Mouse acetylcholinesterase interacts in yeast with the extracellular matrix component laminin-1β

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Abstract Acetylcholinesterase (AChE) is likely to have roles other than the hydrolysis of acetylcholine, e.g., related to developmental processes like neurite outgrowth, differentiation and adhesion. Here, we investigated whether AChE can function as a heterophilic cell adhesion molecule and searched for proteins interacting with it. Using the yeast two-hybrid method and a mouse brain cDNA library, we have identified an interaction between a partial cDNA encoding the globular domain IV of laminin chain β1 and the amino acids 240–503 of mouse AChE. Biochemical co-immunoprecipitation assays confirmed the genetic results. We suggest that AChE, by interacting with laminin-1, is able to exert changes in adhesion signaling pathways.

Keywords: Acetylcholinesterase; Laminin-1; Cell adhesion; Neurite outgrowth

1. Introduction

Acetylcholinesterase (AChE) is known as the enzyme that terminates the action of the neurotransmitter acetylcholine (ACh) at neuromuscular junctions and brain cholinergic synapses. Apart from its catalytic function, strong evidence over the past decade established novel non-catalytic functions of AChE, in particular by affecting neurite outgrowth and cell adhesion [1–6]. AChE is expressed very early in neural development before the onset of cholinergic neurotransmission [7–9], thereby affecting processes of migration, differentiation and synaptogenesis by a mechanism that is unrelated to its classic ACh hydrolyzing activity. Part of the non-catalytic AChE activities is assigned to sequence homologies between AChE and a family of cell adhesion molecules, including the *Drosophila* neurotactin and mammalian neuroligins [10,11]. Although these proteins have a structural organization similar to the enzyme acetylcholinesterase, their cholinesterase-like domain lacks enzymatic activity and functions through a protein–protein interaction motif [12]. Due to these homologies, and also to pharmacological and other molecular studies, it was established that AChE can function as a cell adhesion molecule [1,13,14]. It is generally assumed that an unknown heterologous binding partner for AChE must be involved in its adhesive functions. This partner should be able to transduce signals into cells and be expressed already at early developmental stages.

Laminin-1 is a part of the basement membrane and thus is ideally placed as a potential ligand for AChE in the developing nervous system. Laminin has many and varied functions that are mediated by binding to various components of the basement membrane [15–17]. As a cell attachment factor, it promotes neurite outgrowth and influences neuron migration, growth, morphology, and adhesion, functions important in tissue repair. A number of laminin-binding cellular proteins have been characterized, including a variety of cell surface integrins that mediate the interactions of cells with laminin [18,19]. Recent studies showed that human AChE also binds to mouse laminin-1 and collagen IV in vitro by an electrostatic mechanism [20].

To gain insight into the cell adhesion promoting functions of acetylcholinesterase, we here searched for proteins interacting with it, using a yeast two-hybrid screen. We identified several AChE-binding partners, some seemingly well suited to transduce signals into cells. Here, we report that laminin-1β was identified as a binding partner of mouse AChE using a yeast two-hybrid screening. Co-immunoprecipitation assays confirmed the results of the yeast screen. The interaction between AChE and laminin-1 could well be one of the missing links between AChE and other neuronal cell adhesion molecules like integrins. Possible roles of the AChE laminin-1 interaction in neurite outgrowth and synaptogenesis are discussed.

2. Materials and methods

2.1. Plasmid construction

pVJL11-N583AChE was constructed by inserting the core domain of mouse AChE, encoding the amino acids (aa) 1–583, into pVJL11 vector, a pBTM116 derivative with modified polylinker (kindly provided by Dr. J. Camonis). The bait AChE was amplified by PCR with Pfu polymerase (Stratagene) and cloned in frame at *Eco*RI and *Bam*HI restriction sites of the pVJL11 vector. Two plasmids encoding C-terminally truncated baits were also constructed: pVJL11-N472AChE (aa 1–472) and pVJL11-N263AChE (aa 155–418). pVJL11-laminC and pVJL11-p53 were constructed by the excision of cDNA encoding for the aa 67–230 of human laminC from pLaminC vector (Stratagene) and of cDNA encoding for the aa 72–390 of murine p53 and in-frame ligation in pVJL11. The sequences of all constructs were confirmed by

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Abbreviations: AChE, acetylcholinesterase; ACh, acetylcholine; aa, amino acid; CNS, central nervous system; DO, drop-out

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sequencing. Plasmids pRalAV23 and pRLIP used as positive control for interaction were a gift of Dr. J. Camonis and are described elsewhere [21]. The core domain of AChE (aa 1–583) was expressed using pCDNA3 in HEK293 cells (pCDNA3-AChE kindly provided by Prof. Dr. P. Taylor).

2.2. Yeast two-hybrid screening

The two-hybrid system used in this study has already been described [22,23]. Amino acids 1–583 of the mouse AChE fused to the LexA binding domain were used as bait to screen an adult mouse brain cDNA library fused to Gal4 activation domain in pACT2 vector (Clontech). The yeast strain L40, pre-transformed with the pVJL11-N583AChE recombinant vector, was then transformed with 60 μg cDNA of a mouse brain library. Approximately 2×10^6 yeast transformants were screened. Positive clones were selected for 6 days on drop-out (DO) agar plates (-Leu, -Trp, -His) and assayed for β-galactosidase activity. To assess the specificity of the interaction, positive library recombinant vectors were used to co-transform L40 with pVJL11-N583AChE, pVJL11 empty vector, pVJL11-Lamin or pVJL11-p53. The cDNA inserts from the AChE interacting clones were sequenced using the dideoxy termination method. When two-hybrid results are presented, the results of β-galactosidase test on filter paper are shown. There was no discrepancy between His auxotrophy test and the β-galactosidase test.

2.3. Antibodies

Antibodies used were: polyclonal goat anti-AChE mouse E-19 (Santa Cruz Biotechology, Germany, Product No. sc-6432), raised against the N-terminus of AChE, polyclonal rabbit anti-laminin-1 mammalian species (Sigma, Germany, Product No. L 9393), monoclonal rat anti-laminin 1β mouse (Chemicon International, Germany, Product No. MAB 1928), goat anti-rabbit horseradish peroxidase conjugated (Sigma, Product No. A 0545), goat anti-rat horseradish peroxidase conjugated (Sigma, Product No. A 9037), and rabbit anti-goat horseradish peroxidase conjugated (Sigma, Product No. A 8919).

2.4. Cell culture, transient transfections

HEK293 cells were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 20 units/ml penicillin and 20 μg/ml streptomycin. The cells were grown on 9 cm dishes until 50–60% confluency. They were transfected for 8 h with pCDNA3-AChE, using the DOTAP liposomal transfection reagent (Roche, Germany) and following the manufacturer's instructions. After 48 h, cells were rinsed, collected by centrifugation, and resuspended in phosphate buffered saline, pH 7.4, with 0.1% Triton X-100 plus complete protease inhibitors cocktail (Sigma). Clear supernatants were prepared by centrifugation at $12\,000\times g$, 4 °C, for 10 min. Mouse brain extracts were prepared as follows: mice were euthanized by cervical dislocation and the brain was collected and stored frozen at −80 °C or directly homogenized in 10 ml of 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 0.02% NaN_3 plus fresh protease inhibitors (Sigma) with a sonicator by 4 times 20 s pulses. After ultracentrifugation at $150\,000\times g$ at 4 °C, for 60 min, the pellet was resuspended in 10 vol of 20 mM Na-phosphate, pH 7.0, 1 mM EDTA, 1 M NaCl, 0.5% Triton X-100, and 0.02% NaN_3 plus protease inhibitors, with a sonicator 4 times 20 s pulses. The extract was centrifuged at $150\,000\times g$, 4 °C for 60 min. The supernatant, the final mouse brain membrane extract, was saved, aliquoted and stored at −80 °C. Protein content and acetylcholinesterase activity [24] was determined in different fractions. The brain extract buffer was then changed by dialysis with phosphate buffered saline plus 0.1% Triton X-100, to minimialize the high salt content interference with the binding studies.

2.5. Co-immunoprecipitations and Western blot analysis

HEK-293 cell lysate (1 ml, 2 mg protein/ml) plus 5 μg laminin-1 (Sigma) was incubated with anti-AChE (10 μl, 200 μg/ml stock concentration) or anti-laminin antibodies (5 μg monoclonal, 15 μl polyclonal) at 4 °C rotation for 16 hours, followed by incubation with 100 μl Protein G–agarose (Amersham Pharmacia Biotechnology) for 2 h at 4 °C. Likewise, the brain membranes fraction (1 ml) was subjected to immunoprecipitation by anti-AChE or anti-laminin antibodies. Control immunoprecipitates were carried out using goat pre-immune serum, 1:100. The resin was collected by centrifugation, washed three times by 1 ml phosphate buffered saline, pH 7.4, 250 mM NaCl, plus 0.1% Triton X-100, resuspended in 30 μl SDS gel-loading buffer (50 mM Tris, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol), and boiled for 5 min. After centrifugation, the supernatant was subjected to SDS–PAGE in a 7.5% acrylamide gel. The gel was stained with Coomassie brilliant blue R250. For Western blot analysis, the proteins were electrotransferred from the acrylamide gel to a nitrocellulose membrane (Schleicher & Schuell, Germany). The membrane was blocked in 3% non-fat dried milk in Tris-buffered saline containing 0.05% Tween 20 for 2 h at room temperature, to avoid unspecific binding of the antibody. Polyclonal antibodies against mouse AChE and mouse laminin-1 were diluted at 1:1000 and 1:500, respectively, and incubated with the membrane for 2 h. For detection, horseradish peroxidase-conjugated goat anti-rabbit and anti-rat or rabbit anti-goat antibodies (1:10 000, 1 h) and the ECL chemiluminescence system (Amersham Pharmacia Biotech) were used.

3. Results

3.1. Two-hybrid screen

In order to identify proteins that associate with AChE, we performed a yeast two-hybrid screening of a cDNA library from adult mouse brain, using as bait the core region of AChE, which is common to all AChE molecular forms. Approximately 2×10^6 yeast transformants were screened and plasmid DNAs of the stronger interactions, that were able to grow in selective media (-Trp, -Leu, -His) and displayed a strong β-galactosidase signal, were isolated. 96 colonies were Trp^+, Leu^+, His^+ and $LacZ^+$. Plasmid DNA of the yeast two-hybrid screen positive clones was transformed in *E. coli*, library plasmids were isolated, redundant clones were eliminated by analysis of insert sizes and sequencing. 23 clones that interacted with the AChE fusion protein and not with negative control fusion proteins (e.g., lamin, p53) were identified. One of the 23 remaining clones had 98% homology at the nucleotide sequence level with mouse laminin-1β and interacted specifically with AChE. The clone contains an 898 bp laminin-1β fragment, located closer to the N-terminal region of the chain, and it includes the globular domain IV and part of the cysteine-rich domain III. Fig. 1 shows the signals displayed by AChE and laminin transformants in a β-galactosidase test. To determine the region of AChE involved in interaction, shorter AChE constructs with N- and C-termini deletions were tested. The pVJL11-N263AChE construct was the shortest that could activate the LacZ reporter gene (Fig. 1). The data indicated that in the view of the results of the β-galactosidase filter lift assay, the interaction between these two proteins is moderately strong and that the region containing 240th aa residue to 503th aa residues of AChE is essential for interaction with laminin-1β.

3.2. Co-immunoprecipitation

To verify the yeast two-hybrid interaction of AChE with laminin-1 by an independent technique, we chose the co-immunoprecipitation approach. AChE is a glycosylated extracellular protein that contains three di-sulfide bridges, so that a technique that involves expression in bacteria, like in vitro pull-downs, is not suitable. The HEK-293 cells, being mammalian, allow the correct post-translational modification and expression of AChE.

For co-immunoprecipitation studies, we used mouse brain membrane extracts, or, alternatively, AChE was expressed by transient transfection of pCDNA3-AChE plasmid in HEK-293 cells. The brain extracts expressed high levels of AChE, as

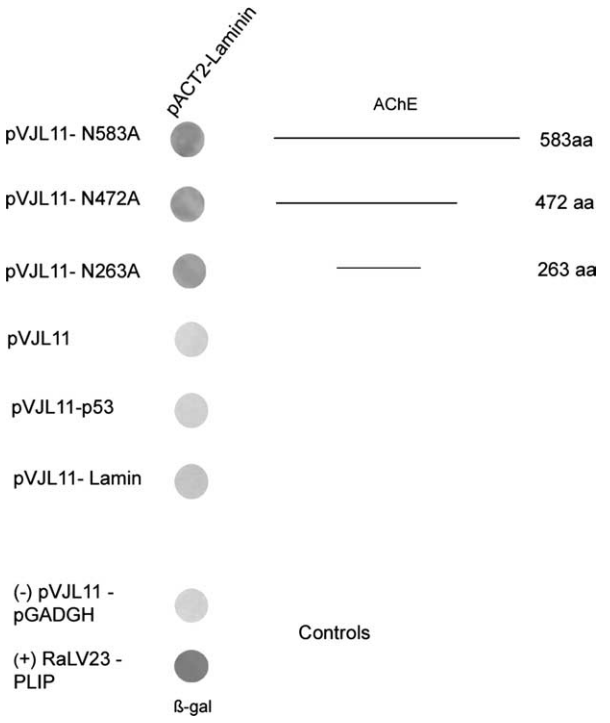

Fig. 1. Yeast two-hybrid interactions of laminin-1β with different AChE constructs. L40 yeast cells were transformed with pair wise combinations of plasmids expressing proteins fused to the LexA binding domain from pVJL11 plasmid and laminin-1β fused to the Gal4 activation domain from the pACT2 plasmid. A β-galactosidase activity assay on yeast colonies was performed. The pVJL11-N583A and pVJL11-N472A constructs activated strongly the LacZ reporter gene when transformed with pACT2-laminin. pVJL11-N263A and pACT2-laminin activated weakly the LacZ reporter gene. pACT2-laminin did not activate LacZ when transformed with pVJL11, pVJL11-p53, or pVJL11-laminC.

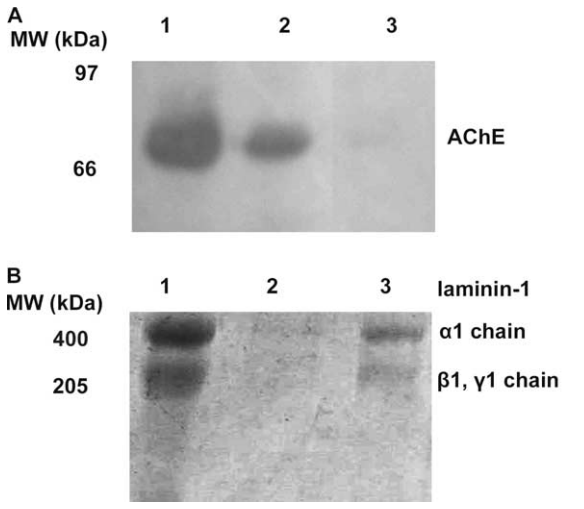

Fig. 2. Co-immunoprecipitation of AChE and laminin-1. (A) AChE co-immunoprecipitates with laminin-1. Proteins were immunoprecipitated with rabbit anti-laminin-1 (lane 1) or goat pre-immune serum (lane 3) from brain membrane extracts containing 1 mg of total protein. After immunoprecipitation they were subjected to immunoblot analysis with an anti-AChE polyclonal antibody. Lane 2 represents a brain extract subjected to immunodetection with a polyclonal anti-AChE antibody. (B) Laminin-1 co-immunoprecipitates with AChE. Proteins were precipitated with pre-immune serum (lane 2) or excess AChE polyclonal antibody (lane 3) from lysates of HEK293 cells containing 1mg of total cellular protein, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-laminin-1 antibody. Lane 1 represents a HEK293 cell lysate containing 20 μg of total cellular protein loaded directly on the gel to test the presence of laminin-1.

Table 1
AChE and laminin-1 form a salt-dependent immunocomplex

Co-immunoprecipitation buffer/salt concentration	Binding of AChE to laminin-1
0.2% Triton X-100, 500 mM NaCl	–
0.2% Triton X-100, 200 mM NaCl	–
0.1% Triton X-100, 100 mM NaCl	–/+
0.1% Triton X-100, 25 mM NaCl	+
0.1% Triton X-100, w/o salt	+

All buffers included 50 mM Tris–HCl, pH 8.0, 1 mM EDTA and 1% protease inhibitors. – represents no binding, '+' binding, '+/–' weak interaction.

measured by the Ellman activity test, but showed low laminin-1 content (Western blot, not shown). Therefore, laminin-1 (Sigma) was added to cell lysates or brain extracts before immunoprecipitation. We then determined whether AChE associates with laminin-1. To this end, proteins from lysates of HEK-293 cells or from brain membrane extract were precipitated with goat pre-immune serum or with a mixture of a polyclonal and a monoclonal antibody to laminin-1. Then, they were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with a polyclonal antibody specific for mouse AChE (Fig. 2A). AChE was detected in brain homogenate (Fig. 2A, lane 1) and in laminin-1 immunoprecipitates (Fig. 2A, lane 2), but not in control immunoprecipitates (Fig. 2A, lane 3). To determine whether laminin-1 co-immunoprecipitates with AChE, proteins from lysates of HEK-293 cells overexpressing AChE were immunoprecipitated with a goat polyclonal anti-mouse AChE and subjected to immunoblot analysis with a polyclonal antibody specific for mouse laminin-1 (Fig. 2B). Laminin-1 (α1 chain 400 kDa corresponding to the upper band, chain β1 and γ1 205 kDa corresponding to the lower band) was detected in cell lysates (Fig. 2B, lane 1), in AChE immunoprecipitates (Fig. 2B, lane 3) but not in control immunoprecipitates (Fig. 2B, lane 2). To optimize the formation of immune complexes, different buffers were used for the step of coupling the antigen to the antibody (see Table 1). High salt concentration of the buffer inhibited the binding. Together, these findings indicated that AChE associates with laminin-1.

4. Discussion

Using a yeast two-hybrid screen, we were able to identify here a fragment of laminin-1β chain as interaction partner of AChE. An obvious concern when using extracellular proteins in a yeast two-hybrid is that these proteins are often glycosylated and contain disulfide bridges. However, AChE was previously successfully expressed in yeast (*Pichia pastoris*), the purified protein having properties comparable with the native AChE, a molecular weight of 67 kDa, suggesting a glycosylation, and exhibited the same inhibition properties [25]. Our co-immunoprecipitation studies confirmed the yeast two-hybrid screen results. Moreover, these studies indicate that the

interaction was also highly dependent on the concentration of NaCl used in the interaction buffer (not shown). High ionic buffers (250–500 mM NaCl) yielded in no binding between these two proteins. These results substantiate earlier indications that an electrostatic mechanism may be involved in binding [20]. Electrostatic complementarity between interacting proteins has been found to be one of the major driving forces for protein complex formation [26]. Examination of the three-dimensional structure of AChE showed the enzyme to be characterized by a marked asymmetric spatial distribution of charged residues. It was proposed that the surface potentials are related to other functions than catalysis, like adhesion [27]. Moreover, AChE is a member of a class of adhesion proteins that, because of their common electrostatic and structural motif, were called electrotactins [27]. Therefore, the high interaction dependence on the salt concentration supports an electrostatic mechanism of this interaction.

The region of AChE essential for the interaction with laminin-1 lacks the catalytic serine 200. That the catalytic triad is not involved in the binding is not unexpected. It was already demonstrated, using specific catalytic site inhibitors, that the developmental functions of AChE, e.g., neurite outgrowth and differentiation, are independent of its enzymatic activity [1–3]. The interaction site on laminin-1 is located on the N-terminal region of the β-chain and includes the globular domain IV and a part of the cysteine-rich domain III. The globular domains are important in laminin self-assembly and also for the binding to extracellular matrix components [28]. To date, the globular domain IV has no specific function. It is therefore a most significant finding that this domain may interact with AChE.

Here, we propose a model for a novel developmental function of AChE, based on its interaction with laminin-1. The most likely mechanism through which AChE sends signals into the cell is the following: during development, the early secreted AChE binds to the laminin chain β1. Laminin-1β is expressed very early and ubiquitously during development. The laminin-1 mRNA is expressed at the two or four cells stage [29], the protein becomes detectable at the morula stage of the mouse [30]. Laminin-1 binds to integrin receptors, a class of cell adhesion molecules known to be involved in neuronal migration during CNS development, most likely by mediating the adhesive interactions between neurons and radial glial fibers. It is well established that cell adhesion molecules such as integrins play an important role in building and maintaining synaptic structure during CNS development [31]. By binding to integrins, laminin-1 could thus send signals intra-neuronally. Possibly, AChE will be also able to signal using this mechanism. The intracellular effect of AChE could lead to changes in signaling pathways and to remodeling of actin cytoskeletal structures.

Further studies, including the co-immunoprecipitation of an AChE–laminin–integrin complex, or disruption of the interaction between the two proteins, will help to validate this model. At any rate, the search for proteins interacting with AChE represents an important tool for further defining its developmental functions.

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