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The C-terminal Region of Human Tau Protein with Ability of Filament Formation

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Tau protein is one of the microtubule-associated proteins in the mammalian brain. In Alzheimer's disease, tau protein is immobilized in the somatodendritic compartment of certain nerve cells, where it forms a part of the paired helical filament (PHF). To understand the role of tau protein in the formation of PHF, a recombinant human tau protein expressed in *Escherichia coli* and five synthetic peptide fragments (peptide 1 to peptide 5), corresponding to the C-terminal region of tau protein, were prepared and their ability in self-assembly to form filamentous structures was examined. The recombinant human tau protein formed short rod-like structures in 0.1 M MES buffer containing 1 mM $MgCl_2$, while a synthetic peptide fragment 1 containing 55 amino acid residues could assemble into a lot of long filamentous structures in water and particularly twisted helical structures in 0.1 M MES buffer containing 1 mM $MgCl_2$. This suggests that the C-terminal region possesses a filament-forming ability and may be related to the formation of the helical structure by providing a powerful filament-forming driving force.

Tau as a microtubule-associated protein promotes microtubule assembly and stability in the nervous system. Tau protein has been co-purified with tubulin from cells of many different species and has been found almost exclusively in axons in the brain (Weingarten et al., 1975; Cleveland et al., 1977). The primary structure of tau protein as predicted from molecular cloning is a stretch of 31 or 32 amino acids that is repeated three to four times in the C-terminal half of the molecule. Additional isoforms exist which contain 29 or 58 amino acid insertions in the N-terminal region in conjunction with three or four repeats, giving rise in humans to a total of six different isoforms identified from full-length cDNA clones (Goedert et al., 1989a). Multiple isoforms are generated from a single gene through alternative RNA splicing, leading to the stage and cell type specific expression of different forms (Goedert et al., 1988, 1989a, b; Himmler, 1989; Himmler et al., 1989; Kanai et al., 1989).

In Alzheimer's disease, tau protein is immobilized in the somatodendritic compartment of certain nerve cells, where it forms a part of the paired helical filament (PHF), the major constituent of the neurofibrillary tangle (Grundke-Iqbal et al., 1986a; Goedert et al., 1988; Wischik et al., 1988; Nieto et al., 1990). Moreover, the level of total tau is about eightfold higher in Alzheimer's disease brain than in control cases (Khatoon et al., 1992) and the repeat region of tau lies in the protease resistant core of the PHF (Jakes et al., 1991). Much of current Alzheimer research is aimed at determining the nature of the PHF deposits in brain. To understand the function of tau both in normal and pathological conditions, it would be required to examine its structure and self-assembly.

In the present study, a recombinant human tau protein and its synthetic peptide fragments corresponding to the C-terminal region were prepared to investigate what portion of the tau protein was important for the formation of PHF-like structures.

Materials and Methods

Purification of the recombinant human tau protein

The preparation of a human tau cDNA clone and expression in *E. coli* were previously reported (Chung et al., 1994). In brief, a human tau cDNA from human brain cDNA library (cloned in λZAPII) was amplified by PCR and cloned in a plasmid DNA (pUC18). A cloned human tau cDNA was identified by DNA sequencing and subcloned into pAR3040 to be expressed in *E. coli*.

For the purification of the recombinant human tau protein, overnight cultures of *E. coli* cells transformed with parental (pAR3040) or recombinant (pSC15) plasmid were diluted 1:10 in fresh NZCYM medium (Sambrook et al., 1989) and grown for 1 h at 37℃. After addition of IPTG to 1 mM and further culture for

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3 h the cells were collected by centrifugation. Expression of recombinant tau protein was checked by resuspending the pellet from 1 ml culture in 100 μl sample buffer (12.5 mM Tris base, pH 6.8, 20% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.001% Bromophenol Blue), boiling for 5 min, and running on a 10% SDS-PAGE followed by staining with Coomassie blue. The highly soluble recombinant human tau protein was isolated on the basis of its resistance to boiling (Weingarten et al., 1975; Hagestedt et al., 1989) and then purified by column chromatography (Cleveland et al., 1977). In order to isolate a recombinant tau protein, the cell lysate in buffer (8% sucrose, 10 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.5% Triton X-100, and lysozyme to 1 mg/ml) containing 20 μl of DNase I (1 mg/ml) per gram of *E. coli* was boiled for 10 min and centrifuged at 27,000 g for 30 min at 4℃ and the supernatant was collected. The protein solution was dialyzed against buffer A (0.1 M MES, pH 6.5, 1 mM dithiothreitol) and applied on a phosphocellulose column pre-equilibrated with buffer A. The column was washed with buffer A and tau protein was then eluted stepwise with 0.1 M, 0.2 M, 0.3 M, and 0.4 M NaCl in buffer A. The tau fractions were pooled, dialyzed against water, and lyophilized. The lyophilized material was reconstituted in water at a concentration of 7 mg/ml and stored at -20℃ until use.

Synthetic peptides

Five peptide fragments (Table 1 and Fig. 1) corresponding to the C-terminal region of an isoform of human tau protein (Chung et al., 1994) were synthesized by solid-phase methodology (Merrifield, 1963) on a peptide synthesizer (Biosearch Model 9500) to elucidate the role of C-terminal region. The resulting peptides were cleaved from the resin with hydrogen fluoride by the Low-High procedure (Tam et al., 1983) and purified by successive chromatography on a gel filtration column (Sephadex G-50F or G-25F), ion exchange column (CM52 or DE52), reverse-phase column (Shim-pack ODS-PREP), and desalting column (Sephadex G-10). The purity of each peptide fragment was confirmed by thin layer chromatography, analytical high pressure chromatography, and amino acid analysis. Peptide sequence was determined by a Gas-Phase Protein Sequencer (Applied Biosystems Model 470A). The molecular weight of peptide fragment was confirmed by a FAB mass spectrometer (JEOL HX100).

N-terminal amino acid sequence determination

Crude protein samples were separated by SDS-PAGE and electro-blotted onto siliconized glass fiber membrane according to Eckerskorn et al. (1988). Protein band corresponding to recombinant tau was cut out of the membrane, washed in distilled water, and dried. Its N-terminal amino acid sequence was determined using Applied Biosystems 477A peptide sequencer.

Table 1. Synthetic peptide fragments used in this study

Peptides	Sequences of amino acid*	Location	Mr
Fragment 1	Ala-Lys-Thr-Asp-His-Gly-Ala-Glu-Ile-Val-Tyr-Lys-Ser-Pro-Val-Val-Ser-Gly-Asp-Thr-Ser-Pro-Arg-His-Leu-Ser-Asn-Val-Ser-Ser-Thr-Gly-Ser-Ile-Asp-Met-Val-Asp-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp-Glu-Val-Ser-Ala-Ser-Leu-Ala-Lys	326~380	5641.24
Fragment 2	Arg-Glu-Asn-Ala-Lys-Ala-Lys-Thr-Asp-His-Gly-Ala-Glu-Ile-Val	321~335	1638.81
Fragment 3	Tyr-Lys-Ser-Pro-Val-Val-Ser-Gly-Asp-Thr-Ser-Pro-Arg-His-Leu	336~350	1642.84
Fragment 4	Ser-Asn-Val-Ser-Ser-Thr-Gly-Ser-Ile-Asp-Met-Val-Asp	351~363	1311.39
Fragment 5	Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp-Glu-Val-Ser-Ala-Ser-Leu-Ala-Lys	364~380	1700.92

*The amino acid sequences were arised from C-terminal region of the recombinant human tau protein (Chung et al., 1994).

Self-assembly and transmission electron microscopy

The recombinant tau proteins and synthetic peptide fragments were dissolved in water or 0.1 M MES buffer (pH 6.5) containing $MgCl_2$ or NaCl at a concentration of 4 mg/ml and incubated for 42 h at 37℃ or 1 h at 60℃. For negatively stained electron microscopic observation, a drop of incubated sample was placed on a carbon-coated collodion grid and 2% uranyl acetate was applied to the grid. Excess stain was removed with Whatman No. 1 filter paper. The grid was allowed to dry and then immediately examined under a JEOL JEM-1200EX electron microscope at 80 kV.

Results and Discussion

Most of insoluble and denatured proteins of *E. coli* cell extract after heat treatment were precipitated and could be removed by centrifugation. The soluble protein was applied to phosphocellulose column and eluted with buffer A containing NaCl. Each fraction was collected and subjected to SDS-PAGE followed by staining with Coomassie blue. The 0.3 M NaCl wash contained the recombinant tau protein. The N-terminal amino acid sequence of the purified recombinant tau protein was determined. The sequence was identical with the expected N-terminal amino acid sequence of the previously reported tau protein. Fig. 2 illustrates the purified recombinant tau protein (molecular weight of approximately 52 kD) on SDS-PAGE.

The present study examined if the recombinant tau

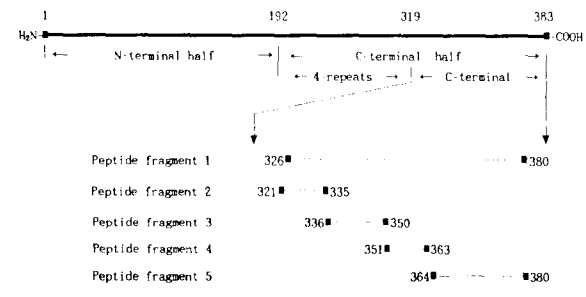

Fig. 1. Schematic diagram of a recombinant human tau protein.

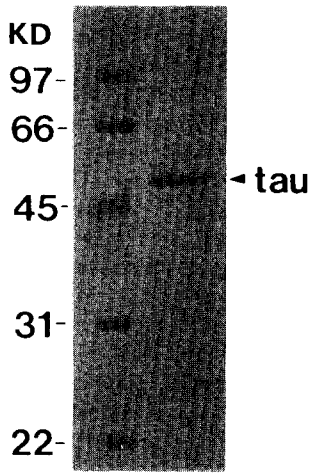

Fig. 2. SDS-polyacrylamide gel electrophoretic analysis of the purified recombinant human tau protein expressed in *E. coli*. The left lane, standard molecular weight marker proteins (Bio-Rad); the right lane, human tau protein purified from phosphocellulose chromatography.

protein and its synthetic peptides were capable of self-assembly in water, 0.1 M MES buffer containing 1 mM $MgCl_2$ (Montejo de Garcini and Avila, 1987) or 10 mM $MgCl_2$, and 0.1 M MES buffer containing 10 mM NaCl or 100 mM NaCl, respectively. In the case of recombinant tau protein no structure was detected in water, but rod-like structures were formed in any other given conditions except for water (Fig. 3). Since tau is a short rod-like molecule (56.1 ± 14.1 nm long) according to Hirokawa et al. (1988), the shorter ones (around 70 nm in length) may be monomers and the longer ones (around 140 nm in length) may be dimers. In the case of synthetic peptide fragments except for peptide fragment 1, no filamentous structure was formed in any conditions.

In the case of peptide fragment 1, a lot of very long filaments were formed after incubation in water at both 37℃ (Fig. 4) and 60℃. The peptide fragment also formed filaments in 0.1 M MES buffer without any salts

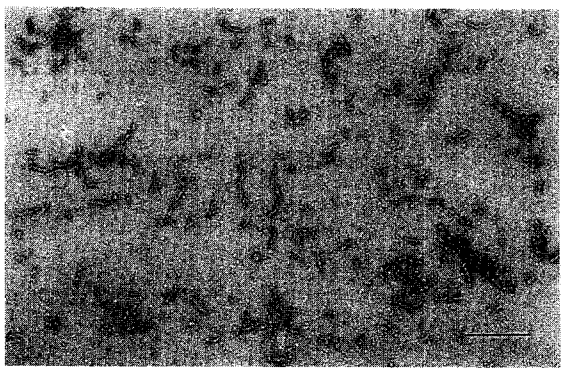

Fig. 3. The rod-like structures formed from purified recombinant human tau protein. The lyophilized recombinant human tau protein was dissolved in 0.1 M MES buffer, pH 6.5, containing 1 mM $MgCl_2$ and incubated at 37℃ for 42 h. Scale bar=200 nm.

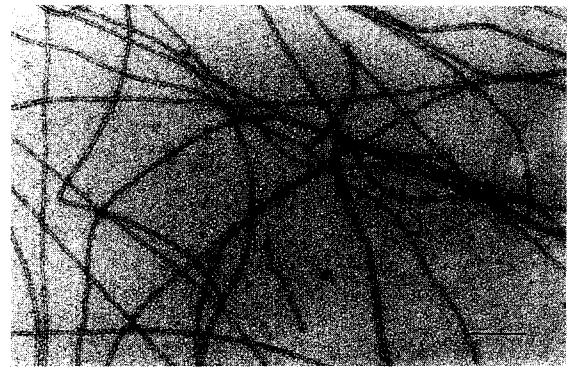

Fig. 4. Long filamentous structures formed from peptide fragment 1 (326 Ala-380 Lys). For filament formation, peptide fragment 1 was dissolved in water, incubated at 37℃ for 42 h. Scale bar=200 nm.

at 37℃, and 0.1 M MES buffer containing 1 mM $MgCl_2$ at 37℃, while a lot of extensively aggregated form of short filaments were formed in 0.1 M MES buffer containing either 1 mM and 10 mM $MgCl_2$, or 10 mM and 100 mM NaCl at 60℃. There was no detectable filamentous structure in 0.1 M MES buffer containing 10 mM and 100 mM NaCl at 37℃.

According to previous electron microscopic studies of Kidd (1964) and Wisniewski et al. (1976), PHF appeared to consist of two filaments wound helically around one another, with a longitudinal spacing between crossovers of 65-80 nm and a width modulated between 27-34 nm at the widest part and 10-15 nm at the narrowest. The dimensions reported for negatively stained PHF was much smaller, ranging from 15-22 nm at the widest to 6-8 nm at the narrowest (Wisniewski et al., 1984). In the present study, some twisted helical filaments which are, however, slightly different from the Alzheimer's PHF, were formed sporadically only in the 0.1 M MES buffer containing 1 mM $MgCl_2$ at 37℃ (Fig. 5).

These results suggest that the higher temperature and salts (NaCl and $MgCl_2$) do not particularly affect the formation of very long filamentous structures, while Mg^{2+} rather than Na^{+} may be involved in the formation of twisted helical structure as illustrated in Fig. 5.

It seems that the recombinant human tau protein cannot form the Alzheimer's PHF in the above given conditions *in vitro*. It may be related with the abnormally phosphorylated status of tau protein, which is a major part of Alzheimer's PHF, as previously described by others (Grundke-Iqbal et al., 1986b; Lee et al., 1991; Biernat et al., 1992). There may be some biochemical differences between recombinant human tau protein and normal tau protein in the human brain, since *E. coli* cells are devoid of post-translational modification systems present in human cells. However, the recombinant human tau protein may have a very similar structure to the normal human tau protein in the context that the recombinant human tau proteins are fully biologically active as assessed by their ability

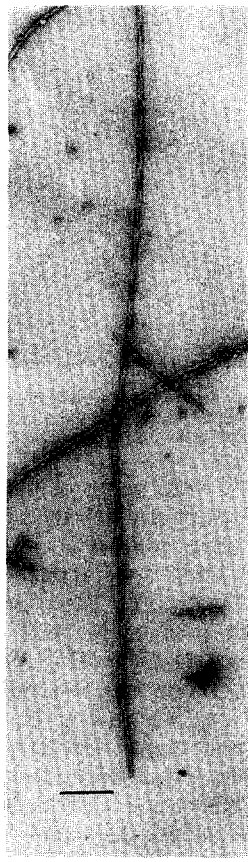

Fig. 5. Helical filamentous structures formed from peptide fragment 1. Peptide fragment 1 was dissolved in 0.1 M MES buffer containing 1 mM $MgCl_2$, incubated at 37℃ for 42 h for filament formation. Some of the filament particles showed a tendency of self-association leading to twisted helical filaments similar to PHFs. Scale bar=200 nm.

to promote microtubule assembly (Goedert and Jakes, 1990). In fact, PHF structure does exist only in the Alzheimer brain, but not in the normal brain. This implies that PHF-like structure cannot be formed by the recombinant human tau protein if three-dimensional structures of recombinant tau and normal human tau are almost identical regardless of the level of phosphorylation. Therefore, other factor(s) may be involved in the formation of PHF. One of the possible factors includes the abnormal phosphorylation of tau protein. Phosphorylation study on tau protein is in progress.

It is unclear whether whole tau protein is integrated into PHF structure or a part of tau protein is. It has been indicated that tau fragments isolated from PHF are restricted to the C-terminal domain of tau (Brion et al., 1991). In some ways, a part of tau protein may play an important role for the formation of backbone of PHF structure. From this point of view, the synthetic peptide fragment 1 (55 amino acid residues) which corresponds to the C-terminal region of human tau protein may offer a driving force to make PHF structure, because peptide fragment 1 possesses a powerful filament-forming ability to make a lot of very long filaments within a short time in water and 0.1 M MES buffer containing 1 mM $MgCl_2$ as illustrated in Figs. 4 and 5.

In the case of blood clotting (Jackson and Nemerson, 1980), a highly soluble fibrinogen molecule is digested by thrombin to make fibrin monomer and fibrinopeptides, after which fibrin monomers become much less soluble than the parent fibrinogen molecules. Fibrin monomers spontaneously associate to form fibrin, which has the form of long, insoluble fibers. One of the possible explanations for their self-association is that release of fibrinopeptides by thrombin gives fibrin monomers a different surface-charge pattern, leading to their specific aggregation (Stryer, 1981). Likewise, we could suppose that a highly soluble full-length tau molecule can be cleaved by a certain proteolytic enzyme which may occur with aging to make some fragments. One of the cleaved fragments (e.g., C-terminal region consisting of the peptide fragment 1 sequences) might then be associated and integrated to provide filamentous structure of PHF. The peptide fragment 1 is interesting in that the N-terminal half is rich in positively charged groups, while the C-terminal half is rich in negatively charged groups. It may be related to the filament formation by providing an electrostatic attraction between molecules.

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