Dual Sorting of the *Saccharomyces cerevisiae* Vacuolar Protein Sna4p

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Sna4p, a vacuolar membrane protein, belongs to a small family of proteins conserved in plants and fungi. It is transported to the vacuolar membrane via the alkaline phosphatase (ALP) pathway, which bypasses the multivesicular bodies (MVBs). Here, we show that transfer of Sna4p by the ALP route involves the AP-3 adaptor protein complex, which binds to an acidic dileucine sorting signal in the cytoplasmic region of Sna4p. In addition, Sna4p can use the MVB pathway by using a PPPY motif, which is involved in the interaction with ubiquitin ligase Rsp5p. Deletion or mutation of the Sna4p PPPY motif or a low level of Rsp5p inhibits the entrance of Sna4p into MVBs. Sna4p is polyubiquitylated on its only lysine, and Sna4p lacking this lysine shows defective MVB sorting. These data indicate that Sna4p has two functional motifs, one for interaction with the AP-3 complex, followed by entry into the ALP pathway, and one for binding Rsp5p, which directs the protein to the MVB pathway. The presence of these two motifs allows Sna4p to localize to both the vacuolar membrane and the lumen.

The proper trafficking of eukaryotic proteins between organelles is mediated by vesicular transport in which newly synthesized signal-bearing proteins are recognized by adaptor or coat elements and actively packed into appropriate vesicle populations, which are then targeted to the correct location. In yeast, secretory proteins are transported to the vacuole through two distinct pathways, the multivesicular body (MVB) and alkaline phosphatase (ALP) pathways (36). MVBs are so-called owing to small vesicles formed by the invagination and budding of the endosomal membrane into the interior of the endosome. Integral membrane proteins intended for degradation are sorted into the internal vesicles of the MVB and ultimately delivered to the interior of the vacuole. However, membrane proteins that do not enter the intralumenal vesicles but remain in the endosomal membrane are delivered to the vacuolar membrane (reviewed in reference 18). In order to be degraded, most membrane proteins require the attachment of ubiquitin to their cytosolic region (16, 18). In *Saccharomyces cerevisiae*, ubiquitin ligase Rsp5p is necessary for the ubiquitylation and degradation of many endocytic cargoes (10, 15, 19, 21, 31). The recognition and sorting of ubiquitylated MVB cargoes into internal vesicles involve a number of endosomal sorting complexes required for transport (ESCRTs) (17). Finally, the MVBs fuse with the vacuole and release the internal vesicles into the vacuole.

Other proteins, such as the membrane-bound vacuolar enzyme ALP and the vacuolar t-SNARE Vam3p, are transported to the vacuole via the ALP pathway, which bypasses the MVBs (8, 27, 29). Transport of proteins to the vacuole by the ALP route involves the AP-3 adaptor protein complex (8, 9, 24, 29).

Mutation in each of the subunits of the AP-3 complex causes mislocalization of ALP and Vam3p but has no effect on the transport of MVB cargo to the vacuole (8, 9). The AP-3 complex interacts with proteins containing acidic dileucine sorting motifs and transfers them into Golgi-derived vesicles, which are targeted to the vacuole (9, 24, 29). Both the membrane-bound vacuolar hydrolase ALP and the vacuolar t-SNARE Vam3p have an acidic dileucine sorting signal in their cytoplasmic domain that is recognized by the AP-3 adaptor protein complex (9, 35).

*S. cerevisiae* Sna4p is a small vacuolar membrane protein of unknown function which comprises two predicted transmembrane spans. It has three close homologues in yeast, Pmp3p/Sna1p, Sna2p, and Sna3p, each localized in a different subcellular compartment (22, 28). Sna3p physically interacts with Rsp5p, and this interaction is essential for sorting of Sna3p to the endosomal pathway. Sna3p is ubiquitylated by Rsp5p and modified by K63-linked ubiquitin chains. Sna3p-Rsp5p interaction was found to be dependent on the Sna3p PPxY motif and on the WW2/WW3 domains of Rsp5p (20, 25, 31, 37). Sna4p contains an acidic dileucine motif, which might be a sorting signal for the ALP pathway (9). In *apm3Δ* mutant cells, which lack the μ subunit of the AP-3 complex, Sna4p is missorted to the vacuolar interior (28). Strikingly, this localization is different from that of ALP and Vam3p in AP-3-deficient cells, where they appear in cytoplasmic vesicles and on the vacuolar membrane (8, 9). This indicates that Sna4p possesses an additional characteristic which targets it to the vacuolar interior.

In this study, we show that the acidic dileucine motif is indeed the signal sorting Sna4p to the vacuolar membrane through the AP3 pathway and that a part of Sna4p is targeted to the vacuole lumen via the MVB pathway. The ability to enter MVBs is linked to the C-terminal PPxY sequence of Sna4p. Indeed, Sna4p interacts with Rsp5p via its PPPY motif, resulting in Sna4p ubiquitylation on lysine 128 and incorporation into MVBs. Thus, Sna4p has two functional sorting signals.
that allow it to use two different pathways directing the protein to the vacuole.

MATERIALS AND METHODS

Strains, plasmids, media, and growth conditions. The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Cells were grown at 28°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in YNB minimal medium containing 0.67% yeast nitrogen base (Difco, Detroit, MI) and 2% glucose and supplemented with the appropriate amino acids required for plasmid maintenance. Cells were transformed by the one-step method developed by Chen et al. (7).

The plasmids used in this study are listed in Table 2. SN44 point mutations and nucleotide deletions were generated by PCR and cloned as HindIII-AgeI fragments into the pRS416 plasmid as a 286-bp insert by using other enzymes for DNA manipulation were from New England BioLabs. Bac-

TABLE 1. Strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| npi1-1 | MATA ura3Δ0 npi1-1 | 13 |
| BY4741 | MATA his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Euroscarf |
| BY4742 | MATA his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Euroscarf |
| BY4741pmpΔ | MATA his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 apm3::kanMX4 | Euroscarf |
| BY4741end3Δ | MATA his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 end3::kanMX4 | Euroscarf |
| BY4742sna4Δ | MATA his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 sna4::kanMX4 | Euroscarf |
| BY4741pmpΔ SNA4 | MATA his3Δ1 leu2Δ0 met15Δ0 pep4::kanMX4 | Euroscarf |
| BY4741doc4Δ | MATA his3Δ1 leu2Δ0 met15Δ0 doc4::kanMX4 | Euroscarf |
| BY4742yps2Δ | MATA his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yps2::kanMX4 | Euroscarf |
| BY4742yps2Δ | MATA his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yps2::kanMX4 | Euroscarf |
| MK1 | MATA his3Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 RSP5::HA-RSP5 | 19b |

Preparation of cell lysates for immunodetection. Yeast cells were grown in synthetic medium (supplemented with appropriate amino acids for plasmid maintenance) to an optical density of 600 nm of 2 and centrifuged at 2,700 × g at room temperature (RT). All but 500 μl of the supernatant was removed, 50 μl of 1.85 M NaOH was added to the pellet, the tube was vortexed and left for 10 min on ice, and then 50 μl of 50% trichloroacetic acid was added and the tubes were again vortexed and left for 10 min on ice. The resulting lysate was centrifuged for 5 min at 12,000 × g at RT, and the supernatant was discarded. The pellet was resuspended in 70 μl of a 1:1 mixture of sample buffer (100 mM Tris HCl [pH 6.8], 4 mM EDTA, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.002% bromophenol blue), 1 M Tris base, and 2% β-mercaptoethanol and heated at 95°C for 10 min, and then the proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore) with a semidry transfer system (Bio-Rad) in 50 mM Tris–40 mM glycine–0.00375% (wt/vol) SDS–20% methanol. The blot was saturated at RT for at least 45 min with 5% (wt/vol) low-fat dried milk dissolved in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.6) containing 0.5% (vol/vol) Tween 20 (saturation buffer). Primary antibody incubation was performed overnight at 4°C in saturation buffer. After several washes with Tris-buffered saline containing 0.1% (vol/vol) Tween 20 and 0.5% (wt/vol) milk, secondary antibody incubation was performed at RT for 1 to 3 h, followed by chemiluminescence detection (Roche Diagnostics). The primary antibodies used were mouse monoclonal antibodies against GFP (clones 7.1 and 13.1; Roche Diagnostics) or ubiquitin (clone P1131; Santa Cruz Biotechnology) or rat mono-
clonal antibody against the hemagglutinin (HA) protein of human influenza virus (clone 3F10; Roche Diagnostics).

Immunoprecipitation. Cells grown to mid-log phase were lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, pH 7.5) supplemented with a protease inhibitor cocktail (Roche Diagnostics) by the glass bead lysis method. The lysate was centrifuged for 2 × 5 min at 960 × g at 4°C, the pellet was discarded, and the supernatant was centrifuged for 30 min at 20,800 × g at 4°C. The final supernatant was preclarified by incubation for 60 min at 4°C with 30 μl of protein G-Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and centrifugation at 2,700 × g for 5 min at

TABLE 2. Plasmids used in this study

| Plasmid | Genotype | Source |
|---------|----------|--------|
| pRS416-SNA4-GFP | CEN URA3 tP1PI-SNA4-GFP | 28 |
| pRS416-pSNA4-SNA4-GFP | CEN URA3 pSNA4-SNA4-GFP | This study |
| pRS425-SNA4-GFP | 2μ LEU2 tP1PI-SNA4-GFP | This study |
| pRS416-SNA4::SNA4-GFP | CEN URA3 tP1PI-SNA4::P135136A-GFP | This study |
| pRS416-SNA4::GFP | CEN URA3 tP1PI-SNA4::GFP | This study |
| pRS416-SNA4-26-GFP | CEN URA3 tP1PI-SNA4::K128R-GFP | This study |
| pRS416-SNA4-286-GFP | CEN URA3 tP1PI-SNA4::ΔC6-GFP | This study |
| pRS416-SNA4-26-GFP | CEN URA3 tP1PI-SNA4::ΔC6-GFP | This study |
| pRS416-SNA4-HA | CEN URA3 tP1PI-SNA4::HA | This study |
| pRS416-SNA4-118b-HA | CEN URA3 tP1PI-SNA4::K128R-HA | This study |
| pRS416-SNA4-286b-HA | CEN URA3 tP1PI-SNA4::ΔC6-HA | This study |
| pRS416-SNA4-62-HA | CEN URA3 tP1PI-SNA4::ΔC6-HA | This study |
| pRS416-SNA4-118b-HA | CEN URA3 tP1PI-SNA4::K128R-HA | This study |
| pRS416-SNA4-286b-HA | CEN URA3 tP1PI-SNA4::ΔC6-HA | This study |
Sna4p is localized to both the membrane and the lumen of the vacuole. Cells transformed by the SNA4-GFP plasmid under the control of the endogenous or *TPPI* promoter were labeled with the vacuolar membrane-staining dye FM4-64 and examined by fluorescence microscopy with Nomarski optics. The VAM3-GFP plasmid was used as a control. The strains used are indicated on the left, and the plasmids used are indicated at the tops of the panels. Plasmid pRS416-*SNA4*-GFP, containing SNA4-GFP under the control of the endogenous *SNA4* promoter, is indicated.

4°C. The precleared lysates were incubated for 4 h at 4°C with 5 μg of monoclonal anti-GFP antibody or 2 μg of monoclonal anti-HA antibody, and then 60 μl of protein G-Sepharose was added and the mixture was incubated at 4°C for 12 h with gentle rocking. The beads were washed four times with the lysis buffer, resuspended in sample buffer, and heated at 56°C for 10 min. The proteins were separated by SDS-polyacrylamide gel electrophoresis, and immunoblot analysis was performed with rat monoclonal anti-HA antibody or mouse monoclonal anti-GFP or anti-ubiquitin antibody.

**RESULTS**

Sna4p is localized to both the membrane and the lumen of the vacuole. It has been previously demonstrated that Sna4p is located in the vacuolar membrane in an AP3-dependent manner (28). To confirm this observation, we expressed Sna4p-GFP in the wild-type (WT) strain and the *apm3Δ* mutant and examined the cells by fluorescence microscopy. Deletion of the *APM3* gene results in an AP-3-deficient complex lacking the μ subunit. In *apm3Δ* mutant cells, Sna4p-GFP was found exclusively in the vacuolar lumen (Fig. 1), whereas in WT cells, most of the fluorescence was seen in the vacuolar membrane, with a significant fraction of the protein still visible in the interior of the vacuole (Fig. 1; see Fig. 5). This dual localization was observed in several transformants and at different growth stages (data not shown) and was not affected by the presence of endogenous Sna4p, as shown with the *sna4Δ* mutant strain (Fig. 1). This dual localization was also seen when Sna4p-GFP was expressed under the control of the *SNA4* promoter in the genome (data not shown) or on a centromeric plasmid (Fig. 1), indicating that overexpression did not affect the localization of Sna4p. Indeed, when SNA4-GFP is expressed on a high-copy plasmid with the *TPPI* promoter, the fluorescence is still visible in both the membrane and the lumen of the vacuole (data not shown).

Surprisingly, when it is expressed in *apm3Δ* mutant cells, the localization of Sna4p-GFP in the vacuolar lumen was different from that of ALP and Vam3p, which appear in cytoplasmic vesicles and on the vacuolar membrane (8, 9; Fig. 1). This difference in localization indicated that Sna4p possesses a sequence, absent in other AP-3 cargoes, that targets it to the vacuolar interior.

The acidic dileucine motif directs Sna4p to the vacuolar membrane. Sna4p-GFP uses the AP-3 pathway and has an acidic dileucine motif in its carboxy-terminal region. To test whether this motif was functional, GFP-tagged Sna4p in which the two leucine residues of the acidic dileucine ExxLL motif were replaced with two alanine residues (Fig. 2A) was expressed in WT cells. The localization of Sna4p<sup>L118/119A</sup>-GFP was similar to that of Sna4p-GFP in *apm3Δ* mutant cells, with additional slight signals coming from the plasma membrane and the vacuolar membrane (Fig. 2B; see Fig. 5). These results confirmed that Sna4p required the complete AP-3 complex for proper sorting to the vacuolar membrane and that disruption of the acidic dileucine motif caused mislocalization of Sna4p-GFP to the vacuolar interior.

Whether the vacuolar interior delivery of Sna4p<sup>L118/119A</sup>-GFP occurred directly from the trans-Golgi network through the MVB pathway or via the plasma membrane with subsequent endocytosis was tested by expression of Sna4p<sup>L118/119A</sup>-GFP in a yeast mutant, the *end3Δ* mutant, defective in the early stage of endocytosis (2). The luminal localization of the protein was not different in the *end3Δ* mutant (Fig. 2C; see Fig. 5), showing that Sna4p<sup>L118/119A</sup>-GFP did not require transit through the plasma membrane to reach the vacuolar lumen and thus traveled directly from the trans-Golgi network to the vacuolar interior. Some signal from Sna4p<sup>L118/119A</sup>-GFP was still observed in the plasma membrane of the *end3Δ* mutant strain, but at a level similar to that in the WT strain. Similarly, the localization of Sna4p-GFP was not affected in the *end3Δ* mutant compared to that in the WT strain (Fig. 2C; see Fig. 5).

Sna4p sorting into the MVB pathway depends on its PY motif and the presence of Rsp5p. To test our hypothesis that Sna4p uses the MVB pathway, we expressed Sna4p-GFP and Sna4p<sup>L118/119A</sup>-GFP in cells lacking Vps27p or Vps24p, two essential components of the ESCRT machinery. The family of Vps (vacuolar protein sorting) proteins is divided into six classes (A to F) according to the vacuole morphology visualized in mutants (5). The *vps24Δ* and *vps27A* mutants belong to class E and are characterized by the presence of a large aberrant MVB (the class E compartment) adjacent to the vacuole,
where proteins using the MVB pathway accumulate (26). In vps24 \( / \) or vps27 \( / \) mutant cells, Sna4p-GFP was localized in the vacuolar membrane and in some surrounding punctate structures, which are likely to be class E compartments (Fig. 3, top two rows). Almost no fluorescence was detected inside the vacuole. The staining of the class E compartment is due to the fraction of Sna4p-GFP using the MVB pathway. The vacuolar membrane staining comes from Sna4p-GFP that uses the AP-3 route or the MVB pathway. In the latter case, Sna4p-GFP cannot be internalized into the interior of the endosomes because of the deficiency in the ESCRT machinery. To test this, we examined the localization of Sna4pL118/119A-GFP expressed from a centromeric plasmid in the mutant strains indicated on the left, and the cells were examined by fluorescence microscopy with Nomarski optics. The vacuolar membrane was labeled with FM4-64.

We then investigated the role of Rsp5p in the delivery of Sna4p-GFP to the vacuole. We used viable npi1 \(-1\) mutant cells, in which Rsp5p expression is reduced because of Ty element insertion in the RSP5 promoter (30). In npi1 \(-1\) mutant cells, Sna4p, like Sna3p (Fig. 2A), contains a PPxY sequence in its C-terminal region that fits the PY motif recognized by WW domain-containing proteins, such as the E3 ubiquitin ligase Rsp5p/Npi1p (14, 37). We introduced mutations in the PPHY motif of Sna4p or removed the PPHY sequence and examined the localization of these Sna4p variants fused to GFP expressed in WT cells. Sna4p-GFP lacking the six C-terminal amino acids including the PY motif was exclusively detected in the limiting membrane of the vacuole (data not shown), as was Sna4pP135/136A-GFP, in which the PPHY sequence was mutated to AAPY (Fig. 4, top row, and 5). In contrast, Sna4p-GFP containing the PPHY sequence was mainly observed in the vacuolar membrane, with part of the protein in the vacuolar interior (Fig. 1). This difference was more striking when observed in apm3 \( \Delta \) mutant cells. Indeed, in these cells Sna4p-GFP was exclusively detected in the vacuolar lumen (Fig. 1) while Sna4pP135/136A-GFP was found in the vacuolar lumen, the vacuolar membrane, and the plasma membrane (Fig. 4). We then investigated the role of Rsp5p in the delivery of Sna4p-GFP to the vacuole. We used viable npi1 \(-1\) mutant cells, in which Rsp5p expression is reduced because of Ty element insertion in the RSP5 promoter (30). In npi1 \(-1\) mutant cells,
Snap-GFP was found only in the vacuolar membrane, with little or no fluorescence from the lumen (Fig. 4, center row, and 5). We then expressed Sna4pL118/119A-GFP in npi1-1 mutant cells, preventing Sna4p from entering both the MVB and AP-3 pathways, and found that it was mainly targeted to the plasma membrane (Fig. 4, bottom row), in contrast to the situation in the WT strain, where it is sorted to the vacuolar interior via the MVB pathway (Fig. 2B). Thus, when the amount of Rsp5p was reduced, Sna4pL118/119A-GFP, which was not able to use the AP-3 pathway, was transported to the plasma membrane by default instead of being incorporated into MVBs. Similarly, a version of Sna4p lacking the last 26 amino acids (Fig. 2A) expressed in WT cells is localized in the plasma membrane and in the vacuolar membrane. All together, these data show that the Sna4p PY motif and Rsp5p are both necessary to target Sna4p to the MVB pathway.

To confirm the data on Sna4p localization obtained by fluorescence, we performed immunodetection of Sna4p-GFP variants in total extracts of WT or sn4a/H9004 mutant cells. As shown in Fig. 6A, anti-GFP antibodies revealed the presence of the fusion proteins Sna4p-GFP (lane 1), Sna4pL118/119A-GFP (lane 3), and Sna4pP135/136A-GFP (lane 5), with apparent molecular masses of around 46 kDa, while Sna4p lacking the last 6 amino acids (PY motif removed) (lane 4) or the last 26 amino acids (acidic dileucine and PY motifs removed) (lane 2) appeared at a reduced molecular mass. The signal at 28 kDa corresponded to free GFP released when GFP-tagged proteins are delivered into the vacuolar interior and exposed to vacuolar proteases. The free GFP signal in Sna4p-GFP- and Sna4pL118/119A-GFP-expressing cells was much stronger than that in cells expressing Sna4p-GFP with a deleted or mutated PY motif. This indicates that a large fraction of Sna4p did not reach the MVB internal vesicles and the vacuolar interior when it lacked a functional PY motif. These observations confirm the data obtained by fluorescence. To confirm that the free GFP signal reflects the transport of Sna4p-GFP into the vacuolar lumen, we showed that the free GFP signal was not detected in pep4Δ or vps4Δ mutant cells (Fig. 6B).

Sna4p physically interacts with Rsp5p via its PY motif. A proteomic study showed that Sna4p is a substrate for ubiquitin ligase Rsp5p (12). The WW domains of Rsp5p mediate interactions through the PY motif located in a variety of proteins (6). The presence of a PPPY sequence in the C-terminal cytosolic domain of Sna4p raised the possibility that it mediated direct association with Rsp5p. We investigated this by performing coimmunoprecipitation with anti-GFP antibodies on lysates of cells expressing Sna4p-GFP under the control of the TPI1 promoter and HA-tagged Rsp5p under the control of its own promoter and analyzing the immunoprecipitated proteins.
by immunodetection. As shown in Fig. 7, HA-Rsp5p was not immunoprecipitated from cells producing untagged Sna4p and HA-Rsp5p (lane 1) but was coimmunoprecipitated with Sna4p-GFP (lane 2) or Sna4pL118/119A-GFP (lane 3). Moreover, Sna4p-GFP in which the PPPY sequence was deleted by removal of the six C-terminal amino acids was unable to bind HA-Rsp5p (lane 4). These experiments demonstrate that Sna4p physically interacts with Rsp5p via its PPPY sequence.

**Sna4p is ubiquitylated by ubiquitin ligase Rsp5p.** Ubiquitylation of most MVB cargo proteins in yeast requires ubiquitin ligase Rsp5p (4, 10, 15, 19, 21). Sna4p has only one lysine, K128, in its C-terminal cytoplasmic region. We generated a mutant Sna4pK128R-GFP construct and expressed it in the vacuolar mutant Sna4pK128R-GFP construct and expressed it in the vacuolar membrane (Fig. 8, top row), in contrast to Sna4p-GFP, which was located in the vacuolar interior in the same mutant cells (Fig. 1). Further studies were performed with cells lacking Doa4p, a ubiquitin isopeptidase responsible for debiquitylation of endocytic vacuolar cargo (11, 34). A lack of Doa4p results in low levels of free ubiquitin, which inhibits many ubiquitylation-dependent processes, including the selective sorting of ubiquitin-tagged MVB cargo (28). In doa4Δ mutant cells, Sna4p lacking the acidic dileucine motif was not able to enter the lumen of the vacuole and was localized in the vacuolar membrane and the plasma membrane (Fig. 8, bottom row). A comparison of this pattern with that in WT cells clearly shows the influence of Doa4p on Sna4p sorting into MVBs. These results suggest that ubiquitylation of Sna4p controls the fate of the tagged protein.

Detection of Sna4p-GFP by Western blotting revealed additional bands at higher molecular masses (Fig. 6B). These bands could represent ubiquitylated forms of Sna4p-GFP. However, the GFP moiety might be ubiquitylated as a result of the interaction of Sna4p with Rsp5p (31). We therefore decided to analyze the HA-tagged Sna4p profile of total extracts prepared from different Sna4p-HA mutant proteins expressed in WT or pep4Δ or npi1-1 mutant cells by immunodetection with anti-HA antibody. Sna4p-HA produced in WT cells (data not shown) or pep4Δ mutant cells (Fig. 9A) displayed bands with a mobility lower than that of the 17-kDa Sna4p-HA protein (lane 2). These low-mobility bands correspond to the successively addition of at least 2 × 8 kDa (the molecular mass of ubiquitin) to the molecular mass of the main signal (17 kDa). In addition, these bands were stronger for Sna4pL118/119A-HA as a result of its exclusively MVB sorting (lane 1). In contrast, no lower-mobility bands were seen for Sna4pK128R-HA (lane 4) or for Sna4p-HA lacking the last PPPY sequence (lane 5). In addition, no such bands were seen for Sna4p-HA expressed in npi1-1 mutant cells (lane 6).

We then performed immunoprecipitation studies with lysates from cells expressing Sna4p-HA variants with anti-HA antibodies. The immunoprecipitated proteins were examined by immunodetection with anti-ubiquitin antibodies. As shown in Fig. 9B, more than five ubiquitylated bands were seen when using Sna4p-HA (lane 2), whereas no ubiquitin conjugates were observed when lysine 128 was mutated to arginine (lanes 3) or when the PPPY sequence was removed (lane 4). We conclude from these experiments that Sna4p is polyubiquitylated on lysine 128 and that this process involves Rsp5p. 

**FIG. 6.** Sna4p-GFP targeting to the vacuolar lumen depends on its PPPY motif. (A, top) Immunodetection of total extracts of sna4Δ mutant cells expressing GFP-tagged versions of Sna4p with anti-GFP antibodies. Lane 1, Sna4p WT; lane 2, Sna4pΔ26; lane 3, Sna4pL118/119A; lane 4, Sna4pΔ6; lane 5, Sna4pK128R/H9004. (A, bottom) Coomassie blue staining as a loading control. The sizes of the molecular mass markers are indicated on the right in kilodaltons. (B, top) Immunodetection of total extracts of WT cells (lane 2), pep4Δ mutant cells (lane 3), and vps4Δ mutant cells (lane 4) expressing GFP-tagged SNA4 with anti-GFP antibodies. Cells expressing untagged SNA4 (lane 1) were used as a control. (B, bottom) Coomassie blue staining as a loading control. The sizes of the molecular mass markers are indicated on the right in kilodaltons.

**FIG. 7.** The Sna4p C-terminal PPPY motif mediates the interaction with Rsp5p. Immunoprecipitation (IP) with anti-GFP antibodies was performed on total extracts of cells producing HA-tagged Rsp5p under the control of its own promoter and transformed by a plasmid encoding GFP-tagged WT or mutant forms of Sna4p under the control of the TPI1 promoter. Cells producing untagged Sna4p (lane 1), Sna4p-GFP (lane 2), Sna4pL118/119A-GFP (lane 3), or Sna4pΔ6-GFP (lane 4) were tested. Immunoprecipitated proteins were detected by immunoblotting with anti-GFP (top) and anti-HA (bottom) antibodies. Molecular masses are indicated on the left in kilodaltons.
mice, and yeast. In yeast, AP-3 function is required for the ALP pathway to the vacuole. Sna4p vacuolar membrane localization is mediated by the AP-3 complex. Lack of either the \( \mu \) subunit of the AP-3 adaptor (\( \text{apm3}^- \) mutant cells) or the acidic dileucine motif of Sna4p results in a similar phenotype, with missorting to the vacuolar interior. The dileucine sorting signal is conserved in some proteins that have been defined as potential AP-3 cargoes. The consensus sequence of the acidic dileucine motif of AP-3 cargo proteins in yeast, such as ALP and Vam3p, is E-XXX-L-[LV], which does not strictly include the ETPLL motif of Sna4p. Thus, this consensus should now be extended to E-X(2,3)-L-[LV], since Sna4p was confirmed as an acidic dileucine motif-possessing AP-3 cargo protein. Moreover, both Sna4p and Vam3p, as well as many other AP-3 cargoes, have a conserved proline at the −1 position of their dileucine sequence (9). These data strongly suggest that Sna4p traffics to the vacuolar membrane through the AP-3 pathway via recognition of its acidic dileucine sequence by the AP-3 complex.

In addition to the dileucine motif, Sna4p has a PY motif, which, together with E3 ligase Rsp5p, is necessary for the localization of Sna4p to the vacuolar membrane. A reduction in the levels of Rsp5p (\( npi1^- \) mutant cells) causes Sna4p to accumulate exclusively in the limiting membrane of the vacuole, and similar localization of Sna4p is observed when its PY motif is deleted or mutated. We found that Sna4p physically interacts with Rsp5p and that its PY motif is important in this process. Most direct substrates of Rsp5p have a PY motif (12), and a number of these have been shown to physically interact with Rsp5p via their PY motif, e.g., Rod1p and Rog3p (1), Bsd2p (15), and Sna3p (20, 25, 31, 37). The interaction between Sna4p and Rsp5p is completely inhibited when the PY motif is deleted from Sna4p, suggesting that Sna4p binds Rsp5p directly.

Sna4p is transported to the vacuole lumen via the MVB pathway, a process that depends on endosomal ESCRT complexes, which are involved in the MVB sorting process. Loss of ESCRT components, such as Vps24p or Vps27p, results in accumulation of Sna4p in the MVB pathway in the class E compartment. In addition, we found that the sorting of Sna4p is dependent on Doa4p, an endosomal deubiquitylation enzyme. In \( \text{doa4}^- \) mutant cells, in which free ubiquitin levels are limiting (34), leading to insufficient cargo ubiquitylation, Sna4p is prevented from entering the MVBs and remains in the vacuolar membrane. These results suggest that ubiquitylation is an important modification that allows Sna4p to enter the MVB pathway. However, the role of Doa4p is possibly more complex, since a recent report pointed to a more direct and positive role for Doa4p in the MVB pathway, in which Doa4p might be needed to deubiquitylate cargoes and/or components of the MVB sorting machinery (23).
We show that Rsp5p targets Sna4p to the MVB, interacts physically with Sna4p, and ubiquitylates Sna4p. Consistent with the fact that ubiquitylation occurs on a lysine on Sna4p, mutation of the only lysine residue in Sna4p leads to a targeting defect, resulting in its localization in the limiting membrane of the vacuole. In addition, we observe a slight Sna4pK128R→K2128R–GFP fluorescence in the vacuolar interior, which is probably due to Sna4pK128R–GFP ubiquitylated on the GFP tag as a result of the physical interaction between Sna4p and Rsp5p. In a previous study, we observed that GFP-tagged Sna3p was still ubiquitylated when all four lysine residues of Sna3p were replaced with arginine and that this was due to ubiquitylation of GFP (31). To avoid this problem, we used HA-tagged Sna4p. Immuno-noprecipitated Sna4p-HA shows several ubiquitin conjugates on Western blots, suggesting polyubiquitylation of Sna4p-HA. No ubiquitin signals are seen when using Sna4pK128R or Sna4p lacking the PY motif, indicating that Sna4p is polyubiquitylated on its target K128 residue by ubiquitin ligase Rsp5p binding to the PY motif of Sna4p. A similar control mechanism of ubiquitylation is observed for Sna3p, since it carries a PY motif that mediates its interaction with Rsp5p, resulting in its Sna3p-dependent polyubiquitylation (20, 25, 31, 37). Both Sna3p and Sna4p possess a PY motif in their cytosolic region, suggesting that they may have similar functions in the MVB pathway. Nevertheless, the dileucine motif is an additional and specific characteristic of Sna4p that could bring some specificity to its function.

In the yeast *Saccharomyces cerevisiae*, only 26 membrane proteins have both an acidic dileucine motif and a PY motif. Of these, only five (besides Sna4p) are located in the vacuolar membrane according to the *Saccharomyces* Genome Database. These are Nvs1p (Niemann-Pick type C related), which is involved in sphingolipid trafficking; Ate2p (calcium-transporting ATPase 2); Pfa3p (protein fatty acyltransferase), which is required for vacuolar membrane fusion; and two proteins with only partial vacuolar localization, Phm7p (phosphate metabolism, unknown function) and Bsd2p (metal homeostasis protein). Bsd2p is particularly interesting, as it is known to interact with Rsp5p and act as an adaptor between this ubiquitin ligase and proteins that require ubiquitylation, such as Cps1p or Smf1p (10a). The acidic di-leucine motif essential for AP-3-dependent sorting and restriction of the functional specificity of the Vac6p vacuolar t-SNARE in *S. cerevisiae*. J. Cell Biol. 142:913–922.

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**REFERENCES**

1. Andoh, T., Y. Hirata, and A. Kikuchi. 2002. PY motifs of Rod1 are required for binding to Rsp5p and for drug resistance. FEBS. Lett. 525:131–134.

2. Bénédetti, H., S. Raths, F. Crausaz, and H. Riezman. 1994. The END3 gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. Mol. Biol. Cell 5:1023–1037.

3. Berger, A. C., G. Salazar, M. L. Sterry, K. A. Newell-Litwa, E. Werner, R. A. Maness, H. Horbett, and V. Faundez. 2007. The subcellular localization of the Niemann-Pick type C proteins depends on the adaptor complex AP-3. J. Cell Sci. 120:3640–3652.

4. Blondel, M. O., J. Morvan, S. Dupré, D. Urban-Grimal, R. Huguenauer-Tsapis, and C. Volland. 2004. Direct sorting of the yeast uracil permease to the endosomal system is controlled by uracil binding and Rsp5p-dependent ubiquitylation. Mol. Biol. Cell 15:883–895.

5. Bowers, K., and T. H. Stevens. 2005. Protein transport from the late Golgi to the vacuole in the yeast Saccharomyces cerevisiae. Biochim. Biophys. Acta. 1744:138–144.

6. Chang, A., S. Cheang, X. Espanel, and M. Sudol. 2000. Rsp5p WW domains interact directly with the carbonyl-terminal domain of RNA polymerase II. J. Biol. Chem. 275:20562–20571.

7. Chen, D. C., C. B. Yang, and T. T. Kuo. 1992. One-step transformation of yeast in stationary phase. Curr. Genet. 21:83–84.

8. Cowles, C. R., G. Odorizzi, G. S. Payne, and S. D. Emr. 1997. The AP-3 adaptor complex is essential for cargo-selective transport to the vacuole. Cell 91:109–118.

9. Darsow, T., J. K. Bruns, S. Pfeffer, and U. Schrezenmeier. 1994. The Rsp5p-Vam3p interaction is required for yeast vacuolar t-SNARE J. Cell Biol. 124:1279–1288.

10. Dupré, S., and R. Huguenauer-Tsapis. 2001. Deubiquitination step in the endocytic pathway of yeast. Traffic 2:468–480.

11. Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19:141–172.

12. Hein, C., J.-Y. Springael, C. Volland, R. Huguenauer-Tsapis, and B. André. 1995. NPI1, an essential yeast gene involved in induced degradation of Gap1 and Fps1p permeases encodes the Rsp5p ubiquitin-protein ligase. Mol. Microbiol. 18:77–87.

13. Hettema, E. H., J. Valdez-Taubas, and H. Pelham. 2004. Bsd2 binds the ubiquitin ligase Rsp5 and mediates the ubiquitination of transmembrane proteins. EMBO J. 23:1279–1290.

14. Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19:141–172.

15. Hein, C., J.-Y. Springael, C. Volland, R. Huguenauer-Tsapis, and B. André. 1995. NPI1, an essential yeast gene involved in induced degradation of Gap1 and Fps1p permeases encodes the Rsp5p ubiquitin-protein ligase. Mol. Microbiol. 18:77–87.

16. Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19:141–172.

17. Hein, C., J.-Y. Springael, C. Volland, R. Huguenauer-Tsapis, and B. André. 1995. NPI1, an essential yeast gene involved in induced degradation of Gap1 and Fps1p permeases encodes the Rsp5p ubiquitin-protein ligase. Mol. Microbiol. 18:77–87.

18. Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19:141–172.

19. Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19:141–172.

20. McNatt, M. W., I. McKittrick, M. West, and G. Odorizzi. 2004. The C2 domain of the acid phosphatase 5p, which is required for the internalization step of endocytosis and sorting of carboxypeptidase S. Mol. Biol. Cell 15:486–490.

21. Kim, Y., Y. Deng, and C. C. Philpott. 2007. GGA2 and ubiquitin-dependent trafficking of Arm1, the ferricoreceptor transporter of Saccharomyces cerevisiae. Mol. Biol. Cell 18:1790–1802.

22. Kwapisz, M., P. Cholbiński, A. K. Hopper, J.-P. Rousseau, and T. Zoladek. 2007. Rsp5p ubiquitin ligase modulates translation accuracy in yeast Saccharomyces cerevisiae. RNA 13:1710–1718.

23. Kwapisz, M., P. Cholbiński, A. K. Hopper, J.-P. Rousseau, and T. Zoladek. 2007. Rsp5p ubiquitin ligase modulates translation accuracy in yeast Saccharomyces cerevisiae. RNA 13:1710–1718.

24. Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19:141–172.

25. Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19:141–172.

26. Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19:141–172.

27. Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19:141–172.
vacuolar and endocytic traffic through a prevacuolar compartment in Saccharomyces cerevisiae. J. Cell Biol. 131:603–618.
27. Piper, R. C., N. J. Bryant, and T. H. Stevens. 1997. The membrane protein alkaline phosphatase is delivered to the vacuole by a route that is distinct from the VPS-dependent pathway. J. Cell Biol. 138:531–546.
28. Reggiori, F., and H. Pelham. 2001. Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. EMBO J. 20:5176–5186.
29. Rehling, P., T. Darsow, D. J. Katzmann, and S. D. Emr. 1999. Formation of AP-3 transport intermediates requires Vps41 function. Nat. Cell Biol. 1:346–353.
30. Springael, J.-Y., and B. André. 1998. Nitrogen-regulated ubiquitination of the Gap1 permease of Saccharomyces cerevisiae. Mol. Biol. Cell 9:1253–1263.
31. Stawiecka-Mirota, M., W. Pokrzywa, J. Morvan, T. Zoladek, R. Hagenauer-Tsapis, D. Urban-Grimal, and P. Morsomme. 2007. Binding to Rsp5p targets to the endosomal pathway the yeast Sna3p, a protein ubiquitylated with lysine-63-linked chains. Traffic 8:1280–1296.
32. Stimpson, H. E. M., M. J. Lewis, and H. Pelham. 2006. Transferrin receptor-like proteins control the degradation of a yeast metal transporter. EMBO J. 25:662–672.
33. Sullivan, J., M. Lewis, E. Nikko, and H. Pelham. 2007. Multiple interactions drive adaptor-mediated recruitment of the ubiquitin ligase Rsp5 to membrane proteins in vivo and in vitro. Mol. Biol. Cell 18:2429–2440.
34. Swaminathan, S., A. Y. Amerik, and M. Hochstrasser. 1999. The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. Mol. Biol. Cell 10:2583–2594.
35. Vowels, J. J., and G. S. Payne. 1998. A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole. EMBO J. 17:2482–2493.
36. Wang, C. W., P. E. Stromhaug, J. Shima, and D. J. Klionsky. 2002. The Ccz1-Mon1 protein complex is required for the late step of multiple vacuole delivery pathways. J. Biol. Chem. 277:47917–47972.
37. Watson, H., and J. S. Bonifacino. 2007. Direct binding to Rsp5p regulates ubiquitination-independent vacuolar transport of Sna3p. Mol. Biol. Cell 18:1781–1789.