Carboxyl-Terminal Fragments of β-Amyloid Precursor Protein Bind to Microtubules and the Associated Protein Tau

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Alzheimer’s disease is a neurodegenerative disorder characterized by protein deposits in intracellular and extracellular spaces in the brain. The intraneuronal deposits are formed by neurofibrillary tangles composed mainly of abnormally phosphorylated tau, a microtubule-associated protein, whereas the major constituent of the amyloid deposited extracellularly in the brain parenchyma and vessel walls is amyloid β-protein (Aβ). The proteolytic processing of the β-amyloid precursor protein (βPP) results in the generation of a complex set of carboxyl-terminal peptides that contain Aβ. In this study, we have used fusion proteins containing carboxyl-terminal fragments of βPP to investigate the association of βPP with cellular components. We demonstrate that specific domains within the carboxyl end of βPP contain binding sites for cytoskeletal components; one, within residues 1 to 28 of Aβ, binds directly to tubulin, and the second one, within sequences carboxyl-terminal to Aβ, binds tau and tubulin. We propose that the two neuropathological hallmarks of Alzheimer’s disease, Aβ deposition and neurofibrillary tangles, represent the residual of a disrupted βPP-tubulin-tau complex. (Am J Pathol 1997, 151:265-271)

β-amyloid precursor protein (βPP) resembles an integral, glycosylated cell surface receptor with a single membrane-spanning region and a short carboxyl-terminal cytoplasmic domain.1–4 Alternative proteolytic processing pathways of βPP result in the generation of multiple fragments including amyloid β-protein (Aβ) and a complex set of carboxyl-terminal peptides that contain Aβ.5–7 Aβ is composed of 28 residues of the putative extracellular domain and 11 to 15 residues of the transmembrane domain of βPP.8 Although mutations resulting in amino acid substitutions in βPP have been found in some families with early-onset Alzheimer’s disease (AD),9–14 there may be additional pathogenic factors, as 1) genetic studies revealed that AD is a heterogeneous disorder,15 2) amyloid fibrils are usually processing products of a normal precursor protein,16 3) a soluble Aβ peptide is constitutively formed and secreted in human cerebrospinal fluid and serum and into culture media of normal cells,17–19 and 4) immunohistochemical studies have identified several proteins within the amyloid deposits.20 Although Aβ and tau are the major constituents of senile plaques and neurofibrillary tangles (NFTs), respectively,8,21 additional minor components were identified within these lesions. Immunohistochemical studies demonstrated that extraneuronal NFTs are immunoreactive with anti-Aβ antibodies.22 An antibody raised to the 20 carboxyl-terminal amino acids of βPP-labeled neurofilaments in axons, paired helical filaments (PHFs) in NFTs, and straight filaments in neurofil threads.23 Moreover, it was suggested that the full-length βPP is a minor component of intraneuronal and extracellular NFTs, based on immunoreactivity with antisera directed to different parts of βPP, including its amino terminus.24,25 Amino acid sequence analysis revealed that tubulin α-4 is a minor component of amyloid fibrils isolated from brain tissue of patients with familial AD.26 In addition, it was shown that βPP immunoreactivity is associated with the cytoskeleton of type I astrocytes.27 βPP is bound to the detergent-insoluble cytoskeleton in both neuronal and glial...
Table 1. Primers Used to Generate the GST-βPP Fusion Proteins

<table>
<thead>
<tr>
<th>GST-βPP fusion proteins</th>
<th>Upstream and downstream primers</th>
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<tbody>
<tr>
<td>βPP&lt;sub&gt;672-770&lt;/sub&gt;</td>
<td>5' CCT CCA TGG ATG CAG AAT TCC GAC AT 3'</td>
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<tr>
<td>βPP&lt;sub&gt;672-713&lt;/sub&gt;</td>
<td>5' CCC TCG AGC TAG TTC TGC ATC ATC TC 3'</td>
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<tr>
<td>βPP&lt;sub&gt;672-699&lt;/sub&gt;</td>
<td>5' CCT CCA TGG ATG CAG AAT TCC GAC AT 3'</td>
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<tr>
<td>βPP&lt;sub&gt;713-770&lt;/sub&gt;</td>
<td>5' CTC CAT GGC GAC AGT GAT C1T CAT C 3'</td>
</tr>
<tr>
<td>βPP&lt;sub&gt;724-770&lt;/sub&gt;</td>
<td>5' CTC CAT GGC CAA GAA GAA ACA GTA CAC A 3'</td>
</tr>
<tr>
<td>βPP&lt;sub&gt;724-770&lt;/sub&gt;</td>
<td>5' CCC TCG AGC TAG TTC TGC ATC ATC TC 3'</td>
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Materials and Methods

Carboxyl-terminal fragments of βPP were expressed as glutathione S-transferase (GST) fusion proteins. DNA fragments were polymerase chain reaction (PCR) amplified using primers (Table 1) that enabled in-frame expression after ligation into pGSTag vector. The fusion proteins were immobilized on glutathione affinity matrix (Sigma Chemical Co., St. Louis, MO) and the concentrations were adjusted by comparison with protein standards visualized by Coomassie blue stain.

Rat brains were homogenized using a polytron in lysis buffer (150 mmol/L sodium chloride, 10 mmol/L Tris, pH 8.0, 1% Nonidet P-40, 0.5% cholic acid, 5 mmol/L EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 mmol/L phenylmethylsulfonyl fluoride) and cleared by centrifugation at 10,000 × g at 4°C for 10 minutes. Tissue culture cell lysates were prepared in lysis buffer from three human cell lines: neuroblastoma IMR-6, astrocytoma U-251, and embryonic kidney 293.

The 40 μg fusion proteins were mixed with 300 μg of protein of rat brain homogenates or tissue culture cell lysates in 500 μl of lysis buffer for 2 hours at 37°C or 4°C. After thorough washing in lysis buffer, the fusion proteins and their bound proteins were released from the insoluble matrix by boiling in sample buffer (1% sodium dodecyl sulfate (SDS), 3.3% glycerol, 1.6% β-mercaptoethanol, and 20 mmol/L Tris/HCl, pH 6.8).

The precipitated proteins from rat brain homogenates were subjected to 5 to 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred (1 hour at 400 mA at 4°C) to ProBlott membrane ( Applied Biosystems, Foster City, CA) using 10 mmol/L 3-cyclohexylamino-l-propanesulfonic acid (CAPS) buffer, pH 11, containing 10% methanol. Membranes were stained with 0.1% Coomassie blue R-250 (BioRad, Richmond, CA) in 40% methanol, 1% acetic acid. All major bands were excised and sequenced on a 477A microsequencer, and the resulting phenylthiohydantoin amino acid derivatives were identified using an on-line 120A PTH analyzer and the standard program (Applied Biosystems).

Immunoreactivity of these proteins was analyzed by fractionation in 8% SDS-PAGE and electrotransfer onto Immobilon-P membranes (Millipore, Bedford, MA) using the above system. The membranes were blocked with 5% nonfat dry milk and 3% bovine serum albumin in TBST (10 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween-20, pH 7.6) overnight at room temperature and then incubated for 2 hours at room temperature with the following primary antibodies: monoclonal mouse anti-α-tubulin and anti-β-tubulin antibodies (1:300; BioGenex, San Ramon, CA), monoclonal anti-tau antibodies (1:100; Tau-1, Boehringer Mannheim, Indianapolis, IN, and Tau-2, Sigma), control mouse ascites fluid (1:200; Sigma), or monoclonal antibody to actin (1:500; Sigma). Horseradish-peroxidase-linked anti-mouse Ig (Amersham, Arlington Heights, IL) was used as secondary antibody (1:5000). Immunoblots were visualized on DuPont Reflection REN film by enhanced chemiluminescence as specified by the manufacturer (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Membranes were washed with TBST and TBS after incubation with primary antibodies and secondary antibody.

Tubulin binding to the fusion proteins was blocked by adding a 500 μmol/L excess synthetic Aβ<sub>1-28</sub> (βPP<sub>672-699</sub>) or Aβ<sub>1-40</sub> (βPP<sub>672-711</sub>) peptides (Sigma) to cell lysates for 2 hours at 4°C before the addition of the fusion proteins.
For immunoblot analysis of immunoprecipitated proteins, tubulin precipitated from tissue culture cell lysates with anti-α-tubulin antibodies and immunobead-anti-mouse IgG was mixed with GST fusion proteins eluted from the affinity matrix with glutathione. After thorough washing, tubulin and the fusion proteins bound to it were separated on Tricine/Tris/SDS/10% PAGE, electrotransferred to Immobilon-P membranes (Millipore), and immunoblotted with monoclonal mouse anti-Aβ1-24 (βPP688–699) antibody (1:2000; 4G8, Senetek), incubated in horseradish-peroxidase-linked anti-mouse Ig (Amersham), and developed with chemiluminescence reagent (Kirkegaard and Perry Laboratories) as described above.

To depolymerize microtubules, cells were treated for 16 hours with 50 μmol/L colchicine (Aldrich Chemical Co., Milwaukee, WI). The 24 μg microtubule proteins isolated from bovine brain by temperature-dependent cycles of assembly and disassembly30 were used in binding experiments with 40 μg of fusion proteins.

Results and Discussion

βPP carboxyl-terminal fragments expressed as GST fusion proteins (Figure 1) and immobilized on agarose beads were mixed with rat brain homogenates. The fusion proteins and any interacting proteins were released from the precipitated agarose beads by boiling in sample buffer, separated on SDS-polyacrylamide gels, and transferred to ProBlott membranes. βPP672-713, encoding the 42 amino acids of Aβ, bound a major protein of ~55 kd identified by microsequencing as α- and β-tubulin, the heterodimeric subunit proteins of microtubules. Immunoblot analysis showed that the 55-kd protein band was immunostained both with the anti-α-tubulin and the anti-β-tubulin antibodies (Figure 2A). Experiments using lysates prepared from three human cell lines, neuroblastoma IMR-6, astrocytic glioma U-251 and embryonic kidney 293, yielded identical results confirming that βPP672-713 binds to tubulin. No binding to GST alone was observed (Figure 2A). The binding of tubulin to βPP672-713 was partially blocked by synthetic Aβ1-24 (βPP672-699) and Aβ1-40 (βPP672-711) peptides, verifying its specificity (Figure 2B). These data, which suggest that the tubulin-binding site resides within residues 1 to 28 of Aβ were confirmed by the presence of the tubulin bands after precipitation with βPP672-699 (Figure 2A).

Three additional fusion proteins were constructed to further study the binding of tubulin to naturally occurring carboxyl-terminal fragments of βPP: βPP724-770 containing the 99 carboxyl-terminal amino acids of βPP, including Aβ; βPP713-770, which starts immediately after Aβ and contains part of the putative transmembrane domain and the cytoplasmic carboxy terminus of βPP; and βPP724-770, which contains only the cytoplasmic carboxyl-terminal portion of βPP (Figure 1). βPP724-770 did not bind tubulin, but βPP713-770 did bind, thus defining an additional binding site within the transmembrane domain of βPP, carboxyl-terminal to Aβ (Figure 2A). To further test Aβ-tubulin binding, tubulin immunoprecipitated with anti-α-tubulin antibodies was mixed with GST fusion proteins eluted from the affinity matrix with glutathione. Immunoblot analysis with monoclonal anti-Aβ antibodies revealed binding of the fusion protein GST-βPP672-713, but not of GST alone, to tubulin (Figure 3).
The proteins including treated cells demonstrated reduced JPP-tubulin the analyzed by mixed with containing chicine, monoclonal anti-a-tubulin and 1PP672-770, suggested and sites revealed Binding experiments using binding a-tubulin was of human a-tubulin to microtubule systems revealed that it was present with anti-Aβ antibody. The arrow is placed at the level of the GST-BPP672-773 immunoreactive band.

In a similar in vitro system, Schulze et al.31 demonstrated binding of β-tubulin to a fusion protein containing the 99 carboxyl-terminal amino acids of βPP and suggested that it was mediated by glyceraldehyde-3-phosphate dehydrogenase. They did not find any binding to a fusion protein containing Aβ1-40 but did not test binding to Aβ1-42. Hughes et al.32 used the two-hybrid system to identify clones capable of interaction with Aβ and found a clone that encodes human α-tubulin (bar1).

To test whether intact microtubules are necessary for βPP-tubulin binding, cells were treated with colchicine, an alkaloid that causes microtubule depolymerization.33 Binding experiments using lysates of treated cells demonstrated reduced binding of tubulin to βPP672-713 (Figure 4A) and to βPP672-699, βPP672-770, and βPP713-770 (data not shown). Binding experiments were also performed with microtubules isolated from bovine brain by temperature-dependent cycles of assembly and disassembly. The twice-cycled microtubule proteins are composed of tubulin and microtubule-associated proteins including tau.30 Immunoblot analysis with monoclonal anti-α-tubulin or anti-β-tubulin antibodies revealed binding of the βPP fusion proteins to isolated microtubules (Figure 4B).

To test the hypothesis that βPP fusion proteins bind a complex of tau and tubulin, cell lysates were mixed with fusion proteins and the precipitates were analyzed by immunoblot using monoclonal anti-tau antibodies. Tau was present in the precipitates together with the complexes formed by tubulin and βPP672-713 or βPP713-770 (Figure 5A). To further determine whether tau mediates βPP-tubulin binding, a mouse monoclonal anti-tau antibody was added to the binding reaction. The antibody diminished tubulin binding to βPP713-770 (Figure 5B) but did not affect its binding to βPP672-699, βPP672-713, or βPP672-770. Control experiments using mouse ascites and monoclonal antibody to actin did not affect the binding, indicating that there is a specific βPP713-770-tau, and tubulin interaction. Immunoblot

![Figure 3](image-url) Immunoblot demonstrating binding of GST-BPP672-713 fusion protein to tubulin immunoprecipitated with monoclonal anti-α-tubulin antibodies. Tubulin did not bind to GST alone. The membrane was probed with anti-Aβ antibody (4G8). The arrow is placed at the level of the GST-BPP672-713 immunoreactive band.

![Figure 4A](image-url) Tubulin binding to βPP fusion proteins requires intact microtubules, studied by analysis of tissue culture cell lines untreated (lanes 1, 3, and 5) or treated with colchicine (lanes 2, 4, and 6). Immunoblot analysis of cell homogenates (lanes 1 and 2) or of cell homogenate proteins bound to GST (lanes 3 and 4) or to BPP672-713 (lanes 5 and 6) were probed with anti-α-tubulin antibody. B: Twice-cycled microtubule proteins binding to βPP fusion proteins. Lane 1, GST; lane 2, BPP672-699; lane 3, BPP672-713; lane 4, BPP672-770; lane 5, BPP713-770; lane 6, BPP770-770. Immunoblot analysis was with anti-α-tubulin antibody.

![Figure 5A](image-url) Tau binds to βPP fusion proteins, as shown by immunoblot analysis of cell homogenates (lane 1) or cell homogenate proteins bound to GST (lane 2), BPP672-713 (lane 3), and BPP713-770 (lane 4) detected by anti-tau monoclonal antibody (tau-1). B: Blocking of α-tubulin binding to BPP713-770 (lane 1) by monoclonal mouse anti-tau-2 antibody (lane 2), probed with anti-α-tubulin antibody.
analysis of the proteins precipitated with the fusion proteins using monoclonal anti-actin antibodies revealed that actin does not bind to any of the fusion proteins. Binding experiments performed at 4°C resulted in reduced binding of βPP fragments to tubulin and tau as compared with binding at 37°C. To test the binding affinities between the proteins, the precipitated complexes were washed with solutions containing 2 mol/L KCl, 2 mol/L urea, or 1 mol/L guanidine hydrochloride. The binding of βPP to tubulin and tau was found to be stable under these different washing conditions but was labile to heat treatment, suggesting a strong but noncovalent reversible interaction.

These results demonstrate that the carboxyl end of βPP contains two binding sites to cytoskeletal components; one, within residues 1 to 28 of Aβ, binds directly to tubulin, and the second one, within sequences carboxyl-terminal to Aβ, binds both tau and tubulin (Figure 1). Binding of tau to a βPP region distal to Aβ was previously reported. Amyloid plaque components including Aβ have been found to interact with tau in vitro. Using four independent techniques, it was demonstrated that tau interacts with a conformation-dependent domain of βPP encompassing residues 714 to 723. Immuno- cytochemical staining with antibodies to βPP 714–733 demonstrated that longer peptides encompassing the tau binding domain of βPP are present in senile plaques. Antibodies to βPP 713–723 labeled PHFs in the brain of AD patients, and a synthetic peptide containing this epitope bound tau proteins. This peptide spontaneously formed fibrils in vitro and, in the presence of tau, generated dense fibrillar assemblies containing both molecules.

As the tau binding domain lies within the putative transmembrane domain of βPP, the interaction between tau and βPP can occur only after disassociation of the latter from the membrane. Indeed, although the majority of βPP seems to be membrane bound, full-length βPP has been reported to be present in the cytosolic fraction of PC12 cells. Similarly, it has also been demonstrated that Aβ, as well as a βPP carboxyl-terminal fragment containing Aβ, is relatively more abundant in the cytosol of lymphoblastoid cells derived from patients with early-onset familial AD than in cells from normal individuals. Furthermore, βPP co-purifies with cytoskeletal proteins in neuronal and glial cells. In situ binding experiments demonstrated that tau bound avidly to senile plaques in AD brains but not to non-AD brains. These data suggest that intraneuronal βPP may be bound to tau or other cytoskeletal molecules.

βPP binding to the microtubules and their associated proteins suggests that full-length βPP and/or its carboxyl-terminal fragments are associated with the cytoskeleton. Intracellular accumulation of βPP carboxyl-terminal fragments in AD patients may therefore result in an increased βPP-microtubule complex formation that can lead to the intraneuronal fibrillar pathology of AD. Carboxyl-terminal fragments of βPP could act similarly as seeds for nucleation of tau into PHFs. Indeed, the disruption of a normal interaction between βPP and the cytoskeleton may be a crucial step in the pathogenesis of both intracellular and extracellular lesions that characterize Alzheimer’s disease and related disorders.

References


