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Expression of a cDNA encoding the glycolipid-anchored form of rat acetylcholinesterase

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We amplified by PCR and characterized a fragment of cDNA from rat spleen, encoding the distinctive C-terminal region of the acetylcholinesterase (AChE) H subunit. A recombinant vector encoding this subunit was constructed and expressed in COS cells: the H subunits produced glycophosphatidylinositol (GPI)-anchored dimers, showing that the spleen cDNA fragment contained a functional GPI cleavage/attachment site. Using PCR, we did not detect mRNAs encoding AChE H in rat muscle or hypothalamus. In the liver of 16-day rat embryos, we found both H and T transcripts, in agreement with the presence of both GPI-anchored dimers and amphiphilic monomers of type II. In addition, we detected 'read-through' (R) transcripts, in which regular introns are spliced, but the intervening sequence between the common exon 4 and the alternative exon 5 (H) is maintained.

Acetylcholinesterase; Glycolipid anchor; Rat; Transfection

1. INTRODUCTION

The molecular forms of acetylcholinesterase (AChE, EC 3.1.1.7), which may be anchored in various ways in synaptic structures, appear to be generated from two types of catalytic subunits, H and T [1–3]. The H and T subunits differ in their C-terminal region [4]. They are encoded by distinct mRNAs, derived from the same gene by alternative splicing [5]. Both types of cDNAs have been characterized in *Torpedo* [6–8]. The H subunits produce glycophosphatidylinositol (GPI)-anchored dimers (amphiphilic forms of type I), whereas the T subunits seem to produce all other homo- or hetero-oligomeric forms, including amphiphilic monomers and dimers of type II [9] and collagen-tailed forms [10].

cDNAs encoding the T subunits of mammalian AChE have been cloned in humans [11], mouse [12] and rat [13]. The genomic region located between the last common exon (exon 4) and the alternatively spliced exon 6 (T) has been analyzed in human and mouse and found to contain another exon, 5 (H), which should encode the C terminus of the GPI anchored H subunit [14].

We presently report the sequence of the corresponding cDNA, obtained from rat spleen mRNA, and we show that expression in transfected COS cells produces GPI-anchored dimers.

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Abbreviations: AChE, acetylcholinesterase; GPI, glycophosphatidylinositol.

2. MATERIALS AND METHODS

2.1. *Amplification of a cDNA region encoding the distinct C-terminal region of AChE H subunit*

Total rat spleen RNA was purified according to the method of Chirgwin et al. [15], and reverse-transcribed with AMV-reverse transcriptase, as previously described [13], using 30 ng of a mouse oligonucleotide primer R_1 (5'-GACCAGAAGGGGAAAGG-3') corresponding to the non-coding region located downstream of exon 5 [14] (see Fig. 3A). The polymerase chain reaction was used to specifically amplify a fragment of cDNA, with the mouse oligonucleotide R_1 and a rat oligonucleotide located in exon 3 (F_C: 5' CTGGGGTGCGGATCGGT-3'). The resulting amplified fragment (approximately 700 nt) was subcloned in the PCR 1000 vector, using the TA cloning kit (Invitrogen), and sequenced.

2.2. *Construction of an expression vector encoding the H subunit of rat AChE*

Using a unique *Bam*HI site, located in exon 3, downstream of oligonucleotide F_C (see Fig. 3A), and a *Not*I site located downstream of the insert in both pEF-BOS [16] and PCR 1000 vectors, we excised a fragment encoding the C-terminal region of subunit H from the PCR 1000/$AChE_H$ clone, and ligated it into the pEF-BOS/$AChE_T$ vector, previously digested with *Bam*HI and *Not*I. We thus obtained a pEF-BOS/$AChE_H$ recombinant vector, containing the complete coding sequence of subunit H. This sequence was verified before expression in COS cells.

2.3. *Transfection of COS cells and analysis of AChE forms*

COS cells were transfected with pEF-BOS/$AChE_H$, using the DEAE-dextran method as previously described [10,13]. The cells were cultured for 3 days at 37°C, before extraction of AChE and analysis by sedimentation in sucrose gradients, as described [10]. Treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), obtained from Immunotech (Marseilles, France), was performed as described by Duval et al. [10].

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A  exon 4 | exon 5H
 L  L  S  A  T ▼ A  T  E  V  P  C  T  C  P  S  P  A  H  G  E  A  A  P  R  P   25
TTGCTCAGCGCCACAGCCACAGAGGTTCCCTGCACCTGCCCAAGCCCCGCCCATGGGGAGGCTGCCCCGAGGCCA  75

 G  P  A  L  S  L  S  L  L  F  F  L  F  L  L  H  S  G  L  R  W  L  *         47
GGGCCCGCCTTATCCCTGTCACTTCTCTTCTTCCTTTTCCTCCTCCACTCCGGGCTTCGGTGGCTCTAA       144

B  exon 4 | R sequence
 L  L  S  A  T ▼ G  R  R  G  V  G  K  Q  G  M  H  K  A  A  R  V  G  R  T  G   25
TTGCTCAGCGCCACAGGTAGGCGCGGGGTGGGGAAGCAGGGAATGCACAAGGCGGCAAGGGTTGGAAGGACGGGA  75

 E  R  K  G  G  K  H  R  M  *                                                 34
GAGAGAAAAGGTGGAAAACACAGGATGTAATCCCTCTCTTCTCTACCTCCCCCA                       129
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Fig. 1. (A) Nucleotide sequence of the 5′ region of the H AChE transcript, and deduced amino acid sequence. (B) Nucleotide sequence of the R transcript, determined from cDNA and from genomic DNA, with the deduced hypothetical amino acid sequence. The limits of the exons are indicated; amino acids differing from the corresponding mouse sequences are indicated in bold type [14].

2.4. *PCR analysis of genomic DNA and mRNAs*

Genomic DNA was prepared as described by Miller et al. [17]. Total RNA was purified from spleen, muscle, hypothalamus and embryonic liver (E-16) and reverse-transcribed as previously described [13], using hexanucleotide primers. Various combinations of the following oligonucleotide primers were used for PCR amplification of the cDNAs, or reamplification of the primary PCR products (see Fig. 3A):

F_C: 5′-CTGGGGTGCGGATCGGT-3′
$F_{C'}$: 5′-TCCCTCACTGAACTACACC-3′
$F_{C''}$: 5′-AATCGTTTTCTCCCCAAATTGCTCA-3′
F_H: 5′-CACAGAGGTTCCCTGCACCTGCCC-3′
R_H: 5′-TTAGAGCCACCGAAGCCCGG-3′
$R_{H'}$: 5′-GCCTCGGGGCAGCCTCCCCATGGG-3′
R_I: 5′-GACCAGAAGGGGAAAGGA-3′
R_R: 5′-CTTCCAACCCTTGCCGCCTTGT-3′
R_T: 5′-TCACAGGTCTGAGCAGCGTT-3′
$R_{T'}$: 5′-CGGCCTTCCACTGGCGCTCCGC-3′

All amplifications were carried out for 35 cycles, consisting of a denaturation step of 1 min at 92°C, an annealing step of 1 min at 65°C and an elongation step of 2 min at 72°C. The amplification products were analysed in 1.2% agarose gels containing ethidium bromide.

3. RESULTS AND DISCUSSION

Because hemopoietic cells synthesize GPI-anchored dimers of AChE, we expected the presence of mRNAs encoding the H subunit in rat spleen. We were indeed able to amplify by PCR a fragment of cDNA, encoding the C-terminal region of the H subunit of AChE. Considering the near-identity between the rat and mouse AChE sequences [12,13], we used a forward primer located in the rat common exon 3 (F_C) and a reverse primer (R_I) designed from the 3′ non-coding region of the mouse exon 5 (H) [14]. Fig. 1 illustrates the amplified H cDNA sequence. The peptidic sequence encoded by exon 5 (H) of rat AChE is very similar to the corresponding sequence of the mouse [14], with only four differences in 42 residues. Both mouse and rat H peptides contain two cysteine residues. In contrast, *Torpedo* and human AChE H subunits possess only one cysteine residue in this region.

By introducing a *Bam*HI/*Not*I fragment of AChE H cDNA in place of the corresponding fragment of the pEF-BOS/AChE$_T$ vector [13], we obtained a recombinant expression vector, pEF-BOS/AChE$_H$, encoding the rat H subunit. Transfected COS cells expressing this subunit were found to produce principally amphiphilic dimers, which were clearly sensitive to PI-PLC and therefore GPI-anchored (Fig. 2). Thus, the cloned H subunit contains a functional GPI cleavage/attachment signal, which is correctly matured in COS cells. This is not surprising, since these cells efficiently process the much more distant H subunit of *Torpedo* AChE [10].

We investigated the presence of mRNAs encoding the

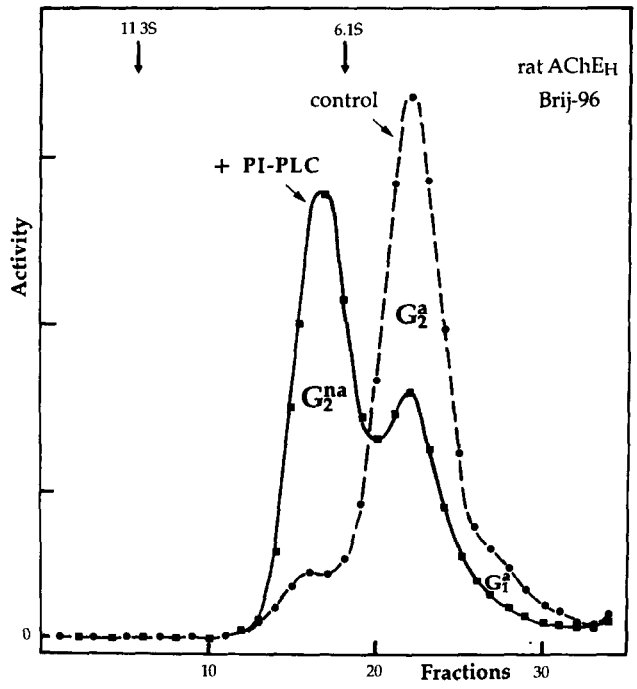

Fig. 2. Sedimentation analysis of AChE forms produced in COS cells, after transfection with the pEF-BOS recombinant vector, expressing the AChE H subunit (pEF-BOS/AChE$_H$). (●) Control; (■) after partial digestion with PI-PLC. In gradients containing 1% Brij-96, the amphiphilic dimer (G_2^a) sediment at 4.5 S, and their non-amphiphilic derivative (G_2^{na} sediments at 6.5 S. The minor monomeric component (G_1^a) is also produced in non-transfected COS cells.

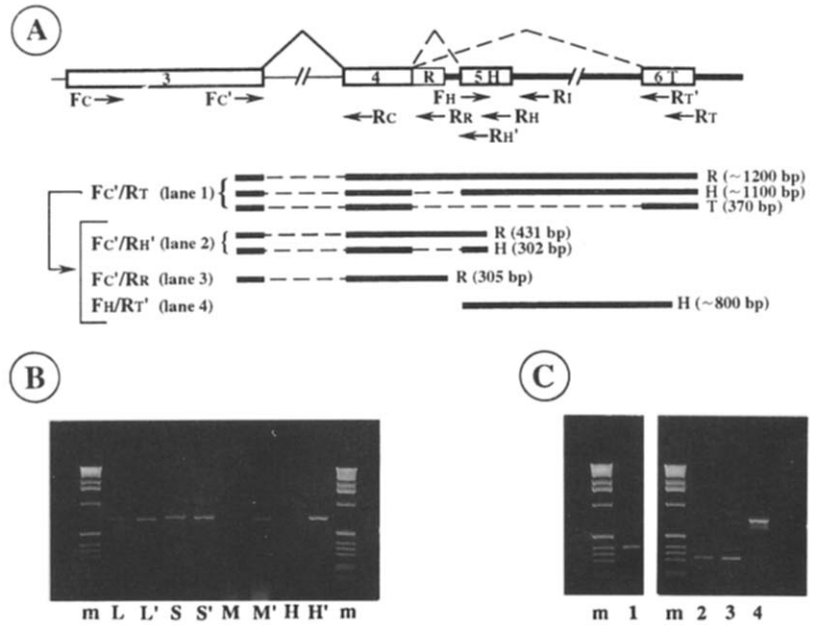

Fig. 3. (A) Splicing scheme of the 3′ coding region of the mammalian AChE gene (after Li et al. [14,18]), using the recommended nomenclature [3]. The coding regions are indicated by boxes, identified by the exon number, and letters H or T for the alternative C-terminal exons; R indicates the potential coding region of the readthrough transcript. The constitutional splicing between the common exons 3 and 4 is indicated by solid lines, and alternative splicing is indicated by dashed lines. The intron separating exons 3 and 4 is shown as a thin line, while the non-coding sequences located downstream of the common exon 4, which are maintained in some transcripts, are shown as thick lines. The positions of oligonucleotide primers used for PCR analysis of the transcripts are indicated (their lengths are not to scale). The products expected for amplification of R, H and T transcripts with selected primer pairs are schematically indicated (see C); the size of the intervening sequence separating 5 (H) and 6 (T) is estimated to be about 600 bp, from the product obtained with $F_H/R_{T'}$ primers. (B) Detection of the H and T transcripts in rat tissues, using $F_{C'}/R_H$ and $F_{C'}/R_T$ primers, respectively: lanes L and L′, embryonic liver; lanes S and S′, spleen; lanes M and M′, muscle; lanes H and H′, hypothalamus. (C) Lane 1, PCR products obtained from embryonic liver cDNA, using $F_{C'}/R_T$ primers; the higher bands at 1100 and 1200 bp are very faint and hardly detectable. Re-amplification of the products with $F_{C'}/R_{H'}$ primers is shown in lane 2; with $F_{C'}/R_R$ primers in lane 3 and with $F_H/R_{T'}$ primers in lane 4. The size markers (lanes m) correspond to the 1 kb ladder from Gibco BRL.

H and T subunits in various rat tissues. We examined the hypothalamus because it contains a high level of AChE mRNAs of different sizes [13], as well as muscle and spleen of adult rats, and liver of 16-day rat embryos. cDNAs obtained by reverse transcription of total RNA were amplified by PCR, using a forward primer located in the common exon 3 (F_C), and specific reverse H or T primers located either in exon 5 (R_H) or in exon 6 (R_T) (Fig. 3A). As shown in Fig. 3B, we obtained a major 676 bp band for embryonic liver and spleen with the F_C/R_H primers, indicating the presence of the H transcript; this band was not detectable in the case of muscle and hypothalamus. With the F_C/R_T primers, we obtained a band of 670 bp, indicating the presence of T transcripts, in all four tissues. We verified that these bands were amplified from cDNA, and not from genomic DNA, by omitting reverse transcription: in this case, they were totally absent.

The simultaneous presence of both types of transcripts in embryonic liver is perfectly consistent with biochemical analyses of its AChE content, which showed comparable proportions of monomers and dimers, together with a smaller proportion of nonamphiphilic tetramers (not shown). The dimers were converted into non-amphiphilic derivatives by PI-PLC, as shown by sedimentation analysis (not shown) and by their migration in non-denaturing electrophoresis (Fig. 4); they are therefore GPI-anchored and derive from H subunits. In contrast, the monomers and tetramers were insensitive to PI-PLC, and, like similar molecules are obtained in transfected COS cells, probably derived from T subunits [13]. It is interesting that we did not find evidence for the presence of H transcripts, or of GPI-anchored dimers in the liver of adult rats (not shown). Our results thus show that while the H transcripts occur in hematopoietic tissues, T transcripts are more extensively expressed (Fig. 3B).

Using primers $F_{C''}$ (located in exon 4, see section 2) and R_I, we amplified the intervening sequence which separates the common exon 4 from the alternative exon 5 (H) in genomic DNA. From this sequence, we designed primer R_R; using $F_{C'}/R_R$, we found that this intervening sequence occurs in a fraction of embryonic liver cDNAs. This sequence is very similar to the 'readthrough' (R) sequence reported in the case of mouse AChE [14]; two gaps, of 1 and 2 nucleotides, did not change the reading frame.

In embryonic liver, when amplification was carried out with primers $F_{C'}$ and R_T, two less intense higher bands could be detected in the PCR products, but are

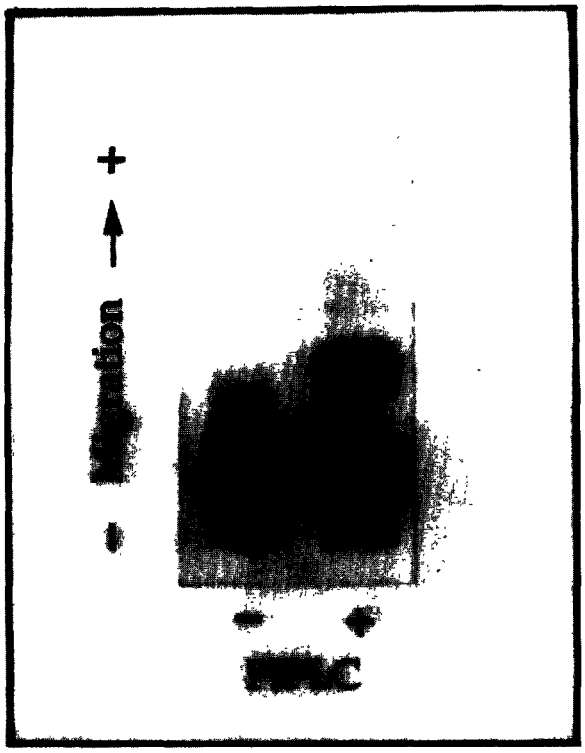

Fig. 4. The G_{a^2} form of AChE from embryonic rat liver is sensitive to PI-PLC. An extract of liver from rat embryos (E-16) was analyzed by electrophoresis in non denaturing polyacrylamide gel containing 0.5% Triton X-100, with or without treatment by PI-PLC, and stained for activity. The band corresponding to the G_{a^2} form is considerably accelerated after digestion, while other components are unaffected.

hardly visible in Fig. 3C, lane 1; these bands could be labeled in Southern blots with oligonucleotide R_T, located in the T sequence (not shown). The presence of these bands could be expected according to the splicing scheme proposed by P. Taylor and his colleagues [14,18], as illustrated in Fig. 3A: one band might correspond to the H transcript, which is thought to retain the downstream T exon, and the other one to the R transcript. In order to confirm the presence of these different cDNA structures, we submitted the PCR products obtained with the F_C/R_T primers to second amplifications with various primers, including $F_C/R_{H'}$, F_C/R_R and $F_H/R_{T'}$, as shown in Fig. 3C. In each case, we obtained the expected products, in agreement with the proposed splicing mechanism.

Using F_C/R_C primers, we could not detect any transcripts containing an intron between exons 3 and 4, showing that the R transcripts did not represent unspliced mRNA precursors. It is interesting that we did not find evidence for the presence of R transcripts in muscle, which seems to contain only T mRNAs (not shown). The question remains whether R transcripts simply arise from a default mechanism, e.g. in cells which produce H transcripts, or whether they represent a functional species, generating an active AChE form. It is interesting that there are three nucleotides more in the mouse than in the rat R transcript, so that the potentially coding sequences are mostly in frame and predict similar hypothetical C-terminal peptides.

The structure of the H C-terminal peptide, as reported here, raises interesting questions, regarding the possible involvement of the two cysteines in intersubunit disulfide bonds, and the exact position of the GPI cleavage/attachment site. At least one of the two cysteines must be maintained in the mature protein, in order to establish the intersubunit disulfide bond. The fact that the H subunit produces only one type of mature molecular form makes it a simple model in which the impact of site-directed mutagenesis on structure and catalytic activity may be analyzed.

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