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ORIGINAL ARTICLE

Spermidine, a sensor for antizyme 1 expression regulates intracellular polyamine homeostasis

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Abstract Although intracellular polyamine levels are highly regulated, it is unclear whether intracellular putrescine (PUT), spermidine (SPD), or spermine (SPM) levels act as a sensor to regulate their synthesis or uptake. Polyamines have been shown to induce AZ1 expression through a unique +1 frameshifting mechanism. However, under physiological conditions which particular polyamine induces AZ1, and thereby ODC activity, is unknown due to their inter-conversion. In this study we demonstrate that SPD regulates AZ1 expression under physiological conditions in IEC-6 cells. PUT and SPD showed potent induction of AZ1 within 4 h in serum-starved confluent cells grown in DMEM (control) medium. Unlike control cells, PUT failed to induce AZ1 in cells grown in DFMO containing medium; however, SPD caused a robust AZ1 induction in these cells. SPM showed very little effect on AZ1 expression in both the control and polyamine-depleted cells. Only SPD induced AZ1 when *S*-adenosylmethionine decarboxylase (SAMDC) and/or ODC were inhibited. Surprisingly, addition of DENSpm along with DFMO restored AZ1 induction by putrescine in polyamine-depleted cells suggesting that the increased SSAT activity in response to DENSpm converted SPM to SPD, leading to the expression of AZ1. This study shows that intracellular SPD levels controls AZ1 synthesis.

Keywords Putrescine · Spermine · *S*-adenosylmethionine decarboxylase (SAMDC) · Cadavarine · *N*-spermidine/spermine acetyltransferase (*N*-SSAT) · Ornithine decarboxylase (ODC) · Intestinal epithelial cells (IEC-6)

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Introduction

Cells maintain intracellular polyamines at optimal levels by regulating synthesis or degradation and by their uptake or release. The intracellular levels of polyamines primarily depend on the activity of ornithine decarboxylase (ODC; EC 4.1.1.17) which catalyzes the first rate-limiting step in polyamine synthesis, forming putrescine from the amino acid, ornithine (Pegg and McCann 1982; Tabor and Tabor 1984; Pegg 2006). Polyamines are inter-convertible after they are taken into cells. *S*–adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) catalyzes the removal of the carboxyl group from *S*–adenosylmethionine (SAM) to form decarboxylated *S*–adenosylmethionine (dcSAM). The propylamine group of dcSAM is transferred to putrescine to form spermidine or from spermidine to form spermine (Pegg and McCann 1982; Tabor and Tabor 1984; Wang et al. 1992; Shantz et al. 1992; Yuan et al. 2000). In a backward conversion, spermine is transformed to *N*-acetylspermine by *N*-spermidine/spermine acetyltransferase (*N*-SSAT) and then oxidized by polyamine oxidase to produce spermidine. Catalyzed by the same enzymes, spermidine is converted to putrescine. The induction and activities of these enzymes are also regulated by polyamines (Casero and Marton 2007; Pegg 2008; Wada and Shirahata 2010). Furthermore, ODC activity itself is highly regulated and varies in response to many different stimuli in different tissues and cells. ODC activity increases rapidly in response to growth factors, amino acids such as asparagine or glutamine, and hypotonic stress (Rinehart et al. 1985; Chen and Canellakis 1977; Heby and Persson 1990a; Ray et al. 1999a, b, 2012). ODC activity is tightly regulated at various levels, namely gene transcription, mRNA degradation, mRNA translation, and protein degradation (Mitchell et al. 1994; Shantz and Pegg 1999; Pegg 2006).

Antizyme is induced by polyamines and inhibits ODC activity by forming a complex with the enzyme. Among the three naturally occurring polyamines, spermidine is regarded as the most potent feedback inhibitor of ODC (Rom and Kahana 1994; Hayashi et al. 1996). However, most studies have shown that spermine is the most, and putrescine the least, effective in supporting various biological processes. Recent studies have implicated AZ1 in the regulation of proliferation and apoptosis and suggested that it may act as tumor suppressor (Feith et al. 2001; Fong et al. 2003; Pegg et al. 2003). Since alterations in polyamine homeostasis are gaining significance in clinical translational research, it is important to understand the role of individual polyamines in the regulation of AZ1 synthesis.

We have shown that asparagine induced ODC activity in salt glucose solution by preventing the synthesis of AZ1 (Ray et al. 1999a, b, 2012). Addition of polyamines to cell extracts containing enzymatically active ODC protein did not inhibit its activity. However, the extract from putrescine-treated cells containing high levels of AZ1 significantly inhibited ODC activity suggesting that AZ1 is the predominant regulator of ODC activity in IEC-6 cells. Although intracellular polyamine levels are highly regulated, it is unclear which particular polyamine plays a pivotal role in regulating AZ1 expression and intracellular polyamine homeostasis. In this study we have demonstrated that spermidine regulates AZ1 and, thereby, the levels of intracellular polyamines in IEC-6 cells.

Materials and methods

Cell culture

The IEC-6 cell line was obtained from the American Type Culture Collection (Manassas, VA) at passage 13. The stock was maintained in T-150 flasks in a humidified, 37 °C incubator in an atmosphere of 90 % air–10 % CO_2. The medium consisted of Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL) with 5 % heat-inactivated FBS, 10 μg/ml insulin, and 50 μg/ml gentamicin sulfate. The stock was passaged weekly and fed three times per week. Passages 15–20 were used in the experiments.

Experimental protocol

For most experiments, the cells were taken up with 0.05 % trypsin plus 0.53 mM EDTA in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}. They were counted and plated (day 0) at 6.25×10^4 cells/cm^2 in DMEM plus 5 % dialyzed FBS, 10 μg insulin, and 50 μg gentamicin sulfate/ml, with or without DFMO (5 mM), DEGBG (1 mM), DFMO + DEGBG, or DFMO + DENspm (10 μM). The cells were fed on day 2 with medium. On day 3, the medium was removed, the cells were washed once with Hanks' balanced salt solution, and the medium was replaced with serum-free DMEM with 10 μg insulin and 50 μg gentamicin sulfate/ml. On day 4, the medium was removed and the cells were washed once with Hanks' balanced salt solution followed by the addition of serum free medium with appropriate treatment. Putrescine (10 μM), spermidine (5 μM), spermine (5 μM), cadaverine (10 μM), and DENSpm (5 μM) were used for the treatment of cells except stated otherwise.

S–adenosyl methionine decarboxylase (SAMDC) activity

Cells grown for 4 days in control and DFMO containing media were left untreated or exposed to 5 μM PUT for 4 h. The activity of the enzyme SAMDC was assayed with a radiometric technique in which the amount of $^{14}CO_2$ liberated from DL- [$^{1-14}$C] *S*–adenosylmethionine was estimated (Wang et al. 1992). Briefly, after experimental treatment, the dishes were placed on ice; the monolayers were washed three times with cold Dulbecco's PBS (DPBS); and 0.5 ml of 1 mM Tris buffer (pH 7.4) containing 1 mM EDTA, 0.05 mM pyridoxal phosphate, and 5 mM dithiothreitol was added. The cells were frozen at − 80 °C until assayed. The buffer was supplemented with 30 μM putrescine to stabilize SAMDC. At the time of assay, the cells were thawed on ice, scraped into microfuge tubes, sonicated, and centrifuged at 12,000 g at 4 °C for 10 min. The SAMDC activity of an aliquot of supernatant was incubated in a stoppered tube in the presence of *S*-[*carboxyl*-^{14}C] adenosyl-L-methionine for 30 min at 37 °C. The $^{14}CO_2$ liberated by the decarboxylation of *S*-[*carboxyl*-^{14}C] adenosyl-L-methionine was trapped on a piece of filter paper impregnated with 20 μl of 2 N NaOH, which was suspended in a center well above the reaction mixture. The reaction was stopped by the addition of TCA to a final concentration of 5 %. The $^{14}CO_2$ trapped in the filter paper was measured by liquid scintillation spectroscopy. Aliquots of the supernatant were assayed for total protein by the method described by Bradford (1976). Enzymatic activity is expressed as picomoles of CO_2 per hour per milligram of protein.

Western blot analysis

After experimental treatments, IEC-6 cells were washed twice with DPBS. The DPBS was removed, 500 μl of mammalian protein extraction reagent (MPER) containing protease and phosphatase inhibitors was added, and the plates were frozen overnight and scraped. The cell lysate was centrifuged at 10,000 rpm for 10 min. The supernatant

was used to determine the protein concentration by the BCA method. Total cell protein (30 μg) was separated on 10 % SDS-PAGE and transferred to a PVDF membrane for Western blotting with a specific primary antibody and appropriate secondary antibody labeled with horseradish peroxidase (HRP). Immune complexes were detected by chemiluminescence system. Rabbit polyclonal antibody against AZ1 was a gift from Dr. Senya Matsufuji (Japan) (Ray et al. 2012).

Results

Which polyamine induces AZ1 in IEC-6 cells?

Polyamines are known to induce AZ by a +1 ribosomal frame-shifting mechanism (Matsufuji et al. 1995; Ivanov and Atkins 2007; Rato et al. 2011). However, the role of particular polyamines in the induction of AZ under physiological conditions is not clearly defined. We have shown that putrescine induced AZ1 within 2 h in glucose salt solution (EBSS), and that ASN decreased PUT-induced AZ1 in EBSS (Ray et al. 2012). Furthermore, amino acids like GLN and ASN inhibited, while LYS, VAL, and ORN increased AZ1 in EBSS. To eliminate the influence of the amino acid constituents of the medium having opposing effects on AZ1 induction, we used EBSS to determine the potency of PUT, SPD and SPM to induce AZ1 in the absence of amino acids. The concentrations of polyamines used were in accordance with physiological growth experiments in which the effects of polyamine depletion using DFMO were prevented by these polyamines (Wang et al. 1991; Wang and Johnson 1991; Ray et al. 1999a, b, 2000). Figure 1 shows that 10 μM PUT increased AZ1 in a time-dependent manner in EBSS. SPD (5 μM) caused a higher induction of AZ1 within 1 h compared to that seen with PUT or SPM and that it began to decrease at 3 h. Interestingly, SPM (5 μM) showed lower levels of AZ1 induction compared to PUT and SPD, which decreased after 1 h similar to that seen with SPD, which began to decrease after 2 h. These results indicate that PUT caused a sustained and SPD and SPM caused a transient induction of AZ1 (Fig. 1b). Furthermore, we exposed cells to various concentrations of PUT and found that 5 μM PUT caused a slight induction of AZ1, which increased at 10 μM and remained constant at doses 15 and 20 μM (Fig. 1c). We have shown that 10 μM PUT or 5 μM SPM prevented the effects of polyamine depletion on growth and migration of IEC-6 cells (Wang et al. 1991; Wang and Johnson 1991; Ray et al. 1999a, b, 2000).

Since putrescine is rapidly converted to spermidine and subsequently to spermine, any of the tested polyamines could induce AZ1. To delineate the role of individual polyamines, it is necessary to completely deplete intracellular polyamines by inhibiting both ODC and SAMDC activities. We have shown that the inhibition of ODC by DFMO (10 mM) depleted PUT within 6 h and SPD within 24 h, and decreased SPM to 40 % within 96 h compared to that seen in control cells (McCormack et al. 1993). We have also shown that the inhibition of S-adenosyl-methionine decarboxylase (SAMDC) by diethylglyoxal bis (guanylhydrazone (DEGBG) caused PUT accumulation and completely depleted SPD and SPM in IEC-6 cells (Yuan et al. 2000). The increased levels of PUT were attributed to increased ODC activity. Therefore, we used cells grown in medium containing DEGBG (1 mM) and DFMO (10 mM) + DEGBG (1 mM) for 4 days and exposed them to PUT (10 μM), SPD (5 μM), or SPM (5 μM) for 4 h to determine the role of each polyamine moiety in the induction of AZ1. Results in Fig. 2a show that only SPD induced AZ1 in cells grown in DEGBG or DFMO + DEGBG containing media. However, cells grown in control medium showed robust induction of AZ1 by PUT and SPD (Fig. 2a). Furthermore, cells grown in control and DFMO containing media for 4 days were exposed to 5 μM concentration of either PUT, SPD or SPM for 4 h to determine the expression of AZ1 (Fig. 2b). AZ1 induction was significantly higher in SPD treated control cells compared to those treated with PUT. Control cells treated with SPM for 4 h had very low levels of AZ1 compared to those exposed to PUT or SPD. However, in polyamine-depleted cells (DFMO), both PUT and SPM failed to induce AZ1 within 4 h while SPD effectively induced AZ1 (Fig. 2b). These results indicate that the inability of PUT and SPM to induce AZ1 might be due to the lack of conversion of PUT to SPD by SAMDC and SPM to SPD by SSAT. These results also indicate that the inter-conversion of polyamines by SAMDC and SSAT plays an important role in maintaining the intracellular SPD levels, and thereby, induction of AZ1.

Like cells exposed to PUT (1,4 diaminobutane) for 4 h, cadaverine (1,5 diamino-n-pentane, Cad) induced AZ1; however, monodansyl (mdC) and didansyl Cadavarine (ddC) failed to do so indicating the requirement of free amino groups for the induction of AZ1 (Fig. 3a). Cells preincubated with DFMO or DEGBG for 1 h to inhibit ODC and SAMDC, respectively, were exposed to CAD or SPD for 4 h. The inhibition of ODC by DFMO (1 h) had no effect on AZ1 induction by Cad or SPD (Fig. 3b). However, inhibition of SAMDC by DEGBG (1 h) completely prevented AZ1 induction by Cad and partially inhibited AZ1 in response to Spd (Fig. 3c). These results indicate that the activity of SAMDC is essential for the induction of AZ1.

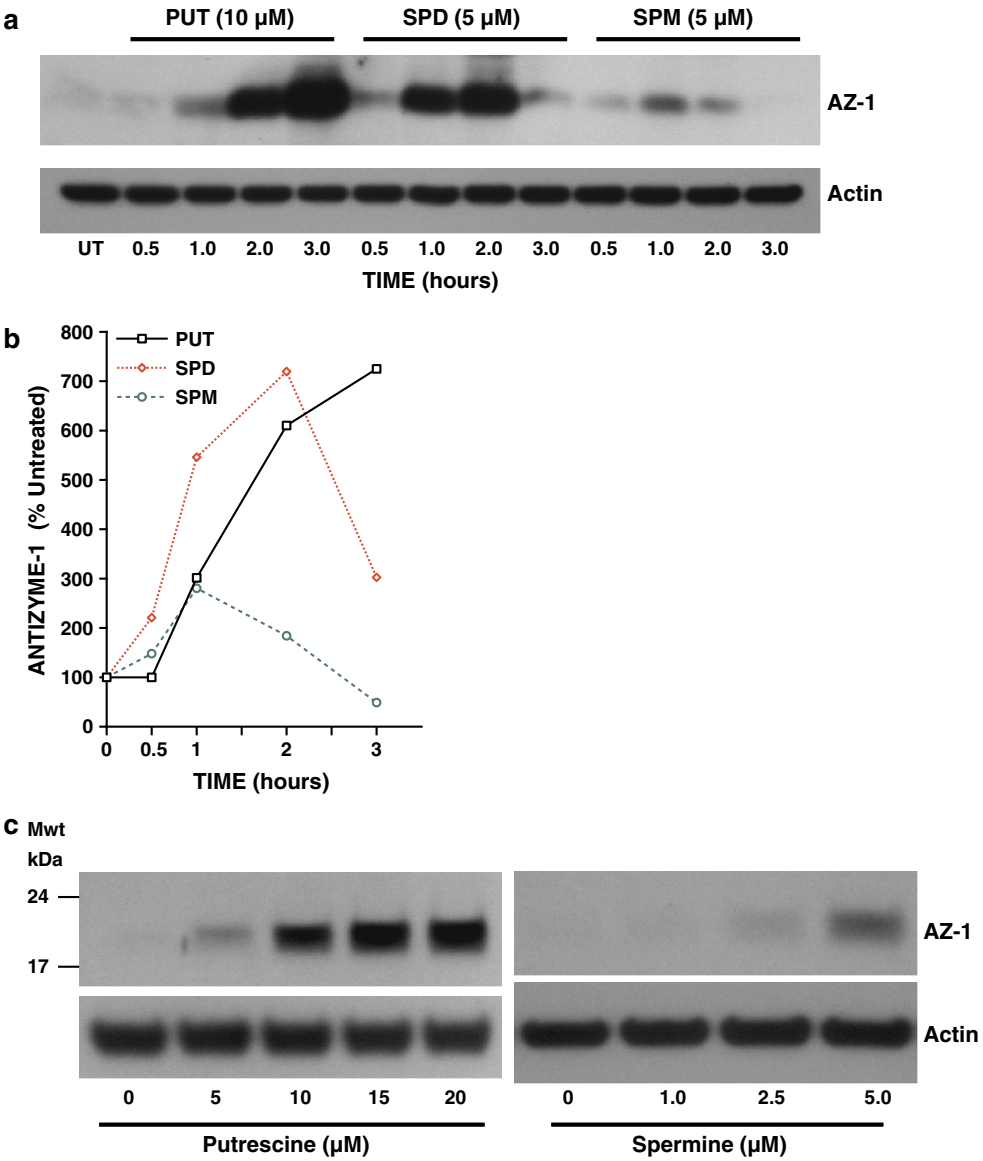

Fig. 1 Serum starved cells grown for 4 days in control medium were incubated with EBSS or EBSS containing polyamines (PUT, SPD, or SPM) for the indicated time period. Cell extracts were analyzed by western blot to determine the levels of antizyme-1 (AZ-1) and actin (**a**). Quantitative analysis of a representative western blot is shown (**b**). Serum starved cells grown for 4 days in control medium were exposed to indicated concentrations of putrescine or spermine for 4 h. Cell extracts were analyzed by western blot to determine the levels of antizyme-1 (AZ-1) and actin (**c**). Blots shown are representative of 3 observations

Since exposure of DEGBG treated cells to PUT, SPM, and CAD failed to induce AZ1, while SPD induced AZ1 in these cells. It appears that SPD is essential for the induction of AZ1. However, it is not clear why PUT failed to induce AZ1 in cells grown in the presence of DFMO for 4 days. We predicted that polyamine depletion by DFMO might influence the activity of SAMDC and, thereby, levels of intracellular SPD required for the induction of AZ1. Therefore, AZ1 levels and SAMDC activity were determined using cells grown for 4 days in control and DFMO containing medium exposed to 5 μM PUT for 4 h. Cells grown in DFMO containing medium had significantly higher SAMDC activity compared to control cells (Fig. 4a). However, 5 μM PUT increased SAMDC activity about sixfold in polyamine-depleted cells (DFMO group) compared to that seen in control cells exposed to PUT (Fig. 4a). These results indicate that rapid conversion of PUT to SPD and subsequently to SPM decreased the SPD pool needed for the induction of AZ1 in these cells. Thus, increasing the activity of SSAT by DENspm in polyamine-depleted cells should convert SPM to SPD and restore the ability of PUT or Cad to induce AZ1. Results in Fig. 4b clearly demonstrate that cells grown in the presence of DFMO + DENspm restored the AZ1 induction by both PUT and Cad to the levels seen with control cells exposed to these polyamines. These results suggest that together the activities of ODC, SAMDC, and SSAT regulate intracellular polyamines, and the level of SPD determines the extent of AZ1 induction. Thus, spermidine acts as a sensor and AZ1 as a response element that controls intracellular polyamine homeostasis.

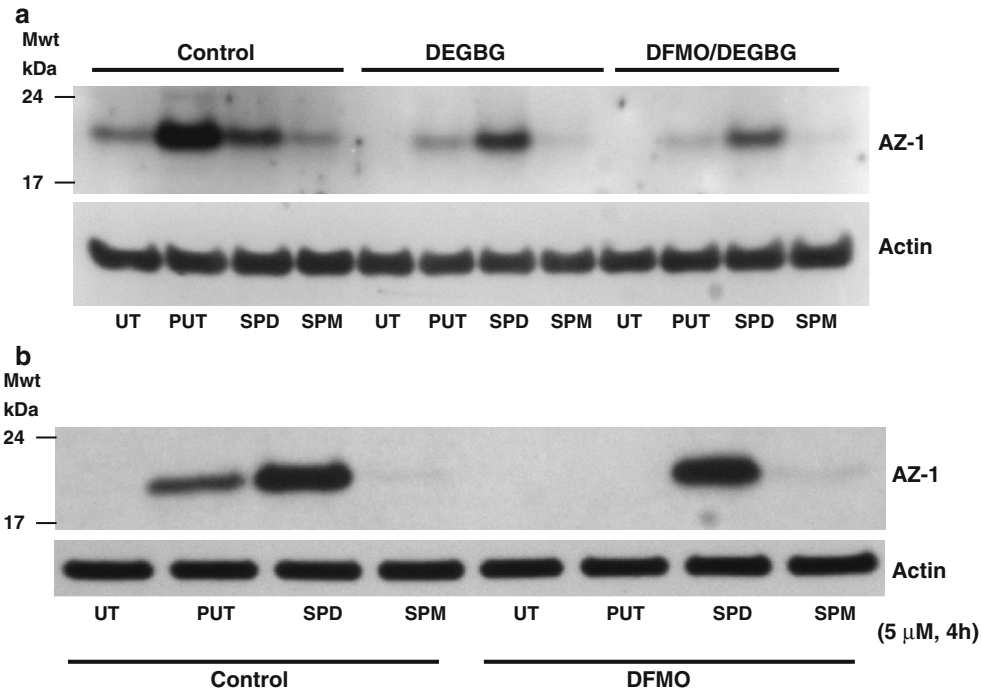

Fig. 2 **a** Serum starved cells grown for 4 days in control, DEGBG or DFMO + DEGBG containing medium were left untreated (UT) or incubated with putrescine (10 μM, Put), spermidine (5 μM, SPD), or spermine (5 μM, SPM) for 4 h. Cell extracts were analyzed by western blot to determine the levels of antizyme-1 (AZ-1) and actin. **b** Serum starved cells grown for 4 days in control or DFMO containing medium were left untreated (UT) or incubated with putrescine (5 μM, Put), spermidine (5 μM, SPD), or spermine (5 μM, SPM) for 4 h. Cell extracts were analyzed by western blot to determine the levels of antizyme-1 (AZ-1) and actin. Blots shown are representative of 3 observations

Discussion

Studies from our group have examined the role of polyamines in the growth and repair of the GI mucosal cells in both rats and cultured normal intestinal cells (Wang and Johnson 1990, 1991; McCormack et al. 1993). The mammalian GI epithelium is especially suitable to study the control of proliferation because of its rapid and continuous renewal (Cheng and Leblond 1974; Yang et al. 1984; Hall et al. 1994). Inhibition of ODC by DFMO depleted polyamines and inhibited the growth of a nontransformed line originally developed from rat crypt cells (IEC-6) by Quaroni et al. (1988) and also prevented the normal repair of mucosal stress ulcers (Wang and Johnson 1990). Depletion of intracellular polyamines inhibited apoptosis accompanied by cell cycle arrest and inhibited cell migration suggesting that polyamines play a crucial role in the regulation of mucosal growth (Ray et al. 1999a, b, 2000, 2003; McCormack et al. 1993).

Polyamines are transported into cells and are obtained by enterocytes from food and intestinal bacteria. Optimal intracellular polyamine levels are maintained by tightly regulated synthesis, degradation, uptake, and release (Grillo 1985; Seiler et al. 1996). Polyamine biosynthesis begins with the conversion of ornithine to PUT by ODC. Although ODC is considered to be the rate-limiting step in the biosynthesis of polyamines, SPD and SPM levels are governed by the activity of SAMDC. Thus, the activities of these two enzymes determine the levels of intracellular spermidine and spermine. ODC activity increases rapidly in response to a variety of stimuli, including growth factors, insulin, stress, and certain amino acids (Rinehart et al. 1985; Mitchell et al. 1998; Ray et al. 1999a, b). ODC has one of the shortest half-lives of any mammalian enzyme and is feedback regulated by polyamines, which induce the synthesis of an inhibitor called antizyme (AZ) (Hayashi et al. 1996). The active form of ODC is a homodimer composed of two 53 kDa subunits. The active site is formed at the interface of the two monomers, the monomers themselves having no enzymatic activity. Antizyme (AZ) inhibits ODC activity by binding to the monomers to prevent the formation of the active homodimer (Pegg 2006).

Although AZ mRNA is constitutively expressed (Coffino 2001), its translation is highly induced in response to polyamines by a +1 ribosomal frameshift (Matsufuji et al. 1995; Ivanov and Atkins 2007). AZ also feedback inhibits the uptake of polyamines by cells. Mitchell et al. (2007) showed that polyamine analogues induced AZ1 and also decreased their own uptake and the uptake of other polyamines (Mitchell et al. 2007). Thus, polyamines can regulate their own synthesis and levels via AZ1. The sensor of polyamine levels in cells is a ribosomal decoding event that is required for antizyme synthesis. AZ mRNAs contain two overlapping reading frames, a short ORF1 and a long ORF2, which lacks a translational initiation codon present in the +1 frame relative to ORF1. When the termination codon for ORF1 enters the A-site of translating ribosomes, the levels of free polyamines determine the outcome of AZ synthesis. At a low concentration of polyamines efficient

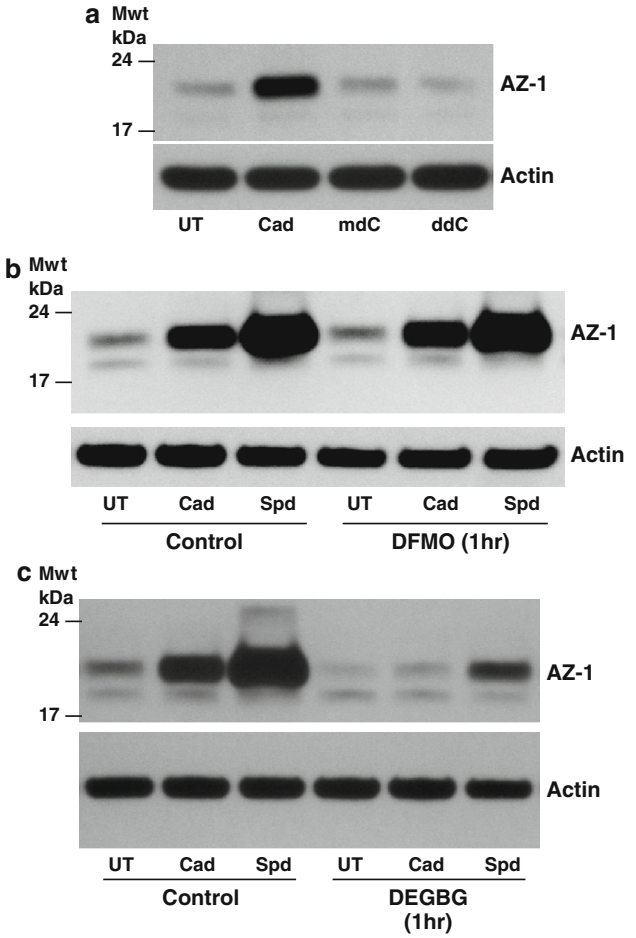

Fig. 3 **a** Serum starved cells grown for 4 days in control medium were left untreated (UT) or incubated with cadavarine (Cad), monodansylcadavarine (mdC), or didansylcadavarine (ddC) for 4 h. Serum starved cells grown for 4 days in control medium were left untreated (UT) or preincubated with DFMO **b** or DEGBG **c** for 1 h followed by the exposure to cadavarine (Cad) or spermidine (Spd) for 4 h. Cell extracts were analyzed by western blot to determine the levels of antizyme-1 (AZ-1) and actin. Blots shown are representative of 3 observations

termination at the end of ORF1 prevents the entry of ribosomes to ORF2, and synthesis of antizyme stops. At high levels of polyamines ribosomes shift to the +1 reading frame and resume standard decoding and synthesize antizyme. Thus, the recoding event regulates AZ synthesis (Matsufuji et al. 1995; Ivanov and Atkins 2007).

AZ1 is degraded by the proteasome, a process influenced by polyamines. Palanimurugan et al. proposed that changes in the levels of polyamines below threshold not only prevent synthesis of AZ1 but also rapidly deplete existing AZ1 and, thereby, allow the resumption of ODC activity. Thus, AZ1 plays a central role in the regulation polyamine homeostasis (Palanimurugan et al. 2004).

Numerous reports indicate that PUT, SPD, and SPM induce AZ synthesis by frameshifting. However, in cells with active polyamine metabolism it is impossible to ascertain which polyamines stimulate AZ synthesis under physiological conditions. Furthermore, synthesis of antizyme by putrescine and spermine in in vitro experiments could be due to the inter-conversion of polyamines by the active SAMDC and SSAT enzymes in the cell extracts. *In vitro* experiments using extracts prepared form the cells lacking SAMDC and SSAT activities or mutant cells could provide clarity regarding the role of individual polyamines in the synthesis of AZ. Recently, Rato et al. used a *S. cerevisiae* quadruple gene knockout strain to prevent interconversion of exogenous polyamines and measured ribosomal frameshifting using a luciferase reporter plasmid. They demonstrated that both SPD and SPM induced AZ frameshifting, while high concentrations of PUT were required for AZ frameshifting (Rato et al. 2011). Although this approach eliminated interconversion of polyamines, the quadruple gene knockout mutant required polyamines for normal growth. Therefore, the issue of endogenous AZ1 levels and their effect on polyamine uptake and efflux under physiological conditions is unknown. Reporter-based assays can provide information regarding the regulatory role of polyamines, but they must address the effects of altered levels of AZ on polyamine uptake.

It is noteworthy that there is only one gene for antizyme in yeasts, while mammalian cells have 3 different AZ genes (AZ1, AZ2, and AZ3). Furthermore, polyamines induce or repress enzymes involved in polyamine biosynthesis depending upon the pool of free intracellular polyamines. In this study, we attempted to analyze AZ1 expression in intestinal epithelial cells to clarify the role of polyamines. We have shown that AZ1 is not only regulated at the level of frameshifting but also by amino acids involving TORC2 activity (Ray et al. 2012). Amino acids like ASN and GLN repress while LYS, ARG, and VAL stimulate AZ1synthesis in EBSS (Ray et al. 2012; Ray and Johnson 2013). Addition of 10 μM PUT stimulated AZ1 expression in EBSS, and both ASN and GLN decreased it. Thus, amino acids influence the levels of polyamine-induced AZ1. It is intriguing that both ASN and GLN induce ODC activity, and thereby, increase polyamine levels but inhibit AZ1 synthesis. These results indicate that besides +1 frameshifting, AZ1 synthesis involves additional regulatory mechanisms.

We used EBSS to eliminate the effects of amino acids on AZ1 expression. Our results clearly demonstrate that all three polyamines tested induced AZ1; however, induction occurred earlier with 5 μM SPD (Fig. 1). AZ1 decreased after 2 h exposure to SPD and SPM, while it remained higher in the case of PUT. It is important to note that the concentrations of both the SPD and SPM were 5 μM, while that of PUT was 10 μM. The levels of AZ1 induction with exposure of the cells to 5 μM PUT were slightly higher

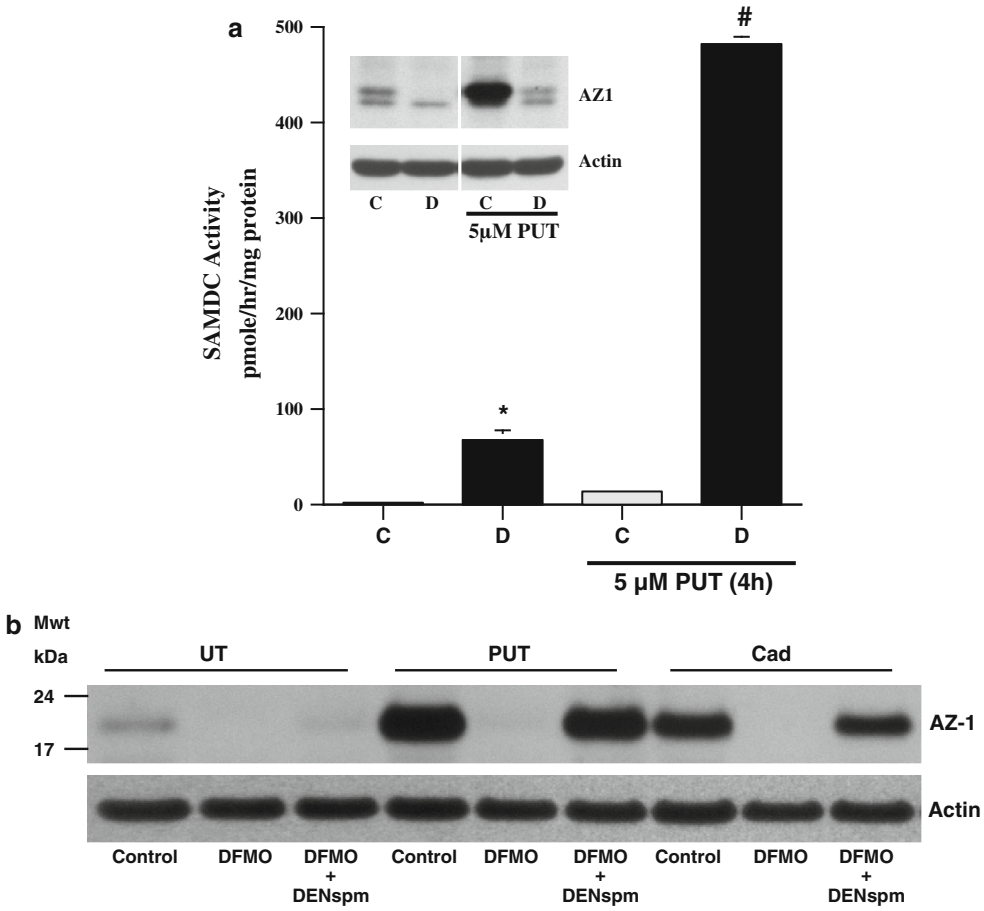

Fig. 4 **a** Serum starved cells grown for 4 days in control C or DFMO D containing medium were left untreated or exposed to 5 μM putrescine (PUT) for 4 h. Cell extracts were used to determine SAMDC enzymatic activity. Western blot analysis was performed to determine the levels of antizyme-1 (AZ-1) and actin. **b** Serum-starved cells grown for 4 days in control, DFMO or DFMO + DENspm containing medium were left untreated (UT) or exposed to putrescine (PUT) or cadavarine (Cad) for 4 h. Cell extracts were used to determine the levels of antizyme-1 (AZ-1) and actin. Blots shown are representative of three observations

compared to those of untreated group, which increased at 10 μM and reached to a maximum levels at 15 and 20 μM (Fig. 1c). With equimolar concentrations of polyamines (5 μM) SPD increased AZ1 more than PUT, and SPM failed to do so indicating that SPD might be the predominant regulator of AZ1 (Fig. 2b, control). Furthermore, the sustained increase in AZ1 stimulated by PUT could be attributed to the continued downstream flow of PUT to SPD. AZ1 induction by SPD in polyamine-depleted cells confirmed that only levels of intracellular SPD regulate AZ1 (Fig. 2b). Inhibition of SAMDC by DEGBG completely depleted SPD and SPM and caused accumulation of PUT in IEC-6 cells and stimulated ODC activity (Yuan et al. 2000). Thus, AZ1 levels in DEGBG treated cells can be considered as a readout for the effectiveness of PUT for AZ1 induction. Results in Fig. 2a show that AZ1 levels were undetectable in cells grown in the presence of DEGBG and that the addition of 10 μM PUT failed to induce it. These results suggest 1) increased intracellular PUT levels are not sufficient to induce AZ1, 2) conversion of PUT to SPD by SAMDC is essential for the induction of AZ1, and 3) like PUT, SPM alone failed to induce AZ1. Furthermore, as in DEGBG treated cells, only SPD induced AZ1 in cells grown in the presence of both the inhibitors of ODC and SAMDC. This clearly indicates that only intracellular SPD levels regulate AZ1 expression.

It is known that PUT stimulates the activity of SAMDC and also stimulates the processing of the SAMDC proenzyme into two subunits (Kameji and Pegg 1987; Stanley and Pegg 1991; Wang et al. 1992). Furthermore, the inhibition of polyamine synthesis causes a compensatory increase in SAMDC activity. However, the supply of excess polyamines decreases its activity to nearly zero (Tabor and Tabor 1984; Heby and Persson 1990a, b; Wada and Shirahata 2010). Wang et al. showed that PUT increased SAMDC activity in IEC-6 cells (Wang et al. 1992). Although PUT can be converted to SPD by SAMDC in polyamine-depleted cells, it is intriguing that PUT could not induce AZ1 in polyamine-depleted cells. We speculated that the excessive induction of SAMDC activity by PUT in polyamine-depleted cells rapidly converted PUT to SPD and further into SPM preventing the accumulation of threshold levels of SPD required for the induction of AZ1 (Figs. 1, 2). The above idea was further supported by the fact that SPM induced less AZ1 in control cells and failed to induce it in DFMO + DEGBG treated cells (Fig. 2).

Indeed, polyamine depletion by DFMO increased SAMDC activity, which was further enhanced 5- to 6-fold by PUT (Fig. 4a). Hibasami et al. have shown that spermidine and spermine biosynthesis is limited by the availability of decarboxylated S-adenosylmethionine (dcSAM) (Hibasami et al. 1980). Thus, an ample supply of dcSAM, due to a sixfold activation of SAMDC by PUT in DFMO treated cells, might stimulate spermidine/spermine synthase activity and rapidly convert PUT into SPM, without producing a significant increase in the levels of SPD.

Spermidine/spermine–N1-Acetyltransferase (SSAT), a highly regulated enzyme plays a crucial role in maintaining polyamine homeostasis (Casero and Marton 2007; Pegg 2008; Wada and Shirahata 2010). SSAT acetylates and converts SPM and SPD to SPD and PUT respectively. Polyamines and their analogs BENspm and DENspm increased SSAT activity and depleted SPM and SPD levels (Svensson et al. 1993; Uimari et al. 2009; Wada and Shirahata 2010; Tian et al. 2012). We reasoned that stimulation of SSAT activity by DENspm in polyamine-depleted cells treated with PUT should convert SPM to SPD and may allow AZ1 induction. As expected, stimulation of both the SAMDC activity by PUT and SSAT activity by DENspm restored AZ1 induction by PUT. These results clearly indicate that the levels of intracellular SPD act as a sensor for the stimulation of AZ1 synthesis. Results depicted in Fig. 3 showing that cadavarine (Cad) induced AZ1 while mono- and di-dansylcadavarine failed to so indicate that primary amine groups are essential for the AZ1 induction and suggest that SAMDC activity may be required for the induction of AZ1. Unlike DFMO, inhibition of basal level SAMDC activity by DEGBG (1 h) prevented Cad from inducing AZ1 and decreased its induction by SPD (Fig. 3b, c). Although it is unclear why SAMDC inhibition (1 h) decreased SPD-induced AZ1, it emphasizes the importance of SAMDC activity in the regulation of AZ1. Further analysis of SAMDC activity and polyamine uptake studies might provide the answer.

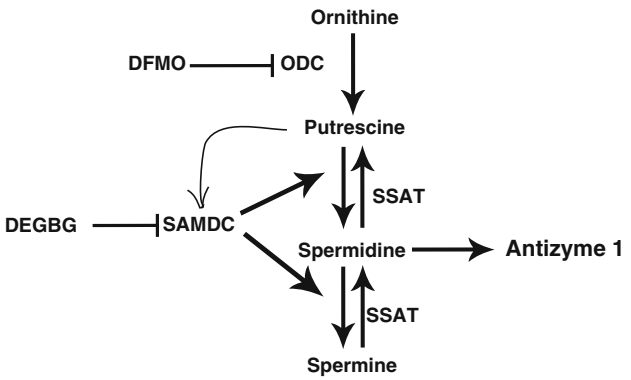

Fig. 5 Coordinated regulation of ODC, SAMDC and SSAT controls intracellular spermidine and AZ-1 levels to maintain cellular homeostasis

Together, results presented in this study clearly show that intracellular SPD levels are sensed by the ribosomal frameshifting mechanism controlling AZ translation (Fig. 5). Thus, AZ1 induction/repression in response to intracellular SPD controls the activity of ODC, and thereby, the levels of PUT which subsequently induce SAMDC allowing the conversion of PUT to SPD and SPM. Thus, coordinated balance of the activities of ODC, SAMDC, and SSAT control AZ1, and thereby, ODC activity, polyamine uptake, and polyamine homeostasis. This is particularly important for homeostasis of the gastrointestinal epithelium because polyamines are synthesized both by the enterocytes and by the gut microflora.

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Conflict of interest The authors declare that they have no conflict of interest.

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