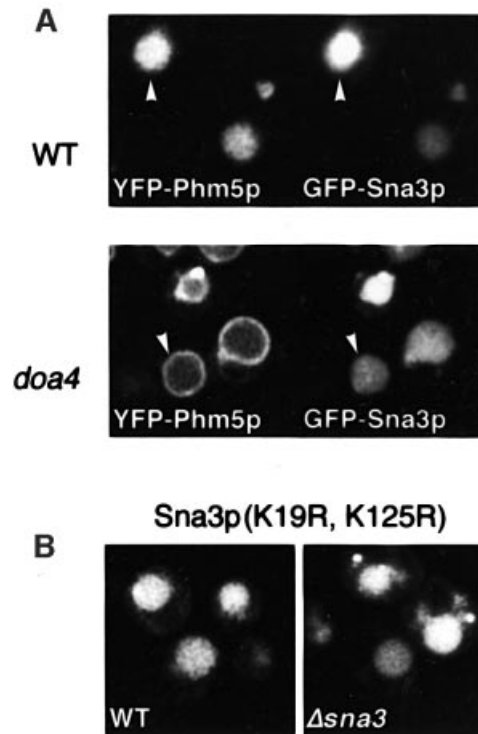
Hmx1p and Sna3p. Three of these are enzymes and none is an obvious candidate for components of the machinery that forms such vesicles, or receptors that might serve to sort proteins into them. Although the difficulties of purification mean that negative results must be interpreted with caution, we found no proteins present in significantly higher molar amounts than these cargo molecules. We therefore consider it likely that the vesicles invaginate without stoichiometric coat components that would end up inside them. Nor are there major transmembrane proteins that could be considered structural. Sorting of proteins into internal vesicles does not, therefore, seem to involve their sequestration either by a vesicle-associated transmem-brane receptor or by a coat. In agreement with this, we have seen little evidence for saturation of the sorting mechanism by overexpression of the proteins (Reggiori *et al.*, 2000 and our unpublished observations).

Ubiquitylation as a sorting signal in late endosomes

Our experiments indicate that Phm5p enters multivesicular bodies because it is ubiquitylated. We show that lysine residues in its cytoplasmic tail are crucial for efficient internalization, that the protein is ubiquitylated, and that the absence of a cytoplasmic lysine can be compensated for by the addition of a single, non-removable, ubiquitin to the N-terminus of the construct. In a *doa4* mutant in which free ubiquitin levels are limiting (Swaminathan *et al.*, 1999), internalization is also blocked. We have also shown that internalization of Cps1p and Hmx1p is affected in

doa4 cells, and that Cps1p is ubiquitylated. Parallel studies by Emr and colleagues have also documented ubiquitin-dependent sorting of Cps1p (Katzmann *et al.*, 2001).

The Phm5p data rule out two alternative interpretations of the *doa4* effect. Thus, it cannot be that *doa4* cells fail to carry out an essential ubiquitylation of some component of the internalization machinery, rather than the cargo proteins themselves, because sorting of Phm5p can be restored in these cells by addition of ubiquitin specifically to Phm5p. It remains possible that other ubiquitylation events are required for endosome function, but if so, they can still occur in *doa4* cells. This is not implausible, since *doa4* cells maintain a low level of ubiquitylation, which is essential for viability. Secondly, complete removal of ubiquitin from the substrates by the Doa4p ubiquitin-specific protease is not essential for their internalization. Such removal does normally occur (Dupré and Haguenauer-Tsapis, 2001), but a non-removable ubiquitin moiety does not prevent invagination of Phm5p.

It is striking that addition of a single ubiquitin to Phm5p is sufficient. In this respect, sorting in endosomes resembles ubiquitin-dependent endocytosis of cell surface receptors and permeases (Terrell *et al.*, 1998; Nakatsu *et al.*, 2000; Hicke, 2001), rather than proteasome-dependent proteolysis, which requires a chain of at least four ubiquitins (Weissman, 2001). Vacuolar proteins can, however, clearly receive multiple ubiquitins, at least when their removal is inhibited by the *doa4* mutation. It may be that extension of ubiquitin chains improves the efficiency of sorting, as has been shown for endocytosis of uracil permease (Galan and Haguenauer-Tsapis, 1997).

The vacuolar proteins do not reach the cell surface in an *end4* mutant, and thus their modification must occur entirely within the cell, either at the late endosome or at an earlier point such as the Golgi. It seems that ubiquitin is used as a signal at multiple points, to induce transport to endosomes from either the plasma membrane or the Golgi, and to induce invagination in endosomes and hence delivery to the vacuole. Indeed, the ubiquitin that marks a plasma membrane protein for endocytosis would, if retained, also ensure its entry into multivesicular bodies and its eventual demise in the vacuole. Diversion from this fate requires specific removal of the ubiquitin, which may therefore be an important regulatory step in protein recycling.

How ubiquitin is recognized in endosomes, and the tagged proteins directed to internal vesicles, remains to be seen. Interestingly, Vps23p, which is required for multi-vesicular body formation, has a domain with homology to ubiquitylating enzymes, yet lacks the Cys residue to which ubiquitin is conjugated in these enzymes (Babst *et al.*, 2000; Bishop and Woodman, 2001). This domain could, therefore, have a recognition, rather than enzymatic, function (Katzmann *et al.*, 2001).

A further remaining question is how the proteins are initially recognized for ubiquitylation. Sequence features of their cytoplasmic tails might provide a signal, although there is no obvious motif common to the various proteins that enter internal vesicles. Alternatively, some other feature such as the TMD might be important (Reggiori *et al.*, 2000), and lysine residues recognized more for their position than their context. Such distinctions will be easier to make when the enzymes responsible for the modification are known, and this is currently under investigation.

A second sorting mechanism

A surprising finding was that Sna3p is sorted by a mechanism that requires neither *doa4*-sensitive ubiquitylation nor cytoplasmic lysine residues. It is possible that Sna3p has some other signal that is specifically recognized by the invagination machinery. However, we favour the idea that the internal membrane differs in its composition and physical properties from the outer endosomal membrane (Kobayashi *et al.*, 1998), and that Sna3p preferentially partitions into this domain due to its own physical properties. This fits with the observation that the related Sna4p is also invaginated when forced to pass through late endosomes, even though it normally uses the AP-3 pathway to the vacuolar membrane and has no need for a specific invagination signal. Other proteins may have similar properties: since completion of this work we have identified one more protein in internal vesicles (corresponding to the band above number 6 in Figure 2B) as Cos4p, a member of a large family of closely related tetraspanning membrane proteins, and this protein also enters multivesicular bodies even in *doa4* cells.

If direct partitioning into the invaginating membrane is possible, it follows that proteins that are selectively retained on the outer endosomal membrane, such as the vacuolar H⁺-ATPase, must have properties that exclude them from this domain. Indeed, we have noticed that Phm5p is not completely absent from the vacuole interior even when its ubiquitylation is inhibited, perhaps because it lacks such properties.

Degradation inside the vacuole is a common fate for proteins that are endocytosed or mislocalized to the endosomal system, and for many foreign or abnormal proteins. Indeed, it is so common that it might be considered a 'default' pathway, with specific signals being required to avoid it. It is now clear, however, that sorting of membrane proteins into the vacuole is a specific process that in many cases requires the selective tagging of the proteins with ubiquitin. This provides many opportunities for regulation, just as the ubiquitin-dependent proteasomal degradation pathway does for cytosolic and nuclear proteins.

Materials and methods

Yeast strains and growth

Yeast strains are described in Table I. All were derived from SEY6210, except the *doa4* strain SD20. The *DOA4* gene was also disrupted in SEY6210 and the distributions of GFP-labelled proteins checked, with identical results to those shown for SD20. Strains were grown in yeast extract–peptone–dextrose (YEPD) or synthetic dextrose (SD) medium as appropriate.

For *YJL151c* (*SNA3*) and *END4* gene disruptions, the entire coding region was replaced with the *HIS5* gene of *Schizosaccharomyces pombe* and the *TRP1* gene, respectively, flanked by coliphage *loxP* sites using PCR primers containing ~50 bases of identity to the regions flanking the open reading frame.

Purification of internal vacuolar vesicles and protein analysis

Vacuoles were purified as described (Haas, 1995) from a Δ*pep4* strain expressing a GFP-tagged *PEP3D* construct [Pep12p with an aspartate in the third position of the TMD (Reggiori *et al.*, 2000)]. Vacuoles from each gradient (600–800 μl) were collected in a microcentrifuge tube, diluted with 700 μl of ice-cold iso-osmotic buffer (10 mM PIPES–KOH

Table I. Yeast strains used in this work

Strain	Genotype	Origin
SEY6210	*Matα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	S.Emr
JHY024	*Matα ura3-52 leu2-3,-112 his3-Δ 200 trp1-Δ901 lys2-801 suc2-Δ9 pep4::HIS3*	Holthuis *et al.* (1998)
SEY4-1	*Matα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 vps4-1*	Babst *et al.* (1997)
FRY034	*Matα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 end4::HIS5spL*	this work
FRY024	*Matα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 apm3::HIS5spL*	Reggiori *et al.* (2000)
JHY005	*Matα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pep12::HIS3L*	Holthuis *et al.* (1998)
SD20	*Matα ade2-1 ura3-1 leu2-3,-112 his3-11 trp1-1 can1-100 doa4::HIS3*	Dupré and Haguenauer-Tsapis (2001)
FRY050	*Matα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 yjl151c::HIS5spL*	this work

pH 6.8, 200 mM sorbitol) and centrifuged at 15 000 *g* for 15 min (4°C). Supernatant was discarded and the pellet resuspended in 20–40 µl of iso-osmotic buffer containing 15% Ficoll. The protein concentration was determined by Bradford assay (Bio-Rad, Munich, Gemany) and adjusted to 1 µg/µl by diluting the samples with the same buffer. Aliquots were then frozen in liquid nitrogen.

Vacuoles (600–800 µg) were thawed on ice and 40 µl aliquots distributed in microcentrifuge tubes. Each aliquot was diluted by pipetting in 460 µl of ice-cold hypo-osmotic buffer [20 mM PIPES pH 6.8, 20 mM EDTA containing 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml pepstatin A and Cømplete™ EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany)] and then centrifuged at 15 000 *g* for 15 min (4°C). Supernatants were pooled and centrifuged again at 15 000 *g* for 15 min (4°C). The final supernatant was transferred to a 3 ml polycarbonate tube (Beckman, Palo Alto, CA) and centrifuged at 500 000 *g* for 30 min (4°C) in a TLA 100.3 Beckman rotor. The supernatant (S500) was removed, 3 ml of hypo-osmotic buffer gently added and the tube inverted 3–4 times before re-centrifugation at 500 000 *g* for 30 min (4°C). The final pellet (P500) was resuspended by sonication in 50 µl of sample buffer containing 2 mM PMSF and Cømplete™ EDTA-free protease inhibitor cocktail; 30 µl were loaded on a preparative gel for mass spectrometry. Tryptic digestion of protein bands and analysis by mass spectrometry were performed as described previously (Siniossoglou *et al.*, 2000). The remaining 20 µl were diluted with 80 µl of sample buffer and 10 µl aliquots were used for western blot analysis. Incubations were carried out with monoclonal antibodies against ALP, Dpm1p and Pgk1 (Molecular Probes, Eugene, OR), and with rabbit polyclonal antibodies against GFP (a gift from Dr Derek McCusker, MRC Cambridge), CPY and Sso2p (Holthuis *et al.*, 1998).

Plasmids

CPS1 was PCR generated and cloned as an *Eco*RI–*Bam*HI fragment into pRS416 vector (Sikorski and Hieter, 1989) behind sequences expressing the mut2 GFP variant (Cormack *et al.*, 1996) and the *TPI1* promoter as described by Wooding and Pelham (1998). The 5′ primer introduced five glycines in front of the starting methionine. *Ylr205c* (*HMX1*) was also generated by PCR and cloned as a *Stu*I–*Bam*HI fragment into the expression plasmid described above, cut with *Eco*RI, blunted with Klenow, and finally digested with *Bam*HI to eliminate *CPS1*. *PHM5* and *PHM5* point mutations were generated by PCR and cloned as an *Asc*I–*Bam*HI fragment in the same expression plasmid in which an *Asc*I site was introduced at the 3′ end of GFP in place of an *Eco*RI site. YFP versions of the same vectors were made by replacing the GFP coding sequences with a PCR-generated *Hin*dIII–*Asc*I fragment encoding YFP.

Yjl151c (*SNA3*), *Yjl151c* point mutations, *SNA1/PMP3*, *Ydr525w-A* (*SNA2*) and *Ydl123w* (*SNA4*) were all generated by PCR and cloned as *Hin*dIII–*Age*I fragments in the pTL321 vector (a gift from T.Levine and S.Munro), creating a C-terminal GFP-tagged Yjl151p under the control of a *TPI1* promoter. A plasmid encoding ubiquitin (Ub) with the point mutations K29R, K48R, K63R and G76V fused to the N-terminus of GFP was kindly provided by E.Hettema. This Ub–GFP fusion was swapped to the GFP–PHM5KR construct as a *Hin*dIII–*Msc*I fragment. To construct a *NOP1* promoter-driven protein A fusion with a TEV cleavage site after the protein A, *CPS1* was cloned by PCR as an *Nde*I–*Bam*HI fragment into the pNOPATA/NPL3 vector (Senger *et al.*, 1998). *PHM5* was cloned as an *Asc*I–*Bam*HI fragment in the same expression plasmid in which an *Asc*I site was introduced at the 3′ end of the protein A coding sequence. In all cases, changes were confirmed by sequencing. All enzymes for manipulation of DNA were from New England Biolabs (Beverly, MA).

Electron microscopy and imaging of live cells

For electron microscopy, *WT* and *Δpep4* cells were grown in YEPD medium to early log phase. Permanganate fixation, dehydratation and embedding in Spurr's resin (Agar Scientific, Stansted, UK) were carried out as described by Kaiser and Schekman (1990). Sections were stained with 5% uranyl acetate for 10 min at 60°C followed by 5 min in Reynold's lead citrate at room temperature. Sections were observed using a Philips CM10 transmission electron microscope.

Living cells were prepared for GFP fluorescence and stained with FM4-64 (Molecular Probes) as described previously (Reggiori *et al.*, 2000). Double-label GFP/YFP analysis was performed as described (Siniossoglou *et al.*, 2000). Purified vacuoles were resuspended in iso-osmotic buffer containing 15% Ficoll and trapped on slides with the coverslip prior to fluorescence imaging.

Affinity purification of protein A fusion proteins for ubiquitylation analysis

Δdoa4 strains expressing protein A fusions were transformed with a plasmid carrying a myc-tagged ubiquitin under the control of a *CUP1* promoter (Ellison and Hochstrasser, 1991). These strains were grown in selective medium containing 0.1 mM CuSO₄, and 3–4 g of cells harvested by centrifugation. Spheroplast lysis, purification on IgG–Sepharose and cleavage with TEV protease (Life Technologies, Paisley, UK) were performed as described (Siniossoglou *et al.*, 2000), except that 1 mM PMSF (Sigma Chemical, St Louis, MO) and 10 mM *N*-ethylmaleimide (Sigma) were included in the lysis buffer. The final supernatants containing the released proteins and the successive bead washes were pooled and successively concentrated using Nanosep columns (MWCO 10 kDa; Pall-Gelman Laboratory, Ann Arbor, MI).

Total extracts and samples were loaded on SDS–PAGE gels and after western blotting were probed either with anti-protein A polyclonal serum (Dako, Glostrup, Denmark) or with anti-myc polyclonal serum (Santa Cruz Biotechnology, Santa Cruz, CA).

Acknowledgements

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