Properties of a polyamine transporter regulated by antizyme

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The regulation of polyamine transport by antizyme, a protein that is involved in the rapid degradation of ornithine decarboxylase (ODC), was studied in FM3A mouse cells overproducing ODC. Both artificial (Z1) and natural antizymes not only inhibited polyamine uptake but also stimulated polyamine excretion. The properties of the polyamine transporter regulated by antizyme were characterized. The uptake of radiolabelled polyamines was inhibited by excess acetylpolyamines and a protonophore, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), whereas the excretion of radiolabelled polyamines was stimulated by unlabelled polyamines, acetylpolyamines and CCCP in the medium. Furthermore, it is shown that polyamines and acetylpolyamines are excreted from cells. On the basis of the results, it is discussed how antizyme regulates polyamine transport negatively.

Key words: eukaryotic cells, excretion, polyamine uptake, spermidine, spermine.

INTRODUCTION

Polyamines are essential for cell growth [1,2]. The polyamine content in cells is regulated by biosynthesis, degradation and transport of polyamines. Ornithine decarboxylase (ODC; EC 4.1.1.17) catalyses the conversion of ornithine into putrescine, the first step in polyamine biosynthesis. The amount of ODC is regulated not only by various growth stimuli but also by polyamines at the levels of transcription, translation and degradation [3]. Eukaryotic cells express 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 leads to a marked increase in the rate 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 small protein of 227 amino acid residues [7], is known to be induced by polyamines and to inhibit the activity of ODC by forming an antizyme–ODC complex [3], leading to the rapid degradation of ODC by the 26 S proteasome without ubiquitination [8–11]. Thus antizyme has an important role in the regulation of polyamine biosynthesis by negative feedback. Furthermore, it was found that antizyme negatively regulates polyamine transport [12–15]. Accumulation of excess intracellular polyamines could be restored to normal levels by transfection of the antizyme gene [14]. Antizyme also delayed the restoration of growth of polyamine-deficient cells that normally occurs after the addition of polyamines to the medium [15]. We have identified the regions of antizyme necessary for the negative regulation of polyamine transport [16]. These regions overlap with the ODC-binding sites on antizyme [17].

In the present study we examined the mechanism of negative regulation of polyamine transport by antizyme. Because a frameshift of antizyme mRNA is necessary for the synthesis of natural antizyme [7], most experiments were performed using Z1 antizyme. This form of antizyme was expressed in ODC-overproducing FM3A mouse cells (AZ-2 cells) under the control of a dexamethasone-inducible promoter without the frameshift of the mRNA [8]. It is known that Z1 antizyme, consisting of 215 residues, retains normal functions because the essential C-terminal 159 residues are identical with those of natural antizyme [7,8]. We found that antizyme not only inhibits polyamine uptake but also stimulates polyamine excretion. Furthermore, we found that the polyamine transporter regulated by antizyme recognized acetylpolyamines in addition to polyamines.

MATERIALS AND METHODS

Cell culture and transfection

ODC-overproducing FM3A (EXOD-1) mouse cells [18] were transfected with pMAMneoZ1 as described previously [14]. The pMAMneoZ1 transfectant (AZ-2) cells thus obtained produced Z1 antizyme in the presence of 1 μM dexamethasone [8]. The AZ-2 cells (5×10^4 cells/ml) were maintained in ES medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 50 i.u./ml streptomycin, 100 i.u./ml penicillin G and 0.5 mg/ml G418 (geneticin), 2 % (v/v) heat-inactivated fetal calf serum and 5 mM α-difluoromethylornithine (DFMO), an inhibitor of ODC [19], at 37 °C in an air/CO_2 (19:1) atmosphere. The cells were cultured without DFMO during experiments. The effects of polyamines were examined in the presence of 1 mM aminoguanidine, an inhibitor of amine oxidase in serum [20]. DFMO was kindly supplied by Hoechst Marion Roussel.

Assay for uptake of polyamines and tetraphenylphosphonium bromide (TPP)

AZ-2 cells were cultured for 2 or 20 h in the absence or presence of 1 μM dexamethasone. Either 30 μM putrescine, 3 μM sper-

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; TPP, tetraphenylphosphonium bromide.

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midine or 1.5 μM spermine was added to the medium to induce endogenous antizyme. After the cells had been washed with NaCl buffer [135 mM NaCl/1 mM $MgCl_2$/2 mM $CaCl_2$/10 mM glucose/20 mM Hepes/Tris (pH 7.2)], the polyamine uptake activity was measured as described [6] by using 10^6 cells in the NaCl buffer. The substrates used were 30 μM [^{14}C]putrescine, 10 μM [^{14}C]spermidine and 10 μM [^{14}C]spermine. The specific radioactivities of the ^{14}C-polyamines were 740 MBq/mmol. The amount of radioactivity in the cells was measured in 10 ml of Triton/toluene scintillant after sonication with 1 ml of 5 % (w/v) trichloroacetic acid. Protein was determined by the method of Lowry et al. [21]. N^1-Acetylspermidine and N^1-acetylspermine were gifts from Dr Z. N. Canellakis (Yale University School of Medicine, New Haven, CT, U.S.A.). The purity of the acetylpolyamines was more than 99 %.

TPP uptake was determined as above for polyamines, with 5 μM [^{3}H]TPP (740 MBq/mmol) [22].

Assay for excretion of polyamines

AZ-2 cells were cultured as described above except that the cells were labelled with 30 μM [^{14}C]putrescine, 3 μM [^{14}C]spermidine or 1.5 μM [^{14}C]spermine. The specific radioactivities of ^{14}C-polyamines were 185 MBq/mmol. The cells were harvested and washed with the polyamine-free culture medium. The cells (5×10^6) were suspended in 3 ml of the polyamine-free culture medium and incubated at 37 °C. At the designated time, a 0.35 ml aliquot of cell suspension was removed and centrifuged. The radioactivity in 0.3 ml of the supernatant was then counted in 10 ml Triton/toluene scintillant.

Measurement of polyamines and their acetyl derivatives

AZ-2 cells (10^6) were harvested and polyamines were extracted with 0.3 ml of 5 % (w/v) trichloroacetic acid. Polyamines were measured in the extract by HPLC as described [23]. The retention times for N^1-acetylspermidine, putrescine, N^1-acetylspermine, spermidine and spermine were 4, 6.5, 8, 12 and 23 min respectively, with slight variations. When ^{14}C-polyamines were measured, radioactivity was counted in 1 ml aliquots of the eluate (1.15 ml/min) from HPLC.

Western blot analysis of ODC and antizyme

Cell lysate and the 100000 ***g*** supernatant were prepared by the method of Touster et al. [24]. The buffer used for cell fractionation consisted of 5 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 1 mM dithiothreitol and 20 μM 6-amino-2-naphthyl-4-guanidinobenzoate dihydrochloride (FUT-175), an inhibitor of protease [25]. FUT-175 was kindly supplied by Torii Pharmaceutical Co. (Tokyo, Japan). The 100000 ***g*** supernatants containing 1 and 20 μg of protein were used for Western blot analyses of ODC and antizyme respectively. Rabbit polyclonal antibodies against ODC and Z1 antizyme were kindly supplied by Dr Y. Murakami and S. Matsufuji (Jikei University School of Medicine, Tokyo, Japan). Western blotting was performed as described [26], with the enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech).

RESULTS

Antizyme induction and polyamine contents in AZ-2 cells

We first determined how the induction of antizyme influences polyamine levels in AZ-2 cells, which express Z1 antizyme under the control of a dexamethasone-inducible promoter. Low concentrations of polyamines (30 μM putrescine, 3 μM spermidine or 1.5 μM spermine), which do not cause an inhibition of cell growth through polyamine accumulation [27], were added to the medium and the accumulated polyamines were measured with or without the induction of Z1 antizyme. As shown in Figure 1, dexamethasone induced Z1 antizyme as early as 3 h after the onset of cell culture, whereas polyamine-dependent natural antizyme synthesis occurred slightly later. Natural antizyme seems to be synthesized from the second initiation codon AUG [7], judging from its molecular mass, and it is termed 'small antizyme (s-AZ)'. The levels of antizyme induced by 30 μM putrescine, by 3 μM spermidine and by 1.5 μM spermine were very similar. A decrease in the level of accumulated polyamines began after the induction of antizyme and was seen earlier after expression of the Z1 antizyme than with natural antizyme (Figure 1). Significant amounts of polyamines (90 %, 60 % and 30 % of added putrescine, spermidine and spermine respectively) still remained in the medium when the accumulated polyamines in cells reached the maximum (results not shown). We previously reported that antizyme negatively regulates polyamine uptake [14,15]. Our results suggest that antizyme might also stimulate polyamine excretion.

The cellular polyamine content during culture for 24 h in the presence and the absence of each polyamine is shown in Table 1. A change in the intracellular level of a particular polyamine occurred mainly after the corresponding polyamine was added to the medium (Figure 1). For example, when 1.5 μM spermine was added to the medium, there was an increase in intracellular spermine concentration but little or no change in the levels of spermidine and putrescine during 24 h in culture. When the cells were cultured in the absence of polyamines for 24 h, an increase in spermidine and putrescine contents was observed owing to the removal of DFMO from the medium.

Natural antizyme is synthesized through a frameshift of antizyme mRNA, which is induced by polyamines [7]. Thus the amount of natural antizyme, but not the amount of Z1 antizyme, was decreased, together with a decrease in polyamines, at 24 h. Antizyme is rapidly degraded by the 26 S proteasome [9]. Therefore the involvement of antizyme in the decrease in cellular polyamine levels was confirmed by using cycloheximide, an inhibitor of protein synthesis, which should rapidly decrease levels of antizyme. The addition of cycloheximide to the medium caused a decrease in the amount of Z1 and natural antizymes, a stabilization of ODC and an accumulation of spermine in AZ-2 cells (Figure 2). ODC in AZ-2 cells was relatively stable even in the absence of cycloheximide, probably owing to the existence of residual DFMO in the cells.

Inhibition of polyamine uptake by antizyme

It is known that accumulated polyamines strongly inhibit polyamine uptake [6]. It is therefore important to adjust polyamine contents in cells to almost the same amounts for the measurement of antizyme effects. Accordingly, the effects of antizyme on polyamine uptake were examined in detail by using AZ-2 cells incubated with polyamine for 2 h in the absence of dexamethasone and AZ-2 cells incubated with polyamine for 20 h in the presence of dexamethasone. The polyamine contents were nearly equal in these two types of cell; high levels of Z1 and natural antizymes were induced in the latter cells (see Figure 1). The K_m values for the uptake of putrescine, spermidine and spermine in FM3A cells were 29, 3.2 and 1.5 μM respectively (results not shown). Thus 30 μM putrescine, 10 μM spermidine and 10 μM spermine were each used as substrates. As shown in Figure 3, the uptake of all three polyamines was greatly inhibited

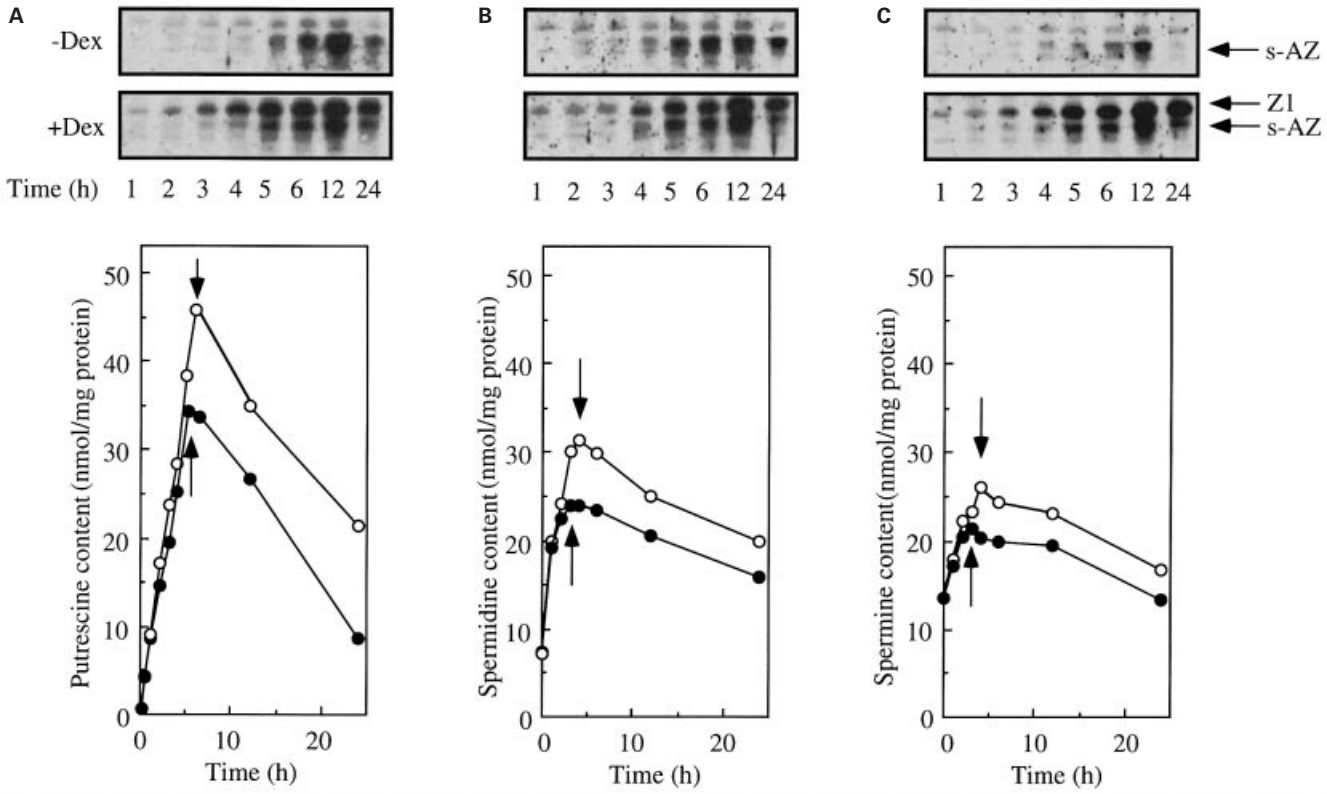

Figure 1 Intracellular polyamine and antizyme levels in AZ-2 cells cultured in the presence of polyamines

Upper panels: the cells were cultured in the presence of polyamines [30 μM putrescine (**A**), 3 μM spermidine (**B**) or 1.5 μM spermine (**C**)] with (●) or without (○) 1 μM dexamethasone (Dex). The amount of antizyme was measured by using 20 μg of protein from the 100 000 ***g*** supernatant by Western blot analysis. Z1 and s-AZ indicate the positions of Z1 and natural antizyme respectively. Lower panels: the change in each polyamine level in cells due to its addition to the medium is shown. The arrow indicates the point of the maximal accumulation of polyamine. Each point is the average for duplicate determinations.

Table 1 Changes of intracellular polyamine levels in AZ-2 cells cultured in the presence of polyamines and dexamethasone

Each value is the average for duplicate determinations.

Polyamine in medium	Duration of incubation (h)	Polyamine content (nmol/mg of protein)		
		Putrescine	Spermidine	Spermine
None	0	0.66	9.05	13.8
None	24	6.77	13.6	14.4
Putrescine (30 μM)	5	34.7	14.2	12.3
Putrescine (30 μM)	10	27.0	14.1	9.02
Putrescine (30 μM)	24	9.59	16.8	8.47
Spermidine (3 μM)	4	0.89	24.0	10.6
Spermidine (3 μM)	10	0.90	21.1	9.78
Spermidine (3 μM)	24	0.39	15.6	8.92
Spermine (1.5 μM)	4	0.59	7.79	22.1
Spermine (1.5 μM)	10	0.36	6.98	16.1
Spermine (1.5 μM)	24	0.14	6.73	14.7

by induction of antizyme. The uptake of arginine, lysine and glutamic acid was not inhibited by the induction of antizyme (results not shown). The uptake of polyamines was inhibited by an ionophore, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), indicating that polyamine uptake is dependent on membrane potential [6]. The uptake was also inhibited weakly by 300 μM N^1-acetylspermine (Figure 3). The plasma membrane potential was measured by TPP uptake. It did not decrease significantly (less than 5%) on the induction of antizyme.

Stimulation of polyamine excretion by antizyme

The excretion of ^{14}C-polyamines (or their metabolites) from cells was then studied to determine whether antizyme affected polyamine excretion as well as polyamine uptake (Figure 4). In AZ-2 cells expressing induced antizyme, significant amounts of [^{14}C]spermidine or [^{14}C]spermine were excreted from cells. Excretion of these polyamines was increased by CCCP, which inhibits the membrane-potential-dependent reuptake of polyamines and extinguishes the membrane hyperpolarization caused by polyamine excretion. Excretion of putrescine in the presence of CCCP was observed in both antizyme-induced and uninduced AZ-2 cells, although the excretion was faster in the antizyme-induced AZ-2 cells. The results suggest that putrescine is more easily excreted from cells than spermidine and spermine. It has been reported that spermidine is excreted by the multidrug transporter *Blt* in *Bacillus subtilis* and that the excretion is inhibited by reserpine [28]. The excretion of polyamines from AZ-2 cells was not inhibited by reserpine (results not shown), supporting the assumption that the excretion system is specific for polyamines.

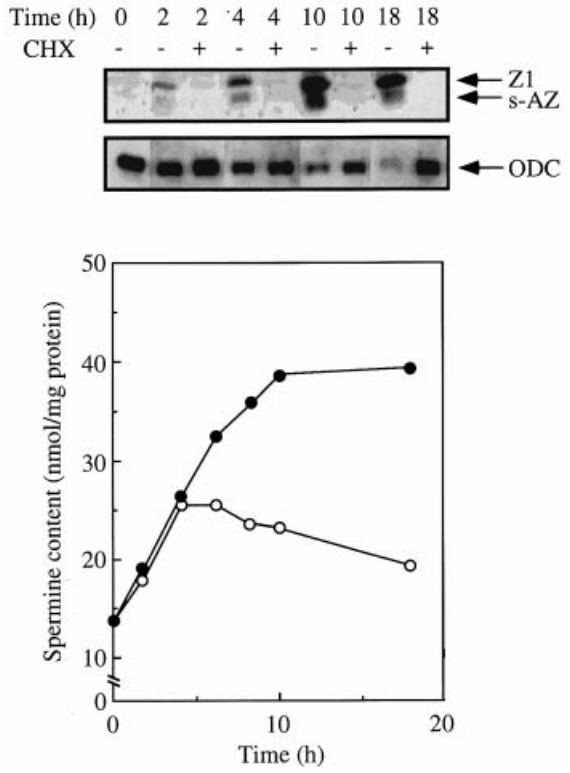

Figure 2 Effect of cycloheximide on antizyme induction and spermine level in AZ-2 cells cultured in the presence of 1.5 μM spermine

Upper panels: the quantities of antizymes and ODC were measured with 20 and 1 μg of protein from the 100 000 ***g*** supernatant by Western blot analysis. Abbreviation: CHX, cycloheximide. Lower panel: the change in the spermine level in cells cultured in the presence of 1.5 μM spermine and 1 μM dexamethasone with (●) or without (○) 50 μg/ml cycloheximide is shown. Each point is the average for duplicate determinations.

Cells were then cultured for 18 h in the absence of spermine and for 2 h in the presence of 1.5 μM [^{14}C]spermine; the excretion of [^{14}C]spermine from the cells was compared with that from the cells cultured for 20 h in the presence of 1.5 μM [^{14}C]spermine and 1 μM dexamethasone. Although spermidine and putrescine contents were different in these two types of cell, the induction of antizyme stimulated the excretion of [^{14}C]spermine (results not shown). Thus antizyme stimulates polyamine excretion regardless of the differences of intracellular conditions.

We next tried to identify the excreted polyamines in AZ-2 cells cultured with ^{14}C-polyamine and 1 μM dexamethasone for 20 h. As shown in Figure 5 (upper panels), putrescine, spermidine and spermine were differentially metabolized during cell culture for 20 h. Putrescine was most easily metabolized; spermine was metabolized least. Thus the percentages of unmetabolized putrescine, spermidine and spermine were approx. 60 %, 65 % and 90 % respectively. Polyamines excreted after 1 h incubation were then identified (Figure 5, lower panels). It was shown that not only ^{14}C-polyamines but also ^{14}C-acetylpolyamines (acetylspermidine and acetylspermine) were excreted from the cells. It is known that some portions of polyamines are excreted as acetyl derivatives in animal cells [29]. The results suggest that acetylpolyamines are excreted through the same polyamine transporter as that which excretes polyamines.

We performed experiments to determine whether polyamine uptake and excretion were catalysed by the same transporter. For this purpose, the effect of the addition of spermine to the medium on the excretion of [^{14}C]spermine was examined by using AZ-2 cells cultured with 1.5 μM [^{14}C]spermine and 1 μM dexamethasone for 20 h (Figure 6). The addition of unlabelled spermine stimulated the excretion of [^{14}C]spermine in the presence and in the absence of CCCP. The excretion of [^{14}C]spermine was also stimulated by N^1-acetylspermine (Figure 6) but was not influenced by ornithine, arginine, lysine or glutamic acid (results not shown). These results can be explained by rapid exchange

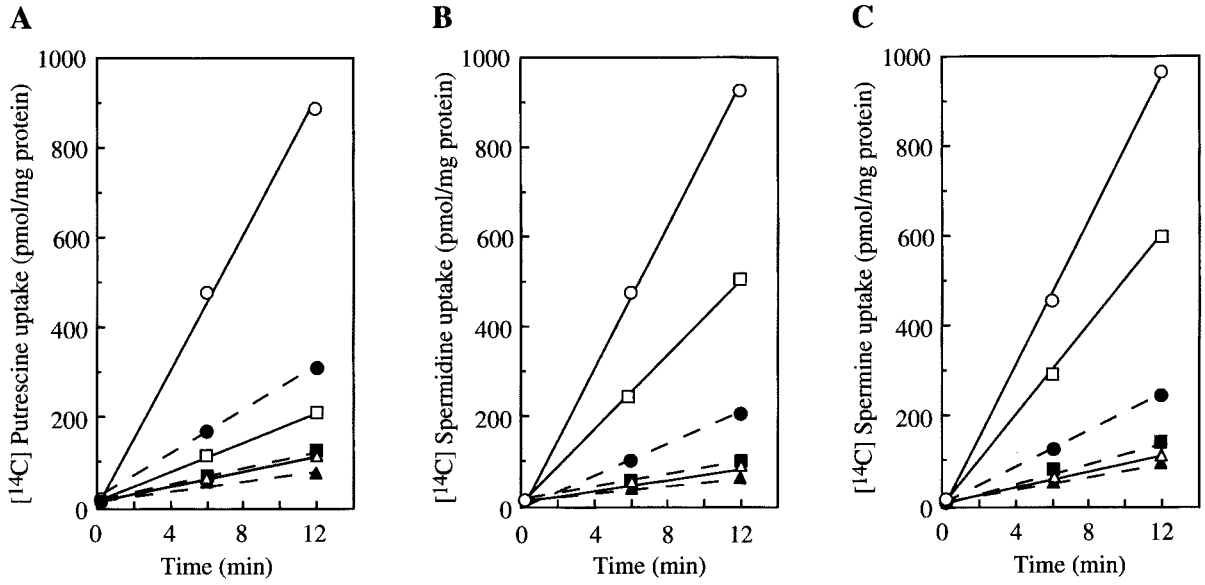

Figure 3 Polyamine uptake by AZ-2 cells

The cells were cultured in the presence of 30 μM putrescine (**A**), 3 μM spermidine (**B**) or 1.5 μM spermine (**C**) for 20 h with 1 μM dexamethasone (●) or for 2 h without dexamethasone (○). Where indicated, 10 μM CCCP (▲,△) or 300 μM N^1-acetylspermine (■,□) was added during the assay of the two kinds of cells (closed symbols, with dexamethasone; open symbols, without dexamethasone). Each point is the average for duplicate determinations.

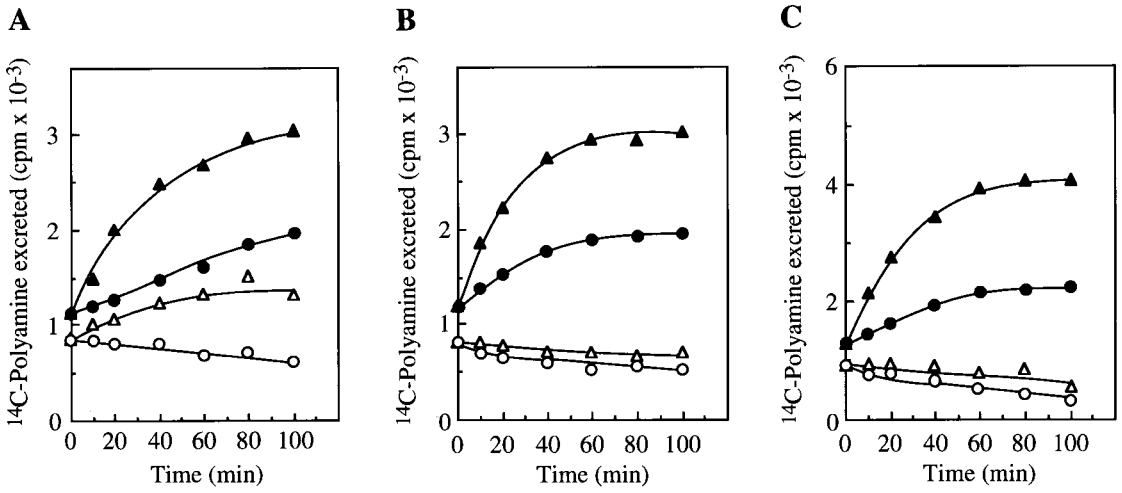

Figure 4 Excretion of ^{14}C-polyamines from AZ-2 cells

The cells were cultured in the presence of ^{14}C-polyamine [30 μM putrescine (**A**), 3 μM spermidine (**B**) or 1.5 μM spermine (**C**)] for 2 h without dexamethasone (△,○) or for 20 h with 1 μM dexamethasone (▲,●). Almost the same radioactivities were accumulated in both cells [(4–5) $\times$ 10^5 c.p.m. of [^{14}C]putrescine, (2.5–3) $\times$ 10^5 c.p.m. of [^{14}C]spermidine and (4–5) $\times$ 10^5 c.p.m. of [^{14}C]spermine in 5 $\times$ 10^6 cells]. Where indicated (▲,△), 10 μM CCCP was added to the assay mixture for the excretion. Each point is the average for duplicate determinations.

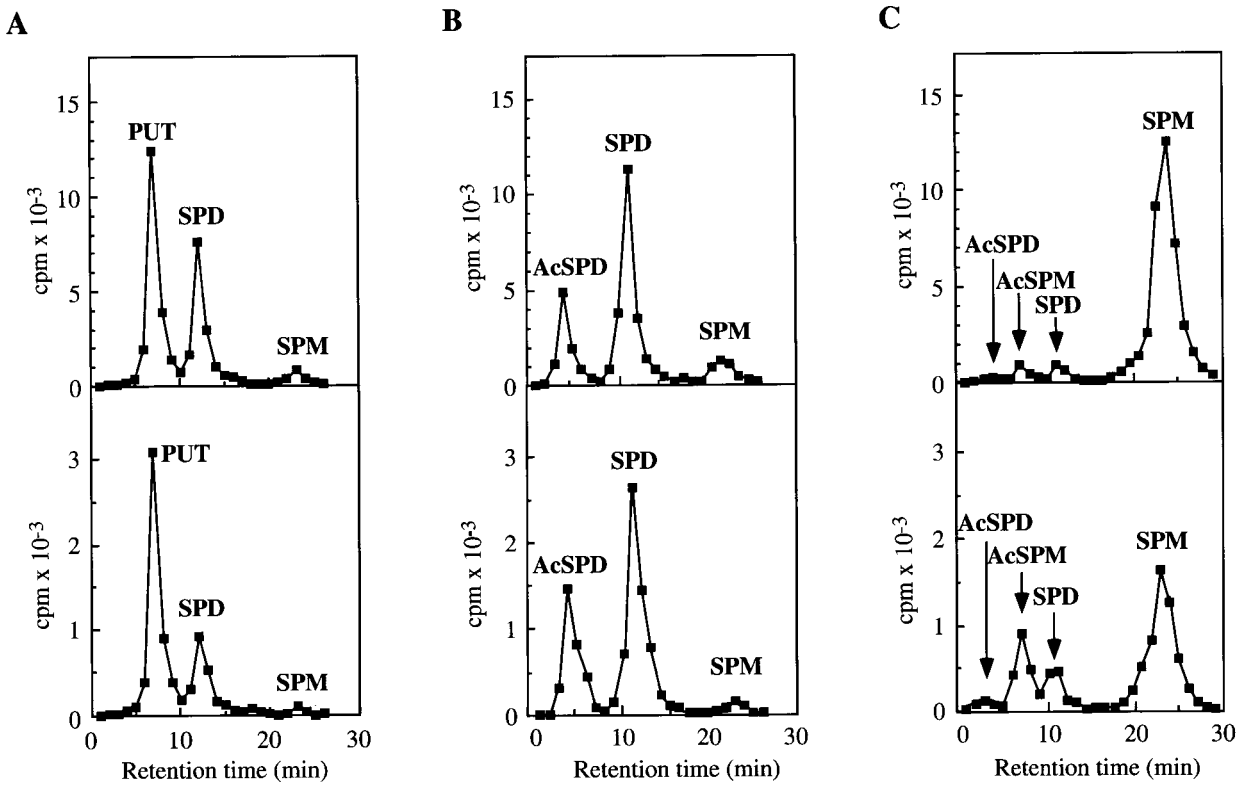

Figure 5 Identification of ^{14}C-polyamines excreted from AZ-2 cells

The cells were cultured in the presence of 30 μM [^{14}C]putrescine (**A**), 3 μM [^{14}C]spermidine (**B**) or 1.5 μM [^{14}C]spermine (**C**) with 1 μM dexamethasone for 20 h. The ^{14}C-polyamine (5.0 $\times$ 10^4 c.p.m.) in cells (upper panels) and the excreted ^{14}C-polyamine (9 $\times$ 10^3 c.p.m.) after incubation for 100 min in the presence of 10 μM CCCP (lower panels) were analysed by HPLC. Abbreviations: AcSPD, N^1-acetylspermidine; AcSPM, N^1-acetylspermine; PUT, putrescine; SPD, spermidine; SPM, spermine.

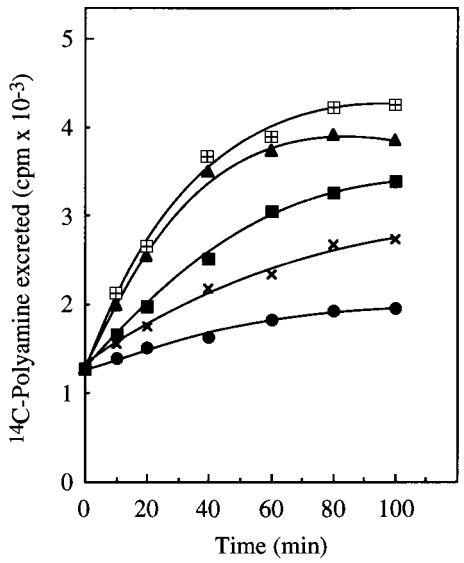

Figure 6 Effect of unlabelled spermine and N^1-acetylspermine on the excretion of [^{14}C]spermine

AZ-2 cells were cultured with 1.5 μM [^{14}C]spermine and 1 μM dexamethasone for 20 h. Where indicated, 0.5 mM spermine (⊞,■), 0.5 mM N^1-acetylspermine (×) and/or 10 μM CCCP (⊞,▲) were added to the assay mixture; ●, no addition. The [^{14}C]spermine accumulated in the AZ-2 cells was 5×10^5 c.p.m. in 5×10^6 cells. Each point is the average for duplicate determinations.

between unlabelled spermine (or N^1-acetylspermine) and [^{14}C]-spermine catalysed by the same polyamine transporter.

DISCUSSION

Our results show that both natural and exogenous antizymes inhibit polyamine uptake and stimulate polyamine excretion. The Z1 and natural antizymes functioned similarly (see Figure 1), indicating that the region of the antizyme protein that affects polyamine transport is in the C-terminal portion of the protein (residues 69–227). This is consistent with results of previous studies in which residues 119–144 and 211–216 were found to be important for the negative regulation of polyamine transport [16]; these regions overlap with the ODC-binding sites in antizyme [17]. Antizyme mRNA has two initiation codons (AUG) [7]. If natural antizyme were to be synthesized from the first of these, the molecular mass of natural antizyme would be greater than that of Z1 antizyme. However, the molecular mass of natural antizyme in cytoplasm was smaller than that of Z1 antizyme (see Figures 1 and 2). Therefore the antizyme in cytoplasm seems to be synthesized from the second initiation codon. If antizyme were synthesized from the first initiation codon, the antizyme might move to mitochondria because of the existence of a signal sequence for mitochondria. Although it has been reported that a larger antizyme form was concentrated in mitochondria [30], we could not judge whether the antizyme exists in mitochondria because of existence of many non-specific bands in our Western blot analysis.

Our results strongly suggest that both uptake and excretion are catalysed by the same polyamine transporter. If different proteins catalyse uptake and excretion, the addition of unlabelled spermine should decrease the excretion of [^{14}C]spermine because unlabelled spermine taken up into cells dilutes [^{14}C]spermine. This argument is valid only if the polyamine concentration in cells is much higher than the K_m. Because the concentration in cells is in the millimolar range, we expect that the concentration is much higher than the K_m values for polyamine excretion, which are probably less than 0.1 mM. Furthermore, the effect of unlabelled spermine should be inhibited by CCCP because the uptake is dependent on membrane potential. Thus the stimulation of the excretion of [^{14}C]spermine by unlabelled spermine is most likely to be due to rapid exchange between unlabelled spermine and [^{14}C]spermine catalysed by the same polyamine transporter. The rapid exchange was also observed with PotE of *Escherichia coli*, which catalyses both the uptake and excretion of putrescine [31,32]. The excretion of putrescine by PotE was stimulated by CCCP [31].

Another possibility is that uptake and excretion are catalysed by different polyamine transporters [4], and this cannot be excluded unequivocally. In this case, the uptake transporter acts as a salvage pathway for excreted ^{14}C-polyamines and the reuptake of ^{14}C-polyamines is inhibited by unlabelled polyamines and CCCP. Under these conditions, the antizyme might function as a negative regulator of the polyamine uptake but not as a positive regulator of the polyamine excretion. If the transporter for polyamine excretion were always present in cells and excreted the synthesized polyamines from cells, it would be difficult to provide a physiological rationale for such an activity. No specific polyamine excretion systems have yet been reported in prokaryotes [33].

Putrescine excretion was observed in the presence of CCCP without antizyme induction (Figure 4A). This result suggests that putrescine might be more easily excreted than spermidine and spermine. There is also a report that an antizyme-independent diamine exporter exists in Chinese hamster ovary cells [34]. Thus the diamine exporter might be involved in the excretion of putrescine together with the antizyme-regulated polyamine transporter.

During the assay for the excretion of polyamines, significant quantities of ^{14}C-polyamines are always present at zero time of the incubation (Figures 4 and 6). It takes some time to mix cells with the polyamine-free culture medium and to isolate the supernatant from cells. Therefore ^{14}C-polyamines might be excreted during this operation.

It is also noted that acetylpolyamines are excreted from cells by the same transporter. In animal cells, excess polyamines are metabolized to acetyl derivatives, which are then excreted [28]. Judging from the results shown in Figure 5, acetylpolyamines might be better substrates for excretion than polyamines themselves, although polyamines are better substrates for uptake than acetylpolyamines. Because the decrease in spermine transport by antizyme was mainly due to the increase in the K_m for spermine [15], it is very likely that the affinity for each substrate in the uptake and excretion changes by the induction of antizyme.

Mouse ODC-overproducing cells were used in our experiments. The levels of antizyme in these cells were higher than those in normal cells. However, negative regulation of polyamine transport by antizyme occurred similarly in both normal and cells overproducing ODC [14,15]. Therefore excess quantities of antizyme in cells overproducing ODC might transiently exist as the ODC–antizyme complex.

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