Isolation of a cDNA clone for a catalytic subunit of *Torpedo marmorata* acetylcholinesterase

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Received 10 October 1985; revised version received 17 October 1985

We have constructed a cDNA library from *Torpedo marmorata* electric organ poly(A^+) RNA in the lambda phage expression vector λgtll. This library has been screened with polyclonal anti-acetylcholinesterase antibodies. One clone, λAChEl, produced a fusion protein which was recognized by the antibodies and which prevented the binding of native acetylcholinesterase in an enzymatic immune assay. These results indicate that λAChEl contains a cDNA insert coding for a part of a catalytic subunit of *Torpedo* acetylcholinesterase. The 200-base-pair cDNA insert hybridized to three mRNAs (14.5, 10.5 and 5.5 kb) from *Torpedo* electric organs. These mRNAs were also detected in *Torpedo* electric lobes.

Acetylcholinesterase *cDNA cloning* *Expression screening* (Torpedo marmorata)

1. INTRODUCTION

The electric organs of *Torpedo* constitute an extremely favourable material for a detailed analysis of the polymorphism of acetylcholinesterase [1], since they contain both collagen-tailed asymmetric and amphipathic globular forms [2–4]. Three types of catalytic subunits have been characterized by SDS-polyacrylamide gel electrophoresis [5,6]: (i) a catalytic subunit of an apparent molecular mass of 76 kDa that is present in the low-salt soluble fraction as a minor component, (ii) the catalytic subunit of the amphipathic dimers present in both low-salt and detergent-soluble fractions (68–69 kDa) and (iii) the catalytic subunit of the asymmetric forms that are solubilized in the high-salt-soluble fraction (72 kDa). The catalytic subunits of the low-salt-soluble fraction of the amphipathic dimer and of the asymmetric forms can be distinguished by proteolytic peptide mapping and affinity for a monoclonal anti-acetylcholinesterase antibody [7,8]. Furthermore, synthesis in vitro produces at least 2 precursors (65 and 61 kDa) for the catalytic subunits of electric organ acetylcholinesterase [9]. These results strongly suggest the existence of multiple mRNAs coding for the catalytic subunits of *Torpedo* acetylcholinesterase. To describe these mRNAs, we have isolated a cDNA clone for a catalytic subunit in an expression library, and used it as a probe in Northern blot hybridization experiments.

2. MATERIALS AND METHODS

2.1. *Construction and screening of a λgt11 cDNA library*

A cDNA library was constructed in the expression vector λgt11 [10,11], from an unfractionated poly(A^+) RNA preparation [9] obtained from electric organs of *Torpedo marmorata*. The library was screened by using a rabbit anti-acetylcholinesterase antiserum [9], as described by Young and Davis [12].

2.2. *Characterization of the fusion protein*

Lysogens were produced in the bacterial strain Y1089 and unlabelled lysogen extracts were obtained as described [12]. To produce labelled pro-

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00145793/85/$3.30

teins, L-[^{35}S]methionine (1000 Ci/mM, Amersham) was added to the heat-shock treated lysogens (using 50 μCi for a 200 μl culture) and the cells incubated for 1 h at 37°C. The labelled bacterial proteins were immunoprecipitated as in [9] and fractionated in a 7.5% SDS-polyacrylamide gel.

The fusion protein produced by one positive clone was characterized using the following enzymatic immune assay. Swine anti-rabbit immunoglobulins were immobilized on 96-well microtiter plates as described [13]. Immunoreaction was performed in these plates, using mixtures containing 50 μl of a $1/10^4$ dilution of anti-acetylcholinesterase antibodies, 50 μl native enzyme (1 Ellman unit/ml [14]) and, as a competitor of the native enzyme, 50 μl of serial dilutions of bacterial lysates. The native enzyme used was either a detergent-soluble or a high-salt soluble fraction of the enzyme. The bacterial lysates possessed no acetylcholinesterase activity. After overnight incubation at room temperature the plates were washed as in [13], and 200 μl Ellman medium [14] containing enzyme substrate was added. The absorbance at 420 nm of each well was measured 30 min later. Non-specific binding of the native enzyme (less than 0.1% of total activity) was evaluated in wells where antibody was omitted, and this value was subtracted for each measurement. Enzyme binding observed in the absence of competitor (B_0) was measured in separate wells using 50 μl buffer instead of bacterial lysate. Results are expressed as B/B_0 (%), where B represents the enzyme activity bound to the solid phase in the presence of competitor. In addition, we also tested the ability of heat-inactivated acetylcholinesterase to compete with the native enzyme in this assay. Pure amphipathic dimer was incubated for 18 h at 56°C and substituted for the bacterial lysate in serial dilutions.

We verified that there was no inhibition of acetylcholinesterase activity after an overnight incubation of the enzyme with serial dilutions of bacterial lysates.

2.3. *RNA filter hybridization*

Poly(A$^+$) RNAs were extracted using the LiCl-urea method [15] and purified by oligo(dT)-cellulose chromatography. Glyoxylated RNAs [16] were fractionated by electrophoresis through 0.6 or 0.8% agarose gel. After transfer to Hybond N membrane (Amersham), the RNAs were prehybridized and hybridized as described [17], using the nick-translated cDNA insert as a probe. The filters were washed at 50°C to a stringency of 0.1 × SSC [18].

3. RESULTS

3.1. *Isolation and characterization of a cDNA clone for a catalytic subunit of* Torpedo *acetylcholinesterase*

Starting from 10 μg poly(A$^+$) RNA, we obtained 500 ng cDNA at the final step of the synthesis. In

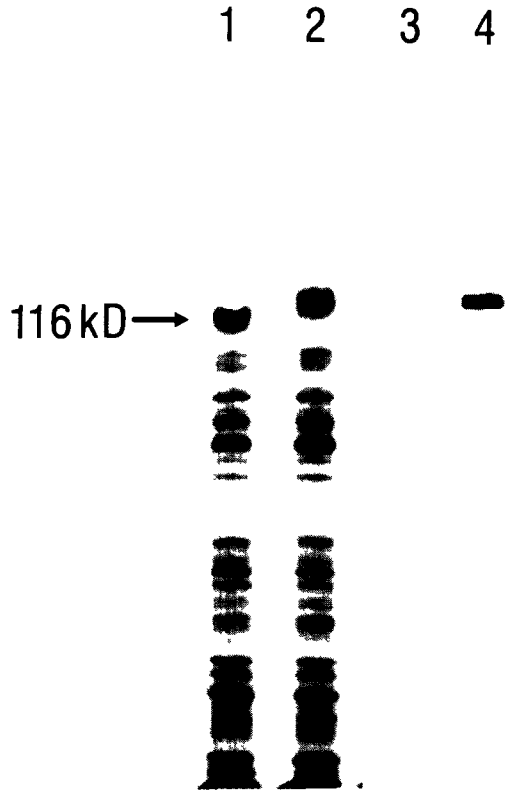

Fig.1. Analysis of λAChE1 fusion protein by SDS-polyacrylamide gel electrophoresis. Labelled lysogen extracts were prepared for λgt11 and λAChE1 and immunoprecipitation with anti-acetylcholinesterase antibodies. Lanes: (1,2) total labelled proteins from λgt11 and λAChE1, respectively; (3,4) corresponding immunoprecipitation by anti-acetylcholinesterase antibodies. The arrow indicates the position of *E. coli* β-galactosidase (116 kDa) deduced from molecular mass standards (BRL).

a ligation reaction, 25 ng of this cDNA were ligated at a molar ratio of 1:2 to the λgt11 arms and packaged, resulting in a library of 40000 independent clones, 60% of which carried inserts. This library was screened by using a polyclonal rabbit antiserum [9]. A single bacteriophage, designated λAChE1, consistently gave a high signal in the antibody-binding assay and was used for further studies. The insert of λAChE1 was contained in a single 200-bp *Eco*RI fragment. To determine whether a fusion protein of the corresponding size was indeed recognized by our antibodies, lysogens of λgt11 and λAChE1 were prepared, induced with isopropylthiogalactoside (IPTG) in the presence of L-[^{35}S]methionine and analyzed by electrophoresis (fig.1). As expected, AChE1 did not synthesize the β-galactosidase detected in λgt11 (lane 1), but instead a slightly heavier fusion protein (lane 2) which was immunoprecipitated by the anti-acetylcholinesterase polyclonal antiserum (lane 4). In the competition assay with native acetylcholinesterase, the λAChE1 lysate prevented the binding to the antibodies of either dimeric amphipathic or asymmetric forms as efficiently as did heat-inhibited enzyme. Fig.2 shows the competition results obtained for the amphipathic dimer. As a control λgt11 lysate did not prevent this binding even at high concentration.

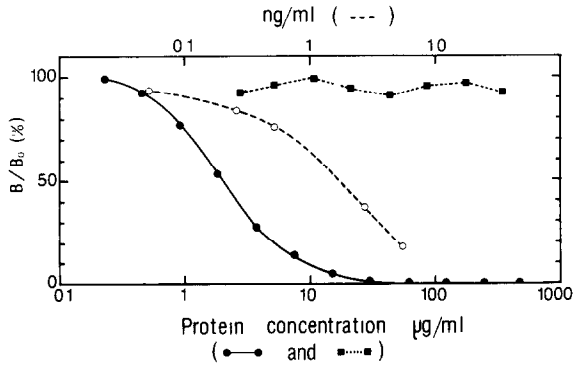

Fig.2. Immunoenzymatic detection of acetylcholinesterase fusion protein in bacterial lysates. For a description of the immunoenzymatic test, see section 2. (●—●) λAChE1 lysate, (■···■) λgt11 lysate, (○---○) heat-inactivated enzyme. The results are plotted as a function of protein concentration expressed as μg/ml for the bacterial lysates or as ng/ml for the heat-inactivated enzyme. Inhibition of native enzyme binding is expressed as B/B_0 (%) where B and B_0 represent the quantity of native enzyme bound in the presence or absence of enzymatically inactive competitor, respectively.

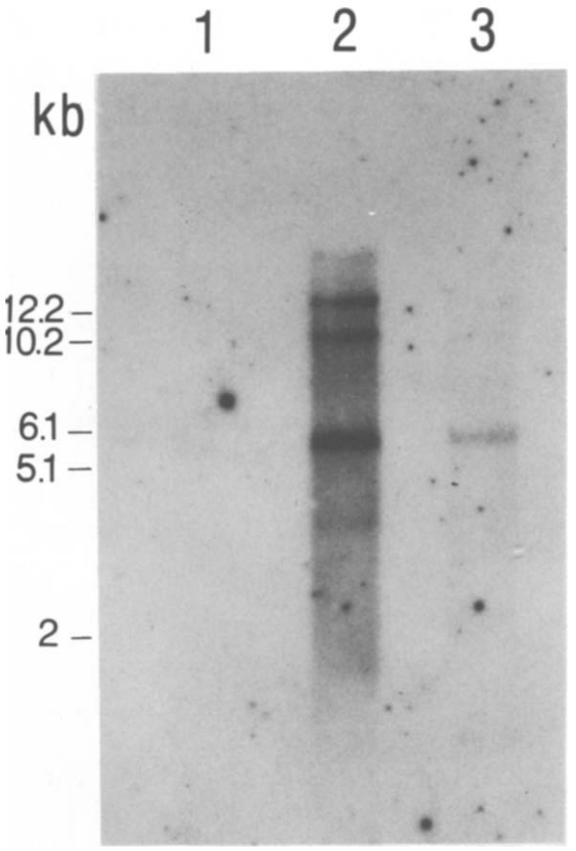

Fig.3. Characterization of acetylcholinesterase mRNAs in *Torpedo* electric organs and electric lobes by Northern blot hybridization. Poly(A)-containing RNA was fractionated by electrophoresis through 0.6% agarose gel. Size markers were BRL I kb ladder and λgt11 DNA. Lanes: (1) 10 μg liver poly(A$^+$) RNA, (2) 10 μg electric organ poly(A$^+$) RNA, (3) 10 μg electric lobe poly(A$^+$) RNA.

3.2. *Northern blot analysis*

When used in Northern blot analysis, the AChE1 200-bp insert did not hybridize to *Torpedo* liver poly(A$^+$) RNA (fig.3, lane 1). Three major bands of 14.5, 10.5 and 5.5 kb were detected in electric organ poly(A$^+$) RNA (fig.3, lane 2). The probe also detected the same transcripts in electric lobe poly(A$^+$) RNA (fig.3, lane 3).

4. DISCUSSION

By immunological screening of our cDNA library with anti-acetylcholinesterase antibodies, we obtained one unambiguously positive clone, λAChE1. We confirmed the identification of the fusion protein produced by the λAChE1 lysogen by testing its cross-reactivity with acetylcholinesterase. λAChE1 lysogen lysates efficiently displaced the active enzyme in an enzymatic immunoassay, whereas λgt11 lysogen lysates were totally ineffective under the same conditions. The high efficiency of the λAChE1 fusion protein in the competition was, in fact, somewhat unexpected since the 200-bp long cDNA insert can only encode 8 kDa of protein. This portion of acetylcholinesterase must therefore contain a substantial proportion of the antigenic sites of the catalytic subunit. The fact that λAChE1 lysates prevented equally well the binding of either dimeric or asymmetric forms of acetylcholinesterase implied that the cDNA insert coded for antigenic determinants common to both types of catalytic subunits.

Northern blot analysis of mRNAs obtained from electric organs, electric lobes or liver showed that the AChE1 insert used as a probe detected the same mRNAs in the 2 cholinergic tissues but none in the liver, which does not contain acetylcholinesterase. Since AChE1 lysates compete efficiently with both globular and asymmetric forms of acetylcholinesterase, we expected to detect at least 2 mRNAs hybridizing with the cDNA probe in the electric organ. We found in fact 3 major transcripts in this organ at 5.5, 10.5 and 14.5 kb. Since these mRNAs appear as discrete bands it is improbable that they represent breakdown products of a higher molecular mass molecule. We may also reasonably exclude the possibility that they represent processing intermediates between a primary transcript and a unique final mRNA, because no mRNA precursors of the acetylcholine receptor chains have been detected in similar experiments [19–22].

The size of the mRNAs detected suggests that they contain very long 3′-untranslated sequences (as described for the δ chain of the acetylcholine receptor) [21]. In fact, the 2 largest mRNAs (10.5 and 14.5 kb) exceed the 7.2 kb encapsidation capacity of phage λgt11 [11]. These 2 factors probably explain why no additional clone was detected when we rescreened the library using this cDNA insert as a probe (not shown).

It is tempting to suggest that the 3 observed transcripts correspond to distinct fractions of acetylcholinesterase. The subunits of the asymmetric forms (72 kDa) and of the amphipathic dimers (68–69 kDa), which have been shown to differ in their peptide sequence [8], are certainly encoded by distinct mRNAs, and a third transcript might correspond to the minor 76 kDa subunit. It is important to note that the 3 transcripts are detected in similar proportions in the electric organs and electric lobes. The electric lobes contain almost exclusively the asymmetric forms of the enzyme, but the motoneurons also produce the globular enzyme that is carried by the axonal flow to the nerve terminals [23,24]. Since we do not know with certainty the relative contributions of the motoneurons and of the electrocytes to the total enzyme activity of the electric organ, and have no estimate of the metabolic turnover of the different enzyme fractions, it is not yet possible to correlate the abundance of the mRNAs with the synthesis of specific acetylcholinesterase forms. In any case, our probe will be an invaluable tool for a further characterization of the different mRNA molecules and their genomic counterparts.

ACKNOWLEDGEMENTS

We thank Drs Florent Soubrier, Anne Devillers-Thiéry and André Klarsfeld for helpful discussions as well as Suzanne Bon and François Rougeon for critical reading of this manuscript. This research was supported by grants from the Centre National de la Recherche Scientifique, the Commissariat à l'Energie Atomique, the Ministère de l'Industrie et de la Recherche and the Direction des Recherches et Etudes Techniques. J.L.S. is the recipient of a Rhône-Poulenc fellowship.

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