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The inhibitory effect of the 5′ untranslated region of muscle acylphosphatase mRNA on protein expression is relieved during cell differentiation

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Abstract Previous experiments suggested that the upstream AUG triplet present in the 5′ untranslated region (UTR) of muscle acylphosphatase mRNA is involved in the regulation of protein expression. In this paper, we study the involvement of the 5′UTR secondary structure and upstream peptide on mRNA stability and protein translation. Our data, obtained using deletion and frame-shift mutants, demonstrate that the 5′UTR controls protein expression regulating translation together with mRNA stability. Furthermore, we demonstrate that the inhibitory effect of the 5′UTR of muscle acylphosphatase is relieved during the differentiation process in agreement with previous data reporting an increase of acylphosphatase content during cell differentiation. Finally, UV cross-linking experiments show that specific mRNA-binding proteins are associated with the 5′UTR of the muscle acylphosphatase mRNA.

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Key words: Acylphosphatase; 5′ Untranslated region; Enhanced green fluorescent protein; Aphidicolin; Phorbol 12-myristate 13-acetate; Differentiation

1. Introduction

Acylphosphatase (EC 3.6.1.7.) is a small (11 kDa) cytosolic enzyme, which catalyzes the hydrolysis of the carboxyl phosphate bond. All animal tissues contain two isoenzymatic forms, called 'muscle type' (MT) and 'common type' acylphosphatase showing about 60% of identities in the amino acidic sequence. The structure of the MT isoform, determined with nuclear magnetic resonance techniques, is composed of two interleaved β-α-β packing units [1]. Both isoforms can hydrolyze in vitro several physiological substrates such as 3-phosphoglyceroyl-phosphate, carbamoyl-phosphate and succinoyl-phosphate [2]. Furthermore, a role of the acylphosphatase in the hydrolysis of the phosphoenzyme intermediate of different membrane pumps, such as the Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase from red blood cell membrane, and the Ca^{2+}/Mg^{2+}-ATPase from sarcoplasmic reticulum of skeletal muscle has been hypotesized [3,4]. It was demonstrated that both isoforms can hydrolyze in vitro either DNA or RNA in an acidic environment [5]. The involvement of this enzyme in cell differentiation was suggested: in particular, myogenesis was associated with an increase of the MT isoform level alone, while an increase of both isoenzymes was observed during erythroid differentiation [6,7]. Furthermore, only the MT isoform migrates in the nucleus during differentiation [8] and is transcriptionally regulated in response to T3 hormone [9]. Recently, we have proposed that the 5′ untranslated region (UTR) of the MT acylphosphatase mRNA may be involved in the regulation of protein expression [10]. Different characteristic elements are present in the 5′UTR, namely a stably secondary structure and an upstream AUG, which is the origin of a 60 codon long open reading frame. In this paper, we demonstrate that the 5′UTR of acylphosphatase is able to regulate protein expression mainly at the translational level and that the upstream AUG triplet plays a key role in this inhibitory mechanism. Furthermore, we demonstrate that cellular differentiation leads to a relief of the inhibitory effect of the 5′UTR and that specific mRNA-binding proteins bind to this region of the mRNA.

2. Materials and methods

2.1. Materials

Unless otherwise specified, all reagents were from Sigma. The synthetic oligonucleotides, the restriction and modifying enzymes, the U.S.E. Mutagenesis kit and the NICK Spin Columns were from Pharmacia. DMEM and RPMI culture medium were from Biowhittaker. The BCA protein Assay Reagent was from Pierce.

2.2. Plasmid construction

The p-5′UTR-EGFP (enhanced green fluorescent protein) plasmid and the p-5′UTRauu-EGFP mutant were obtained as previously described [10]. The deletion mutant (p-del-5′UTR-EGFP) was obtained by polymerase chain reaction (PCR) using the p-5′UTR-EGFP construct as template and the BamGEGFP (5′-TTTTTGGATCCCGGCGGCGGCGAG) and the 5′UTR-del (5′-TTTTTGAATTCGCAAGCAGTCCCATGTGTC) oligonucleotides. The PCR product was inserted in *Eco*RI/*Bam*HI restriction sites of the p-EGFP-N1 vector. The single base insertion mutant (p-ins-5′UTR-EGFP) was obtained using the p-5′UTR-EGFP vector as template with the U.S.E. Mutagenesis kit [11], using a synthetic mutation target oligonucleotide (5′-GTCCCCTCCCTCTCGCGAGCCGCCGCAGTCG), which inserts in the 5′UTR a single base (G) 17 bases downstream the upstream AUG. The presence of mutation was confirmed by nucleotide sequence analysis. For in vitro transcription, the 5′UTR region was inserted in PCRII™ plasmid (PCRII™-5′UTR), downstream the T7 promoter. The acylphosphatase cDNA of *Drosophila melanogaster* [12] was inserted in PCRII™ vector (pCRII™-ACPDro) in the same orientation of the 5′UTR and used as negative control in the UV cross-linking experiments.

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Abbreviations: MT, muscular type isoform of acylphosphatase; 5′UTR, 5′ untranslated region; EGFP, enhanced green fluorescent protein; TPA, phorbol 12-myristate 13-acetate

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2.3. Cell culture and transfection

Cells were cultured with 10% fetal calf serum, at 37°C in a 5% CO_2 humidified atmosphere, in the following media: HeLa cells in DMEM and K562 in RPMI 1640, both supplemented with 50 U/ml of penicillin and 50 μg/ml of streptomycin. Megakaryocytic and erythroid differentiation was induced by treatment of exponentially growing K562 cells with either 5 nM phorbol 12-myristate 13-acetate (TPA) or 5 μg/ml of aphidicolin. Morphological changes occurring during megakaryocytic differentiation were monitored by May–Gimsa staining [13], while benzidine staining was used to detect hemoglobin production. Transient transfection of HeLa and K562 cells was performed with the calcium phosphate and DEAE-dextran method, respectively [14]. A β-galactosidase construct was cotransfected in each transfection assay as control.

2.4. RNA analysis

Extraction of total RNA was carried out using the method of Chomczynski et al. [15] and Northern blot analysis was performed according to Maniatis [14]. The same filter was then hybridized with an actin probe for normalization.

2.5. Cytofluorometric analysis

Analysis of fluorescent cells (2×10^5 cells washed twice in phosphate-buffered saline) was performed in a Becton Dickinson FACScan according to the manufacturer's procedure [16].

2.6. Preparation of RNA transcript

The PCRII™-5′UTR and the pCRII™-ACPDro plasmids were linearized by enzymatic digestion with *Hin*dIII restriction enzyme. In vitro transcription was carried out at 37°C for 1 h in the presence of 1 μg of linearized DNA template, 10 μM dithiothreitol (DTT), 37.5 μM UTP, 12.5 μM [α-^{32}P]UTP, 500 μM of each of the other ribonucleotides, 5 U of RNAguard ribonuclease inhibitor and 3 U of T7 RNA polymerase. After transcription, template DNA was digested with DNase I (RNase free) at 37°C for 15 min and RNA transcripts were purified with NICK Spin Columns. Denaturing electrophoresis of the transcription products on 6% acrylamide gel resolved a single RNA species of appropriate size.

2.7. Preparation of cytosolic protein extracts

Cytosolic protein extracts were obtained by resuspension of the cells with 100 μl of cytoplasmic lysis buffer (10 mM HEPES, 40 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 0.2% Nonidet P40, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM PMSF) and incubated for 10 min in ice. Twenty-five μl of nuclear lysis buffer (20 mM HEPES, 420 mM KCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM PMSF) was added to the supernatant obtained by centrifugation. After incubation of the samples for 30 min in ice, the supernatant was recovered and used for protein assay using the BCA method.

2.8. RNA–protein binding and UV cross-linking

RNA–protein binding and UV cross-linking experiments were carried out at room temperature for 10 min with 70 μg of protein extracts and 5×10^5 cpm of labeled RNA in the presence of 0.5 mg/ml of heparin in a final volume of 10 μl. After irradiation of the mixture by a UV lamp for 15 min in ice from a distance of 1 cm, the sample was treated with RNase (1 mg/ml) for 30 min at 37°C and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

3. Results and discussion

3.1. Involvement of the secondary structure and upstream peptide in protein expression

The 5′UTR of MT acylphosphatase mRNA was previously isolated and computer analysis showed that this region could form in vivo a very stable secondary structure ($\Delta G = -57.6$ kcal/mol). Furthermore, the 5′UTR contains an upstream AUG codon that defines a 60 amino acid long open reading frame, which partially overlaps the MT acylphosphatase coding region. In a previous work, we demonstrated that the

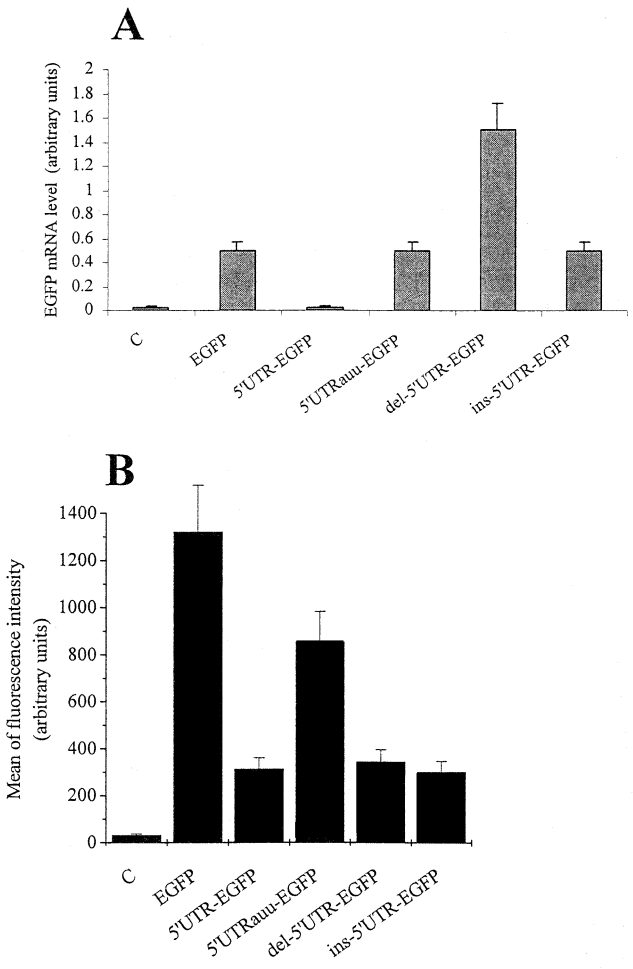

Fig. 1. A: Densitometric evaluation of EGFP mRNA in HeLa transfected cells. Total RNA was extracted from HeLa cells after transient transfection with different plasmids and hybridized with EGFP cDNA as probe. Normalization was performed using actin cDNA as probe. B: Value of the mean fluorescence intensity of the reporter protein EGFP obtained after transfection of HeLa cells with different constructs. Measurements were performed in HeLa cells transfected with the indicated constructs. Fluorescence intensity was determined 72 h after transfection. Transfection efficiency was evaluated either by cytofluorometric analysis or by cotransfection with a β-galactosidase expression vector. In all experiments, transfection efficiency was about 30%. Values are the mean of five independent experiments. S.D. values are indicated.

upstream AUG codon of 5′UTR region of acylphosphatase negatively regulates protein expression by influencing mRNA stability and protein translation [10]. In order to study the importance of the secondary structure and of the upstream peptide in the control of protein expression, two different mutants were obtained: (i) a deletion mutant (p-del-5′UTR-EGFP), that presents the upstream AUG triplet, but should not form any secondary structure since it lacks 80 bases at the 5′ end, and (ii) an insertion mutant (p-ins-5′UTR-EGFP) in which a single nucleotide (+G) was inserted 17 bases downstream the upstream AUG codon, that leads to the translation of a peptide with a different primary structure but maintains the secondary structure of the 5′UTR. The p-del-5′UTR-EGFP and p-ins-5′UTR-EGFP vectors, together with the p-EGFP and the p-5′UTR-EGFP constructs as controls, were used to transiently transfect HeLa cells. Seventy-two h after

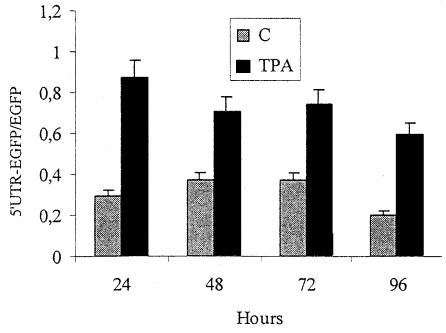

Fig. 2. Role of the 5′UTR in megakaryocytic differentiation. K562 cells were treated with 5 nM TPA for 40 h and then transiently transfected with the p-EGFP or p-5′UTR-EGFP. At different times after transfection, the fluorescence of the EGFP was evaluated by cytofluorometry. The ratio between the fluorescence value retrieved in cells transfected with p-5′UTR-EGFP and cells transfected with the p-EGFP control is reported at different times. Transfection efficiency obtained with the two plasmids was comparable in all experiments. Values are the mean of five independent experiments. S.D. values are indicated.

transfection, the level of the specific EGFP transcript and the amount of the reporter protein EGFP were analyzed. As expected, the amount of the mRNA in the p-5′UTR-EGFP-transfected cells dramatically decreases with respect to the p-EGFP control, while the AUG to AUU mutation of the AUG upstream start codon (p-5′UTRauu-EGFP) abrogates the negative control of the 5′UTR on mRNA level. On the other hand, the deletion mutant (p-del-5′UTR-EGFP) or the introduction of a frame-shift mutation (p-ins-5′UTR-EGFP) are able to increase the EGFP transcript level (Fig. 1A). Cytofluorometric analysis of the expression of fluorescent EGFP reporter protein shows, as expected, that the whole 5′UTR strongly decreases the translation of EGFP (about four-fold), while the AUG to AUU upstream start codon mutation almost restores the protein level of the control. On the other hand, analysis of protein level in cells transfected with p-del-5′UTR-EGFP or p-ins-5′UTR-EGFP plasmids reveals an EGFP expression comparable to that of the p-5′UTR-EGFP-transfected cells (Fig. 1B). These data suggest that the secondary structure and the upstream peptide of the 5′UTR affect only mRNA stability, while they are ineffective on protein translation. It is likely that the 5′UTR of MT acylphosphatase controls protein expression mainly at the translational level but it exerts an additional control on mRNA stability. Hence, we demonstrate that the upstream AUG plays a key role in protein expression since it affects both protein translation and mRNA stability. The secondary structure and the upstream peptide are additional regulatory elements of 5′UTR whose action is restricted to mRNA stability.

3.2. *The inhibitory effect of the 5′UTR on protein expression is relieved during cell differentiation*

Previous results have suggested an involvement of acylphosphatase in cell differentiation, since an increase of the mRNA level and protein amount of both isoforms is associated with this phenomenon [7,8]. This fact suggests that there are regulatory mechanisms which are activated when the cellular differentiation is induced. In order to study whether the 5′UTR plays a role in the regulation of protein level during differentiation, exponentially growing K562 cells were induced towards megakaryocytic lineage using 5 nM TPA for 40 h and then transfected with the p-EGFP or p-5′UTR-EGFP. 24, 48, 72 and 96 h following the transfection, cytofluorometric analysis of the differentiated and transfected cells was carried out. The results are expressed as the ratio of the fluorescence intensity between the p-5′UTR-EGFP- and the p-EGFP-transfected cells: an increase of this ratio indicates the relief of the inhibitory control of the 5′UTR on protein translation (Fig. 2). These data show that TPA treatment leads to an increase of EGFP protein level in the the p-5′UTR-EGFP-transfected cells, very likely as an effect of a relief of the negative control of the 5′UTR region on EGFP expression. In order to better characterize the time course of this phenomenon, a second kind of experiment was performed. K562 cells were treated with 5 nM TPA and immediately transfected with the p-EGFP or p-5′UTR-EGFP. Cytofluorometric analysis performed 24, 48 and 72 h after the treatment demonstrates that the 5′UTR-EGFP/EGFP ratio is only slightly affected by differentiation during the first 48 h but increases later on (Fig. 3A). These results clearly demonstrate that during megakaryocytic differentiation, the inhibitory effect of the 5′UTR on protein expression is relieved.

Moreover, we tested if this phenomenon is limited to the TPA-induced differentiation or it is an effect common to various types of differentiation. K562 cells were differentiated towards erythroid lineage with aphidicolin and transfected with p-EGFP or p-5′UTR-EGFP in an experiment similar

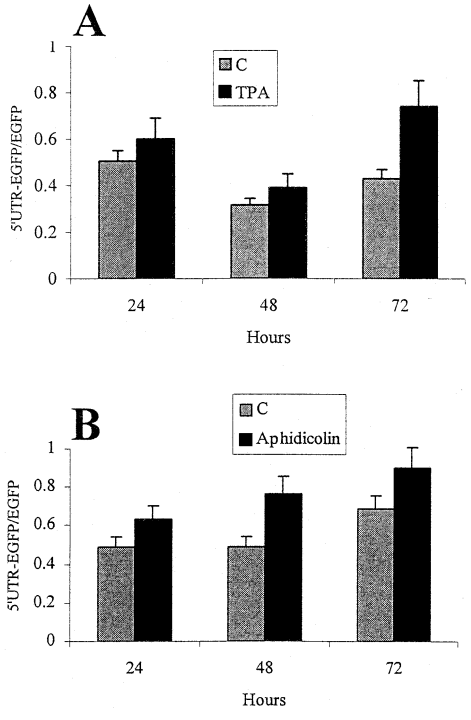

Fig. 3. A: Role of the 5′UTR in early megakaryocytic differentiation. K562 cells were treated with 5 nM TPA and immediately transfected with the p-EGFP or with p-5′UTR-EGFP. 24, 48 and 72 h after the treatment, cells were analyzed by flow cytofluorometry. B: Role of the 5′UTR in early erythroid differentiation. K562 cells were treated with aphidicolin 5 μg/ml and immediately transfected with the p-EGFP or p-5′UTR-EGFP. 24, 48 and 72 h after the treatment, the cells were analyzed by cytofluorometry. Transfection efficiency obtained with the two plasmids was comparable in all experiments. Values are the mean of five independent experiments. S.D. values are indicated.

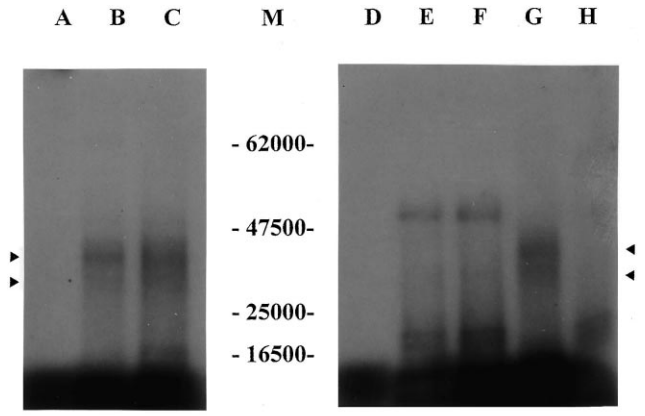

Fig. 4. RNA-binding proteins which link to the 5′UTR of MT acylphosphatase mRNA. Cytosolic extracts of control or TPA-treated K562 cells were incubated with radiolabeled acylphosphatase 5′UTR mRNA and subjected to UV cross-linking (samples from lane A to lane C). Lane A: in vitro transcribed mRNA alone; lane B: cytosolic extracts of control undifferentiated K562 cells; lane C: cytosolic extracts of TPA-treated K562 cells. The specificity of the binding proteins to the 5′UTR was controlled by incubating the same cytosolic extracts using radiolabeled mRNA of ACP *Drosophila* as negative control (samples from lane D to lane F). Lane D: in vitro transcribed mRNA alone; lane E: cytosolic extracts of control undifferentiated K562 cells; lane F: cytosolic extracts of TPA-treated K562 cells. UV cross-linking assay was performed with the cytosolic extracts of HeLa cells incubated with radiolabeled 5′UTR mRNA (lane G) and with radiolabeled mRNA of ACP *Drosophila* (lane H). Lane M: protein marker. The positions of mRNA–protein complexes (indicated by the arrows) are obtained on the basis of the molecular weight of the protein marker.

to that described above. The results (Fig. 3B) show that the inhibitory effect of the 5′UTR on EGFP expression is relieved during erythroid differentiation, although at a lower extent with respect to megakaryocytic differentiation.

Taken together, these observations demonstrate that the inhibitory effect of the 5′UTR on protein expression is relieved during differentiation, although at a different extent, depending on the type of cellular differentiation. It is likely that this effect is responsible for the acylphosphatase level increases that we have observed in K562 cells induced to both megakaryocytic and erythroid lineage [6,7]. These observations show that the 5′UTR acts as a modulator element whose action is very likely strictly linked to cellular changes that occur in the cell after the induction of the differentiation process. The relief of the inhibitory effect of the 5′UTR in megakaryocytic lineage could be the mechanism through which the protein level increases during the differentiation process. It was demonstrated that PDGF expression during megakaryocytic differentiation is regulated in a similar manner. In fact, the inhibition of the translation of PDGF by the 5′UTR of its mRNA was relieved in megakaryocytic lineage [17].

3.3. Identification of 5′UTR mRNA–protein complexes

Many examples of translational regulation by RNA–protein interactions have been reported [18]. In order to identify RNA-binding proteins specific for the 5′UTR region of the MT acylphosphatase mRNA, UV cross-linking of in vitro transcribed ^{32}P-labeled mRNA was performed. Protein extracts from undifferentiated and TPA-differentiated K562 and HeLa cells were incubated with radiolabeled mRNAs, cross-linked by short-wave UV light, and subjected to SDS–PAGE. The cross-linked radiolabeled RNA–protein complexes were detected by autoradiography. The results (Fig. 4) indicate that the pattern of RNA-binding proteins linked to the 5′UTR in cytosolic extracts is similar in undifferentiated and TPA-differentiated K562 cells and in HeLa cells (lanes B, C and G). Two major complexes, with an apparent molecular weight ranging between 35 and 45 kDa, are linked to the 5′UTR in all cytosolic extracts used. These complexes are never present when the radiolabeled mRNA of ACP *Drosophila* is used as negative control (lanes E, F and H). On the other hand, the 50 kDa band to which the mRNA of ACP *Drosophila* links is very likely aspecific. These data demonstrate that specific factors, which probably mediate the expression of the target protein and are unaffected by differentiation, bind to the 5′UTR of the MT acylphosphatase mRNA. The identity of these 35–45 kDa proteins is not yet known. However, some RNA-binding proteins of this molecular weight are able to link to the 5′UTR of different mRNAs, such as some eukaryotic initiation factor, eIF-4A-I, eIF-4A-II and 4A-like Nuk-34, and the ATP-dependent RNA helicase p47. These proteins could be possible candidates for the binding to the 5′UTR of MT acylphosphatase mRNA. On the basis of these data, we suggest that the 5′UTR of muscle acylphosphatase negatively influences the protein expression level mainly through the presence of the upstream AUG codon. Furthermore, we suggest that the relief of the inhibitory role of the 5′UTR in the differentiated lineage process is responsible for the increase of the acylphosphatase level.

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