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## Short Communication

# Immunocytochemical Evidence That the $\beta$ -Protein Precursor Is an Integral Component of Neurofibrillary Tangles of Alzheimer's Disease

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**Amyloid  $\beta$  ( $A\beta$ ) immunoreactivity has been demonstrated in all extracellular neurofibrillary tangles (E-NFT) and most intraneuronal neurofibrillary tangles (I-NFT). We undertook this immunocytochemical study to understand the relationship between  $A\beta$  immunoreactivity localized in NFT and  $\beta$ -protein precursor ( $\beta$ PP). We found epitopes of amino-, mid-, and carboxyl-terminal domains of  $\beta$ PP in I-NFT and the majority of E-NFT. NFT retained  $\beta$ PP after ionic detergent extraction, demonstrating that  $\beta$ PP is an integral component of NFT. Finding  $\beta$ PP in regions of  $A\beta$  immunoreactivity raises the possibility that  $\beta$ PP or its fragments associate with amyloid, and that the stability of  $A\beta$  is responsible for its dominance in amyloid deposits. (Am J Pathol 1993, 143:1586–1593)**

Immunocytochemical studies demonstrate that the primary component of senile plaques, amyloid  $\beta$  ( $A\beta$ ), is also associated with neurofibrillary tangles (NFT).<sup>1–8</sup>  $A\beta$  was reported in only a low percentage of NFT in formalin-fixed, formic acid-denatured samples. However, in tissue not fixed by aldehyde and treated with low concentrations of formic acid, all

extracellular NFT (E-NFT), and many intraneuronal NFT (I-NFT) were  $A\beta$  immunoreactive.<sup>8</sup> These previous studies were limited to antibodies to  $A\beta$  epitopes, which cannot distinguish between  $A\beta$  or a  $\beta$ -protein precursor ( $\beta$ PP) fragment containing  $A\beta$ . The association of  $\beta$ PP fragments with NFT is supported by the presence of  $\beta$ PP epitopes in NFT, which lie outside the  $A\beta$  domain.<sup>9–11</sup> Yet,  $\beta$ PP was only localized to the filaments of I-NFT<sup>10,11</sup> and not to E-NFT.<sup>11</sup> The absence of  $\beta$ PP from E-NFT<sup>11</sup> would suggest that  $\beta$ PP is removed, along with the neuronal cytoplasm, after neuronal death. This interpretation is consistent with the generalized cytoplasmic localization of  $\beta$ PP immunoreactivity in neurons.<sup>9</sup> The peripheral association of  $\beta$ PP with NFT is also consistent with ultrastructural localization of  $A\beta$  to the amorphous covering of E-NFT filaments<sup>3</sup> and a report that some  $\beta$ PP epitopes can be removed from NFT by detergent.<sup>11</sup>

The aim of this study was to determine quantitatively whether  $\beta$ PP-epitopes (amino-, mid-, and carboxyl-terminal domains) are integral or peripheral elements of NFT. The goal was to determine whether the failure to detect  $\beta$ PP epitopes in E-NFT and detergent-extracted NFT was related to poor epitope preservation or exposure. In contrast with previous reports, we show that  $\beta$ PP is specifically associated with the majority of NFT; I-NFT and E-NFT. Furthermore,  $\beta$ PP is, like  $\tau$ , retained after ionic detergent extraction. Therefore, we suggest that the  $\beta$ PP in NFT may provide a source for the  $A\beta$  deposition on NFT. Further, this study suggests that the metabolism of some  $\beta$ PP molecules may occur extracellularly in amyloid deposits.<sup>12,13</sup>

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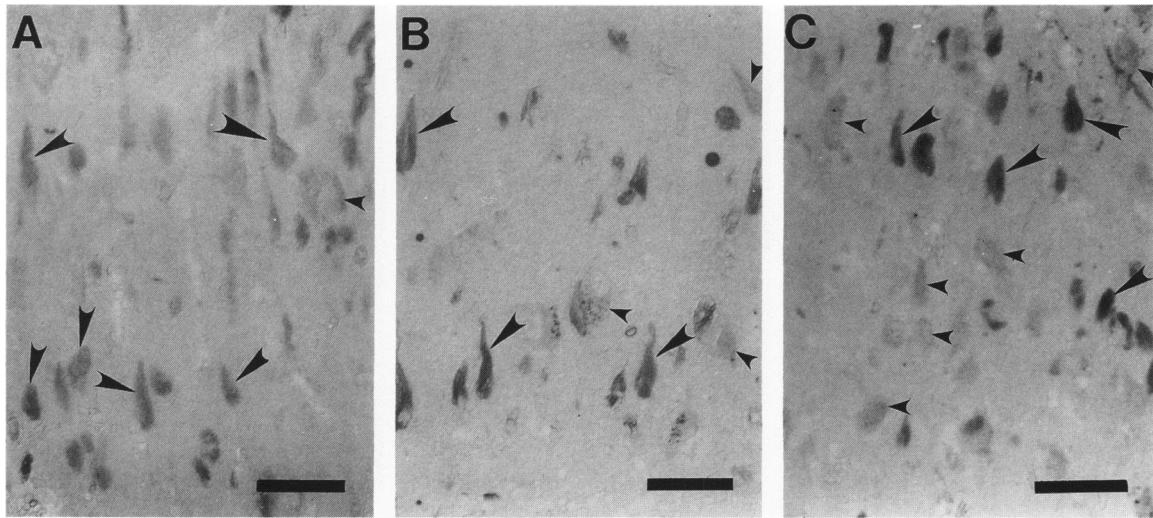


Figure 1. Immunostaining of NFT with anti- $\beta$ PP<sub>45-62</sub> (A) anti- $\beta$ PP<sub>553-570</sub> (B), or anti- $\beta$ PP<sub>648-689</sub> (C) shows that numerous NFT are recognized by antibodies directed to amino-, mid-, and carboxyl-terminal domains of  $\beta$ PP, respectively.  $\beta$ PP antibodies recognized NFT (some indicated by large arrowheads) and neuronal perikarya (some indicated by small arrowheads). Scale bar = 50  $\mu$ m.

## Materials and Methods

### Tissue

The hippocampus and adjacent temporal cortex of five cases of Alzheimer's disease were studied (ages 71, 77, 85, 87, and 90 years). Clinical and pathological diagnoses were based on established criteria. Qualitatively identical results were obtained from all cases. Tissue taken at autopsy was fixed in methacarn (methanol-chloroform-acetic acid, 6:3:1) for 16 hours before paraffin embedding. Six-micron sections were cut.

Homogenates of hippocampal tissue were prepared by gently disrupting subiculum/Somer's sector gray matter (1:10, tissue:buffer ratio) in 50 mmol/L Tris-HCl, pH 7.6, with a Dounce homogenizer (Kontes Glass, Vineland, NJ). Aliquots of the homogenate (10  $\mu$ l) were placed on slides, dried for 16 hours at 37 C, and treated with Tris buffer (50 mmol/L Tris-HCl, pH 7.6), Tris buffer-1% Triton X-100, or Tris buffer-1% sodium dodecyl sulfate (SDS) for 20 minutes at room temperature. The samples were then immunostained.

### Immunological and Biochemical Reagents

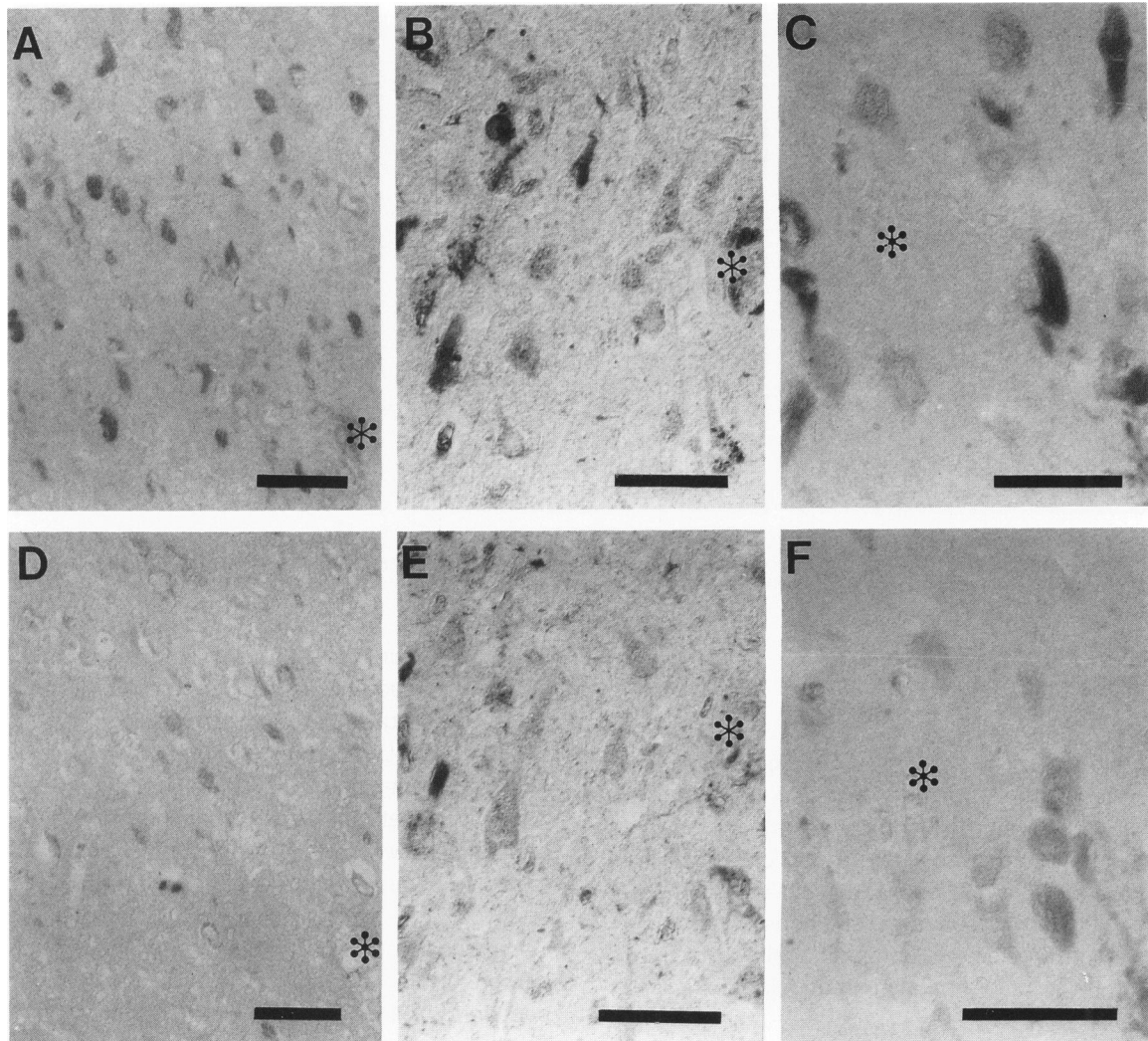
Rabbit antisera raised to synthetic peptides homologous to three domains of  $\beta$ PP were used at a dilution of 1:100: 1) anti- $\beta$ PP<sub>45-62</sub>: amino-terminal, sequences 45-62 of  $\beta$ PP<sub>695</sub>; 2) anti- $\beta$ PP<sub>553-570</sub>: mid-region, sequences 553-570 of  $\beta$ PP<sub>695</sub>; and 3) anti- $\beta$ PP<sub>648-689</sub>: carboxyl-terminal, sequences 648-689 of  $\beta$ PP<sub>695</sub>. None of the  $\beta$ PP antibodies recog-

nized  $\tau$  or neurofilament heavy subunit on immunoblots when used at the same concentration used for immunostaining but, as expected, recognized  $\beta$ PP on immunodots. In preliminary experiments, we found that formaldehyde fixation modified  $\beta$ PP epitopes such that they were not immunostained, and pretreatment with 50% formic acid for 5 minutes at room temperature was optimal for exposing  $\beta$ PP epitopes.

Immunoabsorption consisted of incubating the antiserum with 1 mg/ml of the appropriate synthetic peptide for  $\beta$ PP<sub>553-570</sub> and  $\beta$ PP<sub>648-689</sub> at 4 C for 16 hours before immunostaining. Immunoabsorption of anti- $\beta$ PP<sub>45-62</sub> was performed by passage through a column containing  $\beta$ PP<sub>45-62</sub>.

Heparan sulfate treatment consisted of incubating the sections for 1 hour at 37 C with 1 mg/ml heparan sulfate (molecular weight, approximately 8 kd; Sigma, St. Louis, MO) in 50 mmol/L Tris-HCl, pH 7.6. Heparan sulfate was eluted by treating with 2 mol/L NaCl for 16 hours at room temperature. Heparinase treatment involved incubating sections with heparinase 1 or heparinase 3 (1 U/ml; Sigma) in 10 mmol/L Tris-HCl, pH 7.0, and 20 mmol/L CaCl<sub>2</sub> for 16 hours at room temperature.

Standard markers to quantitatively assess NFT staining were basic fibroblast growth factor (bFGF) binding,<sup>14</sup> a probe for a form of heparan sulfate proteoglycans specific to E-NFT, and Alz-50, a monoclonal antibody that only recognizes I-NFT.<sup>14</sup> The peroxidase anti-peroxidase method using 3,3'-diaminobenzidine as chromogen was used to visualize immunoreactivity. Endogenous peroxidase



**Figure 2.** Specificity of antibody binding was determined by absorption with the immunogen. Immunostaining of hippocampus with anti- $\beta$ PP<sub>45-62</sub> (A), anti- $\beta$ PP<sub>553-570</sub> (B), and anti- $\beta$ PP<sub>648-689</sub> (C) is reduced by preincubation with the respective immunogenic peptide:  $\beta$ PP<sub>45-62</sub> (D),  $\beta$ PP<sub>553-570</sub> (E), and  $\beta$ PP<sub>648-689</sub> (F). A and D, B and E, and C and F are adjacent sections; a landmark is indicated (\*) in each set. Scale bars = 50  $\mu$ m.

activity was quenched by treatment with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes.

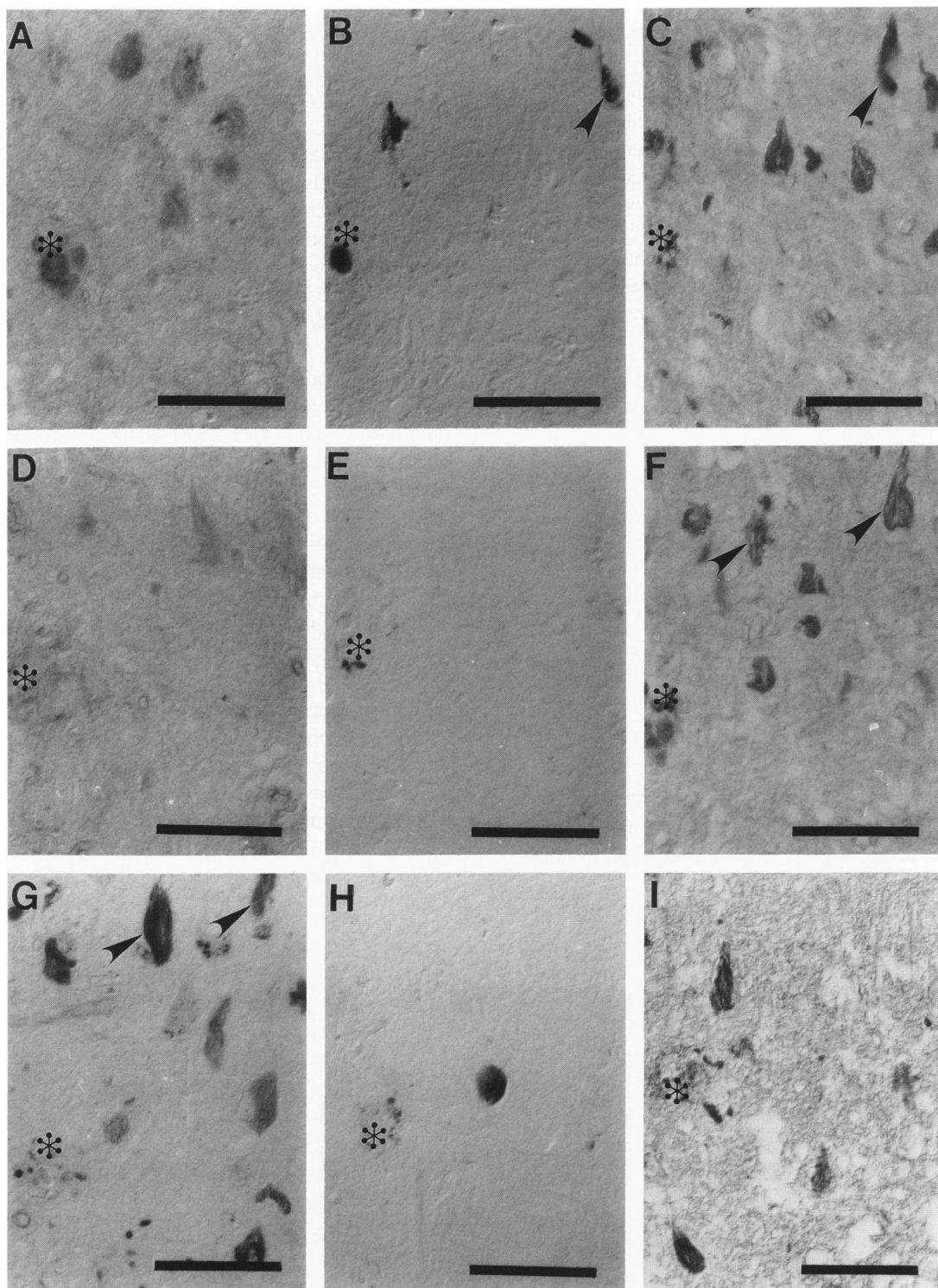
### Quantitation of Stained Structures

The number of E-NFT and I-NFT recognized by the various probes in adjacent sections was determined by counting five fields, 0.2 mm<sup>2</sup> each, of the  $\times 20$  planapochromat objective of an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY). Landmarks such as blood vessels were used to locate the same five fields of the subiculum within each group of adjacent sections. The fields evaluated were chosen in regions of maximum density for NFT. Immunostained NFT were defined by the intense staining of flame-shaped bundles of fila-

ments; structures not displaying a filamentous structure were not counted as NFT. Congo red failed to recognize NFT after formic acid and, therefore, could not be used to augment our criteria to identify NFT. Differential interference contrast (Nomarski) was used to identify the cytoplasm and nucleus surrounding filaments of I-NFT and dispersed filaments of E-NFT.

### Results

The initial goal of our study was immunocytochemical identification of specific  $\beta$ PP domains in NFT. Antibodies to the amino-terminal  $\beta$ PP<sub>45-62</sub> (anti- $\beta$ PP<sub>45-62</sub>), mid-region  $\beta$ PP<sub>553-570</sub> (anti- $\beta$ PP<sub>553-570</sub>), and carboxyl-terminal  $\beta$ PP<sub>648-689</sub> (anti- $\beta$ PP<sub>648-689</sub>)



**Figure 3.** NFT detected by antisera to three  $\beta$ PP domains compared to standards for E-NFT (bFGF binding) and I-NFT (Alz-50; see Figure 4). Adjacent serial sections are shown in order and defined by a senile plaque (\*). A: Anti- $\beta$ PP<sub>45-62</sub>; D: anti- $\beta$ PP<sub>553-570</sub>; G: anti- $\beta$ PP<sub>548-689</sub>; B, E, H: Alz-50; C, F, I: bFGF binding. Although individual NFT cannot always be followed in consecutive sections, the density and type of NFT recognized by these markers can be directly compared. Arrowheads indicate those cases where NFT recognized by different markers are seen in adjacent sections. Scale bars = 50  $\mu$ m.



all recognized NFT (Figure 1). Further, it was readily apparent that  $\beta$ PP is specifically associated with the filaments of NFT in addition to being diffusely distributed throughout neuronal cytoplasm (Figure 1) as previously reported.<sup>11</sup> The specificity of each antibody to  $\beta$ PP was verified by immunoabsorption, which reduced immunoreactivity (Figure 2).

The numbers of NFT recognized by the three  $\beta$ PP antisera were compared to Alz-50<sup>14</sup> (I-NFT) and bFGF<sup>14</sup> binding (E-NFT; Figure 3). A quantitative assessment in Figure 4 shows that  $\beta$ PP antibodies recognizes a majority of E-NFT and many I-NFT.

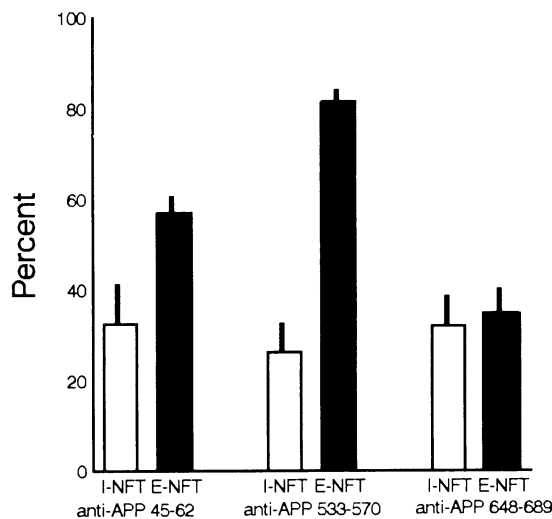


Figure 4. The percentage of NFT containing  $\beta$ PP domains was determined from an area of 1 sq mm compared with the number of NFT stained by bFGF binding (E-NFT) and Alz-50 (I-NFT) in the immediately adjacent section defined by the same landmarks. Values are the mean  $\pm$  SE for three cases.

In a recent study, we showed that the heparan-binding protein cholinesterase, which is associated with NFT and senile plaques, can be specifically dissociated by competition with heparan sulfate.<sup>15</sup> As  $\beta$ PP has a heparan-binding domain,<sup>16,17</sup> we speculated that similar treatment might release  $\beta$ PP or its fragments. Indeed, pretreatment with heparan sulfate reduces immunoreactivity. However, we found that heparan sulfate blocks, rather than removes,  $\beta$ PP, as a high salt treatment after heparan sulfate reexposes  $\beta$ PP epitopes (Figure 5).

The inability to elute  $\beta$ PP with heparan sulfate suggests that  $\beta$ PP has an integral association with NFT. To demonstrate this aspect, we prepared homogenates in 50 mmol/L Tris-HCl from hippocampal grey matter rich in NFT and placed aliquots on microscope slides. The spots were dried and subsequently treated with Tris buffer alone or containing Triton X-100 or SDS. After detergent extraction, which dissolved most of the tissue, the sections were immunostained with the antisera to  $\beta$ PP, a pre-immune serum, or an antiserum to  $\tau$ .<sup>18</sup>

The three  $\beta$ PP domains were retained after non-ionic (Triton X-100) or ionic (SDS) detergent extraction (Figure 6). Quantitative evaluations were made by direct comparison with the number of NFT containing  $\tau$  epitopes. Comparison with  $\tau$  immunostaining is essential, because NFT can be removed from the slide by extraction, and  $\tau$  is retained by NFT after SDS extraction.<sup>19</sup> All three domains of  $\beta$ PP are retained by NFT morphologically identical to those containing  $\tau$  after either Triton X-100 or SDS (Table 1), indicating that  $\beta$ PP, like  $\tau$ , is an integral component of NFT.<sup>19</sup>

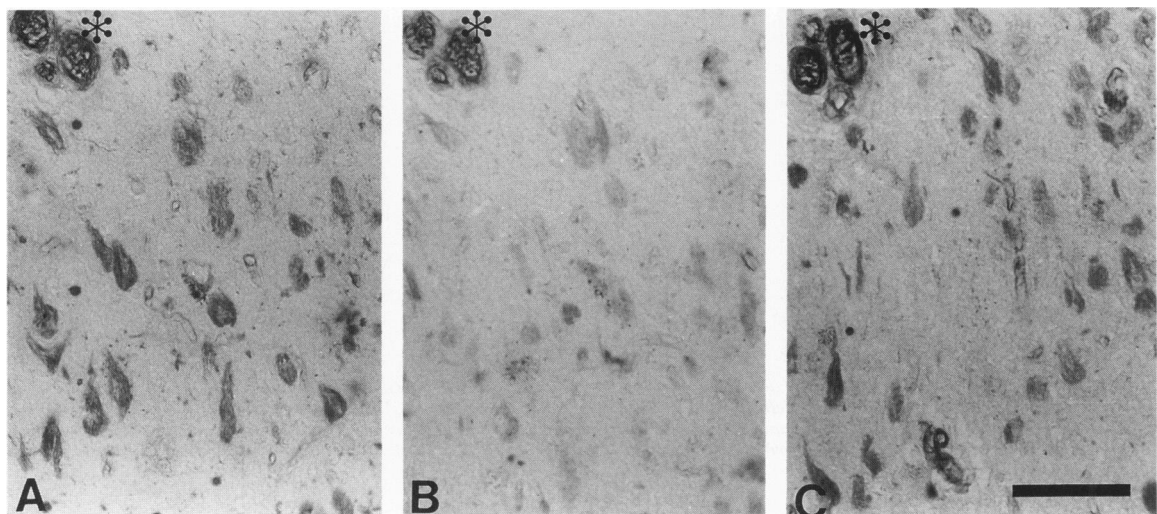
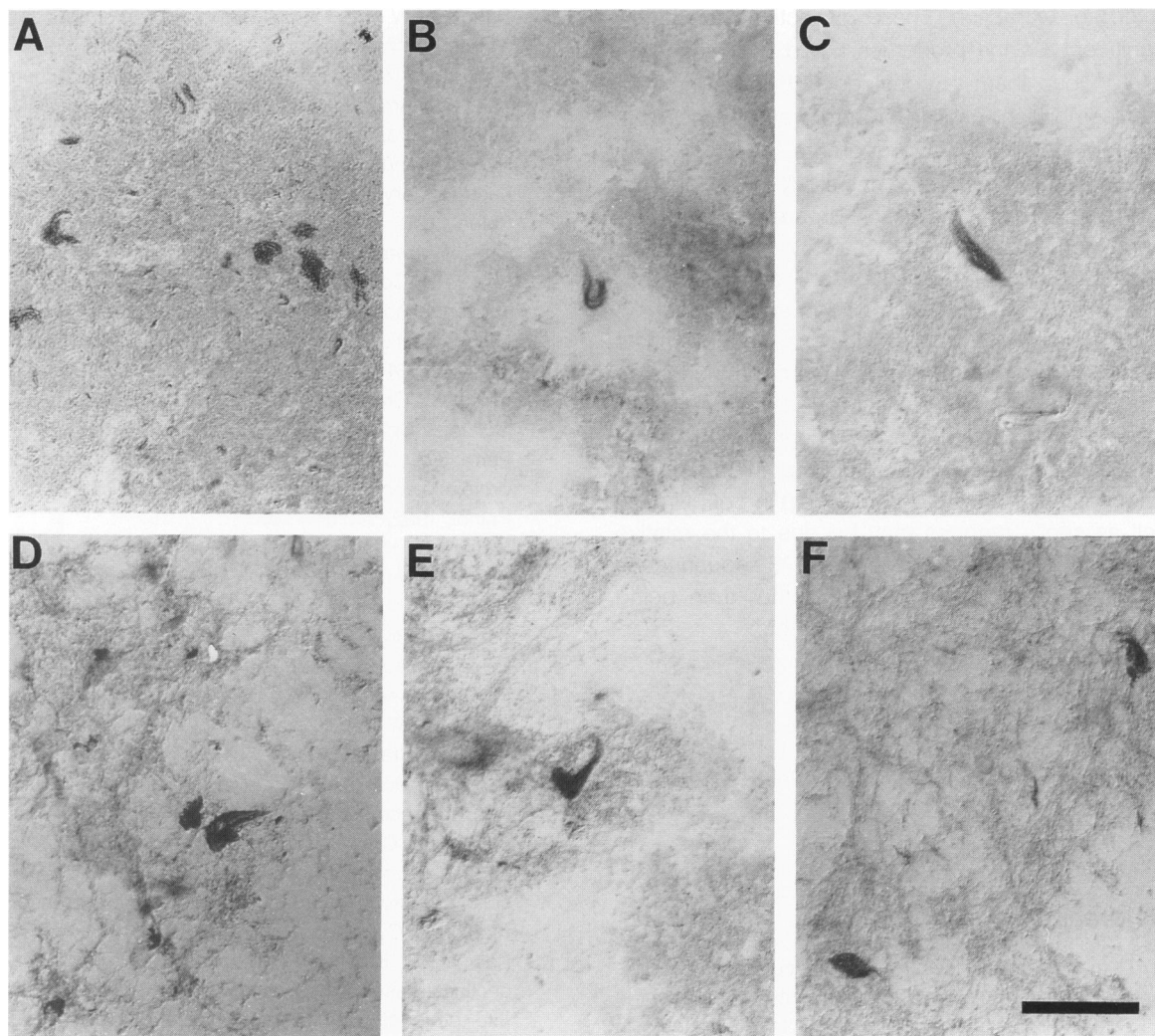


Figure 5. Immunostaining of NFT by anti- $\beta$ PP<sub>553-570</sub> (A) was reduced after incubation with 1 mg/ml heparan sulfate (B), but could be restored by treatment with 2 mol/L NaCl (C). Adjacent sections with the same blood vessel (\*) found in each section are indicated. Scale bar = 50  $\mu$ m.



**Figure 6.**  $\beta$ PP epitopes defined by anti- $\beta$ PP<sub>45-62</sub> (A), anti- $\beta$ PP<sub>553-570</sub> (B), and anti- $\beta$ PP<sub>648-689</sub> (C) were maintained after treatment with Tris buffer as well as after Tris buffer-1% SDS: anti- $\beta$ PP<sub>45-62</sub> (D), anti- $\beta$ PP<sub>553-570</sub> (E), and anti- $\beta$ PP<sub>648-689</sub> (F). Scale bar = 50  $\mu$ m.

**Table 1.**  $\beta$ PP Epitopes Are Retained by NFT after either Nonionic (Triton X-100) or Ionic (SDS) Detergent Extraction to Approximately the Same Extent as  $\tau$  Epitopes

	Anti- $\beta$ PP <sub>45-62</sub> (n = 6)	Anti- $\beta$ PP <sub>553-570</sub> (n = 2)	Anti- $\beta$ PP <sub>648-689</sub> (n = 2)	Anti- $\tau$ (n = 6)	Preimmune (n = 2)
Tris	126.2 $\pm$ 6.9	14.5 $\pm$ 2.1	27	49 $\pm$ 7.5	2 $\pm$ 1.4
Triton	152 $\pm$ 13.6	34 $\pm$ 24	22.5 $\pm$ 6.4	54.8 $\pm$ 13.7	2.5 $\pm$ 0.7
SDS	46.8 $\pm$ 7.9	7 $\pm$ 8.5	30 $\pm$ 25.5	13.3 $\pm$ 4.4	0

Although in some cases there is considerable variability, all data indicate that  $\beta$ PP is retained by NFT. The lower number of NFT after SDS reflects removal of NFT from the slide. Data are expressed as the number of NFT stained in 10- $\mu$ l aliquots of a homogenate (1:10, tissue:buffer ratio). Values are the mean  $\pm$  SE.

## Discussion

Our results demonstrate that three non-contiguous  $\beta$ PP epitopes are found in the majority of NFT. We also show that although heparan sulfate proteoglycans may be responsible for initial  $\beta$ PP incorporation,<sup>16,17,20</sup> it is unlikely that they play a significant role in retaining  $\beta$ PP in NFT, as  $\beta$ PP was not eluted

by heparan sulfate. We speculate that once associated,  $\beta$ PP is retained in NFT by other interactions, such as those described between  $\tau$  and  $\beta$ PP.<sup>21,22</sup>

Our findings differ from those of Yamaguchi and co-workers.<sup>11</sup> We readily detect three distinct  $\beta$ PP domains associated with NFT filaments and show  $\beta$ PP in E-NFT and retention of  $\beta$ PP after ionic

detergent extraction. These distinctions are highly significant, as the properties noted by Yamaguchi et al.<sup>11</sup> lead them to conclude  $\beta$ PP is an insignificant component of NFT, an interpretation inconsistent with the findings reported here.

A provocative aspect of this study is that  $\beta$ PP sequences flanking the A $\beta$  domain are apparently integral components in the majority of E-NFT. This study raises the possibility that  $\beta$ PP or its fragments may directly associate with NFT in addition to amyloid filaments<sup>9,23</sup> in the extracellular space. This is consistent with the results of several immunocytochemical studies that identified  $\beta$ PP epitopes in A $\beta$  deposits<sup>9,23</sup> and with a more recent study identifying  $\beta$ PP epitopes within highly purified amyloid plaque cores.<sup>24</sup> The presence of A $\beta$  in both NFT as well as senile plaques may be due to the relative stability of A $\beta$  compared to other  $\beta$ PP sequences to catabolic degradation,<sup>12,13,25,26</sup> rather than being the result of specific proteolytic events.

### Acknowledgments

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