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Acylphosphatase is involved in differentiation of K562 cells

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Abstract

The level of both isoforms of acylphosphatase was evaluated in the human erythroleukemia K562 cell line during differentiation. K562 cells were treated with PMA, which induces megakaryocytic differentiation, and with aphidicolin or hemin, which stimulate erythrocytic differentiation. While the MT isoform showed an average 10-fold increase independently of the differentiating agent used, only hemin treatment caused a similar increase of the CT isoform, suggesting a different role of the two isoforms in the cell. Treatment with either hemin or aphidicolin of K562 cells overexpressing the two acylphosphatase isoforms suggested the possibility that acylphosphatases play a role in the onset of differentiation.

Keywords: Acylphosphatase, phosphomonohydrolase, hemin, aphidicolin, PMA, differentiation, K562 cell line

Abbreviations: MT, Muscular type and CT, common type, isoforms of acylphosphatase; PMA, phorbol 12-myristate 13-acetate; T_3, tri-iodothyronine

Introduction

Acylphosphatase is a small basic protein widely distributed in animal tissues and especially in skeletal muscle, brain and erythrocytes. Two isoforms of this enzyme are known. These isoenzymes are present at differing ratio in various tissues and have been named 'muscle type' (MT) and 'common type', or 'erythrocyte' form (CT). They are structurally related, showing a 56% sequence homology. Acylphosphatase appears to be present in all vertebrates as well as in some invertebrates, and the amino acid sequence of the two isoenzymes in different animal classes is highly conserved. The enzyme catalyses the hydrolysis of the carboxyl phosphate bond present in physiological compounds like carbamoylphosphate, 1,3-diphosphoglycerate and other acylphosphates, such as β-aspartylphosphate of membrane pump phosphorylated intermediate (for a review see Stefani and Ramponi, 1995). Particularly it has been demonstrated that both isoenzymes are able to *in vitro* hydrolyse the phosphorylated intermediate formed during the action of the Na^+, K^+ ATPase of erythrocyte plasmamembrane (Nassi *et al*, 1993) and of the Ca^{2+}-ATPase of both erythrocyte membrane (Nassi *et al*, 1991) and heart sarcolemma (Nediani *et al*, 1992). In all these cases high affinities and low apparent Km values of the enzyme for the phosphorylated intermediate formed during the activity of the ions pump have been demonstrated (Nediani *et al*, 1995). The action of acylphosphatase on red blood cell membrane causes a significant increase of the rate of ATP hydrolysis and is accompanied by a decrease of the Ca^{2+} transport rate. These effects lead to a reduction in the efficiency of the membrane calcium pump. Similar results have been obtained with the Na^+, K^+ pump in the erythrocyte membrane. Physiological amounts of both isoenzymes significantly reduced the Na^+/ATP stoichiometry of the Na^+, K^+ transport in red blood cell membrane.

The modification of the acylphosphatase level during cell aging and differentiation were determined in various cell types. The concentration and the activity of CT isoenzyme increase in red blood cell with age, and in mature erythrocytes its expression reaches a maximum (Liguri *et al*, 1987). Changes in the level of the MT isoenzyme in cultured myoblasts during myotubes differentiation were also investigated (Berti *et al*, 1992). The enzyme content significantly increases during differentiation in parallel with muscle-specific proteins. The turnover of the MT isoenzyme indicates that the enzyme can be considered to belong to the short lived protein group. It has been also demonstrated that T_3 (tri-iodothyronine), a bland differentiating agent for K562 cells (Arumanayagam and Swaminathan, 1992), can activate the MT acylphosphatase gene, so that the enzyme concentration results to be enhanced after hormone treatment (Chiarugi *et al*, 1995). In contrast, T_3 has no effect on the CT form, indicating that the two genes are differently regulated, and suggesting a distinct role for these two molecules in the cell. As far as the physiological role of this enzyme is concerned, recent data have demonstrated that both isoforms have DNAse and RNAse activity *in vitro* (Chiarugi *et al*, 1996).

In order to better understand the role of acylphosphatase in cellular differentiation, we have carried these studies in K562 cells. This human erythroleukemia cell line is a useful model, because it can differentiate in multiple ways upon exposure to appropriate stimuli (Leary *et al*, 1987; Butler *et al*, 1990; Cheng *et al*, 1994). Erythroid differentiation of these cells can be induced by treatment with several compounds. One of them is hemin (Cioe *et al*, 1984), which has been shown to induce haemoglobin synthesis; however it does not trigger the terminal differentiation of K562 cells. The removal of hemin is, in fact, followed by a rapid reversal of the differentiated erythroid phenotype. Continuous presence of the inducer in the growth medium is necessary to maintain the differentiated state. Another compound capable of inducing erythroid differentiation of K562 cells is aphidicolin (Moore *et al*, 1992). When this

molecule, which is a potent inhibitor of DNA replication, is used as inducer, K562 cells acquire the erythroid phenotype, but in this case they are irreversibly committed to terminal differentiation. This irreversible induction of haemoglobin synthesis is associated with the loss of cell renewal capacity. On the other hand, treatment with phorbol 12-myristate 13-acetate (PMA) induces the expression of megakaryocytic markers (Tabilio *et al*, 1983) and inhibits the erythroid differentiation (Murate *et al*, 1993).

In this paper we study the correlation between the expression of acylphosphatase isoenzymes and erythroid or megakaryocytic differentiation in K562 cells, induced with different specific chemical stimuli.

Results

Acylphosphatase accumulation in differentiating K562 cells

Changes in acylphosphatase isoenzymes expression associated with distinct differentiation processes in K562 cells have been measured. In our experiments the accession of K562 cells to the erythroid phenotype was measured as percentage of benzidine-positive cells, and concentrations of the two acylphosphatase isoenzymes were determined by ELISA. The results obtained using hemin, aphidicolin or PMA as differentiating agents are shown in Figure 1. Little expression of both isoenzymes was observed in K562 cells when undifferentiated. Following exposure to hemin (Figure 1A), the levels of both isoenzymes were found to increase. Their amounts were determined at different times from the addition of the inducer. The increase of the CT isoenzyme was very rapid, reaching 60% of its highest level of expression within the first day of induction. Then it slowly rose to touch maximum during the remaining days. The CT isoenzyme was expressed more than the MT one, showing a 15-fold increase with respect to the basal amount present in undifferentiated cells. During these experiments the cell viability, monitored daily, was always well above 90%.

When aphidicolin was used as inducer (Figure 1B), the level of the MT isoform showed a rapid six-fold increase with respect to undifferentiated controls. In contrast, the CT isoform showed a much lower and delayed increase (about two-fold after 4 days). In the case of aphidicolin, the removal of the inducer from differentiative medium after three days did not affect the induction of erythroid phenotype, and the expression of acylphosphatase isoenzyme was also unaffected.

Changes in the levels of the two acylphosphatase isoenzymes occurring during megakaryocytic differentiation of human erythroleukemia K562 cell line, after PMA induction, were determinated (Figure 1C). This kind of differentiation was monitored by checking the reduction of growth potential and negative haemoglobin staining. Cells became enlarged with large irregularly shaped nuclei and growth was arrested. Morphological changes associated with the megakaryocytic differentiation were followed as reported in Material and Methods. PMA treatment of K562 cell line was characterized by a six-fold increase of the MT isoenzyme within two days, whereas the erythrocyte

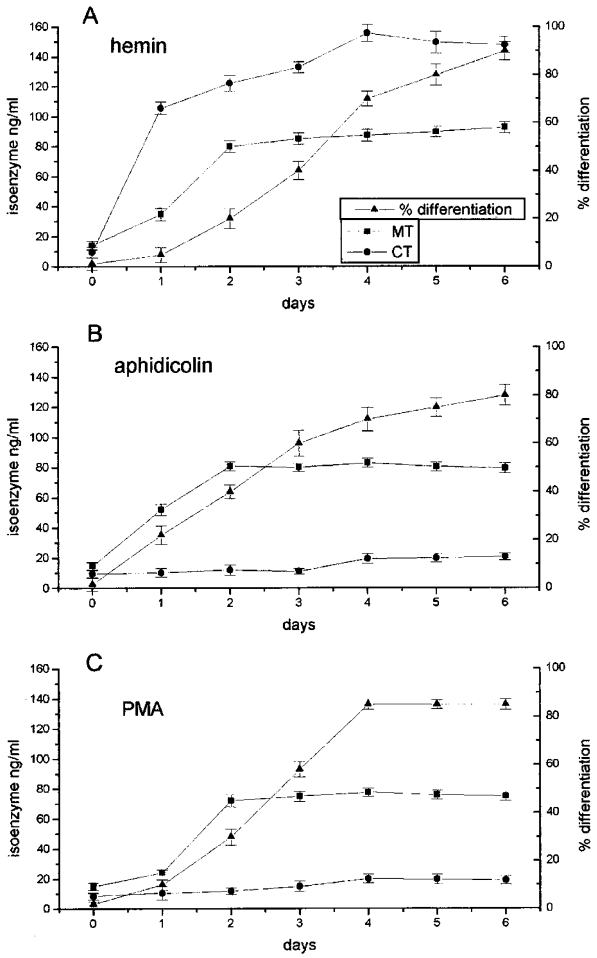


Figure 1 Time course of acylphosphatase levels in K562 cells after addition of hemin (**A**), aphidicolin (**B**) or PMA (**C**). Circles: CT isoenzyme; squares: MT isoenzyme; triangles: percentage of differentiated cells. The results are the mean of three independent experiments. S.D. is indicated.

isoenzyme showed a 2.5-fold increase only after four days of treatment. After three days of PMA treatment, cells were irreversibly committed to the morphological changes of megakaryocytic differentiation. The accumulation of both acylphosphatase isoenzymes did not show any difference according to whether the PMA treatment was continuous, as shown in the figure, or interrupted (data not shown).

Acylphosphatase mRNA levels in differentiating K-562 cells

Total RNA was extracted from control K562 cells and after 1 and 3 days of treatment with differentiating agents such as aphidicolin, hemin and PMA. RNAs were separately hybridized with both human cDNAs coding for the MT and CT isoforms of acylphosphatase (Chiarugi *et al*, 1995; Fiaschi *et al*, 1995). Even if the two probes show some identity (about 65%) it has already been demonstrated that no cross-hybridization occurs (Chiarugi *et al*, 1995). The results of this experiment are shown in Figure 2A. The autoradiography

of a typical Northern blot experiment using the two probes is shown, together with the result of a hybridization of the same filter with an actin probe. In Figure 2B a quantitative evaluation of the same experiment after normalization to actin hybridization is presented. All the differentiating agents used are capable of enhancing the levels of both specific mRNAs about eight times, with respect to the basal level. This effect already reaches a plateau after one day of treatment. The basal levels of the two specific RNA are quite different in this cell line, where the CT isoform mRNA is about 20 times more abundant than that of the MT one. This fact has apparently no consequence on the basal level of the two isoenzymes in the cell, which is very similar, as it can be observed for istance in Figure 1, where T_0 represents a measure on cells before any stimulus . Apart from that, both specific mRNA levels increase about eight times, in comparison to their own basal level, independently of the differentiating treatment used.

Half-lives of the two isoenzymes

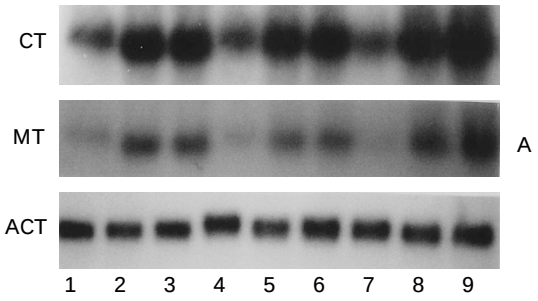
The half-lives of acylphosphatase isoenzymes either in undifferentiated or differentiated K562 cell line were determined by pulse chase method, as described in Materials and Methods. In Table 1 the $t_{1/2}$ values of CT and MT isoenzymes of normal and differentiated cells after treatment with hemin and PMA are presented: the PMA treatment did not affect the half-life of MT isoenzyme ($t_{1/2}$=4.3 h), whereas the CT isoenzyme showed a longer half-life ($t_{1/2}$=11.2 h) than the control value ($t_{1/2}$=8.8 h). Under hemin induction the half-life of the CT isoenzyme was considerably shorter ($t_{1/2}$=4.6 h) with respect to the control ($t_{1/2}$=8.8 h), whereas the half-life of MT isoform was longer ($t_{1/2}$=13.4 h).

Acylphosphatase overexpression in K562 cells

In order to study the possible role of acylphosphatase in differentiation, we overexpressed either one or the other acylphosphatase isoenzyme in K562 cells. To this end, we

Table 1 Half-lives (hours) determination of acylphosphatase MT and CT isoenzymes in K562 cells under hemin treatment (see Materials and Methods) and PMA treatment

Treatment	MT isoenzyme	CT isoenzyme
Control	4.3	8.8
PMA	4.3	11.2
Hemin	13.4	4.6



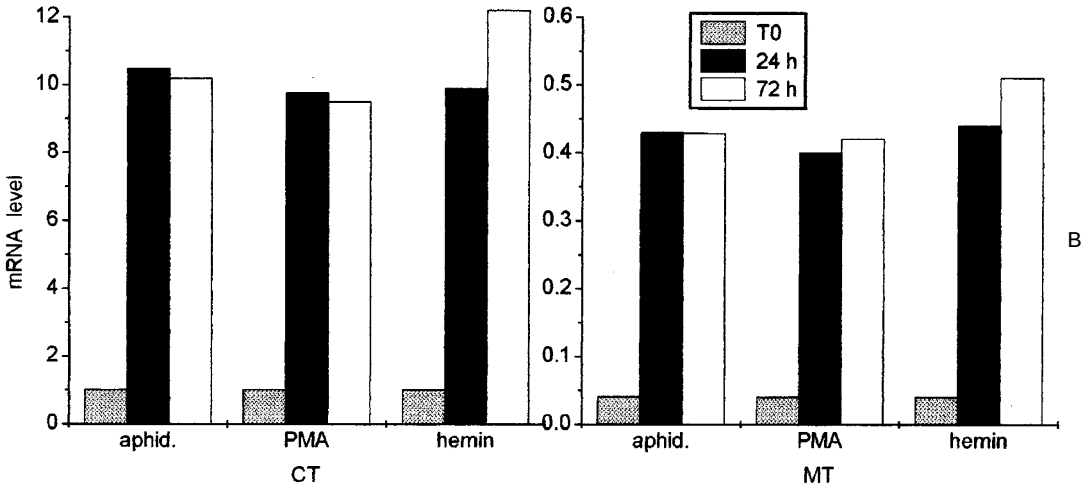


Figure 2 **A**: Typical Northern blot analysis using total RNA from K562 cells, using acylphosphatase MT or CT cDNA probes. Lanes 1, 4 and 7: untreated cells. Lanes 2 and 3: 24 and 72 h of aphidicolin treatment. Lanes 5 and 6: 24 and 72 h of PMA treatment. Lanes 8 and 9: 24 and 72 h of hemin treatment. Hybridization with murine actin cDNA has been used for normalization. Exposure times: 5 days for MT, 24 h for CT and 4 h for actin probe. **B**: Quantitation of the experiment of panel A, after normalization with actin. Two distint graphs are presented fot MT and CT isoforms. Note that the two scales are different.

cloned both acylphosphatase isoform cDNAs under the control of a CMV promoter in the eukaryotic expression vector pRC-CMV, also carrying the neomycin resistance gene. By means of transfection and selection with G418 we created different stable K562 clones overexpressing the MT or CT isoform: clone 6 (overexpressing the MT form) and clone 5 (overexpressing the CT form) were chosen for further experiments. The enzyme content was evaluated by ELISA, using polyclonal antibodies against the two different isoenzymes: the overexpression of both isoforms resulted to be about 10 times higher than the basal level in exponentially growing cells.This level of enzyme is comparable to that reached in the cell during differentiation, as already seen in previous sections. Growth of clones 6 and 5 was tested in comparison to control cells: results showed that the overexpression of acylphosphatase does not have any consequence on the growth rate of the cells.

As a first observation it was possible to note a higher tendency of the clone overexpressing the MT form, and in part also of the clones overexpressing the CT form, to express haemoglobin: in absence of any induction, a part of the exponentially growing cells became benzidine positive, in contrast with the normal or neomycin transfected K562 cells, in which very few benzidine positive cells could be observed in these conditions. These results are shown in Figure 3 (T_0 values): during exponential growth and before any treatment , K562 cell line overexpressing the MT form shows about 30% of haemoglobin containing cells, while only a slight increase (about 4%) is observed in the lines overexpressing the CT form with respect to controls. This latter value too, although very low, is significative because very few benzidine positive cells are observed in neomycin resistant clones (or in non transfected cells). We also wanted to study the differentiative ability (evaluated as accumulation of haemoglobin) of these stable transfected cell lines exposed to differentiating agents such as aphidicolin and hemin. In the previous sections we have showed that aphidicolin induces a marked increase of the MT form (about 10-fold) and only a small rise of the CT form (two-fold), while both the CT and the MT forms are strongly induced by hemin in normal K562 cells (nearly 20 and 10 times respectively). Different behaviour in response to aphidicolin can be shown in cell lines overexpressing the MT or the CT form (Figure 3): after 3 and 5 days the percentage of differentiating cells (evaluated as benzidine positive cells) increases only in the cells overexpressing the MT form, while it is comparable to the control in the cells overexpressing the CT form, suggesting a role of the MT form in the pattern of differentiation induced by aphidicolin. Treatment with hemin leads to an increase of the percentage of benzidine positive cells (evaluated after 3 and 5 days) in both clones overexpressing either the MT or the CT isoform, suggesting that in this case both acylphosphatase isoenzymes are involved in the differentiation induced by this compound. These results indicate that acylphosphatase expression could take a part in the events that lead to differentiation.

In another experiment, very similar to those described above, the haemoglobin accumulation in cell lines overexpressing the MT or the CT isoforms and induced to

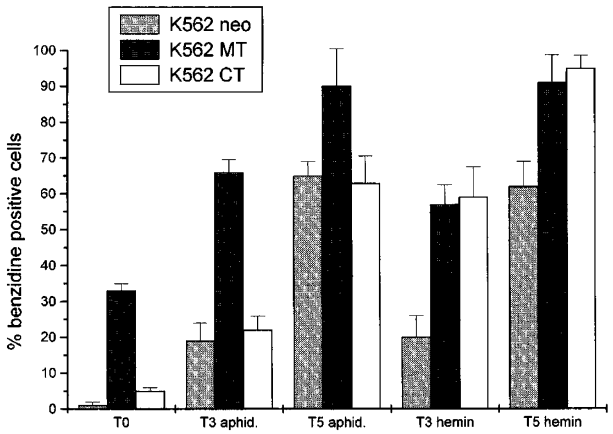


Figure 3 Percentage of benzidine positive K562 cell overexpressing the MT (clone 6) or the CT (clone 5) isoform, and of neomycine resistant clone as control, after treatment with aphidicolin or hemin, for three or five days. The results are the mean of three independent experiments. S.D. is indicated.

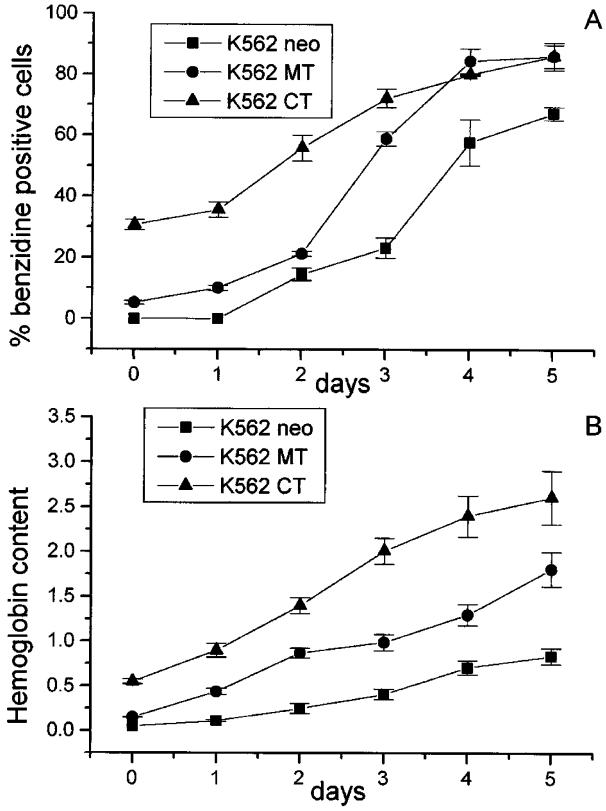


Figure 4 **A**: Time course of the percentage of benzidine positive K562 cells after addition of hemin. **B**: time course of hemoglobin content in the same experiment of panel A. Squares: neomycine resistant clone as a control. Circles: cells overexpressing the MT isoform (clone 6). Triangles: cells overexpressing the CT isoform (clone 5). The results are the mean of three independent experiments. S.D. is indicated.

differentiate with hemin, was evaluated as total content in a cell lysate, normalized for total protein concentration. The percentage of differentiated (benzidine positive) cells was also evaluated. In Figure 4A, the total haemoglobin content is shown up to 5 days of treatment with hemin, in control cells and in cells overexpressing the CT or MT form: it is clear that the increase of haemoglobin content is much higher in the clone overexpressing the CT form than in that overexpressing the MT form. In fact, also considering the graph presented in Figure 4B, while the percentage of differentiated cells (after 5 days) is only slightly different (about 30%), the content of haemoglobin is more than double. Similarly, it is clear that the content of haemoglobin is also increased in the cells overexpressing the MT form in comparison to the control. From data obtained after 3 days of hemin treatment we can also conclude that, with respect to control cells, haemoglobin accumulation is more abundant and rises more quickly not only in the clones overexpressing the CT form, but also, although at a lesser extent, in the clone overexpressing the MT form.

Discussion

In this paper we present results indicating that the two isoforms of acylphosphatase are involved in differentiation of K562 cells, although with different patterns. Previous experiments have already demonstrated that in differentiating myoblasts the level of MT isoform acylphospatase shows a 10-fold increase after 4 days (Berti *et al*, 1992). Here we show that this behaviour is not restricted to muscular tissue cells: also in the K562 erythroblastoma cell line a similar increase can also be observed, following treatment with several inducers, like hemin, aphidicolin and PMA. This increase is independent of the kind of differentiation induced by these compounds: in all cases an 8-fold increase of the protein can be observed, together with an analogous increase of the specific mRNA level, already after one day. A similar increase of mRNA, due to gene activation, has been already observed in this cell line treated with T_3 hormone, but in that case protein content showed only a 2- to 3-fold increase, suggesting that post-trascriptional mechanisms are probably involved in determination of protein level in the cell. The half-life of this isoform in the K562 cell line confirm that this protein can be considered a short-lived protein: the fact that after hemin treatment the half-life increases, could partially justify the increase in enzyme content.

The situation appears more complex if we consider the behaviour of the CT isoform in the same kind of experiments: while hemin treatment strongly increases the protein level (up to about 15 times), this parameter is only partially influenced by aphidicolin or PMA treatment. In contrast, the specific mRNA level shows an eightfold increase, similar to that observed for the MT form, with all treatments. It should be noticed that in this cell line the mRNA basal level of the CT isoform is higher than that of the MT isoform, even if both enzymes show a similar concentration in the cell. It is clear that, at least for the CT form, there is not a direct relationship between the mRNA and the protein level: neither a higher basal level nor an even higher level after induction with aphidicolin or PMA lead to an accumulation of protein. In contrast, with hemin treatment, while mRNA level increases as with aphidicolin or PMA, protein accumulation strongly increases. On the other hand, also in the case of the CT isoform, the half-life of the protein is only marginally influenced by differentiation, irrespective of the kind of treatment used. These results suggest that post-transcriptional regulations may be involved in the mechanism of control of protein expression, especially in the case of the CT isoform.

As a means to characterize the role of acylphosphatase in differentiation, we have overexpressed either one or the other isoform in K562 cells and evaluated the behaviour of these cells concerning differentiation. In the case of cells overexpressing the MT form, even in the absence of specific differentiative stimuli we can notice an enhanced tendency to differentiate, because about 30% of the population is benzidine positive. This percentage is about 4% in the case of overexpression of the CT isoform, and also in this case such tendency is clear; in fact, in control cells the percentage of benzidine positive cells is lower. We believe that this kind of experiment is significative because the level of overexpression reached in these transfected clones (about 10 times the basal level) is not very far from the level that endogenous acylphosphatases reach in response to differentiating agents. Moreover, we have measured the percentage of differentiation under hemin and aphidicolin treatment: the results clearly show that the overexpression of both MT and CT isoforms leads to a quicker and increased rate of differentiation in response to hemin; during aphidicolin treatment, on the other hand, only cells overexpressing the MT form show similar behaviour, while clones overexpressing the CT form are comparable with normal cells. All these results are in agreement with data presented in Figure 1 (panels A and B respectively), where hemin increases the endogenous level of both isoforms while aphidicolin, in contrast, has an influence only on the MT isoform. We can hypothesize that acylphosphatase increase represent an upstream differentiating signal, irrespective of the kind of differentiation: this fact seems to be true at least for muscular and myeloid cells, and in this latter case under stimuli leading to different patterns of differentiation. It is interesting to notice that the two isoforms, at least in the cases here studied, play different roles in these processes: the MT isoform seems to be involved in every kind of differentiation, while the CT isoform role appears to be more restricted. The control seems to be post-transcriptional more than at the level of gene expression or mRNA stability: any differentiating stimulous seems to enhance mRNA level, while this fact is not always true as far as the protein level is concerned. A possible mechanism of post-transcriptional regulation could be due to the 5′ untranslated region of the mRNA, which we have found to be unusually long, covering about 50% of the entire mRNA length and which presents an extra AUG codon (unpublished results): we can hypothesize a regulation similar to that described for the mRNA coding for a subunit of protein phosphatase 2A (Wera *et al*, 1995), where the presence of an additional start codon contribute to the down-regulation of translation. On the other hand also protein stability seems not to play a crucial role in the

control of acylphosphatase level: in fact no significant change in this parameter is observed during differentiation.

More work is necessary to understand what the real action of acylphosphatase is, but we consider it very interesting that these enzymes seem to constitute a signal for cellular differentiation. Recent data obtained in our lab (manuscript in preparation) show a correlation between acylphosphatase and apoptotic pathway induced in K562 cells either with p53 overexpression or by means of oxidative shock. Moreover, *in vitro* nuclease activity of these enzymes has been demonstrated (Chiarugi *et al*, 1996). Taken together, although these findings are still far from giving us a complete understanding of these phenomena, nevertheless they open new perspectives in the role of acylphosphatase in the cell.

Materials and Methods

Cell culture

K-562 cells were grown in RPMI 1640 supplemented with FCS 10% (vol/vol), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) at 37°C in humidified atmosphere with 5% CO_2. Cells were plated at 10^5 cells/ml; in these culture conditions cells exponentially grew during the next four days and the percentage of benzidine positive cells was very low. In differentiation experiments cells at the same density were cultured in presence of the inducer. Treatments with 0.1 mM hemin or 5 nM aphidicolin for a period of 4 to 5 days were used in erythroid differentiation experiments.

Benzidine staining was used to detect haemoglobin production: cells were washed and resuspended in PBS at about 1×10^6 cells/ml; 0.1 ml of cell suspension were added to 50 μl of cold 3% acetic acid solution containing 0.2 % benzidine and 1.5 % H_2O_2. The mixture was kept in ice for 5 min before counting in a Bürker chamber.

Cells were also treated with 1×10^{-8}M PMA and morphological changes occurring during megakaryocytic cell differentiation were monitored by May-Gimsa staining on cytospin slides (Murate *et al*, 1993) Cytocentrifugates were prepared by spinning 1×10^4 cells/ml at 1500 rpm for 10 min. Cell viability was controlled by the trypan blue exclusion test under all culture conditions.

Antibodies production and enzyme-linked immunosorbent assay (ELISA)

The levels of both acylphosphatase isoenzymes in the cells were determined by two different and specific noncompetitive immunoenzymatic assays. Specific antibodies against MT and CT isoenzymes were raised in rabbits using recombinant proteins obtained in *E. coli* as fusion proteins with glutathione S-transferase and purified by affinity chromatography. A microtiter plate was coated with antiacylphosphatase isoenzyme antibodies for 3 h at room temperature. After plate washing, standard antigen dilutions or samples were added to wells and incubated overnight at 4°C. Immunocomplexes were determined by incubating with specific antiacylphosphatase isoenzymes antibodies conjugated to horseradish peroxidase (HRP). Reactions were quantified by using ophenylendiamine as substrate and reading at 492 nm after 30 min of incubation. No cross reaction was detected between the two isoenzymes in our conditions. The lower limit of quantitation was 0.1 ng/ml for the CT and 0.5 ng/ml for the MT isoenzyme respectively. Linearity was kept up to 20 and 40 ng/ml. In these ranges negligible effects of sample dilutions on the assays were observed testing lysates depleted of the enzyme by immunoadsorption. About 2×10^6 cells were used for every determination. Cells were resuspended in 0.5 ml PBS containing 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin. After sonication the lysate was clarified by centrifugation at 18 000 rpm for 20 min at 4°C, and the supernatant was used for both isoenzyme determination in immunoenzymatic assays. Proteins in cellular extracts were determined by the BCA assay (Pierce).

Northern blot analysis

RNA was isolated by the guanidinium thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987). Total RNA was quantified spectrophotometrically, treated with formaldehyde and subjected to electrophoresis in 1.2% agarose gel. The separated RNAs were transferred to Hybond N^+ nylon membrane (Amersham), prehybridized for 4 h and hybridized for 16 h in 4× Standard Saline Citrate, 5× Denhart's solution, 0.1% SDS and 50% formamide. Washes were performed in 4× SSC, 0.1% SDS at 65°C. Filters were subjected to auto radiography at −70°C with Kodak films and intensifying screens. Films with appropriate exposure times (in order to have non saturated signals) were scanned in a Cybertech Image analyzer for quantitation.

Metabolic labelling and immunoprecipitation of the two isoenzymes

Cells in exponential growth or after 4 days of hemin or PMA treatment were washed in methionine-free medium for 45 min. Medium containing 80 μC/ml of L-[^{35}S]methionine was then added. After a 4 h pulse the labelling molecule was removed and chase started by adding complete medium with cold methionine excess. At different chase times, 5×10^6 cells were washed twice with PBS, lysed in 250μl of immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 1 mM PMSF, 0.5% NP-40) and centrifuged. 0.1 ml of cell lysate were incubated in ice with 5μg of the specific antiisoenzyme antibodies in a final volume of 1 ml for 1 h. 25 μl of Protein A-Sepharose (Calbiochem) were then added and the reaction mixture was shacked for 1 h at 4°C. The immunoprecipitate was collected by centrifugation, washed tree times with immunoprecipitation buffer and then resuspended in SDS-polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and boiled. Samples were supplemented with cold standard isoenzymes and electrophoresized on 15% gels. The L-[^{35}S]methionine content in each isoenzyme was measured by scintillation counting of the enzyme band excised from the SDS gel.

Values of the $t_{1/2}$ of acylphosphatase isoenzymes in K562 cell line under different culture conditions were calculated from the specific radioactivity of the immunoprecipitated isoenzyme at varying chase times.

Vectors and transfection

The coding sequences of both CT and MT acylphosphatases (Fiaschi *et al*, 1995) were amplified using polymerase chain reactions and inserted in the HindIII and XbaI restriction sites of pRcCMV eukaryotic expression vector (Invitrogen), also carrying the neomycin resistance gene. Sequences were confirmed by Sanger DNA sequencing. 10^7 K562 cells were transfected with 50 μg of each construct using a Biorad electroporator (950 μF, 0.2 kvolts). Selection of G418 (400 mg/l) resistant clones, was performed starting at 48 h from transfection. Clones were assayed for acylphosphatase isoenzymes expression by ELISA.

Total haemoglobin content determination

Cytosolic hemoglobin was routinely estimated according to Paoletti *et al* (1992) using a spectrometric assay at 414 nM. In order to express hemoglobin concentration in mg/ml, the A_{414} values were multiplied by 0.1290. This conversion factor, close to that previously reported (Kabat *et al*, 1975), was derived by comparing the A_{414} and coulter values of the same standard of human hemoglobin.

Acknowledgements

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