Multiple polyamine transport systems on the vacuolar membrane in yeast

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We recently identified a gene (*TPO1*, *YLL028w*) that encodes a polyamine transport protein on the vacuolar membrane in yeast [Tomitori, Kashiwagi, Sakata, Kakinuma and Igarashi (1999) *J. Biol. Chem.* **274**, 3265–3267]. Because the existence of one or more other genes for a polyamine transport protein on the vacuolar membrane was expected, we searched sequence databases for homologues of the protein encoded by *TPO1*. Membrane proteins encoded by the open reading frames *YGR138c* (*TPO2*), *YPR156c* (*TPO3*) and *YOR273c* (*TPO4*) were postulated to be polyamine transporters and, indeed, were subsequently shown to be polyamine transport proteins on the vacuolar membrane. Cells overexpressing these genes were resistant to polyamine toxicity and showed an increase in polyamine uptake activity and polyamine content in vacuoles. Furthermore, cells in which these genes were disrupted showed an increased sensitivity

to polyamine toxicity and a decrease in polyamine uptake activity and polyamine content in vacuoles. Resistance to polyamine toxicity in cells overexpressing the genes was overcome by bafilomycin A₁, an inhibitor of the vacuolar H⁺-ATPase. Among the four polyamine transporters, those encoded by *TPO2* and *TPO3* were specific for spermine, whereas those encoded by *TPO1* and *TPO4* recognized spermidine and spermine. These results suggest that polyamine content in the cytoplasm of yeast is elaborately regulated by several polyamine transport systems in vacuoles. Furthermore, it was shown that Glu-207, Glu-324 (or Glu-323) and Glu-574 of *TPO1* protein were important for the transport activity.

Key words: spermidine, spermine, vacuoles.

INTRODUCTION

The polyamine content of cells, which is important in cell proliferation and differentiation [1,2], is regulated by biosynthesis, degradation and transport. In *Escherichia coli*, three clones encoding polyamine transport genes (pPT104, pPT79 and pPT71) have been characterized [3]. The system encoded by pPT104 is a spermidine-preferential uptake system; that encoded by pPT79 is a putrescine-specific uptake system. These two systems are ABC (ATP-binding cassette) transporters [4,5], each consisting of four kinds of protein: the pPT104 clone encodes PotA, PotB, PotC and PotD proteins, and the pPT79 clone encodes PotF, PotG, PotH and PotI proteins [6]. In contrast, the putrescine transport system encoded by pPT71 consists of one membrane protein (PotE) with 12 transmembrane segments and is active in the excretion of putrescine from cells through putrescine–ornithine antiporter activity [7–9].

Although multiple polyamine transporters have been identified and extensively characterized in *E. coli*, the properties of polyamine transporters in other systems, including eukaryotic cells, are still poorly understood. We recently reported two serine/threonine protein kinases (PTK1, PTK2) involved in regulating polyamine uptake in yeast [10,11]. Independently, the same kinases were isolated by Poulin and co-workers (PTK1 = STK1, PTK2 = STK2) and an additional serine/threonine kinase essential for reactivation of several permeases was shown to be necessary for the activation of spermidine uptake [12,13]. So far, a gene for a plasma membrane polyamine transporter protein has not yet been isolated from *Saccharomyces cerevisiae* or other eukaryotic cells.

The *Bacillus subtilis* multidrug transporter Blt [14] is responsible for the excretion of spermidine. We identified a yeast gene, *TPO1*, that encodes a protein exhibiting sequence similarity to Blt, which is responsible for the transport of polyamines into

the vacuoles [15]. Overexpression of *TPO1* resulted in increased resistance to polyamine toxicity, whereas the gene-disrupted mutant showed increased sensitivity. *TPO1* possesses 12 potential membrane-spanning helices and belongs to a large group of yeast transporter genes known as the major facilitator superfamily (MFS) [16]. We identified a number of yeast MFS genes that exhibit significant sequence similarity to the *TPO1* protein. Here we report the characterization of these genes with regard to polyamine transport and identify three more genes encoding proteins that confer resistance to polyamine toxicity and that contribute to vacuolar polyamine transport. We also report that three acidic amino acids in *TPO1*, located at positions similar to those of critical acidic residues in PotE [9], are involved in polyamine transport activity.

MATERIALS AND METHODS

Yeast strains and culture conditions

Yeast strain YW5-1B (*MATa*, *trp1*, *ura3-52*, *leu2-3*, *112*) and its polyamine transport-deficient mutant YTM22-8 [10] were grown aerobically at 30 °C in Mg²⁺-limited CSD (completely synthetic medium) supplemented with 0.05 mM MgSO₄ as described previously [17]. Genes to be tested were amplified by PCR by using primers with appropriate restriction sites. After digestion with appropriate restriction enzymes, the PCR products were inserted into the same restriction sites of plasmid YEp351 [18]. The plasmids thus obtained were introduced into YW5-1B by the lithium acetate method of Ito et al. [19]. A list of oligonucleotide primers used for PCR has not been included here but is available from K.I. on request. *TPO2*, *TPO3* and *TPO4* gene-disrupted mutants of yeast strain YPH499 (*MATa*, *ade2-101*, *his3-Δ200*, *leu2-801*, *trp1-Δ63*, *ura3-52*) were generated by one-step gene

Abbreviations used: MFS, major facilitator superfamily; MGBG, methylglyoxal bis(guanyldihydrazone).

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Figure 1 Phylogenetic tree of MFS genes from *S. cerevisiae* exhibiting amino acid sequence similarity to TPO1

The phylogenetic tree was calculated as described in the Materials and methods section.

disruption [20] with the gene::HIS3 PCR products. The *TPO2::HIS3*, *TPO3::HIS3* and *TPO4::HIS3* were constructed by inserting the *NcoI* and *SpeI*, *SpoI*, and *ApaI* fragments of the

HIS3 gene into the same restriction sites of the *TPO2*, *TPO3* and *TPO4* genes respectively. Correct disruption was verified by Southern blot analysis. Yeast strain YPH499 and its gene-disrupted mutants were grown as described above.

Mutagenesis of the *TPO1* gene

Site-directed mutagenesis of the *TPO1* gene for E207Q (containing a glutamine instead of a glutamic residue at position 207), E323Q, E324Q, E574Q and E323Q/E324Q was performed with a QuickChangeTM Site-Directed Mutagenesis Kit (Stratagene). Mutations were confirmed by DNA sequencing with the Seq4 × 4 personal sequencing system (Amersham Pharmacia Biotech). A list of oligonucleotide primers used for mutagenesis has not been included here but is available from K.I. on request.

Polyamine transport assay with intact cells

Yeast cells were harvested during the exponential phase ($D_{540} = 0.5$), washed twice with 5 ml of 20 mM Na/Hepes buffer, pH 7.2, containing 10 mM glucose, then resuspended at 2 mg dry weight/ml in the same buffer and incubated at 30 °C. When wild-type cells were cultured in the presence of 0.3 mM spermine, 5-fold more volume was used and cultured for 40 h and the cells were harvested at $D_{540} = 0.1$. The reaction was started by the addition of [¹⁴C]spermine or [¹⁴C]spermidine (37 MBq/mmol) at a final concentration of 100 μ M; 0.5 ml aliquots were filtered

Figure 2 Effect of various MFS genes on cell growth in the presence of 0.3 mM spermine

Yeast strain YW5-1B transformed with YEp351 (○) or YEp351 containing an MFS gene (●) was cultured in the presence of 0.3 mM spermine. The MFS genes tested are shown in each panel. Cell growth was followed by measuring D_{540} .

Figure 3 Effect of MGBG and Co^{2+} on cell growth of wild-type yeast and MFS gene-transformed yeast

Yeast cells were cultured in the presence of 1 mM MGBG (A) or 0.05 mM CoCl_2 (B). Symbols: $\circ$, wild-type strain; $\blacksquare$, *TPO1* gene-transformed cells; $\bullet$, *TPO2* gene-transformed cells; $\blacklozenge$, *TPO3* gene-transformed cells; $\blacktriangle$, *TPO4* gene-transformed cells. Cell growth was followed by measuring D_{540} .

through cellulose acetate filters (pore size $0.45 \mu\text{m}$) at intervals (20, 40 and 60 min). The reaction was linear during incubation. The radioactivity trapped on the filters was counted in a liquid-scintillation counter.

Measurement of polyamine contents in whole cells, cytosol and vacuoles

Subcellular fractionation into cytosol and vacuoles was performed by the method of Joho et al. [21]. Polyamines in whole cells were extracted by the treatment of cells with 10% (w/v) trichloroacetic acid at 65°C for 1 h. Polyamine contents were

determined by HPLC as described previously [22]. Protein was determined by the method of Lowry et al. [23].

Northern blot analysis of *TPO1*, *TPO2*, *TPO3* and *TPO4* mRNA species

Yeast cells were harvested during the exponential phase ($D_{540} = 0.5$), washed with a buffer containing 10 mM Tris/HCl buffer, pH 8.0, and 1 M sorbitol, then resuspended in the same buffer at 5×10^8 cells/ml. When wild-type cells were cultured in the presence of 0.3 mM spermine, the cells were harvested at $D_{540} = 0.1$ as described above. After incubation with $40 \mu\text{g}/\text{ml}$ zymolyase (Funakoshi, Tokyo, Japan) at 30°C for 60 min, RNA was extracted with an RNAqueous-MiDi Kit (Ambion). Northern blot analysis was performed as described previously [24] with $20 \mu\text{g}$ of total RNA and ^{32}P -labelled probes. PCR products of *TPO1*, *TPO2*, *TPO3* and *TPO4* were labelled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ with BcaBEST[®] Labelling Kit (TaKaRa, Japan) and used as ^{32}P -labelled probes.

Amino acid sequence analysis

The phylogenetic tree was calculated from profile-derived multiple alignments by the CLUSTALW V1.74 program [25] with the use of the neighbour-joining algorithm [26].

RESULTS

Identification of open reading frames of the *S. cerevisiae* genome exhibiting significant sequence similarity to the *TPO1* protein

The *TPO1* protein belongs to the MFS group of drug transporters, primarily studied in prokaryotes [16]. Figure 1 shows a phylogenetic tree of MFS genes from *S. cerevisiae* exhibiting amino acid sequence similarity to *TPO1*. Two of the encoded proteins, *YML116w* and *YKR105c* are more divergent than the others and constitute a separate family (family 2). The other genes, *YOR273c*, *YHR048w*, *YBR008c*, *TPO1* (*YLL028w*), *YGR138c*, *YPR156c* and *YBR180w*, are more closely related and constitute family 1. Each of the encoded proteins of family 1 possesses 12 putative membrane-spanning segments.

Figure 4 Northern blot analysis of *TPO* genes (A), and spermine uptake activity (B)

Cells were cultured in the presence of 0.3 mM spermine. (A) *TPO1*, *TPO2*, *TPO3* and *TPO4* mRNA species of the wild-type strain and *TPO*-gene transformed cells were compared by Northern blot analysis. (B) Spermine uptake was measured with the wild-type strain and *TPO*-gene transformed cells; 100% spermine uptake activity was $65 \text{ pmol}/\text{min}$ per mg dry weight. Values are means $\pm$ S.D. for triplicate experiments.

Figure 5 Effect of bafilomycin A_1 on cell growth cultured with spermine

(A) Wild-type strain; (B) polyamine transport-deficient mutant; (C–F) *TPO* gene-transformed cells, as indicated. Symbols: ○, cells cultured without spermine; ●, cells cultured with 0.3 mM spermine; □, cells cultured with 0.3 mM spermine and 50 μ M bafilomycin A_1 . Cell growth was followed by measuring D_{540} .

Effect of MFS gene overexpression on growth in the presence of spermine

We have shown previously [17] that yeast cell growth was inhibited by polyamines in Mg^{2+} -limited (0.05 mM Mg^{2+}) medium. Resistance to polyamines can be achieved by transporting polyamines into the vacuoles, where they are effectively sequestered from the cellular machinery, as occurred when *TPO1* was overexpressed [15]. Thus we tested the other genes related to *TPO1* (*YLL028w*) to determine whether they could also produce resistance to spermine (Figure 2). Overexpression of the *TPO1*-like MFS genes in yeast indicated that *TPO1* (*YLL028w*), *YGR138c*, *YPR156c* and *YOR273c* conferred resistance to

0.3 mM spermine (Figures 2A–2D); the last three genes were subsequently renamed *TPO2*, *TPO3* and *TPO4* respectively. No significant resistance to spermine toxicity was found after overexpression of *YML116w*, *YBR008c*, *YOR273c*, *YBR180w* and *YKR105c* (Figures 2E–2I) and it is presumed that the products of these genes are not involved in polyamine transport.

We next examined the substrate specificity of the membrane proteins encoded by *TPO1*, *TPO2*, *TPO3* and *TPO4*. Methylglyoxal bis(guanylhydrazone) (MGBG) is a toxic polyamine analogue that is taken up by *TPO1* [15]. We therefore studied the effects of overexpression of the four *TPO* genes on MGBG toxicity. As a control we studied their effects on the toxicity of Co^{2+} , an inorganic bivalent cation that does not affect polyamine transport. Overexpression of each *TPO* gene resulted in growth resistance to 1 mM MGBG (Figure 3A). Overexpression of the non-*TPO* genes *YML116w*, *YBR008c*, *YHR048w*, *YBR180w* and *YKR105c* had no effect on MGBG toxicity (results not shown). None of the four *TPO* genes was able to confer any growth resistance to 0.05 mM $CoCl_2$ (Figure 3B). These results indicate that *TPO1*, *TPO2*, *TPO3* and *TPO4* are polyamine transport proteins and are able to recognize the polyamine analogue MGBG.

Spermine uptake activities in cells overexpressing *TPO* genes

Wild-type cells and those overexpressing *TPO* genes were cultured in the presence of 0.3 mM spermine; the spermine uptake activity of cells overexpressing each *TPO* gene was measured. In these experiments we confirmed the increase in the amount of *TPO1*, *TPO2*, *TPO3* and *TPO4* mRNA species by Northern blot analysis (Figure 4A). The four *TPO* mRNA species were strongly induced when cells were cultured with spermine, in comparison with the mRNA species in cells cultured without spermine (results not shown). In parallel, the spermine uptake activity of cells overexpressing *TPO* genes was increased approx. 3-fold (Figure 4B). These results are in accordance with the interpretation that spermine accumulated in the vacuoles of the cells overexpressing *TPO* genes.

To confirm that the proteins encoded by *TPO1*, *TPO2*, *TPO3* and *TPO4* are vacuolar membrane proteins, we studied the effects of bafilomycin A_1 , an inhibitor of vacuolar H^+ -ATPase [27], on cell growth. It is known that bafilomycin A_1 inhibits polyamine uptake of vacuolar membrane vesicles [28]. As shown in Figures 5(A) and 5(B), bafilomycin A_1 did not influence the cell growth of the wild-type strain or that of the polyamine-transport-deficient mutant YTM22-8 in the presence of 0.3 mM spermine. However, the attenuation of spermine toxicity by *TPO1*, *TPO2*, *TPO3* and *TPO4* was abolished by bafilomycin A_1 (Figures 5C–5F).

Sensitivity to spermine of *TPO* gene-disrupted mutants

Yeast strains were produced in which each of the four *TPO* genes was disrupted and growth of the mutants was examined. In the absence of added polyamines, the initial growth rate of the *TPO1* mutant was lower than that of the *TPO2*, *TPO3* and *TPO4* mutants and the wild-type strain but the cell growth of the *TPO1* mutant finally reached the same level as those of the other mutants (Figure 6A). The rate of cell growth of the wild-type strain was decreased by 0.2 mM spermine to approximately one-third but cells could grow significantly (Figure 6B). The presence of 0.2 mM spermine caused a severe decrease in overall growth for each *TPO* mutant in comparison with the wild-type strain (Figure 6B). After 40 h, growth of the *TPO* mutants was 5–20 % of that of the wild-type strain, indicating clearly that the increased sensitivity to 0.2 mM spermine of each of the gene-

Figure 6 Effect of spermine and spermidine on cell growth of *TPO* gene-disrupted cells

Cells were cultured in the absence of spermine or spermidine (**A**) and in the presence of 0.2 mM spermine (**B**) or 2.5 mM spermidine (**C**). Symbols: ○, wild-type strain; ■, *TPO1* gene-disrupted cells; ●, *TPO2* gene-disrupted cells; ◆, *TPO3* gene-disrupted cells; ▲, *TPO4* gene-disrupted cells. Cell growth was followed by measuring D_{540} .

Table 1 Spermine and spermidine uptake activities of *TPO* gene-disrupted cells

Disrupted genes are indicated by Δ . Cells were cultured in the absence of polyamines. Results are means $\pm$ S.D. for triplicate experiments.

Strain	Uptake activity (pmol/min per mg dry weight)	
	Spermine	Spermidine
Wild-type	150 $\pm$ 3.4	110 $\pm$ 3.8
$\Delta TPO1$	76.7 $\pm$ 1.3	53.2 $\pm$ 1.7
$\Delta TPO2$	111 $\pm$ 1.8	113 $\pm$ 4.1
$\Delta TPO3$	108 $\pm$ 1.5	112 $\pm$ 4.7
$\Delta TPO4$	124 $\pm$ 2.1	86.3 $\pm$ 2.9

disrupted mutants might have been due to the decreased transport of polyamines to the vacuoles whereby toxicity of the polyamines was attenuated. However, cell growth of the *TPO4* mutant was recovered significantly at 60 h, suggesting that spermine transport activity of *TPO4* might have been weak.

Spermidine toxicity was then examined (Figure 6C). Although 2.5 mM spermidine caused a severe decrease in overall growth for the *TPO1* and *TPO4* mutants, cell growth of the *TPO2* and *TPO3* mutants finally reached the same level as that of the wild-type strain. These results suggest that the transporters encoded by *TPO2* and *TPO3* recognize spermine specifically among polyamines.

Polyamine uptake activities of *TPO* gene-disrupted mutants

The spermine uptake activity of each mutant with a disrupted *TPO* gene was determined in intact cells (Table 1). Because there were four polyamine transport proteins on vacuoles, the effect of disruption of each of the *TPO* genes was small. The greatest decrease was seen with the *TPO1* mutant, in which spermine uptake was decreased by 50 %, whereas for the *TPO2* and *TPO3* mutants the uptake of spermine was decreased by approx. 25 % and that for *TPO4* by 15 %. The smallest decrease seen with *TPO4* was parallel with the smallest toxicity of spermine on cell growth with *TPO4* out of the four *TPO* gene-disrupted mutants (see Figure 6B).

In contrast with effects on spermine, the uptake of spermidine was decreased in the *TPO1* and *TPO4* mutants but not in the *TPO2* and *TPO3* mutants (Table 1). These results indicate that *TPO1* and *TPO4* recognize both spermidine and spermine but that *TPO2* and *TPO3* recognize only spermine.

Spermine contents in whole cells, cytosol and vacuoles

To make sure that proteins encoded by *TPO1*, *TPO2*, *TPO3* and *TPO4* were vacuolar membrane proteins, spermine content in whole cells, cytosol and vacuoles was measured. As shown in Table 2, an increase in spermine content in whole cells was

Table 2 Spermine content in whole cells, cytosol and vacuoles

Cells were cultured for 60 h in the presence of 0.3 or 0.2 mM spermine. Fractionation into cytosol and vacuoles was performed by the method of Joho et al. [21]. Spermine content was measured with wild-type strain, with *TPO* gene-transformed cells and with *TPO* gene-disrupted cells and is expressed as nmol/ 10^8 cells. Results are means $\pm$ S.D. for triplicate experiments.

Strain	Spermine in medium (mM)	Spermine content (nmol/10 ⁸ cells)		
		Whole cells	Cytosol	Vacuoles
<i>TPO</i> gene-transformed cells				
Vector	0.3	42.3 ± 2.3	17.5 ± 1.5	25.2 ± 2.5
TP01	0.3	83.6 ± 4.8	15.8 ± 1.7	67.1 ± 3.4
TP02	0.3	80.1 ± 6.1	16.2 ± 1.3	63.7 ± 2.9
TP03	0.3	81.9 ± 4.3	16.1 ± 1.8	64.2 ± 5.2
TP04	0.3	79.8 ± 5.2	16.4 ± 2.2	61.5 ± 4.5
<i>TPO</i> gene-disrupted cells				
Wild-type	0.2	40.3 ± 3.9	17.1 ± 1.2	24.0 ± 2.2
Δ <i>TP01</i>	0.2	24.5 ± 1.5	18.7 ± 1.6	7.5 ± 1.0
Δ <i>TP02</i>	0.2	33.8 ± 3.3	18.0 ± 2.0	14.2 ± 1.7
Δ <i>TP03</i>	0.2	31.4 ± 1.9	18.2 ± 1.3	13.1 ± 1.1
Δ <i>TP04</i>	0.2	37.1 ± 4.2	18.1 ± 0.8	19.3 ± 1.9

Figure 7 Alignment of the deduced amino acid sequences of TPO1, TPO2, TPO3 and TPO4

Putative transmembrane segments are indicated by I–XII. Identical and similar residues in TPO1 to TPO4 are shown by boxes. The putative calcineurin recognition sequence is shown by a rectangle; the asterisk shows the putative Thr residue that is phosphorylated by PK1 or PK2. Three glutamate or aspartate residues that might interact with polyamines are indicated by black boxes with white lettering.

observed in cells overexpressing any one of the four *TPO* genes, whereas a decrease in spermine was observed in *TPO* gene-disrupted mutants. The content of spermine in the cytosol was nearly equal among wild-type, *TPO* gene-overexpressing and *TPO* gene-disrupted cells, although the content was slightly lower in *TPO* gene-overexpressing cells and was slightly higher in *TPO* gene-disrupted cells than in wild-type cells. In contrast, spermine content in vacuoles was higher in *TPO* gene-overexpressing cells, and lower in *TPO* gene-disrupted cells, than in wild-type cells. These results clearly indicate that TPO1, TPO2, TPO3 and TPO4 proteins are vacuolar membrane proteins.

Sequence comparisons of the TPO proteins

The amino acid sequences of the four TPO proteins suggest that there are 12 putative membrane-spanning helices in each protein (Figure 7). A long N-terminus is present in each protein and it is noticeable that each sequence is rich in serine and threonine residues, indicating possible regulation by protein kinases. In addition, there is a sequence (Arg-Arg-Xaa-Thr) [29] that is recognized by calcineurin (a Ca^{2+} /calmodulin-dependent protein phosphatase) in TPO1. Relative to the other proteins, TPO4 possesses an insertion in the ninth loop between membrane-spanning regions IX and X, and the C-terminus is approx. 100 residues longer. Three glutamate or aspartate residues (indicated by black boxes with white lettering) that might interact with polyamines are located in positions similar to those of the critical acidic amino acid residues in PotE, a putrescine-excreting protein in *E. coli* [9]. The amino acid sequence similarity of TPO1 to TPO4 was higher than to TPO2 and TPO3. This might explain the difference in the substrate specificities of these polyamine transporters.

Figure 8 Effect of TPO1 gene mutants on cell growth in the presence of spermine

Yeast cells transformed with normal TPO1 and mutant TPO1 genes were cultured in the presence of 0.35 mM spermine. Cell growth was followed by measuring D_{540} . Symbols: ●, normal TPO1; Δ, TPO1 E207Q; ▲, TPO1 E323Q; ■, TPO1 E324Q; □, TPO1 E323Q/E324Q; ◇, TPO1 E574Q; ○, vector (YEp351).

Effect of three glutamic residues [Glu-207, Glu-324 (or Glu-323) and Glu-574] in TPO1 on growth in the presence of spermine

The function of three glutamic residues in TPO1, located in positions similar to those of the critical acidic residues in PotE [9], was examined. As shown in Figure 8, no significant resistance

to spermine toxicity was observed in cells overexpressing *TPO1* E207Q or *TPO1* E574Q. However, the cells overexpressing *TPO1* E324Q produced resistance to spermine. A glutamic residue also exists at position 323. Although the cells overexpressing *TPO1* E323Q produced resistance to spermine, no significant resistance to spermine toxicity was observed in cells overexpressing *TPO1* E323Q/E324Q. The results indicate clearly that Glu-207, Glu-324 (or Glu-323) and Glu-574 are important for spermine transport.

DISCUSSION

Our results indicate that there are multiple polyamine transport systems (TPO1 to TPO4) in the yeast vacuolar membrane. This suggests that the polyamine content in the cytoplasm of yeast is elaborately regulated by these transport systems and that a single polyamine transport system might not be enough to maintain an optimal polyamine content in the cytoplasm if high concentrations of polyamines exist outside cells. The sum of each effect of the gene-disrupted mutants on the uptake of spermine became nearly 100%, suggesting that the four *TPO* genes are responsible for most of the spermine transport requirements of the yeast vacuoles. However, it remains possible that other amines might be the real substrates for some of these transport systems. Further studies are necessary to clarify the significance and biological role of the four polyamine transporters.

In a previous paper [15] we reported that the initial rate of spermine uptake in intact cells did not change significantly in wild-type cells, in cells overexpressing the *TPO1* gene and in the *TPO1* gene-disrupted mutant. Spermine uptake activity was measured in cells cultured without spermine. In the present study, spermine uptake was measured in cells cultured in the presence of 0.3 mM spermine. Under these conditions, TPO mRNA species in cells overexpressing the *TPO* genes were clearly increased and we showed that spermine uptake activity in cells overexpressing polyamine transport genes was increased in parallel with the increase in TPO mRNA species (see Figure 4). The increase in TPO mRNA species was not significant in cells cultured without spermine (results not shown). Although the spermine uptake activity of wild-type cells cultured with spermine was significantly lower than in those cultured without spermine (results not shown), this was probably due to feedback inhibition by spermine accumulated in cells. As for the measurement of spermine uptake activity of gene-disrupted mutants, the accuracy was increased by incubating the reaction mixture for 1 h instead of 20 min because the reaction was linear during incubation. These results show that spermine uptake activities of the vacuolar membrane reflect the activities in cells. Furthermore, it was confirmed that spermine content in vacuoles was increased in cells overexpressing *TPO* genes and was decreased in *TPO* gene-disrupted mutants.

The increase in the quantity of mRNA species in cells overexpressing *TPO* genes was greater than the increase in spermine uptake activities in cells (see Figures 4A and 4B). This might be due to regulation by protein kinases (PTK1 and PTK2) of the activities of polyamine transport. Poulin and co-workers suggested that calcineurin regulates polyamine transport together with protein kinases [12]. There is a sequence (Arg-Arg-Xaa-Thr) in TPO1 that is recognized by calcineurin (see Figure 7); TPO1 mRNA was the most strongly expressed of the four mRNA species encoding polyamine transport proteins in the wild-type strain cultured in the absence of spermine (see Figure 4A).

When wild-type, *TPO* gene-overexpressing and *TPO* gene-disrupted cells were cultured in the presence of spermine, the content of spermine in the cytosol was slightly lower in *TPO*

gene-overexpressing cells and was slightly higher in *TPO* gene-disrupted cells than that in wild-type cells. Experiments are now in progress to clarify whether such a small difference in spermine content in the cytosol is correlated with a significant difference in cell growth in these three kinds of cell.

Our results also indicate that Glu-207, Glu-324 (or Glu-323) and Glu-574, located in positions similar to those of the critical acidic amino acid residues in PotE [9], are important for spermine transport. We recently identified more amino acid residues of PotE involved in putrescine transport and constructed a model of the putrescine recognition site on PotE [30]. In these experiments, levels of all mutated PotE proteins expressed on the plasma membrane were almost the same as that of wild-type PotE. In TPO1, E323Q and E324Q proteins are thought to be correctly folded and targeted because the mutated proteins functioned similarly to wild-type TPO1 protein (Figure 8). Thus we expect that other TPO1 mutants are also correctly folded and targeted. It is of interest to determine whether these three glutamic residues are involved in the recognition of polyamines or have fundamental roles in maintaining the active structure of the transporter.

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