Cloning and expression of the cDNA coding for the erythrocyte isoenzyme of human acylphosphatase

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Abstract **Three independent cDNAs coding for the erythrocyte isoform of human acylphosphatase were isolated and characterized. All the clones were incomplete at the 5′ end, but Northern blot analysis using the cDNA as a probe showed the presence of an unusually long mRNA 5′-untranslated region. The transcript was present in a variety of human cell lines of different origins, although at different levels. Southern blot analysis on DNA from different individuals revealed a simple hybridization pattern. Large amounts of pure enzyme with kinetic characteristics very similar to those of the native protein were expressed in *E. coli*.**

Key words: Acylphosphatase; Recombinant protein; cDNA; Polymerase chain reaction

1. Introduction

Acylphosphatase (EC 3.6.1.7) is a small (M_r 11,000) and cytosolic enzyme widely distributed in several tissues of vertebrates. The enzyme catalyzes the hydrolysis of the carboxyl-phosphate bond of acylphosphates. These substrates can be either physiological (1,3-bis-phospho-glycerate, carbamoyl phosphate, succinoyl phosphate, acetyl phosphate, β-aspartyl phosphate) or synthetic (benzoyl phosphate, *p*-nitrobenzoyl phosphate). Two isoenzymes have been isolated, called 'muscle' and 'erythrocyte' acylphosphatase, due to their main localization. The two isoforms show 60% homology in the amino acid sequence and exhibit the same substrate specificity, even if the erythrocyte isoform has higher catalytic activity. For a review see Stefani and Ramponi [1]. Using NMR techniques the three-dimensional structure in solution of the skeletal muscle acylphosphatase has been determined [2]. The enzyme consists of two interleaved β-α-β packing units. The construction of a synthetic gene coding for the muscle isoform of acylphosphatase [3] allowed us to produce a large amount of catalytically active enzyme both in *E. coli* and *S. cerevisiae* and to begin to study the structure of the active site with in vitro mutagenesis experiments [4,5]. The physiological function of acylphosphatase is still being debated. Previous studies have shown that the enzyme is able to hydrolyze the phosphoenzyme intermediate of different membrane pumps such as Na^+,K^+-ATPase, Ca^{2+},Mg^{2+}-ATPase and Ca^{2+}-ATPase [6,7]. This effect is probably due to the dephosphorylation of an aspartate residue [8].

Several results have suggested that acylphosphatase may be involved in cellular differentiation mechanisms. It has been shown that the muscle isoenzyme level increased during the differentiation of L6J1 myoblasts into myotubes, in parallel with some other proteins known to represent differentiating markers: this fact suggests a possible role of the enzyme during this phenomenon [9]. A possible involvement of the acylphosphatase in metabolic variations in hyperthyroidism has been postulated. Several studies have evidenced that the amount of the enzyme increased in muscle, liver and erythrocytes of triiodothyronine-treated rabbits. This effect may be due to a biosynthesis of acylphosphatase induced by the T_3 hormone [10]. Recent studies have demonstrated that T_3 controls transcription of the acylphosphatase gene. In particular, results have shown increased levels of skeletal muscle isoform mRNA in T_3 treated K562 cells. In contrast, the hormone seems unable to regulate the amount of the erythrocyte isoenzyme mRNA [11].

In order to obtain a useful tool for elucidating the physiological role of erythrocyte acylphosphatase isoenzyme, we have cloned the cDNA coding for this protein and expressed the functional enzyme in *E. coli*. Using the cDNA as a probe we have evaluated the level of the transcript in different cell lines and performed a Southern blot analysis on human DNAs.

2. Materials and methods

2.1. Materials

Restriction endonucleases and *Thermus aquaticus* (*Taq*) DNA polymerase were from Promega. Sequenase was obtained from USB. λgt11 human placental cDNA library was a gift of Maria Luisa Melli (Biocine, Siena, Italy). cDNA Synthesis System Plus, cDNA rapid adaptor ligation module, cDNA rapid cloning module, in vitro Packaging module and nitrocellulose membrane Hybond-N^+ were from Amersham. mRNA purification Kit was from Quiagen. Synthetic oligonucleotides and pGEX-KT prokaryotic vector were from Pharmacia. IPTG was from Boeringher and ampicillin, lysozyme, bovine thrombin and glutathione-linked agarose resin from Sigma. Bluescript SK prokaryotic vector was from Stratagene. Human cell lines SH-SY5Y (neuroblastoma), RD (rhabdomyosarcoma, embryonic), A549 (carcinoma, lung), Hep G2 (carcinoma, liver), JURKAT (leukaemia, T cell), IMR-90 (lung, embryonic), K562 (leukaemia, chronic myeloid) and HL-60 (peripheral blood, leukaemia, promyelocytic) was a gift of Lucia Magnelli (Institute of General Pathology, Florence, Italy).

2.2. cDNA isolation

In an early experiment, a 500 ng DNA sample from a human placental cDNA library in λgt11 phage vector, was used as a template in the polymerase chain reaction (PCR), primed with two degenerate oligonucleotides for 40 temperature cycles in 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 15 mM Tris-HCl, pH 8.4. Each cycle consisted of 1 min

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Abbreviations: PCR, polymerase chain reaction; bp, base pair; NMR, nuclear magnetic resonance; T_3, triiodothyronine.

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at 95°C, 42°C and 72°C in an MJ Research Minicycler. The sequences of the two oligonucleotides were 5′-GTNGAC(T)TAC(T)GAG(A)A-TA(CT)TTC(T)GG and 5′-TTG(A)AAG(A)TTG(A)TTG(A)CTC(T)-TTC(T)CA, which correspond to the amino acids 9–15 (Val-Asp-Tyr-Glu-Ile-Phe-Gly) and 79–85 (Asn-Phe-Asn-Asn-Glu-Lys-Val) of the human erythrocyte acylphosphatase.

A cDNA library was prepared from K562 mRNA. Double-stranded cDNA was synthesized using 2 μg of purified poly(A)$^+$ mRNA obtained from K562 cells using the cDNA Synthesis System Plus and ligated to an *Eco*RI adaptor according to the cDNA rapid adaptor ligation module. The cDNA was then inserted into the cloning vector Moss *lox* and packaged in vitro using the cDNA rapid cloning module and in vitro Packaging module. The primary cDNA library contained about 1×10^6 independent clones. Screening was performed with standard methods [12]. The positive clones were sequenced by the Sanger method using a Sequenase kit.

2.3. RNA preparation and analysis

Total RNA was purified using the method of Sacchi et al. [13]. Poly(A)$^+$ mRNA fraction was isolated using mRNA Purification Kit. Northern blot analysis was performed according to Solito et al. [14],

2.4. Southern blot analysis

Genomic DNA was isolated from human blood according to Kunkel et al. [15]. Hybridization was performed using the R clone cDNA insert as probe, ^{32}P-labelled with the random-priming method [12] in 50% formamide, 10 × Denhart's, 2 × SSC, 0.2% SDS at 42°C overnight. Filters were washed in 2 × SSC, 0.2% SDS at 65°C. For low-stringency conditions, filters were hybridized at 37°C and washed at 60°C. Autoradiography lasted for 6 days, with intensifying screens.

2.5. Expression and purification of recombinant erythrocyte acylphosphatase

Two synthetic oligonucleotides (5′-TTTGGATCCGCAGAGGGA-AACACCCTG-3′ and 5′-CTTAGAAAACTTGAATTCAGG-3′), containing *Bam*HI and *Eco*RI restriction sites respectively, were used in a PCR experiment using R clone as a template. The resulting fragment, containing the entire coding sequence of the enzyme, was digested with *Bam*HI and *Eco*RI restriction enzymes and inserted into the unique sites *Bam*HI and *Eco*RI of the pGEX-KT vector, downstream and in frame with the glutathione-*S*-transferase coding sequence.

The expression of the fusion protein and the purification of the enzyme was according to Taddei et al. [16].

2.6. Characterization of recombinant enzyme

Acylphosphatase activity was measured using benzoyl phosphate as substrate at 25°C in 0.1 M acetate buffer, pH 5.3, according to Ramponi et al. [17]. K_m was calculated by plotting the reciprocal of the initial velocities measured at various substrate concentrations vs. the reciprocal of substrate concentrations according to Lineweaver and Burk [18].

3. Results and discussion

3.1. Isolation and characterization of three cDNA clones

In an early experiment two degenerate oligonucleotides were synthesized, based on the primary structure of the protein [1], corresponding to the 9–15th and 79–85th amino acid sequences, respectively. The two oligonucleotides were utilized in a polymerase chain reaction (PCR) experiment using DNA from a λgt11 human placental cDNA library as a template. The PCR product, of the predicted length of about 230 bp was subcloned in the Bluescript SK vector. DNA sequence analysis from both strands with the Sanger method shows that it corresponds to the desired erythrocyte acylphosphatase cDNA fragment. This fragment was used as a probe for screening a λ phage cDNA library constructed using the poly(A)$^+$ mRNA fraction of K562 cell. This human erythroblastoid cell line was chosen because it expresses abundant mRNA coding for the erythrocyte acylphosphatase. 5×10^5 independent plaques were screened and three independent positive clones were isolated. DNA sequence analysis of the isolated clones (named R, H and D) were performed on both strands and the resulting sequence is shown in Fig. 1. Clone R, the longest one, is 556 base pairs in length and contains 54 bases of 5′-untranslated region, an open reading frame of 294 bp encoding the whole amino acid sequence of the enzyme, 201 bases of 3′-untranslated region and a poly(A) tail. A putative polyadenylation signal, AATAAA, is located 15 nucleotides upstream of the poly(A) tail. Clone H is a little shorter than clone R, having only 49 bases of 5′-untranslated region. Apart from this fact the two sequences are completely identical. Clone D is even shorter, starting at codon 28 of the coding region. The sequence of this clone is identical to clones H and R, except for the 3′-end, where an additive fragment, 16 nucleotides in length, is present just before the poly(A) tail, perhaps representing a 3′-end polymorphism of acylphosphatase mRNA. This variability in the polyadenylation site position in eukaryotic mRNA has been found in mRNAs from human fetal liver [19]. Our results suggest that this variability might be the result of an alternative processing of the primary transcript of the erythrocyte acylphosphatase gene.

According to a Northern blot experiment (see next section), the predicted mRNA length is about 1400 bases. Because of the fact that even the two longest isolated cDNA clones are both only about 600 bases in length and result to contain the 3′-end, we must conclude that they are largely incomplete at their 5′-end. The failure to isolate the full-length cDNA may be due to the inability of reverse transcriptase to copy efficiently this mRNA molecule. In fact, other attempts to isolate the 5′-end with the use of the RACE technique [20] have always given fragments of similar length: elongation always seems to stop about 50 bases upstream from the ATG codon, perhaps in an area where particularly stable mRNA secondary structure may be present. Considering the results of Northern blot experiments, it is clear that the 5′-untranslated region of this mRNA is unusually long (about 800 bp). Previous studies have suggested a possible regulatory role on translation of certain eukaryotic mRNA by some structural elements present in the 5′-untranslated region [21–24]. Also in the case of erythrocyte acylphosphatase this region may be important for the translational regulation, as it has been demonstrated for other proteins [25,26]. For these reasons new attempts to isolate the full-length cDNA will be made

3.2. Expression of erythrocyte acylphosphatase mRNA in human cell lines

A Northern blot analysis on total RNA purified from K562 cells was performed, using the R clone as a probe in order to assess the approximate transcript length: as shown in Fig. 2A, a single band of about 1400 bases in length is visible. The specific mRNA, as expected, is relatively abundant in these cells which represent a precursor of erythrocytes, the kind of cells where this enzyme is maximally expressed. In order to evaluate the erythrocyte acylphosphatase mRNA levels, total RNA was purified from a number of human cell lines originated from different tissues and used in Northern blot experiments. The results show that the specific transcript is always expressed, although at different levels. In Fig. 2B, densitometric evaluation of the signal corresponding to the 1400 base-long specific band is presented as percentage of mRNA level in K562 cells. As shown in Fig. 2, the level of mRNA is also quite high in lung

A

				-54	CAT	GGG	TCC	CGG	AGT	GAT	CCT	GGC	AGC	CGG	TGG	GAA	GAC	AAG	GAG	GGT	TTG	AGC	ATG	3
																						Met	
	Ala	*Glu*	*Gly*	*Asn*	*Tyr*	*Leu*	*Ile*	*Ser*	*Val*	*Asp*	*Tyr*	*Glu*	*Ile*	*Phe*	*Gly*	*Lys*	*Val*	*Gln*	*Gly*	*Val*	*Phe*	*Phe*	
4	GCA	GAG	GGA	AAC	ACC	CTG	ATA	TCA	GTG	GAT	TAT	GAA	ATT	TTT	GGG	AAG	GTG	CAA	GGG	GTG	TTT	TTC	70
	Arg	*Lys*	*His*	*Thr*	*Gln*	*Ala*	*Glu*	*Gly*	*Lys*	*Lys*	*Leu*	*Gly*	*Leu*	*Val*	*Gly*	*Trp*	*Val*	*Gln*	*Asn*	*Thr*	*Asp*	*Arg*	
71	CGT	AAG	CAT	ACT	CAG	GCT	GAG	GGT	AAA	AAG	CTG	GGA	TTG	GTA	GGC	TGG	GTC	CAG	AAC	ACT	GAC	CGG	136
	Gly	*Thr*	*Val*	*Gln*	*Gly*	*Gln*	*Leu*	*Gln*	*Gly*	*Pro*	*Ile*	*Ser*	*Lys*	*Val*	*Arg*	*His*	*Met*	*Gln*	*Glu*	*Trp*	*Leu*	*Glu*	
137	GGC	ACA	GTG	CAA	GGA	CAA	TTG	CAA	GGT	CCA	ATC	TCC	AAG	GTG	CGT	CAT	ATG	CAG	GAA	TGG	CTT	GAA	202
	Thr	*Arg*	*Gly*	*Ser*	*Pro*	*Lys*	*Ser*	*His*	*Ile*	*Asp*	*Lys*	*Ala*	*Asn*	*Phe*	*Asn*	*Asn*	*Glu*	*Lys*	*Val*	*Ile*	*Leu*	*Lys*	
203	ACA	AGA	GGA	AGT	CCT	AAA	TCA	CAC	ATC	GAC	AAA	GCA	AAC	TTC	AAC	AAT	GAA	AAA	GTC	ATC	TTG	AAG	268
	Leu	*Asp*	*Tyr*	*Ser*	*Asp*	*Phe*	*Gln*	*Ile*	*Val*	*Lys*	—												
269	TTG	GAT	TAC	TCA	GAC	TTC	CAA	ATT	GTA	AAA	TAA	TGG	CCT	GAA	TTT	AAG	TTT	TCT	AAG	ATA	AAC	TCA	334
335	GTG	GTT	TGG	TTT	TTA	TTA	TTA	ATA	GAG	ATA	GAA	CTA	TTG	TGT	GTT	AAT	ATT	AGC	ATT	AGT	CAA	TAA	400
401	GTT	ATT	TTA	ATG	TCA	GAT	TTT	TGA	ATG	TTA	TAT	ATA	TTA	CCT	GTA	TGA	TGG	AAG	GAT	TAC	CAC	TGT	466
467	ACA	CAA	ATC	TAA	TCA	ATA	AAA	ACG	TTA	GAA	CCT	CT(A)$_n$											

B

500 CTT GCT TAG AGT ACT TTT(A)$_n$

Fig. 1. (A) Nucleotide and deduced amino acid sequence of acylphosphatase cDNA, clone R. Position 1 corresponds to the A of start codon. Putative poyadenylation signal is underlined. (B) Additive fragment present at the 3'-end of clone D (in bold).

cell lines A549 and in HL-60, while it decreased greatly in IMR-90. In contrast, the liver cell line HepG2 shows a lower mRNA level. All the autoradiographic data presented were normalized to an internal control: for this purpose the same filters were hybridized with a human actin probe. These results are in agreement with the quantity of protein present in the corresponding human tissues.

3.3. Genomic blot analysis

A Southern blot analysis of human genomic DNA purified from leukocytes from three unrelated donors was performed using ^{32}P-labelled R clone cDNA. DNAs were treated with *Bam*HI, *Eco*RI or *Hin*dIII restriction endonucleases until complete digestion. Autoradiography evidenced that a single positive band is present in all three digested DNAs, approximately 12 kb in length for *Bam*HI and *Hin*dIII and 3 kb for *Eco*RI digestion, as determined for comparison with molecular weight markers (data not shown). Hybridization shows a simple pattern: this fact suggests that a single gene coding for erythrocyte acylphosphatase may be present per haploid genome. The same filter was hybridized again with the cDNA probe under low-stringency conditions in order to evaluate the possible presence of sequences closely related to that of acylphosphatase: no additional positive band appears even after long exposure.

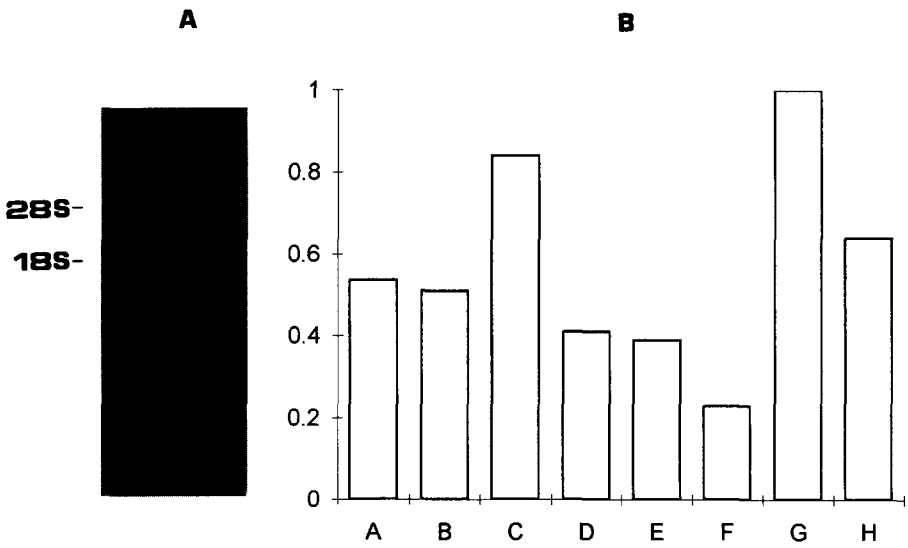

Fig. 2. (A) Northern blot analysis of K562 total RNA using human erythrocyte acylphosphatase cDNA as a probe: 20 and 5 μg were used respectively. 28S and 18S rRNA positions are shown. Exposure time was 48 h at −80° C with intensifying screen. (B) Erythrocyte acylphosphatase mRNA level in different human cell lines (see section 2), evaluated with Northern blot analysis using human erythrocyte acylphosphatase cDNA as a probe. Densitometric scanning data are expressed as percentage of the value obtained with K562 cells, after normalization with an internal control. Lane A = SH-SY5Y, lane B = RD, lane C = A549, lane D = Hep G2, lane E = Jurkat, lane F = IMR-90, lane G = K562 and lane H = HL-60.

Table 1
Comparison of the kinetic parameters of native (A), glutathione-*S*-transferase erythrocyte acylposphatase fusion protein (B) and purifyied recombinant enzyme (C) Standard deviation values are indicated

	Specific activity (U/mg)	K_m (mM)
A	7500 ± 320	0.15 ± 0.08
B	4000 ± 125	0.15 ± 0.09
C	4000 ± 140	0.15 ± 0.07

DNA from the three unrelated donors give identical results with all the restriction enzymes used: it is likely that no extensive polymorphism of this locus is present in our population, at least in relation with the restriction enzymes used in this test.

3.4. *Expression in* E. coli *and characterization of erythrocyte acylphosphatase isoenzyme*

The sequence encoding the human erythrocyte acylphosphatase was cloned into the pGEX-KT prokaryotic expression vector. The correct insertion of the sequence was checked by DNA sequence analysis. In the pGEX-KT vector the coding sequence was under the control of the IPTG-inducible *tac* promoter and the protein was obtained as a fusion, where the carboxyl-terminus of the glutathione-*S*-transferase was linked to the recombinant amino-terminus of acylphosphatase. The presence of a thrombin cleavage site at the joining point of the chimeric protein permitted the cleavage of the recombinant enzyme from the fusion protein using purified bovine thrombin. Purification of the protein by affinity chromatography allows the purification of about 7 mg of pure recombinant acylphosphatase per litre of culture. The catalytic activity of recombinant erythrocyte acylphosphatase was investigated using benzoyl-phosphate as a substrate. Kinetic parameters were determined using either the recombinant purified enzyme or the protein still fused with glutathione-*S*-transferase, before thrombin treatment, and compared to the same parameters obtained with the protein purified from human erythrocytes (Table 1): the K_m values (0.15 mM) are identical for all the samples. On the other hand, a two-fold decrease of the specific activity (4000 against 7500 U/mg) can be noticed by comparing the recombinant and native enzymes. No difference in the catalytic activity can be seen between the purified recombinant enzyme and the fusion protein. These results show that the K_m value of the recombinant enzyme is identical to that of the native enzyme where, in contrast, the specific activity is reduced to 60%. This fact could be correlated with the acetylation present on the N-terminal residue of the native protein: in fact the protein obtained from *E. coli* does not have this modification. A very similar feature has already been observed for the muscle form expressed in bacteria [3]. It is interesting to notice that kinetic parameters are not influenced by the presence of the glutathione-*S*-transferase sequence fused to the enzyme.

4. Conclusions

Isolation of erythrocyte acylphosphatase cDNA have demonstrated an unusual feature of the acylphosphatase mRNA molecule that may assume an important role in the regulation of the enzyme level in the cell. Recent results seems to point out that muscle isoenzyme may have different roles in the cell respect to erythrocyte acylphosphatase: for this reason it could be interesting to investigate if also the muscle isoform presents similar characteristics. With the availability of this cDNA as a probe it will be possible to perform experiments on the regulation of transcription of this gene, while the possibility of producing the recombinant protein will allow us to design a mutagenesis strategy in order to define the active site and the catalytic mechanism of this enzyme.

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