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Amyloid β Protein and Its 3-kDa Fragment Are Present in the Axoplasm Fraction of the White Matter in Human Brain

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Production of the soluble amyloid β-protein (Aβ) precedes abnormal accumulation of Aβ amyloid in the brains of subjects with Alzheimer's disease. To determine the cellular source and generating mechanisms of soluble Aβ in the human brain, we separated an axoplasm fraction from the cerebral white matter and analyzed it. The axoplasm fraction contained secretory isoforms of β-amyloid precursor protein (APP) and 11.5 kDa Aβ-bearing carboxyl-terminal fragments (CTFs) of APP. Furthermore, soluble 4 kDa Aβ and 3 kDa fragments of Aβ (p3) were obtained from the axoplasm fraction. These results suggest that amyloidogenic 4 kDa Aβ is intracellularly produced in cerebral neurons and carried through the axons in human brain.

The 39–43 amino acid polypeptide Aβ, derived from a set of much larger transmembrane glycoprotein precursors termed APP (1), is a major component of amyloid deposits in the brains of patients with Alzheimer's disease (AD) and Down's syndrome (DS) (2–4). Identification of familial AD with mutations in the APP gene (5) provides strong evidence for Aβ having a critical role in the development of AD. It is now well established that Aβ is produced in soluble form even during normal cellular metabolism and exists in human biological fluids (6–8). This means that Aβ is generated as a soluble protein but turns into an insoluble polymer in the development of Aβ deposition (9). Thus, generation and release of soluble Aβ still remain initial and important events in the development of AD pathology. The actual cellular source and generating mechanisms of soluble Aβ in the human brain, however, have yet to be determined. The restricted deposition of Aβ only in the brain and the finding that APP undergoes fast axonal transport (10–12) support a neuronal origin for Aβ deposits. On the basis of these observations, we consider that the major source of soluble Aβ in the human brain is the cerebral neurons, in which intracellular trafficking of APP is involved in Aβ production. To test this hypothesis we examined axoplasm fraction derived from cerebral white matter of the human brains and now report that soluble Aβ and its precursors exist in the axons of the human brains.

MATERIALS AND METHODS

Cases. Brain tissues used for the present study were obtained from a patient with DS (55-year-old female), who had been shown to have numerous senile plaques and neurofibrillary tangles in the cerebral cortex (13), and from a non-demented control subject (78-year-old female) with no amyloid deposit in the brain.

Antibodies. Antibodies used for immunoblotting of APP and its carboxyl-terminal fragments (CTFs) were raised to purified synthetic polypeptides corresponding to subsequences of APP; N-terminal T97 (residues 18–38 of APP) and C-terminal R37 (residues 681–695 of APP695) (14). The monoclonal antibody 6E10 raised to Aβ 1–17 (15) was also employed. Antibodies used for immunoprecipitation of Aβ and p3 peptide were raised to synthetic Aβ1–28, Aβ1–40 and Aβ1-42 (16).

Preparation of axoplasm fractions, microscopic examination and immunoblotting. Myelinated axons were isolated and purified from the cerebral white matter of human brain by the axonal flotation method (17). Frontal and temporal white matter of DS brain (5.0 g) (flash frozen at autopsy and stored at −80°C) was gently Dounce-homogenized in 9 vols. of 10

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mM phosphate buffer at pH 6.5/0.1 M NaCl/1 mM EDTA/0.85 M sucrose (PBS/S). The homogenate was centrifuged at 10,700g for 30 mim (HIMAC centrifuge, HITACHI), which caused myelinated axons to float to the top of the tubes in the form of paste-like material that could be separated from the supernatant (supA fraction). The float and pellet were collected separately, fixed in 1% osmium tetroxide, then embedded in epoxy resin. Transverse sections 1 μm thick were cut and stained with 1% toluidine blue for microscopic examination.

The float consisting of myelinated axons was intensively rehomogenized in a Potter-type homogenizer in 5 vols. of the PBS/S buffer to which 1% Triton X-100 was added, then centrifuged at 10,700g for 1 hour. After removal of the myelin float, the supernatant (axoplasm fraction) was pooled. To detect APP isoforms and CTFs of APP, the axoplasm fraction was analyzed by SDS-PAGE and immunoblotting as described previously (12,18). We used a 7.5% Laemmli gel and 10–16.5% gradient gel (Tris-Tricine system) for separation of APP isoforms and CTFs, respectively.

Immunoprecipitation of Aβ-related peptides and immunoblotting. One ml aliquots of the axoplasm and supA fractions were made 1× in RIPA (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% Nonidet P−40, 0.5% sodium deoxycholate, 0.1% SDS) buffer. Twenty μl of each capturing antibody (anti-Aβ1–28, anti-Aβ1–40, anti-Aβ1–42 antiserum) and the RIPA-washed protein A-agarose slurry (25 μl) was added, and the whole incubated at 4°C for 24 hours. The immunoprecipitated proteins were separated by 10/20% Tris-Tricine SDS-PAGE and analyzed by immunoblotting using anti-Aβ1–42 antiserum or antibody 6E10.

RESULTS AND DISCUSSION

We attempted to purify the axoplasm from the cerebral white matter of the DS and control brains. The float fraction separated by the axonal flotation method (17) was almost entirely composed of fragments of myelinated axons (Fig. 1A). The nuclei of the glial cells and fragments of the vessels were pelleted in the PBS/S (Fig. 1B). Rehomogenization of the float fraction with PBS/S buffer containing 1% Triton X-100 (PBS/S/T) made the axoplasmic material soluble, after which we could, by centrifugation, separate the supernatant axoplasm fraction from the floating myelin. This axoplasm fraction contained several APP isoforms and 9–12 kDa CTFs of APP (Figure 2A). Immunoreactive APP isoforms had molecular weights of 93–97, 105–112, 115–125, and 130–145 kDa. The two former weights and the two latter ones respectively were the same as those of the secretory and full-length APPs obtained from the cerebral gray matter of human brains reported previously (19). Together with the fact that APP undergoes fast axonal transport (10–12), the existence of secretory APP isoforms in the axoplasm fraction suggests that some full-length APPs are processed to secretory APPs while being transported anterogradely in the axon. Furthermore,

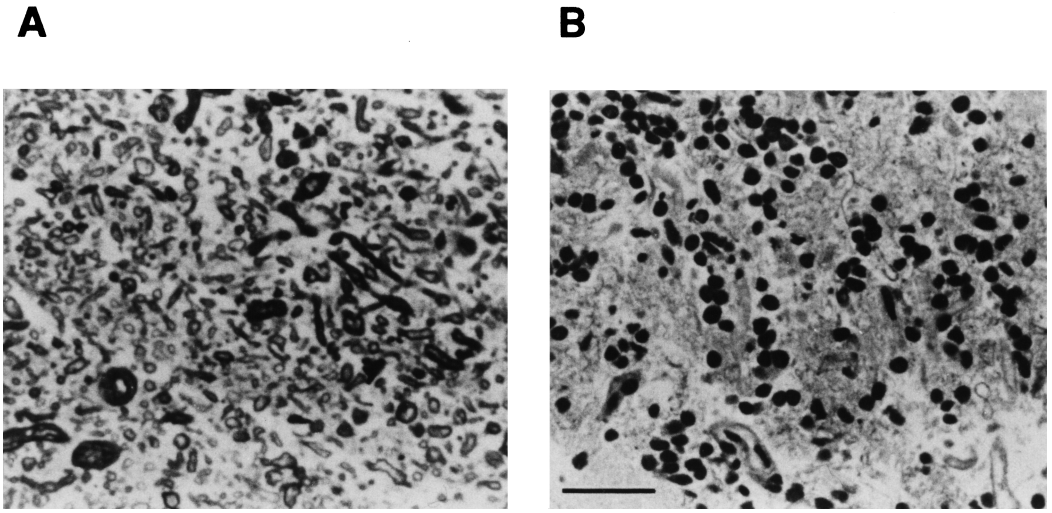

FIG. 1. Preparation and characterization of the fraction composed of axoplasm. Photomicrographs of the floated axons (A) and the pellet (B) obtained by the axonal flotation method from the frontal white matter of the DS brain. Myelinated axons were floated by centrifugation after homogenization of the white matter in phosphate buffer containing 0.85 M sucrose. Scale bar, 20 μm.

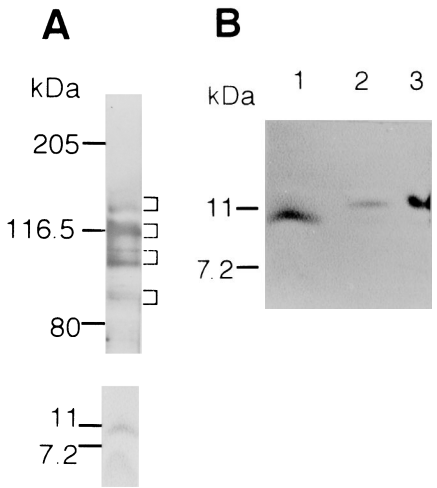

FIG. 2. (A) Immunoblot analysis of axoplasm fraction derived from the DS brains with antisera to T97 (upper) and R37 (lower). (B) Identification of the CTFs of APP in the axoplasm derived from the DS brain by immunoblotting. The axoplasm fraction (lanes 1,2) and APP-C100 (20) (lane 3) were immunoblotted with an antiserum to R37 (lanes 1,3) and an antibody 6E10 (lanes 2). The immunoblotted CTFs had molecular weights of 9–12 kDa (lane 1) and 11.5 kDa (lane 2).

some secretory APP isoforms are known to leave behind Aβ-bearing amyloidogenic CTFs when processed from the full-length APP (12,18). We obtained such 11.5 kDa CTF that reacted with 6E10 and comigrated with APP-C100 peptide, a peptide composed of carboxyl-terminal 100 residues of APP (20), in the axoplasm fraction (Fig. 2B). This amyloidogenic CTF in the axon is therefore considered to be produced by β-secretase cleavage of full-length APP transported anterogradely.

Several reports have shown that 4 kDa Aβ is produced from Aβ-bearing CTFs (8,21,22). The existence of Aβ-bearing CTFs in the axon suggests that 4 kDa Aβ could be produced from these CTFs while they are transported through the axon. In the axoplasm fraction of the DS brain, we identified a 4 kDa and a 3 kDa peptide immunoprecipitated by an anti-Aβ1–42 antiserum, previously shown to bind 4 kDa Aβ (6,16), and by two other antisera raised to synthetic Aβ1–28 and Aβ1-40 peptide (Fig. 3A). The 4 kDa peptide was obtained from the axoplasm fraction but not from the supA fraction which contains substance of white matter other than the axons (Fig. 3B). Monoclonal antibody 6E10 raised to synthetic Aβ1–17 detected the 4 kDa but not the 3 kDa peptide (Fig. 3B). Furthermore, the 4 and 3 kDa peptides comigrated respectively with synthetic Aβ1–40 and Aβ17–42 (Fig. 3C). These findings indicate that the 4 and 3 kDa peptides from the axoplasm fraction respectively were soluble Aβ (6,8) and p3 peptide beginning at residue 17 of Aβ (6,23). These peptides were also present in the axoplasm from the control brain (Fig. 3C). It should be noted that the p3 peptide was more abundant than the 4 kDa Aβ in the brains examined. Densitometric determination showed that the axoplasm fraction of the DS brain contained 4- to 6-fold the p3 peptide than 4 kDa Aβ.

Our analysis of human cerebral white matter showed the presence of the 4 kDa Aβ and p3 peptides intracellularly in cerebral neurons. This is the first evidence that cerebral neurons in human brains contain amyloidogenic soluble Aβ peptide intracellularly. We also emphasize that Aβ peptide was obtained from the axoplasm of the white matter in which no amyloid deposition had been detected, indicative that there is intracellular trafficking of the soluble Aβ through the axons in the brain. There are two possible routes of intracellular trafficking for this axonal transport of Aβ; anterograde and retrograde Aβ trafficking. The former and the latter respectively imply the production of soluble Aβ in secreting (19,24) and endocytotic/transcytotic vesicles (25,26). In the

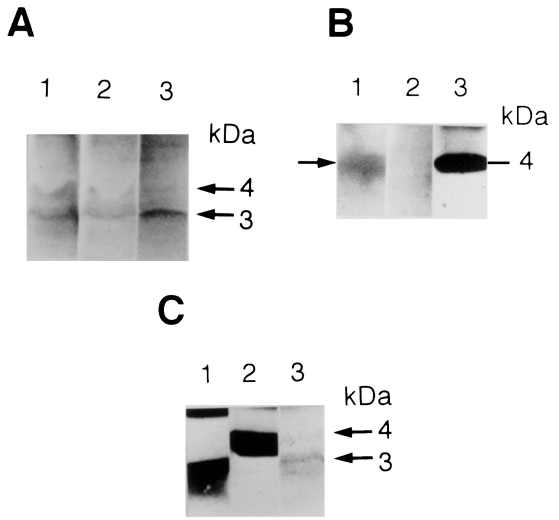

FIG. 3. (A) Immunoprecipitation and immunoblotting of 4 kDa Aβ and p3 peptide in the axoplasm fraction of the DS brain. These peptides were immunoprecipitated with anti-Aβ1-42 antiserum (lane 1), anti-Aβ1-28 antiserum (lane 2) or anti-Aβ1-40 antiserum (lane 3), then electrophoresed and immunoblotted with anti-Aβ1-42 antiserum. The 4 kDa Aβ and 3 kDa p3 peptide are indicated (arrows). (B) Identification of 4 kDa Aβ by immunoprecipitation and immunoblotting in the axoplasm fraction (lane 1) but not in the supA fraction (lane 2) derived from the DS brain. The 4 kDa Aβ was immunoblotted with antibody 6E10 (lane 1, arrow). Synthetic Aβ1-42 (lane 3) was used as a control. (C) Identification of the 4 kDa Aβ and p3 peptide in the axoplasm fraction of the control brain (lane 3). Synthetic Aβ17-42 (lane 1) and Aβ1-40 (lane 2) were used as controls. Immunoprecipitation and immunoblotting were performed with anti-Aβ1-42 antiserum. The 4 kDa Aβ and 3 kDa p3 peptide are indicated (arrows).

secreting vesicles Aβ may be produced from Aβ-bearing CTFs that are generated when full-length APPs are cleaved by β-secretase. Alternatively, in the endosomes there are full-length APPs and/or Aβ-bearing CTFs re-internalized from the presynaptic nerve terminals (26) and targeted through the axons again to the cell surface by transcytosis (26), during which APPs and CTFs could be processed to Aβ within retrogradely transported endosomes.

In the axoplasm fraction, we detected Aβ-bearing CTFs produced from anterogradely transported full-length APP (18,27). We therefore speculate that Aβ is produced from these Aβ-bearing CTFs in the secreting vesicles. Previous reports suggested that Aβ is generated in an acidic cellular compartment other than the lysosome; e.g., the early endosomes and late Golgi (23,25). The trans-Golgi and/or post-Golgi compartment were shown to be the secretory cleavage sites of APP (28,29). Thus, we speculate that late Golgi and/or Golgi-derived secretory vesicles are more likely to be the compartment where soluble 4 kDa Aβ is produced. The recently identified *S182* gene on chromosome 14 (30) encodes a protein (presenilin) that shares substantial homology with the protein of *Caenorhabditis elegans*, SPE-4 (30). SPE-4 is a constituent of Golgi-derived organelles of spermatocytes and essential for correct intracellular transport of various proteins during meiotic cell division in *C. elgans* sperm. This fact supports our speculation that the Golgi-derived vesicles and the intraneuronal trafficking of APP could be involved in the production of 4 kDa Aβ.

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