A recent study filling the gap in the genome sequence in the left arm of chromosome 2 of *Schizosaccharomyces pombe* revealed a homolog of budding yeast Vba2p, a vacuolar transporter of basic amino acids. GFP-tagged Vba2p in fission yeast was localized to the vacuolar membrane. Upon disruption of vba2, the uptake of several amino acids, including lysine, histidine, and arginine, was impaired. A transient increase in lysine uptake under nitrogen starvation was lowered by this mutation. These findings suggest that Vba2p is involved in basic amino acid transport in *S. pombe* under diverse conditions.

Key words: *Schizosaccharomyces pombe*; vacuolar transporter; amino acid; nitrogen starvation

Yeast vacuoles function as digestive compartments and also as storage compartments. Vacuolar compartmentalization of amino acids plays an important role in the maintenance of intracellular amino acids at appropriate levels. In *Saccharomyces cerevisiae*, the bulk of the basic amino acids is usually concentrated in the vacuoles, while acidic amino acids are concentrated primarily in the cytosol.  

Active transport of various amino acids into the vacuoles is driven by a proton electrochemical gradient across the vacuolar membrane generated by the action of V-ATPase, and a H+/amino acid cotransport mechanism has been proposed.  

Some vacuolar amino acid transporters have been identified in *S. cerevisiae.* In the amino acid transport (AVT) family, which belongs to the amino acid/auxin permease family, a vacuolar importer (Avt1p) and exporters (Avt3p, Avt4p, and Avt6p) have been found, but the function of the other AVT members, Avt2p, Avt5p, and Avt7p, has not been determined.  

In the vacuolar basic amino acids transporter (VBA) family, another subfamily of the major facilitator superfamily, Vba1p, Vba2p, and Vba3p were found to be involved in the vacuolar transport of basic amino acids, but the function of the other VBA members, Vba4p and Vba5p, is still unknown.  

In the fission yeast *Schizosaccharomyces pombe*, there are also large amounts of basic amino acids compartmentalized in the vacuoles, and small but significant amounts of acidic amino acids are retained in the organelles. Our reverse genetics study suggested that Fnx1p and Fnx2p of *S. pombe*, two Vba2p homologs, are involved in the vacuolar uptake of lysine and isoleucine. Based on phylogenetic analysis of the *S. cerevisiae* and *S. pombe* genome databases, we found homologs of *S. cerevisiae* AVT members, Avt3p and Avt5p, in *S. pombe.*  

S. pombe* Avt5p is involved in the vacuolar uptake of a variety of amino acids.* It is noteworthy that Avt5p was required for the sporulation of *S. pombe.* This suggests a correlation between vacuolar amino acid transport and cellular response to nitrogen starvation.

Recently Sasaki et al. reported a gap-filling sequence between SPBPB21E7.09 and SPBPB10D8.01 on the left arm of chromosome 2 of *S. pombe*, harboring five ORFs. The deduced amino acid sequence of SPBC460.03, one of those ORFs, showed 50% identity with *S. cerevisiae* Vba2p, and phylogenetic analysis revealed that it was the closest relative of Vba2p in *S. pombe* (Fig. 1A). Here we examined the role of SPBC460.03 (vba2+) in vacuolar amino acid transport.

First we performed RT-PCR to check the expression of vba2+ in wild-type cells. The *S. pombe* wild-type strain used in this study was ARC039 (h+ leu1–32 ura4–C190T). Total RNA was prepared from the cells at logarithmic phase in YES medium by the hot phenol method. First-strand cDNA was prepared with SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA). Total RNA was treated with Recombinant DNase I (Takara Bio, Ohtsu, Japan) before use. PCR was done for 25 cycles using ExTaq (Takara Bio), and act1 was amplified from ARC039 genomic DNA by PCR. The oligonucleotides used in this study are listed in Supplemental Table 1 (see *Biosci. Biotechnol. Biochem.*).
Several vacuolar transporters have been characterized directly with isolated vacuolar membrane vesicles, but it is difficult to isolate vacuoles from S. pombe cells. It has been established that active transport into the vacuoles is critical for amino acid uptake by whole S. cerevisiae cells. Investigation with concanamycin A (CCA), a specific potent inhibitor of vacuolar H+−ATPase (V-ATPase), and the vma1Δ mutant, in which the vma1 gene encoding the A subunit of V-ATPase, has suggested that V-ATPase-dependent vacuolar compartmentalization is also involved in amino acid uptake by whole S. pombe cells. In order to determine the involvement of Vba2p in vacuolar amino acid transport, the uptake activities of various amino acids by whole cells were examined. Wild-type and vba2Δ cells were cultured in YES medium, and harvested during logarithmic phase, and then amino acid uptake was measured as described previously. Uptake reactions were initiated by the addition of specific [3H]-labeled substrate at a final concentration of 40 μM, and the values of the uptake activities were averaged for two independent experiments (Fig. 2 and Table 1). Figure 2A shows the time course of the uptake of 2-deoxyglucose (2-DG), lysine, and tyrosine by wild-type and vba2Δ cells. 2-DG is a substrate of the glucose transporter (Ght1p) on the plasma membrane, but the uptake of lysine, and not of tyrosine, notably decreased in the vba2Δ mutant, indicating that Vba2p is critical for vacuolar amino acid transport.

Fig. 1. Summary of Vba2p.
A, Dendrogram of VBA proteins from S. pombe with homologous proteins from S. cerevisiae. B, RT-PCR analysis of vba2+. PCR was performed using 1st-strand cDNA as template prepared from total RNA. RNAs were prepared from wild-type (lane W) and vba2Δ mutant (lane D) cells. C, Fluorescence microscopy of vba2Δ mutant cells expressing the GFP-Vba2p fusion protein. Vacuoles were visualized with FM4-64.

Web site). The PCR product was cloned into pTN54 derived from pREP41. GFP-tagged Vba2p was expressed under the control of the attenuated nmt1 promoter. Cells transformed with this plasmid were grown in MM medium without leucine at 30°C for 20 h. Vacuoles were labeled with lipophilic dye FM4-64 (Invitrogen) as described previously. After exposure to FM4-64, cells were incubated with sterilized distilled water at 4°C overnight to induce vacuolar fusion, and then examined with an Olympus IX-71 fluorescence microscope using appropriate filter sets (Olympus, Tokyo). GFP-Vba2p was found in the vacuolar membrane, and was co-localized with FM4-64 (Fig. 1C). Vba2p was found to be a vacuolar membrane protein.

To determine the role of Vba2p in amino acid transport, disruption mutant vba2Δ was constructed. The 5′- and 3′-flanking regions of the vba2+ ORF and ura4+ cassette were amplified by PCR and sequentially cloned into pBluescriptII-SP-K+ (Stratagene, La Jolla, CA). The S. pombe wild-type strain was transformed with PCR products amplified from this construct. Gene disruption was confirmed by colony-PCR. The expression of vba2+ in this mutant was also checked by RT-PCR. Specific amplification of vba2+ did not occur in the vba2Δ mutant, indicating that vba2 was completely knocked out (Fig. 1B).

In S. cerevisiae, many vacuolar transporters have been characterized directly with isolated vacuolar membrane vesicles, but it is difficult to isolate vacuoles from S. pombe cells. It has been established that active transport into the vacuoles is critical for active transport into the vacuoles. The uptake of lysine, and not of tyrosine, notably decreased in the vba2Δ mutant, indicating that Vba2p is critical for vacuolar amino acid transport.
activity of histidine, arginine, valine, and asparagine as well as lysine was lower in the \textit{vba2Δ} cells, but that of tyrosine, isoleucine, serine, glutamine, aspartate, and glutamate was not. These results suggest that Vba2p is involved in the uptake of amino acids with a substrate specificity especially for basic amino acids. Vba2p is thus an ortholog of \textit{S. cerevisiae} Vba2p that is a vacuolar importer of arginine, histidine, and lysine.\textsuperscript{3,5)}

\textit{S. cerevisiae} Vba2p mediates V-ATPase-dependent vacuolar uptake of basic amino acids.\textsuperscript{3,5)} The effect of CCA on uptake of lysine and 2-DG by \textit{S. pombe} cells was examined (Fig. 2B). Cells were preincubated for 30 min at 30 °C with 10 μM CCA or DMSO vehicle before initiation of the reaction. 2-DG uptake by wild-type and \textit{vba2Δ} cells was little affected by 10 μM CCA. On the other hand, about 45% of lysine uptake by the wild-type cells was inhibited by CCA, in accordance with our notion that V-ATPase-dependent (CCA-sensitive) vacuolar compartmentalization is partially involved in the cellular uptake of amino acids.\textsuperscript{7,8)} It is likely that CCA-insensitive lysine uptake reflects cytosolic accumulation \textit{via} the plasma membrane transport system. Lysine uptake by \textit{vba2Δ} cells was also particularly inhibited by CCA, suggesting that the activity of V-ATPase-dependent vacuolar transport of lysine still remained in \textit{vba2Δ} cells. In our previous paper, we reported that Fnx1p is a major determinant of lysine uptake.\textsuperscript{17)} Vba2p should be minor in the vacuolar transport of lysine by \textit{S. pombe} cells cultured in standard medium. CCA-insensitive lysine uptake was lower than that of the parent strain (Fig. 2B). When we examined the CCA sensitivity in lysine uptake by wild-type, \textit{fnx1Δ}, and \textit{avt5Δ} cells, CCA-insensitive lysine uptake by mutant cells was nearly equal with that by the wild-type cells,\textsuperscript{19,20)} suggesting that Fnx1p and Avt5p are involved in the uptake of amino acids with a substrate specificity especially for basic amino acids. CCA insensitive lysine uptake reflects cytosolic accumulation \textit{via} the plasma membrane transport system. Lysine uptake by \textit{vba2Δ} cells was also particularly inhibited by CCA, suggesting that the vacuolar amino acid transporter on the plasma membrane and thereby affects the balance between CCA-sensitive and

| Table 1. Relative Uptake Activities of Various Amino Acids in \textit{vba2Δ} Cells |
|---------------------------------|-----------------|-----------------|
| Amino acid | (Uptake activity) | Relative activity |
| Lys | 10.26 | 72 |
| His | 4.51 | 77 |
| Arg | 8.97 | 87 |
| Tyr | 2.81 | 108 |
| Ile | 0.81 | 103 |
| Val | 0.73 | 82 |
| Ser | 2.42 | 109 |
| Asn | 3.75 | 81 |
| Gln | 1.98 | 112 |
| Asp | 0.83 | 103 |
| Glu | 0.75 | 101 |

\textsuperscript{a}Data are expressed as nmol/mg of cell dry weight after 60 min.

CCA-insensitive lysine uptake, but further investigation is required to understand the details.

Many amino acid transporters and permeases respond to environmental stress.\textsuperscript{17)} In \textit{S. cerevisiae}, several vacuolar amino acid transporters respond to nitrogen starvation.\textsuperscript{18)} It is to be determined how vacuolar amino acid transporters contribute to the maintenance of amino acid homeostasis.\textsuperscript{7,8)} We found in \textit{S. pombe} that amino acid uptake by whole cells transiently increased about 2–3 fold during periods of nitrogen starvation.\textsuperscript{3,5)} This increase in the uptake of amino acids was inhibited by CCA, suggesting that the vacuolar amino acid transporter is responsive to nitrogen starvation. To determine the role of \textit{S. pombe} Vba2p in response to nitrogen starvation, we examined lysine uptake by wild-type and \textit{vba2Δ} cells in nitrogen-poor (MM-N) medium (Fig. 3). When wild-type cells were shifted to the MM-N medium, lysine uptake varied: after 1 h of incubation, uptake increased three-fold, but upon further incubation, this increase declined, and after three hours uptake was close to 55% of the level observed during growth on rich medium (Fig. 3). In the absence of Vba2p, nitrogen starvation also induced a transient 2.6-fold increase in lysine uptake, but overall uptake activities were reduced to 70% of that of wild-type cells, in line with the expected transport defect caused by the lack of Vba2p (Fig. 2A and Table 1). These results suggest an important role of Vba2p in lysine uptake under nitrogen starvation.

Additionally, our previous study indicated that histidine uptake by \textit{avt5Δ} cells decreased to 50% of that of wild-type cells,\textsuperscript{18,19)} suggesting that Avt5p, rather than Vba2p, is the major factor in histidine transport. Confirming this hypothesis, the transient increase in histidine uptake fell to 60% in \textit{vba2Δ} cells during nitrogen starvation, while it was more severely limited to 30% in the \textit{avt5Δ} mutant (unpublished results). The multiple mutants of \textit{vba2Δ}, \textit{avt5Δ} and/or \textit{fnx1Δ} genes should be useful in investigating the roles of the individual vacuolar amino acid transporters, and construction and characterization of these multiple mutants is now in progress.

The experiments on the effects of CCA on lysine uptake (Fig. 2B) suggest that loss of Vba2p downregulates the CCA-insensitive activity of the amino acid transporter on the plasma membrane and thereby affects the balance between CCA-sensitive and
Gap1p, undergoes translocation in response to nutrient conditions. Aat1p molecules localized to the Golgi apparatus in rich medium were transported from the Golgi to the plasma membrane upon a shift to nitrogen-free medium. Prolonged incubation in nitrogen-limited condition resulted in transport of Aat1p into vacuoles by endocytosis. This is in good agreement with our observations of amino acid uptake by whole cells (Fig. 3). The vacuolar transporters as well as the plasma membrane transporters contributed to a transient increase in amino acid uptake during nitrogen starvation (Fig. 3). It would be interesting to investigate the regulatory mechanism of amino acid transporters under nitrogen starvation. To address these issues, characterization of these amino acid transporters at the molecular level and biochemical analysis in purified vacuoles are required.

Acknowledgments

We thank Ms. Mai Nakase for technical assistance, and Dr. Taro Nakamura for providing *S. pombe* plasmid. Part of this work was performed at the Venture Business Laboratory of Ehime University and it was supported in part by a Grant-in-Aid for Scientific Research (to Y.K.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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