Polyamine Uptake by DUR3 and SAM3 in Saccharomyces cerevisiae

Received for publication, December 4, 2006, and in revised form, January 8, 2007 Published, JBC Papers in Press, January 11, 2007, DOI 10.1074/jbc.M611105200

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It has been reported that GAP1 and AGP2 catalyze the uptake of polyamines together with amino acids in Saccharomyces cerevisiae. We have looked for polyamine-preferential uptake proteins in S. cerevisiae. DUR3 catalyzed the uptake of polyamines together with urea, and SAM3 was found to catalyze the uptake of polyamines together with S-adenosylmethionine, glutamic acid, and lysine. Polyamine uptake was greatly decreased in both DUR3- and SAM3-deficient cells. The $K_m$ values for putrescine and spermidine of DUR3 were 479 and 21.2 $\mu$M, respectively, and those of SAM3 were 433 and 20.7 $\mu$M, respectively. Polyamine stimulation of cell growth of a polyamine requiring strain of S. cerevisiae was activated by phosphorylation of Thr250, Ser251, and Thr684 of polyamine transport protein kinase 2.

Polyamines (putrescine, spermidine, and spermine) in cells, which are essential for cell growth, are regulated by biosynthesis, degradation, and transport (1–4). With regard to polyamine transport, the properties of four polyamine transport systems were characterized in Escherichia coli (5–8). They include spermidine-preferential and putrescine-specific uptake systems as well as PotE (involved in the excretion of putrescine by a putrescine-ornithine antiporter activity) and CadB (involved in the excretion of cadaverine by a cadaverine-lysine antiporter activity). The former two transport systems function at neutral pH (2), whereas the latter two transport systems at acidic pH (9). In Saccharomyces cerevisiae, we identified four genes that encode polyamine excretion proteins TPO1–TPO4, mainly located on the plasma membrane (10–12). We also found that UGA4 (located on vacuoles) can catalyze the uptake of $\gamma$-aminobutyric acid and putrescine (13), and TPO5 (located on Golgi or post-Golgi secretory vesicles) can catalyze the excretion of polyamines (14). Furthermore, we reported that GAP1, located on the plasma membrane, can catalyze the uptake of putrescine and spermidine together with the uptake of amino acids (15). Although it has been reported that AGP2 can selectively catalyze the uptake of spermidine (16), there is also a report that AGP2 functions as an amino acid permease (17). In this study, we looked for proteins that can preferentially catalyze the uptake of polyamines in S. cerevisiae. We found that DUR3 can catalyze the uptake of polyamines together with urea, and SAM3 (which belongs to the family of amino acid polyamine-organocation transporters (18)) can catalyze the uptake of putrescine and spermidine together with glutamic acid, lysine, and S-adenosylmethionine. Our results indicate that DUR3 and SAM3 are strongly involved in polyamine uptake in yeast.

EXPERIMENTAL PROCEDURES

Plasmids—For construction of YEpDUR3, the gene for the DUR3 open reading frame and its upstream region (19) was amplified by PCR from yeast X2180-1A (MATa SUC2 malo 12 CLIP1) genomic DNA as a template using primers DUR3F (5'-CGCGGAATTCCCTAGCCAAGACCAAGGTT-TCT-3') and DUR3R (5'-CGGGGTACCGAGACGAGAT-GCAAAAATG-3'). The resulting DNA fragment was digested with BamH1 and Kpn1 and inserted into the same restriction sites of the plasmid YEp352 (20). Plasmid YCp-DUR3-EGFP, which encodes enhanced green fluorescent protein (EGFP) 2 at the C terminus of the DUR3 open reading frame, was constructed as follows. The gene for DUR3 lacking the termination codon was amplified by PCR using primers DUR3F and DUR3-StopR (5'-CGGGGTACCAATTAT-TTATACACCTTGCCG-3'), digested with BamH1 and Kpn1, and inserted into the same sites of plasmid YEpEGFP (14). The DNA-encoding DUR3-EGFP fusion protein was amplified by PCR using primers DUR3F and BamHI-UGA4-R (5'-CGCGGATCCATCTGGCCATTAACATTCCC-3'), digested with BamH1 and inserted into the same site of the single copy plasmid YCp50 (21). For construction of YEpSAM3, the gene for the SAM3 open reading frame and its upstream region (22) was amplified by PCR from yeast

2 The abbreviations used are: EGFP, enhanced green fluorescent protein; CSD, completely synthetic dextrose; MES, 4-morpholineethanesulfonic acid; DUR3, urea transporter; SAM3, S-adenosylmethionine transporter; PTK2, polyamine transport protein kinase 2.
Polyamine Transport in Yeast

X2180-1A genomic DNA as a template using primers SAM3F (5′-CGCGGATCCCTTGAAGTGAAATATAGCGC-3′) and SAM3R (5′-CGGGGTACCTCGAGAGCGTGTACTTTGTCAT-3′). The resulting DNA fragment was digested with BamHI and KpnI and inserted into the same restriction sites of the plasmid YEp352. Plasmid YEpSAM3-EGFP was constructed as YEpDUR3-EGFP. Plasmid YEpGAP1 was prepared as described previously (15). For the construction of YEpAGP2, the gene for GAP1 was prepared as described previously (15). For the construction of YEpAGP2, the gene for GAP1 was prepared as described previously (15).

restriction sites of the plasmid YEp352. Plasmid YCpSAM3-GAP1 was prepared as described previously (15). For the construction of YEpAGP2, the gene for GAP1 was prepared as described previously (15). For the construction of YEpAGP2, the gene for GAP1 was prepared as described previously (15).

Yeast Strains and Culture Conditions—S. cerevisiae YPH499 (MATa ade2–101 his3–200 leu2–1 lys2–801 trp1–1Δ63 ura3–52)-carrying plasmid was cultured in Mg 2+ -limited CSD medium (24), which contains 50 μM MgSO4 instead of 2 mM at 30 °C. In CSD medium, 20 mg/liter Arg, Met, Tyr, and Ile, 50 mg/liter Phe, 100 mg/liter Glu and Asp, 150 mg/liter Val, 200 mg/liter Thr, and 500 mg/liter Ser were included (24). DUR3 and SAM3 gene-disrupted mutants of YPH499 were generated by one-step gene disruption (25) with the DUR3::hisG-URA3-hisG and SAM3::hisG-URA3-hisG PCR products. The DUR3::hisG-URA3-hisG and SAM3::hisG-URA3-hisG constructs were generated by inserting the DNA fragment encoding the hisG-URA3-hisG gene amplified by PCR from pNK51 (26) using the primer set of HUHF (5′-GGAAGATCTATGTTAGACACACCGCGTTCACTATAAAAAGC-3′) and HUHR (5′-GGAAGATCTTCACCTCATCACTCTTCTCGA-3′) in the BglII site of the DUR3 and SAM3 genes on YEpDUR3 and YEpSAM3. Plasmid pNK51 was kindly supplied by Dr. N. Kleckner, Harvard University. DUR3 and SAM3 double gene-disrupted mutant was generated according to the method of Alani et al. (26). The yeast strain carrying SAM3::hisG-URA3-hisG at the SAM3 locus was cultured in the presence of 0.1% 5-fluoro-orotic acid. Surviving cells showing URA − phenotype were used as the host strain for disruption of the DUR3 gene by the DUR3::hisG-URA3-hisG fragment. Resulting double gene-disrupted mutant was obtained by the selection of a 5-fluoro-orotic acid-resistant mutant, and DUR3 and SAM3 double gene-disrupted cells were obtained as URA − cells. The AGP2 or PTK2 gene-disrupted mutant of YPH499 was similarly generated by one-step gene disruption (25) with

FIGURE 1. Polyamine uptake by DUR3 and SAM3. Putrescine (A) and spermidine (B) uptake was measured as described under “Experimental Procedures” using DUR3- or SAM3-transformed cells and DUR3 and/or SAM3 gene-disrupted cells. Data shown are the mean ± S.E. of triplicate determinations. Left two panels, ○ represents gene-transformed cells (YPH499/YEpDUR3 or YPH499/YEpSAM3), and □ represents control cells (YPH499/YEp352). Right three panels, ○ represents DUR3 and/or SAM3 gene-disrupted YPH499 cells, and □ represents control YPH499 cells.

Figure 2. Effect of 0.5 mM amino acid, urea, and S-adenosylmethionine on putrescine uptake by DUR3 and SAM3. Amino acid mixture (nonpolar: 0.5 mM each of Gly, Ala, Val, Leu, Ile, Met, Pro, Phe, and Trp; polar uncharged: 0.5 mM each of Ser, Thr, Tyr, Asn, Glu, and Cys; polar charged: 0.5 mM each of Lys, Arg, His, Asp, and Glu) or 0.5 mM individual amino acid, S-adenosylmethionine, or urea shown in the figure was added to the reaction mixture, and putrescine uptake of YPH499/YEpDUR3 or YEpSAM3 was measured using 0.5 mM [14C]putrescine as substrate. Activity of YPH499/YEp352 was subtracted from the activity of YPH499/YEpDUR3 or YEpSAM3. The 100% value of putrescine uptake by DUR3 and SAM3 was 0.93 ± 0.03 and 0.61 ± 0.03 nmol/min/mg protein, respectively. Data shown are the mean ± S.E. of triplicate determinations.

### TABLE 1

| Gene | Substrate | $K_{m}$ μM | $V_{max}$ nmol/min/mg protein | Inhibitor | $K_{i}$ μM |
|------|-----------|------------|-------------------------------|-----------|------------|
| DUR3 | Putrescine | 479 ± 80   | 2.27 ± 0.32                  | Spermidine| 23.2 ± 2.5 |
|      | Spermidine| 21.2 ± 4.3 | 0.85 ± 0.13                  | Spermine  | 6.91 ± 1.1 |
|      |           | 433 ± 84   | 1.10 ± 0.25                  | Urea      | 60.3 ± 9.5 |
| SAM3 | Putrescine | 20.7 ± 8.7 | 0.76 ± 0.12                  | Spermidine| 39.1 ± 6.7 |
|      | Spermidine| 3.34 ± 1.3 | 26.2 ± 7.9                  | SAM       | 26.2 ± 7.9 |

*Data shown are the mean ± S.E. of triplicate determinations.

* S-Adenosylmethionine.
the AGP2::HIS3 or PTK2::HIS3 PCR product. AGP2::HIS3 or PTK2::HIS3 was constructed by inserting the XbaI fragment of the HIS3 gene into the same restriction site of the AGP2 or PTK2. The XbaI site was introduced into the AGP2 gene in YEpAGP2 by PCR using primers XbaI-YEpAGP2F (5′-GCTCTAGAGGAAACGTGTGAAGATAC-3′) and XbaI-YEpAGP2R (5′-GCTCTAGATGACCCAGAAGGTTCCAA-3′). The SPE1 gene-disrupted mutant of YPH499, YPH499 Δdur3, YPH499 Δsam3, YPH499 Δdur3 Δsam3, YPH499 Δgap1 (15), and YPH499 Δgap2 were generated by the same method using the SPE1::LEU2 construct. The SPE1::LEU2 construct was amplified by PCR using the primer sets of SPE1F (5′-GCAGCAACACTTCGTATCTTTCCA-3′) and SPE1R (5′-GCAGCAACACTTCGTATCTTTCCA-3′) from genomic DNA of Y363 (MATa ura3–52 his6 leu2Δ his1–52 leu2Δ esp1::LEU2) (27), kindly provided by Dr. H. Tabor, National Institutes of Health. Correct disruption of genes was verified by PCR. As the occasion demanded, 20 mg/liter of adenine sulfate, uracil, His, Leu, Lys, and/or Trp were added to the medium.

The XbaI site was introduced into the AGP2 gene in YEpAGP2 by PCR using primers XbaI-YEpAGP2F (5′-GCTCTAGAGGAAACGTGTGAAGATAC-3′) and XbaI-YEpAGP2R (5′-GCTCTAGATGACCCAGAAGGTTCCAA-3′). The SPE1 gene-disrupted mutant of YPH499, YPH499 Δdur3, YPH499 Δsam3, YPH499 Δdur3 Δsam3, YPH499 Δgap1 (15), and YPH499 Δgap2 were generated by the same method using the SPE1::LEU2 construct. The SPE1::LEU2 construct was amplified by PCR using the primer sets of SPE1F (5′-GCAGCAACACTTCGTATCTTTCCA-3′) and SPE1R (5′-GCAGCAACACTTCGTATCTTTCCA-3′) from genomic DNA of Y363 (MATa ura3–52 his6 leu2Δ his1–52 leu2Δ esp1::LEU2) (27), kindly provided by Dr. H. Tabor, National Institutes of Health. Correct disruption of genes was verified by PCR. As the occasion demanded, 20 mg/liter of adenine sulfate, uracil, His, Leu, Lys, and/or Trp were added to the medium.

Site-directed Mutagenesis of the DUR3 Gene—Site-directed mutagenesis of the DUR3 gene was carried out with the QuickChange site-directed mutagenesis kit (Stratagene).
Mutations were confirmed by DNA sequencing using the CEQ8000 genetic analysis system (Beckman-Coulter). A list of oligonucleotide primers used for mutagenesis has not been included but is available from the authors upon request.

Sequencing of PTK2 Gene—The DNA fragment encoding the PTK2 gene amplified by PCR from the genomic DNA of YTM22-8 cells (28) using primer sets of PTK2F (5′-CAGTTTTTTACAGGTTAACCC-3′) and PTK2R (5′-AAGAGGAATTAAAGAAGTCC-3′) was cloned into pST-Blue-1 cloning vector with the Perfectly Blunt cloning kit (Novagen). The sequence of the PTK2 gene was analyzed by the CEQ8000 genetic analysis system.

Transport Assay—Yeast cells were harvested at the exponential growth phase (A540 = 0.5), washed three times with 20 mM Na/MES buffer, pH 5.0, containing 10 mM glucose, suspended at 0.2 mg protein/ml in the same buffer, and incubated at 30 °C for 5 min. The reaction was started by the addition of labeled substrates. After incubation for 20, 40, or 60 min, a 0.5-ml aliquot of reactions was filtered through a cellulose acetate filter (pore size 0.45 μm Advantec) and washed twice with 2 ml of the buffer described above containing 10-fold concentrations of non-labeled substrates. The radioactivity on the filter was counted in a liquid scintillation counter. When substrate specificity was tested, an amino acid mixture or a single amino acid was added to the reaction mixture at the concentration of 0.5 mM. The concentration and specific activities of substrates were as follows: 0.5 mM [14C]putrescine (37 MBq/mmol, American Radiolabeled Chemicals) and 0.1 mM [14C]spermidine (37 MBq/mmol, GE Healthcare).

Where indicated, the SPE1 gene-disrupted mutant was used instead of YPH499 cells. The mutant was cultured in CSD medium in the absence and presence of 1 mM putrescine, 0.05 mM spermidine, or 0.01 mM spermine and harvested at A540 = 0.5.

Measurement of Polyamine Contents in Whole Cells—YPH499, Δspe1, ΔdurΔspe1, Δsam3Δspe1, Δgap1Δspe1, and Δgap2Δspe1 mutants were grown in the CSD medium in the absence and presence of 0.05 mM putrescine and harvested at A540 = 0.25. Polyamines were extracted by treatment with 10% (w/v) trichloroacetic acid at 70 °C for 1 h. Polyamine contents were determined by high pressure liquid chromatography as described previously (29). Protein was determined by the Bradford method (30).
Northern Blot Analysis of DUR3, SAM3, GAP1, and AGP2 mRNAs—The SPE1 gene-disrupted mutants and YPH499 cells carrying YEpDUR3, YEpSAM3, YEpGAP1, or YEpAGP2 were harvested at the exponential growth phase (A540 = 0.25), washed with a buffer containing 10 mm Tris-HCl (pH 7.5) and 1 m sorbitol, and then resuspended in the same buffer at 5 × 10^6 cells/ml. After incubation with 40 μg/ml zymolyase 20T (Seikagaku Corporation, Tokyo) at 30 °C for 30 min, RNA was extracted from the spheroplast with an RNAqueous MiDi-Kit (Ambion). Northern blot analysis was performed as described previously (31) using the ECL direct nucleic acid labeling and detection system (GE Healthcare) with 20 μg of total RNA. Genes of DUR3, SAM3, GAP1, AGP2, and ACT1 were amplified by PCR using primer sets of DUR3F and DUR3R for DUR3, SAM3F and SAM3R for SAM3, GAP1F and GAP1R for GAP1 (15), AGP2F and AGP2R for AGP2, ACT1-P1 (5′-GGATAAATAGGGGCTTG-3′) and ACT1-P2 (5′-AAGAGATTTCGACGCGGT-3′) for ACT1, respectively, and genomic DNA of X2180-1A as a template. The size of the DUR3, SAM3, GAP1, AGP2, and ACT1 PCR products was 2.9, 2.5, 2.4, 2.0, and 0.9 kbp, respectively. PCR products were used as templates for probes. Chemical luminescense was detected by a LAS-1000 plus luminescent image analyzer (Fuji Film).

Western Blot Analysis—YPH499 and YPH499 Δptk2 cells carrying either YEpDUR3-EGFP or YEp352 were cultured and harvested at A540 = 0.5. Membranes were prepared according to the method of Graschopf et al. (32). Twenty micrograms of protein of the membranes were separated on a 8.5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Immobilon P; Millipore). DUR3-EGFP and phosphoserine and phosphothreonine of DUR3-EGFP were detected by the ECL plus Western blot analysis system (GE Healthcare) using anti-GFP (clone GFP-20; Sigma), anti-phosphoserine (Zymed), anti-phosphothreonine (Zymed), respectively.

Fluorescence Microscopy—DUR3 and SAM3 gene-disrupted YPH499 cells carrying plasmid YCpDUR3-EGFP or YCpSAM3-EGFP were grown to the early logarithmic phase (A540 = 0.25) in CSD medium and mounted on the poly-l-lysine-coated slide glass (Muto-Glass). EGFP fluorescence was observed under an Olympus BX51 microscope equipped with fluorescence optics U-LH100 HGAPO. Images were acquired directly with a cooled CCD camera DP70 (Olympus).

**RESULTS**

Polyamine Uptake by DUR3 and SAM3—We searched for genes encoding a polyamine-preferential transporter among the genes that code for proteins with at least twelve putative transmembrane segments. These included DUR3 and SAM3. As shown in Fig. 1, cells overexpressing DUR3 and SAM3 had a higher uptake of putrescine and spermidine compared with the parent strain. Conversely, cells in which the DUR3 or SAM3 gene was disrupted had lower uptake of putrescine and spermidine than the parent strain. When both the DUR3 and SAM3 genes were disrupted, putrescine and spermidine uptake activities were clearly decreased compared with the parent strain (Fig. 1). These results indicate that DUR3 and SAM3 are involved in polyamine transport, presumably functioning directly as polyamine transporters, although DUR3 and SAM3 have been reported to be an urea and an S-adenosylmethionine transporter, respectively (22, 33).

Kinetic parameters were then determined. As shown in Table 1, the K_m and V_max values of DUR3 for putrescine and spermidine uptake were 479 and 21.2 μM and 2.27 and 0.85 nmol/min/mg protein, respectively. The K_m and V_max values of SAM3 for putrescine and spermidine uptake were 433 and 20.7 μM and 1.10 and 0.76 nmol/min/mg protein, respectively. These values were similar to those reported for GAP1 (15). Urea and spermidine inhibited putrescine uptake by DUR3 with K_i values of 60.3 and 23.2 μM, respectively. S-Adenosylmethionine and spermidine inhibited putrescine uptake by SAM3 with K_i values of 26.3 and 39.1 μM, respectively. The results suggest that urea was less effectively recognized than spermidine by DUR3,
Polyamine Transport in Yeast

Phosphorylation site:
[S or T]XX[R or K]
[S or T]XX[D or E]

B

Cell: YPH499

Pretrescine uptake (% activity of wild type)

Wild Type  T119A  T250A  S251A  T250/S251A  T524A  T489A  T684A  T690A  S718A  T250E  T250E  S251E  T250/S251E  T684E

In

C

Cell: YPH499 Δptk2

Pretrescine uptake (% activity of wild type cells)

Wild type  T250E  S251E  T250/S251E  T684E

D

Western blotting

kDa

83

DUR3-EGFP

83

P-Ser

83

P-Thr
and S-adenosylmethionine was recognized similar to spermidine by SAM3. Spermine uptake activity by DUR3 and SAM3 was low (data not shown), so the correct \( K_m \) value for spermine could not be obtained. However, judging from the \( K_m \) value of spermine for putrescine and spermidine uptake, the \( K_m \) value of DUR3 and SAM3 for spermine is probably between 4 and 10 \( \mu M \).

The effects of amino acids on putrescine transport by DUR3 and SAM3 were studied. As shown in Fig. 2, putrescine transport by DUR3 was not influenced by amino acids each at 0.5 mM. However, putrescine transport by SAM3 was inhibited by five kinds of polar and charged amino acids at 0.5 mM each. Thus, the effects of five individual amino acids on putrescine uptake by SAM3 were tested. It was found that putrescine uptake by SAM3 was inhibited by glutamic acid and to a lesser extent by lysine. The results suggest that DUR3 is a selective polyamine transporter, and SAM3 is a polyamine preferential uptake protein, because urea and S-adenosylmethionine do not normally exist at high levels in the environment.

**Subcellular Localization of DUR3 and SAM3—**Localization of DUR3 and SAM3 was determined by fluorescence microscopy using DUR3-EGFP and SAM3-EGFP fusion proteins. As shown in Fig. 3, strong fluorescence was observed on the plasma membrane with both DUR3-EGFP and SAM3-EGFP. For DUR3, fluorescence was also observed in vacuoles, suggesting that DUR3 may be rapidly degraded. The uptake of putrescine in cells expressing the DUR3-EGFP and SAM3-EGFP constructs was similar to that observed with DUR3 and SAM3 (data not shown).

To confirm that DUR3 and SAM3 exist on the plasma membrane but not on the vacuolar membrane, we next compared the effect of azide, an uncoupler of the respiratory chain in mitochondria, with that of bafilomycin A1, a specific inhibitor of vacuolar \( \text{H}^+ \)-ATPase (34). As shown in Fig. 4, putrescine uptake by DUR3 and SAM3 was strongly inhibited by azide but not by bafilomycin A1. The results confirm that SAM3 and DUR3 are located on the plasma membrane but not on the vacuolar membrane and that polyamine uptake is energy-dependent.

**Repression of DUR3 and SAM3 mRNAs by Polyamines—**It is known that polyamine uptake by *E. coli* (35) and mammalian cells (36) is repressed by intracellular polyamines through the inhibition of mRNA synthesis. In a polyamine-requiring mutant, which is deficient in ornithine decarboxylase (\( \text{spe1} \) mutant), polyamine uptake by DUR3 and SAM3 was also repressed by polyamines (Fig. 5A). Thus, the level of DUR3 and SAM3 mRNAs in cells cultured in the presence or absence of polyamines was measured. As shown in Fig. 5B, overexpression of DUR3 mRNA or SAM3 mRNA was observed in cells carrying YEpDUR3 or YEpSAM3 and the level of DUR3 mRNA was strongly repressed and that of SAM3 mRNA was weakly repressed by polyamines in both cells, whereas levels of GAP1, AGP2, and ACT1 mRNAs were not affected by polyamines. The decrease in putrescine uptake activity due to the accumulation of polyamines in cells was parallel with the decrease in the expression of DUR3 and SAM3 mRNAs (Fig. 5). These results further support the conclusion that DUR3 and SAM3 are indeed polyamine transporters.

**Significance of DUR3, SAM3, GAP1, and AGP2 for Polyamine Transport—**To date, the existence of four polyamine uptake proteins in yeast, DUR3, SAM3, GAP1, and AGP2 has been reported. Thus, the relative importance of each of these four proteins for polyamine uptake was estimated using cells transformed with each gene. Expression of GAP1 mRNA was slightly higher than that of AGP2, DUR3, and SAM3 mRNAs in YPH499 cells (Fig. 5B). However, polyamine uptake was higher in cells transformed with DUR3 or SAM3 than in cells transformed with GAP1 or AGP2 (Table 2). Furthermore, polyamine uptake in cells transformed with GAP1 or AGP2 was greatly decreased when 20 amino acids at 10 \( \mu M \) each were added to the reaction mixture.

**Stimulation of the cell growth of a polyamine-requiring mutant \( \text{spe1} \) YPH499 by putrescine was then tested. As shown in Fig. 6A, stimulation of cell growth by 0.05 mM putrescine was observed in \( \text{spe1} \), \( \Delta \text{spe1} \Delta \text{gap1} \), and \( \Delta \text{spe1} \Delta \text{gap1} \) mutants, but cell growth of \( \Delta \text{spe1} \Delta \text{dur3} \) and \( \Delta \text{spe1} \Delta \text{sam3} \) mutants in the presence of 0.05 mM putrescine was slow. In the \( \Delta \text{spe1} \Delta \text{dur3} \) \( \Delta \text{sam3} \) triple mutant, stimulation of cell growth by putrescine was negligible. Cell growth was parallel with polyamine content in cells (Fig. 6B). These results confirm that DUR3 and SAM3 are major polyamine uptake systems in yeast.

**Regulation of the Polyamine Uptake Activity of DUR3 by PTK2—**We previously reported that the polyamine uptake activity of a mutant YTM22-8, which has low polyamine uptake activity (28), was recovered by transforming the PTK2 gene encoding a Ser/Thr protein kinase (37). Thus, the nucleotide sequence of PTK2 in YTM22-8 was determined. It was found that Gly-735, which is located on a close position of the active site of PTK2 (37), was mutated to Asp (Fig. 7A), and the mutated PTK2 did not recover the polyamine uptake activity of YTM22-8 (Fig. 7B). Thereafter, it was tested whether polyamine uptake activity of DUR3, SAM3, GAP1, and AGP2 was regulated by PTK2. As shown in Fig. 7C, polyamine uptake activity by DUR3 (but not by SAM3, GAP1, and AGP2) was low in the \( \Delta \text{ptk2} \) mutant, indicating that DUR3 activity is regulated by PTK2.

Thereafter, phosphorylation site(s) of DUR3 by PTK2 was determined by replacing seven candidate Ser and Thr residues.
Polyamine Transport in Yeast

It has recently been reported that GAP1 (15) and AGP2 (16) can catalyze the uptake of polyamines together with amino acids in yeast. However, polyamine uptake by these proteins was greatly decreased when amino acids were present in the medium. Thus, we tried to identify polyamine-preferential transporters and found that DUR3 and SAM3 are polyamine-preferential transporters. DUR3 and SAM3 were originally reported as transporters for urea and S-adenosylmethionine (19, 33). However, normally urea and S-adenosylmethionine do not exist at high levels outside cells. Thus, DUR3 and SAM3 normally function as polyamine transporters. This became much clearer with the finding that polyamine stimulation of the cell growth of a polyamine-requiring mutant Δasp1 YPH499 was inhibited by disrupting DUR3 and SAM3 genes but not by GAP1 and AGP2 genes. Furthermore, expression of DUR3 and SAM3 mRNAs was repressed in the presence of polyamines. Thus, accumulation of polyamines by DUR3 and SAM3 in the presence of relatively high concentrations of polyamines was reduced. Such autoregulation of the expression of mRNAs for polyamine transporters was also observed in E. coli and mammalian cells (35, 36). This characteristic is presumably effective in maintaining an optimal concentration of polyamines in cells.

In experiments using cells with disruption of both the DUR3 and SAM3 genes, the total uptake of putrescine could be accounted for by the sum of putrescine uptake by DUR3 and SAM3. However, in the case of spermidine, total uptake could not be explained by the sum of uptake via DUR3 and SAM3. Spermidine uptake was not significantly decreased in the presence of 20 amino acids at 10 μM each, indicating that residual uptake of spermidine was not due to the activity of GAP1 and AGP2. Thus, another, as yet unidentified, spermidine uptake protein probably exists in yeast. Experiments are in progress to identify that transporter.

It is noted that polyamine uptake by DUR3 is regulated by phosphorylation by PTK2. We previously reported that polyamine transport activity is low with the PTK2-deficient mutant YTM22-8 (37). This was because of the inhibition of DUR3 activity. PTK2 enhanced the polyamine uptake activity of DUR3 through phosphorylation of Thr-250, Ser-251, and Thr-684 in DUR3. Similarly, polyamine excretion activity of TPO1 was regulated by phosphorylation of Ser-19, Thr-85, and Ser-342 in TPO1 by protein kinase C, casein kinase 1, and AMP-dependent protein kinases 1 and 2 (12). Thus, it is clear that optimal concentration of polyamines in cells is regulated by phosphorylation with various protein kinases.

The location and activities of polyamine transport proteins thus far identified in yeast are summarized in Fig. 9. Uptake of polyamines is mainly catalyzed by DUR3 and SAM3, and excretion of polyamines is mainly catalyzed by TPO5 on Golgi or post-Golgi secretory vesicles (14) and TPO1 on the plasma membrane (12, 39), because expression of TPO2, TPO3, and TPO4 is low compared with TPO1–5. UGA4 may also be important for accumulation of putrescine in vacuoles when cytoplasmic polyamine levels are high.

Acknowledgments—We thank Dr. K. Williams for help in preparing the manuscript. We also thank Drs. H. Tabor and N. Kleckner for kindly supplying the S. cerevisiae strain Y363 and plasmid pNK51.
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MARCH 9, 2007 • VOLUME 282 • NUMBER 10
JOURNAL OF BIOLOGICAL CHEMISTRY 7741