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Acylphosphatase Is a Strong Apoptosis Inducer in HeLa Cell Line

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Acylphosphatase (AcP) is a low-molecular-weight protein widely distributed in many vertebrate tissues with a yet unknown physiologic function. To study the *in vivo* behavior of AcP, HeLa cells were transiently transfected with a vector expressing the AcP/EGFP fusion protein. Analysis of the transfected cells showed a high level of cellular death in cells expressing the AcP/EGFP fusion protein with respect to control cells expressing EGFP alone. Flow cytometry and time lapse analysis of AcP/EGFP transfected cells evidenced a typical pattern of apoptosis. Surprisingly, cells transfected with a mutated form of AcP, with negligible *in vitro* acylphosphatase activity, undergo apoptosis as well as cells transfected with wild-type protein, suggesting that the physiologic role of AcP could be not related to this catalytic activity.

Key Words: **acylphosphatase; apoptosis.**

Acylphosphatase (AcP) is a 11-kDa protein that is found in many vertebrate tissues predominantly as a cytosolic enzyme [1–3]. AcP exists as two isoenzymes sharing over 50% sequence identity, suggesting that both are derived from a common ancestor by gene duplication [4, 5]. One isoform called muscle type is expressed mainly in skeletal and heart muscle while the other, the common type isoenzyme, is expressed in all tissues, although its expression is particularly high in erythrocytes, brain and testis [5]. The AcP isoenzymes have a similar three-dimensional structure [6, 7], a globular α/β fold consisting of a β-sheet with five antiparallel strands and two α-helices packed parallel on the same side of the sheet, but they differ in the conformation of the loops. AcP catalyses, *in vitro*, the hydrolysis of acylphosphates such as 1,3-bisphosphoglycerate, carbamoylphosphate, succinylphosphate and β-aspartylphosphate [3]. Site-directed mutagenesis experiments have suggested that arginine 23 and asparagine 41 are essential residues of the AcP active site. Arginine in position 23 appears to be the main phosphate-binding site, whereas mutation at residue 41 resulted in an almost complete inactivation of the enzyme with no effects on substrate binding capability. It has been proposed that arginine 23 is implied in the binding of phosphate moiety of the substrate whereas asparagine 41 could be involved in binding and orienting the catalytic water molecule [3].

However, very little is known about AcP physiological function. It has been suggested a role of this protein in cellular calcium and sodium homeostasis since the acylphosphatase activity of this enzyme hydrolyses *in vitro* the aspartyl-phosphate intermediate of different membrane pumps [8–11]. In addition, some evidences suggested that AcP is involved in cellular differentiation process. In fact, increase of both isoforms can be observed in the K562 erythroid cell line after induction of differentiation [12]. In a recent paper, Fiaschi *et al.* [13] have shown that the 5′-untranslated region (5′-UTR) of human AcP muscle isoform has an inhibitory effect on AcP expression. In fact, the 5′-UTR of the AcP mRNA presents an AUG triplet upstream the protein start codon and a very stable stem-loop structure. Both these features contribute to the inhibition of mRNA translation [13]. Modulation of translation rate has long been recognized as a mechanism for controlling global synthetic activity of the cell. More recently, it has been found that specific genes can also be regulated through particular features of their mRNA molecules such as a stable secondary structure or initiation codons and upstream open reading frame (uORF) [14, 15]. In fact, the inhibitory effect on mRNA translation of these AUG triplets allows a fine-tuning of gene expression. This tight regulation of AcP expression, usually reserved to proto-oncogenes products, could be a clue of a very important role that this ubiquitous protein plays in cellular processes. In fact, the results reported in this paper indicate that the expres-

Abbreviations used: AcP, acylphosphatase; N41Q-AcP, N41Q-mutated acylphosphatase; EGFP, enhanced green fluorescent protein; 5′-UTR, 5′-untranslated region.

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sion level of AcP is critical for HeLa and NIH-3T3 cell surviving, since ectopic expression of this protein leads to apoptotic cell death induction.

MATERIALS AND METHODS

Materials. Unless specified all reagents were obtained from Sigma. HeLa and NIH-3T3 cells were purchased from ATCC; pEGFP-N1 vector and anti-EGFP antibodies were from Clontech; propidium iodide and G418 was from Calbiochem; Enhanced Chemi-Luminescence kit was from Amersham; restriction enzymes, synthetic oligonucleotides and the unique site elimination mutagenesis kit were from Pharmacia; Trizol reagent was from Gibco Life Technologies.

Construction of AcP/EGFP expression vector. To express the cDNA coding for muscle type AcP as a fusion protein with EGFP (enhanced green fluorescent protein), we performed a site directed mutagenesis on pEGFP-N1 vector in order to eliminate the ATG start codon of the fluorescent protein. The oligonucleotide Mut1 (5′GCAAGCAGTCCCATTTGTCCCCTCCC), which contains the codon GGG for glycine, instead of ATG triplet, was used in unique site elimination method together with the oligonucleotide Mut2 (5′GGCTTTTTTGGAGGGGTAGGCTTTTGC), which eliminates the *Stu*I restriction site in the pEGFP-N1 vector. The cDNA for AcP were amplified by PCR using two specific oligonucleotides, which maintain the ATG triplet and eliminate the stop codon. In particular, the AcP cDNA was amplified using the primers "direct" (5′TTTTTGAATTCATGAGCACCGCCCAAAGCC) and "reverse" (5′TTTTGGATCCACGCCATAGCGAATAGAAAA) containing the restriction site for *Eco*RI and *Bam*HI respectively. The PCR products were digested with the two restriction enzymes and cloned in the mutated form of pEGFP-N1 vector upstream the coding sequence of the EGFP protein. The vector expresses a fusion protein in which the C-terminal of AcP is linked to the N-terminal of EGFP protein. Cloning of the AcP N41Q mutant in pEGFP-N1 vector was performed following the same procedure used for the wild type protein.

Cell culture and transfections. HeLa and NIH-3T3 cells were routinely cultured in DMEM added with 10% fetal calf serum (FCS) in 5% CO_2 humidified atmosphere. Transfections were performed according to the method of Chen and Hiroto [16]. Briefly, exponentially growing cells were seeded at 5×10^5 cells per 10-cm plate and incubated overnight in 10 ml of growth medium. 20 μg of plasmid DNA were mixed with 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml of 2× BBS (50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4). After incubation for 20 min at room temperature, the calcium-phosphate DNA solution was added dropwise to the cells and incubated for 24 h at 37°C under 3% CO_2. The medium was removed, the cells were rinsed twice with PBS buffer, reefed and incubated at 37°C under 5% CO_2. In transient transfection experiments the cells were trypsinized at different time after transfection and analyzed by flow cytometry in order to evaluate the transfection efficiency. The pool of stable transfected cells were obtained after incubation for several days in the presence of G418 (400 μg/ml).

Growth rate determination. Cell viability was measured by trypan blue exclusion staining. 2×10^5 cells were seeded into 6-cm dishes and transfected with the vectors expressing AcP/EGFP, N41Q AcP/EGFP and EGFP alone as control. 24, 48 and 72 h after transfection floating and adherent cells were mixed with trypan blue (1:1 v/v). After 5–10 min of incubation, the number of cell excluding or staining positively for uptake of trypan blue were counted. The percentage of died cells was calculated at different times after transfection.

Flow cytometric analysis. Flow cytometric analysis was performed according to Liu [17]. Briefly, 5×10^5 cells were seeded into 10-cm dishes and transfected with the three different constructs (see Figs. 2 and 3) using the calcium-phosphate method (see above). After 24 h from transfection cells were rinsed twice with cold PBS. In necrosis determination experiment (Fig. 2) cell were harvested in PBS containing 5 mg/liter PI and analyzed. In DNA fragmentation experiment (Fig. 3) cells were lysed in 1 ml Na-citrate 50 mM 0.1% Triton X-100 containing 50 mg/liter of propidium iodide (PI). For each sample were acquired and analyzed 20,000 cells. Analysis was performed in a Becton–Dickinson FACScan using the Lysis II and Cell Fit Cell Analysis Software according to the manufacturer's procedure.

Time-lapse videomicroscopy. Apoptotic cell death was scored by means of a Zeiss inverted microscope equipped with a time-lapse videomicroscopy apparatus (JVC BR9030 time-lapse videorecorder and Panasonic CCD cameras). Cells were transfected with the AcP/EGFP expressing construct or with pEGFP-N1 control vector, according to the protocol described above. Sixteen hours after transfection the cells were rinsed twice with PBS, refed and monitored for additional 24 h. The optic field was chosen randomly and the cell number at the beginning of the experiment was similar in each transfection (see the results for details).

RESULTS AND DISCUSSION

Analysis of AcP/EGFP and N41Q-AcP/EGFP Expression in HeLa Cell Line

Previous experiments evidenced a great difficulty to obtain stable cell lines overexpressing AcP, suggesting that a high level of expression of the protein could be harmful for cell survival. Hence, we decided to perform

transient transfection experiments on HeLa cells using muscle type AcP as a fusion protein with EGFP. This kind of construct allows monitoring of AcP expression level at different times after transfection by evaluation of fluorescence intensity of the reporter protein.

We transfected both wild type and a mutant form of AcP (N41Q), which is almost inactive [18], in order to investigate the role of AcP catalytic activity within the cell. Flow cytometric analysis 24 h after transfection revealed that transfection efficiency with either AcP/EGFP, N41Q-AcP/EGFP or EGFP vectors were comparable (data not shown). Measurement of growth rate of cells transfected with the three different constructs showed that cells expressing either AcP/EGFP or N41Q-AcP/EGFP possess a very reduced growth rate in comparison to that of EGFP-transfected cells (Fig. 1A). On the same cells trypan blue viable staining revealed that both AcP/EGFP and N41Q-AcP/EGFP expressing cells showed a very high mortality rate with respect to EGFP transfected cells at all considered times (Fig. 1B).

Interestingly, the rate of mortality induced by AcP transient expression does not depend upon its acylphosphatase activity, since N41Q-AcP mutant (which retains a negligible activity on benzoylphosphate or ATP) acts as the wild-type enzyme. Hence, we have tried to establish stable transfected HeLa cell lines overexpressing AcP; three weeks after transfection we were able to detect, by means of cytometric and Western blot analysis, an AcP/EGFP expression about 50 times lower with respect to cells transfected with EGFP alone. Hence, these data suggested that cell survival may be linked to very low levels of ectopic AcP expression. The same kind of experiment was repeated using NIH-3T3 cell line with very similar results, indicating that AcP-induced cell death is not restricted to a particular cell line (data not shown).

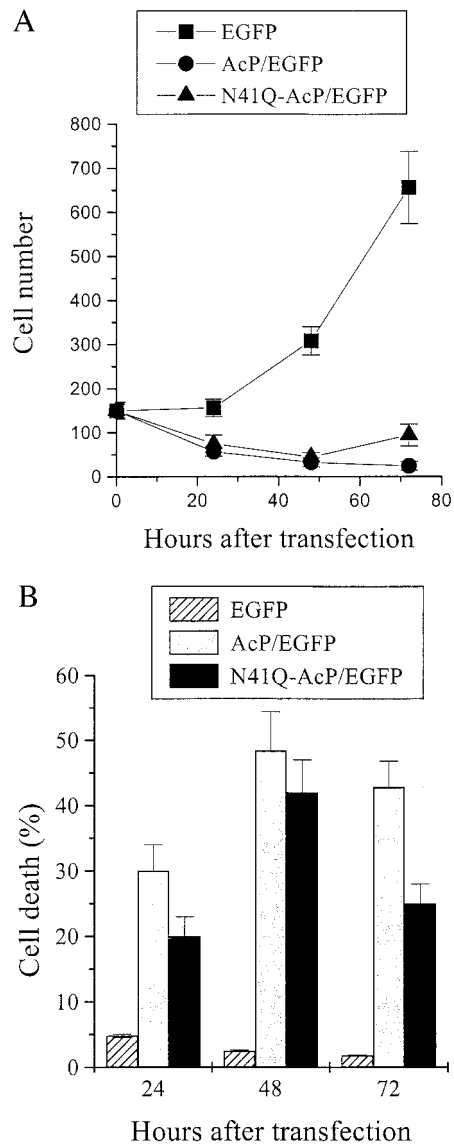

FIG. 1. (A) Growth rate of transiently transfected HeLa cells. Exponentially growing HeLa cells were transfected with AcP/EGFP, N41Q-AcP/EGFP or EGFP expressing vectors. 24, 48, and 72 h after transfection the number of viable cells was determined by trypan blue exclusion staining as described under Materials and Methods. (B) Percentage of death in cell expressing AcP/EGFP, N41Q-AcP/EGFP, or EGFP alone. Death rate, evaluated by trypan blue staining, was measured at 24, 48, and 72 h after transfection with the three different constructs.

Flow Cytometric Analysis of Necrotic Pattern in AcP-Transfected Cells

The overexpression of AcP even at very low level is not compatible with cell survival. Two alternative modes of cell death can be distinguished, apoptosis and accidental cell death, generally defined as necrosis [19]. Necrosis is a passive degenerative process whose early stage is mitochondrial swelling followed by rupture of plasma membrane and release of cytoplasmic content. Hence, one of the characteristic features of necrosis is the alteration of plasmalemma's permeability. We have performed a bidimensional cytometric analysis using propidium iodide (PI), a molecule that is normally excluded from viable cells. PI was used in isotonic condition to evaluate the percentage of necrotic cells in AcP/EGFP transient transfected cells. The analysis was performed at various times after transfection. Figure 2 shows the bidimensional analysis of the cells 24 h after transfection, a time at which we observed a very high mortality rate in cell transfected with either wild-type or N41Q-mutant AcP (Fig. 1B). The cells present in the II quadrant are both positive for: (i) green (FL1) fluorescence, and consequently positives for EGFP expression either alone or fused with AcP; (ii) red fluorescence due to PI (FL2), that is an evidence of necrotic cell death. Hence, Fig. 2 shows that the necrotic pattern is very similar in cells

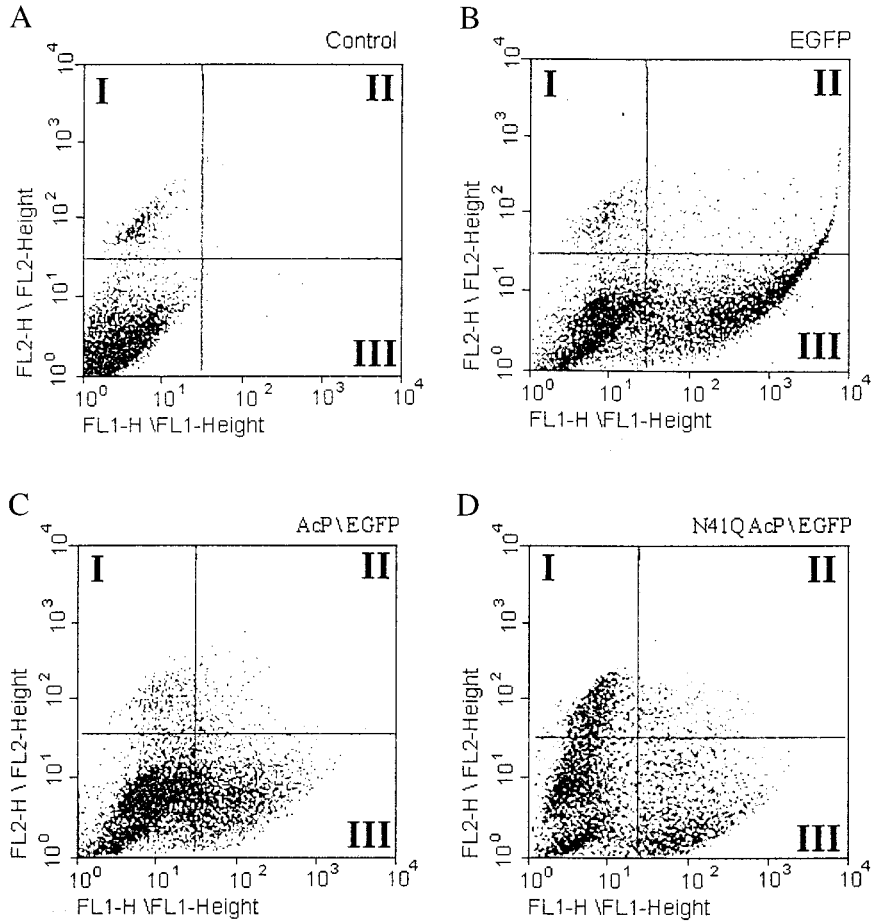

FIG. 2. Bidimensional flow cytometric analysis of transiently transfected HeLa cells 24 h after transfection. The green fluorescence intensity of EGFP (FL1, *X* axis) is reported against the red fluorescence intensity of PI (FL2, *Y* axis). The percentage of necrotic cells positive for both FL1 and FL2 fluorescence (II quadrant) in AcP/EGFP (1.2%) and N41Q-AcP/EGFP (3.1%) transfection is similar to that of EGFP alone transfected cells (2.8%). The control mock-transfected cells, without any green fluorescence, show a similar amount of PI-positive necrotic cells (I quadrant). The overall number of cells acquired for each sample is 20,000. The transfection efficiency (sum of cells in quadrant II and III) is similar in all the experiments (about 30%). The results are representative of three independent experiments.

transfected with the different constructs. In fact the percentage of necrotic cells (II quadrant) in EGFP-expressing HeLa cells is 2.8%, comparable with the values obtained for AcP/EGFP and N41Q-AcP/EGFP expressing cells (1.2 and 3.1% respectively). It can be noted that transfection efficiency (which is represented by the sum of cells that are present in quadrant II and III) is about 30% in all transfected cells (either with AcP/EGFP, N41Q-AcP/EGFP or EGFP alone). These results show that, very likely, the overexpression of AcP does not induce cell death by activating the necrotic process.

Flow Cytometry and Time-Lapse Analysis of AcP-Transfected Cells

Our data indicate that AcP transient overexpression induce cell death but not with the typical feature of necrosis. We have tested the hypothesis that AcP could instead induce apoptosis. Apoptosis, or programmed cell death, is a genetically controlled multistep mechanism that leads to the selective elimination of unwanted cells. Apoptosis is involved in a variety of biological processes including embryonic development, immune system regulation, tissue homeostasis and in the prevention of malignancies such as tumor growth and viral infection. A cell triggered to undergo apoptosis activates a cascade of molecular events that ultimately lead to cell disintegration. There are many cellular changes that are peculiar of the apoptotic process that can be used as markers to identify this mode of cell death. One of the early events is cell dehydration that leads to a condensation of cytoplasm followed by changes in cell shape and size. Another peculiar modification is condensation of nuclear chromatin followed by nuclear fragmentation. In addition, at least during the initial phase of apoptosis, the plasma membrane structure and function are preserved. Hence, apoptotic cells undergo to an extensive DNA cleavage that can be

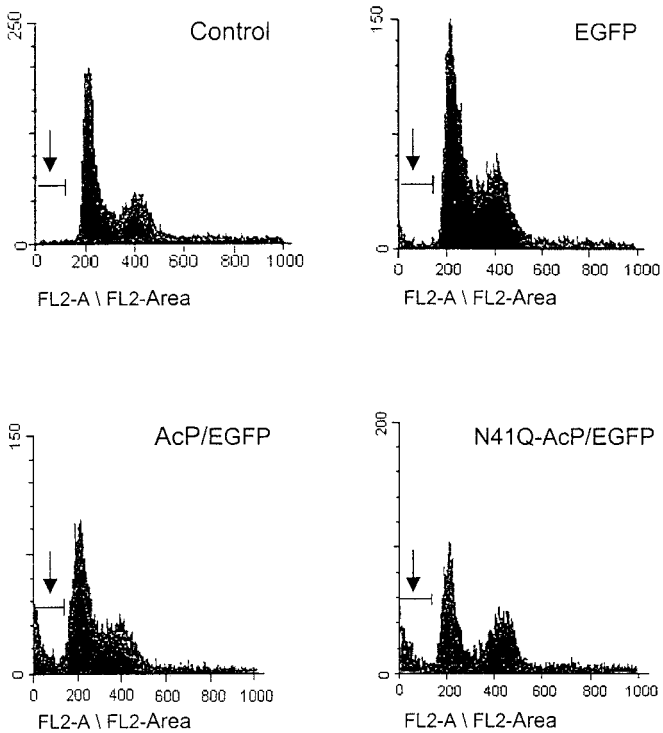

FIG. 3. Cytometric analysis of DNA fragmentation of transiently transfected HeLa cells 24 h after transfection. The red fluorescence intensity of PI (FL2, *X* axis) is reported against the number of fluorescent cells (*Y* axis). The percentage of DNA fragmentation, correspondent to sub-G_1 area (indicated by the arrow), in both AcP/EGFP and N41Q-AcP/EGFP-expressing cells (about 40 and 31%, respectively) is greater than DNA fragmentation of EGFP and mock-transfected cells (about 4 and 7%, respectively). The transfection efficiency is similar in all experiments (about 30%). The results are representative of three independent experiments.

evidenced as a "sub-G1" peak in cytometric analysis [20–22]. Moreover, we have evaluated the presence of DNA fragmentation, a typical marker of apoptotic cell death, in transient AcP/EGFP-transfected HeLa cells. This analysis was performed 24 h after transient transfection of AcP/EGFP, N41Q-AcP/EGFP or EGFP alone using propidium iodide staining in hypotonic conditions. The pattern of DNA fragmentation showed significant differences among differently transfected cells, even if transfection efficiency was comparable for all tested cells (about 30%). As shown in Fig. 3, EGFP-expressing cells showed a percentage of about 7% of DNA fragmentation, similar to the mock transfected cells (4%). This value is highly increased in cells harboring the AcP recombinant constructs. In fact, AcP/EGFP expressing cells show about 40% of DNA fragmentation, similar to the value of the cells transfected with the N41Q-AcP mutant (31%). These results clearly indicate that the overexpression of AcP leads to an at least 4-fold increase of DNA fragmentation with respect to the control cells expressing EGFP alone. In addition, in order to confirm the data obtained by DNA fragmentation analysis, 16 h after transient transfection the EGFP and AcP/EGFP expressing cells were monitored by time-lapse videomicroscopy for an additional period of 24 h (Fig. 4). Within the period of observation, the cells expressing AcP lost adherence to the tissue culture dish and exhibited the main characteristic hallmarks of apoptosis such as cell condensation, membrane blebbing and apoptotic bodies formation (Fig. 4B). These effects were not observed in EGFP expressing cells, which maintained the typical phenotype of living cells (Fig. 4A), also in the presence of the

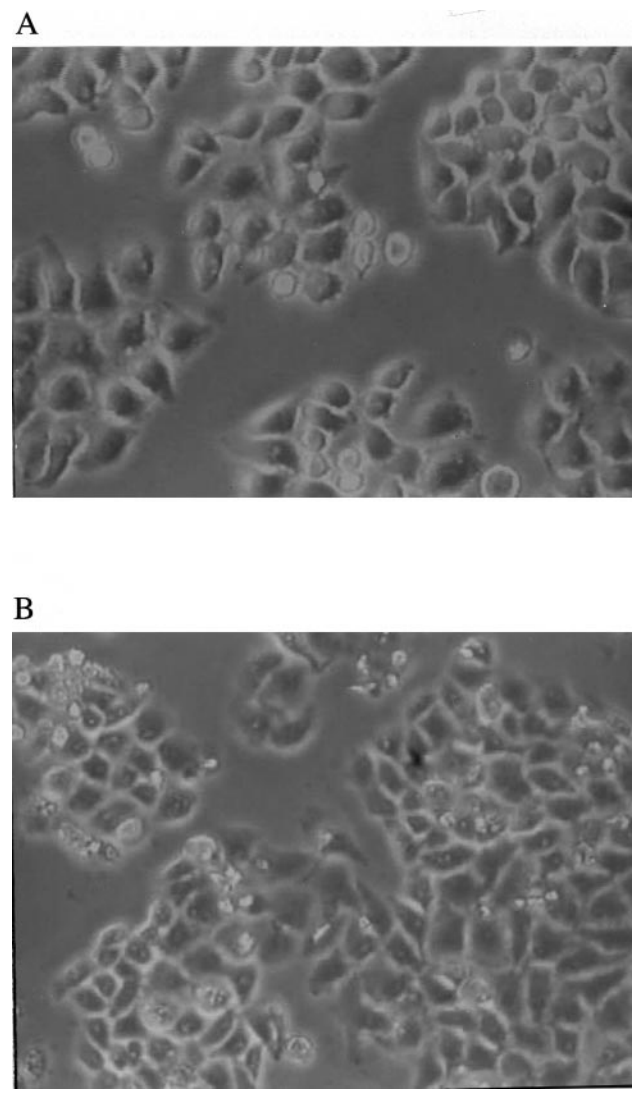

FIG. 4. Time-lapse videomicroscopy of HeLa cells after transient transfection with AcP/EGFP. These images were acquired 24 h after transfection and reveal that a significant number of AcP/EGFP expressing cells (B) exhibit morphological changes typical of apoptotic cells with respect to EGFP expressing ones (A), which maintain the normal phenotype of living cells. During the overall period of analysis (24 h) we observed 31 apoptotic events in AcP/EGFP expressing cells while only 3 apoptotic events were counted in control EGFP expressing cells. The total number of analyzed cells was similar in the two samples (see text).

same transfection efficiency of AcP/EGFP-transfected cells. In addition, within 24 h of observation, the cells transfected with pEGFP-N1 showed only 3 apoptotic events in contrast with the 31 observed in the AcP/EGFP expressing cells. We underline that the number of cells contained in the optic field at the beginning of time lapse analysis was similar in EGFP expressing cells and AcP/EGFP expressing ones (98 and 76, respectively). Furthermore, we assessed that during the period of analysis the AcP/EGFP expressing cells present a lower growth rate (1.9-fold) with respect to control cells (2.7-fold) reaching 184 cells and 212 cells respectively. According to previous results, the time lapse data confirm that the overexpression of AcP leads to cell death involving the activation of apoptotic machinery.

The ectopic expression of a limited number of proteins, such as p53, cyclins, some Bcl-2 family members, etc., is able to induce apoptotic cell death. We have now found that AcP too can induce apoptosis in transient transfected HeLa and NIH-3T3 cells. Surprisingly, the N41Q-AcP mutant, a protein that has lost most of its *in vitro* acylphosphatase activity on benzoyl-phosphate [18] and ATP [23], behaves exactly as the wild-type enzyme for what cell survival is concerned, thus suggesting that the well characterized *in vitro* acylphosphatase activity of this protein is not necessary for AcP-induced cell death. The results that we have exposed in this work are very interesting in the light of an important observation of Fiaschi *et al.* [13] on the regulation of AcP expression mediated by the 5′-UTR of its mRNA. The primary target for translational control is at the initiation step, and control of translational initiation on individual mRNA is determined primarily by the structural properties of the mRNA, particularly the 5′-UTR [24]. The most important 5′-UTR features that control mRNA translation are its secondary structures, the presence of additional initiation AUG codons and of an upstream open reading frame. Fiaschi *et al.* [13] have found that the 5′-UTR of AcP has an inhibitory effect on protein expression because possess both an upstream AUG and a very stable stem-loop structure. Interestingly, only less than 10% of the vertebrate mRNAs contains initiation codons upstream the major open reading frame [25]. In addition, the population of mRNAs containing upstream AUG is strongly biased toward proto-oncogenes products and genes encoding growth factors and cell-surface receptors [26–29]. An explanation for this kind of translational inhibition of AcP is that AcP expression must be strictly controlled in order to avoid apoptotic cell death.

In our opinion, the findings described in this work shed a new light on a protein whose physiologic role, probably not linked to its acylphosphatase activity, could be very important in regulating key aspects of cell proliferation and/or survival.

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