The sorting of the yeast proteases proteinase A and carboxypeptidase Y to the vacuole is a saturable, receptor-mediated process. Information sufficient for vacuolar sorting of the normally secreted proteinase A has in fusion constructs previously been found to reside in the propeptide of proteinase A. We found that sorting of such a hybrid protein is dependent on the vacuolar protein-sorting receptor Vps10p. This was unexpected, as strains disrupted for Vps10p sort more than 85% of the proteinase A to the vacuole. Consistent with a role for Vps10p in sorting of proteinase A, we found that 1) overproduction of Vps10p suppressed the missorting phenotype of a carboxypeptidase Y, 3) vacuolar sorting of proteinase A in a vps10Δ strain was readily saturated by modest overproduction of proteinase A, and 4) Vps10p and proteinase A interact directly and specifically as shown by chemical cross-linking. Interestingly, overexpression of two telomere-linked VPS10 homologues, VTH1 and VTH2 suppressed the missorting phenotypes of a vps10Δ strain. However, disruption of the VTH1 and VTH2 genes did not affect the sorting of proteinase A. We conclude that proteinase A utilizes at least two mechanisms for sorting, a Vps10p-dependent path and a Vth1p/Vth2p/Vps10p-independent path.

The yeast vacuole contains a number of soluble hydrolases that are delivered to this organelle via the endoplasmic reticulum and the Golgi complex. Thus, both vacuolar and secretory proteins transit through these early compartments of the secretory pathway on their way to their final destination. Sorting takes place in a late subcompartment of the Golgi complex, the trans Golgi network, where soluble vacuolar proteins are diverted to the late endosome by an active, saturable mechanism (1–4). Carboxypeptidase Y (CPY)1 and proteinase A (PrA) have been the model enzymes in most studies of the biosynthesis of soluble yeast vacuolar proteins. These enzymes are synthesized as precursor forms that upon arrival in the vacuole are activated by proteolytic removal of an N-terminal propeptide. The vacuolar sorting signal of CPY is located in the propeptide (5–8), and the VPS10 gene encodes the receptor (Vps10p), which interacts with this signal and is responsible for the sorting of CPY (9). Disruption of VPS10 results in complete mislocalization of CPY but does not strongly affect vacuolar sorting of PrA, indicating that PrA can be sorted to the vacuole by an alternate mechanism (9). The 54-amino acid propeptide of PrA can direct the normally periplasmic enzyme into the vacuole, indicating that it contains sorting information (10). More precise identification of this propeptide-located sorting signal turned out to be difficult, as the propeptide proved to be essential for folding, and thus also endoplasmic reticulum exit, of PrA (11). In a previous study, the role of the PrA propeptide in folding of the enzyme was investigated by random substitution of either the N-terminal or the C-terminal half of the propeptide. In the subsequent screen for functional mutant PrA forms, many were found to produce active PrA, and these mutant PrA forms were also localized to the vacuole (12). This raised the possibility that the propeptide was not essential for the sorting of PrA, a hypothesis that we have investigated further in the present study. We find that the PrA propeptide contains a recognition site for Vps10p and that PrA can be sorted to the vacuole by a Vps10p-dependent mechanism as well as a Vps10p-independent mechanism. Furthermore, we describe a Vps10p homologue, Vth1p, that can also function as a sorting receptor for both PrA and CPY.

**Experimental Procedures**

Strains, Media, and Materials—Saccharomyces cerevisiae strains used were JRY20–2cΔapc1 (MATα ura3–52 leu2–3, 112 his3–1200 Δapr1–1::HIS3) (7), SEY6210 (MATα leu2–3, 112 ura3–52 his3–1200 trp1–1901 lys2–801 suc2–a29) (13), TVY1 (MATα leu2–3, 112 ura3–52 his3–1200 trp1–1901 lys2–801 suc2–a29 pep4–LEU2) (derived from SEY6210, kindly supplied by T. Vida), EMY3 (MATα leu2–3, 112 ura3–52 his3–1200 trp1–1901 lys2–801 suc2–a29 pep3–HIS3) (9), M4039 (MATα leu2–3, 112 ura3–52 his3–1200 trp1–1901 lys2–801 suc2–a29 pep101–1::HIS3) (14), M4040 (MATα leu2–3, 112 ura3–52 his3–1200 trp1–1901 lys2–801 suc2–a29 pep4–1137), and M4040 (MATα leu2–3, 112 ura3–52 his3–1200 trp1–1901 lys2–801 suc2–a29 pep9–1137) (13). pFV99 contains a recognition site for Vps10p and can be sorted to the vacuole by a Vps10p-dependent mechanism as well as a Vps10p-independent mechanism. Furthermore, we describe a Vps10p homologue, Vth1p, that can also function as a sorting receptor for both PrA and CPY.

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1 The abbreviations used are: CPY, carboxypeptidase Y; PrA, proteinase A; VPS, vacuolar protein sorting; VTH, Vps ten homologue; WT, wild type.

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the presence, and 59 codons of the enzyme region) and pBVH17 (containing the PEP4 promoter, the presence, the consequence, and 59 codons of the enzyme region) (11) were subcloned into pFW99, resulting in pFW20 (encoding PrA137-pro-Inv) and pFW23 (encoding PrA137-Inv), respectively. The plasmids pFW42, pFW47, pFW50, pFW5, pFW9, pFW18 (encoding PrA137-dl-Inv), PrA137-dl-Inv (PrA137-dl-Inv, half of the propeptide, and a construct with the wild-type propeptide (PrA137-Inv) were fused to invertase, introduced into a Δsuc2 strain (which was also deleted for PEP4 to avoid competition for sorting from wild-type PrA). After growth at 30 °C, extracellular and total invertase activities were determined. Consistent with previous data (10) transformants producing the control invertase fusion (PrA137-Inv, containing the wild-type propeptide and the first 59 residues of mature PrA) secreted less than 5% of their invertase activity (Table I, VPS10). The three hybrid proteins containing random sequence in the N-terminal half of the propeptide (PrA137-rd6-Inv through PrA137-rd3-Inv) showed a degree of secretion similar to that of a strain producing PrA137-Inv. However, most of the invertase activity was secreted from the three strains that produced hybrid proteins containing random sequence in the C-terminal half of the propeptide (PrA137-rd4-Inv through PrA137-rd6-Inv), indicating that the C-terminal part of the wild-type propeptide contains a vacuolar sorting signal. However, the strains containing PrA137-rd4-Inv through PrA137-rd6-Inv sorted more than a strain containing PrA61-Inv, suggesting that the 59 residues of the PrA enzyme region that were still present in these constructs contained sorting information. Indeed, a significant amount (50%) of the peptide-lacking construct PrA137-plpro-Inv was sorted to the vacuole, indicating that the 97 N-terminal residues of the mature enzyme region also contain sorting information.

Together, the data indicate that the 137 most N-terminal residues of pro-PrA contain at least two sorting signals, one in the C-terminal half of the propeptide (as was earlier proposed by Klionsky et al. (10)) and one in the 59 most N-terminal residues of the mature enzyme region.

Sorting of the PrA-invertase Fusion Proteins Is Vps10p-dependent—A strain producing PrA137-rd6-Inv reproducibly secreted slightly less of its invertase activity than strains producing the other PrA-invertase hybrid proteins with an altered C-terminal half of the propeptide. The random sequence in the propeptide of PrA137-rd6-Inv contained the tetrapeptide QRIL, a sequence resembling the vacuolar sorting signal of pro-CPY (QRPL; Ref. 8). This suggested that perhaps PrA137-rd6-Inv was sorted more efficiently than the other similar mutant (QRPL; Ref. 8). This suggested that perhaps PrA137-rd6-Inv was sorted more efficiently than the other similar mutant forms due to interaction with Vps10p, the sorting receptor for CPY (9). In a Δvps10 strain this difference in sorting should be abolished. Surprisingly, all PrA-invertase hybrid proteins were almost completely (80–90%) missorted in the Δvps10 strain (Table I, Δvps10) including PrA137-Inv, which contains only wild-type pro-PrA sequences.

Because PrA is efficiently sorted to the vacuole in a strain disrupted for VPS10 (9), there must be a Vps10p-independent mechanism for PrA sorting. We constructed a fusion consisting of the entire pro-PrA sequence fused to invertase (PrA405-Inv). In order to allow correct folding of both enzymes, the two domains in the hybrid were separated by a linker peptide consisting of five glycine residues. Sorting of PrA405-Inv in strains lacking or containing Vps10p was investigated. In a VPS10 strain, 5% PrA405-Inv was secreted to the extracellular space. In contrast, in a strain lacking Vps10p, 60% of the invertase activity was secreted (Table I). The presence of a considerable amount of PrA405-Inv inside the cells suggests that at least a portion of this fusion can be sorted to the vacuole via a VPS10-independent mechanism. The fact that strains expressing PrA405-Inv from a centromere plasmid produced as
much PrA activity as a similar plasmid construct producing wild-type PrA (data not shown) suggests that the PrA domain of the hybrid protein folded efficiently into an active conformation and that the cell-associated material is targeted and activated normally in the vacuole.

Overproduction of Vps10p Can Prevent Mislocalization of PrA—The observation that the PrA-invertase fusions were sorted to the vacuole in a VPS10-dependent manner suggested that wild-type PrA could also be sorted by Vps10p. Overproduction of PrA results in saturation of the vacuolar sorting machinery, and a large fraction of the PrA molecules are secreted to the extracellular space (1). If Vps10p can sort PrA to the vacuole, then it might be expected that wild-type PrA could also be sorted by Vps10p. Overproduction of Vps10p sorted PrA to the vacuole in a strain that overproduced PrA 3–5-fold relative to the chromosomal copy of PrA (Fig. 1). PrA was overexpressed up to 15% of the total invertase activity that was secreted (Fig. 1).

To test this, we chose to use a strain disrupted in the structural gene for Prc1 (JHRY20–2C-Δprc1) for this experiment. A plasmid pBVH17 (WT) expressed approximately the same, less of it was in the extracellular (E) and intracellular (I) fractions. Labeled protein was separated by 10% SDS-PAGE. The strain (JHRY20–2C-Δprc1) devoid of CPY and expressed PEP4 from a centromeric plasmid pBVH17 (WT + CEN-PEP4). CENTEP4 indicates that this PEP4-carrying plasmid expresses 3–5-fold as much PrA as the chromosomal copy of PEP4. The cells contained a multicopy plasmid with (pVW197; lanes 3 and 4) or without (pRS425; lanes 1 and 2) VPS10. PseudoPrA is the product of the autodigestion of pro-PrA in the extracellular media fraction (15) and is indicated by an asterisk.

Overproduction of PrA Leads to Mislocalization of CPY—If Vps10p can sort PrA to the vacuole, then it might be expected that PrA and CPY compete for Vps10p. To test this by transforming a strain which was wild-type for PrA and CPY with a multicopy plasmid containing PEP4, a centromere plasmid containing PEP4, or a control plasmid lacking PEP4. By pulse labeling and immunoprecipitation of intra- and extracellular PrA and CPY, it was found that higher levels of PrA production resulted in greater amounts of CPY secretion (Fig. 2). Quantitation of the bands showed that, while the total amount of CPY remained roughly the same, less of it was in the intracellular vacuum form upon overproduction of PrA (Fig. 2).

PrA Sorting in Δvps10 Cells Is Easily Saturated—In order to further investigate the role of VPS10 in PrA sorting, strains producing approximately 2 times the normal amount of PrA were constructed by introducing a PEP4-carrying centromere plasmid pSEY306-PEP4 (unlike plasmid pBVH17, which expresses PrA at levels 3–5-fold that of the chromosomal copy of PEP4, pSEY306-PEP4 expresses approximately the same amount of PrA as the chromosomal PEP4 gene). PseudoPrA was introduced into both a wild-type strain and a Δvps10
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Fig. 2. Sorting of pro-CPY is affected by overproduction of pro-PrA. CPY was immunoprecipitated and prepared from pulse-labeled cells as described in the legend to Fig. 1 using 8% SDS-PAGE. The intracellular material is indicated by I, while extracellular material (medium + periplasm) is marked E. The cells expressed PEP4 at various gene dosages: single chromosomal copy (WT), single copy + low copy centromere plasmid pBVH17 (WT + CENPEP4), single copy + multicopy 2-μm plasmid pPA2 (WT + 2 μP4). The quantitation of CPY protein expressed as a percentage of the total is shown below each lane. Intensities of bands were quantitated using the PhosphorImager. CENPEP4 indicates that this PEP4-carrying plasmid expresses 3-5-fold as much PrA as the chromosomal copy of PEP4.

Fig. 3. A Δvps10 strain has a lower capacity for PrA sorting than a wild-type strain. Cells were pulse labeled with 35S-labeled amino acids for 10 min and chased for 30 min. PrA antigen was immunoprecipitated from intracellular (I) and extracellular (E) fractions. Labeled protein was separated by 8% SDS-PAGE. Panel A, first and second lanes, a wild-type strain (SEY6210); third and fourth lanes, a wild-type strain (SEY6210) carrying a centromere plasmid pSEYC306-PEP4 containing PEP4. Panel B, first and second lanes, a Δvps10 strain (EMY3); third and fourth lanes, a Δvps10 strain (EMY3) carrying a centromere plasmid pSEYC306-PEP4 containing PEP4.* indicates that this PEP4-carrying plasmid expresses 3-5-fold as much PrA as the chromosomal copy of PEP4.

Fig. 4. Chemical cross-linking of Vps10p and pro-PrA. Spheroplasts were pulse labeled with 35S-labeled amino acids and lysed in a way that retained the integrity of organelles. Lysates were then treated with the membrane-permeable cross-linker diithio-bis(succinimidylpropionate), and treated with Vps10p-specific antibodies. The precipitated immune complexes were treated with a reducing and denaturing buffer to release cross-linked proteins and antibodies. Finally, the samples were treated with PrA-specific antibodies, and the precipitated proteins were analyzed by SDS-PAGE. Lanes 1 and 2, strain SEY6210 (VPS10); lanes 3 and 4, strain EMY3 (Δvps10); lanes 5 and 6, strain TVY1 (Δpep4). The positions of the endoplasmic reticulum and Golgi-modified forms of PrA are indicated by proPrA, and the position of the mature enzyme is indicated by mPrA. For molecular weight comparison, the rightmost lane shows all of the PrA antigens.

Fig. 5. Schematic representation of Vps10p and Vth1p. The 14 boxes in each molecule represent Asp boxes (23) with the sequence (S/T)(D/E)GGX(T/S)W/F. Domains 1 and 2 of each molecule are approximately 20% identical to each other. The spacing of the cysteines in the cysteine-rich motifs is perfectly conserved between the two proteins. These motifs are likely to be structural elements. The percentage of identity between the various domains of the proteins is indicated by the numbers between the molecules. Gray boxes indicate the transmembrane domains.

Vps10p

Vth1p

Homologues of Vps10p—In a BLAST search two S. cerevisiae genes were identified, which show approximately 70% identity to VPS10 over the entire open reading frame (Fig. 5). The two genes are mutually identical with the exception of one nucleotide and have been given the names hypothetical protein Y19402.02 (chromosome IX) and hypothetical protein HRC 1549 (chromosome X). Here we refer to them as Vth1p and Vth2p, respectively (VTH for VpsTen Homologue). Contrary to VPS10, both genes were located close to the telomere of their respective chromosomes. It is not uncommon to have genes repeated on different chromosomes in the telomeric regions (20). Given the degree of sequence similarity, we thought it likely that the homologues could function as vacuolar sorting receptors when overproduced. A wild-type strain, a Δvps10 strain, and a Δvps10 strain carrying a multicopy plasmid containing VTH1 were pulse-labeled. Lysates from these cells were

noprecipitated when cross-linker was present (lane 2) but not in the absence of cross-linker (lane 1). As would be expected for a ligand interacting with a vacuolar sorting receptor in the trans Golgi network, only the pro form of PrA could be detected. Co-immunoprecipitation of the pro-PrA antigen with Vps10p was dependent on the presence of both Vps10p and PrA, as experiments performed in a Δvps10 strain (lanes 3 and 4) or a Δpep4 strain (lanes 5 and 6) did not result in the detection of any protein with the same molecular weight.

Vps10p Physically Interacts with Pro-PrA—The data presented thus far are all consistent with a model in which PrA directly interacts with Vps10p. In order to test this model, chemical cross-linking experiments were undertaken. Spheroplasts from wild-type, Δvps10, or Δpep4 strains were pulse-labeled for 10 min. As shown in Fig. 4, pro-PrA was co-immu
then probed with Vps10p-specific antiserum (9). In the wild-type strain (Fig. 6, lane 1), the antiserum recognized a protein of approximately 190 kDa, which has previously been shown to be Vps10p (9). This protein is absent in the Δvps10 strain (Fig. 6, lane 2). In the Δvps10 strain with the multicopy plasmid carrying VTH1, a protein that is slightly smaller than Vps10p was present (Fig. 6, lane 3), indicating that Vth1p is indeed made and cross-reacts with the antibody directed against Vps10p. This latter result also implies that the failure to detect Vth protein in a Δvps10 strain (lane 2) must be due to much lower levels of Vth production in strains that only produce Vth proteins from their normal chromosomal locus. The possibility that Vth is normally much more scarce than Vps10p is supported by the observation that even when increasing amounts of Vps10p-specific antisera were used, only a very small amount of Vth protein could be seen in the Δvps10 strain (data not shown). We then investigated whether Vth1p was capable of sorting proteins to the vacuole. Cells from a Δvps10 strain and a Δvps10 strain with a multicopy plasmid containing VTH1 were pulse-labeled. After chase, CPY was immunoprecipitated. It was found that while the Δvps10 strain could only properly localize about 10% of the CPY molecules, the strain overexpressing VTH1 could sort about 60% of the CPY molecules to the vacuole (Fig. 7A). This indicates that Vth1p can act as a vacuolar sorting receptor for CPY. In an attempt to see whether Vth1p could also sort PrA, a centromere plasmid containing PEP4 was introduced into a Δvps10 strain and a Δvps10 strain overexpressing Vth1p. Pulse labeling and immunoprecipitation of PrA was performed. As was shown earlier in Fig. 3, the Δvps10 strain could only properly localize approximately half of the PrA molecules (Fig. 7B). However, the Δvps10 strain that overexpressed Vth1p sorted and matured almost all of the PrA (Fig. 7B). Taken together, these data indicate that Vth1p is capable of sorting both CPY and PrA. To investigate the role of the VTH genes, we constructed a Δvps10, Δvth1, Δvth2 triple-deletion strain and compared its sorting of PrA with that of a Δvps10 single-deletion strain. No significant differences were found, i.e., the triple-deletion strain still sorted most of its newly synthesized PrA molecules correctly (data not shown). This indicates that the Vth proteins do not play a major role in the Vps10p-independent vacuolar sorting of PrA.

**DISCUSSION**

Genetic screens have identified a large number of mutants defective in vacuolar sorting of pro-CPY or of pro-CPY-invertase fusions. The majority of the mutants isolated have pleotropic defects in vacuolar protein sorting (see Ref. 21 for review). However, a few (vps10, vps29, and vps35) turned out to be more specifically defective in the localization of pro-CPY (9, 22). It has since been shown that one of these genes, VPS10, encodes the receptor that interacts with CPY and sorts this ligand to the vacuole. Deletion of VPS10 results in the missorting of more than 90% of the newly synthesized pro-CPY. However, during normal vegetative growth, less than 10% of PrA or proteinase B is missorted (9). This observation raised the question of whether an independent sorting mechanism existed for each individual vacuolar protein.

The sorting signal of pro-CPY resides in a sequence (Gln-Arg-Pro-Leu, or QRPL) close to the N terminus of the propeptide (6, 8). Similarly to pro-CPY, fusion of the PrA propeptide to invertase could direct the fusion protein to the vacuole (10). Unlike the case of pro-CPY, however, cis-dominant mutations leading to missorting of pro-PrA have not been identified. Indeed, pro-PrA appeared to be sorted correctly to the vacuole even when those parts of the propeptide sufficient for targeting of invertase were exchanged with completely unrelated sequences (12). In principle, these unrelated sequences could all contain targeting information, although this would seem unlikely. To investigate this problem further, we fused some mutant PrA prosequences, randomized either in the N-terminal or the C-terminal half, to invertase and analyzed the sorting phenotype. Three additional random inserts within each of the two segments were also tested (data not shown), and they showed the same phenotype as those shown in Table I. This analysis confirmed earlier evidence for propeptide-directed sorting (10). Thus, PrA-propeptide-invertase fusions that contained sequences unrelated to pro-PrA in place of the C-terminal half of the propeptide were secreted, even though the same mutant prosequences were sorted correctly when fused to the mature region of PrA (i.e., in a normal PrA context). Together these data strongly suggested that PrA can be sorted by two independent signals, one of which is found in the propeptide. The central role for PrA as the primary activating protease for numerous vacuolar proenzymes may explain why there are at
least two independent receptors that direct vacuolar delivery of this protease. It furthermore allows for independent regulation of the sorting of CPY and PrA.

The presence of a QRPL-like sequence in one of the constructs prompted us to test whether Vps10p might be responsible for the somewhat better sorting of this construct. To our surprise we found that all of the PrA propeptide-Inv fusion constructs were secreted in a Δvps10 strain. The observation that Vps10p appeared to be involved in the sorting of the fusion constructs suggested that it interacted with, or was involved in the sorting of, native pro-PrA. All further experiments confirmed this hypothesis. Δvps10 strains exhibit a strongly reduced sorting capacity for PrA, and overproduction of Vps10p is able to suppress the missorting phenotype associated with moderate overproduction of PrA. Moreover, functional interaction between pro-CPY and Vps10p would be expected to subject to competition by pro-PrA. Indeed, overproduction of pro-PrA leads to significant secretion of pro-CPY. Importantly, chemical cross-linking experiments show pro-PrA and Vps10p to directly interact.

QRPL constitutes the core of the Vps10p recognition sequence in pro-CPY. The C-terminal half of the PrA propeptide, which confers Vps10p-dependent vacuolar sorting of invertase, contains the similar sequence KQYL. However, this probably is not the signal for Vps10p-dependent sorting, as the constructs PrA137-rd1-Inv through PrA137-rd3-Inv, which have a Gln to Glu mutation at this position, are sorted as efficiently as PrA137-Inv. One possibility is that Vps10p has more than one binding site and binds the two ligands via different binding sites. Mutational analysis of the QRPL sequence and its surroundings in pro-CPY have shown that Vps10p has a fairly broad specificity (17). Thus, it may well be that sequences seemingly unrelated to QRPL can function as ligands for the same binding site on Vps10p in a way analogous to that by which a peptide interacts with the binding site of a protease. This would require that they are exposed and/or in a dynamic/disordered structure. Such extended structures are likely to be unaffected by fusion to invertase. The notion that many disordered structures are substrates for Vps10p interaction is supported by the observation that PrA137-prop-Inv, containing only the 59 N-terminal residues of the mature PrA sequence, is sorted with approximately 50% efficiency in a Vps10p-dependent manner. Regarding the Vps10p-independent sorting signal, it is likely that it is localized outside the propeptide, since e.g. the PrA137-Inv fusion is completely missorted in a Δvps10 strain. Possible receptor candidates for the Vps10p-independent sorting of PrA were Vth1p and Vth2p. These two proteins are identical in sequence and show close homology to Vps10p. Vth1p is indeed a vacuolar sorting receptor, as it can suppress the missorting of PrA and CPY when overproduced in a Δvps10 strain. However, the Vth proteins appear to be produced at very low levels from their normal chromosomal loci. This indicates that the Vth proteins normally only play a minor role in vacuolar sorting of PrA and CPY, a conclusion that is supported by the fact that, even though Vth1p can sort CPY, more than 90% of the newly synthesized pro-CPY molecules are missorted in a Δvps10 strain. Finally, a strain having a triple deletion of VPS10, VTH1, and VTH2 sorted PrA with the same efficiency as a Δvps10 single-deletion mutant. This indicates that yeast contains a sorting mechanism for PrA that is independent of VPS10, VTH1, and VTH2.

The nature of the VPS10, VTH1, VTH2-independent mechanism of PrA sorting remains as yet unclear. An interesting recent observation in this context is that two vps mutants (vps29 and vps35), which sort PrA correctly (9, 22), exhibit a general deficiency in recycling of Vps10p and other Golgi-located proteins to the Golgi complex. This might suggest that the alternative mechanism of PrA sorting involves a carrier protein that does not cycle between the late endosome and the Golgi complex but rather remains trapped in the late endosome or proceeds to the vacuole. Indeed, PrA may “piggyback” a ride on a normal integral membrane protein of the vacuole. While this remains speculative at present, a consequence of the redundancy of the sorting mechanism is that it has been difficult to identify vps mutants affecting pro-PrA specifically. It should be possible to overcome this problem by employing a Δvps10 strain in future genetic screens.

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