Antizyme Regulates the Degradation of Ornithine Decarboxylase in Fission Yeast Schizosaccharomyces pombe

STUDY IN THE spe2 KNOCKOUT STRAINS

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The mechanism of the regulatory degradation of ornithine decarboxylase (ODC) by polyamines was studied in fission yeast, Schizosaccharomyces pombe. To regulate cellular spermidine experimentally, we cloned and disrupted S-adenosylmethionine decarboxylase gene (spe2) in S. pombe. The null mutant of spe2 was devoid of spermidine and spermine, accumulated putrescine, and contained a high level of ODC. Addition of spermidine to the culture medium resulted in rapid decrease in the ODC activity caused by the acceleration of ODC degradation, which was dependent on de novo protein synthesis. A fraction of ODC forming an inactive complex concomitantly increased. The accelerated ODC degradation was prevented either by knockout of antizyme gene or by selective inhibitors of proteasome. Thus, unlike budding yeast, mammalian type antizyme-mediated ODC degradation by proteasome is operating in S. pombe.

Polyamines (putrescine, spermidine, and spermine) are biologically ubiquitous compounds that have been implicated in many aspects of growth and development in a wide range of organisms (1–3), but their precise function is largely unknown. The biosynthesis of polyamines in yeast and most of animals depends on the decarboxylation of ornithine to putrescine by ornithine decarboxylase (ODC), EC 4.1.1.17. Subsequent attachment of an aminopropyl moiety forms spermidine, and the second aminopropyl transfer to spermidine yields spermine. Decarboxylated S-adenosylmethionine serves as the aminopropyl donor, which is produced by S-adenosylmethionine decarboxylase (AdoMetDC, EC 4.1.1.50). Both the decarboxylating enzymes are highly regulated and subject to control by cellular polyamines.

In mammalian cells, ODC is negatively regulated in response to the increase in cellular polyamines mainly through a unique mechanism mediated by a protein termed antizyme (4, 5). Synthesis of antizyme requires translational frameshifting, which occurs at the end of the initiating frame or open reading frame 1 (ORF1) (6, 7). The ribosome shifts its reading frame to the +1 frame and continues to decode the second ORF to synthesize the entire antizyme protein. The second ORF of antizyme encodes all the known functions of the protein (8–10). Polyamines stimulate the frameshifting and thus control the level of antizyme. Antizyme binds to ODC monomer preventing formation of the homodimeric active enzyme. The ODC associated with antizyme is rapidly degraded by the 26 S proteasome without ubiquitination (11–13). In addition, antizyme negatively regulates the polyamine transporter (14, 15). Mammalian cells contain another regulatory protein, antizyme inhibitor, a homolog of ODC without the decarboxylating activity (16). It binds to antizyme with a higher affinity than ODC and releases active ODC from the inactive antizyme-ODC heterodimer.

At least in two lower eukaryotes, polyamine-induced destabilization of ODC has been studied in detail. In Saccharomyces cerevisiae, spermidine-induced ODC destabilization seems to be dependent on protein synthesis (17, 18), and the 26 S proteasome is responsible for the proteolysis of ODC (18, 19). However, computer search of an antizyme homolog in the completed genome of S. cerevisiae has been negative (20). In a filamentous fungus, Neurospora crassa, spermidine and putrescine accelerate the turnover of ODC protein (21). Recently, a presumed antizyme homolog has been noted in the N. crassa genome (AF291578, MIPS Neurospora data base).

Although S. cerevisiae and the fission yeast, Schizosaccharomyces pombe, are both ascomycetous fungi, they are almost as divergent each other as between these and mammals (22). S. pombe is often more similar to mammalian cells than S. cerevisiae in various aspects. Interestingly, the frameshift signal of mammalian antizyme directs the correct +1 shift in S. pombe, but aberrant −2 shift in S. cerevisiae (23, 24). Very recently, Ivanov et al. (25) identified an antizyme gene in S. pombe demonstrating its frameshift induction by polyamines, involvement in the cellular polyamine control, and inhibitory activity on ODC. Deletion of antizyme gene (SPA) from the fission yeast genome did not bring about apparent phenotypes, but resulted in the increase in putrescine, spermidine, and cadaverine, which was more prominently observed in the cells at stationary phase than in exponentially growing cells, whereas overexpression of SPA led to large reduction in the cellular level of polyamines (25). A role for antizyme in ODC degradation in S. pombe cells, however, was not demonstrated.

In the present paper, we studied the mechanism of ODC repression by spermidine in S. pombe. In order to be able to...
regulate cellular spermidine experimentally, we cloned and disrupted *S. pombe* gene for AdoMetDC (spe2). We show that the null mutant of spe2 (Δspe2) is devoid of spermidine and spermine and contains a high ODC activity, which is rapidly repressed in response to addition of spermidine through antizyme-dependent ODC degradation catalyzed by proteasome. To our knowledge, this is the first experimental evidence of antizyme being involved in the regulation of ODC degradation in single-celled eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Materials—**Proteasome inhibitors, clasto-lactacystin β-lactone and MG132 (Z-LIIL-CHO), were purchased from Boston Biochem (Cambridge, MA) and Peptide Institute (Osaka, Japan), respectively. Restriction enzymes were obtained from New England Biolabs. Oligodeoxynucleotides were from Amersham Pharmacia Biotech. α-Deoxyfluoromethylornithine (DFMO) was kindly provided by Merrell Dow Research Institute (Cincinnati, OH). Media components were from Difco. Other chemicals and reagents were obtained from Sigma unless otherwise mentioned.

**Yeast Strains and Culture—** *S. pombe* strain JY745 (ura4-D18 leu1-32 ade6-M210 his1-200 trp1-289) was a kind gift from Dr. M. Yamamoto. An anti-sense (5′-TTTGGAGCNYNGAARYTYNTYG-3′) and anti-sense (5′-GTNTNCACCRRGNTNWDRAT-3′), where R = A or G; Y = T or C; W = A or T; D = A, G, or T; H = A, C, or T; N = A, G, C, or T, were designed based on multiple alignment of AdoMetDC from several eukaryotic organisms on the data base and used to amplify a fragment of spe2 gene from *S. pombe* genomic DNA with PCR. The product was labeled with [α-32P]dCTP (3,000 Ci/mmol, PerkinElmer Life Sciences) using Random Primer DNA Labeling kit (Takara). An *S. pombe* cDNA library in pCD2 vector (a gift from Dr. H. Nojima) was screened with the standard colony hybridization protocol (27). Positive clones were isolated, subcloned in the BamHI site of pBluescript SK (−) (Stratagene), and sequenced using Prism cycle sequencing kit (ABI) with T3 and T7 primers. Additional primers were designed to sequence the entire cDNA in both directions. The cDNA was then used in screening an *S. pombe* genomic DNA library constructed in pBluescript KS (−) vector (a gift from Dr. H. Nojima). One of the positive clones (R7A, 5.2 kb) was digested with EcoRI, subcloned into pBluescript SK (−), and sequenced as above. Sequence comparison was carried out with GENE- TSY-MAC program (Software Development).

**Disruption of spe2—**One-step gene replacement was performed to disrupt spe2 as follows (Fig. 1B). *S. pombe* ura4 gene was taken from pREP2 (28) as a HindIII fragment and cloned into pBluescript SK (−) that had been modified by reversing the positions of the EcoRI and PstI sites with an oligonucleotide pair. The 3′-flanking region of spe2 (453 bp) was then inserted downstream of the *ura4* gene at the *PstI*-EcoRI sites. The 5′-flanking region (840 bp) was then subcloned into pBluescript SK (−) at the EcoRI site, and then *KpnI* (on the vector)-BamHI fragment (containing the 740-bp EcoRI-BamHI fragment) was ligated upstream of *ura4* at the *KpnI*-HindIII sites of the earlier construct. In each step, subclones with the proper orientation were selected with restriction analysis and confirmed by sequencing. Thus, in the final construct, almost the entire coding region of spe2 was replaced by *ura4*. The construct was digested with EcoRI followed by gel purification and used to transform JY745 or Δspe strains with modified lithium acetate method (29). The stable URA′ transformants were selected. Homologous recombination (spe2::ura4) was confirmed first by colony PCR, and then by Southern analysis of genomic DNA digested with HindIII using the standard techniques (27). The PstI-EcoRI fragment of *spe2* genome at the 5′ vicinity of the disruption construct (1.0 kb) was used as a probe.

**Enzyme and Polysyme Assays—**For AdoMetDC assay, cells were grown as described above and harvested at an *A*~600~ of 1.0. Cells from 2 ml of culture were collected with centrifugation at 10,000 × g, 4 °C for 10 min, and were suspended in 0.3 ml of the extraction buffer containing 50 mM Tris-HCl (pH 7.2), 1 mM DTT, 0.1 mM EDTA, 0.3 mg/ml bovine serum albumin, 0.1 mM putrescine, and 8 μM S-adenosyl-l-cysteine (Sigma, Adolph, at a dilution of 1:10,000 essentially following the method of Mierendorff et al. (31). To measure the polyamines, *S. pombe* cells were collected from 2 ml of the culture with centrifugation at 1,000 × g, 4 °C for 10 min and washed twice with phosphate-buffered saline (20 mM sodium phosphate, 140 mM NaCl, pH 7.4). Each pellet was resuspended in 0.2 ml of phosphate-buffered saline, and a part (50 μl) was mixed with the same volume of 8% perchloric acid. The mixture was vortexed, kept on ice for 5 min, and centrifuged at 12,000 × g, 4 °C for 5 min. The supernatant (10 μl) was subjected to polyamine analysis on high performance liquid chromatography and fluorometry as described (31).

To our knowledge, this is the first experimental evidence of antizyme being involved in the regulation of ODC degradation in single-celled eukaryotes.

**ODC activity was measured using l-[14C]ornithine as a substrate essentially as described (30). The cells were harvested at the indicated time and disrupted as above in extraction buffer containing 50 mM Tris-HCl (pH 7.5) and 1 mM DTT. One unit of ODC activity is defined as the amount releasing 1 nmol of CO2 from ornithine/h at 37 °C. To measure the polyamines, *S. pombe* cells were collected from 2 ml of the culture with centrifugation at 1,000 × g, 4 °C for 10 min and washed twice with phosphate-buffered saline (20 mM sodium phosphate, 140 mM NaCl, pH 7.4). Each pellet was resuspended in 0.2 ml of phosphate-buffered saline, and a part (50 μl) was mixed with the same volume of 8% perchloric acid. The mixture was vortexed, kept on ice for 5 min, and centrifuged at 12,000 × g, 4 °C for 5 min. The supernatant (10 μl) was subjected to polyamine analysis on high performance liquid chromatography and fluorometry as described (31).

**Production of Antibody against ODC and Western Blotting—** E. coli BL21 carrying pGEX-SPO was grown in the presence of 0.5 mM isopropl-1-thio-b-galactopyranoside at 18 °C overnight to induce glutathione S-transferase (GST)-ODC fusion protein. The expression product was purified on a glutathione-Sepharose 4B-affinity column (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Rabbits were injected with 0.5 mg of the protein mixed with Freund’s complete adjuvant. Two booster doses were given with the same amount of protein using Freund’s incomplete adjuvant 1 and 2 months later. The antisera were taken 7 days after the second booster and absorbed by an extract of ODC (spe1) gene-disrupted *S. pombe* cells. The same *S. pombe* extracts (20 μg) that were used for ODC assay were fractionated on 10% SDS-PAGE. Immunoblotting was performed according to a standard protocol using Immobilon-P membrane (Millipore). Immunodetection was carried out with the primary antibody (one of the antisera) against GST-ODC at a dilution of 1:5,000 and secondary antibody, alkaline-phosphatase conjugated anti-rabbit IgG (chain-specific, Sigma), at a dilution of 1:10,000 essentially following the method of Mierendorff et al. (33).

**Measurement of Inactive ODC Complex—**The untagged ODC was induced by 0.3 mM isopropyl-1-thio-b-galactopyranoside in BL21 (DE3) carrying pET3a-SPO and purified from the extract by DEAE- Cellulofine (Seikagaku Kogyo) chromatography as described previously (30). The partially purified ODC (5,500 units) was incubated with 2 mM DFMO, in a mixture containing 10 μM pyridoxal phosphate, 5 mM DTT, 40 mM Tris-HCl (pH 7.4), and 0.01% Tween 80 in a total volume of 100 μl at 37 °C for 2 h. The residual free DFMO was removed by gel filtration through a NAP 5 column (Amersham Pharmacia Biotech).

**2 M. K. Chattopadhyay, K. Mita, and S. Matsufuji, unpublished data.**
The resultant preparation did not contain detectable level of ODC activity. For each ODC assay, 15 units of DFMO-ODC (as initially determined) was mixed and kept on ice for 30 min before enzyme reaction. The amount of ODC complex was calculated from the gain of ODC activity. For each ODC assay, 15 units of DFMO-ODC (as initially determined) was mixed and kept on ice for 30 min before enzyme reaction. The amount of ODC complex was calculated from the gain of ODC activity.

**RESULTS**

**Cloning of S. pombe spe2 Gene**—A region of spe2 containing the proenzyme cleavage site was amplified with degenerated PCR. The product (249 bp) was used in screening of an S. pombe cDNA library as the probe. Several clones with the identical cDNA insert of 1,421 bp were isolated. The cDNA contains a small upstream ORF (38). An ORF that encodes a tetrapeptide, MTIF, located closely to the main ORF with only one nucleotide gap (Fig. 1A). There is no leader amino acid residues are indicated on the left and right, respectively. The tetrapeptide uORF is a common feature in the peptide sequence specified by uORFs (39). Some of the used restriction sites are shown: H, HindIII; P, PstI; E, EcoRI; BA, BsaAI. The EcoRI-BsaAI and PstI-EcoRI fragments indicated with the bold lines at the bottom were used to flank the ura4 gene to make the disruption construct.

Screening of the S. pombe genomic DNA library identified seven overlapping clones encompassing an 8.2-kb region that covers the entire ORF. Comparison of the genomic and the cDNA sequences revealed that spe2 gene contains no intron (Fig. 1B). Searching the data base showed that spe2 is located at the close 3′ vicinity of the cut14 gene on the S. pombe genome (39).

Disruption of spe2—The spe2 gene was disrupted by the single-step gene replacement (Fig. 1B). Southern hybridization confirmed the replacement of the single-copy spe2 gene with homologous recombination (data not shown). AdoMetDC activity in the extracts of exponentially growing cells was measured.

Δspe2 mutant cells contained no detectable activity. Wild type cells exhibited 423 pmol of CO₂/h/mg of AdoMetDC protein. Δspe2 mutant and wild type cells were grown in the polyamine-free medium for two passages. Twenty hours after the second passage, the mutant cells contained no detectable levels of spermidine and spermine and accumulated 11.7 times putrescine and 6.0 times cadaverine over those detected in wild type cells (Fig. 2). Thereafter, the mutant cells ceased growing without addition of spermidine or spermine (Fig. 3). When putrescine and cadaverine were added to the culture medium at 100 μM concentration, they restored growing at a doubling time comparable to that of wild type cells after several hours of time lag. Addition of putrescine (Fig. 3), cadaverine, or diaminopropionate (data not shown) had no supportive effect on the growth of Δspe2 cells.

Changes in ODC Activity in Wild Type and Δspe2 Cells—Wild type cells showed virtually no ODC activity after growing in polyamine-free medium overnight (Fig. 4A). At this time, the...
cells were collected and resuspended in the same volume of fresh polyamine-free medium. ODC activity was rapidly induced reaching a peak in 4 h, and then a sharp decay was observed. Addition of spermidine (100 μM) 4 h after changing the medium caused faster ODC decay. On the other hand, Δspe2 cells contained a high ODC activity after overnight culture in the polyamine-free medium (Fig. 4B). Changing medium resulted in a much larger and more prolonged ODC induction in Δspe2 cells than in wild type cells. Addition of spermidine caused rapid decay of ODC activity also in Δspe2 cells. Thus, in Δspe2 cells, ODC was derepressed despite a large accumulation of putrescine. The mechanism of the accelerated ODC decay by spermidine was studied further in Δspe2 cells.

Protein Synthesis-dependent Degradation Accounts for Accelerated ODC Decay—Spermidine (100 μM) was added to the Δspe2 culture 4 h after the medium change, and cells were harvested at the indicated time to measure ODC activity. In Fig. 5A the total ODC activities (the sum of free ODC and complex form ODC activities measured in the presence of DFMO-ODC, see below) are plotted. Addition of spermidine reduced 98% of ODC activity in 6 h with a half-life of 65 min. This decay was much faster than after addition of cycloheximide (50 μg/ml), the half-life of which was more than 6 h. When cycloheximide was added together with spermidine, the acceleration of ODC decay was completely prevented. Actinomycin D (2.5 μg/ml), in contrast, did not change the effect of spermidine. Western blot analysis of the same cell extracts depicted that the decrease in ODC activity was associated with decrease in the amount of ODC protein (Fig. 5B). Addition of...
cycloheximide, but not actinomycin D, prevented this accelerated decrease of the protein. These results are consistent with a model that a short-lived protein that is induced by spermidine at a posttranscriptional level mediates the accelerating decay of ODC. Since it has been shown earlier that S. pombe antizyme is expressed through polyamine-inducible translational frameshifting (25), the protein is very likely to be antizyme.

Detection of Complex Form of ODC and Increase in Its Ratio by Spermidine—In mammalian cells, antizyme binds to ODC, forming an inactive heterodimer from which active ODC can be released by adding an excess amount of DFMO-ODC (30) or antizyme inhibitor (16). To test if a similar ODC-antizyme complex exists in S. pombe, we prepared S. pombe ODC inactivated with DFMO and used it in the competitive assay. A significant increase in ODC activity was observed when DFMO-ODC was added to the cellular extracts (Fig. 6A). Addition of spermidine to the culture medium increased the ratio of the gain over the total ODC activity. Cycloheximide blocked the increase. A virtually identical result was obtained by the use of rat antizyme inhibitor instead of DFMO-ODC (Fig. 6B). Spermidine thus increased the fraction of S. pombe ODC that forms an inactive complex. The ratio of the complex form to the total ODC was up to 25% after spermidine treatment, although 98% of the initial ODC activity disappeared in 6 h after the addition of spermidine. It is therefore likely that the ODC associated with antizyme is degraded rapidly.

Involvement of Antizyme in ODC Degradation—To confirm that antizyme is the protein factor that is induced by spermidine and promotes the degradation of ODC, we employed a knockout strain of S. pombe antizyme (Δspa) (25). The spe2 gene in the mutant strain was further disrupted by the single-step gene replacement. Addition of spermidine to the culture medium of Δspe-Δspe2 double knockout cells caused only a very slow ODC decay (Fig. 7). Approximately 90% of the activity remained even 6 h after addition of spermidine in the double knockout mutant compared with 2% in the Δspe2 single mutant. The result clearly indicates that antizyme is necessary for a major part of ODC decay that is stimulated by spermidine. It was also noted that about 10% of the initial ODC activity reproducibly disappeared in 6 h after the addition of spermidine in the absence of antizyme. Almost no ODC reduction was observed in the double mutant to which cycloheximide was added 4 h before spermidine. Almost no ODC reduction was observed in the double mutant to which cycloheximide was added together with spermidine. Simultaneous addition of actinomycin D did not block the ODC decay.

Effects of the Proteasome Inhibitors on ODC Degradation—The 26S proteasome has been shown to catalyze ODC degradation in both animals and S. cerevisiae (11, 18, 19). We tested if the proteasome is also involved in the accelerated ODC decay in S. pombe using inhibitors of proteasome. clasto-Lactacystin β-lactone or MG132, both at 50 μM, was added 4 h before spermidine. Both did not affect the cell growth at this concentration. As shown in Fig. 8, the rapid decay of ODC caused by spermidine was effectively inhibited by both inhibitors. This result strongly suggests that the 26 S proteasome catalyzes spermidine-induced degradation of ODC in S. pombe.

**DISCUSSION**

Mammalian antizyme represents a unique regulatory protein with a number of novel features, namely (i) expression and induction through translational frameshifting, (ii) the function as a non-ubiquitin stimulator for proteolysis of ODC by the proteasome, and (iii) dual activity on ODC and polyamine...
transporter. The frameshift induction serves as a polyamine sensor. The feedback system allows both the maintenance of the cellular polyamines within a certain range and their appropriate fluctuation. The molecular mechanisms of antizyme functions, however, have not been fully understood. In addition to the availability of genetics, recent identification of antizyme in *S. pombe* (25) makes the organism particularly an attractive system to study the mechanisms of antizyme functions.

The wild type *S. pombe* cells contain a high endogenous level of spermidine, which is not readily changed by exogenous spermidine. To study the feedback control of ODC by exogenous polyamines, use of mutants lacking spermidine synthesis appears to be useful as shown in *S. cerevisiae* (17, 18). Therefore, we intended to clone and disrupt *sponge* gene for AdoMetDC, spe2.

The Δspe2 mutant (spe2::ura4) cease dividing after several generations in polyamine-free medium. The mutant contained a large excess of putrescine and cadaverine, indicating indispen-
sability of spermidine and spermine for growth in the organ-
ism. The essential role of spermidine and spermine was also demonstrated in *S. cerevisiae* and *N. crassa* (40, 41). Δspe2 mutant of the budding yeast showed an increase in cell size, a decrease in budding, accumulation of vesicle-like bodies, and abnormal distribution of actin-like material (40). In the pre-

cence of oxygen, a rapid cessation of cell growth and associated cell death were observed (42). Similar studies in *S. pombe* are yet to be performed.

Wild type *S. pombe* cells contain very low basal level of ODC. It was induced by changing medium, but subject to rapid re-
pression due probably to increase in the endogenous poly-
amines (Fig. 4A). In Δspe2 cells ODC is elevated (Fig. 4B) despite a large accumulation of putrescine (Fig. 2). Spermidine added to the culture medium could rapidly repress ODC (Fig. 4B). It is of interest to note that *S. pombe* ODC is repressed by spermidine, but not effectively by putrescine, in connection with the observation that spermidine or spermine, but not putrescine, supports cellular growth. In *S. cerevisiae*, ODC decay is accelerated by putrescine to much less extent than by spermidine (43), whereas *N. crassa* ODC is strongly destabilized by both spermidine and putrescine (21).

The half-life of ODC activity was greatly shortened by spermidine in Δspe2 cells (Fig. 5A). The ODC decay is mostly attributable to a change in ODC protein (Fig. 5B). The acceler-
ated ODC decay was prevented by the inhibitor of protein synthesis, but not by the inhibitor of RNA synthesis. In addition, it was found that a part of ODC existed as an inactive complex, from which active ODC is released by the competitors such as DFMO-ODC or antizyme inhibitor (Fig. 5).

Although the fraction of ODC forming the inactive complex was increased such as DFMO-ODC or antizyme inhibitor (Fig. 6). Although complex, from which active ODC is released by the competitors, it was found that a part of ODC existed as an inactive synthesis, but not by the inhibitor of RNA synthesis. In addi-
tion, attributing to a change in ODC protein (Fig. 5B).

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