Cytosolic and nuclear spermidine acetyltransferases in growing NIH 3T3 fibroblasts stimulated with serum or polyamines: relationship to polyamine-biosynthetic decarboxylases and histone acetyltransferase

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The expression (mRNA level or enzymic activity) of cytosolic and nuclear spermidine acetyltransferases was studied in NIH 3T3 fibroblasts, either (1) serum-starved and stimulated to grow by serum refeeding, or (2) treated with inhibitors of ornithine decarboxylase (ODC) (MDL 72.175) and *S*-adenosylmethionine decarboxylase (AdoMetDC) (MDL 73.811) and stimulated to grow by spermidine. Expression of the known growth-regulated genes for ODC, AdoMetDC and histone acetyltransferase was also examined. The mRNA for spermidine/spermine *N*¹-acetyltransferase (SAT) accumulated after serum refeeding (between 6 and 16 h) and even more after spermidine addition (16 h). Histone

acetyltransferase activity increased after both growth stimuli, whereas spermidine *N*⁸-acetyltransferase activity remained unchanged. After serum stimulation, the ODC mRNA level and activity rose between 6 and 16 h, whereas AdoMetDC mRNA accumulation occurred later (16 h) than the increase in enzyme activity (6 h). Stimulation of ODC and AdoMetDC activities was suppressed by the inhibitors added alone or in combination with spermidine, whereas mRNA accumulation was down-regulated by spermidine. These results indicate that the expression of SAT was growth-controlled and that SAT mRNA level was regulated by polyamines.

INTRODUCTION

In cells stimulated to proliferate from quiescence, there is early accumulation of polyamines [1] and mRNAs encoding ornithine decarboxylase (EC 4.1.1.17; ODC) and *S*-adenosylmethionine decarboxylase (EC 4.1.1.50; AdoMetDC), the rate-limiting enzymes for polyamine biosynthesis [2]. A second polyamine peak is detectable in the late G₁–S phase, as it is in continuously dividing cells [1,3]. Stimulation of polyamine biosynthesis is critical for the commitment of quiescent cells to DNA replication [4], and also plays a role in S-phase progression of the cell cycle [1,3].

The involvement of cytosolic and nuclear polyamine acetyltransferases in the genetic programme for growth activation of resting cells has never been studied. The cytosolic spermidine/spermine *N*¹-acetyltransferase (SAT) catalyses the key step in the interconversion pathway of spermidine and spermine to putrescine, and it is inducible after various growth stimuli [5]. The nuclear enzyme(s) which acetylate spermidine at *N*-8 [6,7] may act synergistically with histone acetyltransferase to destabilize inactive chromatin structure [8–10].

We have investigated the expression of cytosolic and nuclear polyamine acetyltransferases and their relationships with the polyamine-biosynthetic decarboxylases and histone acetyltransferase after stimulation of quiescent NIH 3T3 fibroblasts to grow.

The results show that expression of the SAT gene was growth-regulated and dependent on intracellular polyamines. Histone

acetyltransferase activity was stimulated immediately after growth induction, but not spermidine *N*⁸-acetyltransferase activity.

EXPERIMENTAL

Materials

[³H]Thymidine (6.7 Ci/mmol), [³²P]dCTP (3000 Ci/mmol), DL-[1-¹⁴C]ornithine (58 Ci/mol), *S*-adenosyl-L-[carboxyl-¹⁴C]methionine (53 Ci/mol) and [1-¹⁴C]acetyl-CoA (50 Ci/mol) were purchased from Amersham International (Amersham, Bucks., U.K.). (2*R*,5*R*)-Hept-6-yne-2,5-diamine hydrochloride [MDL 72.175; (2*R*,5*R*)MAP], 5'-[[(*Z*)-4-aminobut-2-enyl]methylamino]-5'-deoxyadenosine [MDL 73.811; (*Z*)AbeAdo] and α-difluoromethylornithine (DFMO) were generously given by Marion Merrell Dow Research Institute (Strasbourg, France). All the other chemicals used were of the highest grade available.

Cell cultures and inhibitor treatments

NIH 3T3 fibroblasts were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % horse serum (HS) (Flow Laboratories, Irvine, Scotland, U.K.), 100 units of penicillin/ml, 100 µg of streptomycin/ml, 10 mM Hepes buffer, 1 % L-glutamine, 1 mM sodium pyruvate, at 37 °C in a 5 % CO₂ atmosphere. HS was used instead of fetal-calf serum because of its lower content of amine oxidases [11]. Fibroblasts used for the experiments had undergone 5–11 population doublings in this

Abbreviations used: ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; SAT, spermidine/spermine *N*¹-acetyltransferase; DFMO, α-difluoromethylornithine; (2*R*,5*R*)MAP, (2*R*,5*R*)-hept-6-yne-2,5-diamine (MDL 72.175); (*Z*)AbeAdo, 5'-[[(*Z*)-4-aminobut-2-enyl]methylamino]-5'-deoxyadenosine (MDL 73.811); HS, horse serum; DMEM, Dulbecco's modified Eagle's medium.

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culture system. The cells were plated at a concentration of 3.3×10^4 cells/cm² in DMEM containing 10% HS, and were allowed to attach to 100 mm-diameter Petri dishes. The experimental design included four treatment conditions. For each condition, the fibroblasts were seeded in 20–30 dishes to obtain enough cells to be utilized for cell counts, preparation of total RNA, assay of polyamines and enzyme activities. (1) Cells were made quiescent by 44 h cultivation in serum-deprived medium (DMEM supplemented with 0.5% HS), which decreased [³H]-thymidine incorporation to 5% within 24 h (the present paper, and [12]). (2) Serum-starved cells were then transferred to medium containing 20% HS for the times indicated in the text. (3 and 4) Cells in the exponential phase of growth (16 h after serum refeeding) were treated with the combination of 100 μ M (2*R*,5*R*)MAP plus 25 μ M (Z)AbeAdo for 72 h; at this time, for treatment (4), fresh medium containing 20 μ M spermidine plus the combination of the two inhibitors was added for the time periods reported in the text. Preliminary experiments were carried out with either 25 μ M or 50 μ M (Z)AbeAdo in combination with 100 μ M (2*R*,5*R*)MAP for 72 h to study the dose-dependent effects on cell growth.

For experiments measuring the incorporation of [³H]thymidine, fibroblasts were cultivated in multi-well plates and pulse-labelled with 1 μ M [³H]thymidine/well during the last 8 h of culture.

Flow-cytometric analysis

Cell samples were stained by the standard propidium iodide method and analysed by flow cytometry [13].

Northern-blot analysis and molecular hybridization

Preparation of total RNA and Northern-blot analysis were performed as previously described [14–16].

DNA probes

The 539-base-pair fragment of SAT cDNA was obtained by reverse transcriptase-PCR amplification of normal human liver cDNA [17], using oligo-primers synthesized on the basis of the published SAT sequence [18]. The 750-base-pair *Pst*I fragment of ODC cDNA was obtained from the pOD48 plasmid [19]. The 180-base-pairs *Sau*3A fragment of AdoMetDC cDNA was derived from the pSDC 1.35 plasmid [20]. The 'house-keeping' gene LLRep 3 was used to normalize RNA gel contents [21].

Isolation of nuclei

Cells from 6–8 dishes were harvested by scraping with a rubber policeman, broken [22], and used for preparation of nuclei without addition of detergents [23]. Nuclei were examined for purity, and were frozen at -80°C [22].

Determination of polyamines and their acetyl derivatives

Cell extracts were prepared in 2.5 vol. (w/v) of 0.2 M HClO₄ and analysed by h.p.l.c. [24,25] with a C₁₈ column (5 μ m particle size, 150 mm $\times$ 4 mm) (Perkin–Elmer Italia, Milano, Italy), post-column derivative formation with *o*-phthalaldehyde and fluorescence detection.

ODC and AdoMetDC activity assays

We measured the release of CO₂ from [1-¹⁴C]ornithine and from *S*-adenosyl-L-[carboxyl-¹⁴C]methionine, by the methods of Jänne and Williams-Ashman [26,27] with modifications. Cells were sonicated into 2 vol. (w/v) of 20 mM Tris/HCl (pH 7.1) containing 0.25 M sucrose, and centrifuged at 18000 *g* for 30 min. The reaction mixture for ODC (final volume 500 μ l) contained the cytosolic supernatant (about 400 μ g of protein), 50 mM Tris/HCl (pH 7.1) and 1 mM [¹⁴C]ornithine (sp. radioactivity 1 mCi/mmol) [16,24]. The reaction mixture for AdoMetDC (final volume 125 μ l) contained the cytosolic supernatant (about 200 μ g of protein), 2.5 mM putrescine, 0.4 mM labelled *S*-adenosylmethionine (sp. radioactivity 2.28 mCi/mmol). Blanks contained DFMO [28] in the ODC activity assay, or were without cytosolic supernatant in the AdoMetDC activity assay.

Spermidine *N*⁸-acetyltransferase and histone acetyltransferase activity assays

As previously described [22,24,29,30], we measured the incorporation of the labelled acetyl group from 50 μ M [1-¹⁴C]acetyl-CoA (sp. radioactivity 32 mCi/mmol) into spermidine (3 mM) or histones (50 μ g). The 100 μ l reaction mixture contained sonicated nuclei corresponding to 30 μ g of protein. For some spermidine *N*⁸-acetyltransferase activity assays, the spermidine acetyl derivatives formed were analysed by h.p.l.c. [24]. The reaction product was 80% *N*⁸-acetylspermidine.

Protein determination

Protein content was determined by the method of Lowry et al. [31].

Statistical analysis

Data were analysed by ANOVA, with *P* < 0.05 considered significant.

RESULTS

Polyamine contents of quiescent and growth-stimulated NIH 3T3 fibroblasts

We first defined the experimental condition to make serum-stimulated fibroblasts quiescent by co-treatment with (2*R*,5*R*)MAP and (Z)AbeAdo, inhibitors of polyamine-bio-synthetic decarboxylases [32–35]. We observed by cytofluorimetric analysis that 72 h treatment with 100 μ M (2*R*,5*R*)MAP plus 25 μ M (Z)AbeAdo caused cytostasis, with 70% of the cells in G₀/G₁ phase, 7% in S phase and 23% in G₂/M phase. By comparison, the serum-stimulated cells were distributed about 30% in each of these three phases of the cell cycle (results not shown). The inhibitory treatment did not cause cell death, as assessed by Trypan Blue exclusion, whereas the combination of 100 μ M (2*R*,5*R*)MAP plus 50 μ M (Z)AbeAdo had a definite cytotoxic effect (50% cell death) that rendered it useless. Addition of exogenous spermidine produced rapid recovery of fibroblast growth and restored the cell population doubling time to about 20 h, which was the same as for control cells stimulated to grow by serum (results not shown). Thus the inhibitory effect seemed to be related to a decrease in the intracellular pool of polyamines, which was replenished by spermidine.

To see whether or not, and to what extent, the combined inhibitor treatment caused polyamine depletion, cell polyamine and *N*¹-acetylspermidine levels were measured (Figure 1). *N*⁸-

Figure 1 Changes in polyamine levels in cells activated from quiescence

Polyamines and N¹-acetylspermidine were assayed in HClO₄ cell extracts by h.p.l.c., as reported in the Experimental section. ○, Confluent cells; ●, serum-starved cells (0.5% HS); ▲, serum refeeding (20% HS); ▲---▲, treatment with 100 μM (2*R*,5*R*)MAP plus 25 μM (Z)AbeAdo; △, 20 μM spermidine in addition to 100 μM (2*R*,5*R*)MAP plus 25 μM (Z)AbeAdo. Abbreviation: n.d., not detectable (N¹-acetylspermidine was undetectable in serum-starved cells and in inhibitor-treated cells).

Acetylspermidine was undetectable in our experimental conditions, probably due to its rapid hydrolysis in the cytosol, catalysed by N⁸-acetylspermidine deacetylase [22,36].

In serum-starved cells, N¹-acetylspermidine (to below the limit of detection) and spermine (by 50%) were decreased as compared with confluent cells. Addition of 20% HS to quiescent cells increased putrescine content about 4-fold at 6 and 16 h, whereas spermidine and spermine were increased 1.6-fold only at 6 h. N¹-Acetylspermidine was also measurable 6 h after serum refeeding. Since the levels of spermidine, spermine and N¹-acetylspermidine were lower at 16 h than at 6 h, whereas putrescine remained constant, activation of the interconversion pathway of higher polyamines to putrescine might have occurred. In cells refed with serum, further increases in spermidine, spermine and N¹-acetylspermidine were observed between 48 and 72 h (2.0–2.5-fold), while putrescine reached a peak at 72 h (11-fold).

The combined treatment with 100 μM (2*R*,5*R*)MAP and 25 μM (Z)AbeAdo for 72 h decreased putrescine and spermidine in serum-stimulated cells to 30%, spermine to less than 20% and N¹-acetylspermidine to an undetectable value. This inhibitory treatment depleted spermine more completely than reported for the ODC inhibitor DFMO [4]. Figure 1 also shows that addition of spermidine to the 72 h-inhibitor-treated cells restored intracellular spermidine content and resulted in a rise in putrescine (1.8 times) and an accumulation of N¹-acetylspermidine 6 h later. At 16 h putrescine had rapidly declined, whereas N¹-acetylspermidine and spermine had doubled.

Figure 2 Expression of SAT, ODC and AdoMetDC mRNAs

Northern-blot analysis of the RNA extracted from the fibroblasts, under the different experimental conditions, was performed as reported in the Experimental section. Hybridization was carried out with the probes for (a) SAT, (b) ODC, (c) AdoMetDC and (d) LLRep 3' house-keeping gene. Lanes: 1, confluent cells; 2, 44 h-serum-starved cells (0.5% HS); 3, 6 h after serum refeeding (20% HS); 4, 16 h after serum refeeding (20% HS); 5, 6 h after addition of 20 μM spermidine to the inhibitor-treated cells; 6, 16 h after spermidine addition to the inhibitor-treated cells; 7, treatment with 100 μM (2*R*,5*R*)MAP plus 25 μM (Z)AbeAdo for 72 h.

mRNA levels for SAT, ODC and AdoMetDC in cells stimulated to grow after quiescence

Northern-blot analysis (Figure 2a) showed that the SAT mRNA level diminished to about one-half when confluent cells (lane 1) were serum-starved (lane 2), and to about one-third when serum-stimulated cells (lane 4) were treated with the polyamine-biosynthetic-enzyme inhibitors (lane 7). Accumulation of SAT mRNAs was observed in growth-stimulated cells. Serum refeeding doubled SAT mRNA levels between 6 and 16 h (Figure 2a, lanes 3 and 4). However, there was greater expression of SAT mRNA at 16 h (5-fold) (Figure 2a, lane 6) than at 6 h (2-fold) (Figure 2a, lane 5) after spermidine addition to inhibitor-treated cells.

The same Northern blot was then hybridized with ODC and AdoMetDC probes (Figures 2b and 2c). The AdoMetDC probe should hybridize two mRNA species corresponding to the approximate sizes of 3.6 and 2.4 kb [2,20]. There was less of the 2.4 kb mRNA, and its detection required longer film exposure. ODC and AdoMetDC mRNA levels were similar in both serum-starved (Figures 2b and 2c, lane 2) and confluent cells (Figures 2b and 2c, lane 1). Detection of ODC mRNA in serum-starved cells is consistent with previously published data [2], and supports the idea that ODC mRNA is stable in quiescent cells [1]. As expected, serum refeeding increased ODC and AdoMetDC mRNA levels [2]. We found that ODC mRNA had increased at 6 h (4-fold) (Figure 2b, lane 3), and remained well over the control value at 16 h (2-fold) (Figure 2b, lane 4). The peak of AdoMetDC mRNA was reached at 16 h (2-fold) (Figure 2c, lane 4). After the

Figure 3 ODC and AdoMetDC activities

ODC and AdoMetDC activities were assayed in the fibroblast cytosol fraction under the various experimental conditions used, as reported in the Experimental section. Values are the means of duplicate experiments carried out with two different cell pools, each prepared from five dishes. AdoMetDC activity is not shown in (b), since it was undetectable in the inhibitor-treated cells either in the presence or in the absence of spermidine. (a) ODC activity in (○) confluent cells, (△) serum-starved cells (0.5% HS), (●) 20% serum-refed cells; AdoMetDC activity in (△) confluent cells, (△) serum-starved cells (0.5% HS), (▲) 20% serum-refed cells. (b) ODC activity (□) in 72 h-inhibitor-treated cells and (■) after spermidine addition to the inhibitor-treated cells.

Figure 4 Spermidine *N*⁶-acetyltransferase and histone acetyltransferase activities

The enzymes were assayed in cell nuclei under the various experimental conditions used, as reported in the Experimental section. Each point represents the mean $\pm$ S.E.M. of 3 different experiments carried out in nuclei purified from 3 cell pools, each prepared from 6–8 dishes. Where the bars that represent the standard errors are not reported, they lie within the symbols. (a) Spermidine *N*⁶-acetyltransferase activity in (△) confluent cells, (△) serum-starved cells (0.5% HS), (●) 20% serum-refed cells; histone acetyltransferase activity in (○) confluent cells, (○) serum-starved cells (0.5% HS), (●) 20% serum-refed cells. (b) Spermidine *N*⁶-acetyltransferase activity (◇) in 72 h-inhibitor-treated cells and (◆) after spermidine addition to the inhibitor-treated cells; histone acetyltransferase activity (□) in 72 h-inhibitor-treated cells and (■) after spermidine addition to the inhibitor-treated cells. **P* < 0.05.

treatment with the inhibitors (Figures 2b and 2c, lane 7), AdoMetDC mRNA increased (about 3-fold), whereas the ODC mRNA remained at the level seen in serum-stimulated cells. Addition of spermidine to the inhibitor-treated cells (Figures 2b

and 2c, lanes 5 and 6) decreased the levels of ODC and AdoMetDC mRNAs, the last being undetectable at 16 h.

ODC and AdoMetDC activities in cells stimulated to grow from quiescence

The effectiveness on the target enzymes of treatment with the inhibitors was confirmed by testing ODC and AdoMetDC activities (Figure 3). After serum refeeding, ODC and AdoMetDC activities increased at 6 h and 16 h (i.e. 50 h and 60 h after the beginning of the treatments) by about 6-fold and 2.5-fold respectively (Figure 3a). When growing cells were treated with the inhibitors (Figure 3b), ODC activity fell below the value for confluent cells (to about 50%) and AdoMetDC activity became undetectable. Exogenous spermidine did not modify the activities of the two enzymes (Figure 3b). Polyamines are, in fact, known to inhibit translation of ODC and AdoMetDC mRNAs and to favour the degradation of the two enzyme proteins [37].

Spermidine *N*⁶-acetyltransferase and histone acetyltransferase activities in cells activated from quiescence

To examine further some regulatory aspects of nuclear spermidine *N*⁶-acetyltransferase and histone acetyltransferase in relationship to the cell's growth state, the patterns of these enzyme activities were examined in fibroblasts activated from quiescence by serum or spermidine.

When confluent cells were deprived of serum, the activity of spermidine *N*⁶-acetyltransferase did not change, whereas histone acetyltransferase activity increased about 3-fold (Figure 4a). It has been published that synthesis and acetylation of histones increase transiently between 24 h and 48 h of starvation [12]. Serum refeeding further stimulated histone acetyltransferase activity, which reached its maximum at 6 h (4-fold increase) and declined within 48 h (i.e. within 92 h after the beginning of treatments) to the value in confluent cells. In contrast, spermidine *N*⁶-acetyltransferase activity was unaffected. Addition of spermidine to cells pre-treated with (2*R*,5*R*)MAP plus (*Z*)AbeAdo caused a 12-fold increase in histone acetyltransferase activity (16 h), spermidine *N*⁶-acetyltransferase activity remaining unchanged (Figure 4b).

DISCUSSION

Using two different models to induce fibroblast quiescence and re-entry into the cell cycle, we obtained evidence that the interconversion pathway of polyamines is particularly active during the G₀/G₁ transition. In fact, addition of either serum or spermidine to quiescent cells caused accumulation of *N*¹-acetyl-spermidine which was associated with modifications of SAT gene expression. The expression of SAT mRNA was growth-controlled by the cell polyamines spermidine and/or spermine through a mechanism that might be transcriptional or post-transcriptional. Changes in SAT mRNA levels are probably responsible for increases in SAT enzyme protein and activity. It is known that in rat tissues and other cell types SAT activity can be modified by treatment with exogenous polyamines or polyamine analogues [38–40].

After serum-induced transition from G₀ to G₁, ODC mRNA increased as expected at early times [2], whereas AdoMetDC mRNA had different kinetics, since its accumulation was delayed. The increase in ODC activity was higher than that in ODC mRNA level, suggesting post-transcriptional control. The increase in AdoMetDC activity 6 h after serum refeeding was not associated with mRNA accumulation, probably because the high

level of putrescine in cells emerging from quiescence favoured activation of the proenzyme [37].

The long-lasting accumulation of ODC mRNA confirmed the high stability of the transcript and was in contrast with the very short half-life of ODC protein [37,41]. This property differentiates ODC from the other primary response ('competence') genes, such as the proto-oncogenes *c-myc* and *c-fos*, which have very rapid turnover of both protein and mRNA [2,41,42].

Our findings are in agreement with the idea that polyamines, especially spermidine, negatively control the transcription of AdoMetDC [37,43]. Polyamines are also known to inhibit ODC and AdoMetDC translation and favour their degradation [37,43].

After serum refeeding, nuclear spermidine *N*⁸-acetyltransferase activity remained unchanged for 48 h. By contrast, there was rapid induction of histone acetyltransferase activity. Acetate incorporation into histones of fibroblasts refed with serum is reported to be biphasic, and to affect mostly newly synthesized histones [12]. Acetylated histones are thought to regulate the major changes in structure and function of chromatin during the cell cycle [8,12], whereas the role of *N*⁸-acetylspermidine in these processes still remains elusive.

The data reported here are not compatible with the existence of two nuclear enzymes, either of which can catalyse both histone and spermidine acetylation [44]. They support our previous data demonstrating that spermidine *N*⁸-acetyltransferase and histone acetyltransferases are regulated differently in regenerating rat liver [24]. The heterogeneity of these nuclear acetyltransferases is also indicated by data from preliminary purification studies, which suggest that different subunit associations may confer preferential specificity towards spermidine or histones [45]. We were interested to see that spermidine addition to fibroblasts which had been made quiescent by polyamine depletion induced only histone acetylation in nuclei. This effect might be related to an increase of spermidine levels in certain specialized cell compartments in which spermine was lacking because of inhibition of polyamine biosynthesis. Spermidine might substitute for spermine, e.g. in interaction with chromatin [8–10], and become inaccessible for acetylation, with histone acetylation favoured as a consequence. We consider that spermidine acetylation at N-8 position is not a key step in cell progression from quiescence into S phase.

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