NISR Research GRANT
2010 Research Grant

The mechanism for vacuolar amino acid compartmentalization and its regulation in the budding yeast *Saccharomyces cerevisiae*

Takayuki SEKITO

Faculty of Agriculture, Ehime University

Research aims

The vacuole is an organelle, which serves as a storage compartment of ions, secondary metabolites, and toxic substances as well as a digestive compartment of macromolecules such as proteins. Vacuoles in *Saccharomyces cerevisiae* contain more than 50% of cellular amino acids. This raises the possibility that vacuoles play some important roles in amino acid metabolism. Recent studies are particularly focusing on the role in autophagic protein degradation induced under nitrogen starvation. Amino acids generated in vacuoles by autophagy, have been thought to be recycled for protein synthesis. However, those export machineries from vacuoles have not been understood well. In this study, to elucidate the mechanism of formation and regulation of vacuolar amino acid pool, we investigated the *in vivo* function of Avt3 and Avt4, amino acid transporters involved in the export of glutamine, tyrosine and isoleucine from vacuoles. In addition, we carried out a comprehensive analysis to identify transporters that localize to the vacuolar membrane. Vacuolar amino acid amounts were measured for the mutants of newly identified vacuolar transporters.

Methods

Amino acid composition analysis

The budding yeast *S. cerevisiae* cells were cultured in YPD (1% yeast extract, 2% polypeptone, 2% dextrose) to early logarithmic phase. For nitrogen-starvation, logarithmically growing cells in YPD were washed and suspended with SD-N (0.17% yeast nitrogen base w/o amino acids and ammonium sulfate, 2% dextrose), and further cultured for 6 hours. Cells were collected and subjected to cupric ion treatment to extract vacuolar amino acid pool as described previously[1,2]. Amino acid amounts were measured by using Hitachi L-8800 automatic amino acid analyzer.

Microscopic analysis

GFP fluorescence was observed by using Olympus IX71 fluorescent microscope equipped with Metamorph imaging software.

Results

1. Effect of *AVT3* and/or *AVT4* disruption on vacuolar amino acid composition

In vitro analysis using isolated vacuolar membrane vesicles identified several vacuolar amino acid transporters in the budding yeast *S. cerevisiae*[3–5]. Avt3 and Avt4 have been suggested to extrude glutamine, tyrosine and isoleucine from vacuoles in a redundant manner[3]. To examine their *in vivo* functions, vacuolar amino acid compositions were compared among wild-type, *avt3*Δ, *avt4*Δ, and *avt3*Δ*avt4*Δ cells (Figure 1A). Consistent with previous report, in *avt3*Δ*avt4*Δ vacuoles, amounts of glutamine, tyrosine and isoleucine increased. In addition, it is found that most of the other neutral amino acids, including threonine, serine, asparagine, glycine, alanine, valine, methionine, leucine, and proline, also increased. Either single disruption of *AVT3* or *AVT4* gene resulted in a minor change of these amino acid amounts. Levels of acidic and basic amino acids were little changed by either single or double gene disruption. These suggest that Avt3 and Avt4 export a variety of neutral amino acids from vacuoles in a redundant manner. We assumed that the extrusion of vacuolar amino acids by Avt3 and Avt4 contributes to amino acid recycling during autophagy. In agreement with this, under nitrogen-starved condition, which induces autophagy, *avt3*Δ*avt4*Δ vacuoles contained more neutral amino acids than wild-type vacuoles (Figure 1B). Basic amino acids, which are abundant in vacuoles in nutrient-rich condition, were greatly reduced in wild-type and *avt3*Δ vacuoles in response to nitrogen starvation (compare Figure 1A and B). On the other hand, more than 20 fold of basic amino acids was retained in *avt4*Δ and *avt3*Δ*avt4*Δ vacuoles when compared with wild-type vacuoles (Figure 1B). These suggest that both Avt3 and Avt4 are involved in the recycling of neutral amino acids during autophagy. In addition, Avt4 may have a distinct function from Avt3, which is the export of basic amino acids from vacuoles.

2. Function of N-terminal hydrophilic region of Avt4

In predicted topology models, Avt4 possesses a long N-terminal hydrophilic region, of which length is about 300 amino acids. In some cases, such long hydrophilic region of transporter is post-translationally modified and/or interacts

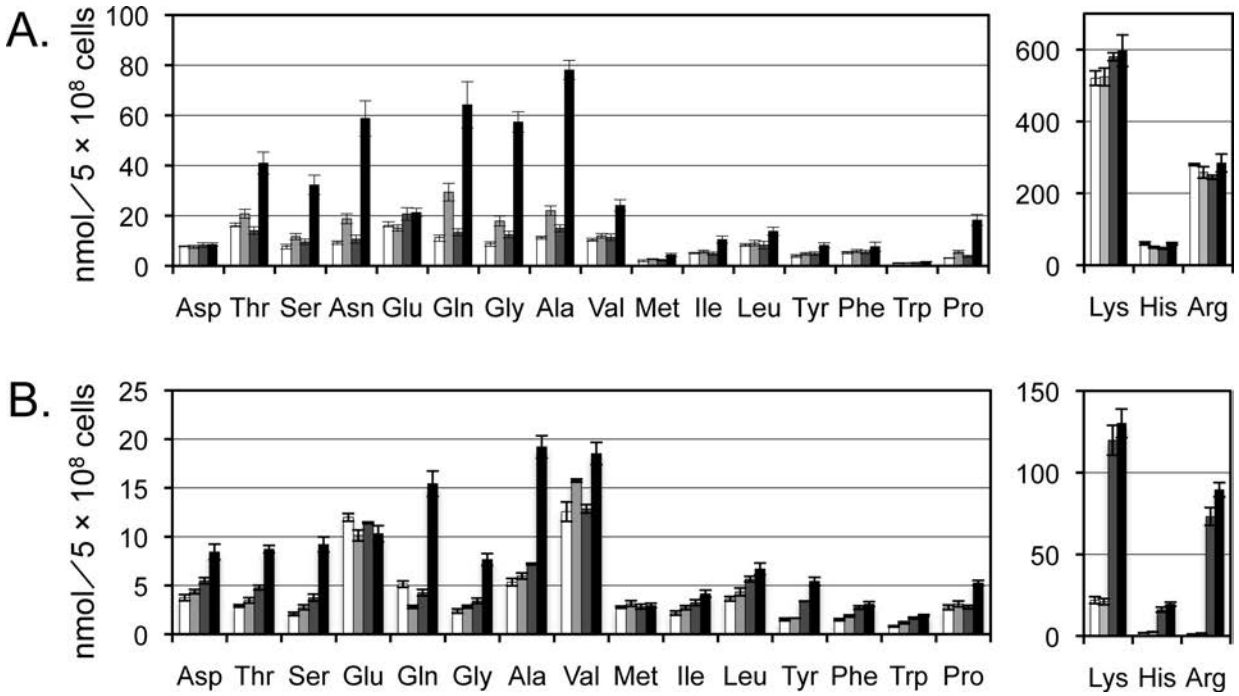

Figure 1 Vacuolar amino acid amounts in cells cultured in nutrient-rich (A) and in nitrogen-starved (B) conditions. Wild-type (white bar), *avt3*Δ (light gray bar), *avt4*Δ (dark gray bar), and *avt3*Δ*avt4*Δ (black bar) cells were cultured and treated with cupric ion to extract those vacuolar amino acid pools as described in "Methods". The amounts of basic amino acids, which were abundant in vacuoles, were presented in separate graphs (right).

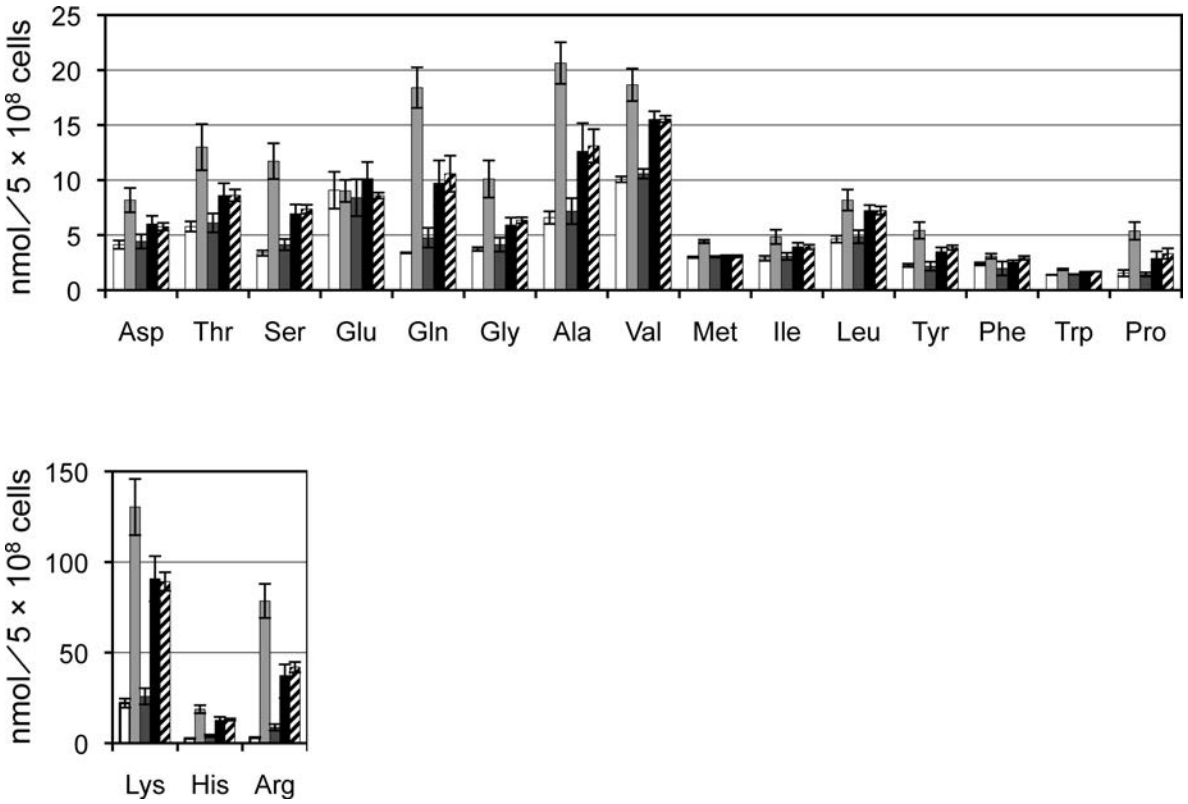

Figure 2 Effect of the truncation of N-terminal hydrophilic region from Avt4 on vacuolar amino acid amounts under nitrogen starvation. Vacuolar amino acid amounts in *avt3*Δ cells carrying empty plasmid (white bar) and *avt3*Δ*avt4*Δ cells carrying either empty plasmid (light gray bar) or plasmid encoding GFP-Avt4$^{1\text{-}713}$ (dark gray bar), GFP-Avt4$^{101\text{-}713}$ (black bar), or GFP-Avt4$^{201\text{-}713}$ (hatched bar) were measured as in Figure 1B.

with the other protein to regulate its transport activity. To elucidate the function of the N-terminal region of Avt4, GFP-fused truncated Avt4 variants, GFP-Avt4$^{101\text{-}713}$, GFP-Avt4$^{201\text{-}713}$, and GFP-Avt4$^{301\text{-}713}$, in which the N-terminal 100, 200, and 300 amino acids were deleted, respectively, were expressed in *avt3*Δ*avt4*Δ cells, and examined for their cellular level and subcellular localization under nitrogen starvation. Western blot analysis and microscopic observation revealed that, the level of GFP-Avt4$^{301\text{-}713}$ was much less than that of full-length Avt4 (GFP-Avt4$^{1\text{-}713}$). In addition, vacuolar membrane localization of GFP-Avt4$^{301\text{-}713}$ was defective when compared with GFP-Avt4$^{1\text{-}713}$. On the

other hand, GFP-Avt4$^{101\text{-}713}$ and GFP-Avt4$^{201\text{-}713}$ exhibited similar cellular levels to that of GFP-Avt4$^{1\text{-}713}$ and no defect in the vacuolar membrane localization. Next, we compared vacuolar amino acid amounts in *avt3*Δ*avt4*Δ cells expressing GFP-Avt4$^{101\text{-}713}$ or GFP-Avt4$^{201\text{-}713}$ with GFP-Avt4$^{1\text{-}713}$ under nitrogen starvation. As shown in Figure 2, vacuolar amino acid amounts in cells expressing either truncated Avt4 were more than 2-fold of those in cells expressing GFP-Avt4$^{1\text{-}713}$. This implicates that the N-terminal region of Avt4 plays an important role in amino acid export from vacuoles.

3. Identification of vacuolar transporters

Thirty putative transporters encoded in *S. cerevisiae* genome, which have not been characterized, were fused to GFP at either N- or C-terminal end and examined for the subcellular localization by fluorescent microscope. Among them, six transporters were found to localize to the vacuolar membrane. However, disruption of each gene did not change the vacuolar amino acid content. It is possible that the defect caused by the disruption of each transporter gene was compensated by overlapping function of the other transporters. Amino acid composition analysis is currently in progress for strains with multiple gene disruptions, in which some of the newly identified six transporter genes together with other transporter genes, including *AVT* genes, were disrupted, as well as for strains overexpressing each of the six transporters.

Conclusion

Amino acid composition analysis suggested that both Avt3 and Avt4 extrude variety of neutral amino acids from vacuoles. Furthermore, we obtained the data suggesting that Avt4 is involved in the export of basic amino acids from vacuoles during nitrogen starvation. Results of amino acid transport assay using isolated vacuolar membrane vesicles were consistent with the suggested broad substrate specificity of Avt3 and Avt4 (data not shown). We speculate that the vacuolar amino acid transport system may participate in the maintenance of cellular amino acids at optimal level in a concerted manner with other systems, such as amino acid transporters at the plasma membrane and enzymes involved in amino acid metabolism. Considering available data, it is likely that activity of vacuolar amino acid transporters is regulated in response to change in the cellular amino acid level. In this study, we found that cells expressing truncated Avt4, in which the N-terminal hydrophilic region was deleted, retained more amino acids in vacuoles than cells expressing full-length Avt4 during nitrogen starvation. Since the cellular level and the vacuolar membrane localization were not affected by the truncation, this result suggests that the N-terminal region is required for the amino acid transport activity of Avt4. We expect that the N-terminal region of Avt4 is involved in the regulation of activity. This study also revealed that six uncharacterized putative transporters were located at the vacuolar membrane. Microarray analysis suggested that some of them were induced by nitrogen/amino acid starvation. This implies the close relation of these transporters to amino acid metabolism. Further investigation is in progress.

References

1) Ohsumi, Y., Kitamoto, K., and Anraku, Y. (1988) Changes induced in the permeability barrier of the yeast plasma membrane by cupric ion. *J. Bacteriol.* **170**: 2676–2682.
2) Chahomchuen, T., Hondo, K., Ohsaki, M., Sekito, T., and Kakinuma, Y. (2009) Evidence for Avt6 as a vacuolar exporter of acidic amino acids in *Saccharomyces cerevisiae* cells. *J. Gen. Appl. Microbiol.* **55**: 409–417.
3) Russnak, R., Konczal, D., and McIntire, S. L. (2001) A family of yeast proteins mediating bidirectional vacuolar amino acid transport. *J. Biol. Chem.* **276**: 23849–23857.
4) Shimazu, M., Sekito, T., Akiyama, K., Ohsumi, Y., and Kakinuma Y. (2005) A family of basic amino acid transporters of the vacuolar membrane from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**: 4851–4857.
5) Sekito, T., Fujiki, Y., Ohsumi, Y., and Kakinuma, Y. (2008) Novel families of vacuolar amino acid transporters. *IUBMB Life* **60**: 519–525.