Volume 261, number 2, 303-306 FEBS 08155 February 1990

A 15 kDa proteolipid found in mediatophore preparations from *Torpedo* electric organ presents high sequence homology with the bovine chromaffin granule protonophore

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Received 22 December 1989

Upon SDS PAGE of isolated mediatophore, an acetylcholine-translocating protein, a doublet at 15 kDa was identified. Amino acid sequencing after CNBr cleavage gave a 17 residue-long peptide completely homologous with a sequence of the proton-translocating proteolipid from bovine chromaffin granules. A 51-mer oligodeoxynucleotide corresponding to this sequence was used to screen a library of electric lobe cDNAs constructed in λZap II. A positive recombinant clone was isolated and found to encode the complete sequence of a 15.5 kDa protein highly homologous to the bovine chromaffin or yeast vacuolar ATPase proteolipid. In vitro translation of sense RNA transcripts of the clone indeed yielded a single 15 kDa proteolipid. Northern blot analysis showed that the 1.3 kb mRNA encoding this protein is significantly expressed in nervous tissues but not in electric organ or liver of *Torpedo marmorata*.

Mediatophore; Acetylcholine release; Proteolipid; H^+-ATPase; cDNA cloning; (*Torpedo marmorata*)

1. INTRODUCTION

The mediatophore is an oligomeric transmembrane protein isolated from *Torpedo* electric organ synaptosomes. This protein is characterized by its virtue of translocating acetylcholine in a calcium-dependent manner when reconstituted in liposomes [1,2]. Several experimental results, both physiological [3] and pharmacological [4], argue for a direct role of this protein in acetylcholine release from stimulated cholinergic nerve endings [5,6]. Electrophoresis and negative staining data suggested that the quaternary structure of the mediatophore resulted from the association of numerous, at least 10, subunits of apparent molecular weight around 15 kDa [2]. However, we do not know whether all the subunits are identical. This article reports on the primary structure of a 15 kDa hydrophobic protein present in mediatophore preparations. This proteins shares large homologous segments with a recently sequenced 16 kDa subunit of the vacuolar H^+-ATPase from bovine chromaffin granules [7] or yeast [8].

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2. MATERIALS AND METHODS

2.1. *Preparation of* Torpedo *mediatophore protein and subunits*

The mediatophore was isolated from *Torpedo marmorata* electric organ and extensively delipidified as described previously [9]. SDS-PAGE was performed in 8-18% (w/v) gradient gels [10]. The bands were electroeluted according to [11]. Afterwards, the material was dialysed against water to remove as much SDS as possible.

2.2. *Amino-acid sequencing*

Peptides were sequenced with an Applied Biosystems apparatus. Reproducible results were obtained by first applying the mediatophore protein or electroeluted material to the polyethylenimine-treated glass filter of the sequenator and then cleaving by CNBr: overnight treatment at room temperature with 15% CNBr (w/v) in 80% formic acid (v:v).

2.3. *cDNA cloning and library screening*

RNA was isolated from *Torpedo* brain electric lobes (that contain the cell bodies of cholinergic neurons innervating the electric organ) by the guanidine isothiocyanate method [12] and enriched in poly $(A)^+$ [13]. After size-separation by ultracentrifugation in a 5-20% sucrose gradient, mRNAs of 1-2 kb were used to synthesize oligo(dT)-primed cDNAs [14], which were ligated with EcoRI linkers and then inserted into λZAPII vectors [15]. About 400 000 recombinants were plated and duplicate filters were prepared [16]. They were screened with a ^{32}P end-labelled synthetic oligonucleotide: d(CAGGGAGT-TGGCAATGAGGACTGCCACCACCAGACCATAGATGGCGA-TGAT). After two rounds of screening, two strongly positive phage clones were excised in vivo using the helper phage R408 as described in the Stratagene λZAPII kit [15]. The recombinant plasmids thus obtained were amplified and purified by CsCl/ethidium bromide equilibrium gradient.

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00145793/90/$3.50

2.4. *Other methods*

Plasmid DNA was sequenced after alkaline denaturation [17,18] on both strands by annealing with universal or appropriately synthesized 17-mer oligonucleotides.

The λZAPII vector contains the promoters for T3 and T7 RNA polymerases in opposite orientations [15]. Pure sense capped RNA transcripts were obtained by in vivo transcription [19] of the plasmids (linearized by *Xho*I) as described in the Stratagene RNA transcription kit. RNA transcripts were either in, vitro translated in wheat germ extract or injected in *Xenopus* stage VI oocytes.

Northern blot analysis was essentially conducted according to [20]. Probes were ^{32}P labelled oligonucleotides or nick-translated plasmids.

3. RESULTS

The oligomeric structure of the mediatophore clearly appears after negative staining of the purified fraction (Fig.1A,B). Previous works suggested an apparent molecular mass of 150–200 kDa for this protein [2,21]. However, upon dissociation by SDS, the only subunit detected after gel electrophoresis was a 15 kDa protein, frequently appearing as a doublet band (Fig.1C,D). N-terminal amino-acid sequencing of the mediatophore or of the electroeluted subunits were never successful: this was probably due to amino-terminal blockage. Trying to sequence CNBr-cleaved peptides was also unsuccessful. A 17-mer peptide could be sequenced by applying CNBr directly to the filter of the sequenator (see section 2). Its sequence (Ile-Ile-Ala-Ile-Tyr-Gly-Leu-Val-Val-Ala-Val-Leu-Ile-Ala-Asn-Ser-Leu) was found to be identical to that of a hydrophobic segment of the recently cloned proteolipid subunit of the bovine chromaffin granule H^+-ATPase [7]. Starting from this

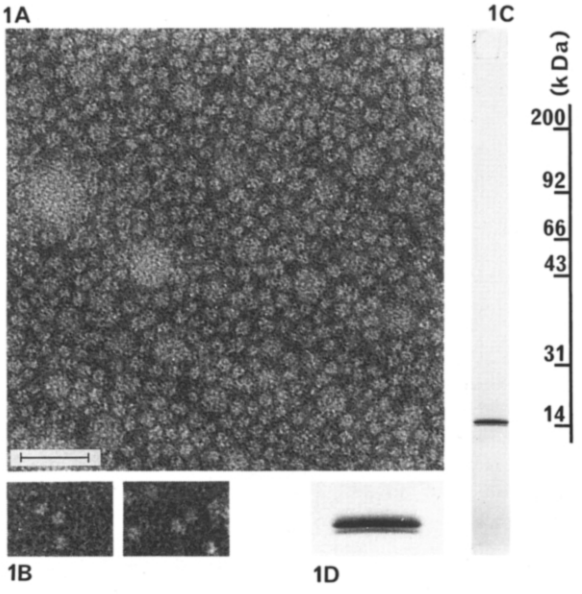

Fig.1. Morphological aspect and electrophoresis pattern of the mediatophore. (A,B) Electron micrographs obtained after negative staining of the purified mediatophore fraction. The pentameric structure of the protein is very apparent in B. C: The SDS-PAGE of the same preparation exhibits a 15 kDa doublet band, which is magnified in D. Coomassie blue staining. Bar in A = 30 nm for A and 40 nm for B.

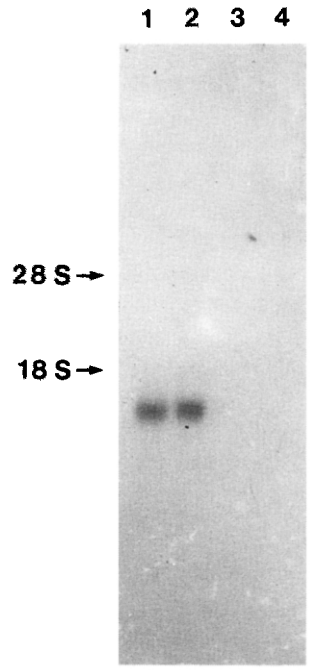

Fig.2. RNA blot hybridization with the synthetic oligonucleotide probe (autoradiogram). Poly (A^+) RNA from *Torpedo* tissues was fractionated by electrophoresis, transferred to nitrocellulose filters and hybridized with a ^{32}P end-labelled oligonucleotide probe corresponding to the sequenced peptide. Lanes: 1, size-selected (1–2 kb) mRNA from electric lobe (0.7 μg); 2, electric lobe mRNA (2 μg); 3, electric organ mRNA (2 μg); 4, *Torpedo* liver mRNA. Position of ribosomal RNA migration markers is shown on the left. The size of the hybridizing RNA is approximately 1.3 kb.

similarity, we decided to screen a *Torpedo* electric lobe cDNA library with a long 51-mer oligonucleotide probe whose sequence (see section 2) was exactly complementary to that of the chromaffin granule proteolipid mRNA. This oligonucleotide was found to hybridize in

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                                                        CGTGTAC
ACCACTTCCCCGGCACCAAGCCGCCCGCCCCGTGTCCACCCGCGCCGAGCAGCTGACGAG

ATGTCCACTCCCGGAGCTCCCGAGTATTCGGCCTTCTTCGGTGTGATCGGCGCCTCGGCG      60
M  S  T  P  G  A  P  E  Y  S  A  F  F  G  V  I  G  A  S  A

GCCATGGTCTTCAGCGCTCTAGGAGCTGCTTATGGAACAGCCAAGAGTGGGACAGGAATT     120
A  M  V  F  S  A  L  G  A  A  Y  G  T  A  K  S  G  T  G  I

GCTGCCATGTCGGTCATGAGGCCTGAACTTATCATGAAATCCATCATTCCTGTCGTCATG     180
A  A  M  S  V  M  R  P  E  L  I  M  K  S  I  I  P  V  V  M

GCAGGTATCATTGCTATCTATGGACTGGTGGTGGCAGTACTAATTGCCAACTCACTCACA     240
A  G  I  I  A  I  Y  G  L  V  V  A  V  L  I  A  N  S  L  T

GAAGATATATCACTGTTCAAGAGCTTCCTTCAGCTGGGTGCTGGTCTGAGCGTGGGACTG     300
E  D  I  S  L  F  K  S  F  L  Q  L  G  A  G  L  S  V  G  L

AGTGGTCTTGCTGCTGGTTTTGCCATTGGCATAGTAGGTGATGCTGGAGTTCGCGGAACT     360
S  G  L  A  A  G  F  A  I  G  I  V  G  D  A  G  V  R  G  T

GCACAGCAGCCCCGATTATTCGTCGGGATGATCCTCATCCTCATTTTTGCTGAAGTGTTG     420
A  Q  Q  P  R  L  F  V  G  M  I  L  I  L  I  F  A  E  V  L

GGTCTGTACGGCTTGATTGTTGCCCTGATCCTTTCCACAAAATAAATTCCACAACTATAA     480
G  L  Y  G  L  I  V  A  L  I  L  S  T  K

AATTCAGCTCCATCCTTATGGTCTTAACTCCAGAATGTGTACAGTTGTCTCAATTTGGTA     540

GTCAATCTC...
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Fig.3. Nucleotide sequence of *Torpedo* electric lobe cDNA clone and deduced amino-acid sequence of the 15 kDa proteolipid. The peptide obtained by amino-acid sequencing is underlined. Amino-acids are in standard one-letter symbols.

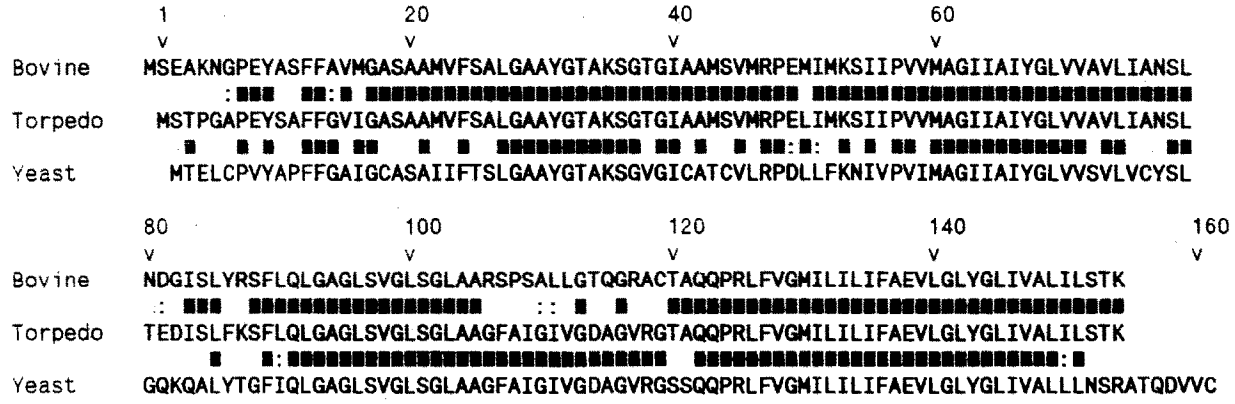

Fig.4. Aligned sequence of *Torpedo*, bovine and yeast proteolipids. Identical amino-acids are indicated (■). E–D, G–A and I–L are considered as conservative replacements (:). Bovine and yeast sequences are from [7] and [8] respectively.

northern blots with a single electric lobe mRNA of about 1.3 kb, but not with *Torpedo* electric organ or liver mRNA (fig.2).

A cDNA library was constructed in λZAPII from size-selected small mRNAs (1–2 kb in length). Two positive recombinant clones (insert size: 1.0 and 1.2 kb respectively) were partly sequenced and proved to be identical. The cDNA sequence contained an open reading frame encoding a 15 496 Da protein and significant non-coding regions (fig.3).

Comparison of the primary structure of the *Torpedo* 15 kDa protein and bovine and yeast vacuolar ATPase proteolipids revealed high sequence homologies (fig.4). A segment not found in the chromaffin granules protein (amino-acids 106–119) is, interestingly, present in the yeast. Moreover, the hydropathy profiles are superposable for the three proteins (not shown), with four putative transmembrane domains [7].

Sense RNA transcripts obtained from the clone containing the largest insert were in vivo translated, yielding a major 15 kDa protein (fig.5A). After injection of this pure RNA in *Xenopus* oocytes, the 15 kDa protein was found to be incorporated into membranes and could be extracted by organic solvents, as expected from its proteolipid nature (fig.5B).

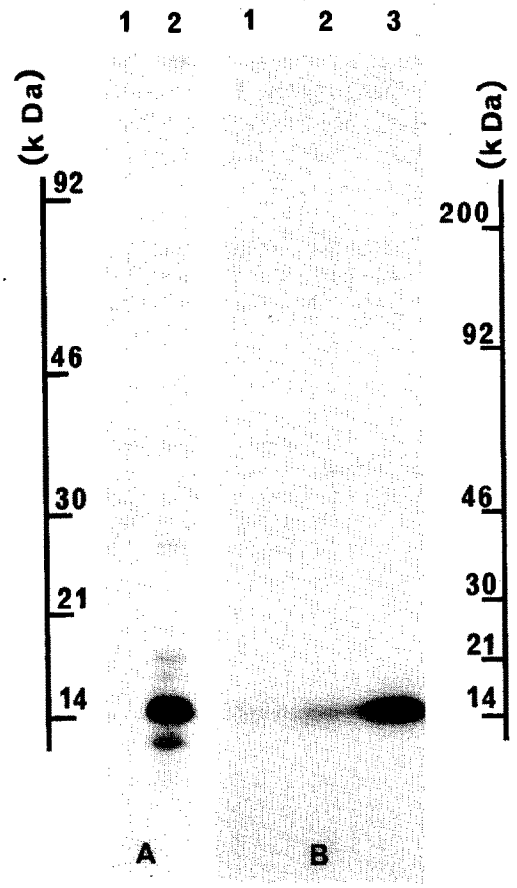

Fig.5. In vitro translation and oocyte injection of sense RNA transcripts of the electric lobe cDNA clone (autoradiograms). (A) In vitro translation with a wheat germ extract. RNA was translated in the presence of [^{35}S]methionine; the protein synthesized was submitted to gel electrophoresis and an autoradiogram was obtained from the gel. Translation: 1, without added RNA; 2, in the presence of sense RNA transcripts. (B) Oocyte injection. RNA was injected in *Xenopus* oocytes. After 4 days of incubation in the presence of [^{35}S]methionine, membranes were isolated and extracted with chloroform–methanol 1:1 (v/v). The extract was precipitated by 5 vol. of diethylether and submitted to SDS–PAGE. Lanes: 1, no injection; 2, *Torpedo* electric lobe poly (A^+) RNA (40 ng per oocyte); 3, sense RNA transcripts of the clone (8 ng per oocyte). Four oocytes per condition.

4. DISCUSSION

The extensive sequence homology found between the *Torpedo* 15 kDa proteolipid and the related bovine and yeast proteins (fig.4) suggests that differences are most probably species-related. Furthermore, a glutamic acid residue (Glu^{138}), suspected to be a binding site for N,N′-dicyclohexylcarbodiimide (DCCD) [7], is conserved in the three proteins, and we have found that ^{14}C-DCCD treatment of purified *Torpedo* electric organ synaptic vesicles indeed led to labelling of a 15 kDa protein (S. Birman and M.F. Diebler, unpublished results). Thus, it is likely that the protein sequenced here is part of the H^+-translocating vacuolar ATPase

recently shown to be present in cholinergic synaptic vesicles [22].

Nevertheless, the negative staining of the purified mediatophore seemed to be that of a homogeneous fraction, showing a single type of particle, and a prominent 15 kDa protein always copurifies with the mediatophore activity (fig.1). Since the only peptide we have presently succeeded in sequencing after CNBr treatment of mediatophore preparations belongs to the protonophore family, we are led to regard this 15 kDa proteolipid as a potential subunit of the mediatophore protein.

It has been recently reported [23] that a major 16 kDa protein coisolating with gap junctions from diverse vertebrate tissues [24] is also homologous to the vacuolar H^+-ATPase proteolipid. This peptide might then be part of the junctional protein (the connexon). That the mediatophore may be involved in the formation of gap junction-like pore structures has been previously hypothesized [25]. These results suggest that the vacuolar proteolipid may play a more diversified role in intercellular communications than had been initially expected.

Acknowledgements: We thank Dr J. Mallet and his collaborators for their helpful advice on cloning methodologies. This work was supported by a grant from the Association Française contre les Myopathies.

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