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Regulation of spermidine/spermine N^1-acetyltransferase by intracellular polyamine pools

Evidence for a functional role in polyamine homeostasis

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Through its role in polyamine acetylation and the back-conversion pathway, spermidine/spermine N^1-acetyltransferase (SSAT) has the potential to control intracellular polyamine pools by facilitating their catabolism and/or excretion. The possibility that the enzyme is subject to regulation by intracellular polyamine pools was investigated in MALME-3 human melanoma cells. Increases in intracellular polyamine pools by treatment with 3 μM exogenous spermidine or spermine for 48 h caused SSAT activity to increase 111% and 226%, respectively, and SSAT-specific mRNA to rise 19% and 66%, respectively. Decreases in polyamine pools by treatment with inhibitors of polyamine biosynthesis caused SSAT activity to decrease by 46% and mRNA to fall by 89%. Both SSAT activity and mRNA were more sensitive to changes in spermine than spermidine. The identification of a positive regulatory relationship between SSAT and intracellular polyamine pools further implicates this enzyme in a proposed model for polyamine pool homeostasis.

Polyamine; Polyamine homeostasis; Spermidine; Spermine; Spermidine/spermine N^1-acetyltransferase

1. INTRODUCTION

Under steady-state growth conditions, the intracellular polyamine pools of any given cell type are maintained within a relatively narrow range, the upper limits seemingly determined by the potential cellular toxicity of polyamine excess and the lower limits by the polyamine requirement for cell growth. To a large degree, this range is controlled by changes in polyamine biosynthesis and uptake, both of which are negatively regulated by intracellular polyamine pools [1–3]. Through their respective functions, uptake and biosynthesis have the potential to actively raise polyamine pools. The polyamine acetylating enzyme, spermidine/spermine N^1-acetyltransferase (SSAT), serves as a rate-limiting step in a polyamine back-conversion pathway [4]. Because polyamine acetylation and the back-conversion pathway facilitate polyamine excretion and catabolism [4–6], respectively, SSAT has the potential to participate in polyamine pool homeostasis by lowering intracellular polyamine pools [5].

At present, the regulatory relationship between intracellular polyamine pools and SSAT gene expression is not well defined. SSAT activity is known to be inducible by increases in intracellular polyamine pools [7–9]. More impressive increases in SSAT activity are produced with structurally similar analogs of polyamines [8–12]. Analog-induced increases in SSAT activity have recently been found to be accompanied by the accumulation of SSAT-specific mRNA [13–16]. In this report, we demonstrate that the natural polyamines can mediate increases and decreases in SSAT activity and mRNA levels. These findings further substantiate a probable cellular role for SSAT in polyamine pool homeostasis. Portions of this work have been reported in abstract form [17].

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Abbreviations: AMA, *S*-(5′-deoxy-5-adenosyl)-methylthioethylhydroxylamine; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; PUT, putrescine; SAMDC, *S*-adenosylmethionine decarboxylase; SPD, spermidine; SPM, spermine; SSAT, spermidine/spermine-N^1-acetyltransferase.

2. EXPERIMENTAL

The human melanoma cell line, MALME-3M, was chosen for study because of its extreme SSAT inducibility by polyamine analogs [12,18]. Cells were grown as monolayers in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% NuSerum (Collaborative Research Products, Bedford, MA), 2% HEPES/MOPS buffer, 60 U/ml penicillin and 60 μg/ml streptomycin. Cells were treated with polyamines and/or inhibitors 24 h after plating. In experiments where polyamines were added, the medium was supplemented with 1 mM aminoguanidine to inhibit serum amine oxidase [19].

The relationship between intracellular polyamines and SSAT activity and mRNA levels was evaluated by selectively increasing and

decreasing individual polyamine pools. PUT was found to have a minimal regulatory effect on SSAT activity or mRNA (data not shown). Pools were increased by treatment with 3 μM exogenous SPD or SPM for 48 h and decreased by treatment with specific and irreversible inhibitors of polyamine biosynthetic enzymes in the presence and absence of exogenous polyamines. Typically, the ODC inhibitor, DFMO (at 1 mM), depletes PUT and SPD pools [20], and the SAMDC inhibitor, AMA (at 0.1 mM) elevates PUT pools and lowers SPD and/or SPM pools [21]. Addition of SPD or SPM (at 3 μM to avoid toxicity) to the combination of DFMO and AMA selectively augmented those particular polyamine pools. Cells were treated with the inhibitors for 48 h.

SSAT activity was measured via biochemical assay as previously described [22]. It should be noted that this enzyme assay also detects other non-specific acetylase activities which, under basal conditions, account for about 70% of the total measured activity in MALME-3M cells, as determined by immuno-precipitation [12]. Polyamine pools were determined on perchloric acid cell extracts using a high performance liquid chromatography system described elsewhere [12].

RNA was extracted with guanidinium isothiocyanate on cesium chloride gradients, electrophoresed with ethidium bromide on 1.5% agarose formaldehyde gels, transferred to Duralon membrane (Stratagene, LaJolla, CA), crosslinked (Stratagene) and probed with ^{32}P-labeled human SSAT cDNA (provided courtesy of R. Casero Jr., Johns Hopkins Oncology Center, Baltimore, MD; [14,23]). Transferred RNA was visualized by UV irradiation to assess the integrity of 28 S and 18 S rRNA and uniformity of loading for each sample. Northern blots were quantitated by densitometry of phosphoimaging analysis (Phosphoimager, Molecular Dynamics, Sunnyvale, CA).

3. RESULTS

A comparison of the effects of exogenous polyamines on SSAT activity in MALME-3M cells treated for 48 h revealed that exogenous SPD and SPM increased enzyme activity (Table I) while PUT has a minimal effect (data not shown). Treatment with SPD increased SPD pools by 47% and SSAT activity by 111% while SPM increased SPM pools by 40% and SSAT activity by 226%. Due to various compensatory responses (see section 4), SPM decreased when SPD increased and vice versa, so that the overall polyamine content of treated cells remained relatively constant during treatment (i.e. 2.10–2.25 nmol/10^6 cells). Both treatments resulted in the accumulation of N^1-acetylSPD, a product of SSAT, indicating that the induced enzyme was functional in the cell. The effect of SPM on SSAT activity and N^1-acetylSPD was consistently greater than that of SPD and, although subtle, this trend was reinforced by similar increases in SSAT mRNA.

Northern blot analysis of mRNA from treated cells revealed three bands which hybridized to the SSAT cDNA probe (~ 3.5, 1.5 and 1.3 kb). The 3.5 kb SSAT transcript was typically found in much lower abundance than the 1.5 and 1.3 kb species, and appears to represent an unprocessed precursor based on its size similarity to the SSAT gene and because it is the most prominent mRNA form in nuclear preparations [16]. For the purpose of quantitating SSAT mRNA levels, densitometric measurements of the B and C bands were combined. As with SSAT activity, treatment with exogenous SPD or SPM consistently increased SSAT mRNA, with SPM being the more effective (Fig. 1, Table I).

In addition to being up-regulated by increases in polyamine pools, SSAT was found to be down-regulated by decreases in polyamine pools, as achieved by treatment with the ODC inhibitor, DFMO, and the SAMDC inhibitor, AMA. Treatment with 1 mM DFMO depleted PUT pools and reduced SPD by 40% while leaving SPM pools relatively unaffected (Table I). Under these conditions of SPD depletion, SSAT activity and mRNA were reduced by 37% and 51%, respectively. Treatment with AMA increased PUT pools 16.5-fold, and decreased SPM pools by 60% while SPD pools were relatively unchanged. This treatment produced a 36% reduction in SSAT activity and a 74% decrease in SSAT mRNA. Thus, inhibiting polyamine biosynthesis decreases SSAT activity. It should be noted that since the polyamine pools following treatment with SPM or

Table I

Modulation of polyamine pools, SSAT activity and mRNA in MALME-3M cells

Treatment (48 h)	Polyamine Pools[a] (nmol/10^6 cells)				SSAT activity[b] (% control)	SSAT RNA[d] (Bands B+C; % control)
	PUT	SPD	SPM	N^1-AcSPD		
Control	0.07 ± 0.013	0.84 ± 0.083	1.26 ± 0.023	0.04 ± 0.004	100	100
3 μM SPD	nd[c]	1.19 ± 0.095	1.04 ± 0.182	0.94 ± 0.362	211 ± 41	119
3 μM SPM	nd	0.32 ± 0.032	1.77 ± 0.152	0.23 ± 0.063	326 ± 110	166
1.0 mM DFMO	0.01 ± 0.015	0.53 ± 0.066	1.55 ± 0.271	0.01 ± 0.014	63 ± 15	49
0.1 mM AMA	1.19 ± 0.107	0.80 ± 0.046	0.55 ± 0.091	nd	64 ± 12	26
AMA+DFMO	0.34 ± 0.039	0.92 ± 0.072	0.71 ± 0.096	nd	54 ± 10 (100)[e]	19 (100)[e]
AMA+DFMO+SPD	0.19 ± 0.070	2.25 ± 0.459	0.45 ± 0.106	0.39 ± 0.089	107 ± 48 (198)	121 (636)
AMA+DFMO+SPM	0.01	0.23	1.91	0.14	303 ± 81 (561)	158 (832)

[a]Mean ± S.D. of 3 flasks; except AMA+DFMO+SPM, mean of 2 flasks.
[b]Mean ± standard deviation from duplicate determinations of 3–5 experiments; control SSAT activity, 44 pmol/min/mg.
[c]nd, not detectable.
[d]Relative densitometric scanning of representative Northern blot probed with human SSAT cDNA.
[e]Percent of DFMO+AMA value.

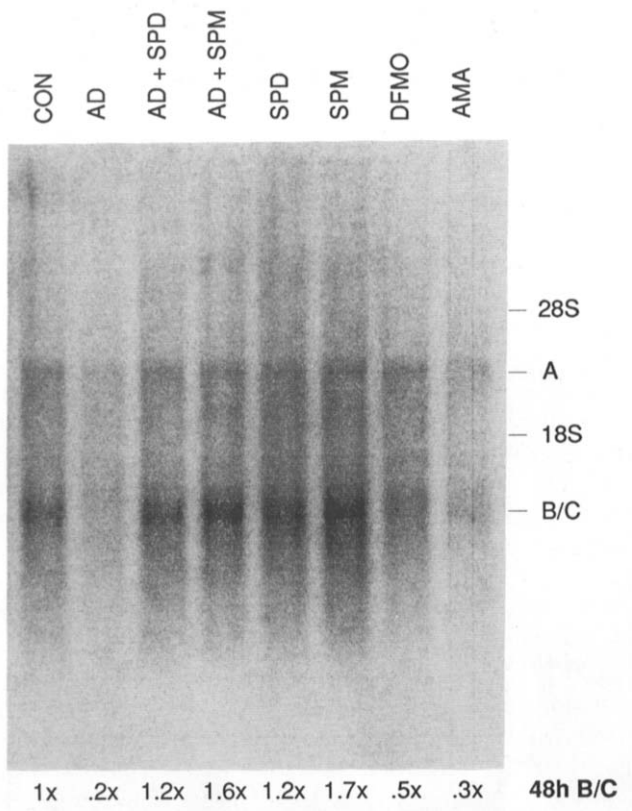

Fig. 1. Northern blot phosphoimage of MALME-3M human melanoma cells treated for 48 h with exogenous SPD and SPM in the presence and absence of the biosynthetic inhibitors, 0.1 mM AMA (A) and/or 1.0 mM DFMO (D) (AMA plus DFMO = AD). Blots loaded with 15 μg RNA per lane, were probed with human SSAT cDNA. Typically, the SSAT cDNA hybridizes with three transcripts [16], but in this phosphoimage, bands B and C are not resolved. Values at the bottom are based on densitometry of phosphoimage analysis of band B/C relative to control RNA (100%).

DFMO were very similar, the different SSAT responses reflect the direction in which the pools were pressured to change during treatment. As will be discussed, the final polyamine pools represent the net effects of several compensatory responses (including that of SSAT).

In order to further confirm that pool modulation was regulating SSAT mRNA and activity, experiments were performed in which the inhibitors, DFMO and AMA, were used in combination with exogenous SPD and SPM to examine the combined effects of polyamine depletion with selective pool maintenance. Overall, the DFMO/AMA inhibitor combination was far less effective in lowering polyamine pools in MALME-3M cells than in L1210 leukemia cells where near-total SPD and SPM pool depletion has been reported [21]. In part, this is probably because the MALME-3M cells have a doubling time that is 4-fold longer than L1210 cells so that during a 48 h incubation, there is less dilution (depletion) of pools by cell division. In MALME-3M cells, the inhibitor combination lowered SPM pools by 56% and increased SPD pools by 10% while preventing PUT pools from rising by as much as with AMA alone. This combination treatment produced the greatest decrease in both SSAT activity and mRNA (46% and 81%, respectively) seen with any treatment. Although greater pool perturbations were obtained with individual inhibitors (i.e. AMA), it is again relevant to consider that the polyamine pools measured at the end of treatment with two inhibitors probably reflect responses which attempt to maintain a normal pool profile. Results with the inhibitor–polyamine combinations confirmed earlier indications that, relative to SPD, SPM exhibited a greater capacity to induce SSAT. The combination of DFMO, AMA and SPD markedly elevated the SPD pool while further lowering the SPM pool. Under these conditions, SSAT activity increased by 10% while mRNA levels increased by 21%. When considered relative to the DFMO+AMA combination, these same values increased to 98% and 536%, respectively. Substitution of SPM for SPD in the DFMO+AMA combination lowered intracellular SPD pools further while markedly increasing the SPM pool. With this treatment, SSAT activity increased by 203%, and mRNA rose by 58%. Relative to the DFMO+AMA combination, these values increased by 461% and 732%, respectively. These trends were consistently obtained in three separate experiments. Thus, under the combined pressures of polyamine depletion and polyamine excess, the predominant SSAT response was in the direction of up-regulation, presumably to counter polyamine excess.

4. DISCUSSION

SSAT activity was found to be sensitively regulated by both increases and decreases in intracellular polyamine pools. The magnitude of these changes is probably underestimated since only about 30% of the basal activity (and probably less than that under conditions of down-regulation) represents authentic SSAT activity in MALME-3M cells [12]. Although increases in SSAT activity in response to polyamines have been previously reported [7–9], this present study reveals that decreases in SSAT activity occur in response to inhibition of polyamine biosynthesis and the subsequent lowering of polyamine pools. This finding is substantiated by parallel decreases in SSAT-specific mRNA. These data indicate that, under basal conditions, steady-state levels of SSAT expression are probably maintained in a partially up-regulated state by basal polyamine pools since a decline in these pools results in a decrease in SSAT activity and mRNA. Polyamine transport and the polyamine biosynthetic enzymes, ODC and SAMDC, seem to be similarly maintained under basal conditions but via an inverse relationship since a decline in pools causes an increase in their expression [1,26] and vice versa [1,2].

The observation that SSAT induction can be accompanied by increases in mRNA accumulation was first

directly demonstrated in studies with the polyamine analogs which subsequently lead to the cloning of the SSAT cDNA from human [14,23], mouse [27] and hamster [28] sources. This present study illustrates that the natural polyamines can also produce increases in SSAT mRNA. The significance of this observation is 3-fold: (i) it indicates that the observed changes in SSAT activity are truly regulatory responses as opposed to direct effects on the enzyme; (ii) it strongly suggests a probable role for SSAT in polyamine pool homeostasis (as discussed below); and (iii) it confirms that expression of the SSAT gene is positively regulated by the natural polyamines at the level of mRNA. This last feature renders SSAT unique among known genes. Although the steady-state levels of c-*myc* and c-*fos* in COLO 320 cells have been shown to decrease with polyamine depletion [29], the inverse relationship, whereby mRNA levels increase with the addition of polyamines to intact cells, has not been demonstrated. Also, when these same cells are treated with a polyamine analog, N^1,N^{12}-bis(ethyl)spermine, which is known to mimic natural polyamines in down-regulating ODC [30] and transport [31,32] and by up-regulating SSAT [8–12], the steady-state mRNA levels of these proto-oncogenes decreases instead of increasing [29]. Thus, unlike SSAT, the expression of these genes does not appear to be regulated by polyamines via a truly positive relationship. The only other gene which is known to be positively regulated by exogenous polyamines in intact cells is an ODC-binding protein termed antizyme, which contributes to the rapid degradation of that enzyme [33]. Its induction, however, is not mediated by increases in antizyme-specific mRNA but by changes at the level of translation [34].

As noted above, the nature of the regulatory relationship between polyamine pools and SSAT gene expression, as indicated by activity and mRNA, is distinctly positive – as pools increase, SSAT also increases and vice versa. The former was apparent from experiments with exogenous polyamines and the latter by pool depletion with specific enzyme inhibitors. The biosynthetic enzymes, ODC and SAMDC [1], and the polyamine transport system [2,31,32,35], are also sensitively up-regulated and down-regulated by changes in intracellular polyamine pools. Unlike SSAT, however, the regulatory relationships for these systems are negative in nature – as pools decrease, enzyme and transport activities increase and vice versa. Taken together, these various systems can be integrated into a model for polyamine homeostasis (Fig. 2) consisting of three key effectors: the biosynthetic enzymes (ODC and SAMDC), the transport apparatus, and SSAT. All three are characterized by being specific for polyamines, rate-limiting in their respective activities and pathways, and by being sensitively up-regulated or down-regulated by changes in intracellular polyamine pools. The biosynthetic enzymes and the transport system can actively respond to polyamine *insufficiency* by synthesizing and taking-up,

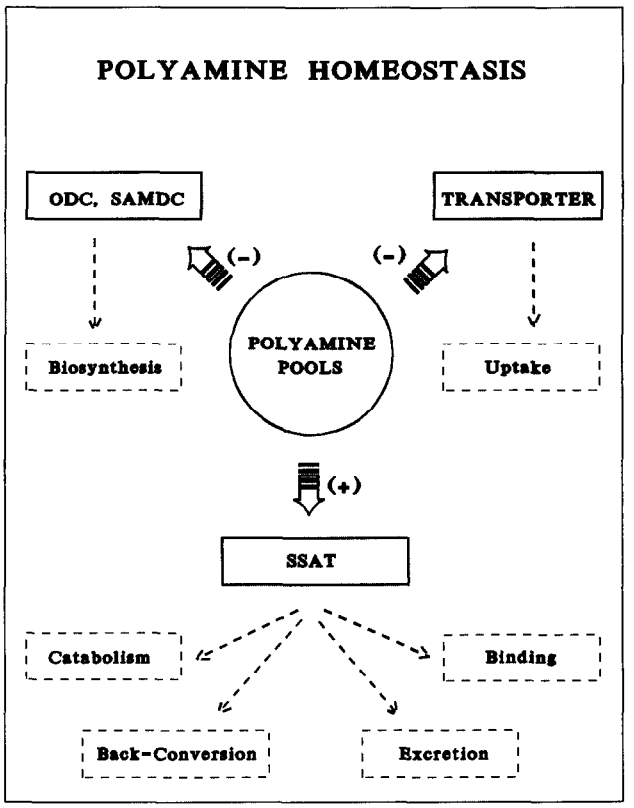

Fig. 2. Proposed model for polyamine homeostasis showing the critical role of polyamine pools in regulating three homeostatic effectors (solid boxes) and the various activities (broken boxes) which the latter control. SSAT differs from other effectors in that it has the potential to control several activities. Polyamine binding is included among these since acetylation reduces the net positive charge of a polyamine by one and would therefore be expected to limit its binding capabilities. The parentheses beside the large arrows indicate the nature of the regulatory relationship between the pools and the effector: in a positive relationship, the effector is *down-regulated* when pools go down and vice versa; in a negative relationship, the effector is *up-regulated* when pools go down and vice versa. The model predicts that depletion of polyamine pools by enzyme inhibitors would up-regulate the putative transporter and the biosynthetic enzymes, ODC and/or SAMDC (depending on which if either enzyme is targeted by the inhibitor), and down-regulate SSAT. By contrast, polyamine excess, as achieved with exogenous polyamines, or *apparent* polyamine excess, as achieved by polyamine analogs, would down-regulate the transporter and biosynthetic enzymes and up-regulate SSAT. (Reproduced with kind permission of Kluwer Academic Publishers, Lancaster, England, from [5].)

respectively, more polyamines. By contrast, SSAT can actively respond to polyamine *excess* by acetylating SPD and SPM and thereby facilitating their excretion out of the cell and/or promoting their back-conversion to PUT which can be catabolized by various PUT-directed oxidases [4,6]. These same systems can respond passively as follows: during polyamine excess, biosynthesis and uptake can decrease, and during polyamine insufficiency, SSAT-mediated catabolism and excretion can decrease.

It is important to emphasize that the proposed model is a working hypothesis which requires additional vali-

dation by experimentation. Studies with polyamine analogs are supportive of the model. Cells treated with such analogs respond as though polyamine excess exists, namely ODC and SAMDC are down-regulated [12], transport is down-regulated [31,32], and SSAT is markedly up-regulated [8–12]. Acetylated polyamines are excreted into the medium [12,36]. It is interesting to note that during treatment with polyamine analogs, intracellular polyamines are decreased (presumably via halted synthesis and increased excretion) as the analog is taken up, so that a relatively constant total polyamine content (including analog) is maintained by the cells [32,37]. These observations would seem to confirm the integrated potential of the various responses in polyamine pool homeostasis.

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