Excretion of Putrescine and Spermidine by the Protein Encoded by YKL174c (TPO5) in Saccharomyces cerevisiae*

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Ken Tachihara, Takeshi Uemura, Keiko Kashiwagi, and Kazuei Igarashi‡
From the Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8675, Japan

The properties of the protein encoded by YKL174c (TPO5) were studied. It was found that TPO5 excretes putrescine effectively and spermidine less effectively. γ-Aminobutyric acid slightly inhibited the excretion of putrescine, but basic amino acids did not affect excretion, suggesting that TPO5 preferentially recognizes polyamines. Accordingly, yeast cells transformed with the plasmid encoding YKL174c (TPO5) were resistant to toxicity caused by 120 mM putrescine or by 3 mM spermidine, and a mutant with a disrupted YKL174c (TPO5) gene was sensitive to toxicity by 90 mM putrescine. The growth of this mutant was faster than that of the wild-type strain. In parallel, there was an increase in putrescine and spermidine content of the YKL174c (TPO5) mutant compared with wild-type. It is noted that TPO5 functions as a suppressor of cell growth by excreting polyamines. The level of YKL174c (TPO5) mRNA was increased by the addition of polyamines to the medium.

The degree of induction of the mRNA was spermine > spermidine > putrescine. The subcellular localization of TPO5 was determined by immunostaining of hemagglutinin-tagged TPO5, and it was found on Golgi or post-Golgi secretory vesicles. Excretion of putrescine and spermidine by TPO5 was reduced in cells that have mutations in the secretory or endocytic pathways, indicating that both processes are involved in the excretion of polyamines.

EXPERIMENTAL PROCEDURES

Plasmids—For construction of YepYKL174c, the gene for the YKL174c open reading frame and its upstream region was amplified by PCR from yeast X2180-1A (MATa SUC2 ma10 go12 CUP1) genomic DNA as template, using primers HindIII-YKL174cF (5′-CCCAAGCT-TCATGACACACCATTTATATCC-3′) and BamHI-YKL174cR (5′-CCGGGATCCCATCATCACATCGAGAAAGG-3′). The resulting 2.5-kb fragment was digested with HindIII and BamHI, and inserted into the same restriction sites of the plasmid Yep532 (12). For construction of YepYKL174c-HA, which encodes five glycine residues and three copies of Haemophilus influenzae HA3 epitope at the C terminus of YKL174c lacking the termination codon, PCR was performed as described above using primers HindIII-YKL174cF and SalI-YKL174cR (5′-TAAACGGCGCATGACTACTATCATACATCGAGGCGCA-3′). The product was digested with HindIII and SalI and inserted into the same sites of YepYKL174c-HA1, which catalyzes the transport of putrescine into vacuoles (10). UGA4 is classified into the family of amino acid-polyamine-organocation transporters (11). The name “polyamine” of amino acid-polyamine-organocation transporters is originated from polyamine transporter PotE. Among the family, there is only one gene (YKL174c) whose function has not yet been identified. In this study, we tried to clarify properties of the protein encoded by YKL174c. We found that YKL174c excretes putrescine and spermidine from cells and that the protein is located on Golgi or post-Golgi secretory vesicles. Thus, we termed the YKL174c gene as TPO5, which is involved in transport of polyamines.

Polymers, aliphatic cations present in almost all living organisms, are necessary for normal cell growth (1, 2). Intracellular polyamine levels are elaborately regulated by biosynthesis, degradation, and transport (3). With regard to transport, the properties of three polyamine transport systems were characterized in Escherichia coli (4–6). They include spermine-preferential and putrescine-specific uptake systems, which belong to ATP-binding cassette transporters, and a protein, PotE, involved in the excretion of putrescine by a putrescine-ornithine antiporter activity. In Saccharomyces cerevisiae, we identified four genes which encode polyamine transport proteins TPO1–TPO4 (7, 8). Among the four polyamine transporters, those encoded by TPO2 and TPO3 were specific for spermine, whereas those encoded by TPO1 and TPO4 recognized putrescine, spermidine, and spermine. Furthermore, we recently reported that UGA4, which catalyzes the transport of γ-aminobutyric acid (9), is located on vacuoles and also catalyzes the transport of putrescine into vacuoles (10). UGA4 is classified into the family of amino acid-polyamine-organocation transporters (11). The name “polyamine” of amino acid-polyamine-organocation transporters is originated from polyamine transporter PotE. Among the family, there is only one gene (YKL174c) whose function has not yet been identified. In this study, we tried to clarify properties of the protein encoded by YKL174c. We found that YKL174c excretes putrescine and spermidine from cells and that the protein is located on Golgi or post-Golgi secretory vesicles. Thus, we termed the YKL174c gene as TPO5, which is involved in transport of polyamines.
Northern blot analysis was performed using 20 µg of total RNA as described previously (26) using ECL direct nucleic acid labeling and detection systems with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Probes for YKL174c and ACT1 mRNAs were amplified by PCR using HindIII-YKL174cF and BamHI-YKL174cR for YKL174c, ACT1-P1 (5'-GGATTTATGGGCTTGTA-3') and ACT1-P2 (5'-AAAGATTGAGCAGCCTG-3') for ACT1 as primers, and genomic DNA of X2180-1A as a template. Chemical luminescence was detected by a LAS-1000 plus luminescence image analyzer (Fuji Film).

Preparation of Phylogenetic Tree—The phylogenetic tree was calculated from profile-derived multiple alignments by the ClustalW V1.8 program including bootstrapping (27) with the use of the neighboring algorithm (28).

RESULTS

Excretion of Putrescine and Spermidine by YKL174c (TPO5)—Fig. 1 shows a phylogenetic tree of the family of amino acid-polyamine-organocarrier transporters in S. cerevisiae. The phylogenetic tree was calculated as described under “Experimental Procedures.” The accession number of YKL174c and UGA4 was Z28174 and X66472, respectively (the Entrez Nucleotide data base). The function of proteins encoded by these genes can be seen in Saccharomyces genome data base, Stanford genomic resources.

Northern blot analysis was performed using 20 µg of total RNA as described previously (26) using ECL direct nucleic acid labeling and detection systems with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Probes for YKL174c and ACT1 mRNAs were amplified by PCR using HindIII-YKL174cF and BamHI-YKL174cR for YKL174c, ACT1-P1 (5'-GGATTTATGGGCTTGTA-3') and ACT1-P2 (5'-AAAGATTGAGCAGCCTG-3') for ACT1 as primers, and genomic DNA of X2180-1A as a template. Chemical luminescence was detected by a LAS-1000 plus luminescence image analyzer (Fuji Film).

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RESULTS

Excretion of Putrescine and Spermidine by YKL174c (TPO5)—Fig. 1 shows a phylogenetic tree of the family of amino acid-polyamine-organocarrier transporters in S. cerevisiae. Among these proteins, there is one, termed YKL174c, whose function is unknown but which is highly homologous with UGA4, a γ-amino butyric acid and putrescine transport protein on the vacuolar membrane (10). In light of this, we carried out experiments to determine whether YKL174c has polyamine transport activity. We looked for activity using YPH499 cells transformed with YEPYKL174c (or the vector YEp352 for control cells) and cells in which the native YKL174c gene is disrupted (Δykl174c). As shown in Fig. 2, uptake of putrescine and spermidine was lower in YPH499/YEPYKL174c cells than in control YPH499/YEP352 cells. Uptake of spermidine was not affected by transformation with YEPYKL174c. Furthermore, uptake of putrescine was enhanced in Δykl174c cells compared with wild type (Fig. 2B). The results suggest that YKL174c catalyzes the excretion of putrescine and, to a lesser degree, of spermidine. Thus, the YKL174c gene is hereafter termed TPO5. Excretion of putrescine by TPO5 was examined directly by using cells preloaded with [14C]putrescine. As shown in Fig. 3, the excretion of putrescine was enhanced by TPO5; that is, excretion of [14C]putrescine was reduced in wild-type cells than Δykl174c cells. The excretion of paraquat, a polyamine analogue (8), was not influenced by TPO5 (data not shown). The results indicate that TPO5 preferentially excretes putrescine. The increased putrescine uptake seen in cells with a disrupted YKL174c gene (equal to putrescine excretion activity by TPO5) was slightly inhibited by non-labeled γ-amino butyric acid (1 mM), but not by lysine, arginine, histidine, and ornithine (Fig. 4), indicating that TPO5 recognizes polyamines preferentially.

The effects of TPO5 on polyamine toxicity were then measured using cells transformed with YEPYKL174c (TPO5). As
mental Procedures." The level of preloaded \[14C\]putrescine in wild-type (YPH499/YEp352; YPH499; \(\Delta ykl174c\)) cells was 80.4 \pm 5.2 and 130.4 \pm 18.2 n mole/mg protein, respectively. Data are shown as mean \pm S.E. of triplicate determinations.

**FIG. 2.** Inhibition of putrescine and spermidine uptake activity by TP5 protein encoded by \(YKL174c\). Polyamine uptake activities were measured as described under "Experimental Procedures." A. ○, YPH499/YEp352; ●, YPH499/YEpYKL174c. B. ○, YPH499; ●, \(\Delta ykl174c\). PUT, putrescine; SPD, spermidine; SPM, spermine. Data are shown as mean \pm S.E. of triplicate determinations.

**FIG. 3.** Excretion of preloaded \[14C\]putrescine by TP5 protein encoded by \(YKL174c\). The assay for excretion of preloaded \[14C\]putrescine by TP5 was performed as described under "Experimental Procedures." The level of preloaded \[14C\]putrescine in wild-type (○) and \(\Delta ykl174c\) (●) cells was 80.4 \pm 5.2 and 130.4 \pm 18.2 n mole/mg protein, respectively. Data are shown as mean \pm S.E. of triplicate determinations.

**FIG. 4.** Effect of amino acids on putrescine excretion activity by TP5 protein encoded by \(YKL174c\). Putrescine uptake activity of wild-type and \(\Delta ykl174c\) cells was measured in the presence of 0.5 mM \[14C\]putrescine and 1 mM amino acid shown in the figure. Increase in putrescine uptake activity by \(YKL174c\) gene-disrupted cells (equal to putrescine excretion activity by TP5) was shown. 100% activity without non-labeled amino acid was 0.82 \pm 0.13 n mole/min/mg of protein. Data are shown as mean \pm S.E. of triplicate determinations. GABA, \(\gamma\)-aminobutyric acid.

shown in Fig. 5, cells transformed with YEYpTPO5 grew faster than control cells (containing the vector) in the presence of 120 mM putrescine or 3 mM spermidine. In addition, the \(\Delta ykl174c\) mutant showed strong sensitivity to 90 mM putrescine (Fig. 5). According to the finding that spermidine is not effectively effluxed. An effect of polyamines on cell growth was clearly observed, because the time course for cell growth was followed for more than 150 h, whereas polyamine uptake activity was followed for only 60 or 120 min.

It should be noted that the \(\Delta ykl174c\) mutant grows faster than the wild-type strain in the absence of polyamines (Fig. 5B). Such a growth difference was observed even in the presence of 0.3 mM putrescine (Fig. 6A). Under these conditions, cellular polyamine content was measured (Fig. 6B). Levels of polyamines in the \(\Delta ykl174c\) mutant cultured without putrescine increased from 2.97 and 30.4 (wild-type) to 21.5 and 60.0 n mole/mg of protein (mutant) for putrescine and spermidine, respectively. The amount of putrescine and spermidine accumulated in the \(\Delta ykl174c\) mutant corresponds to 1.84 and 5.13 mM, respectively, if the intracellular water space is estimated as 11.7 \(\mu\)l of cell volume/mg of protein (15). Even if 0.3 or 1 mM putrescine was added to the medium, the levels of putrescine and (or 0.3 mM putrescine) spermidine were still higher in the \(\Delta ykl174c\) mutant than in wild-type. These results suggest that wild-type yeast having TP5 gene do not maintain an optimal level of polyamines necessary for cell growth under these conditions.

Subcellular Localization of TP5—Subcellular localization of TP5 was determined by indirect immunofluorescence microscopy using HA-tagged TP5 and an anti-HA antibody. As shown in Fig. 7A, TP5 was located on small vesicles existing in the vicinity of plasma membrane. The vesicles were different from vacuoles, which were observed by differential interference contrast and from nuclei judged by DNA staining with propidium iodide. Accordingly, localization of TP5 with SEC7, a marker of the Golgi complex (20), was examined. As shown in Fig. 7B, most of TP5 was colocalized with SEC7, indicating that TP5 is located on Golgi or post-Golgi secretory vesicles. Subcellular localization of TP5 was then analyzed by sucrose density gradient centrifugation. As shown in Fig. 8, most of TP5-HA, was localized in the low density position similar to Golgi complex, estimated by SEC7, and vacuoles, estimated by VPH1 (a subunit of vacuolar proton ATPase), and very small amount of TP5 was localized in the high density position similar to plasma membrane, estimated by PMA1.
triplicate determinations. Independent experiments were repeated three times and reproducible results were obtained. B, polyamine content at A₅₄₀ = 0.5. Open column, YPH499; closed column, Δyk1174c, PUT, putrescine; SPD, spermidine; SPM, spermine. Data are shown as mean ± S.E. of triplicate determinations.

(plasma membrane ATPase 1). Some portion of SEC7 was also located in the high density portion. This was probably because of the coagulation of SEC7 during the preparation of cell lysate, because SEC7 was not observed on plasma membrane (see Fig. 7). These results are in accordance with an idea that TPO5 is located mainly on Golgi or post-Golgi secretory vesicles.

To confirm the location of TPO5 on Golgi or post-Golgi secretory vesicles, polyamine transport activity was measured using a temperature sensitive mutant deficient in the process of secretion (sec6) (17) and a mutant deficient in endocytosis (Δend4) (14). In the sec6 mutant, fusion of the secretory vesicles with the plasma membrane during the final exocytosis process is inhibited at non-permissive temperatures (17), and exocytosis of a zinc transporter ZRT1 is deficient in Δend4 mutant (14). As shown in Fig. 9A, putrescine and spermidine excretion activity was lower with a sec6 mutant containing YEpYKL174c (TPO5) than with its parent strain containing YEpYKL174c (TPO5) at a non-permissive temperature (37 °C), but the activity of these two strains containing YEpYKL174c (TPO5) was nearly equal at a permissive temperature (23 °C). Excretion activity of putrescine and spermidine was also lower with the endocytosis mutant containing YEpYKL174c (TPO5) than its parent strain containing YEpYKL174c (TPO5) (Fig. 9B). These results indicate that excretion of putrescine and spermidine by TPO5 was not observed in the exocytosis and endocytosis mutants and that both exocytosis of polyamines by TPO5 and endocytosis of TPO5 for recycling are involved in the activity of TPO5.

Induction of TPO5 mRNA by Polyamines—We next tested whether TPO5 mRNA is induced by polyamines. Northern blot analysis was carried out using total RNA isolated from YPH499/YEpYKL174c cultured in the absence and presence of polyamines. As shown in Fig. 10, TPO5 mRNA was most strongly induced by 0.2 mM spermine, even though spermine is not a substrate for TPO5. Spermidine and putrescine also caused induction of TPO5 mRNA. As a control, the level of ACT1 mRNA encoding actin 1 was measured. It was not influenced by polyamines. Because the effective concentration is in the order putrescine > spermidine > spermine in terms of the stimulation of protein synthesis (29), it is thought that induction of TPO5 mRNA depends on the effective polyamine concentration in cells.

We studied the function of a protein encoded by an unidentified gene YKL174c, which has high homology with UGA4, a member of the family of amino acid-polyamine-organocation transporters in S. cerevisiae. We found that YKL174c catalyzes the excretion of putrescine and spermidine, and thus termed this protein as TPO5. The homology of an amino acid sequence between TPO5 and UGA4 was 42%, and the major difference was observed in the C-terminal regions of the two proteins. Thus, the cellular localization of these two proteins (TPO5 is found on Golgi or post-Golgi secretory vesicles, and UGA4 is found on vacuoles) may be determined by the C termini of these proteins.

Our data clearly indicate that TPO5 is mainly located on Golgi or post-Golgi secretory vesicles and that both processes of exocytosis and endocytosis are involved in the secretion of putrescine and spermidine. This is the first report that shows the necessity of these two processes for secretion of small molecules in yeast. In yeast, it has been reported that the process of exocytosis is necessary for the sorting of tryptophan permease to the plasma membrane (17), and also the process of endocytosis is necessary for degradation in vacuoles of the zinc transporter ZRT1 (14), a general amino acid permease (30) and a uracil permease (31) on plasma membrane. However, there is no report thus far stating that both processes are necessary for the function of a specific protein. It has been reported that both exocytosis and endocytosis are necessary for neurotransmitter release from synaptic vesicles in mammalian cells (32). At present, we do not know the exact reason why TPO5 mainly exists on Golgi or post-Golgi secretory vesicles. It may be better for TPO5 to exist on Golgi or post-Golgi secretory vesicles to excrete polyamines more effectively once polyamines are accumulated in secretory vesicles like neurotransmitters accumulated in synaptic vesicles.

Polyamine transporters previously characterized were not specific for polyamines in S. cerevisiae. TPO1 can excrete other substances that are not related to polyamines (33–35), and UGA4 has higher affinity for γ-aminobutyric acid rather than putrescine (10). Thus, TPO5 is the only protein studied to date that recognizes putrescine and spermidine preferentially. Because TPO2 and TPO3 only recognize spermine among polyamines, experiments are in progress to test whether TPO2 and TPO3 specifically recognize spermine. The existence of spermine (or acetyl spermine) oxidase has been reported recently in yeast (36). If the enzyme also catalyzes the conversion of spermidine to putrescine, excess polyamines may be excreted effectively by TPO5.

It is surprising that cells with a disrupted TPO5 gene (Δyk1174c) grow more rapidly than wild-type cells. Under these conditions, the accumulation of putrescine and spermidine in Δyk1174c cells was observed. The results suggest that the existence of TPO5 slows down cell growth through excretion of putrescine. It has been reported that excess spermidine inhibits protein synthesis by binding to ribosomes in E. coli (37). Acetylation of polyamines decreases their ability to stimulate protein synthesis and their toxicity (38). Acetytransferases of polyamines are present in both mammalian cells and E. coli (1). However, the existence of acetytransferase(s) of polyamines has not been reported in S. cerevisiae, although the whole genome sequence was determined. TPO5 together with spermine oxidase (36) may function for detoxification of polyamines in yeast.

When cells were cultured in the presence of high concentrations of putrescine (120 mM) or spermidine (3 mM), it took 50–100 h until reaching the logarithmic phase of cell growth (see Fig. 5). It may take a long time to induce the expression of
the YKL174c (TPO5) gene under these experimental conditions. Induction of TPO5 mRNA was measured in the presence of 50 mM putrescine or 2 mM spermidine (Fig. 10). Under these conditions, the lag time was within 24 h, and the difference in cell growth between cells transformed with YEpYKL174c (TPO5) and YEp352 in the presence of polyamines was not clearly observed (data not shown).

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