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Presenilin-1 Is Associated with Alzheimer's Disease Amyloid

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Mutations in presenilin (PS)-1 and -2, located on chromosome 14 and 1 respectively, are the major association with early-onset familial Alzheimer's disease (FAD). FAD has also been linked to mutations in the amyloid β precursor protein (βPP), and the presence of the apolipoprotein E4 allele is a risk factor for late-onset AD. The role of PS in FAD and in sporadic AD is unclear. We previously reported the presence of a PS-1 carboxyl-terminal epitope in neuritic plaques (Wisniewski T, Palha JA, Ghiso J, Frangione B: S182 protein in Alzheimer's disease neuritic plaques. Lancet 1995, 346:1366). In the present study, we examined a number of biochemically different cerebral and systemic amyloidoses, finding the PS-1 carboxy epitope only in association with amyloid β (Aβ) lesions. We confirm the presence of this epitope ultrastructurally in neuritic plaques. In addition, biochemical and amino acid sequence data are presented for an association of the 18-kd carboxy fragment of PS-1 with neuritic plaques with a start at residue 300. Three of the proteins with linkage to AD have now been found as components of neuritic plaques. It remains to be determined whether all of these proteins are involved in the same or different pathological pathway(s) and which of these proteins is the most important for the common, late-onset form of AD. ***(Am J Pathol 1997, 151:601–610)***

Alzheimer's disease (AD) is the most common cause of late-life dementia and is characterized neuropathologically by the presence of neurofibrillary tangles and the deposition of amyloid β (Aβ) in the form of neuritic plaques and congophilic angiopathy.[1] AD can be divided into an early-onset form (onset before 60 years of age) and the more common late-onset (after 60 years) form. So far, three genes have been linked to early-onset AD, including the β-amyloid precursor protein (βPP) on chromosome 21,[2,3] presenilin (PS)-1 on chromosome 14,[4] and PS-2 on chromosome 1.[5,6] The majority of early-onset AD is thought to be related to mutations in PS-1 and -2. To date, over 35 mutations have been reported in PS-1 and 2 in PS-2 among familial early-onset AD pedigrees.[7,8] Recently we have found a novel PS-1 mutation (P117L) in a Polish early-onset AD kindred (unpublished observations). How presenilin is involved in the pathogenesis of AD remains unclear. As the neuropathological features of PS-linked familial AD (FAD) is similar to the more common late-onset AD, it is presumed that understanding the role of PS in FAD will have implications for elucidating the pathology of all forms of AD.

The PS-1 gene encodes a predicted protein of 467 amino acids with six to nine putative transmembrane domains.[7] PS-1 mRNA has been found in many different tissues and cell lines, with high levels in the brain and neurons.[4,9,10] In the normal brain, immunohistochemical studies have shown the presence of this protein predominantly in neuronal cells, where it is preferentially concentrated in the cytoplasm and dendrites.[11–14] Studies of PS-1 processing in cell lines and in brain tissue have shown that a portion of PS-1 undergoes endoproteolytic cleavage to at least two major fragments: an 18-kd carboxy fragment and a ~28-kd amino terminal fragment.[15]

In 1995 we reported that an antibody raised to the carboxy terminus of PS-1 immunoreacted with some neuritic plaques.[16] This immunoreactivity was found in neuritic plaques of both PS-1-linked FAD patients and in late-onset, sporadic AD, suggesting a general role for presenilin in AD. Since then, other reports using antibodies raised to residues in the 18-kd carboxy fragment of PS-1 have immunohistochemically confirmed the presence of PS in association with the amyloid of AD neuritic plaques.[11,12,17] However, numerous proteins have been reported to be associated with all or many of the biochemically distinct forms of amyloid deposits. In this report we have extended these observations and immunohistochemically studied a number of biochemically distinct cerebral and systemic amyloidoses to determine whether the carboxyl-terminal PS immunoreactivity is specific for Aβ-related deposits or is present in all types of systemic and/or cerebral amyloid deposits. In addition, we have documented the immunoelectron microscopic co-localization of Aβ and PS-1 in AD neuritic plaques. A recent report has also documented anti-PS-1 immunoreactivity associated with neurofibrillary tangles, using an

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antibody to residues 263 to 280, which is on the amino-terminal fragment of PS-1.[18] Given the large size of the PS-1 protein, its cleavage into at least two major fragments and the possible existence of several homologous presenilin proteins, it is not surprising that antibodies to different PS epitopes may show distinct distributions. However, these somewhat diverse reports of immunohistochemical localization underscore the need for biochemical studies to confirm these observations. Therefore, in this study we have also purified Aβ from neuritic plaques and identified the co-purifying carboxyl PS-1 fragment by amino-terminal peptide sequencing to confirm our immunohistochemical observations.

Materials and Methods

Tissue Samples

Paraffin-embedded 6-μm tissue sections fixed in formalin or PLP or frozen tissue sections were obtained from the following patients: 10 AD patients (8 late-onset, sporadic AD patients and 2 with linkage to PS-1 on chromosome 14), 5 patients with Down's syndrome (DS; ages at death, 65, 60, 58, 50, and 35 years), 5 patients with hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D),[19] 5 patients with familial amyloid polyneuropathy, type 1 (FAP) with leptomeningeal amyloid deposits, 1 patient with Hungarian amyloidosis,[20] 2 patients with hereditary cerebral hemorrhage with amyloidosis, Icelandic type (HCHWA-I),[21] 2 cases with Gerstmann-Straussler syndrome (GSS), 1 cases of Creutzfeldt-Jacob disease with amyloid deposition, 1 case of familial amyloidosis, Finnish type,[22] 3 cases of light-chain-related systemic amyloidosis, and 3 cases with amyloid A (AA)-related systemic amyloidosis. Brain tissue sections from three aged normal control patients were also analyzed. The GSS, Hungarian amyloidosis, and FAP tissue was obtained from sources listed in the acknowledgments. All the rest of the cases were obtained through the Departments of Pathology at New York University Medical Center or the Department of Neuropathology at Columbia-Presbyterian Medical Center. All patients had complete neuropathological diagnostic evaluations. For the cases of AD, DS, HCHWA-D, HCHWA-I, Hungarian amyloidosis, GSS, Creutzfeldt-Jacob disease, and FAP, sections of the frontal cortex, occipital cortex, hippocampus, and cerebellum were examined. For the cases of familial amyloidosis, Finnish type and light-chain and AA systemic amyloidoses, kidney sections were examined. For the biochemical studies of AD neuritic plaques, frozen brain tissue was used from three AD patients, ages 72, 75, and 80 years at death, from the brain bank at Columbia-Presbyterian Medical Center.

Antibodies

Synthetic peptides were made by custom order by Research Genetics (Huntsville, AL) and coupled to multiple antigen peptide resin for immunization of two rabbits per peptide. Peptides corresponding to the following residues of PS-1 were used for immunization: residues 48 to 70 (anti PS-1–48), residues 331 to 355 (anti PS-1–331), residues 395 to 410 (anti PS-1–395), and residues 448 to 467 (anti PS-1–448; see Figure 1). The sequence for PS-1–48 and PS-1–331 is not homologous to PS-2, whereas the sequence of PS-1–448 and PS-1–395 are homologous to PS-2. Sera were analyzed for reactivity to the peptide immunogens by enzyme-linked immunosorbent assay and for reactivity to the intact PS-1 by Western blotting. All antibodies were subject to affinity purification against the peptide conjugated to epoxy-activated Sepharose 6B (Pharmacia, Uppsala, Sweden) following the manufacturer's instructions. To test for reactivity to intact PS-1, total protein lysates were harvested from human kidney 293 cells stably transfected with human PS-1 and control, untransfected cells from two different laboratories (kindly provided by Dr. S. A. Sisodia, John Hopkins University, Baltimore, MD, and Dr. N. Robakis, Mount Sinai Medical Center, New York, NY).[15]

Immunohistochemistry

The deparaffinized tissue sections were pretreated with 98% formic acid for 1 hour, followed by quenching of endogenous peroxidase activity with 0.3% H_20_2 in methanol and blocked in 10% fetal calf serum in phosphate buffer, pH 7.4, 0.05% Tween-20 for 1 hour at room temperature. The anti-PS-1–48, -PS-1–331, -PS-1–395, and -PS-1–448 antibodies were diluted in the same buffer at 1:100. To assess Aβ immunoreactivity, monoclonal 4G8 antibodies were used.[23] The tissue sections were incubated with primary antibodies overnight at 4°C, followed by application of secondary biotinylated species-specific antibodies (Amersham Corp., Arlington Heights, IL) and horseradish-linked streptavidin (Sigma Chemical Co., St. Louis, MO) in the same buffer as above. Sections were developed with 0.01% 3,3′-diaminobenzidine in phosphate buffer, with or without cobalt hexachloride ions (0.005%; both reagents from Sigma). For controls, sequential sections were incubated with the preimmune serum or with primary antibody preabsorbed with the specific peptide. Some sequential sections were also Congo red stained to determine whether the Aβ-immunoreactive lesions were pre-amyloid. We defined pre-amyloid lesions as Aβ-immunoreactive lesions that, on adjacent Congo-red-stained sections, did not give the characteristic apple-green birefringence of amyloid when viewed under polarized light.[24]

Immunoelectron Microscopy

Immunoelectron microscopy was performed according to a modification of Phend et al.[25] Small pieces of frontal cortex were fixed in 0.5% depolymerized paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.2) for 12 hours in the cold. Pieces were washed in buffer and post-fixed for 1 hour with 1% osmium oxide in the same buffer. Osmicated pieces were rinsed in buffer, dehydrated in graded series of ethanols into propylene oxide, infiltrated with Epon 812, and poly-

merized at 60°C. Thin sections were cut with a diamond knife. Grids were floated for 2 hours on drops of 4% sodium metaperiodate in phosphate-buffered saline (PBS), followed by blocking with 1% bovine serum albumin in PBS for 2 hours. The grids were then incubated in PS-1–448 at 1:50 overnight in a moist chamber, followed by several washes in Tris-buffered saline with 0.05% Tween-20 (TBST). Next, protein A-gold 5-nm particles 1:50 were applied for 2 hours, followed by washing. The grids were then incubated in paraformaldehyde vapors and blocked again with 1% bovine serum albumin/PBS. 4G8 antibody was then applied in the same buffer at 1:20 and incubated overnight. After several washes, anti-mouse IgG conjugated to 15-nm gold particles were applied for 2 hours. The grids were then washed, dried, and stained with 1% uranyl acetate and Reynolds lead citrate.

Amyloid Extraction

From three late-onset, sporadic AD patients separate neuritic plaque extractions were performed as previously described.[26] In brief, approximately 30 g of frontal cortex was dissected free of large-vessel contamination, followed by homogenization in a Dounce homogenizer in 5 vol of 20 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.02% sodium azide, 200 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin at 4°C and centrifuged at 100,000 × *g* for 1 hour. The pellets were rehomogenized in a Dounce homogenizer in 10 vol of TB buffer (0.1 mol/L Tris/HCl, pH 7.4, 5 mmol/L EDTA, with sodium azide and protease inhibitors as above). This solution was filtered through a series of nylon meshes to remove small-vessel contamination, followed by centrifugation at 135,000 × *g* for 2 hours at 20°C. The pellet was washed in 0.1 mol/L Tris/HCl, pH 8.0, four times and digested by collagenase (CLS-3; Worthington; 0.2 mg/ml) and DNAseI (Worthington; 10 μg/ml) in the presence of 2 mmol/L $CaCl_2$ for 16 hours at 37°C. After digestion, the material was pelleted at 5000 × *g* and washed in 0.1 mol/L Tris/HCl, pH 8.0, four times. The pellet was then suspended in 1 mol/L sucrose and layered over a discontinuous density gradient of 1.8, 1.2, 1.15, 1.1, and 1.05 mol/L sucrose. The 1.2/1.8 mol/L sucrose was amyloid enriched by Congo red staining. This material was washed in distilled water with protease inhibitors and resuspended in 0.32 mol/L sucrose with protease inhibitors and 1% sodium dodecyl sulfate (SDS). The sucrose was brought to 1 mol/L and layered over a discontinuous gradient of 2.2 and 1.1 mol/L sucrose, followed by centrifugation at 5000 × *g* for 1 hour. The amyloid was found mainly at the 1.1/2.2 mol/L interface. All steps of the purification procedure were followed by Congo red staining. The purified amyloid fraction was assessed for the presence of Aβ peptide and PS-1 by SDS-PAGE and immunoblotting. A portion of the amyloid fraction was lyophilized and solubilized in Laemmli sample buffer under reducing conditions. Samples were run both boiled and nonboiled on a 12% Tris Tricine SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system. The separated proteins were transferred onto a nitrocellulose membrane by electroblotting in Tris/glycine buffer with 10% methanol for 90 minutes at a constant current of 450 mA. The membranes were blocked by 5% nonfat milk in 150 mmol/L NaCl, 20 mmol/L Tris, 0.05% Tween-20, pH 7.4 (TBST) and probed with anti-PS-1–331 and monoclonal antibody 4G8 (directed against residues 17 to 24 of Aβ).[23] After incubation with horseradish-peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin, antibody binding was visualized with an enhanced chemiluminescence detection system (ECL, Amersham Corp. Arlington Heights, IL).

Immunoprecipitation and Amino Acid Sequencing Studies

The purified amyloid was subject to suspension in 6 vol of 1% SDS, PBS with protease inhibitors as above. This suspension was mixed end over end overnight at 4°C followed by centrifugation at 1000 × *g*. To the supernatant 50 μl of affinity-purified anti-PS-1–331 antibodies were added, followed 1 hour later by 50 μl of anti-rabbit conjugated magnetic beads (Dynal Lake Success, NY). This was mixed for 16 hours at 4°C. The beads were separated with a magnet, followed by five washes in PBS, 0.05% Tween-20 (PBST) and one in PBS. In addition to anti-PS-1–331, peptide-absorbed anti-PS-1–331, preimmune serum, and a mixture of monoclonal anti-Aβ antibodies 4G8/6E10[23] were used for immunoprecipitation. The monoclonal antibodies were precipitated with anti-mouse conjugated magnetic beads (Dynal). The immunoprecipitated protein was separated by 12% Tris Tricine SDS-PAGE as above and electrotransferred to either nitrocellulose or Immobilin P (Millipore, Bedford, MA). For Western blotting, the nitrocellulose membranes were blocked with 5% nonfat milk and probed with either anti-PS-1–331 or 4G8 overnight at 4°C. Fluorograms were prepared as described above. To identify the 18-kd band that was immunoprecipitated with the anti-PS-1–331 antibody and was also recognized by this antibody on fluorograms, the 18-kd band was cut out from Coomassie-stained, Immobilin P membranes and subject to NH_2-terminal sequencing on a 477A protein sequencer, and the resultant phenylthiohydantoin derivatives were identified using an on-line phenylthiohydantoin derivative analyzer (Applied Biosystems, Foster City, CA).

Results

Immunohistochemistry

Four antisera were raised against PS-1 homologous synthetic peptides, described in the Materials and Methods. These sera are referred to as anti-PS-1–48, anti-PS-1–331, anti-PS-1–395, and anti-PS-1–448 (see Figure 1). The best immunohistochemical results were obtained with these antibodies when formic acid pretreatment was used on the sections. No immunostaining was obtained when preimmune serum was used or when peptide-absorbed antibodies were used. Both anti-PS-1–395 and

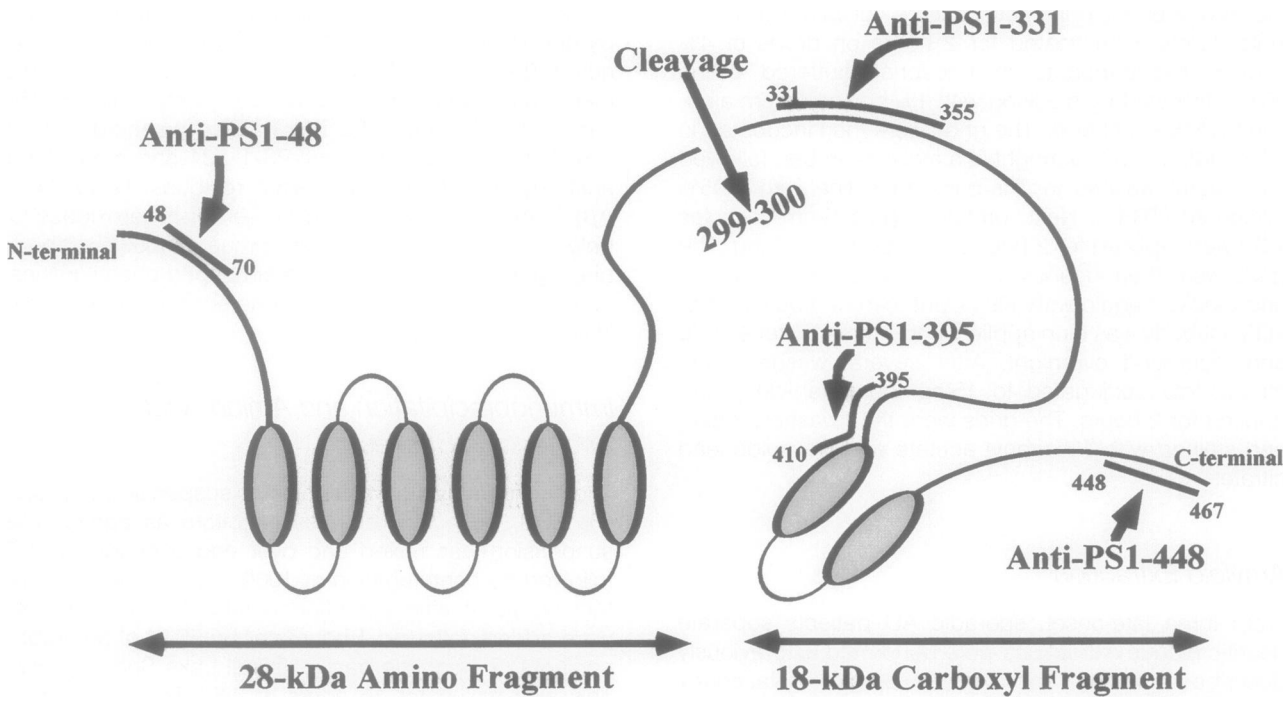

Figure 1. PS-1 residues that were used to produce our antibodies and the cleavage site of PS-1, which we determined by amino-terminal sequencing of the 18-kd PS-1 fragment isolated from AD neuritic plaque. Each ellipse represents a putative PS-1 transmembrane domain. Eight transmembrane domains are shown; however, this topology remains to be clarified.[46,47,48]

anti-PS-1–448 immunoreacted with approximately 30 to 40% (Figure 2) of neuritic plaques. This immunoreactivity included the amyloid core as well as amyloid in the vicinity of the core. This co-localized with the immunoreactivity seen with the monoclonal anti-Aβ antibody 4G8 (Figure 2, A–C). The immunoreactivity seen with the 4G8 antibody was always more intense than that seen with the anti-PS-1–448. The anti-PS-1–448 gave better immunoreactivity than anti-PS-1–395. Both antibodies immunoreacted faintly with the cytoplasm of neurons. No difference was noted in the immunoreactivity with these antibodies in the two cases of early-onset FAD with linkage to chromosome 14 *versus* the late-onset sporadic AD cases. The anti-PS-1–448 also immunoreacted with occasional pre-amyloid deposits, which is seen in sections of HCHWA-D (Figure 2, D–F). The anti-PS-1–395 did not immunoreact with pre-amyloid deposits. The anti-PS-1–448 and the anti-PS-1–395 also immunoreacted with 30 to 40% of Aβ cerebral vessel deposits, which was best seen in HCHWA-D sections (Figure 2, D–F) but was also noted in the congophilic angiopathy of AD (data not shown). The anti-PS-1–448 or anti-PS-1–395 did not react with non-Aβ-related cerebral vessel deposits (Figure 2, G and H), cerebral parenchymal deposits, or systemic amyloid deposits. The lack of immunoreactivity in a case of HCHWA-I is illustrated in Figure 2, G and H. Anti-PS-1–48 and anti-PS-1–331 did not immunoreact with Aβ amyloid deposits or other types of amyloid deposits. Both of these antibodies immunoreacted with the cytoplasm of neurons as well as the proximal segment of basal and apical dendrites (Figure 2I). Axonal staining was not present. In addition, occasional immunoreactivity of astrocytes was noted (data not shown). Immunoreactivity of neurofibrillary tangles was not observed with any of these anti-PS-1 antibodies.

Immunoelectron Microscopy

Immunoelectron microscopy was done with the anti-PS-1–448 and the monoclonal anti-Aβ antibody 4G8.[23] In Figure 3A, the extensive labeling of extracellular neuritic plaque amyloid in a sporadic, late-onset AD case with the anti-PS-1–448 (5-nm gold particles; see arrows) is seen. This co-localized with the labeling with 4G8 (15-nm gold particles; see arrowheads). This contrasts with the few 5-nm gold particles noted when single labeling was done with peptide-absorbed anti-PS-1–448 on similar tissue from the same case (see Figure 3B).

Western Blot and Amino Acid Sequencing Studies

The anti-PS-1–48, anti-PS-1–331, and anti-PS-1–448 affinity-purified antibodies each recognized PS-1 from transfected cell lysates on Western blots. The anti-PS-1–331 gave the best results on Western blots, whereas the anti-PS-1–395 did not work well for Western blotting. In Figure 4A, lanes 1, 3, and 5, 25 μg of cell lysates from human 293 cells transfected with full length PS-1 was run. A 50-kd band is evident with the anti-PS-1–48 (Figure 4A, lane 1), the anti-PS-1–448 (Figure 4A, lane 3), and the anti-PS-1–331 (Figure 4A, lane 5). As can be appreciated, the anti-PS-1–331 gave the most intense reaction. In addition to the 50-kd full-length band, the anti-448 and the anti-PS-1–331 antibodies detected a carboxy cleav-

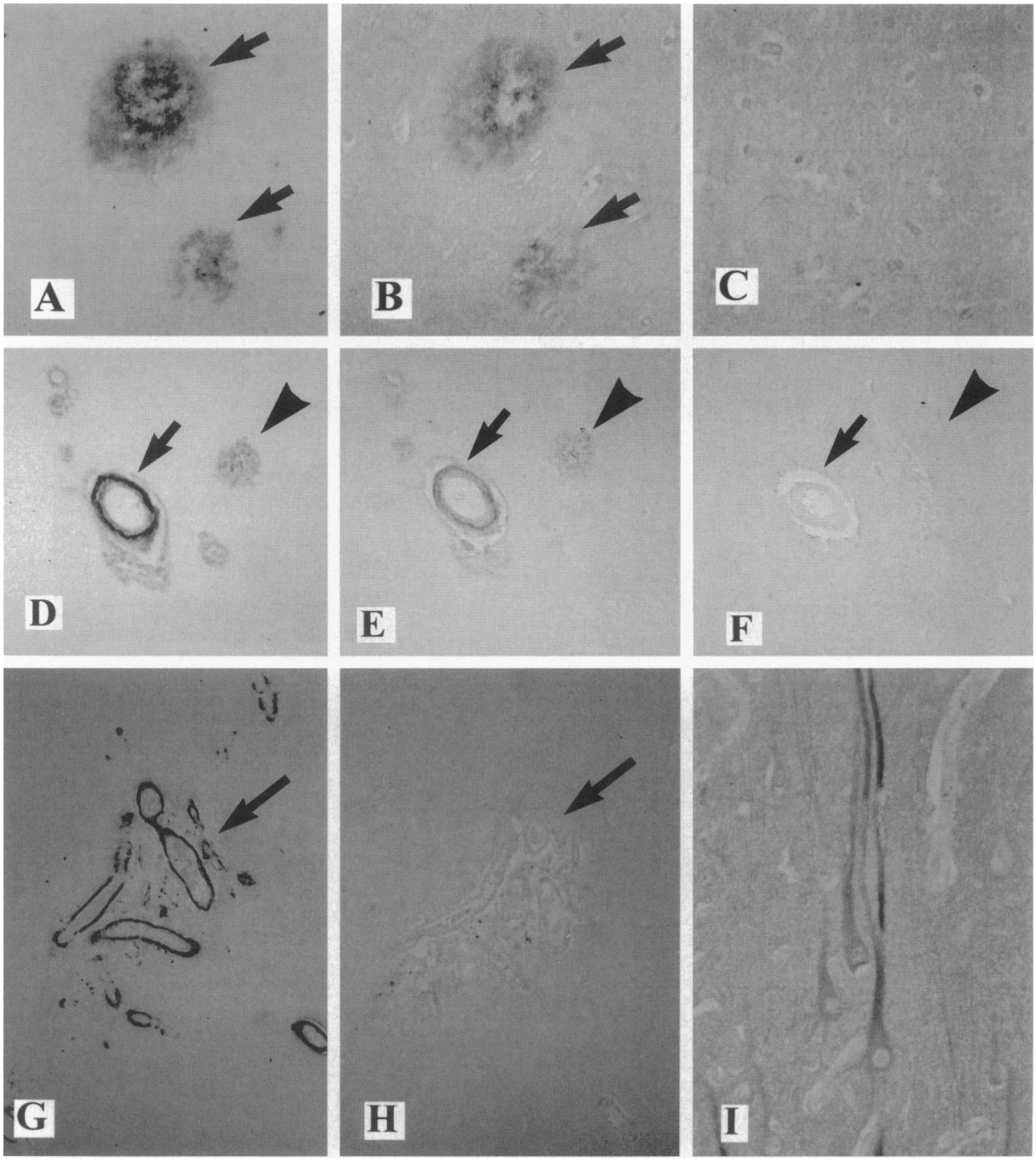

Figure 2. Immunohistochemical studies with anti-PS-1 antibodies. **A** to **C**: Sequential sections of neuritic plaques of a late-onset, sporadic AD patient immunoreacted with monoclonal anti-Aβ antibody 4G8 (**A**), anti-PS-1–448 (**B**), and peptide-absorbed anti-PS-1–448 (**C**). The **arrows** show two neuritic plaques in **A** and **B**, which are not seen in the control (**C**). Original magnification, ×400. **D** to **F**: Sequential sections from a HCHWA-D patient, immunoreacted with 4G8, anti-PS-1–448, and peptide absorbed anti-PS-1–448, respectively. Original magnification, ×200. The **arrow** shows an amyloid-laden vessel, with is stained in **D** and **E**. The **arrowhead** shows a pre-amyloid lesion (Congo red negative in a sequential section, data not shown), which is stained by 4G8 (**D**) and anti-PS-1–448 (**E**). **G** and **H**: Sequential sections from a patient with HCHWA-I, immunoreacted with anti-cystatin C (**G**) and anti-PS-1–448 (**H**). Original magnification, ×100. **I**: Section of frontal cortex from a non-AD, aged control patient, immunoreacted with anti-PS-1–331, showing neuronal cytoplasmic and dendritic staining. Original magnification, ×400.

age fragment of PS-1 at 18 kd (Figure 4A, lanes 3 and 5), which was not detected by the anti-PS-1–48 antibody (Figure 4A, lane 1). This band is much more clearly seen with the anti-PS-1–331. Twenty-five micrograms of protein from control untransfected 293 cell lysates was run in Figure 4A, lanes 2, 4, and 6. This was reacted with the affinity-purified anti-PS-1–48, anti-PS-1–448, and anti-PS-1–331, respectively. The anti-PS-1–48 and anti-PS-1–

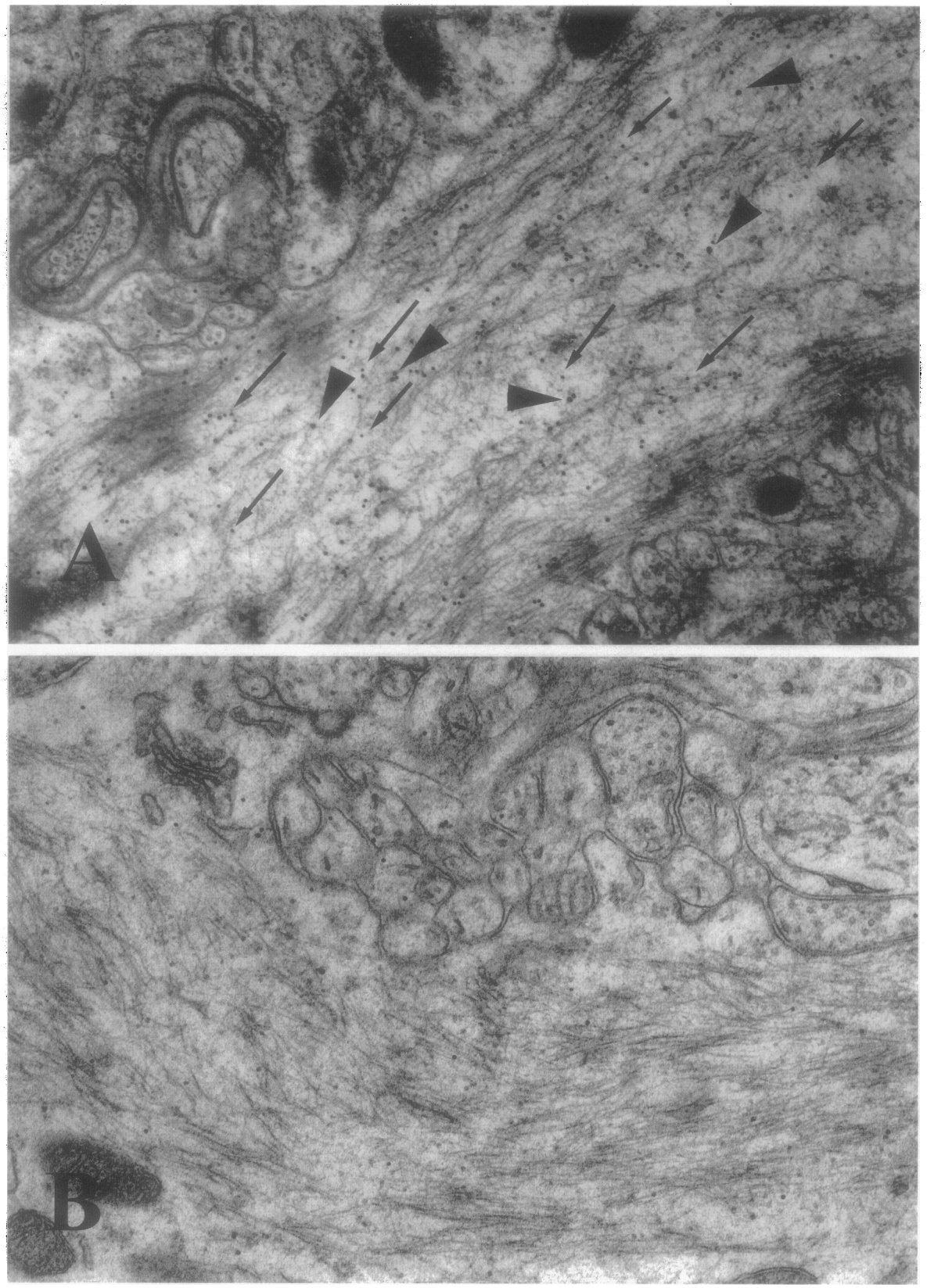

Figure 3. A: Electron micrographs of neuritic plaque amyloid immunoreacted with anti-PS-1–448 (visualized with 5-nm gold particles, see **arrows**) and monoclonal anti-Aβ 4G8[23] (visualized with 15-nm gold particles, see **arrowheads**). Original magnification, ×36,000. Most of the immunogold particles are on amyloid fibrils and not on surrounding structures, indicating the specificity of the immunolabeling. **B:** Control experiment, where peptide-absorbed anti-PS-1–448 was used to label the amyloid, from the same tissue used in **A**. Only rare 5-nm gold particles are evident. Original magnification, ×36,000.

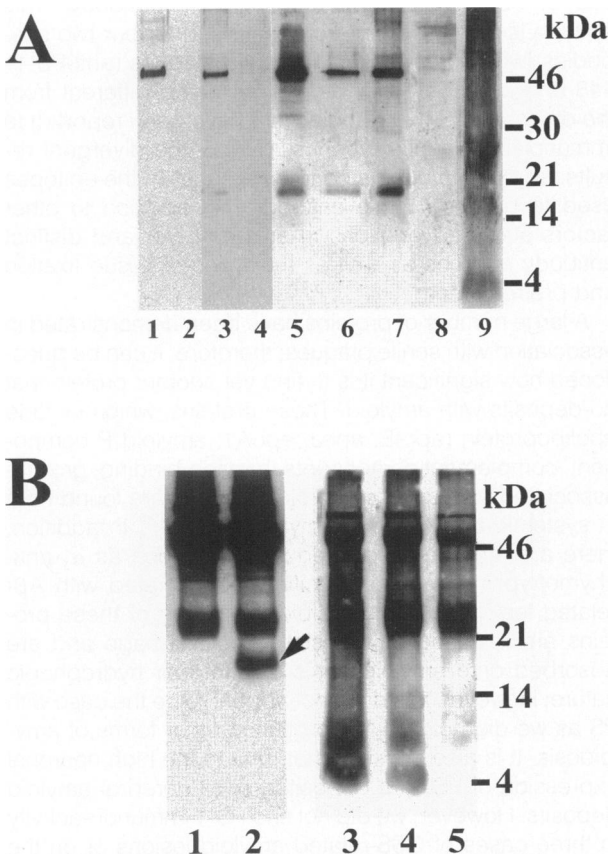

Figure 4. A: Western blots using anti-PS-1 antibodies. In **lanes 1, 3,** and **5,** 25 μg of cell lysates from 293 cells transfected with full-length PS-1 was run. A 50-kd band is evident with the anti-PS-1–48 (**lane 1**), the anti-PS-1–448 (**lane 3**), and the anti-PS-1–331 (**lane 5**). As can be appreciated, the anti-PS-1–331 gave the most intense reaction. In addition to the 50-kd full-length band, the anti-448 and the anti-PS-1–331 antibodies detected a carboxy degradation fragment of PS-1 at 18 kd (**lanes 3** and **5**). This band is much more clearly seen with the anti-PS-1–331. In **lanes 2, 4,** and **6,** 25 μg of protein from control untransfected 293 cells lysates was run. This was reacted with the affinity-purified anti-PS-1–48, anti-PS-1–448, and anti-PS-1–331, respectively. The anti-PS-1–48 and anti-PS-1–448 did not recognize any protein in the control cell lysate, whereas the anti-PS-1–331 with its higher sensitivity was able to detect lower levels of the full-length and 18-kd carboxy fragment in the wild-type cells. Due to the greater sensitivity of the anti-PS-1–331, it was used for the rest of the Western blot studies. Approximately 5 μg of the Aβ purified from neuritic plaques, as described in Materials and Methods was run in **lanes 7, 8,** and **9**. In **lane 7**, the primary antibody was anti-PS-1–331. In **lane 8**, the primary antibody was peptide-absorbed anti-PS-1–331. In **lane 9**, the amyloid material was reacted with 4G8. With the 4G8 monoclonal antibody, it can be seen (**lane 9**) that most of this material consisted of aggregated Aβ as well as some monomeric 4-kd Aβ. This purified Aβ also contained the 18-kd carboxy fragment of PS-1 as well as some 50-kd PS-1-immunoreactive material (**lane 7**). The 50- and 18-kd bands evident in the amyloid material, seen in **lane 7** are specific as they are not present when the same material was reacted with peptide-absorbed anti-PS-1–331 (**lane 8**). **B:** Western blot from the immunoprecipitation experiments. The neuritic plaque amyloid extracted material was suspended in 1% SDS/PBS, as described in Materials and Methods. This material was pelleted, and the supernatant was immunoprecipitated with anti-PS-1–331. This immunoprecipitated material was separated on SDS-PAGE and in **lanes 1** and **2** was immunoreacted with the anti-PS-1–331, revealing a specific 18-kd band seen in **lane 2** (see **arrow**). When preimmune serum was used for the immunoprecipitation, no 18-kd band is evident (**lane 1**). In **lanes 1** and **2**, the same strong 23- and 50-kd bands are seen, which represent heavy and light chain of IgG. The 18-kd band seen in **lane 2** was also transferred to Immobilin membranes and subject to amino-terminal amino acid sequencing. The following sequence was obtained: EGDPE. This corresponds to residues 300 to 304 of PS-1. The supernatant of the neuritic plaque amyloid material suspended in SDS/PBS was also immunoprecipitated with monoclonal anti-Aβ antibodies 4G8/6E10, peptide-absorbed anti-PS-1–331, and preimmune serum from the rabbit used to produce anti-PS-1–331. This material was immunoblotted with anti-Aβ monoclonal antibody 4G8.[23] The Western blot of the amyloid supernatant immunoprecipitated with 4G8/6E10 is seen in **lane 3**, whereas the supernatant immunoprecipitated with anti-PS-1–331 and preimmune serum is seen in **lanes 4** and **5**, respectively. The 4- and 8-kd bands corresponding to Aβ, which were immunoprecipitated with 4G8/6E10 and detected with 4G8, are evident in **lane 3**. Similar but less intense bands are seen in **lane 4**, which were immunoprecipitated with anti-PS-1–331 and detected with 4G8. These bands are specific as they were not obtained when the preimmune serum was used for the immunoprecipitation, as seen in **lane 5**.

448 did not recognize any protein in the control cell lysate, whereas the anti-PS-1–331 with its higher sensitivity was able to detect lower levels of the full-length and 18-kd carboxy fragment in the wild-type cells. Due to the greater sensitivity of the anti-PS-1–331, it was used for the rest of the Western blot studies. The observations that untransfected cell lysates were not reactive with either anti-PS-1–48 or anti-PS-1–448, whereas the reactivity with anti-PS-1–331 was several-fold greater in transfected cells, provides strong evidence for each of these antibodies being specific for PS. Approximately 5 μg of the Aβ purified from neuritic plaques, as described in the Materials and Methods was run in Figure 4A, lanes 7 to 9. In lane 7, the primary antibody was anti-PS-1–331. In lane 8, the primary antibody was peptide-absorbed anti-PS-1–331. In lane 9, the amyloid material was reacted with 4G8. With the 4G8 monoclonal antibody, it can be seen (Figure 4A, lane 9) that most of this material consisted of aggregated Aβ as well as some monomeric 4-kd Aβ. This purified Aβ also contained the 18-kd carboxy fragment of PS-1 as well as some 50-kd PS-1-immunoreactive material (Figure 4A, lane 7). The 50- and 18-kd bands evident in the amyloid material (Figure 3A, lane 7) are specific as they are not present when the same material was reacted with peptide absorbed anti-PS-1–331 (Figure 4A, lane 8).

The neuritic plaque amyloid extracted material was suspended in 1% SDS/PBS, as described in Materials and Methods. Supernatant was immunoprecipitated with anti-PS-1–331, and the immunoprecipitate was separated on SDS-PAGE, followed by immunoblotting with the anti-PS-1–331. This revealed a specific 18-kd band seen in Figure 4B, lane 2 (see arrow). When preimmune serum was used for the immunoprecipitation, no 18-kd band was evident (Figure 4B, lane 1). In Figure 4B, lanes 1 and 2, the same strong 23- and 50-kd bands are seen, which represent heavy and light chains of IgG. The 18-kd band seen in Figure 4B, lane 2, was also transferred to Immobilin membranes and subjected to amino-terminal amino acid sequencing. The following sequence of low yield was obtained: EGDPE. This corresponds to residues 300 to 304 of PS-1. This is very similar to the recently reported major amino-terminal sequence of the carboxy fragment of PS-1 from cells in tissue culture, which started at residue 299 of PS-1.[27] It was also attempted to obtain amino acid sequence data for the 50-kd anti-PS-1–331 immunoreactive band from the amyloid purified from neuritic plaques, seen in Figure 4A, lane 7. However, no sequence data was obtained from this source either due to insufficient material or the amino terminus being blocked.

In addition, we show that the anti-PS-1–331 immunoprecipitate also contained Aβ. The amyloid fraction isolated from neuritic plaques was suspended in 1% SDS/

PBS and immunoprecipitated with one of the following: monoclonal anti-Aβ 4G8/6E10, anti-PS-1–331, peptide-absorbed anti-PS-1–331, or preimmune serum from the rabbit used to produce anti-PS-1–331. This material was immunoblotted with anti-Aβ monoclonal antibody 4G8.[23] The Western blot of the amyloid supernatant immunoprecipitated with 4G8/6E10 is seen in Figure 4B, lane 3, whereas the supernatant immunoprecipitated with anti-PS-1–331 and preimmune serum is seen in lanes 4 and 5, respectively. Clear 4- and 8-kd bands corresponding to Aβ, which were immunoprecipitated with 4G8/6E10 and detected with 4G8, are evident in Figure 4B, lane 3. Similar but less intense bands are seen in Figure 4B, lane 4, which were immunoprecipitated with anti-PS-1–331 and detected with 4G8. These bands are specific as they were not obtained when the preimmune serum was used for the immunoprecipitation, as seen in Figure 4B, lane 5. Other control antibodies were also used for immunoprecipitation under these conditions, such as rabbit anti-apoA1 (data not shown). These antibodies did not immunoprecipitate either Aβ or PS.

Discussion

We have demonstrated the association of a PS-1 carboxyl-terminal fragment with AD-related amyloid deposits. This finding is specific by several criteria. First, we found immunohistochemically that two different anti-PS carboxy fragment antibodies label neuritic plaques and that this could be absorbed using the specific synthetic peptides to which the antibodies were raised. Other groups have also demonstrated the immunoreactivity of a portion of AD neuritic plaques (from 20 to 50%) using other anti-PS-1 carboxy fragment antibodies.[11,12,17] Second, we have co-localized Aβ and carboxy fragment PS-1 epitopes on AD neuritic plaque amyloid fibrils ultrastructurally. Third, our biochemical studies of partially purified neuritic plaque amyloid have confirmed the presence of a carboxy fragment of PS-1, as determined by amino-terminal amino acid sequencing. The sequence we obtained is very similar to a recent study in which it was found that the main cleavage site of PS-1 occurs between residues 298 and 299, in cells transfected with human PS-1.[27] Fourth, we have found that anti PS-1–331 antibodies can immunoprecipitate Aβ along with the carboxy 18-kd PS-1 fragment from neuritic plaque amyloid that is partially dissolved in SDS buffer. This immunoprecipitation is specific in that preimmune serum or peptide-absorbed anti-PS-1–331 do not precipitate Aβ or the carboxy fragment of PS-1 under the same conditions. The above provides extensive evidence for the presence of PS within neuritic plaques. However, some other reports using anti-PS-1 antibodies have not found immunoreactivity in neuritic plaques.[14,18] In the paper by Weber et al,[14] an antibody raised to PS-1 residues 331 to 350 was used, which is very similar to our anti-PS-1–331. Our anti-PS-1–331 also does not immunoreact with neuritic plaques, with the immunoreactivity being present mainly in neuronal cytoplasm, in agreement with this previous report. In the report by Murphy et al,[18] residues 263 to 280 of PS-1 were used to raise their antibody. This epitope is quite different from that used for our two antibodies, which do react with neuritic plaques (anti-PS-1–448 and anti-PS-1–395) as well as being different from the other anti-PS-1 antibodies that have been reported to immunolabel neuritic plaques. Hence, the divergent results are likely to be related to differences in the epitopes used to produce these antibodies in addition to other factors such as variations in antibody titer and distinct antibody affinities as well as the types of tissue fixation and pretreatment.

A large number of proteins have been demonstrated in association with senile plaques; therefore, it can be questioned how significant it is to find yet another protein that co-deposits with amyloid. These proteins, which include apolipoprotein (apo)E, apoJ, apoA1, amyloid P component, complement components, heparin-binding, growth-associated molecule, and proteoglycans, are found both in systemic and cerebral amyloidoses.[28–32] In addition, there are amyloid-associated proteins, such as α_1-antichymotrypsin, that are specifically associated with Aβ-related lesions.[33] It is possible that some of these proteins are released as a result of cell damage and are absorbed onto amyloid fibrils due to their hydrophobic nature. However, this does not appear to be the case with PS as we did not find this protein in other forms of amyloidosis. It is also possible that, due to the high neuronal expression of PS, it is deposited in all cerebral amyloid deposits. However, we did not find PS-1 immunoreactivity in three cases of GSS-related amyloid lesions or on the transthyretin-related sub-pial deposits of Hungarian amyloidoses (data not shown).[20] This suggests a more specific role of PS in the pathogenesis of AD-related neuritic plaques. As PS is present in both early- and late-onset AD lesions, this role is not limited to familial AD cases with linkage to chromosomes 14 or 1. Our biochemical studies have corroborated these immunohistochemical findings, showing the co-purification and binding of Aβ and PS. However, due to the sticky nature of amyloid, it is possible that some of the co-purifying PS becomes associated with Aβ during the extraction procedure. Cleavage fragments of other proteins linked to AD have also been found in neuritic plaques. Aβ itself is a cleavage product of βPP. We have previously reported the biochemical co-purification of a carboxy fragment of apoE in neuritic plaques,[34] a finding that was corroborated by another group.[35] *In vitro* experiments have shown that similar carboxy fragments of recombinant apoE are fibrillogenic. Interestingly, it has recently been shown that some synthetic peptides homologous wild-type and mutated PS can make amyloid-like fibrils *in vitro*, with the presence of the mutation greatly enhancing fibrillogenesis.[36] It is not known whether the 18-kd PS-1 fragment, which we found in neuritic plaque, is also able to form amyloid-like fibrils or is directly involved in amyloid fibril formation. We found immunohistochemically that only 30 to 40% of neuritic plaques stained with our PS-1 antibodies. The lack of staining in the rest of the neuritic plaques may be due to a lack of sensitivity of these antibodies or it may be related to the PS-1 epitopes being hidden within neuritic plaques.

A possible interaction between βPP and PS is that PS may influence the production of the more highly amyloidogenic Aβ1–42 form of Aβ. Higher levels of Aβ42 have been found in the plasma and cultured fibroblast media of PS-1 and -2 mutation carriers.[37] This increase has also been noted in brains of transgenic mice and transfected cells.[38–40] Furthermore, immunohistochemical studies using antibodies for the carboxy terminus of Aβ42 have shown an increase of Aβ42 deposits in the brains of AD patients with PS-1 mutations.[41] However, the elevation of Aβ42 in PS-1 mutation patients is relatively modest. How essential Aβ1–42 is for neuritic plaque development is also not fully resolved, as Aβ1–42 deposits have also been documented in pathological situations where extensive Aβ pre-amyloid lesions are found but progression to neuritic plaques is rare. These include the DS cerebellum, HCHWA-D patient brains, and the aged canine model of AD.[26,42,43] Another not mutually exclusive possibility is that the processing of PS may be important in AD. Some PS-1 and one of the PS-2 mutations have been shown to alter PS processing with an increase in the production of the PS carboxy fragment.[7,15,44] However, this has not been a consistent finding with all of the PS-1 FAD-linked mutations. For example, the Cys411Tyr PS-1 mutation shows no quantitative alteration of the amino and carboxy fragments in brain tissue. Hence, it remains unclear what the role of PS is in the pathogenesis of AD. PS-1 knockout transgenic mice have demonstrated that PS has an important role in central nervous system development.[45] These mice die during late gestation due to massive hemorrhages that are limited to the brain and spinal cord. This hemorrhage is present beneath the primordial dura and leptomeninges as well as within the ventricles and in the brain parenchyma.[45] As loss of PS function may not be compatible with life, this suggests that PS mutations may lead to a gain of negative function. Additional experiments in cell lines and transgenic animals in which wild-type PS and mutant PS is overexpressed, along with βPP, may provide insights into the possible role of PS in AD.

Our data show that the carboxy terminus of PS-1 as well as some full-length PS-1 is specifically deposited in AD-related amyloid lesions. Three of the genes that have been linked to AD, including PS-1, βPP, and apoE, are now known to be components of senile plaques. This raises at least two possibilities: either each of these proteins are part of one pathway with amyloid formation as a final causative pathogenic event or amyloid deposition in AD is a reactive process related to dysfunction of a number of different central nervous system proteins.

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