CMLS, Cell. Mol. Life Sci. 61 (2004) 1775–1784
1420-682X/04/141775-10
DOI 10.1007/s00018-004-4192-y
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CMLS Cellular and Molecular Life Sciences

Research Article

Acylphosphatase overexpression triggers SH-SY5Y differentiation towards neuronal phenotype

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Received 3 May 2004; accepted 25 May 2004

Abstract. An acylphosphatase (AcPase) overexpression study was carried out on SH-SY5Y neuroblastoma cells, using a green fluorescent fusion protein (AcP-GFP), with GFP acting as a reporter protein. The cellular proliferation rate was significantly reduced by overexpression of AcPase by a factor of ten. In contrast, clones transfected with two inactive AcPase mutants showed a growth rate comparable to control cells. This suggests that AcPase catalyzes the proliferative down-regulation. AcPase-overexpressing clones showed a physiological mortality rate as assessed by an MTT reduction test and by evaluation of necrotic markers. DNA fragmentation analysis and assays of caspase-3 and poly (ADP-ribose) polymerase (PARP)-active fragments showed no evidence of any apoptotic pattern. AcPase overexpression led to a marked increase in PARP activity as well as Bcl-2 content; these are commonly up-regulated during differentiative processes in neuronal cells. In fact, the typical differentiation marker, growth-associated-protein 43, was significantly up-regulated. Microscopic observations also showed a clear increase in the differentiative phenotype in AcPase-overexpressing cells. Our results clearly show that AcPase plays a primary causative role in neuronal differentiation.

Key words. Acylphosphatase; SH-SY5Y neuroblastoma cell; differentiation; apoptosis; PMA.

Acylphosphatase (AcPase) is a small cytosolic enzyme (11 kDa) distributed widely in vertebrate tissues in two different isoenzymatic forms, muscle type (MT) and organ common type (CT), which share over 55% sequence homology [1–4]. It is a globular protein consisting of a five-stranded antiparallel twisted β sheet facing two antiparallel α helices. AcPase catalyzes the hydrolysis of compounds containing a carboxylphosphate bond, such as 1,3-bisphosphoglycerate, carbamoylphosphate, succinylphosphate, acetylphosphate and β-aspartylphosphate [4–7]. Site-directed mutagenesis studies suggest that arginine 23 and asparagine 41 are essential residues of the AcPase active site [8, 9]. In particular, arginine 23 appears to be the main phosphate-binding residue, whereas asparagine 41 is involved in catalytic water molecule binding.

Although the three-dimensional structure and catalytic properties of enzymes such as AcPase are well known, its role remains unclear. There is increasing experimental evidence that the phosphoaspartyl intermediate of Ca^{2+}/Mg^{2+}-ATPase is the physiological substrate of AcPase [10]. An increase in AcPase expression during erythrocyte aging and also in cellular differentiation processes in several cell types has been reported [11–14]. For example, we observed a progressive increase in CT isoenzyme content during retinoic acid (RA) and phorbol-12-myristate-13-acetate (PMA) differentiation in SY5Y neuroblastoma cells [12]. In particular, the MT isoenzyme remained unaffected by RA stimulation, whereas it showed a marked initial increase accompanied by a restoration of the basal level upon PMA differentiation. Moreover, AcPase is involved in the differentiation of L6J1 rat myoblasts and K562 human erythroid cells

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[13, 14]. The MT isoform showed a tenfold increase during megakaryocytic and erythrocytic differentiation processes, but only hemin treatment caused a similar increase in the CT isoenzyme. These observations, taken together, indicate that the two AcP isoenzymes are subject to different regulatory mechanisms during differentiation. As an example, thyroid hormone treatment induces the MT isoform but exerts no influence upon the CT isoform in K562 cells [15]. Translation of MT AcPase mRNA is regulated by the presence of an additional upstream AUG codon and a very stable stem-loop structure in the 5′ untranslated (UTR) region, which provides stringent control of AcPase expression. The existence of such an inhibitory mechanism on MT AcPase expression suggests that a high enzyme content is harmful for cell survival. In fact, previous efforts to derive HeLa and NIH-3T3-overexpressing AcPase always failed or resulted in clones that transiently expressed the transgene at low levels [16]. In particular, much experimental evidence indicates that MT AcPase overexpression leads to a strong increase in DNA fragmentation and to cell condensation, membrane blebbing and formation of apoptotic bodies. Moreover, the MT isoenzyme in response to various apoptotic stimuli showed a nuclear migration in K562 and Jurkat cells, but the CT isoenzyme did not show any change in its cellular content and localization. Nuclear accumulation also appeared to be a characteristic feature of MT AcPase alone during differentiation in C2C12 and K562 cells [17]. Significant AcPase nuclease activity on DNA has also been observed in an acidic environment [18]. Since many different DNAse activities are involved in typical apoptotic DNA fragmentation, an MT AcPase has been proposed as an isoenzyme as part of a multimolecular protein complex which hydrolyzes DNA during apoptotic processes [19, 20].

To settle the role of MT AcPase in differentiation processes, rather than its potential role in the apoptotic cell death machinery, we have stably overexpressed the wild-type enzyme and two almost inactive mutants of MT AcPase as fusion proteins with a green fluorescent protein (GFP) in SH-SY5Y neuroblastoma cells.

Materials and methods

Materials

All reagents were of analytical grade or the highest purity available. Unless otherwise stated, chemicals were purchased from Sigma (St. Louis, Mo.).

Cell culture treatment and transfection procedure

SH-SY5Y neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1 mM glutamine and antibiotics at 37 °C in a humidified atmosphere. Neuronal differentiation was obtained by addition of 100 nM PMA to the culture medium, which was renewed every 2 days. The cDNAs coding for wild-type AcPase and for the R23Q and N41Q AcPase mutants were cloned in the pEGFP-N1 vector (BD Biosciences Clontech, San Jose, Calif.) upstream of the coding sequence of the GFP protein [16]. These three vectors, AcP-GFP, R23Q-AcP-GFP and N41Q-AcP-GFP, express three fusion proteins in which the AcPase C terminal is linked to the GFP N terminal. Transfections with 10 µg of the three different constructs or with pEGFP-N1 alone (GFP) were performed by the calcium phosphate procedure. The stable clones were obtained by selection with Geneticin G418 (400 µg/ml). As GFP emits bright-green light when exposed to UV or blue light, AcPase expression levels were assessed by flow cytometric analysis of chimeric protein florescence in a Becton-Dickinson FACScan on SY5Y stably transfected cells.

Morphological analysis

To assay the neurite outgrowth, five randomly chosen fields of investigated clones were photographed under a phase-contrast microscope and neurite number and length were measured. Green fluorescence micrographs of SY5Y cells were observed under UV illumination in an epifluorescence inverted microscope (Nikon, Diaphot TMD-EF) with an appropriate filter set.

AcPase immunoassay

Cell plates were harvested in 50 mM Tris-HCl, pH 7.2 containing 0.1 mM phenylmethylsulfonylfluoride (PMSF), 10 µg/ml leupeptin and 10 µg/ml aprotinin prior to storage at –80 °C until use. After plasma membrane rupture by three freeze-thaw cycles, the cytosolic fraction was obtained by centrifugation at 36,000 g for 60 min. Protein content was evaluated by the Bradford assay using bovine serum albumin as a standard. Equal amounts of cytosolic fractions were subjected to 15% SDS-PAGE, then blotted to nitrocellulose membranes (Sartorius, Goettingen, Germany) and immunorecognition was performed by polyclonal anti-AcPase antibodies. Specific antibodies against MT AcPase were raised in rabbit using a recombinant fusion protein with glutathione S-transferase and purified by affinity chromatography [21]. Incubation with secondary antibodies conjugates with horseradish peroxidase (Calbiochem, Darmstadt, Germany) and development with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Milan, Italy) followed. The band densities were quantified as densitometric units/25 µg protein (the constant protein amount applied on SDS-PAGE) using the program for image analysis and densitometry Quantity One (Bio-Rad, Milan, Italy).

AcPase activity assay

AcPase activity was measured on soluble fractions by a high-sensitivity continuous fluorimetric method at

340 nm excitation/390 nm emission and 25 °C using 2-methoxybenzoyl phosphate (2MBP) as substrate, based on the difference in fluorescence emission at 390 nm between 2MBP and 2-methoxybenzoic acid (2MBA) [22]. The unit of activity is defined as the amount of the enzyme that liberates 1 µmol of 2MBA/min at 25 °C and pH 5.3. The concentration of 2MBP ranged from 0.1 to 5.0 mM; the values of apparent K_m were calculated by a nonlinear fitting of the hyperbolic Michaelis-Menten equation using the computer program Fig. P (Biosoft, Cambridge, UK).

AcPase mRNA analysis

For Northern blot analysis on AcPase-transfected clones, RNA was isolated by the guanidinium thiocyanate phenol chloroform method [23]. Total RNA was quantified spectrophotometrically, treated with formaldehyde and subjected to electrophoresis in a 1.2% agarose gel. The separated RNAs were transferred to Hybond N^+ nylon membrane (Amersham), prehybridized for 3 h and hybridized for 16 h in 4 × standard saline citrate (SSC), 5 × Denhart's solution, 0.1% SDS and 0.2% EDTA with the ^{32}P-labeled AcPase cDNA probe at 65 °C. Washes were performed in 4 × SSC, 0.1% SDS at 65 °C. Filters were subjected to autoradiography at –80 °C with Kodak films and intensifying screens for 6–7 days.

Proliferation rate assay

SH-SY5Y human neuroblastoma cells were plated at a density of $4 \times 10^4/cm^2$ on 12 × multiwells and maintained in the exponential phase of growth at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was renewed 24 h after seeding and this time was considered zero time. The cells were labeled with 1 µCi/ml of [^{3}H]thymidine pulse (1 µCi/well) for 15 h, after which they were incubated twice with cold 16% trichloacetic acid (TCA) at 4 °C for 15 min each time. After 1 h of 0.2 N NaOH incubation, an aliquot was measured by liquid scintillation counting. Disintegrations per min (DPM)/µg protein content per well were then calculated. Growth rate and cumulative population doubling at each passage were also determined by a crystal violet test.

Differentiative marker analysis

Differentiative markers were assessed by a Western blot analysis of growth-associated-protein (GAP43) and Bcl-2 protein levels. Soluble fractions obtained from cell lysates were subjected to 15% SDS-PAGE and then blotted to nitrocellulose membranes (Sartorius, Göttingen, Germany). Immunorecognition was performed by monoclonal mouse anti-GAP43 antibodies (Affiniti, Nottingham, UK) and by monoclonal mouse anti-Bcl-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif.). SKN cell lysate was employed as a positive control of GAP43. Incubation with secondary antibody conjugates with horseradish peroxidase (Calbiochem, Darmstadt, Germany) and development with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Milan, Italy) followed.

Poly(ADP-ribose)polymerase (PARP) activity was assessed by an immunodot blot method which detects poly(ADP-ribosylated) proteins [24]. Preparation of homogenates and purification of the nuclear fraction from SH-SY5Y human neuroblastoma cells were obtained by three freeze-thaw cycles in ice-cold 50 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA, 1mM dithiothreitol, 50 mM NaCl, 0.25 M sucrose, 0.2 mM PMSF and 10 µg/ml of aprotinin and leupeptin. The homogenate was centrifuged at 600 g for 10 min; the pellet, containing the nuclear fraction, was washed with the homogenizing buffer, then suspended in 50 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$, 0.1 mM PMSF and finally sonicated on ice. In brief, an aliquot of nuclear suspension was diluted in 0.4 M NaOH containing 10 mM EDTA and loaded on a Hybond N+ nylon membrane (Amersham) previously rinsed with water. The membrane was then washed once with 0.4 M NaOH, and saturated in PBS-MT (PBS, pH 7.4, containing 5% nonfat dried milk and 0.1% Tween 20) and then incubated overnight with the first antibody LP96-10 (Alexis, San Diego, Calif.). The membrane was then washed with PBS-MT and incubated for 30 min with peroxidase-conjugated anti-rabbit IgG (Amersham). The blot was again washed with PBS-MT followed by washes in PBS prior to analyzing by chemiluminescence. Image analysis of the dot blot was performed by the program for image analysis and densitometry Quantity One.

Evaluation of necrotic markers

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrzolium bromide (MTT) reduction inhibition assay allowed physiological stress evaluation [25]. One hundred microliters of a stock MTT solution in PBS was added to give a final concentration of 0.5 mg/ml and incubated for a further 4 h. One hundred microliters of cell lysis buffer (20.0% SDS, 50.0% N,N-dimethylformamide, pH 4.7) was added to each well and the samples were incubated overnight at 37 °C in a humidified incubator. Absorbance values of blue formazan were determined at 590 nm with an automatic plate reader.

Moreover, cell death was assessed by the trypan blue internalization test [26]. SH-SY5Y neuroblastoma cells were treated with trypan blue and survival was quantified by counting (three fields per well, two wells per clone, an average of 50 cells per field). Lactate dehydrogenase (LDH) release was also measured in the culture medium after 48 h from cell seeding by routine laboratory spectrophotometric methods at 340 nm.

Evaluation of apoptotic markers

For DNA fragmentation analysis, cells were lysed in 1 ml Na-citrate 50 mM, 0.1% Triton X-100 containing 50 mg/

ml propidium iodide (PI). For each sample 20,000 cells were acquired and analyzed. Analysis was performed in a Becton-Dickinson FACScan using the Lysis II and Cell Fit Analysis Software according to the manufacturer's procedure.

Cellular DNA fragmentation was also determined using the cell death detection ELISA reagent (Boehringer Mannheim, Mannheim, Germany) following the manufacturer's instructions. DNA fragmentation was expressed as the enrichment of histone-associated mono- and oligonucleosomes released into the cytoplasm. The enrichment factor (EF) was calculated according to absorption at 405 nm, which represented the enrichment of histone-associated DNA fragmentation and accounted for apoptosis of SH-SY5Y neuroblastoma cells.

The apoptotic pattern was also assessed by a Western blotting analysis of caspase-3- and PARP cleaved fragments. For the PARP assay, an aliquot of sonicated nuclear suspension was diluted in Laemmli's sample buffer and incubated at 65 °C for 15 min. Proteins (10 µg) were separated on 10% SDS-PAGE. For caspase-3 determination, total homogenate was sonicated in ice and centrifuged at 14,000 g. The obtained supernatant was diluted in Laemmli's sample buffer, boiled for 5 min and separated on 15% SDS-PAGE. After blotting, whose completeness was checked by suitable staining, the nitrocellulose membranes were blocked in 5% bovine serum albumin and then probed using C2-10 anti-PARP monoclonal antibody (Oncogene Research Products, Cambridge, Mass.) and anti-caspase-3/CPP32 polyclonal antibody (Biosource International, Camarillo, Calif.) for 2 h. Incubation with the horseradish peroxidase (HRP)-conjugated secondary antibody and ECL procedure followed. The band densities were quantified as densitometric units/10 µg protein (the constant protein amount applied on SDS-PAGE) using the program for image analysis and densitometry Quantity One (Bio-Rad).

Measurement of cellular redox status

To assess the rate of lipid peroxidation, malonaldehyde (MDA) and 4-hydroxyalkenal (4-HNE) concentrations were determined in the supernatant of the homogenate prepared as described above. Measurements were performed by a colorimetric method based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA or 4-HNE at 45 °C [27].

To assess the protein oxidation levels, the protein carbonyl content was determined using the 2,4-dinitrophenylhydrazine method of Levine et al. [28]. In brief, cytosolic samples (200 µg protein) were dried in a vacuum centrifuge, after which, 500 µl of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl was added to each tube and then the cited procedure was followed. Spectrophotometric measurement was performed at 375 nm considering 22,000/M per centimeter as the molar absorption coefficient.

ATP measurement was achieved by a highly sensitive bioluminescence assay (Kit HS II; Roche, Basel, Switzerland). Briefly, it uses the ATP dependency of the light-emitting luciferase-catalyzed oxidation of luciferin for the measurement of extremely low concentrations of ATP [29].

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test. A p value less than 0.05 was considered statistically significant.

Results

Analysis of AcP-GFP, R23Q-AcP-GFP and N41Q-AcP-GFP expression in SY5Y neuroblastoma cells

The invariable failure to derive HeLa and NIH-3T3 overexpressing AcPase suggests an apoptotic role of this enzyme as a DNase effector with a very stringent expression regulation [16]. To study the physiological role of AcPase, we introduced chimeric genes, containing either the entire coding region of the human wild type or the inactive mutant R23Q and N41Q forms of MT AcPase fused at its C terminus to the N terminus of the GFP coding region, into SY5Y neuroblastoma cells. Stable cell lines expressing the fusion protein were isolated using the geneticin resistance marker. We believed that the transfection of SY5Y cells might be successful since we have previously demonstrated a significant AcPase increase during PMA neuronal differentiation [12]. GFP protein allowed monitoring of the AcPase expression level and its localization, by determining the fluorescence intensity of the reporter chimeric proteins in positively transfected clones. Different green fluorescence intensities were measured in various positive clones, suggesting a variable level of construct expression. Stably transfected clones expressing the highest level of the chimeric proteins were selected for use in the present study. These clones possessed very similar levels of fluorescence of both wild-type and the R23Q-AcP-GFP and N41Q-AcP-GFP inactive mutant forms (table 1), whereas the fluorescence intensity of the GFP

Table 1. GFP fluorescence intensity in stably transfected clones.

Clones	GFP intensity (relative units) (mean ± SD)	fluorescent cells (%)
GFP	1345 ± 35	82.5
AcP-GFP	159 ± 15	87.1
R23Q-AcP-GFP	162 ± 28	79.5
N41Q-AcP-GFP	157 ± 60	78.2

Data are from four independent experiments.

clone was rather higher. The lower fluorescence in chimeric transfectants might be due to a quenching effect on GFP florescence emission by the AcPase moiety of the construct or to a strict selection of clones overexpressing AcPase protein up to 10 times, the maximum enzyme level allowing cell survival. The proportion of fluorescent cells was high in all selected clones. In figure 1A, a fluorescence micrograph of an AcPase-overexpressing cell shows a strong GFP-associated fluorescence over the cytoplasmic region. Since no nuclear localization was observed, there is a clear discrepancy with previous published reports [17]. The more than tenfold increase in chimeric protein content relative to the native form of AcPase in the cytosolic compartment of all selected clones was evaluated by Western blot using anti-AcPase antibodies (fig. 1B). The AcP-GFP fusion protein retains an enzymatic activity on the synthetic substrate 2MBP that is very similar to that of the native enzyme expressed in SY5Y cells, as assessed by a high-sensitivity fluorimetric method [22]. In particular, the K_m value of the reporter chimeric protein was approximately the same (0.68 ± 0.09 mM) as that of native MT AcPase (0.73 ± 0.10 mM) in cytosolic fractions. It follows that fusion of GFP at the C terminus did not modify the enzymatic properties of AcPase. Total AcPase activity was significantly higher in AcPase-overexpressing clones (0.296 ± 0.023 U/mg protein; $p < 0.001$) than in GFP-expressing clones (0.043 ± 0.016 U/mg protein) or control untransfected cells (0.041 ± 0.019 U/mg protein). In contrast, both mutant proteins were almost completely unable to catalyze the hydrolysis of the substrate, based on the absence of any significant change in 2MBP AcPase activity in R23Q-AcP-GFP- (0.043 ± 0.007 U/mg protein) or in N41Q-AcP-GFP- (0.054 ± 0.010 U/mg protein) expressing cells. These results indicate that these mutants may be useful for investigating the physiological function of the enzyme. The presence of the AcP-GFP-specific transcript in transfected cells was evaluated by Northern blot analysis. Total RNA was extracted from AcP-GFP selected clones and control SY5Y cells and was hybridized with the ^{32}P-labeled human AcPase cDNA as a probe. Film analysis revealed a specific AcP-GFP mRNA band in transfected clones (fig. 1C).

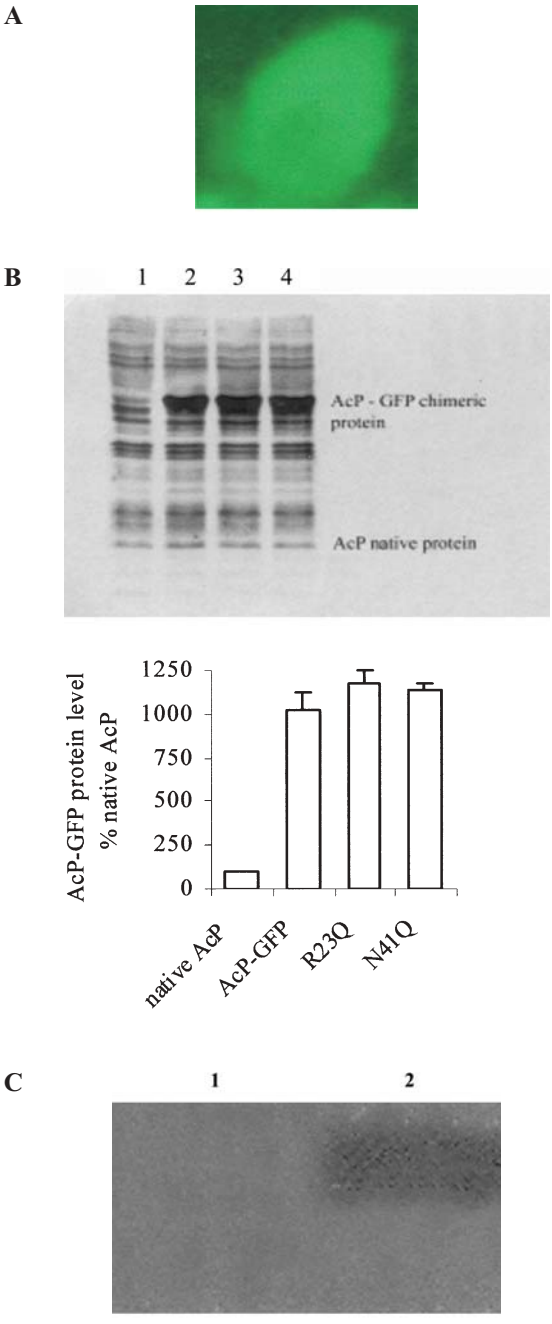


Figure 1. (*A*) Fluorescence micrograph of a typical AcP-GFP-expressing cell with cytoplasmic chimeric protein localization. (*B*) Top: representative Western blot analysis of native and chimeric AcPase contents in the cytosolic compartment using anti-AcPase antibodies. Lanes: 1, GFP clone; 2, AcP-GFP clone; 3, R23Q-AcP-GFP clone; 4, N41Q-AcP-GFP clone. Bottom: quantitative data. The band densities of the chimeric protein were quantified as densitometric units by the Quantity One program and are expressed as a percentage of the native AcPase content in each clone. Each bar represents the mean ± SD of four independent blots. (*C*) Evaluation of the AcP-GFP mRNA in control cells (1) and in AcP-GFP-transfected clones (2) by Northern blot analysis as described in Material and methods. These results are representative of three independent experiments.

Proliferation rate analysis

According to our previous report, AcPase-overexpressing cells grew significantly more slowly than GFP-transfected clones [16]. In particular, a marked decrease in [^{3}H]thymidine incorporation in AcPase-overexpressing cells was observed up to 72 h after seeding (fig. 2A). Analysis of proliferative activity by crystal violet also showed a dramatic inhibition of the growth rate induced by high levels of enzyme production (fig. 2B). A reduced cumulative population doubling was also found in another stably transfected clone (AcP-GFP1), confirming that this is a common feature in all AcP-GFP clones. In-

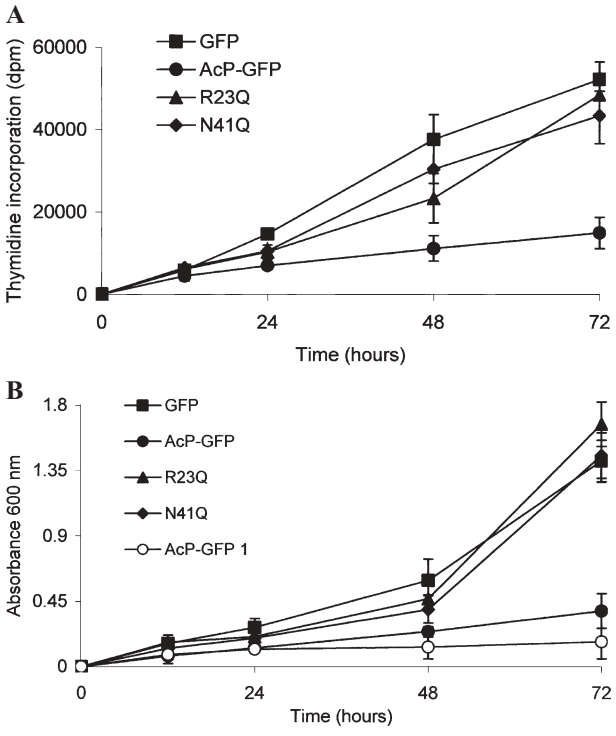


Figure 2. [^{3}H]thymidine incorporation in stably transfected SY5Y clones (*A*) and growth rate analysis of transfected clones by crystal violet staining (*B*). Values are means ± SD of four independent experiments, each performed in duplicate.

terestingly, the cell growth rate in both R23Q-AcP-GFP and N41Q-AcP-GFP clones was not affected by inactive protein expression, so that the proliferative decrease depends on AcPase enzymatic activity.

Evaluation of differentiative markers

Since the stable AcP-GFP transfectants proliferate more slowly, the reason might be a differentiative process. Morphological analysis by optical microscopy showed a typical differentiative phenotype in AcP-GFP-expressing cells. In particular, in AcP-GFP clones, both the number and length of neurites (0.45 ± 0.12 neurites/cell and 28.3 ± 5.1 µm; $p < 0.05$) were significantly higher than in GFP clones (0.32 ± 0.11 neurites/cell and 20.3 ± 3.9 µm; mean ± SD of three independent experiments, in which five randomly chosen fields were photographed) (fig. 3A). Bcl-2, which is usually up-regulated during differentiative processes, was also expressed more (about 45% increase) in AcP-GFP clones than in GFP-expressing cells or control cells, based on Western blot analysis using specific anti-Bcl-2 antibodies (fig. 3B). To further confirm the involvement of AcPase activity in activating the differentiative process, we checked another marker, namely PARP activity in AcPase-overexpressing cells. The ADP ribosylation levels of PARP substrates were 30% higher in the AcP-GFP-overexpressing clone than in control cells. Moreover, we observed an approximately twofold increase in expression of the typical neuronal differentiative marker GAP43 in the AcP-GFP line relative to GFP-expressing clones and to SY5Y untransfected cells during the exponential growth phase (fig. 3C). In contrast, GAP43 content was unaffected by inactive mutant AcP expression in R23Q-AcP-GFP and N41Q-AcP-GFP clones. Since we have previously reported GAP43 up-regulation in PMA-differentiated SY5Y cells [12], we also looked at the differentiative ability of transfected lines upon treatment with this differentiative agent. The AcP-GFP clone showed an earlier increase in GAP43 content during differentiation upon PMA exposure relative to GFP clones, confirming a greater tendency of the AcPase-overexpressing clone to develop a mature neuronal phenotype (fig. 3D).

Evaluation of necrotic and apoptotic markers

The previous unsuccessful effort to obtain cell lines overexpressing AcPase suggested an apoptotic role for AcPase [16]. To preclude the possibility that AcPase triggers necrotic or apoptotic pathways as the main cause of the reduced proliferation activity in AcP-GFP clones, we analyzed stress markers in this cell type. Physiological stress evaluation by an MTT test revealed that AcPase overexpression does not significantly modify cell viability (fig. 4A). Trypan blue viable staining confirmed a very low mortality level in all AcPase-overexpressing lines, very similar to that in SY5Y untransfected cells (data not shown). Moreover, LDH release in the culture medium at 48 h after cell seeding was not increased by AcPase overexpression (0.61 ± 0.11 U/ml against 0.67 ± 0.08 U/ml). The proportion of DNA fragmentation, as assessed by cytometric assay, was not significantly different in AcP-GFP (about 5% of total DNA content) from its value in cells expressing GFP (about 4% of total DNA content) or from R23Q-AcP-GFP (about 4% of total DNA content) or N41Q-AcP-GFP (about 3% of total DNA content) (fig. 4B). Analysis of the enhancement factor of histone-associated oligonucleosomes released in the cytoplasm confirmed these data. In regard to protein markers, Western blot analysis of caspase-3 and PARP fragments found no apoptotic pattern in stably transfected cells (fig. 4C, D).

Cellular redox status analysis

Since alterations in intracellular redox status and energetic potential are common features of necrotic and apoptotic cells, we decided to analyze these parameters in AcPase-overexpressing clones in order to assess their involvement in the cellular proliferative decrease. No experimental evidence of lipid or protein oxidation was found in any transfected clones (see table 2). In particular, levels of MDA plus 4-HNE, which is one of the most reactive end-products of lipid peroxidation, were virtu-

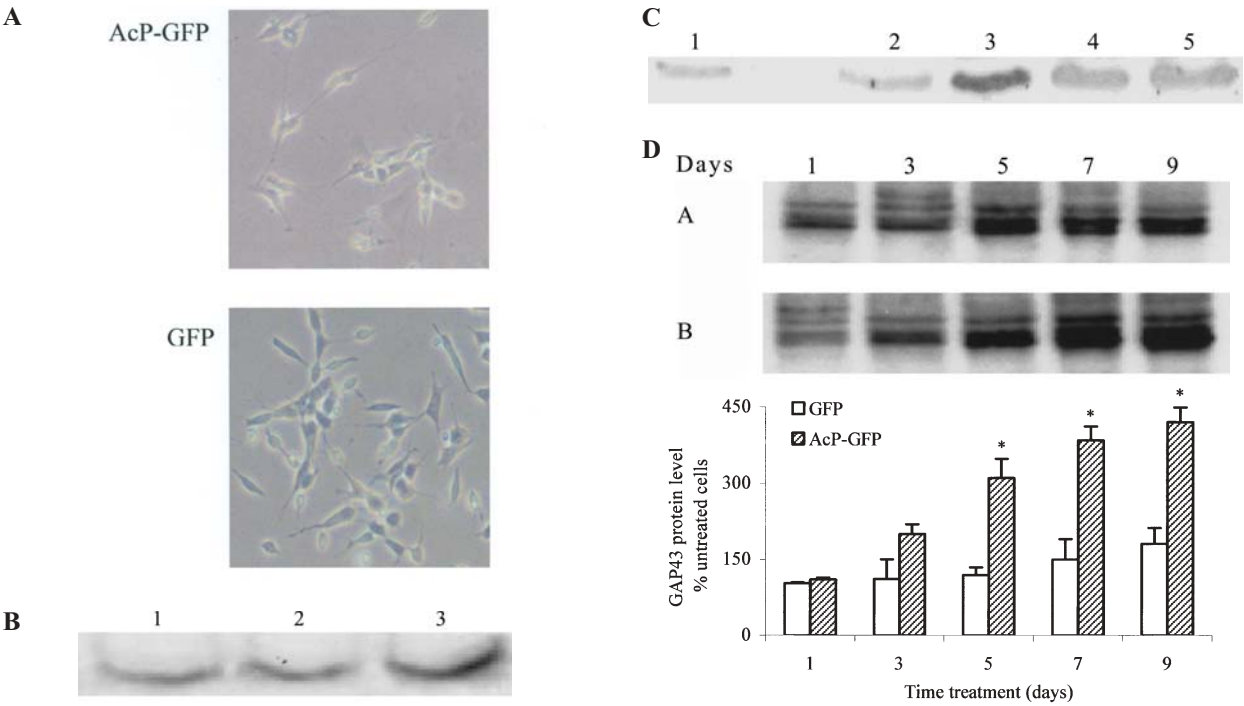


Figure 3. (*A*) Images of AcP-GFP- and GFP-expressing cells by light microscopy. Five randomly chosen fields of investigated clones were photographed using a Nikon phase contrast microscope and then neuritis number and length were measured. (*B*) Representative Western blot analysis of Bcl-2 in control cells (1), GFP- (2) and in AcP-GFP- (3) expressing clones during the exponential growth phase. (*C*) Representative Western blot analysis of neuromodulin GAP43 in SY5Y control cells (1), in GFP- (2), in AcP-GFP- (3), in R23Q-AcP-GFP- (4) and in N41Q-AcP-GFP- (5) expressing clones during the exponential growth phase. (*D*) Top: typical time course of GAP43 expression levels in GFP (A) and in AcP-GFP (B) clones during PMA-induced differentiation. Bottom: quantitative data. The band densities of the GAP43 protein were quantified as densitometric units by the Quantity One program and are expressed as a percentage of the untreated-cell value. Each bar represents the mean ± SD of three independent blots. * Significant difference ($p < 0.05$) vs GFP. For details see Material and methods.

Table 2. Markers of energetic and cellular redox status in stably transfected clones and control cells.

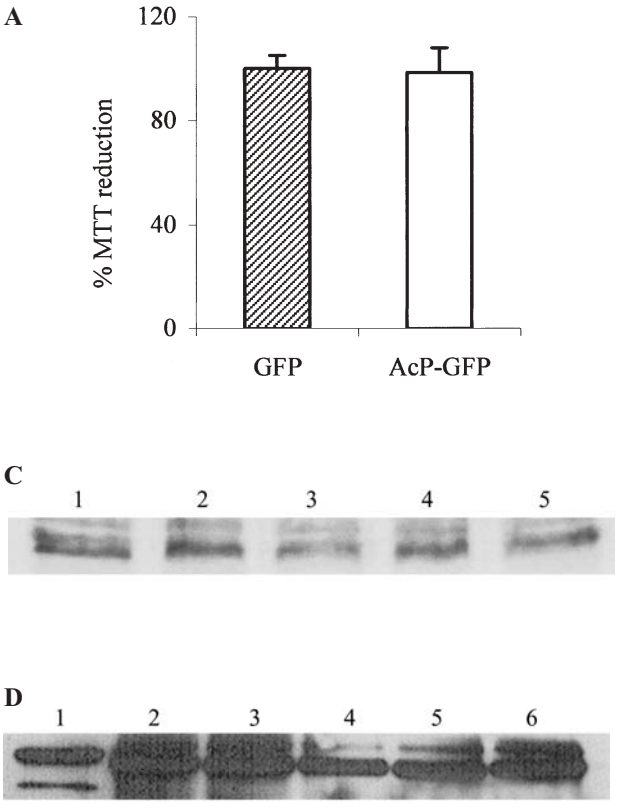
Cell type	MDA plus 4-HNE (nmol/mg protein)	Protein carbonyls (nmol/mg protein)	ATP (nmol/mg protein)
Control	0.28 ± 0.08	31.69 ± 6.53	5.01 ± 0.48
GFP	0.33 ± 0.06	32.26 ± 4.55	4.71 ± 0.48
AcP-GFP	0.27 ± 0.05	25.47 ± 3.97	5.36 ± 1.17
R23Q-AcP-GFP	0.19 ± 0.02	30.00 ± 7.29	4.92 ± 0.85
N41Q-AcP-GFP	0.28 ± 0.15	29.03 ± 3.14	3.11 ± 1.47

Data are means ± SD of two independent experiments, each performed in duplicate.

ally indistinguishable in AcPase (both wild type and mutants) and GFP clones. Likewise, we found no increase in protein carbonyl content in the AcPase-overexpressing clone. Nevertheless, the effectiveness of transfected cells remained unchanged, as assessed by a high-sensitivity bioluminescence assay of the ATP level.

Discussion

Evidence for a role of AcPase in differentiation pathways comes from several previous experimental studies of various cell types [11–14]. Since significant AcPase nuclease activity on DNA has been observed in an acidic environment, a role has been proposed for AcPase as part of a multimolecular protein complex, which hydrolyzes DNA during apoptotic processes [16, 19, 20]. There is also published evidence of an AcPase nuclear migration in response to various apoptotic stimuli in K562 and Jurkat cells. This supports the hypothesis that the isoform MT AcPase cooperates in vivo with other factors in DNA degradation processes [30]. In particular, coimmunoprecipitation experiments in Jurkat cells showed an association between AcPase and two other higher-molecular-weight DNAses.



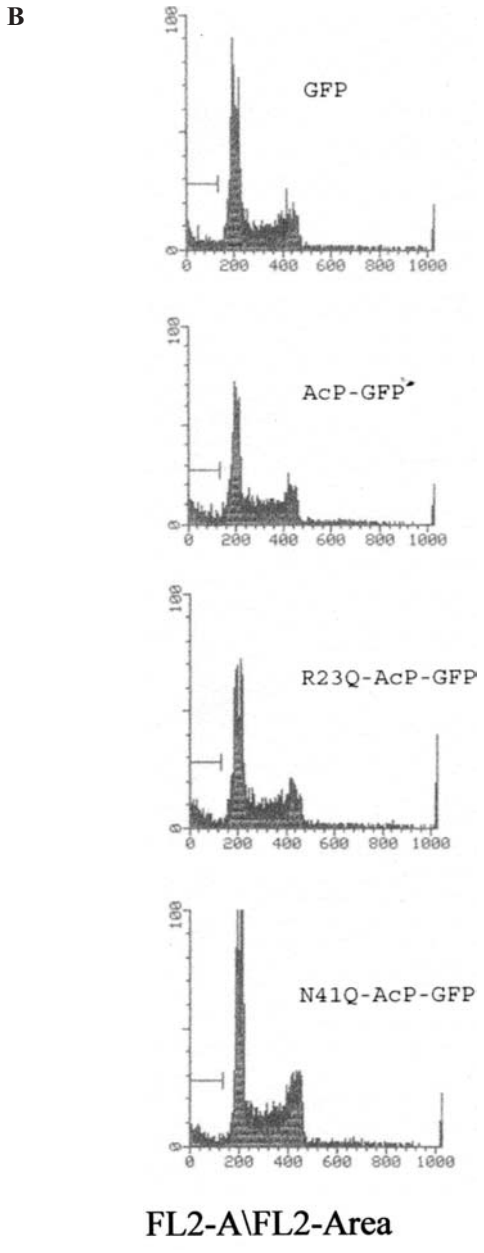


Figure 4. (*A*) Physiological stress evaluation by an MTT test in AcP-GFP clones, taking the MTT value in the GFP clone as 100%. (*B*) Cytometric analysis of DNA fragmentation corresponding to the sub-G_1 area (indicated by the bar) in stably transfected SY5Y cells. The red fluorescence intensity of PI (FL2-A/FL2-area, x-axis) is plotted against the number of fluorescent cells (y-axis). (*C*) Representative Western blot analysis of caspase-3/CPP-32 cleavage patterns; lanes: 1, control untransfected cells; 2, GFP clone; 3, AcP-GFP clone; 4, R23Q-AcP-GFP clone; 5, N41Q-AcP-GFP clone. (*D*) Representative Western blot analysis of PARP fragments; lanes: 1, typical PARP fragmentation resulting from staurosporine-inducted apoptosis in SY5Y cells; 2, control untransfected cells; 3, GFP clone; 4, AcP-GFP clone; 5, R23Q-AcP-GFP clone; 6, N41Q-AcP-GFP clone.

The present study targets the role of AcPase in neuronal differentiation pathways rather than in the apoptotic cascade. This was done by overexpressing a green fluorescent fusion protein of the wild type and two inactive mutant forms of the enzyme in SY5Y neuroblastoma cells. Geneticin-resistant clones, showing higher green fluorescence intensity, elicited an increase of at least tenfold in AcPase protein levels in the cytosolic compartment relative to control cells expressing GFP alone. Previous efforts to derive HeLa and NIH-3T3 overexpressing AcPase always failed [16], so that this is the first time the physiological role of AcPase has been studied in a cellular model using the transgene approach. We also considered the AcPase subcellular distribution as additional evidence to suggest the potential substrates of the enzyme. Since there is no evidence in this study of AcPase nuclear localization in SY5Y cells, we can exclude, at least in this cell type, involvement of this enzyme in typical apoptotic DNA fragmentation. Nevertheless, the observation that the C-terminal fusion with GFP does not change the AcPase enzymatic activity compared to the native enzyme suggests that the overexpression of chimeric protein can suitably mimic a physiological increase in AcPase level. Also, both mutant forms of AcPase were completely inactive in overexpressing cells, providing a useful tool for studying the physiological function of the enzyme.

In our cellular model, the proliferation rate was significantly reduced by AcPase overexpression, whereas SY5Y

transfected with both inactive AcPase mutants or GFP alone showed a growth rate comparable to control untransfected cells. This indicates the involvement of AcPase catalytic activity in down-regulating proliferation. Interestingly, compared to control cells, all AcPase-overexpressing clones showed a physiological mortality rate as assessed by the MTT reduction test and by trypan blue viable staining analysis and LDH release. Thus the reduced growth rate cannot be ascribed to impairment by AcPase of cell survival or induction by AcPase of necrotic processes. Furthermore, flow cytometric analysis of the hypodiploid DNA content, DNA fragmentation testing, and Western blot assays of caspase-3 and PARP active fragments all showed no evidence of an apoptotic pattern in SY5Y transfected cells. Moreover, the absence of significant alterations in energetic and cellular redox status in all transfected clones excludes their involvement in down-regulating proliferation. These data differ from previous results showing a typical apoptotic pattern during ectopic AcPase expression in the HeLa cell line [16]. These differences are probably due to some peculiar features of neuroblastoma cells. Neuroblastoma cells are characterized by increased levels of survivin, a recently described member of the IAP family which is involved in inhibition of the apoptotic response, and by a deficiency of procaspase 8, a key intermediate in the programmed cell death cascade [31]. Consequently, the neuroblastoma line is probably more resistant to programmed cell death. All selected clones nevertheless showed an increase in AcPase content up to tenfold, suggesting that this is the maximum enzyme level allowing cell survival.

In contrast, AcPase overexpression led to a marked increase in PARP activity and in Bcl-2 content, the latter being frequently up-regulated during differentiative processes [32]. In the present study, differentiation processes taking place in AcPase-overexpressing clones during proliferative down-regulation were also confirmed by the increased level of GAP43, which is a neuronal differentiation marker. A similar increase of neuromodulin GAP43 content has been already observed in this cell line during differentiation induced by exposure to retinoic acid and PMA [12]. Moreover, the present microscopic observations show a clear increase in differentiative phenotype in AcPase-overexpressing clones. These results all agree with our previous observation that both the number and length of neuritis increase during neuronal differentiation in PMA-treated SY5Y cells [12]. To better understand the role of AcPase in the differentiation pathway, we have measured the level of specialization under PMA treatment. Differentiative capability was increased in AcPase-overexpressing cells relative to GFP-expressing cells, confirming the greater tendency of AcPase-overexpressing clones to develop a mature neuronal phenotype.

To conclude, this is the first report showing a primary causative role for AcPase in differentiation processes of neural-type cells in vivo. The mechanism by which AcPase triggers the differentiation process needs further study.

Acknowledgements. This study was supported by grants from MIUR (Ministero dell'Istruzione dell'Università e della Ricerca) and from Cassa di Risparmio di Firenze.

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