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Short Communication

Presenilin Binding Protein Is Associated with Neurofibrillary Alterations in Alzheimer's Disease and Stimulates Tau Phosphorylation

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A novel presenilin binding protein, PBP, has recently been identified. PBP is localized to the particulate fraction of extracts of Alzheimer's disease brain but is found in the soluble fractions of brain from age matched normal controls. It is shown here that PBP is associated with neurofibrillary tangles in Alzheimer's disease brain. In addition, the expression of PBP increases the phosphorylation of tau in cultured cells. Therefore PBP may have a regulatory role in tau phosphorylation and in the genesis of neurofibrillary tangles. ***(Am J Pathol 2001, 159:1597–1602)***

The neurodegenerative process in Alzheimer's disease (AD) is characterized by the progressive and irreversible deafferentation of the limbic system, association neocortex and basal forebrain,[1–6] accompanied by neurofibrillary pathology. While the causes of sporadic AD are not clear, recent studies have shown that familial forms of AD are associated with rare mutations in the amyloid precursor protein (APP) and presenilin (PS) 1 and 2. Presenilins are membrane-associated proteins potentially involved in the cleavage of APP as well as several signaling pathways.[7] The normal biological activities of the presenilins are, however, currently unclear. Using the yeast two hybrid system and immunoprecipitation techniques, it has been shown that presenilins interact with over a dozen other proteins.[8] Among them are the serine/threonine protein kinase glycogen synthase kinase-3β (GSK-3β)[9] and a novel presenilin binding protein named PBP.[10] GSK-3β was initially purified as an enzyme that phosphorylates and inactivates glycogen synthase, but it was later shown that it has the ability to phosphorylate the neurofibrillary protein tau.[11] PBP was identified by its ability to bind to the large cytoplasmic loop of PS1 in the yeast two-hybrid system. PBP also co-immunoprecipitates with PS1 in hippocampal cell lysates, is found largely in areas of the brain prone to AD pathology, and becomes insoluble in fractions of AD brain tissue relative to age matched controls.[10] PBP is a 240-kd protein with 40% homology to the adapter protein Dock 180. It binds CRK and has one SH_3 domain, suggesting that it may be involved in the regulation of protein kinases. To better understand the potential role of PBP in AD, we analyzed the patterns of expression of PBP in the AD brain and the effect of PBP on tau phosphorylation. We found that PBP increases tau phosphorylation and is closely associated with neurofibrillary tangles (NFT), suggesting that PBP might be involved in the neurodegenerative process in AD.

Materials and Methods

Samples

Twenty-two autopsy cases from the Alzheimer Disease Research Center at the University of California, San Diego were used in the present analysis. Fourteen of the cases had clinical histories of AD, confirmed at autopsy. The average age of the AD cases was 79 ± 8 years, with a postmortem delay of 5 ± 2 hours. The other eight control cases were clinically and histopathologically free of neurological disease. The average age of these control cases was 69 ± 15 years with a postmortem delay of 8 ± 4 hours. The studies were carried out in blocks taken from the frontal cortex and posterior hippocampus that were

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fixed in 2% buffered paraformaldehyde for 72 hours at 4°C and serially sectioned at 40 μm. Adjacent paraffin sections of cortical and subcortical regions stained with hematoxylin/eosin, thioflavine-S and cresyl violet were used for routine histopathological examination and morphometric analysis.

Immunohistochemistry, Morphometry, and Western Blot Analysis

Briefly, Vibratome sections from control and AD cases were first washed in phosphate-buffered saline (PBS) (pH 7.4), blocked with 10% normal goat serum, and incubated overnight at 4°C with anti-PBP affinity purified antibody which recognizes amino acid residues 2012–2027 of PBP.[10] Serial antibody dilutions (1:20, 1:50, 1:100, 1:500) were used to find the optimal antibody concentration. The free-floating sections were then washed and incubated with secondary biotinylated antibody, followed by avidin D-HRP (ABC Elite, Vector Laboratories, Inc., Burlingame, CA) and reacted with diaminobenzidine (DAB) (0.2 mg/ml) in 50 mmol/L Tris buffer (pH 7.4) with 0.001% hydrogen peroxide. Additional frontal cortex and hippocampal sections, immunostained with anti-PBP, were counterstained with thioflavine-S. For positive controls, experiments were performed using Vibratome sections from mouse brains. Negative control experiments were performed by incubating sections from both the AD and age-matched control group with the preimmune serum or the immunizing peptide.

The antibody against PBP was generated in rabbits against a peptide derived from the PBP protein and purified by affinity chromatography.[10] Analysis of the specificity of anti-PBP in human brain was done by Western blot analysis. Lysates from human 293 cells expressing PBP and vector alone were assayed for protein content and were loaded (20 μg per lane) onto SDS-polyacrylamide gels and immunoblotted with anti-PBP. Figure 1 shows that anti-PBP immunostains predominately a single band of 240 kd in the transfected cells (lane 2) and little in the control cell lysate (lane 1). Immunoblotting in the presence of 500 μg/ml peptide blocked the reaction (Figure 1, lane 3).

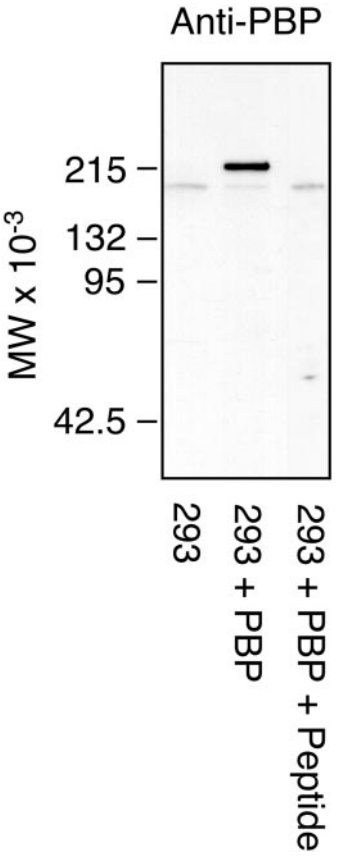

Figure 1. Specificity of antiserum. Rabbits were immunized with the PBP peptide and the antiserum affinity purified as described in Materials and Methods. Lysates (20 μg) from HEK293T cells transfected with plasmid alone (**lane 1**) or PBP (**lane 2**) run on SDS acrylamide (10%) gels, transferred to Immobilon, and immunoblotted with anti-PBP. As a blocking control, the immunoblotting of the PBP lysate was done in the presence of 500 μg/ml peptide (**lane 3**).

Double Immunolabeling and Laser Confocal Imaging

Forty-μm-thick Vibratome sections from control and AD cortex were double-immunolabeled (2,55) with the rabbit polyclonal against PBP and the mouse monoclonal against tau2 (Sigma, St. Louis, MO). Sections were then incubated with the goat anti-rabbit biotinylated antibody (1:100, Vector) followed by a mixture of FITC-conjugated horse anti-mouse IgG (1:75) and avidin Cy 5 D (1:150) (Jackson ImmunoResearch Labs, West Grove, PA). The double-labeled sections were transferred to SuperFrost slides (Fisher Scientific, Tustin, CA) and mounted under glass coverslips with antifading media containing 4% *N*-propyl gallate (Sigma). The sections were studied with the Bio-Rad MRC-1024 laser scanning microscope mounted on an Axiovert Zeiss body. This system permits the simultaneous analysis of double-immunolabeled samples in the same optical plane. Serial optical sections, 0.5 μm thick, of the neocortex displaying the tau-immunolabeled neurites, neurons, and tangles were recorded in the FITC channel, as well as the corresponding serial images of the anti-PBP in the Texas red channel. The aperture, black, and gain level were initially adjusted manually to obtain images with pixel intensity within a linear range.

Tau Phosphorylation and GSK-3β Assay

The analysis for tau phosphorylation was carried out as described by Shackelford and Nelson.[12] Briefly, HEK293T and HEK293T(PBP) cells were cultured to 80% confluence, collected, and lysed in cell lysing buffer containing 1% Triton, 50 mmol/L HEPES pH 7.5, 50 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L Na_3VO_4, 50 mmol/L NaF, 10 MM NaP_2O_7, plus a mixture of protease inhibitors (Complete Mini, Roche, IN). The same amount of protein (500 μg) was used for immunoprecipitation using a monoclonal tau antibody (MAB3420, also called tau1,[13]

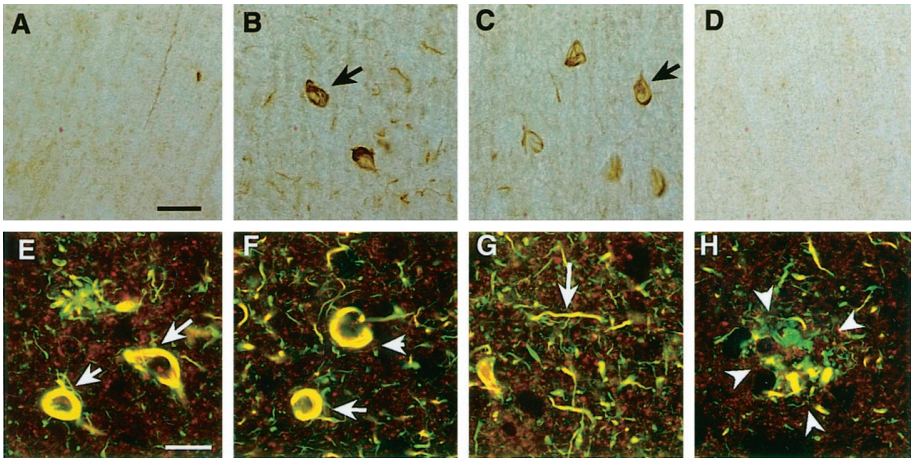

Figure 2. Immunocytochemical analysis of PBP immunoreactivity in human brain. **A:** Control PBP mildly immunolabeled the neuropil and occasionally the pyramidal cells. In the neuropil, some neuritic processes and presynaptic boutons were labeled. Scale bar, 15 μm. **B** and **C:** In AD frontal cortex and hippocampus, PBP antibody strongly immunolabeled the NFT (**arrowheads**), the neuropil threads, and a subpopulation of the neuritic component of the plaques. **D:** Preimmune serum labeled little material. **E** and **F:** Laser scanning confocal imaging. Sections were double labeled with an antibody against phosphorylated tau (AT8, green) and PBP (red). Scale bar, 15 μm. Colocalization of yellow and **arrows** of PBP and tau tangles (**E** and **F**), in the neuropil threads (**G**), fusiform neurites in the plaque (**H**).

Chemicon, Temecula, CA), which binds to all known electrophoretic species of tau, and collected by incubating with anti-mouse IgA agarose beads (Sigma). The beads were washed three times and resuspended in 200 μl of 100 mmol/L Tris pH 8.0, 5 mmol/L MgCl, 2 mmol/L PMSF, and 20 μg/ml leupeptin. The immunoprecipitates were incubated in the presence or absence of 30 U of alkaline phosphatase at 34°C for 45 minutes. The beads were collected, dissolved in sample buffer, and analyzed by SDS-PAGE and immunoblotting using a set of antibodies (pT181; pS199; pS202; pT205; pT212; pS214; pT217; pT231; pS262; pS396) (Biosource, Camarillo, CA) which recognize specific tau protein phosphorylation sites. Of this set, only pS199 antiserum showed a significant positive change with respect to controls. GSK-3β enzymatic activity assays were done exactly as described previously.[14]

Statistical Analyses

Statistical analyses of the results were conducted using the StatView II software package running on a Macintosh computer. Statistical comparisons among the different groups of the control and AD cases were done with the unpaired, two-tailed, Student's *t*-test (values expressed as means ± SEM). Simple linear regression analysis was performed to assess the correlation between PBP and anti-tau immunostained structures.

Results

PBP Is Found in Intracellular NFT

In normal control cortex, the antibody against PBP mildly immunolabeled the neuropil and occasionally the pyramidal cells. In the neuropil, some neuritic processes and presynaptic boutons were labeled (Figure 2A). In AD frontal cortex and hippocampus, PBP antibody strongly immunolabeled intracellular NFT, the neuropil threads, and a subpopulation of the neuritic component of the plaques (Figure 2, B and C). Ghost tangles showed mild or no PBP immunoreactivity. Control experiments where sections were labeled with preimmune serum showed minimal labeling (Figure 2D), as did blocking experiments with peptide (not shown). These results show that PBP is made by neurons and that it has the potential to participate in early intracellular NFT formation.

The pattern of neurofibrillary alterations immunostained by PBP was indistinguishable from that of antibodies against tau. However, detailed quantitative assessment showed that anti-tau immunostained an average of 41 ± 6 NFT per mm^2, while PBP immunostained an average of 11 ± 3 NFTs per mm^2. Linear regression analysis of NFT counts immunolabeled with PBP and tau antibodies showed a significant positive correlation ($r = 0.7$, $P < 0.05$, $n = 14$) between these two probes. Immunolabeling of non-AD cases with NFT pathology such as progressive supra nuclear palsy and Pick's disease showed minimal or no PBP immunoreactivity. Similarly, the PBP antibody did not label Lewy bodies of Parkinson's disease.

Laser scanning confocal microscopy of sections double immunolabeled with antibodies against PBP and tau showed that both of these proteins were closely colocalized in the fibrillary meshwork of intracellular NFTs (Figure 2, E and F), the neuropil threads (Figure 2G) and a subpopulation of fusiform neurites in the plaque (Figure 2H). Occasionally, anti-tau immunolabeled in a diffuse fashion the cell bodies of pyramidal neurons (so-called pre-tangles). In this type of neuron, PBP immunoreactivity was either very low or not present.

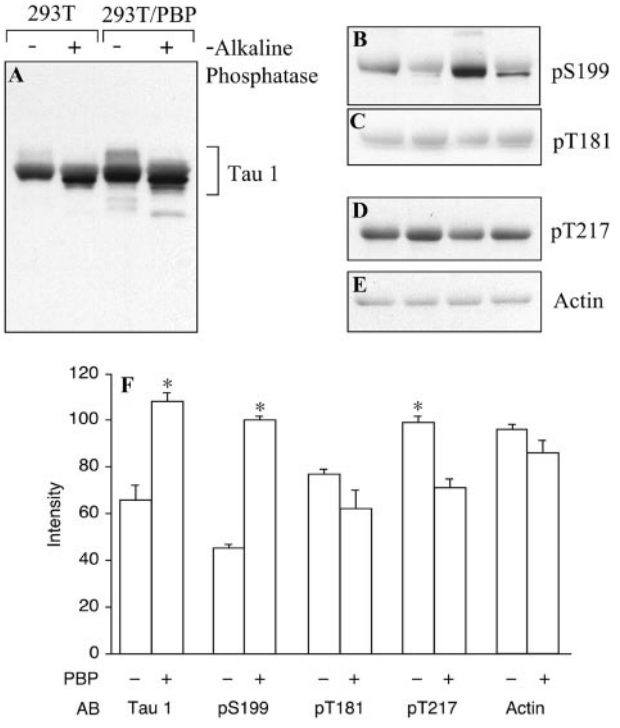

Figure 3. The effect of PBP on tau phosphorylation. The same amount of protein extracts (500 μg) isolated from HEK293T and HEK293T(PBP) cells were immunoprecipitated by the antibody MAB3420, treated with or without alkaline phosphatase, and analyzed by Western blotting using the antibodies MAB3420 (**A**); pS199 (**B**); pT181 (**C**); pT217 (**D**); Other sites which were not changed as defined by site-specific sera were pS202, pT205, pT212, pS214, pT231, pS262 and pS396. Actin in the total lysate served as a loading control (**E**). **F:** The Western blots of 293T and 273T (PBP) were quantitated by scanning using NIH Image, and the data presented as the mean of triplicate determinations plus or minus the SEM. The relative intensities of each band are shown. *, Significantly different from other member of pair, $P \leq 0.01$.

PBP Increases the Phosphorylation of Tau Protein at a Specific Site

Since PS1 binds both tau and GSK-3β to regulate tau protein phosphorylation,[9] and PS1 binds PBP, we examined the effect of PBP on tau phosphorylation. The protein extracts from HEK293T cells and HEK293T(PBP) cells stably expressing PBP were immunoprecipitated with an antibody against tau (MAB3420) which binds to all known electrophoretic species of tau at an epitope at or near a phosphorylation site.[13] Immunoprecipitation was complete as assayed by the lack of immunoreactivity remaining in the supernatant as defined by Western blotting (data not shown). One-half of the immunoprecipitate was treated with alkaline phosphatase and both halves subjected to immunoblotting with specific antibodies recognizing tau proteins phosphorylated at different amino acid residues. The expression patterns of tau were different between untransfected cells and cells transfected with PBP (Figure 3A). Cells expressing PBP contained more tau protein bands which were shifted to higher molecular weights compared to the control HEK293T cells. MAB3420, which is sensitive to tau phosphorylation, also reacts with tau from PBP expressing cells more strongly than from control cells. These bands were shifted to a lower molecular weight after alkaline phosphatase treatment, showing that the increase in the apparent size of the protein was due to phosphorylation. We further identified a site for PBP enhanced tau phosphorylation by using a set of phosphorylation site-specific antisera. PBP specifically increased tau phosphorylation at ser199, and this effect was reversed by alkaline phosphatase treatment (Figure 3B). In contrast, many sites were not affected by PBP, including pT181, and there was a slight but significant decrease in the phosphorylation of pT217 (Figure 3, C and D, respectively). The results are quantitated in Figure 3F. These data suggest that PBP specifically increases tau phosphorylation at ser199, and possibly at other sites which were not assayed.

Tau Phosphorylation Is Not via GSK-3β Activation

GSK-3β has the ability to phosphorylate tau proteins.[11] However, the phosphorylation of tau seen in PBP-transfected HEK293T cells is probably not through GSK-3β activation because the activity of GSK-3β is decreased by about 50% in cells expressing PBP. In a typical experiment, GSK-3β stimulated substrate phosphorylation with a specific activity of 274 $\pm$ 8 cpm/min/mg protein in HEK293T cells and 116 $\pm$ 17 cpm/min/mg protein in HEK293T cells expressing PBP, resulting in a 58% decrease in enzyme activity (n = 3).

Discussion

The neurofibrillary pathology in AD is characterized by the accumulation of paired helical filaments (PHF) in neuronal cell bodies (tangles),[15–17] dendrites (neuropil threads),[18–20] and axons (dystrophic neurites).[21] The PHF is mainly composed of hyperphosphorylated tau, a microtubule-associated protein predominantly expressed by neurons.[22–26] The mechanisms leading to neurofibrillary pathology in AD are currently under intense scrutiny. Among the explanations proposed is the possibility that tau might be abnormally expressed or processed.[27,28] Supporting this possibility, recent studies have shown that frame-shift mutations in tau can be found in neurons of AD patients.[29] In addition, mutations in the human tau gene cause fronto-temporal dementia and Parkinsonism linked to chromosome 17.[30–32] Some mutations, including mutations in exon 10, induce increased levels of the four-repeat tau protein isoform, leading to neurodegeneration.[33–35] Another possibility is that tau hyperphosphorylation might be a secondary neuronal response to amyloid toxicity and amyloid accumulation. In support of this, *in vitro* studies have shown that treatment of nerve cells with β amyloid promotes tau hyperphosphorylation.[36,37] Similar results have been reported *in vivo* by intracerebral injection of β amyloid.[38–40] The fact that PBP colocalizes with tau suggests that it may have some role in its function or secondary modification.

PBP is a large protein with sequence homology to the adapter protein Dock 180.[10] Adapter proteins of this type frequently modulate protein kinase activities.[41] PBP is found almost exclusively in areas of the brain which are susceptible to NFT formation and nerve degeneration associated with AD.[10] Tau can be phosphorylated by a large number of protein kinases in cell free systems, but the exact mix of kinases which are responsible for tau phosphorylation *in vivo* are not known.[42] GSK-3β is one kinase that is able to phosphorylate tau.[11] The expression of PBP increases tau phosphorylation in the human HEK293T fibroblast cell line but decreases GSK-3β activity. Several of the putative GSK-3β sites are unchanged and one, pS199, is increased. It is therefore likely that in this cell line the PBP-dependent increase in tau phosphorylation is mediated by a different kinase. Individual sites on tau can be phosphorylated by multiple enzymes.[42] The modifications in kinase activities caused by PBP expression are not known, but are under investigation. Presenilin can co-immunoprecipitate with both GSK-3β and tau.[9] The fact that PBP also binds presenilin and significantly decreases the activity of GSK-3β in human cells suggests that a large complex between these molecules may exist in which the very large 240-kd PBP plays a central role. Additional kinases that alter tau phosphorylation may also be part of this complex.

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