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Amyloid precursor-like protein 1 accumulates in neuritic plaques in Alzheimer's disease

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Abstract The Alzheimer's disease (AD) β-amyloid precursor protein (APP) and the amyloid precursor-like protein 1 (APLP1) and 2 (APLP2) are members of a superfamily of proteins that appear functionally related. Although APLPs are highly homologous to APP in the N- and C-terminal domains, they lack the βA4/amyloid peptide, i.e., the main constituent of neuritic plaques in AD. To assess a potential role of APLP1 in AD, we have determined its immunohistochemical distribution in human hippocampal formation, a structure which is strongly affected in AD, and compared it with APP immunoreactivity. There was a considerable overlap of APP and APLP1 regional expression patterns. Significant APLP1 immunoreactivity was observed in neuritic plaques. Large pyramidal neurons of the subiculum showed an accumulation of APLP1 protein in their dendritic compartment. Some astrocytes elicited perinuclear APLP1 staining, but this was observed in both AD and control brains. These findings raise the possibility that APLP1 may contribute to the pathogenesis of AD-associated neurodegeneration.

Key words Alzheimer's disease · Amyloid precursor-like protein 1 · Amyloid precursor protein · Plaques

Introduction

Alzheimer's disease (AD) is characterized by abundant neuritic plaques, neurofibrillary tangles in the cerebral cortex and hippocampus, and by congophilic angiopathy. Tangles are predominantly composed of abnormally phosphorylated tau protein. βA4/β-amyloid or Aβ peptide is the major constituent of both neuritic plaques and vascular deposits in the walls of cerebral and meningeal arterioles and arteries. The amyloid deposits are mainly composed of aggregates of a peptide with 39–43 residues [8, 19], which is proteolytically derived from the large β-amyloid precursor protein (APP) [13, 28]. APP has the structure of a type I integral membrane glycoprotein with a major extracellular component and a short cytoplasmic tail [13, 24]. Alternative splicing of exons 7, 8 and 15 gives rise to eight isoforms, which are expressed in a cell-type specific manner. The discovery of amyloid precursor-like proteins (APLPs) related to APP classifies APP as a member of a multigene family [21, 26, 27, 30, 31]. The βA4/β-amyloid domain, however, is unique to APP and absent in other members of the superfamily.

Studies on the physicochemical properties of APP and its novel APLP homologues indicated that these molecules represent a family of zinc-modulated, heparin-binding proteins [4] that mediate cell–cell and cell–substratum interactions [20, 22]. More recently, a report on human APLP 1 (APLP1) revealed that APLP1 is subjected to *N*- and *O*-glycosylation and proteolytic cleavage, suggesting that all soluble derivatives of APP and APLPs are secreted and mature through a secretory pathway [21, 26, 32]. Interestingly, APLP1 elicits an exclusively neural expression profile. Neurons and glial cells have been found to harbor APLP1 mRNA [16]. APLP1 protein has been shown in postsynaptic densities. Its levels appear to be increased during neurogenesis [14, 16].

In this study we have analyzed the subcellular, cellular and regional distribution of APLP1 protein in the hippocampal formation, a region heavily affected in AD brain. To discriminate APP from the highly homologous APLP1, an antipeptide antibody was employed which specifically recognizes APLP1. None of our antisera showed cross-reactivity to APP, a prerequisite for detecting significant differences in the expression of APP and APLP1.

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Materials and methods

Antiserum to APLP1

A polyclonal antiserum against APLP1 was raised in New Zealand white rabbits with an unconjugated peptide from APLP1 [amino acids (aa) 499–557]. Briefly, primary immunization was carried out with a total of 200 µg peptide in Freund's incomplete and Freund's complete adjuvant. Two subcutaneous sites were injected. Boost immunizations were carried out after approximately 30-day intervals, and terminal bleeds were collected approximately 10 days after the fourth boost. Serum was prepared immediately and stored at –20° C.

Detection of APLP1 protein in transfected cells

COS7 cells were stably transfected with the APLP1 cDNA and analyzed for APLP1 expression as described [21]. For immunoprecipitation, whole cell lysates were used and the proteins were precipitated by the addition of 20 µl of the corresponding antiserum together with approximately 3 mg of protein A-Sepharose (Sigma) prepared in 1 M phosphate-buffered saline (PBS). For detection of precipitated proteins, horseradish peroxidase-conjugated rabbit anti-mouse IgG (Promega) and the ECL detection system (Amersham) were used.

Immunoblotting analysis of APLP1 protein in human hippocampus

For immunoblotting, frozen hippocampal tissue from normal human brain was resuspended in lysis buffer on ice [50 mM TRIS pH 8.0, 100 mM EDTA, 10% Nonidet P40, 100 mM phenylmethylsulfonyl fluoride (PMSF)]. Samples of normal brain without clinical or neuropathological signs of a brain disorder were obtained at autopsy. After boiling and addition of 3 × Laemmli sample buffer, proteins were electrophoretically separated on a 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS) and blotted onto a nitrocellulose filter. After washing with PBS, the filter was incubated in blocking buffer overnight (5% non-fat dry milk, 0.02% Tween-20 in PBS), and rinsed with antibody dilution buffer (0.1% bovine serum albumin and 0.02% NaN_3 in PBS). The polyclonal antiserum to APLP1, pAb 25104 (diluted 1:100), was added and the filter was incubated at room temperature for 2 h. After subsequent incubation with a mouse anti-rabbit antibody, goat anti-mouse antibody and alkaline phosphatase complex, protein bands were visualized using an alkaline phosphatase-driven color reaction with 4-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) as substrate.

Immunohistochemical analysis of human hippocampal specimens

Archival formalin-fixed and paraffin-embedded tissue blocks of human hippocampus from six patients (between 58 and 90 years of age) with a histologically confirmed diagnosis of AD and from three age-matched normal human brains without neurological disorder were retrieved from the files of the Department of Neuropathology, University of Bonn Medical Center.

For immunohistochemical analysis, we used an APLP1-specific polyclonal rabbit antiserum against residues 499–557 diluted 1:50 in 1 M PBS. APP staining was achieved using monoclonal antibody 8E5 to aa 444–592 of full-length APP (diluted 1:1000), which recognizes the three major APP isoforms (APP 695, 751 and 770; Athena Neuroscience). To enhance the immunoreactivity, sections were heated in a microwave oven [5] in 0.01 M citrate buffer pH 6.0. Treatment in 88% formic acid did not improve staining. Sections were treated with normal goat serum prior to the addition of polyclonal antibodies to block nonspecific binding sites. This was followed by an overnight incubation with the primary antibody at room temperature. Staining was visualized using the avidin-biotin-peroxidase complex method, with a Vectastain kit (Vector Laboratories, Burlingame, USA). Counterstaining was carried out with hematoxylin. Control experiments were performed (1) without the primary antibody, (2) with an unrelated rat IgG fraction, and (3) with peptide preabsorption of the primary antiserum prior to binding (overnight incubation with the corresponding peptide, 1–10 mg/ml).

Results

The polyclonal antiserum to APLP1 is highly specific

The specificity of the anti-APLP1 antiserum was verified using a combination of immunoprecipitation and Western blotting analysis. COS7 cell lysates stably transfected with an APLP1 construct carrying a myc epitope at the C terminus, or mock-transfected cell lysates were subjected either to immunoprecipitation using polyclonal antiserum raised against the C terminus of APP (anti-APP-CT) or by the anti-APLP1 serum (pAb 25104). Since the C termini of APP and APLP1 are highly homologous at the protein level (Fig. 1), both endogenous APP and ectopic APLP1 were precipitated (Fig. 2A, lanes 2 and 4). The immunocomplexes were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a nitrocellulose membrane and probed with the monoclonal anti-myc antibody 9E10 (Fig. 2A). Both antisera precipitated APLP1 (Fig. 2A, lanes 2 and 4), whereas no protein was detected in mock-transfected cells (Fig. 2A, lanes 1 and 3). On SDS-polyacrylamide gels APLP1 appeared as a protein doublet of approximately 84–86 kDa, corresponding to the immature *N*-glycosylated forms, and 95–98 kDa, corresponding to the mature *N*-and *O*-glycosylated forms [21]. APLP1 was selectively immunoprecipitated by the anti-APLP1 antiserum since a non-homologous region was selected to generate the antiserum. This notion was verified by stripping the antibodies used for detection from the nitrocellulose membrane and reprobing the filter

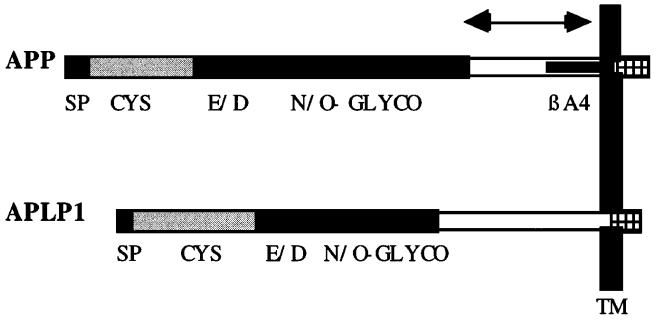

Fig. 1 Schematic drawing of APP and APLP1 (not drawn to scale). The domain organization is illustrated. Regions of high homology are indicated by the same pattern. The extracellular domain adjacent to the TM region displays the highest extent of sequence variations (*arrow*). Note that the βA4 domain is not present in APLP1 (*APP* amyloid precursor protein, *APLP1* amyloid precursor-like protein 1, *SP* signal peptide domain, *CYS* conserved cysteine-rich domain, *E/D* less conserved acidic domain, *N/O-GLYCO N*- and *O*-glycosylation domain, *βA4* βA4/β-amyloid domain of APP, *TM* transmembrane domain)

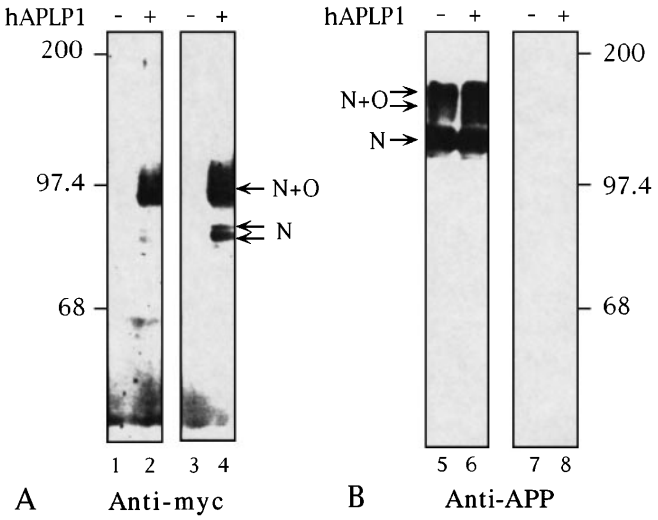

Fig. 2A, **B** The anti-APLP1 antiserum does not cross-react with APP. Proteins from lysed COS7 cells stably transfected either with the empty expression vector (–) or with the cDNA encoding a C-terminal myc epitope-tagged APLP1 variant (+) were subjected to immunoprecipitation either with an antiserum raised against the entire highly homologous intracellular C terminus of APP and APLP1 (anti-APP-CT; *lanes 1, 2, 5, 6*) or with the APLP1-specific antiserum pAb 25104 (*lanes 3, 4, 7, 8*). The immunoprecipitated proteins were separated by 8% SDS-PAGE and analyzed by immunoblotting. **A** myc-tagged APLP1 was detected with the monoclonal anti-myc antibody 9E10. **B** APP was detected with the monoclonal antibody 22C11. The *arrows* indicate the position of the *N*- and *O*-glycosylated forms of APP and APLP1, respectively. The *numbers* indicate the positions of the molecular weight markers

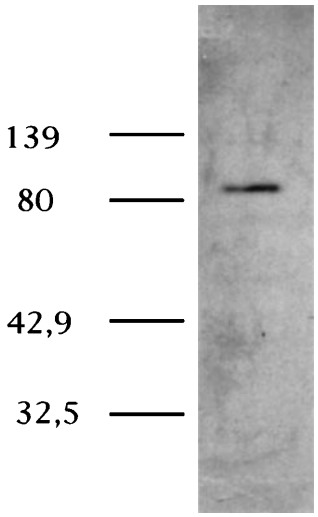

Fig. 3 Western blot analysis of human hippocampus with APLP1 antiserum. Human hippocampus lysate (25 µg total protein) was electrophoretically separated and blotted onto a nitrocellulose membrane. The polyclonal APLP1 antiserum reacts specifically with a band segregating with human APLP1 protein. The *numbers* indicate the positions of the molecular weight markers

with the anti-APP monoclonal antibody 22C11 to detect endogenous APP (Fig. 2B). As expected, anti-APP-CT immunoprecipitated APP (Fig. 2B, lanes 5 and 6), but not the anti-APLP1 immunoprecipitated material contained endogenous APP (Fig. 2B, lanes 7 and 8). Moreover, the APLP1 antiserum recognized only one protein band of approximately 90 kDa using Western blot analysis of human hippocampus (Fig. 3). The anti-APLP1 antiserum did not show any cross-reactivity with APLP2 (not shown).

APLP1 immunoreactivity in normal and AD hippocampal formation

In normal human hippocampus, APLP1 immunostaining was found in most if not all neurons of the hippocampus, dentate gyrus, subiculum and entorhinal cortex. APLP1 immunoreactivity was abundant in neuronal cytoplasm and in some neuritic processes of large pyramidal neurons. Astrocytes revealed a weaker perinuclear staining. In AD brains, there was a similar pattern with significant immunoreactivity in virtually all neurons. A subset of senile plaques harbored APLP1 deposits. In addition, dendrites of pyramidal neurons showed abundant immunoreactivity in normal and AD hippocampi. APLP1 precipitates were present in dystrophic neurites of plaques in the CA1 segment, subiculum and entorhinal cortex of AD brains (Fig. 4). The astrocytic APLP1 signal was identical in controls and AD specimens. Blood vessels did not stain significantly, even in AD cases with established congophilic angiopathy (not shown).

APP immunoreactivity in normal and AD hippocampal formation

APP immunoreactivity has been studied previously in detail in brains of normal aged, AD and Down's syndrome individuals [1, 2, 10–12], as well as in non-human primates [17, 25]. For the detection of APP, we used monoclonal antibody 8E5, which recognizes the three major isoforms of APP 695, 751 and 770 (Athena Neuroscience). Normal hippocampal pyramidal neurons in all fields of the Ammon's horn (CA), in subicular areas and in granule cells of the dentate gyrus were strongly immunoreactive. In the pyramidal layer, immunoreactivity for APP was detected in the soma of virtually all neurons. In the hippocampus of AD patients, the same neuronal population showed expression of APP, but in a more patchy distribution. In addition, dystrophic neurites in neuritic plaques were strongly positive with 8E5. Notably, we did not detect prominent APP accumulation in apical dendrites of pyramidal neurons within the CA1, subiculum or entorhinal cortex.

Discussion

To elucidate a potential involvement of APLP1 in the pathogenesis of AD, we raised a polyclonal antiserum that specifically recognizes APLP1. The antiserum to APLP1 was analyzed by immunoprecipitation, Western blot analysis and immunohistochemical methods. APLP1 belongs to a family of APP-related proteins which also includes APLP2. All three proteins share homologous protein domains. It was, therefore, necessary to use an antipeptide antibody to a subtype-specific epitope of APLP1. The detailed characterization of the antipeptide serum used in this study clearly indicates that it specifically recognizes APLP1.

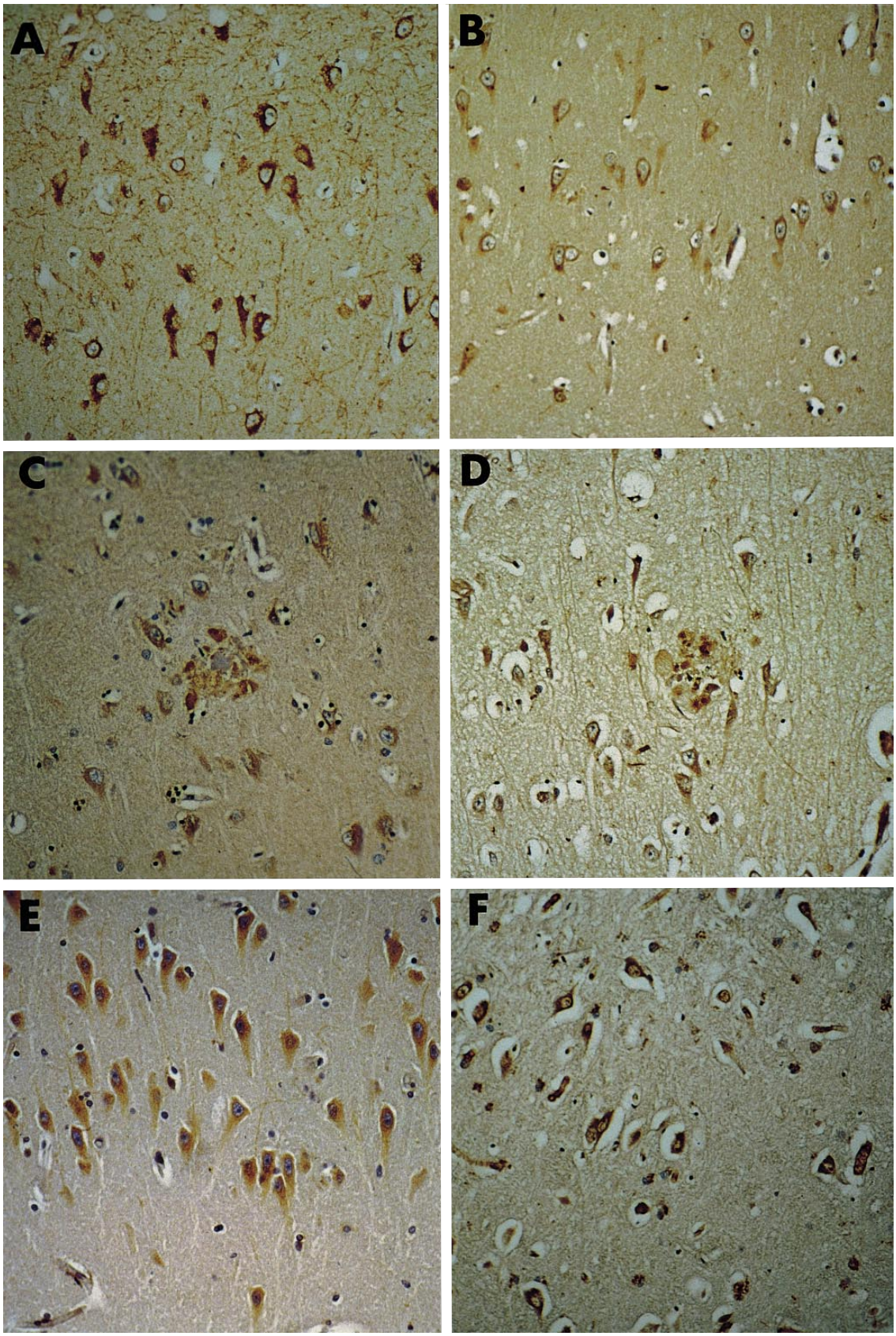

Fig. 4 Immunohistochemical analysis of normal (**A**, **B**) and Alzheimer's disease (AD) hippocampal formation (**C**–**F**) incubated with anti-APLP1 antiserum (**B**, **D**, **F**) or with anti-APP antibody (**A**, **C**, **E**). **A** Ubiquitous APP expression in the soma of pyramidal neurons. **B** APLP1 immunoreactivity of large pyramidal neurons in CA1. **C** APP immunoreactivity in a neuritic plaque. **D** APLP1 immunoreactivity of a neuritic plaque. **E** APP expression in the soma of pyramidal neurons in CA1 of an AD brain. **F** APLP1 immunoreactivity in large neurons and astrocytes of the CA4 field (AD patient). **A**–**F** × 50

APP is thought to play a major role in the pathogenesis of AD. However, the mechanisms leading to the severe neurodegeneration in AD brains are poorly understood. Evidence for direct β-amyloid toxicity in vivo has not been demonstrated convincingly [7, 9, 18], despite several hints from in vitro experiments. Neuritic plaques with APP as a major component have recently received considerable attention in AD [1, 2, 10–12]. Since APP travels to the synapse via the rapid axonal flow [15] and synapses contain a high amount of transmembranous APP [23], there is a growing body of evidence that abnormal APP transport, resulting in synaptic impairment and neuronal death, may occur during early stages of AD. Studies of human brain biopsy samples [29] and of aged canine and primate specimens [17, 33, 34] have shown that dystrophic neurites precede deposition of β-amyloid in the formation of neuritic plaques. More recently, retroviral transfer of human APP cDNAs into fetal rat brain transplants has revealed that plaque induction can occur in the absence of β-amyloid deposition [3].

Other members of the APP gene family may also play a role in AD. Considering the high degree of homology among the different members of the APP-related family, some of their functions could be redundant. APP knockout mice support this notion because only mild phenotypes with reactive gliosis and locomotion abnormalities were observed [35]. Recently, increased levels of APLP2 have been reported in AD brains. APLP2 has been detected in both neuritic plaques and dystrophic neurites [6]. There is only limited information available on the potential functions of APLP1. The detection of immunoreactive APLP1 in a subpopulation of neuritic plaques and in prominent pyramidal dendrites of AD-affected brains would be compatible with the hypothesis that APLP1 plays a role in the development of AD neuropathological changes. Factors accounting for the preferential dendritic accumulation of the molecule include an impairment of dendritic APLP1 transport, APLP1 deposition as a consequence of the neurodegenerative alterations and abnormal processing of the APLP1 molecule. Additional studies will be required to characterize its potential role.

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