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Molecular machinery and regulatory system of vacuolar amino acid compartmentalization as a new target for yeast engineering

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Research aims

The vacuole, which is a membrane-bound organelle present in all eukaryotic microorganisms and plants, serves as a compartment for the storage of metabolic products and sequestration of xenobiotics. It also functions as a digestive compartment for macromolecules such as proteins, which is a common feature with lysosomes in mammalian cells. In recent years, the digestive role of vacuoles has attracted increasing attention, specifically, the process of autophagy that is induced during nitrogen starvation. More than 50% of the cellular amino acids are found in the vacuoles. Although it is plausible that vacuoles play a critical role in amino acid metabolism, including protein synthesis and degradation, the molecular mechanism of vacuolar amino acid compartmentalization and regulation, and its association with amino acid metabolism remain unclear. In this study, we used the budding yeast *Saccharomyces cerevisiae* as a model organism to establish the molecular basis of vacuolar amino acid compartmentalization for application in the fermentation industry. We also analyzed the regulatory mechanism of the vacuolar amino acid transporter Avt4 and identified novel vacuolar amino acid transporters.

Methods

Strains and growth conditions

The derivatives of *S. cerevisiae* strain X2180-1B (*MATα SUC2 mal mel gal2 CUP1*) were used in this study. The cells were cultured in SD+CA (0.17% yeast nitrogen base w/o amino acids and ammonium sulfate, 0.5% casamino acids, 0.5% ammonium sulfate, and 2% dextrose) to the early logarithmic phase. For sporulation, the cells were washed two times, suspended in sporulation medium (0.1% yeast extract, 0.05% glucose, and 1% potassium acetate), and then cultured at 25°C for 6 days.

Analysis of amino acid composition

The cells were collected and subjected to cupric ion treatment to extract the vacuolar amino acid pool, as described previously.[1] The amount of the amino acids was measured using Hitachi L-8900 automatic amino acid analyzer.

Amino acid transport assay using vacuolar membrane vesicles

Vacuolar membrane vesicles were prepared by the Ficoll floating method, as previously described.[2] The efflux of amino acids from vesicles was measured according to the previous report.[3] Briefly, the reaction was initiated by adding 2 mM ATP to vesicles preloaded with ^{14}C-labeled amino acids and then terminated by diluting with ice-cold buffer. Radioactivity of the vesicles recovered by vacuum filtration on cellulose acetate membrane filters was determined using a liquid scintillation counter. ATP-dependent uptake of amino acids and calcium was assayed as described previously.[2, 4]

Results

1. Regulation of vacuolar amino acid transport activity by the N-terminal hydrophilic region of Avt4

Avt4 mediates the extrusion of various neutral amino acids from the vacuoles in a redundant manner with its closest homologue Avt3. In addition, Avt4 is involved in the extrusion of basic amino acids that are highly accumulated in the vacuoles.[3, 5] When cells are subjected to conditions of nitrogen starvation, the content of vacuolar basic amino acids is rapidly reduced. Since this reduction is significantly suppressed in *avt4*Δ cells, it is likely that the vacuolar amino acid extrusion by Avt4 increases under these conditions.[3] The hydrophilic region of transporter proteins is known to regulate their activity. Topology prediction of Avt4 suggests the presence of a long hydrophilic region at the N-terminus (Fig. 1A). Fungal homologues of Avt4 also possess a similar long hydrophilic region and an alignment of the two proteins reveals five conserved motifs (motif I-V) in this region (Fig. 1A). The expression level and vacuolar membrane localization of Avt4 mutants deleted for each of the five motifs (Avt4ΔI-ΔV) and expressed under the native *AVT4* promoter in *avt3*Δ*avt4*Δ cells were almost the same as the wild-type Avt4. The content of neutral amino acids in the vacuoles was increased by the expression of Avt4ΔI, ΔII, ΔIII, or ΔIV, but decreased by the expression of Avt4ΔV, compared to that in the vacuoles expressing wild-type Avt4 (Fig. 1B). The content of basic amino acids in the vacuoles was also highly reduced by Avt4ΔV expression (Fig. 1B). In contrast, the content of

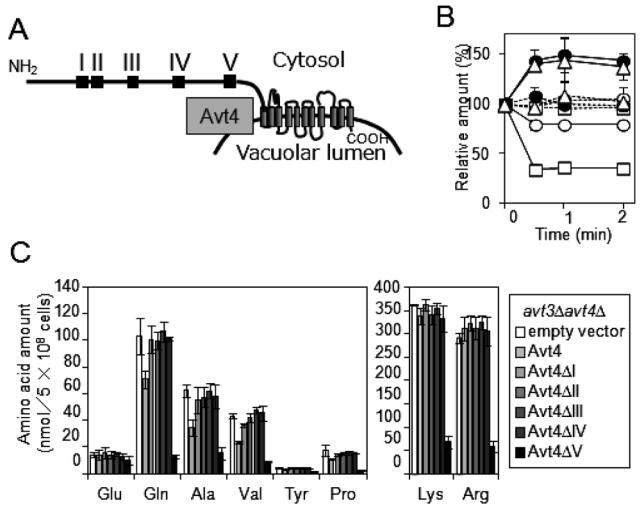

Fig. 1. Functional analysis of the N-terminal hydrophilic region of Avt4.
(A) Predicted topology and the conserved motifs (I-V) in the N-terminal hydrophilic region of Avt4. (B) Changes in the alanine transport activity of vacuolar membrane vesicles by the deletion of the conserved motif. ^{14}C-labeled alanine was preloaded into vacuolar membrane vesicles isolated from *avt3*Δ*avt4*Δ cells carrying an empty vector (*closed circles*) or a vector expressing wild-type Avt4 (*open circles*), Avt4ΔIV (*open triangles*), or Avt4ΔV (*open squares*), and further incubated in the presence (*solid lines*) or absence of ATP (*dotted lines*). The amount of preloaded ^{14}C-labeled alanine (0 min) was considered to be 100%. The relative amount of alanine in the vesicles at each time point was plotted. The results are expressed as mean±SD of three independent experiments. (C) Vacuolar amino acid content in *avt3*Δ*avt4*Δ cells expressing the Avt4 mutant deleted for the conserved motif. Vacuolar amino acids extracted from the indicated strains were analyzed with an amino acid analyzer. The results are expressed as mean±SD of three independent experiments.

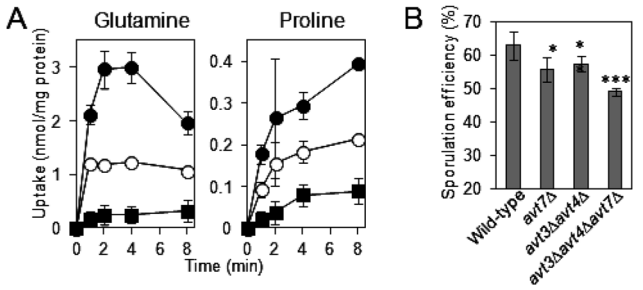

Fig. 2. Effect of *AVT7* expression on the net uptake of amino acids by vacuolar membrane vesicles and sporulation efficiency.
(A) Vacuolar membrane vesicles were prepared from the *avt3*Δ*avt4*Δ/empty vector (*open circles*), *avt3*Δ*avt4*Δ*avt7*Δ/empty vector (*closed circles*), and *avt3*Δ*avt4*Δ*avt7*Δ/pGPD-AVT7 (*closed squares*). [^{14}C]glutamine (*left*) or [^{14}C]proline (*right*) were preloaded into the vacuolar membrane vesicles. After the addition of 2 mM ATP, the vesicles were filtered through a cellulose acetate filter (pore, 0.45 μm) at the indicated time points and the radioactivity remaining on the filter was determined using a liquid scintillation counter. The net uptake activity was calculated by subtracting the amount of amino acids in the preloaded vesicles (0 min) from that at each time point. (B) After culturing the diploid cells in sporulation medium for 6 d, the total number of cells and asci was counted under a microscope to calculate the sporulation efficiency. The results are represented as mean±SD of five independent experiments. *, P=0.019; **, P=0.049; and ***, P=0.0013.

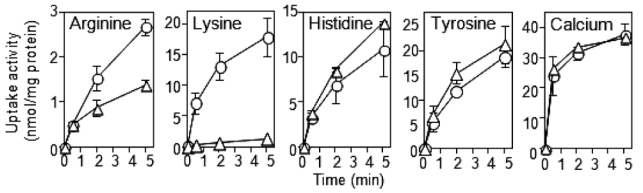

Fig. 3. ATP-dependent uptake of amino acids and calcium by vacuolar membrane vesicles.
Uptake assays were performed for the indicated amino acids and calcium into the vacuolar membrane vesicles isolated from wild-type (*open circles*) and *ypq1*Δ (*open triangles*) cells. The results are represented as mean±SD of three independent experiments.

acidic amino acids such as glutamate and aspartate remained unchanged. Following the addition of ATP, a slight efflux of alanine from the vacuolar membrane vesicles isolated from *avt3*Δ*avt4*Δ cells expressing wild-type Avt4 was observed and was further accelerated by the expression of Avt4ΔV (Fig. 1C). In contrast, alanine was taken up into the vesicles expressing Avt4ΔIV as well as vesicles isolated from *avt3*Δ*avt4*Δ cells carrying an empty vector (Fig. 1C). These results demonstrate that the N-terminal hydrophilic region of Avt4 regulates vacuolar amino acid extrusion both positively and negatively.

2. Identification and characterization of novel vacuolar amino acid transporters[6, 7)]

The *S. cerevisiae* genome encodes for seven members of the AVT transporter family. Among them, Avt1, Avt3, Avt4, and Avt6 are known to be involved in vacuolar amino acid transport,[5)] while Avt2, Avt5, and Avt7 are uncharacterized. We found that GFP-fused Avt7 localizes to the vacuolar membrane. To explore the possibility of its involvement in vacuolar amino acid transport, we examined the effect of *AVT7* disruption on vacuolar amino acid content. When *AVT7* was disrupted in *avt3*Δ*avt4*Δ strain, in which vacuolar amino acid extrusion is minimized, the content of various neutral amino acids in the vacuoles was found to increase. Among them, the increase in glutamine and proline was remarkable. In addition, the ATP-dependent uptake of these amino acids into vacuolar membrane vesicles isolated from *avt3*Δ*avt4*Δ*avt7*Δ cells also increased, when compared with vesicles from *avt3*Δ*avt4*Δ cells (Fig. 2A). On the other hand, overexpression of *AVT7* resulted in decreased uptake of glutamine and proline into vacuolar membrane vesicles (Fig. 2A). These results suggest that Avt7 is a novel vacuolar amino acid transporter, which is involved in the extrusion of neutral amino acids from vacuoles. The recycling of vacuolar amino acids is particularly important for protein synthesis under conditions of nitrogen starvation. The content of various neutral amino acids in the vacuoles of *avt3*Δ*avt4*Δ*avt7*Δ cells was considerably higher than in those of *avt3*Δ*avt4*Δ cells in this condition. The efficiency of sporulation, which is induced by nitrogen starvation, was slightly reduced in *avt7*Δ as well as *avt3*Δ*avt4*Δ diploid cells, and was significantly decreased by *avt3*Δ*avt4*Δ*avt7*Δ triple disruption (Fig. 2B). These data demonstrate the involvement of vacuolar amino acid recycling in the adaptation to nutrient starvation.

Ypq1, the *S. cerevisiae* homologue of lysosomal basic amino acid transporter LAAT-1/PQLC2 in nematode/rat, localizes to the vacuolar membrane.[8)] We compared the ATP-dependent uptake of basic amino acids such as lysine, arginine, and histidine into the vacuolar membrane vesicles isolated from wild-type and *ypq1*Δ cells. *YPQ1* disruption drastically decreased lysine uptake, partially impaired arginine uptake, but did not largely affect histidine uptake (Fig. 3).

The uptake of tyrosine and calcium remained unaffected in the vesicles of *ypq1*Δ cells (Fig. 1). In addition, the vacuolar uptake of basic amino acids depends on the proton motive force generated by V-ATPase.[2] To assess the effect of *YPQ1* disruption on the V-ATPase activity, vacuolar membrane vesicles were subjected to Western blot analysis for V-ATPase subunit Vph1. Both the wild-type and *ypq1*Δ vesicles exhibited similar expression of Vph1 (data not shown). Measurement of the quenching of quinacrine fluorescence showed that a pH gradient was generated in *ypq1*Δ as well as wild-type vesicles (data not shown). Thus, *YPQ1* disruption does not appear to affect the V-ATPase activity. These results suggest that Ypq1 is specifically involved in the vacuolar uptake of lysine and arginine.

Conclusions

This study demonstrates that, among the five conserved motifs found in the N-terminal hydrophilic region of Avt4, motifs I, II, III, and IV facilitate the vacuolar extrusion of amino acids, whereas motif V represses it. It is likely that the amino acid transport activity is regulated by Avt4, thereby providing the first insight into the regulatory mechanism of vacuolar amino acid transport. Our results also show that Avt7, a member of the AVT transporter family, mediates the extrusion of various neutral amino acids from vacuoles. The amino acids extruded by Avt7, Avt3, and Avt4 are probably utilized for the process of sporulation during nitrogen starvation. In addition, Ypq1, a homologue of the nematode/rat lysosomal amino acid transporter, participates in the uptake of lysine and arginine into the vacuoles. Further research is required to identify and explore the regulation of vacuolar amino acid transporters to reveal the crosstalk between vacuolar amino acid transport and amino acid metabolism and to understand the physiological importance of the vacuolar amino acid pool.

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