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# Identification of Regulatory Region of Antizyme Necessary for the Negative Regulation of Polyamine Transport

Kaori Sakata, Tomomi Fukuchi-Shimogori, Keiko Kashiwagi, and Kazuei Igarashi[1]
*Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan*

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**Antizyme is a negative regulator of ornithine decarboxylase (ODC) and of polyamine transport. Regions of antizyme necessary for the negative regulation of polyamine transport were determined by transfecting ODC-overproducing EXOD-1 cells with mutant antizyme genes containing different size deletions in the $NH_2$- and COOH-terminal of antizyme (AZ69-227). When peptide 119-144 or peptide 211-216, which are responsible for the binding of ODC, were deleted from antizyme, the mutant antizyme could not reverse the inhibition of growth of EXOD-1 cells produced by spermine. In parallel with the decrease in antizyme effect on cell growth, spermine transport activity and the accumulation of spermine in EXOD-1 cells were not significantly altered by the mutant antizyme, whereas wild-type antizyme decreased spermine transport and accumulation. When the peptide 69-118, which is responsible for the degradation of ODC, was deleted from antizyme, the mutant antizyme showed a smaller effect compared with the normal antizyme in terms of the inhibition of spermine transport and the recovery from the spermine inhibition of cell growth. The results indicate that regions 119-144 and 211-216 in antizyme are necessary for the negative regulation of polyamine transport and that these regions overlap with ODC binding sites.** 

Polyamines are essential for cell growth (1,2). The polyamine content in cells is maintained by both biosynthesis and transport of polyamines. Ornithine decarboxylase (ODC, EC 4.1.1.17) catalyzes the conversion of ornithine to putrescine, the first step of polyamine biosynthesis. The amount of ODC is regulated not only by various growth stimuli but also polyamines themselves at several levels, namely transcription, translation, and degradation (3). Eukaryotic cells generally contain an inducible and saturable transport system that incorporates all three polyamines with $K_m$ values in the micromolar range (4). A decrease in the polyamine content of cells, caused by inhibitors of polyamine biosynthesis, leads to a marked increase in the velocity of polyamine uptake, apparently without affecting the affinities for the substrates (5,6). Subsequently, as the polyamine content of cells increases, the uptake activity decreases.

Antizyme, a 227 amino acid protein (7), is known to be induced by polyamines and to inhibit the activity of ODC by forming an antizyme-ODC complex (8). It has also become clear that antizyme is involved in the rapid degradation of ODC by the 26S proteasome (9-13). Therefore, antizyme plays an important role in the negative feedback regulation of polyamine biosynthesis. Furthermore, it has been suggested that antizyme may function as a negative feedback regulator of polyamine transport (14). Indeed, it was recently demonstrated that antizyme negatively regulates polyamine transport (15,16), and that the abnormal accumulation of polyamines, which is a specific characteristic of ODC-overproducing cells, was restored to a normal level by transfection of the antizyme gene (15). Antizyme, through its negative regulation of polyamine transport (17), also delayed the restoration by spermine of growth of polyamine-deficient cells. In this study, we examined the effect of several mutant forms of antizyme, with different size deletions in the $NH_2$- and COOH-terminal, on the negative regulation of polyamine transport. We found that regions 119-144 and 211-216 in antizyme are necessary for the negative regulation of polyamine transport as well as the binding to ODC.

## MATERIALS AND METHODS

*Plasmids.* The plasmids containing mutant antizyme genes fused with the gene for maltose-binding protein in pMal-c2 (New England Biolabs, Inc.) (18) were kindly supplied by Drs. Y. Murakami and S. Hayashi. The mutant antizyme genes were amplified from the above plasmids by PCR using the following nucleotides 5′-CCAACAGGT-

[1] To whom correspondence should be addressed. Fax: +81-43-290-2900. E-mail: iga16077@p.chiba-u.ac.jp.



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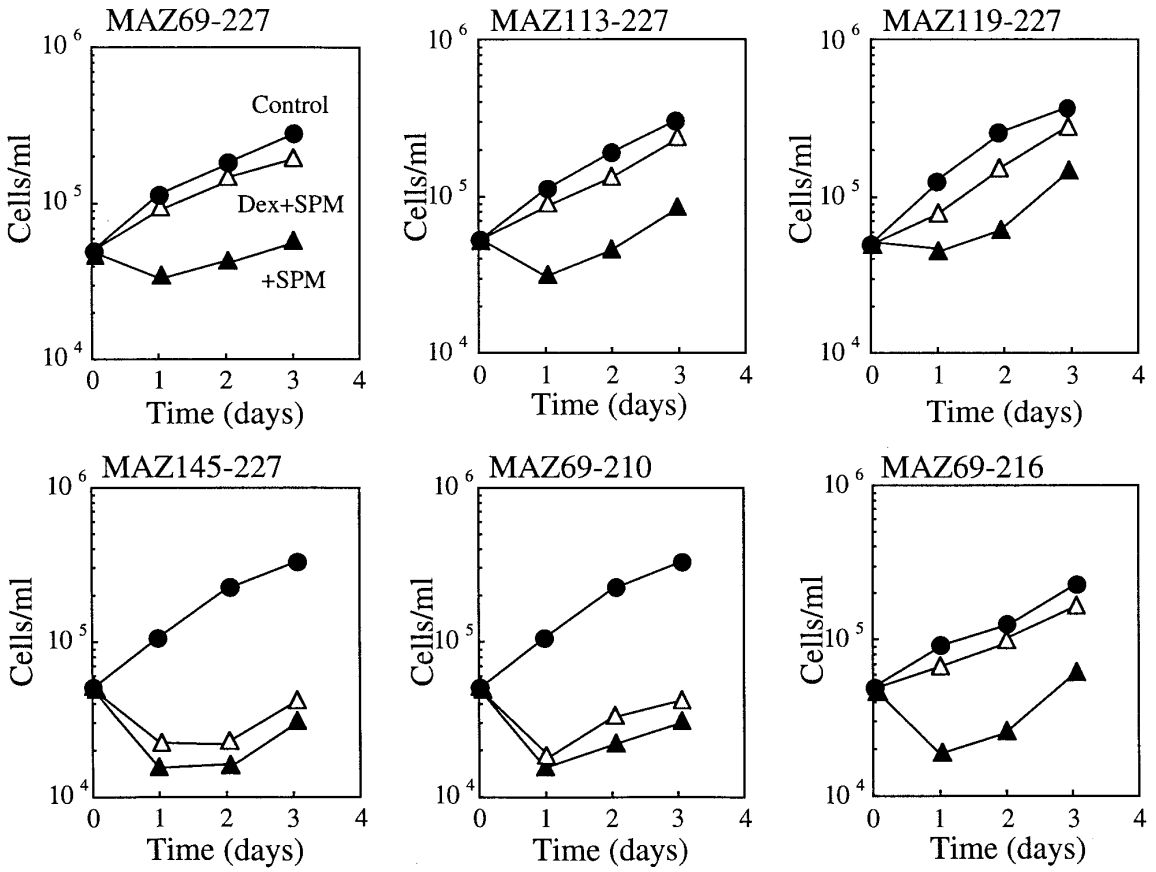


**FIG. 1.** Effect of spermine and mutant antizymes on growth of EXOD-1 cells. EXOD-1 cells, transfected with various mutant antizyme genes, were cultured under standard conditions. Where dexamethasone was used, cells were pretreated with 1 $\mu$M for 24 h to induce antizyme synthesis. Dexamethasone did not influence cell growth as described (15). ●, control; ▲, 30 $\mu$M spermine; △, 30 $\mu$M spermine and 1 $\mu$M dexamethasone. Mutant antizymes used are shown at the top of the figure.

ACCATAGATTATGAAAA-3′ and 5′-TTCCTCGAGTCACGACGT-TGTAAAACG-3′, containing *Kpn*I and *Xho*I cutting sites, respectively. The products were digested with *Kpn*I and *Xho*I, and inserted into the same restriction sites of pMAM2-*BSD* (Funakoshi). Transformation of *Escherichia coli* DH5$\alpha$ with the plasmids and culture of *E. coli* were carried out as described by Maniatis *et al.* (19). The plasmids in *E. coli* were purified using QIAGEN Plasmid Midi Kit (QIAGEN) according to the manufacturer's protocol. The structure of all constructs were verified by sequence analysis.

*Cell culture and transfection.* ODC overproducing mouse FM3A cells (EXOD-1) were previously isolated as described (20). The cells ($5 \times 10^4$ cells/ml) were cultured in ES medium (Nissui Pharmaceutical) containing 2% heat-inactivated fetal calf serum and 5 mM $\alpha$-difluolomethylornithine (DFMO) as described previously (15). The *Nru*I digested pMAM2-*BSD*, containing mutant antizyme genes, was transfected into EXOD-1 cells by electroporation according to the method of Kimura *et al.* (21). The antizyme was induced by preincubation of the transfectants with 1 $\mu$M dexamethasone for 24 h. The effect of spermine on cell growth was then examined in the presence of 1 mM aminoguanidine, an inhibitor of amine oxidase in serum (22). DFMO was kindly provided by Hoechst Marion Roussel Inc.

*Assay for spermine transport.* Antizyme gene transfected EXOD-1 cells were cultured in the presence and absence of 1 $\mu$M dexamethasone for 24 h. After washing the cells with NaCl buffer (135 mM NaCl / 1 mM $MgCl_2$ / 2 mM $CaCl_2$ / 10 mM glucose / 20 mM Hepes, pH adjusted to 7.2 with Tris), the spermine transport activity was measured as described (6) with $1 \times 10^6$ cells and 5 $\mu$M [$^{14}$C]spermine (740 MBq/mmol). The amount of radioactivity in the cells was measured in 10 ml Triton/toluene scintillant after sonication with 1 ml of 5% trichloroacetic acid. Initial rates of the transport were measured by incubation of the reaction mixture for 10 min. Protein was determined by the method of Lowry *et al.* (23).

*Measurements of polyamines.* Antizyme gene transfected EXOD-1 cells ($1 \times 10^6$), cultured for 12 h after spermine addition, were harvested and extracted with 0.3 ml of 5% trichloroacetic acid. Polyamines (putrescine, spermidine, and spermine) were measured as described (24) using the extract.

*Western blot analysis of ODC and antizyme.* Cytosol containing 0.5 $\mu$g and 40 $\mu$g of protein were used for Western blot analysis of ODC and antizyme, respectively. Rabbit polyclonal antibodies for mouse FM3A ODC and recombinant Z1 antizyme were kindly supplied by Drs. S. Matsufuji and S. Hayashi. Western blot analysis of ODC and antizyme was performed as described (25) using the ProtoBlot Western Blot AP System (Promega).

## RESULTS

*Effect of mutant antizymes on toxicity of spermine in EXOD-1 cells.* Induction of transfected antizyme in EXOD-1 cells was low. Therefore, we constructed a se-

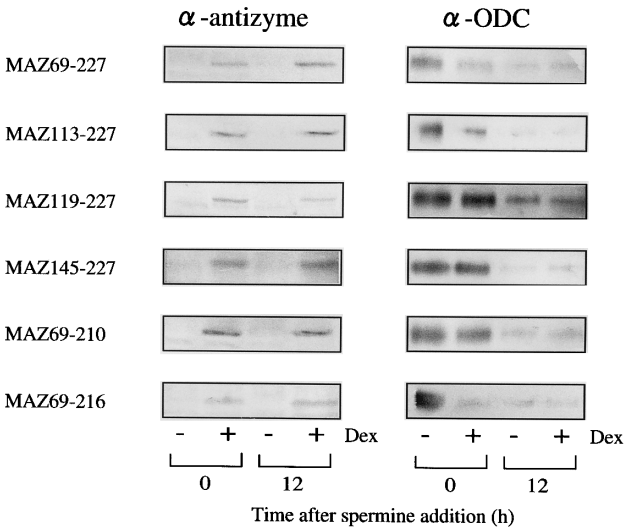


**FIG. 2.** Western blot analysis of ODC and antizyme. EXOD-1 cells, transfected with various mutant antizyme genes, were cultured in the presence (+) and absence (−) of 1 $\mu$M dexamethasone (Dex) as shown for 24 h, and then 30 $\mu$M spermine was added.

ries of deletion mutants of rat antizyme cDNA fused with the gene for maltose binding protein (MBP). By making MBP-fused mutant antizymes, almost equal amounts of the various mutant antizymes could be induced in EXOD-1 cells (see Fig. 2). Because antizyme retains normal functions even after truncation of the N-terminal 68 amino acids (26), antizyme (AZ69-227), encoding amino acids 69-227, was used as the starting material. We constructed six MBP-fused mutant antizymes (MAZ69-227, MAZ113-227, MAZ119-227, MAZ145-227, MAZ69-210, and MAZ69-216), which have different size deletions of peptide in the NH2- and COOH-terminal of antizyme.

We have shown previously that exposure of ODC-overproducing cells to micromolar levels of spermine caused abnormal accumulation and toxicity of spermine, and that induction of antizyme caused a decrease in spermine accumulation and promoted the recovery of cell growth (15). The effects of six mutant antizymes on toxicity of spermine in EXOD-1 cells were first examined (Fig. 1). When 30 $\mu$M spermine was added to the medium, inhibition of cell growth was observed in these six transfectants, as in untransfected EXOD-1 cells. When mutant antizymes were induced in the presence of 1 $\mu$M dexamethasone, recovery of growth inhibition was observed with MAZ69-227, MAZ113-227, and MAZ69-216, and partially with MAZ119-227 transfectants. A region which is necessary to promote ODC degradation (the peptide 113-118) is missing in MAZ119-227 (see Fig. 3B and ref. 18,27,28). Recovery of growth inhibition was, however, not observed with MAZ145-227 and MAZ69-210. Regions which are necessary to bind to ODC (the peptide 122-144 and the peptide 211-218) are missing in these two mutant antizymes (see Fig. 3B and ref. 18,27,28). The results suggest that the binding sites in antizyme for the polyamine transport system are overlapped with the binding sites for ODC.

Induction of the six mutant antizymes by dexamethasone was confirmed by Western blot analysis using antibody for antizyme (Fig. 2) or for MBP (data not shown). Degradation of ODC induced by mutant antizymes was observed with MAZ69-227, MAZ113-227 and MAZ69-216, by comparing the amount of ODC with or without dexamethasone before spermine addition (Fig. 2). When spermine was added to the medium, spermine dependent synthesis of natural antizyme occurred in EXOD-1 cells (15) by frameshifting in decoding antizyme (7). Thus, ODC was degraded at 12 h after spermine addition with or without dexamethasone (Fig. 2), and recovery of growth inhibition due to the natural antizyme was observed with all transfectants from 24 h after spermine addition (Fig. 1).

*Effect of mutant antizymes on spermine accumulation and transport of EXOD-1 cells.* The effect of mutant antizymes on spermine accumulation and transport of EXOD-1 cells was examined (Table I). Polyamine contents were measured at 12 h after addition of spermine. Abnormal accumulation of spermine was reduced by MAZ69-227, MAZ113-227 and MAZ69-216 (more than 13 nmol/mg protein), and slightly disturbed by MAZ119-227. However, accumulation of spermine was not reduced by MAZ145-227 and MAZ69-210 (Table I), mutants that also affect the ability of antizyme to rescue cell growth (Fig. 1). Spermine uptake activity of EXOD-1 cells was also examined after induction of mutant antizymes by dexamethasone. Spermine uptake activity decreased with MAZ69-227, MAZ113-227 and MAZ69-216 (more than 15 pmol/min/mg protein), and slightly decreased with MAZ119-227. However, spermine uptake activity did not change significantly with MAZ145-227 and MAZ69-210 (Table I).

The correlation between recovery from spermine toxicity and inhibition of spermine uptake is shown in Fig. 3A. The recovery from spermine toxicity was almost parallel with the decrease in spermine uptake activity, confirming that inhibition of cell growth was due to the abnormal accumulation of spermine, and that antizyme negatively regulates spermine transport. The results also suggest that degradation of the spermine transport system by antizyme may not be necessary for the inhibition of spermine transport, since the region to promote ODC degradation was not strongly involved in the inhibition of spermine transport (Fig. 3B) and was absolutely necessary for ODC degradation (18).

## DISCUSSION

In this study, we have identified regions of antizyme that are necessary for the negative regulation of poly-

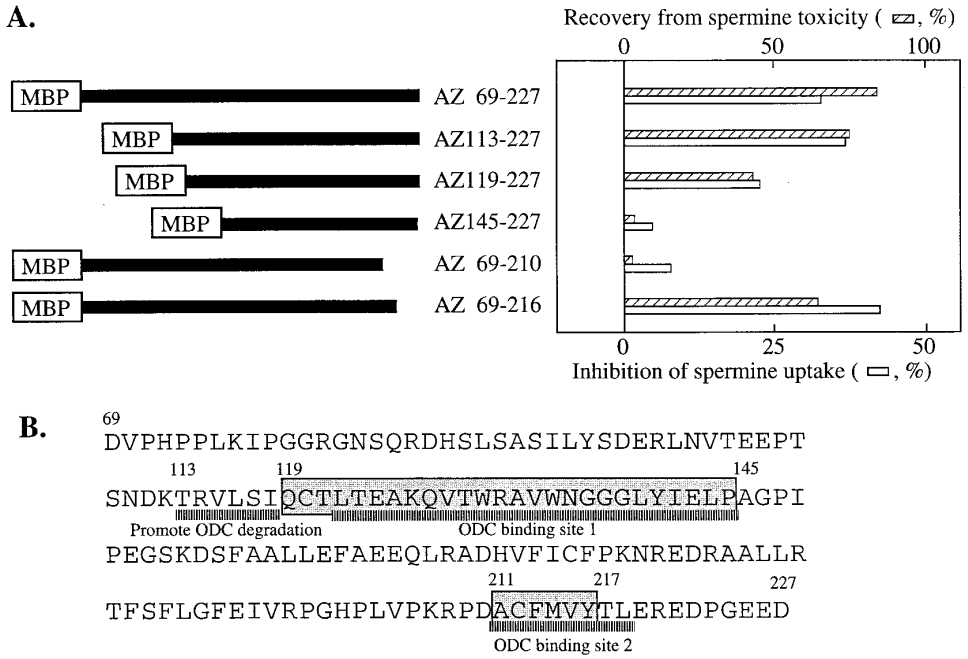


**FIG. 3.** Relationship between recovery from spermine toxicity and inhibition of spermine uptake (A) and functional regions of antizyme necessary for negative regulation of polyamine transport (B). A: The left panel represents structures of mutant antizymes used. The right panel shows the recovery from spermine toxicity by mutant antizymes at 24 h after the addition of 30 $\mu$M spermine (▨) and inhibition of spermine uptake by antizyme mutants at 24 h after the addition of 1 $\mu$M dexamethasone (□). Percentage was calculated as follows: Recovery from spermine toxicity, 100 × [(No. of cells treated with spermine and dexamethasone) − (No. of cells treated with spermine)] / [(No. of control cells) − (No. of cells treated with spermine)]. Inhibition of spermine uptake, 100 × (spermine uptake activity of cells treated with dexamethasone) / (spermine uptake activity of cells without dexamethasone). B: The sites 1 and 2 of ODC binding and the region to promote ODC degradation were cited from the data of Ichiba *et al.* (18). Shadowed peptides were involved in the negative regulation of polyamine transport.

amine transport. The results indicated that two regions of antizyme, peptides 119-144 and 211-216, are necessary for the inhibition of transport (Fig. 3B). These regions are also necessary for binding to ODC (18,27,28). It is notable that antizyme utilizes the same functional regions for negative regulation of polyamine transport as well as biosynthesis. This may explain why only ODC-overproducing cells easily accumulate polyamines leading to cell death because ODC competes with the polyamine transport system as for anti-

**TABLE 1**

Polyamine Contents and Spermine Uptake Activities of EXOD-1 Cells Transfected with Mutant Antizyme Genes

| Antizyme gene | Dex treatment | Polyamine content (nmol/mg protein)[a] | | | Spermine uptake[a] (pmol/min/mg protein) |
|---|---|---|---|---|---|
| | | Putrescine | Spermidine | Spermine | |
| MAZ 69-227 | − | <0.1 | 3.15 | 34.5 | 53.0 |
| | + | <0.1 | 2.45 | 21.1 | 37.2 |
| MAZ113-227 | − | <0.1 | 2.96 | 52.6 | 60.1 |
| | + | <0.1 | 2.10 | 30.9 | 38.0 |
| MAZ119-227 | − | <0.1 | 3.81 | 40.5 | 41.0 |
| | + | <0.1 | 2.32 | 33.3 | 31.7 |
| MAZ145-227 | − | <0.1 | 5.65 | 34.5 | 57.9 |
| | + | <0.1 | 5.25 | 33.7 | 53.9 |
| MAZ 69-210 | − | <0.1 | 3.62 | 43.1 | 58.6 |
| | + | <0.1 | 2.96 | 40.7 | 53.9 |
| MAZ 69-216 | − | <0.1 | 3.97 | 35.8 | 59.6 |
| | + | <0.1 | 2.79 | 22.8 | 33.8 |

[a] Each value is the average of two determinations. Polyamine contents and spermine uptake activities were measured as described in "Materials and Methods".

zyme binding. Thus, a decrease in free antizyme levels, caused by overexpression of ODC, would be expected to lead to an increase in transport activity.

Experiments were performed with MBP-fused antizyme because induction of transfected antizyme in EXOD-1 cells was low. Thus, the possibility remains that MBP may influence the tertiary structure of antizyme. Furthermore, we used AZ69-227 instead of intact antizyme, although AZ69-227 retained normal functions in terms of the ODC inhibition and the promotion of ODC degradation (26). It is known that the NH2-teminal 68 amino acids of antizyme are hydrophobic (7). The NH2-teminal peptide may also influence the affinity of antizyme for the polyamine transport system and for ODC. Thus, the exact affinity of antizyme for the polyamine transport system, as well as for ODC, remains to be determined.

ODC still existed when AZ119-227, lacking the region to promote ODC degradation, was induced in EXOD-1 cells, but spermine transport and accumulation were significantly disturbed with this transfectant. These results support the idea that antizyme is a negative regulator of polyamine transport, and that ODC itself does not function as a positive regulator of transport.

We have thus far discussed the findings that antizyme interacts directly with the polyamine transport system and affects the rate of polyamine uptake without influencing the $K_m$ values of polyamines for the transport protein when the molar ratio of antizyme to ODC increases. However, the possibility that antizyme also acts in some indirect manner on polyamine transport cannot be ruled out.

## ACKNOWLEDGMENTS

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