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Short Communication

Antibodies to Presenilin Proteins Detect Neurofibrillary Tangles in Alzheimer's Disease

Greer M. Murphy, Jr.,* Lysia S. Forno,† William G. Ellis,‡ David Nochlin,§ Ephrat Levy-Lahad,‖ Parvoneh Poorkaj,¶ Thomas D. Bird,# Zhiling Jiang,** and Barbara Cordell**

From the Department of Psychiatry and Behavioral Sciences, Stanford University Medical Center, Stanford, California; the Department of Pathology,† Veterans Affairs Palo Alto Health Care System, Palo Alto, California; the Department of Pathology,‡ School of Medicine, University of California, Davis, Davis, California; the Departments of Pathology§ and the Department of Medicine,‖ University of Washington, Seattle, Washington; the Geriatrics Research Education and Clinical Center¶ and the Division of Neurology,# Veterans Affairs Medical Center, Seattle, Washington; and Scios Inc.,** Mountain View, California*

Mutations in the presenilin (PS)-1 and PS-2 genes have been shown to be linked with the development of Alzheimer's disease (AD). We examined Alzheimer's brain tissue by immunohistochemistry using a set of antibodies raised to sequences shared between PS-1 and PS-2 proteins. These antibodies reacted exclusively with a subset of neurofibrillary tangles and not with neuropil threads or dystrophic neurites. Detection of the presenilin epitope in neurofibrillary tangles was observed in sporadic Alzheimer's disease brain samples and in samples from individuals carrying PS-1 and PS-2 mutations with no qualitative difference. These data indicate that both wild-type and mutant PS proteins are involved in a common pathogenic pathway in AD. (Am J Pathol 1996, 149:1839–1846)

The involvement of genetic components in the pathophysiology of Alzheimer's disease (AD) has been clearly demonstrated by epidemiological studies. A number of human pedigrees have been identified in which early-onset AD segregates as a fully penetrant autosomal dominant trait. Two genes involved in the development of familial AD have been recently identified at the molecular level using genetic linkage studies. These genes, located on chromosomes 14 and 1, encode novel proteins referred to as presenilin (PS)-1 and PS-2, respectively. A frequent association of the defective gene on chromosome 14 with familial AD was first reported by Schellenberg and co-workers[1] followed by identification of the primary sequence of this gene by Sherrington et al.[2] A second and different familial AD locus was mapped to chromosome 1.[3,4] The gene on chromosome 1 was independently identified by Levy-Lehad et al[5] and Rogaev et al,[4] who both described extensive primary structural homology between PS-2 and the PS-1-encoded protein sequence on chromosome 14.

Both PS-1 and PS-2 appear to be members of an evolutionarily conserved family of membrane-spanning proteins as judged by their predicted structures and by homology with proteins encoded by two genes in *Caenorhabditis elegans*, *sel-12*[6] and *spe-4*.[7] The PS proteins localize to membranes of the intracellular secretory pathway[7,8] and appear to form a structure with 10 transmembrane-spanning domains (our observations). Mutations in *spe-4* suggest that PS proteins may be involved in protein trafficking.[7]

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Address reprint requests to Dr. Barbara Cordell, Scios Inc., 2450 Bayshore Parkway, Mountain View, CA 94043.

Dr. Levy-Lahad's present address: Shaare Zedek Medical Center, Jerusalem, Israel.

More recent information indicates that mammalian PS proteins may function as an effector of apoptosis.[9] Mutations in PS-1 and PS-2 result in the development of AD pathology, although the mechanism by which these mutations lead to the development of AD is unknown. In this report, we demonstrate the association between PS protein and a major histopathological component of AD brain, the neurofibrillary tangle (NFT).

Materials and Methods

Tissue Samples

Case material fixed in methacarn and embedded in paraffin included 14 cases with clinical and neuropathological diagnoses of AD, 2 cases of AD with cortical Lewy bodies (AD/LB), 2 cases with mixed AD and Parkinson's disease (AD/PD), 1 case with PD, and 1 69-year-old with Wernicke-Korsakoff disease. Formalin- or paraformaldehyde-fixed, paraffin-embedded material included 9 cases with AD, 6 cases with AD/LB, 4 cases with mixed AD/PD, 1 case with PD, 2 cases with incidental LB in substantia nigra, and 3 cases greater than 70 years of age with no evidence of neurological disease. All of the above cases were obtained through the neuropathology services of the Palo Alto Veterans Affairs Health Care System or the University of California, Davis, Medical Center, and all had complete neuropathological diagnostic evaluations. Additional formalin-fixed, paraffin-embedded case material was obtained through the University of Washington Alzheimer's Disease Research Center. These included 7 cases with chromosome-14-linked familial AD encompassing three different pedigrees, 3 cases with chromosome-1-linked familial AD representing three pedigrees, 2 cases of sporadic AD, and 2 cases of familial dementia with NFT but no senile plaques. For AD, AD/LB, AD/PD, and control cases, sections from the medial temporal lobe (amygdala, hippocampus, and parahippocampal gyrus) and superior temporal gyrus were studied. For AD/LB, AD/PD, PD, and incidental LB cases, sections were examined from the midbrain, including the substantia nigra.

Antibodies

A synthetic peptide was prepared corresponding to residues 263 to 280 of the PS-1 published sequence[2]. An identical sequence is encoded by PS-2.[3,4] The native cysteine at position 263 corresponding to the amino terminus of the peptide was used to conjugate the peptide to keyhole limpet hemocyanin with sulfo-maleobenzoic sulfide. The peptide conjugate was used to repeatedly immunize three rabbits. Sera samples were analyzed for reactivity to the peptide immunogen and for reactivity to the native protein by Western blotting. To detect native and recombinant human PS, total protein was harvested from Chinese hamster ovary (CHO) cells grown in culture as previously described[10]. Equivalent amounts of total CHO protein from each sample was fractionated using electrophoresis on a 10% polyacrylamide gel. For Western blotting, a 1:200 dilution of serum was used followed by detection with horseradish peroxidase (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Expression of human PS-1 protein in CHO cells was made by inserting a full-length cDNA encoding PS-1 into a mammalian cell expression plasmid behind the cytomegalovirus promoter (Invitrogen Corp., San Diego, CA). Adaptor oligonucleotides were used to insert the PS-1 cDNA (beginning with 29 bases upstream of the initiator methionine codon through 7 bases after the termination codon) into the vector polylinker. Plasmid DNA was used to transfect CHO cells in culture by the lipofectamine method (Gibco BRL, Gaithersburg, MD). Individual CHO cell clones were isolated and were assayed for the presence of PS protein by Western blot analysis.

Immunohistochemistry

Immunostaining on methacarn sections was performed using the peroxidase-antiperoxidase method or the avidin-biotin method (Vectastain ABC kit, Vector Laboratories) as previously described.[11,12] The PS 2.3 antiserum was used at dilutions of 1:200 or 1:275. The PS 2.1 and 2.2 antisera were used at a dilution of 1:75. Corresponding preimmune sera were used at identical dilutions. For formalin- and paraformaldehyde-fixed sections, signal intensity was increased by using a gold/silver detection method (Zymed Laboratories, South San Francisco, CA). Treatment with 80% formic acid for 5 minutes was used for both detection systems and for all types of fixed tissue sections. Sections adjacent to those used for PS immunostaining were reacted at a dilution of 1:750 with a rabbit antiserum to sodium-dodecyl-sulfate-isolated paired helical filaments[13] (PHFs) or at a dilution of 1:500 with a tau mouse monoclonal antibody (clone tau-1, Boehringer Mannheim, Indianapolis, IN), and bound antibody was detected with the peroxidase anti-peroxidase method. Immunohistology involving peptide competition was performed by preincubating PS 2.2 or 2.3 antisera with 250 or 500 μg/ml synthetic peptide in

Table 1. *Quantitation of NFT Subset Identified by PS Antisera*

Diagnosis	Region	PHF-NFT	PS-NFT	Ratio
AD F77	CA1 Mid-hippocampus	458	69	6.6
AD/LB F89	CA1 Mid-hippocampus	543	155	3.5
AD F86	Entorhinal cortex	375	99	3.8
AD M87	CA1 Posterior hippocampus	407	79	5.2
Mean				4.8 ± 0.72

phosphate-buffered saline at 37°C for 2 hours and then at 4°C for 15 hours, after which the antiserum solution was used for immunohistochemistry. Antisera identically treated, except that the peptide was omitted, served as controls.

Quantitation

To compare the numbers of PS-labeled and PHF-labeled NFTs, adjacent sections from four cases (3 AD and 1AD/LB) were examined. For each pair of slides, a region of interest was demarcated on each section based on cytoarchitecture. All immunolabeled NFTs were then counted using a standard research microscope at ×100 magnification.

Results

Three antisera were raised against a synthetic peptide corresponding to a sequence conserved between PS-1 and PS-2. This sequence is located within the third hydrophilic loop of this membrane-spanning protein. These sera (referred to as 2.1, 2.2, and 2.3 or collectively as PS antibodies) were utilized to examine AD brain by immunohistochemical methods. Each serum was found to be qualitatively identical, revealing intense immunoreactivity to NFT (Figure 1). Preimmune sera showed no reactivity with any pathological structures on any of the sections (Figure 1). The 2.3 antiserum showed the strongest reactivity of the set, and therefore, this reagent was used more extensively than the other sera. As seen in Figure 1, the PS antibodies labeled a subset of NFTs, but not all, in methacarn-, formalin-, and 4% paraformaldehyde-fixed AD brain. Both globose and flame-shaped NFTs reacted with the PS antibodies (Figure 2). Almost all NFTs labeled by each of the three PS antibodies were intraneuronal; rarely, a ghost tangle was detected. Comparison with adjacent sections reacted with the PHF and tau reagents revealed that there was a large population of extraneuronal and intraneuronal NFTs, especially in sections from the medial temporal lobe, which were not labeled with the PS sera. In four cases in which NFT were quantified, there was a mean of 4.8 (SE = 0.72) times as many PHF-labeled tangles as PS-labeled tangles (Table 1). Furthermore, there was no labeling of dystrophic neurites in plaques or neuropil threads by the PS antibodies, although the PHF and tau antisera clearly decorated these structures (Figures 1 and 2). Reactivity of the PS sera with β-amyloid peptide (β-AP) deposits in plaques or vessels was absent, even after treatment with formic acid, although numerous β-AP-containing plaques have been previously detected in these cases.[11]

In sections from the medial temporal lobe, PS-labeled NFTs were found most frequently in the corticomedial division of the amygdala and in the entorhinal and parahippocampal cortices. Many NFTs labeled by the PS antibodies in these regions were globose (Figures 2 and 3). Numerous flame-shaped NFTs in the pyramidal layer of Ammon's horn also reacted with the PS antibodies (Figure 1D and Figure 2D). In the entorhinal and parahippocampal cortices, PS-labeled NFTs were most common in layers II and V. In neocortical sections from the superior temporal gyrus, PS- reactive NFTs were found most frequently in layers V and VI, although PHF and tau antiserum often revealed the presence of additional NFTs in layers II and III.

AD cases from individuals carrying mutations in PS-1 or PS-2 were examined using the PS antibodies. Three different pedigrees, and PS mutations (W120D, A260V, and G209V), were represented in the chromosome-14-linked familial AD cases, and three different pedigrees, each with a N141I mutation, were included for the chromosome-1-linked cases. In both chromosome-1- and chromosome-14-linked AD cases, the antibodies reacted exclusively with numerous NFTs. Like the pattern of immunoreactivity observed with other AD cases, no reactivity was seen to dystrophic neurites, to neuropil threads, or to β-AP-containing structures in the cases harboring PS mutations (Figures 1 and 2). Qualitatively, all

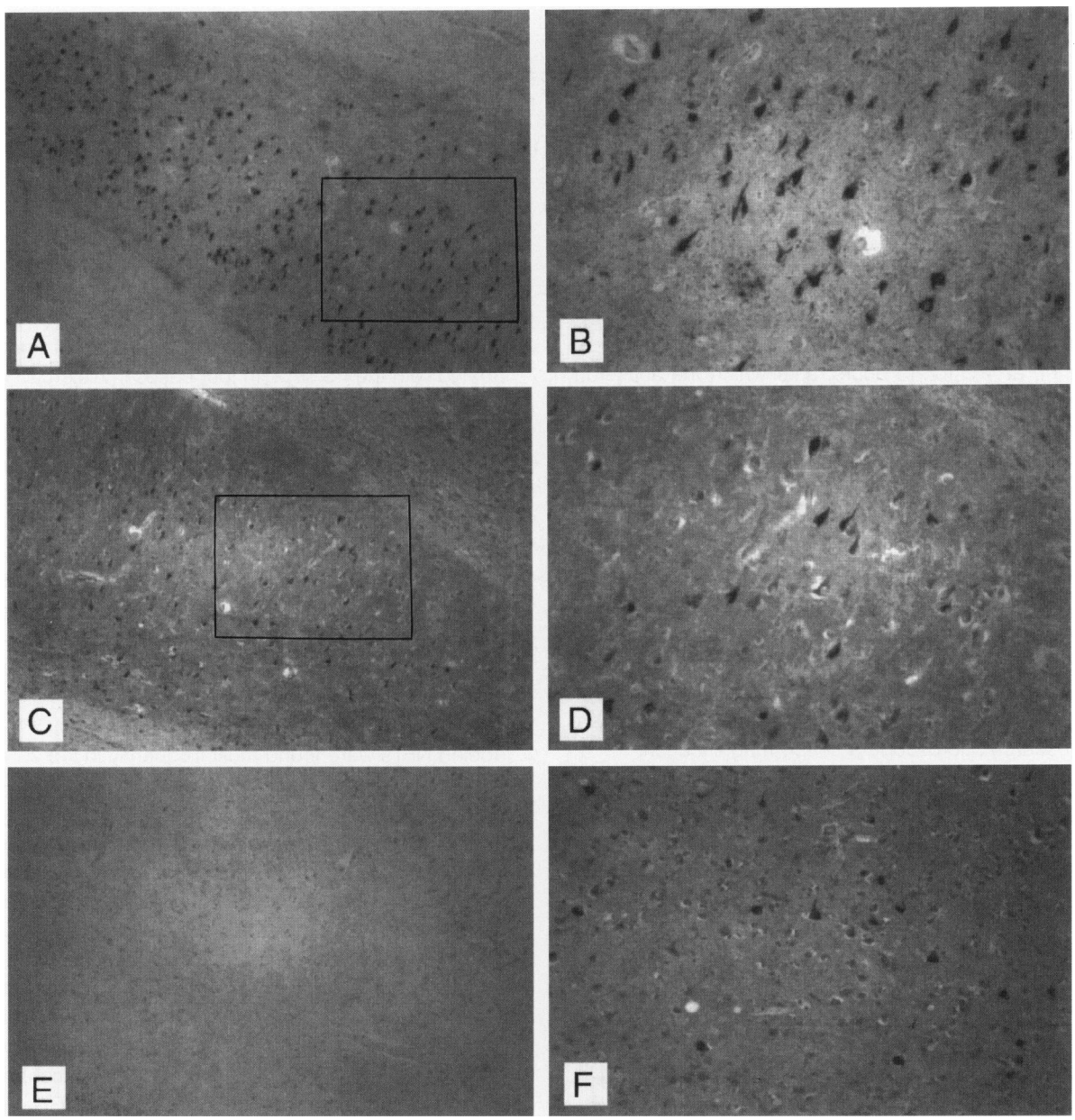

Figure 1. *PS immunoreactivity in AD brain.* **A**, **C**, *and* **E**: *Low-power (×20) view of junction between CA1 and subiculum from an 89-year-old AD case with cortical Lewy bodies showing NFT labeled with antiserum to PHF epitopes* (**A**) *and with PS 2.3 antiserum* (**C**). *Note that the PS antiserum labels only a subset of NFT detected by the PHF antiserum.* **E** *shows the absence of labeling by preimmune serum from the rabbit used to generate the PS 2.3 antiserum. Gold/silver immunostaining technique on formalin-fixed sections was used.* **B** *and* **D**: *Higher-power (×50) views of the same case and region as in* **A** *and* **C**, *respectively* (**boxed areas**). *The selectivity of PS immunoreactivity is apparent. Flame-shaped intraneuronal tangles are typically labeled in this region with PS antiserum.* **F**: *View (×50) of parahippocampal cortex from a 52-year-old male chromosome-14-linked AD case with known PS-1 mutation reacted with PS 2.3 antiserum. The morphology of the NFT is similar to that in sporadic AD cases.*

AD cases, including chromosome-1- and chromosome-14-linked cases, exhibited similar reactivity to the PS antibodies. A total of 35 AD cases were surveyed in this study, with known chromosome-1- and chromosome-14-linked cases comprising 10.

Numerous intraneuronal inclusions were labeled by the PS 2.3 antiserum in the cortical nucleus of the amygdala and adjacent regions in the AD/LB cases. Most were identified as globose tangles, but some resembled cortical Lewy bodies. However, in the two Parkinson's disease cases, the two incidental LB cases, and the AD/LB cases, the 2.3 reagent did not label Lewy bodies in the substantia nigra, although these were clearly visible on adjacent sections stained with hematoxylin and eosin. Likewise, in the six mixed AD/PD cases, there was no labeling of midbrain Lewy bodies by the 2.3 reagent. Cases free of neurological disease and the Wernicke-Korsakoff

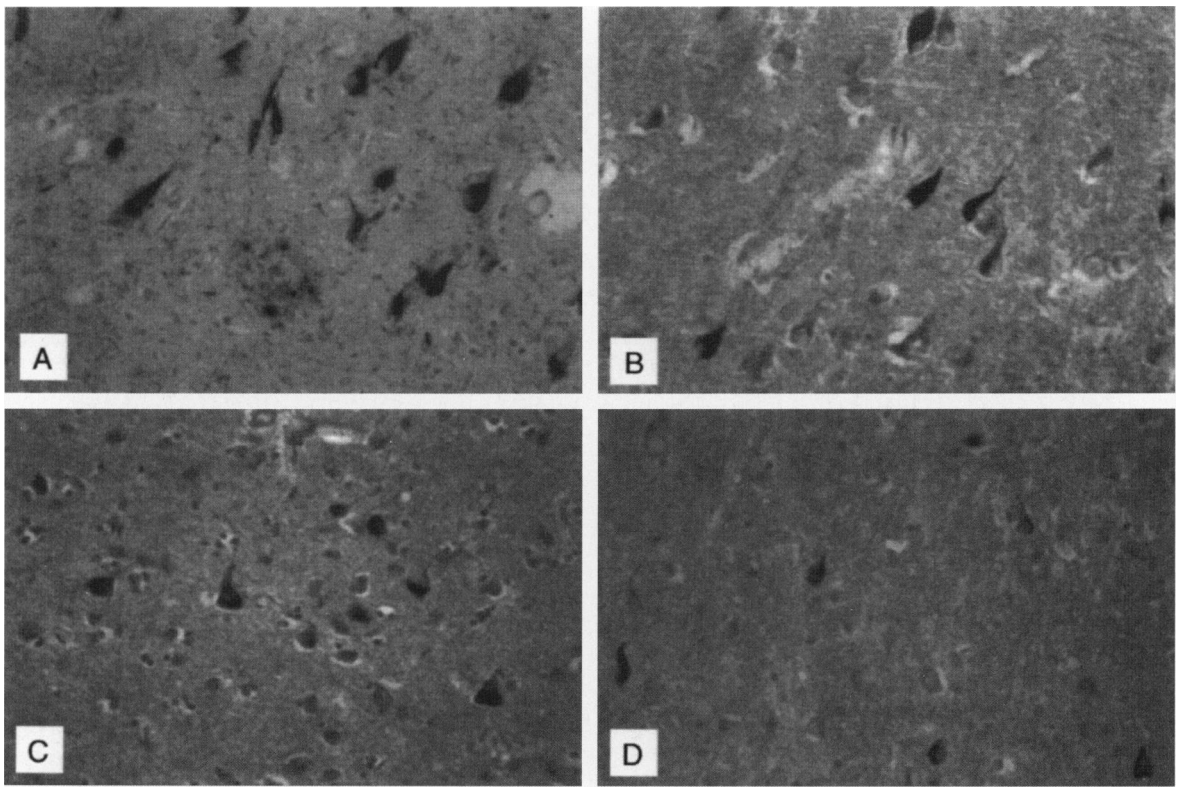

Figure 2. *PS immunoreactivity in AD brain.* **A** *and* **B:** *View (×100) of CA1-subiculum junction in an 89-year-old female with AD and cortical Lewy bodies.* **A:** *PHF antiserum.* **B:** *PS 2.3 antiserum. Note that the PHF antiserum labels plaque neurites and neuropil threads, whereas the PS reagent does not. Gold/silver immunostaining technique on formalin fixed tissue was used.* **C:** *Higher-power view (×100) of same cortical field shown in Figure 1F, showing labeling of intraneuronal globose and flame-shaped tangles with PS 2.3 antiserum.* **D:** *NFT in Ammon's horn from a 77-year-old female AD case labeled with PS 2.2 antiserum viewed at ×50. Gold/silver immunostaining technique was used.*

case were negative except for a few NFTs labeled by the PS antiserum in one of the elderly control brains.

The specificity of the PS antibodies was demonstrated in several ways. First, the immunoreactivity to NFT could be eliminated by prior treatment of either PS 2.2 or 2.3 antisera with cognate peptide followed by immunohistochemistry (Figure 3). Using Western blotting, the PS 2.2 serum reacted with a ~60-kd protein in extracts prepared from cultured CHO cells (Figure 4). This ~60-kd signal was reduced by treatment of the PS antibody with cognate peptide before Western blotting (data not shown). Recombinant expression of the human PS-1 cDNA in CHO cells results in the staining of a new major protein band at ~45 kd (Figure 4). In addition to the prominent endogenously and exogenously expressed PS proteins, the immune 2.2 serum uniquely identifies a set of proteins >100 kd. The apparent molecular weight of all of the proteins specifically reacting to the PS antisera in CHO cells is in agreement with the single report by Kovacs et al,[8] who describe a 50- to 55-kd and >100-kd set of recombinant PS proteins in COS cells after exogenous introduction of a tagged PS-2 human cDNA.

Discussion

Using antibodies directed to an epitope shared by PS-1 and PS-2, we have identified PS immunoreactivity with NFTs in AD brain. This reactivity was shown to be specific by several criteria: 1) three individual antisera displayed identical profiles by immunohistochemical analysis; 2) by Western blotting, the antibodies detect an endogenous CHO cell protein with a molecular weight consistent with that previously reported[8] as well as a newly acquired abundant protein of ~45 kd when the CHO cells are genetically engineered to express human PS-1; 3) no reactivity was seen with preimmune sera using immunohistochemical and Western blotting procedures; and 4) the immunoreactivity could be abolished by pretreatment of the PS antibodies with cognate peptide. Furthermore, the peptide sequence used to raise the PS antibodies was compared with known protein components of NFTs with no homology revealed. PS reactivity in NFTs may reflect the presence of intact PS proteins, a fragment of the molecule, or both. Curiously, only a subset of the NFTs identified by tau and PHF immunoreactivity were labeled by the PS

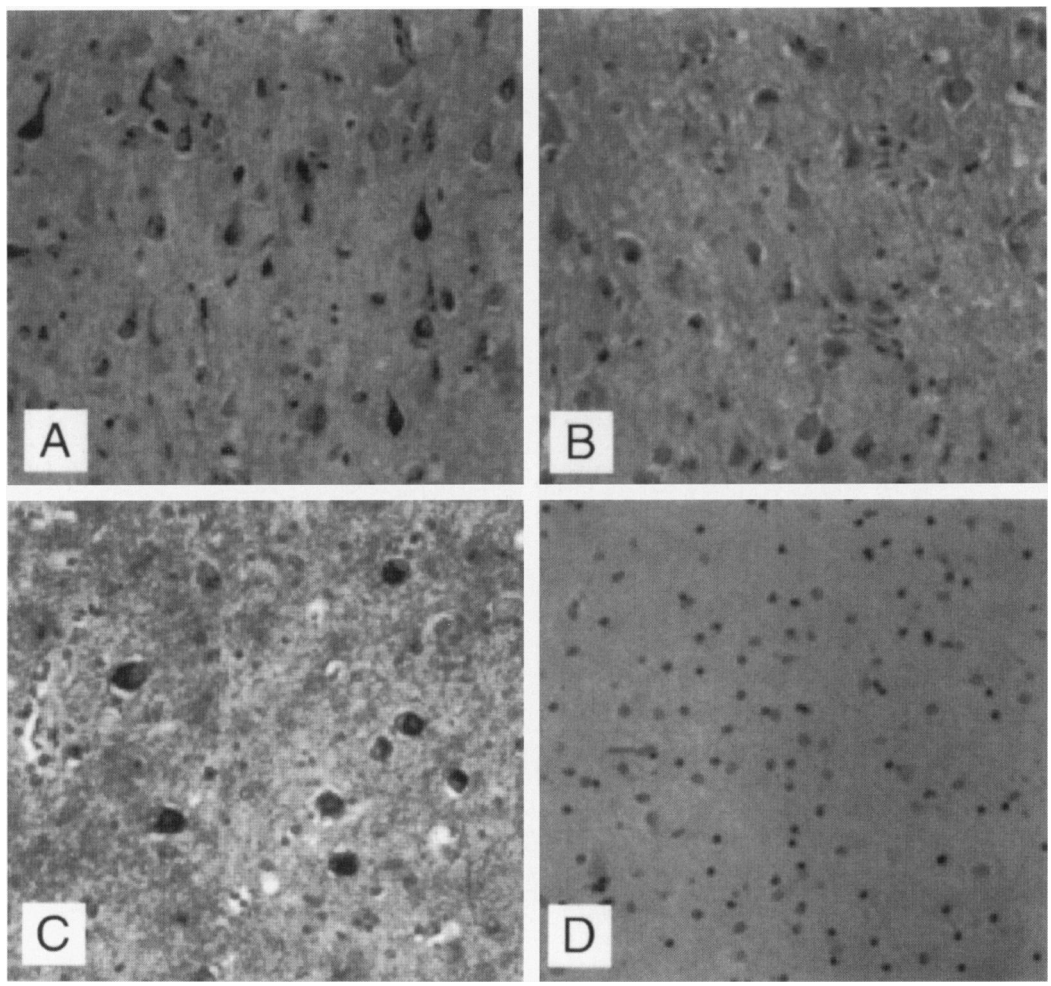

Figure 3. *Competition of NFT reactivity by cognate PS peptide.* A: *NFT identified by the 2.3 antiserum and peroxidase detection.* B: *Same as* A *but with 2.3 antiserum reacted with the synthetic peptide used to raise antibodies before immunohistochemistry.* C: *Globose tangles identified by the 2.2 antiserum and gold/silver detection.* D: *Same as* C *but with 2.2 antiserum reacted with cognate peptide before performing immunohistochemistry. All panels at* ×*100.*

antisera. This could suggest that PS proteins are expressed only in a subset of nerve cells that develop NFTs. Alternatively, this could reflect expression and/or accumulation of these proteins during a limited time interval during NFT development. To our knowledge, this is the first identification of NFT subtypes. We can only speculate as to the basis for the association between PS and NFT. Tau protein, the major constituent of NFTs, displays abnormal cellular localization in AD neurons apparently due to an aberrancy in protein transport. If PS proteins are involved in intracellular protein trafficking, a function exhibited by their homologue in *C. elegans*,[7] these two proteins may have direct interactions.

The absence of neuritic PS labeling is intriguing. Although dystrophic neurites and neuropil threads contain PHF and tau and were clearly labeled by antibodies directed against these epitopes in our material, they were not labeled with the PS antisera. Thus, the PS epitopes detected by our reagent appear to be limited to the neuronal cell body and proximal cell processes. The PS reactivity in AD brain may simply reflect the localization of PS protein to the endoplasmic reticulum and Golgi apparatus[8].

In contrast to a previous report[14], we did not detect PS reactivity in plaque amyloid deposits. In some AD cases, we observed faint plaque reactivity with the PS antisera, but similar labeling was seen with the preimmune sera, thereby reflecting a nonspecific interaction. It is important to note that the reagent used by Wisniewski et al[14] was directed against a different epitope on the PS-1 molecule than our reagents. It is possible that individual subdomains of PS proteins accumulate in different neuropathological entities in AD.

We observed that the pattern of immunolabeling by PS antisera in the chromosome-1- and chromosome-14-linked familial AD cases was no different

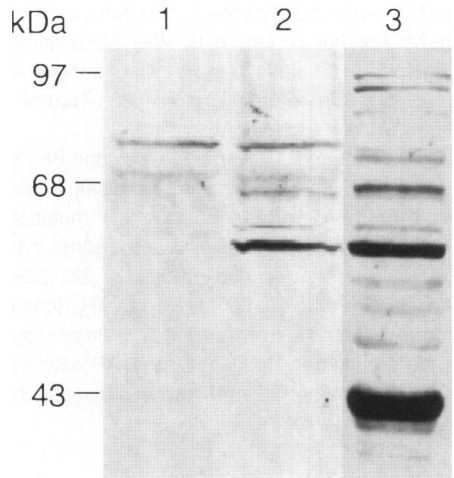

Figure 4. *Western blot of PS proteins. Protein from CHO cell lysates was analyzed by polyacrylamide gel electrophoresis and Western blotting using PS 2.2 preimmune serum* (lane 1) *and PS 2.2 immune antiserum* (lane 2). *A prominent endogenous ~60-kd protein and minor >100-kd proteins specifically react with the PS 2.2 antiserum. A prominent ~45-kd protein from CHO cell lysates expressing exogenously introduced human PS-1 was detected by the 2.2 antiserum by identical methods* (lane 3).

from that seen in the other AD material. As these familial cases are known to be caused by mutations in the PS genes, this finding may be unexpected. However, it should be noted that the neuropathology of the chromosome-1- and chromosome-14-linked cases is not different from that found in AD in general.[15,16] Similarly, the neuropathology of AD cases linked to mutations in the β-amyloid precursor protein gene is also unremarkable.[17,18] Thus, although mutations in PS or β-amyloid precursor protein genes predispose to an early onset of the disease, they do not appear to alter the fundamental histopathology. The qualitatively similar presence of PS protein in NFTs in cases with PS mutations and in other AD cases demonstrates that this is a common molecular feature of both sporadic and familial AD.

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