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C-Terminal Fragments of α- and β-Tubulin Form Amyloid Fibrils *in Vitro* and Associate with Amyloid Deposits of Familial Cerebral Amyloid Angiopathy, British Type

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Familial amyloidosis, British type, is an autosomal dominant disease characterized by progressive dementia, spastic paralysis and ataxia. The identity of the accumulating amyloid is not known, thus preventing the definitive classification of the disease. Biochemical methods were used to characterize the nature of the amyloid deposits from the brain tissue of one individual who died with this disease. The purified tissue material was subjected to trypsin digestion and subsequent N-terminal sequence analysis. Major tryptic fragments yielded the sequences VGINYQPPTVVPGGDLAK, FDLMYAK, GLTVPEL and GYLTVAAVFR, which are all tryptic fragments of the C-termini of human tubulin subunits α and β. Synthetic peptides based on the sequences of these fragments formed amyloid fibrils *in vitro* fitting the characteristic definition of amyloid. These findings suggest that the C-terminal fragments of both α- and β-tubulin are closely associated to the amyloid deposits of familial amyloidosis, British type.

The major neuropathological features of familial amyloidosis, British type (FAB) are extensive deposition of amyloid in small cerebral arteries and arterioles, mainly non-neuritic parenchymal amyloid plaques, and ischemic white matter damage (1,2). The age of onset varies from 40 to 57 years, while the age of death is between 50 and 66 years. Clinical features include dementia, spasticity and ataxia (1). A recent immunohistochemically based study suggested that the amyloid from these patients is not related to any of the sixteen known amyloid proteins (3). Positive immunohistological results were obtained with anti-amyloid P component, and anti-apolipoproteins E and J. These are all amyloid-associated proteins that are found in all biochemical types of amyloidoses (4). In addition, a faint immunoreactivity was found to cystatin C. A mutated form of cystatin C is associated with hereditary cerebral hemorrhage, Icelandic type (HCHWA-I) (5). However, normal cystatin C is known to be co-deposited with cerebral amyloid proteins, such as amyloid β (6).

In this study, we used a classical method for amyloid purification followed by micropreparative gel electrophoresis and microbore high performance liquid chromatography (HPLC) to biochemically characterize FAB (7). Amino terminal sequence of tryptic fragments of the isolated amyloid material identified the C-terminal region of both α- and β-tubulin. In order to study the amyloidogenicity of these tubulin fragments, we synthesized homologous peptides and show that these peptides can form amyloid-like fibrils *in vitro.*

MATERIALS AND METHODS

Materials. The reagents for Tris-Tricine SDS-PAGE were purchased from Bio-Rad Inc. (Richmond, CA). The prestained low molecular weight proteins markers were purchased from Amersham Life Science (Arlington Heights, IL). Sequencing grade trypsin was purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). The Micro-Prep separation

Abbreviations: ATP, adenosine triphosphate; GTP, guanosine triphosphate; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PHF, paired helical filaments; 4-VP, 4-vinyl pyridine.

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unit is based on a modification of the Bio-Rad Mini Protean II 2D Cell as described (8). Synthetic peptides of tubulin (MLSNTTAIAEAWARL, positions 377 to 391 of tubulin α-chain; GYLTVAAVFRG, amino acids 310 to 320 of tubulin β-chain) were custom synthesized by Bio-Synthesis, Inc. (TX, USA).

Purification procedure. Human brain tissue of case V41 (1) stored at −70°C, was subjected to repeated homogenization in 0.15 M NaCl (pH 7.2) containing protease inhibitors (200μg/ml PMSF, 1μg/ml leupeptin, 1μg/ml pepstatin and 1μg/ml aprotinin), followed by centrifugation at 70,000 × g for 1 hour at 4°C until the OD_{280} of the supernatant reached the level of less than 0.1. The remaining pellet was resuspended in 50 mM Tris-HCl pH 7.0, containing 10 mM $CaCl_2$ and 3 mM NaN_3, incubated at room temperature overnight with collagenase Type IV (Sigma) 1:100 (w/w) and centrifuged as above. The remaining pellet was washed twice with 0.15 NaCl in water, suspended in 1% SDS in water and delipidated in methanol/chloroform (1:2). The phase between both layers was recovered and used for further experiments.

Micropreparative gel electrophoresis. 30 μg of total protein from the partially purified fraction was mixed with the original Laemmli sample buffer and applied to the Micro-Prep apparatus on a high-resolution Tris-Tricine SDS-PAGE without any further treatment. A 1:100 diluted lower gel buffer (final concentration: 15 mM Tris-HCl, pH 8.8) was used as the elution solvent. The electrophoresis was performed as described (8).

HPLC analysis. Microbore reverse-phase HPLC was performed on an Applied Biosystems 130A protein/peptide separation system (Applied Biosystems/Perkin Elmer, Foster City, CA). Samples were analyzed on either a Vydac C-18 or C-4 microbore reverse phase column (218TP52 or 214TP52, respectively; Vydac Separations Group, Hercules, CA) using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid in water (0-60% in 50 min or 5-65% in 50 min).

Enzymatic cleavage. The HPLC purified material was first subjected to reduction and alkylation, using the following method: 20μg of purified FAB amyloid was dissolved in 50 μl of 0.5 M Tris-HCl pH 7.5, containing 6M guanidine-HCl/2 mM EDTA, followed by the addition of 5 μl of 1.4 M dithiotreitol (DTT). After 10 min incubation at room temperature, 1 μl of 4-vinylpyridine was added (9) followed by an additional 5 μl of DTT after 10 min incubation under the same conditions. The reaction was stopped after 20 min by the addition of 1μl trifluoroacetic acid. For tryptic digestion approximately 10 μg of the HPLC purified, reduced and alkylated FAB amyloid was dissolved in 0.1 M ammonium bicarbonate pH 8.2, and incubated with TPCK-Trypsin (1/20; w/w) at 37°C for 12 hours. For pepsin digestion, approximately 5 μg of the HPLC purified FAB amyloid was dissolved in 0.2% HCl (pH 2.4) and incubated with pepsin (1/1; w/w) at RT for 12 hours. The resulting peptide fragments were separated by microbore HPLC.

Protein sequence analysis. Sequence analysis was performed by automated Edman degradation using an Applied Biosystems 477A sequencer and the resulting phenylthiohydantoin (PTH) derivates were identified using an on-line PTH derivative analyzer (Model 120A, Applied Biosystems).

Immunohistochemistry. Parraffin embedded brain tissue sections from case V41 (1) were immunoreacted using a polyclonal anti-tubulin antibody (BT-578, Biomedical Tech. Inc., Stoughton, MA), which was raised against intact tubulin. Standard methods were used (3).

Amyloid fibril formation in vitro. HPLC purified synthetic peptides of α- and β- subunit of tubulin were incubated at room temperature for 96 h at 2 mg/ml concentration in 0.1 M Tris-HCl, pH 7.3. After incubation, fibril formation was assessed by electron microscopy (10) and Congo red staining.

RESULTS

Amyloid fibrils were isolated from the brain of one individual with FAB. After NaCl extraction, collagenase digestion and delipidation, Congo red positive, partially purified amyloid material was applied onto a micropreparative Tris-Tricine SDS-PAGE as described in Materials and Methods. Two major fragments with apparent molecular weights of 3 kDa and 6 kDa, respectively, were detected by Coomassie blue and silver staining after re-analyzing the eluted fractions on a Tris-Tricine SDS-PAGE. These major fragments were further purified on microbore reverse phase HPLC (Vydac C-4), resulting in two partially overlapping peaks as shown in Figure 1. Attempts to identify the nature of these peaks by direct N-terminal sequence analysis failed. In addition, both fractions were unaffected by enzymatic digestion with trypsin prior to reduction and alkylation. Tryptic digestion of the reduced and alkylated material followed by microbore reverse phase HPLC (Vydac C-18) yielded several peaks which were subjected to automated N-terminal sequence analysis. Four major sequences were identified: VGINYQPPTVVPGGDLAK and FDLMYAK, corresponding to residues 353-370 and 395-401 of α-tubulin, respectively; and GLTVPEL and GYLTVAAVFR identified as amino acids 283–289 and 310-319 of the β- tubulin, respectively (Figure 2). No other sequences in addition to tubulin could be detected.

In order to verify that the C-terminal fragments of tubulin are not present in other amyloidoses but are specific for the British type, we used Alzheimer's amyloid and amyloid of the Finnish type

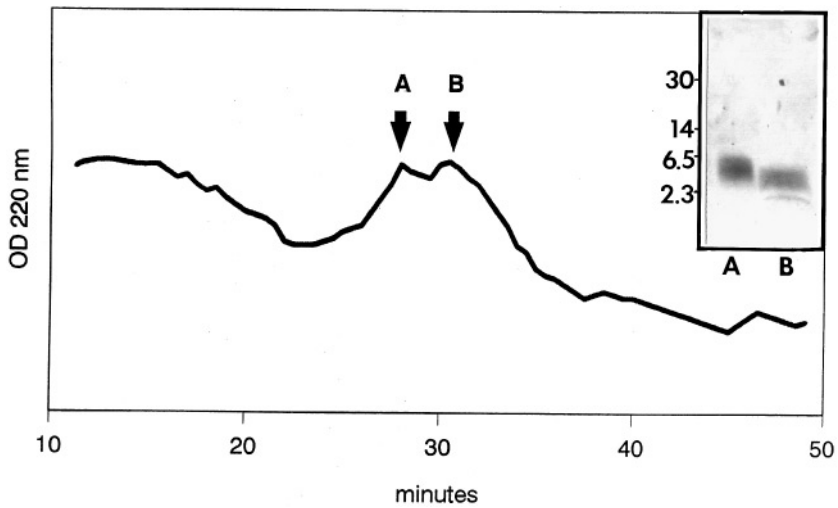

FIG. 1. Shows the microbore reverse phase HPLC analysis of the micropreparative purified fragments of FAB amyloid (seen in the inset) using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid in water (5–65% in 50 minutes); the column was a Vydac C-4; 214 TP 52. The inset in the top right corner shows a silver stain of a Tris-Tricine SDS-PAGE gel that was loaded with partially purified amyloid material after collecting the eluting fractions from the micropreparative device. A 6kDa band and a 3kDa band are seen in lanes A and B, respectively. Molecular weights are given in kDa to the left of the inset.

as controls. We were not able to co-purify any tubulin fragments in the purified control amyloid tissue material, indicating that the presence of the C-terminal fragment of tubulin is specific for the FAB tissue. However, the polyclonal anti-tubulin antibody, which was raised to the whole tubulin protein, did not label the FAB amyloid deposits, suggested that the intact tubulin is not present in the deposits or that the epitope which this antibody recognizes is hidden.

Synthetic peptides based on both, the sequences identified by N-terminal analysis and the apparent molecular mass of the FAB amyloid (Figure 2), formed amyloid-like fibrils *in vitro* as shown in the electron micrograph in Figure 3. In addition, the fibrils showed the characteristic apple-green birifringence of amyloid, after Congo Red staining (not shown).

DISCUSSION

We have recently shown that antibodies against the known amyloid proteins, as well as to other proteins such as tau and neurofilament all failed to react with the plaques of the British amyloid (3).

α-tubulin:

353 370

338 KRSIQFVDWCPTGFK**VGINYQPPTVVPGGDLAK**VQRAVC

395 401

377 MLSNTTAIAEAWARLDHK**FDLMYAK**RAFVHWYVGEGMEEGE

β-tubulin:

283 289

271 APLTSRGSQQYR**GLTVPEL**TQQMFDAKNMMAACDPRHGR

310 319

310 **GYLTVAAVFR**GRGRMSMKEVDEQMLSVQSKN

FIG. 2. Shows a partial amino acid sequence of α and β tubulin. The peptides which were identified by amino terminal sequencing of the tryptically digested, purified FAB amyloid are shown in the larger, bold type. The size of the fragments shown is hypothetical and only reflects the approximate location and size of the putative amyloid fragment. The synthetic peptides used for *in vitro* amyloid fibril formation studies are underlined. Both peptides are included in the potential amyloid fragments and show the most probable amyloid "core" sequence to form β-pleated sheet structure as calculated according to Chou and Fasman (18).

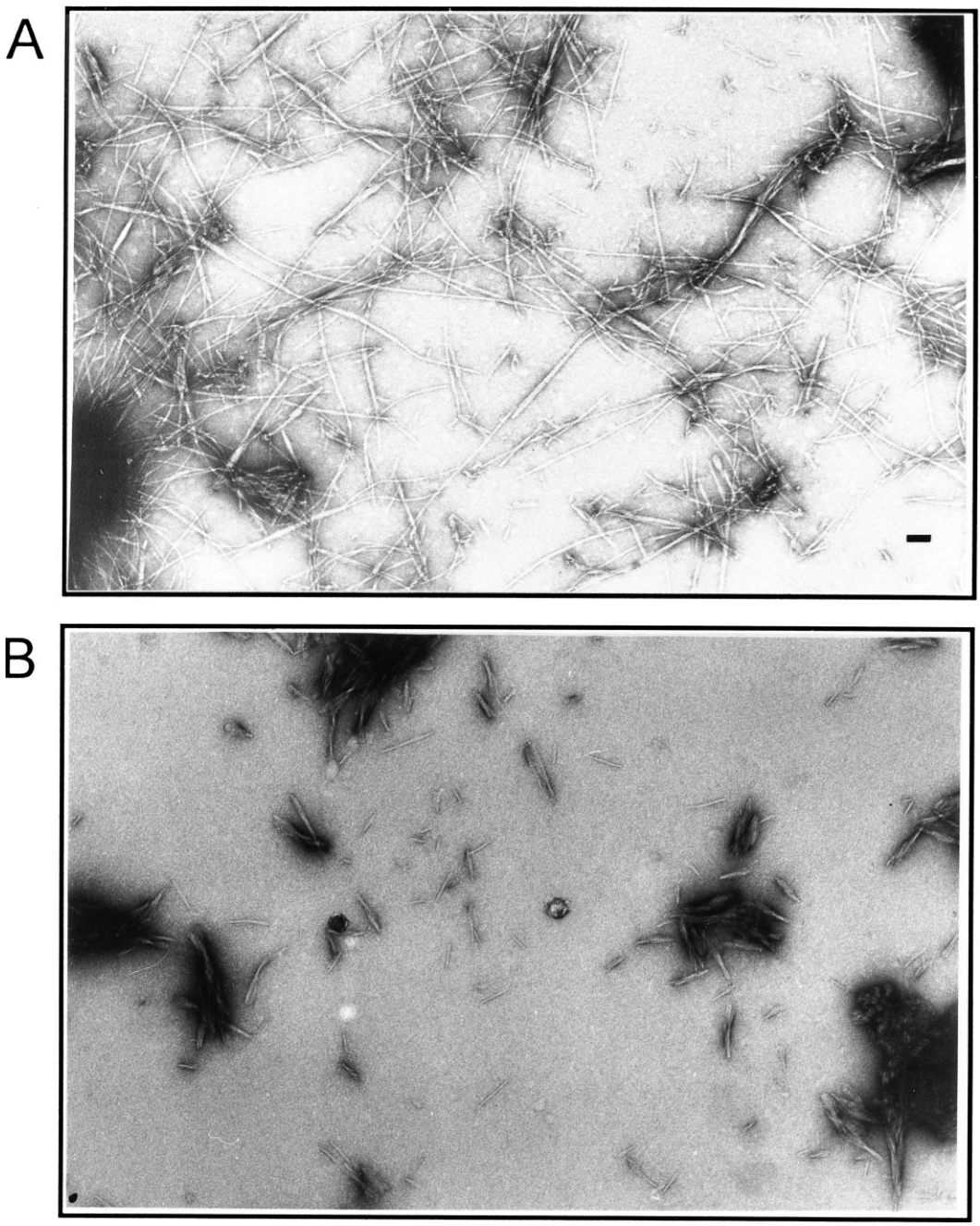

FIG. 3. Electron micrographs of amyloid fibril formation by the synthetic peptides of tubulin: A) α-tubulin peptide; B) β-tubulin peptide (bar = 100 nm).

In agreement with these results we now show that none of these proteins are found in the biochemically purified British amyloid. The only peptide sequences identified were localized to the C-terminal region of α- and β-tubulin. Accumulation of these fragments seems to be specific for the British amyloid, as indicated by the fact that we did not find these peptides in two other biochemical types of amyloid deposits, using the same purification procedures. However, we

cannot completely rule out the possibility that the C-terminal fragments of tubulin that we isolated are non-specifically associated with the FAB amyloid. To assess for such a possibility, further extractions from FAB patients would need to be done, but such brains are currently unavailable.

Moreover, the C-terminal domains of both tubulin subunits contain sequences which favor β-pleated sheet structure, thus raising the possibility for these fragments to be amyloidogenic (Figure 2). The ability of these tubulin fragments, which contain the tryptic peptides identified in this study, formed amyloid fibrils *in vitro* (Figure 3), thus providing strong evidence for the possible accumulation of identical or similar sequences in the amyloid deposits of cerebral amyloid angiopathy, British type. Although, the anti-tubulin antibody which we used failed to label the amyloid, this may only indicate that the intact tubulin is not present in the lesions.

Tubulin has a molecular mass of approximately 50 kDa containing $\sim$450 amino acid residues with small variations depending on the isoform (11). The α and β subunits of tubulin make up microtubule fibrils (11). Tubulin consists of multiple functional domains with distinct properties which are mediated by the binding of numerous agents, e.g. ATP, GTP (12), microtubule associated proteins (13,14), heat-shock proteins (15), kinesin (14) and colchicine (16). Modifications of tubulin have been implicated in cellular aging, although the function of these post-translational modifications is still unclear (17). Our data indicate that carboxy-terminal fragments of tubulin are present in FAB amyloid deposits but cannot determine whether they play a primary or a secondary role in the development of the disease.

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