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Calcyclin binding protein and Siah-1 interacting protein in Alzheimer's disease pathology: neuronal localization and possible function

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ABSTRACT

The calcyclin binding protein and Siah-1 interacting protein (CacyBP/SIP) protein was shown to play a role in the organization of microtubules. In this work we have examined the neuronal distribution and possible function of CacyBP/SIP in cytoskeletal pathophysiology. We have used brain tissue from Alzheimer's disease (AD) patients and from transgenic mice modeling 2 different pathologies characteristic for AD: amyloid and tau. In the brain from AD patients, CacyBP/SIP was found to be almost exclusively present in neuronal somata, and in control patients it was seen in the somata and neuronal processes. In mice doubly transgenic for amyloid precursor protein and presenilin 1 there was no difference in CacyBP/SIP neuronal localization in comparison with the nontransgenic animals. By contrast in tau transgenic mice, localization of CacyBP/SIP was similar to that observed for AD patients. To find the relation between CacyBP/SIP and tau we examined dephosphorylation of tau by CacyBP/SIP. We found that indeed it exhibited phosphatase activity toward tau. Altogether, our results suggest that CacyBP/SIP might play a role in AD pathology.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder. It is characterized by a progressive loss of neurons and cognitive functions. Pathologic hallmarks of AD are extracellular amyloid plaques, composed of the amyloid-β (Aβ) peptide and intracellular neurofibrillary tangles (NFTs) that predominantly comprise tau protein (Braithwaite et al., 2012). The pathogenic mechanism of AD is still poorly understood and thus further studies on factors or proteins that might be important in the development of AD are necessary. One such factor might be calcyclin binding protein and Siah-1 interacting protein (CacyBP/SIP), a protein expressed at high levels in the brain (Jastrzebska et al., 2000) and having an age-dependent subcellular localization in neurons (Filipek et al., 2008).

CacyBP/SIP was discovered as a S100A6 (calcyclin) target (Filipek and Kuznicki, 1998; Schneider and Filipek, 2011) but later shown to interact with some other S100 proteins (Filipek et al., 2002a) and with Siah-1 (Matsuzawa and Reed, 2001), Skp1 (Bhattacharya et al., 2005), tubulin (Schneider et al., 2007), actin (Schneider et al., 2010), and extracellular signal-related kinase (ERK)1/2 (Kilańczyk et al., 2009). CacyBP/SIP is present in various tissues with the highest level in brain (Jastrzebska et al., 2000) and in some cell lines such as mouse neuroblastoma NB2a (Filipek et al., 2002b). The high level of CacyBP/SIP in neurons (Jastrzebska et al., 2000) and its interaction with tubulin (Schneider et al., 2007) suggested its involvement in developmental changes of the neuronal cytoskeleton. This supposition has been confirmed by the finding that subcellular compartmentalization of CacyBP/SIP in rat brain neurons depends on age. In young rats CacyBP/SIP was present in the cytoplasm and in neuronal processes, and in aged animals it was seen only in the cytoplasm and not in neuronal processes (Filipek et al., 2008). Such changes in localization of CacyBP/SIP were similar to those of tau, a major neuronal microtubule-associated protein, and suggested a possible role of CacyBP/SIP in cytoskeletal physiology and pathophysiology (Filipek et al., 2008). Interaction of CacyBP/SIP with actin, another cytoskeletal protein (Schneider et al., 2010), might

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also implicate a role for CacyBP/SIP in cytoskeletal organization. Furthermore, a substantial sequence similarity between CacyBP/SIP and phosphatases from the mitogen activated protein (MAP) kinase phosphatase family and its ability to dephosphorylate ERK1/2 kinase (Kilanczyk et al., 2011) suggest that the involvement of CacyBP/SIP in cytoskeletal dynamics might rely on its dephosphorylating activity toward key cytoskeletal components.

Microtubules, composed predominantly of α- and β-tubulin subunits, are the major structural components of the neuronal cytoskeleton that plays an important role in neurodevelopment, aging, and neurodegenerative disorders (Baas and Qiang, 2005; Brandt et al., 2005). Tau protein is important in the assembly and disassembly of dynamic microtubules and is associated normally with axonal rather than somatodendritic microtubules (Binder et al., 1985). AD is characterized by a substantial redistribution of tau into the somatodendritic compartment as neurofibrillary pathology, which is in the form of intra- and extracellular tangles, and as dystrophic neurites either throughout the neuropil or within neuritic plaques (Harrington, 2012). Neurofibrillary tau pathology involves the formation of paired helical filaments (PHFs) that comprised tau protein. Tau is an ideal substrate for phosphorylation by multiple kinases in the somatodendritic compartment in AD (Gong et al., 2005).

In the present work we have examined immunohistochemically the neuronal localization of CacyBP/SIP in association with phosphorylated tau (p-Tau) and β-tubulin, in the brains from AD patients and from 2 lines of mice separately transgenic for amyloid and tau. Moreover, we checked the level of CacyBP/SIP and the ratio of the major CacyBP/SIP forms in control and pathologic mouse brain material by applying sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 2-dimensional (2D) electrophoresis, respectively. Because CacyBP/SIP has recently been shown to exhibit phosphatase activity toward ERK1/2, we also analyzed whether it could dephosphorylate tau, a protein that becomes hyperphosphorylated in AD pathology.

2. Methods

2.1. Human material

Human brain tissues from 10 patients (age 72–94 years) with neuropathologically diagnosed AD and from 7 control patients (age 43–85 years) were analyzed. Following the Austrian legal procedures, every patient who dies in a hospital can undergo autopsy for diagnostic and/or scientific purposes. Based on the request from the Ethical Committee of the State of Upper Austria upon hospital admission, patients sign a written consent for scientific evaluation of their brain after death. The neuropathologic diagnosis of AD was rendered using the Consortium to Establish a Registry for Alzheimer's Disease (Mirra et al., 1993) and National Institute on Aging-Reagan diagnostic criteria (1997). The Braak staging system for tangle formation was also used (Braak and Braak, 1991). Briefly, after removal from the skull, the brains were fixed for 1 week in 4% formaldehyde. Brain regions were then cut and embedded in paraffin. For immunohistochemical analysis, slices of hippocampal and adjacent cortex of 5 μm thickness were used.

2.2. Animal models

All mice (control and transgenic) were housed in an environmentally controlled room (temperature 23 °C ± 1 °C, 12 hours light/12 hours dark cycle) in groups of 4 per cage. The study was conducted with the approval of the local ethics commission (Polish Law on the Protection of Animals) and carried out in accordance with the Principles of Laboratory Animals Care (NIH publication No. 86-23). All efforts were made to minimize the number of animals used for experiments and their suffering.

Mice B6C3-TgN(Prn-APP695)$_3$Dbo Tg(PSEN1)$_5$Dbo/J, designated as APP+PS1, with overexpression of mutant amyloid precursor protein (K595N/M596L) and mutant presenilin 1 (A246E) were used as a model of Aβ pathology and B6C$_3$F$_1$/J mice were used as controls (The Jackson Laboratory, Jax Mice and Service). Characterization of these mice is given by Borchelt et al., (1997). The expression of both mutated proteins together accelerates the deposition of Aβ in mice, with deposits appearing by the age of 9 months.

A transgenic model of tau aggregation in vivo (line 1 tau mice) was created in which the mouse expresses truncated tau (amino acids 296–390 of the longest human 441-residue tau form) that is fused with a membrane localization signal sequence to nucleate aggregation (Zabke et al., 2008). Expression in neurons is under the control of the murine Thy1-promoter. The tau transgenic model (referred to as line 1 tau mice throughout the text) was established in mice of the outbred NMRI strain (Harlan–Winkelmann, Paderborn) that served as a wild type control. The truncated tau species represent the proteolytically stable core of the PHF left after exogenous proteolysis (Wischik et al., 1988) and a fragment that is capable of generating proteolytically stable tau aggregates in vitro (Wischik et al., 1996). The brain of line 1 tau mice shows an age-dependent increase in tau immunoreactivity. The tau aggregates are neither argyrophilic nor thioflavin-S positive, but similar to the amorphous aggregates described in the early stages of AD. The anatomic pattern and progression of the tau-immunoreactivity in these mice is similar to that described by Braak staging in AD (Braak and Braak, 1997), initiating in the hippocampus and entorhinal cortex before progressing to the neocortex. Mice are not impaired in motor function but display cognitive impairment in spatial learning in the T-maze (Zabke et al., 2008).

2.3. Perfusion and processing of mouse brain

Female mice were deeply anesthetized with Vetbutal and the perfusion procedure was begun when all reflex responses to cutaneous stimulation were absent. Generally, tissues were fixed by intra-aortic perfusion with cold (4 °C) phosphate buffered saline (PBS), containing heparin (5 IU of heparin per 1 mL of buffer), followed by 4% paraformaldehyde in PBS (fixative solution), and then followed by 5% glycerol and 2% dimethyl sulphoxide (DMSO) in PBS. The brains were removed, placed for 1 hour in the fixative solution and then immersed for cryoprotection in 10% glycerol and 2% DMSO and subsequently in 20% glycerol and 2% DMSO. All perfusion solutions contained 1 mM Na_3VO_4 (sodium orthovanadate), a phosphatase inhibitor, to minimize postmortem dephosphorylation of proteins.

For immunohistochemical analysis, sections were cut coronally through the forebrain at a 40 μm thickness with a freezing stage microtome. The consecutive sections were collected throughout the hippocampal region (from −0.94 mm to −4.04 mm posterior to Bregma; Franklin and Paxinos, 2008) of each mouse. From 4 series of the sections collected, 1 set was processed for CacyBP/SIP immunoreactivity, the second for p-Tau(Ser-404) immunoreactivity, the third for β-tubulin immunoreactivity, and the fourth corresponding series of sections was stored at −20 °C in a cryoprotective solution as a reserve.

2.4. Immunohistochemistry and immunofluorescence

For immunohistochemical and immunofluorescence analyses the following antibodies were used: polyclonal anti-CacyBP/SIP (developed and described by Filipek et al. [2002b]), rabbit polyclonal antibodies to neuron specific β III tubulin (clone name ab 18207, Abcam), polyclonal anti-p-Tau(Ser-404) (Santa Cruz),

and monoclonal AT8 (p-Tau(Ser-202/Thr-205)), recognizing PHF (Thermo Scientific).

For sections from frozen mouse brains, antibodies against CacyBP/SIP and β-tubulin were each used at 1:1000 and anti-p-Tau(Ser-404) at 1:200 dilution. Sections tested for CacyBP/SIP and β-tubulin were incubated in PBS and those for p-Tau(Ser-404) in PBS supplemented with 0.4 mM Na_3VO_4 (PBS-V). Incubation with primary antibodies was performed for 1 hour at room temperature (RT) followed by overnight continuous agitation at 4 °C. Sections were then incubated for 1 hour at RT with goat anti-rabbit biotin conjugated IgG (diluted 1:1000, Chemicon, Int., Inc.) or goat anti-mouse biotin conjugated IgG (diluted 1:1000, Sigma). Sections for CacyBP/SIP and β-tubulin staining were incubated for 1 hour at RT with peroxidase conjugated with streptavidin (diluted 1:500, The Binding Site Ltd.). Sections for p-Tau(Ser-404) staining were washed 3 times for 5 minutes with PBS-V, incubated for 30 minutes with peroxidase conjugated with streptavidin, washed again, and treated for 10 minutes at RT with the tyramide signal amplification reagent (PerkinElmer LAS, Inc.). Sections were then washed and incubated again for 30 minutes with peroxidase conjugated with streptavidin. To visualize the reaction, sections stained for CacyBP/SIP, β-tubulin, and p-Tau(Ser-404) were incubated for 5 minutes in PBS or PBS-V containing diaminobenzidine (DAB) and H_2O_2 at a concentration of 0.05% and 0.01%, respectively.

For staining of human paraffin-embedded sections, antibodies were diluted as follows: anti-CacyBP/SIP at 1:250; anti-β-tubulin at 1:500, and AT8 (for immunohistochemistry and immunofluorescence) at 1:50. The sections stained for CacyBP/SIP were incubated in PBS, those for PHFs in PBS-V, and those for β-tubulin in Tris buffered saline. Incubation with primary antibodies was performed for 1 hour at RT followed by overnight continuous agitation at 4 °C. Sections were then incubated for 1 hour at RT with goat anti-rabbit biotin-conjugated IgG (diluted 1:1000, Chemicon, Int., Inc.) or goat anti-mouse biotin-conjugated IgG (diluted 1:1000, Sigma), followed by incubation for 1 hour at RT with peroxidase conjugated with streptavidin (diluted 1:500, The Binding Site Ltd.). Sections were then stained for 5 minutes in PBS or PBS-V containing DAB and H_2O_2 at a concentration of 0.05% and 0.01%, respectively. For immunofluorescence, streptavidin was conjugated with fluorescein isothiocyanate (FITC) or Texas Red rather than peroxidase.

Sections stained with DAB were analyzed using a bright field microscope (Nikon Eclipse E 400) and sections stained with the fluorescent dyes were analyzed with a Leica confocal laser fluorescence microscope (Leica TCS SP5 Spectral Confocal with STED). In all cases control sections were included in which the primary or secondary antibodies were omitted. No immunopositive reaction was observed for any control slide (data not shown).

2.5. Analysis of CacyBP/SIP in line 1 tau transgenic brain: preparation of protein extract, SDS-PAGE, Western blot, and 2D electrophoresis

Fresh samples of the hippocampus and parietotemporal cortex derived from brains of 8-month-old mice (line 1 tau transgenic or control) were homogenized 4 times for 30 seconds using a Polytron homogenizer at 6000 rpm in a solution containing 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), 150 mM NaCl, protease inhibitors, and 50 mM Tris (pH 7.5). The homogenates were then centrifuged at 21,500 *g* for 45 minutes and protein concentration in the supernatant was estimated by the Bradford procedure with bovine serum albumin as a standard (Bradford, 1976). Protein (70 μg) was subjected to SDS-PAGE, which was performed by the method of Laemmli (1970), using 10% polyacrylamide gels. Proteins were transferred electrophoretically onto nitrocellulose and the level of CacyBP/SIP was studied using monoclonal antibodies against CacyBP/SIP (Abcam) diluted 1:1000 and against glyceraldehyde 3-phosphate dehydrogenase (Chemicon International Inc.) diluted 1:5000. Then the blots were allowed to react with secondary antibodies conjugated to horseradish peroxidase (Sigma) and developed with the ECL chemiluminescence kit (Amersham Biosciences) followed by exposure against an x-ray film. The intensities of protein bands on immunoblots were quantified using the Ingenius densitometer and the Gene Tools from SynGene with glyceraldehyde 3-phosphate dehydrogenase as a reference protein.

For 2D electrophoresis, protein extracts from whole brain of line 1 tau transgenic or control mouse, aged 3 or 8 months were obtained as described above. Proteins (120 μg) were separated by isoelectric focusing on linear pH gradient (pH 3–10) strips (BioRad) and then by 10% SDS-PAGE followed by immunoblot development with monoclonal anti-CacyBP/SIP antibodies, as described above.

2.6. Cell-based experiments

Mouse neuroblastoma NB2a cells were cultured as described by Schneider et al. (2007). Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. To overexpress tau, CacyBP/SIP or S100A6, cells were cotransfected with 1.8 μg of p3xFLAG-CMV-14-tau (a generous gift from Dr. K. Nieznanski, Nencki Institute of Experimental Biology, Poland), obtained as described by Osiecka et al. (2011), 1.8 μg of p3xFLAG-CMV-10-CacyBP/SIP (Schneider et al., 2007), or 1.8 μg of pcDNA3.1.-S100A6-c-myc (Spiechowicz et al., 2007). Cells were harvested 24 hours after transfection and protein extracts were obtained by incubation of cells in radioimmunoprecipitation assay buffer (RIPA) buffer containing 20 mM Tris, pH 7.5; 150 mM NaCl, 1mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.5% NP-40, 1% Triton-X 100, supplemented with protease and phosphatase inhibitors (Roche) for 30 minutes on ice. Then, the lysate was centrifuged at 20,000 *g* for 20 minutes and protein (120 μg) from the supernatant fraction was subjected to 2D electrophoresis as described above.

2.7. In vitro tau dephoshorylation

Phosphorylated bovine tau protein (2 μg), purchased from Cytoskeleton Company (Cat. No. TA01), was incubated with recombinant CacyBP/SIP purified as described by Filipek et al. (2002b) in a buffer containing 20 mM Tris (pH 7.5) and 50 mM NaCl for 30 minutes at 30 °C. Then samples were subjected to 2D electrophoresis followed by immunoblotting with monoclonal phospho-independent antibody recognizing a C-terminal tau epitope (404-441; T46, Sigma) as described above.

In another approach, tau protein (2 μg) was incubated with CacyBP/SIP for 1 hour at 30 °C in the buffer containing 20 mM piperazine-N-N'-bis(2-ethanesulfonic acid) (PIPES) (pH 7.5) and 150 mM NaCl. Inorganic phosphate release was assessed using the Malachite Green Phosphate Detection kit (R&D Systems) according to the manufacturer's instruction.

3. Results

3.1. Subcellular localization of CacyBP/SIP in human AD brain

Previously we have shown that CacyBP/SIP changes its subcellular localization in brain neurons in an age-dependent manner (i.e., it translocated from neuronal processes to somata with advancing age) (Filipek et al., 2008). Interestingly, the altered localization of CacyBP/SIP resembled that of tau protein in aged brain. Because tau pathology is closely associated with clinical AD (Harrington, 2012) and is an early occurrence in brains (Dubois et al., 2010), we have

examined the localization of CacyBP/SIP and its relationship with the distribution of p-Tau and a microtubule marker, β-tubulin, in AD human brain and in brains from mice which represent 2 models of the pathologies characteristic of this disease.

In humans we found that the age-matched control subjects differed from the AD patients in terms of cellular CacyBP/SIP, β-tubulin, and PHF staining in brain regions analyzed (Fig. 1). In control subjects, CacyBP/SIP staining of moderate intensity was concentrated in soma and processes of neurons found in the CA1 area of the hippocampus and in the posterior parahippocampal (PPH) cortex (Fig. 1A-a and B-a). In contrast, CacyBP/SIP staining in AD patients was greatly enriched in the soma and neuronal fibers were devoid of labeling (Fig. 1A-b and B-b). For β-tubulin, hippocampal and PPH cortical neurons of control subjects showed much stronger immunolabeling in dendrites, forming a characteristic striped pattern (Fig. 1A-c and B-c). By contrast, in AD patients, β-tubulin immunostaining in the hippocampus and PPH cortex was absent in neuronal processes but strong β-tubulin immunoreactivity was observed in the visibly dystrophic perikarya (Fig. 1A-d and B-d). For human control sections stained with AT8 antibodies, tangles were absent from hippocampus and the adjacent PPH cortex (Fig. 1A-e and B-e). In some control patients tangles were occasionally seen but at a much lower intensity (not shown). In AD sections stained for PHF, numerous neurons were labeled in all areas of the hippocampal formation and in the adjacent PPH cortex (Fig. 1A-f and B-f). Furthermore, as investigated by double immunofluorescence staining, shown in Fig. 2, CacyBP/SIP colocalized with the PHF structures.

3.2. Subcellular localization of CacyBP/SIP in brain of APP+PS1 transgenic mice

In order to obtain more detailed information on CacyBP/SIP characteristics we studied its distribution in the brains of mice transgenic for APP+PS1 that represent a model of amyloidosis (Fig. 3). No changes in CacyBP/SIP, β-tubulin, and p-Tau(Ser-404) compartmentalization in neurons of the hippocampus and cortex between control and APP+PS1 mice was observed.

3.3. Subcellular localization of CacyBP/SIP in brain of line 1 tau transgenic mice

The pattern of CacyBP/SIP, β-tubulin and p-Tau(Ser-404) immunoreactivity differed between control and line 1 tau transgenic mice (Fig. 4). These differences were observed in the hippocampus and neocortical regions. CacyBP/SIP, β-tubulin and p-Tau(Ser-404) in the hippocampus of the control mice were confined mainly to the fibers of pyramidal cells in the CA1 area (Fig. 4A-a, c, and e). By contrast, there was no clearly detectable immunoreactivity in the CA1 pyramidal cell fibers in line 1 tau transgenic mice (Fig. 4A-b, d, and f). A similar distribution of CacyBP/SIP, β-tubulin, and p-Tau(Ser-404) immunoreactivity was observed in the parietotemporal cortex (Fig. 4B). In control mice β-tubulin and p-Tau(Ser-404) antibodies preferentially stained neuronal fibers, and CacyBP/SIP immunoreactivity was observed in both somata and fibers (Fig. 4B-a, c, and e), and in line 1 tau mice CacyBP/SIP, β-tubulin, and p-Tau(Ser-404) labeling in cell fibers was absent (Fig. 4B-b, d, and f). In general, in the control mice, expression of all 3 proteins was found predominantly in dendrites or axonal processes, and in the line 1 tau mice CacyBP/SIP, β-tubulin and p-Tau(Ser-404) were almost exclusively localized to the neuronal cell bodies. The greater intensity of cytoplasmic immunoreactivity in line 1 tau transgenic mice suggests that the examined proteins could be sequestered from the axodendritic compartment to neuronal somata.

3.4. Properties of CacyBP/SIP in brain of line 1 tau transgenic mice

To check whether the more intensive staining seen in neuronal somata might be because of an increased level of CacyBP/SIP, we

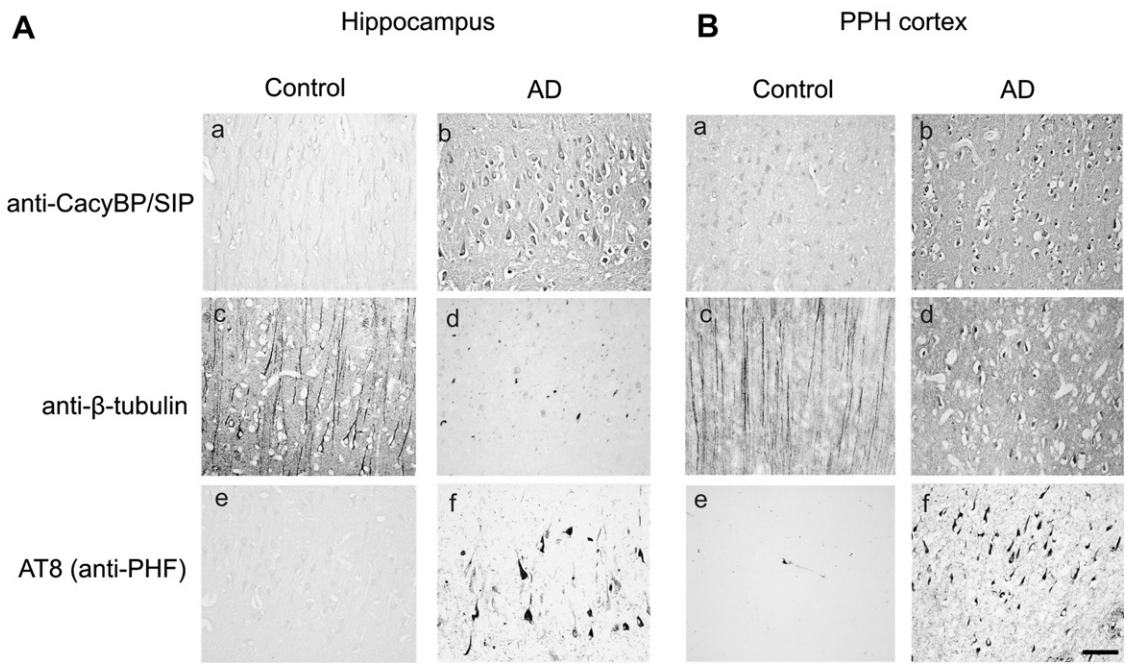

Fig. 1. Immunohistochemical localization of calcyclin binding protein and Siah-1 interacting protein (CacyBP/SIP), β-tubulin, and paired helical filament (PHF) in human brain slices, control (a, c, e), and Alzheimer's disease (AD) (b, d, f). (A) Hippocampus and (B) parahippocampal (PPH) cortex, stained with anti-CacyBP/SIP (a, b), anti-β-tubulin (c, d) and anti-phosphorylated tau (p-Tau) (Ser-202/Thr-205) recognizing PHF (AT8) (e, f) antibodies. Representative images are shown from 1 case, out of 7 and 10 cases analyzed, for control and AD, respectively. Scale bar, 100 μm.

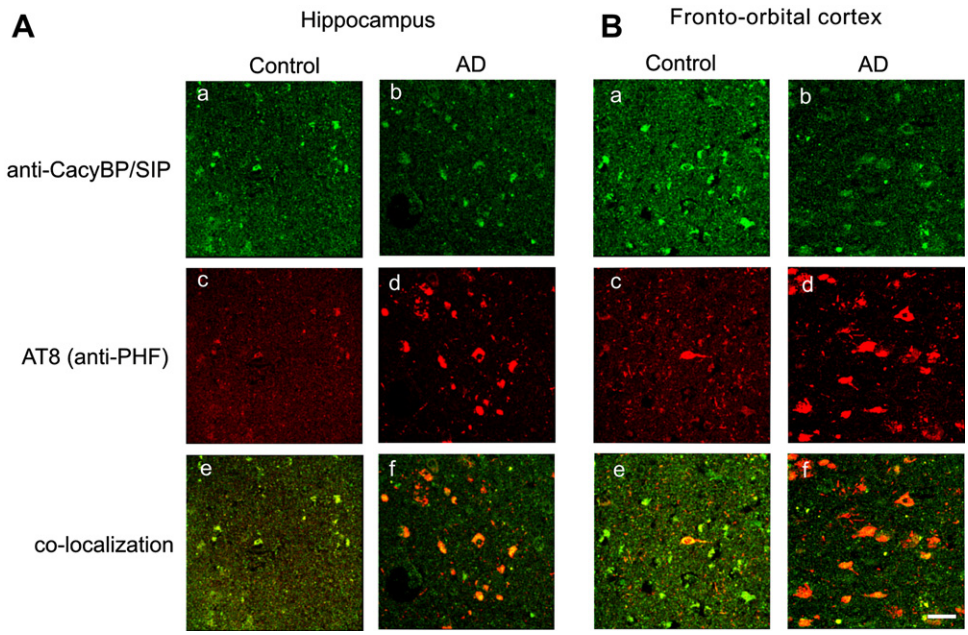

Fig. 2. Immunofluorescence staining of calcyclin binding protein and Siah-1 interacting protein (CacyBP/SIP) and paired helical filament (PHF) in human control (a, c, e) and Alzheimer's disease (AD) (b, d, f) sections. (A) Hippocampus and (B) fronto-orbital cortex. Staining with anti-CacyBP/SIP (a, b), AT8 (c, d), and merged images (e, f). Representative images are shown from 1 case, out of 2 analyzed, of each group. Scale bar, 100 μm.

performed immunoblotting using samples derived from the control and line 1 tau transgenic mouse brain. More precisely, we analyzed the CacyBP/SIP level in the hippocampus and the parietotemporal cortex. The amount of CacyBP/SIP is similar in the control and line 1 tau transgenic material (Fig. 5), suggesting that the more intensive staining seen in neuronal somata is because of concentration of CacyBP/SIP within the cytoplasm.

Because posttranslational modifications very often determine the subcellular distribution of proteins, we checked CacyBP/SIP forms by 2D electrophoresis. The ratio of the 2 major CacyBP/SIP forms having different isoelectric points (pI) was similar in control and line 1 tau transgenic brains derived from 3-month-old mice (Fig. 6A). In the case of 8-month-old mice (Fig. 6B), the amount of the more basic CacyBP/SIP form was much greater in the line 1 tau transgenic brain (lower panel, dashed circles) than in the control tissue suggesting a different type or extent of posttranslational modifications of CacyBP/SIP in aged line 1 tau mice. These modifications might play an important role in the cellular redistribution of CacyBP/SIP to neuronal somata.

Recently, we have reported that CacyBP/SIP exhibits phosphatase activity toward ERK1/2 (Kilanczyk et al., 2011). This observation together with the fact that CacyBP/SIP colocalized with PHFs in

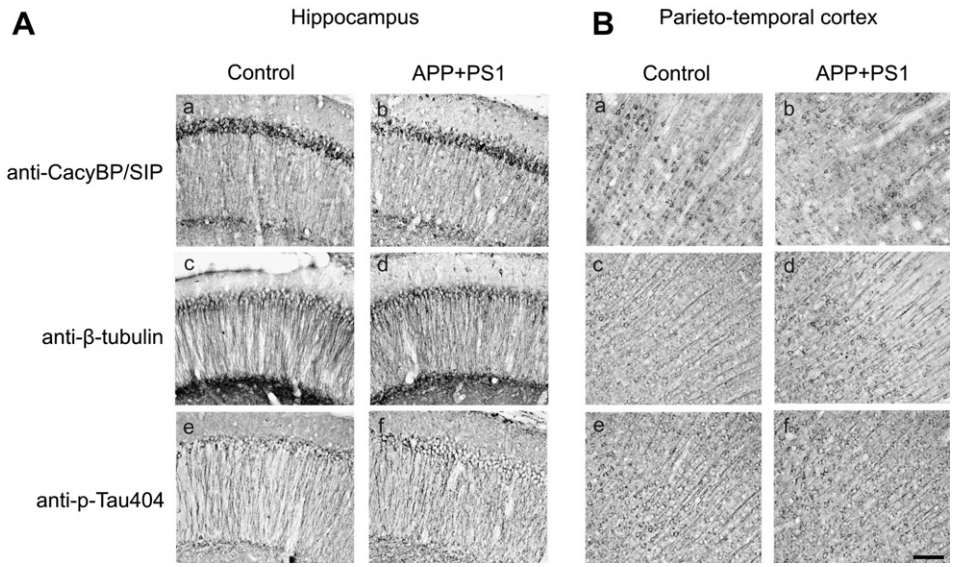

Fig. 3. Immunohistochemical localization of calcyclin binding protein and Siah-1 interacting protein (CacyBP/SIP), β-tubulin, and phosphorylated tau (p-Tau) (Ser-404) in B6C3-TgN(Prn-APP695)$_3$Dbo Tg(PSEN1)$_5$Dbo/J (APP+PS1) mouse brain. (A) Hippocampus and (B) parietotemporal cortex of control (a, c, e) and APP+PS1 (b, d, f) 8-month-old mice. Staining with anti-CacyBP/SIP (a, b), anti-β-tubulin (c, d) and anti-p-Tau(Ser-404) (e, f) antibodies. Representative images are shown from 1 animal, out of 4 analyzed, of each group. Scale bar, 100 μm.

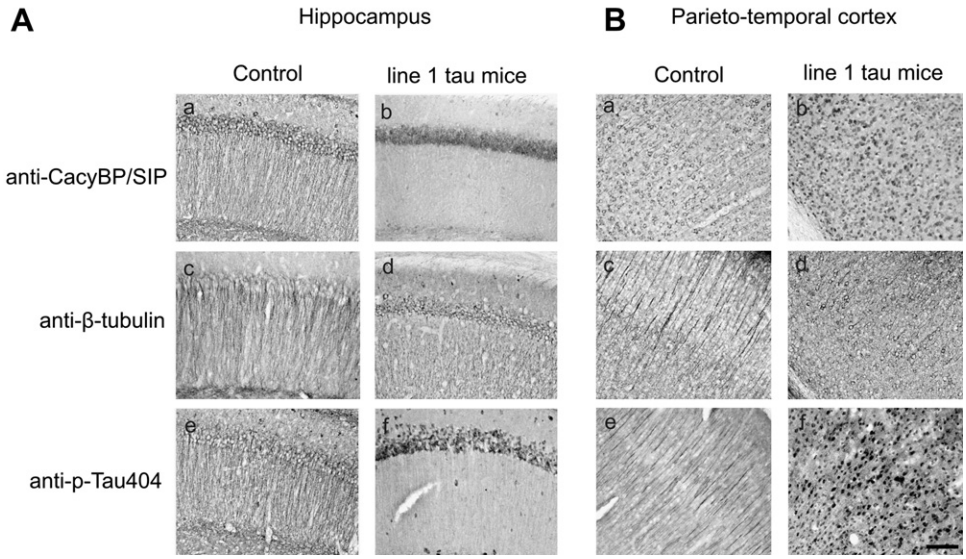

Fig. 4. Immunohistochemical localization of calcyclin binding protein and Siah-1 interacting protein (CacyBP/SIP), β-tubulin, and phosphorylated tau (p-Tau) (Ser-404) in brain of line 1 tau transgenic mice. (A) Hippocampus and (B) parietotemporal cortex of control (a, c, e) and line 1 tau e (b, d, f) 8-month-old mice. Staining with anti-CacyBP/SIP (a, b), anti-β-tubulin (c, d), and anti-p-Tau(Ser-404) (e, f). Representative images are shown from 1 animal, out of 4 analyzed, of each group. Scale bar, 100 μm.

AD prompted us to see whether CacyBP/SIP might have an effect on the level of tau phosphorylation. To examine this, a sample of phosphorylated tau protein isolated from bovine brain was incubated in the presence or absence of recombinant CacyBP/SIP and then analyzed by 2D electrophoresis. CacyBP/SIP changed the pattern of tau forms (Fig. 7A). A shift in the direction of higher pI is seen (lower panel, dashed circle) that is consistent with tau dephosphorylation. In another approach we have monitored inorganic phosphate release from tau using Malachite Green Phosphate Detection kit. We found that 1 nmol of inorganic phosphate was released from 1 nmol of phosphorylated tau after incubation with CacyBP/SIP. To check whether tau dephoshorylation can be observed in the cell, neuroblastoma NB2a cells were transfected either with plasmids encoding tau and CacyBP/SIP or with plasmids encoding tau, CacyBP/SIP, and S100A6. The level of different forms of tau was again analyzed by 2D electrophoresis (Fig. 7B). Overexpression of CacyBP/SIP resulted in the appearance of a tau form having a high pI (middle panel, dashed circle). This finding suggests that CacyBP/SIP might be involved in dephosphorylation of tau protein in the cell. Interestingly, overexpression of S100A6 in NB2a cells inhibited the CacyBP/SIP phosphatase activity toward tau (Fig. 7B), suggesting that S100A6 had an effect on CacyBP/SIP activity (lower panel). In accordance with it we have observed that overexpression of S100A6 in NB2a cells affected the 2D electrophoretic pattern of CacyBP/SIP (Fig. 7C) as demonstrated by the appearance of a more basic form of CacyBP/SIP at the expense of the acidic one (lower panel, dashed circle).

4. Discussion

AD is the most common form of dementia among elderly people (Mayeux, 2010; Querfurth and LaFerla, 2010). The pathologic features of this disease include deposition of intracellular neurofibrillary tangles composed of tau protein, and extracellular plaques of amyloid-β peptides (Braak and Braak, 1998; Dickson, 1997).

In the present work we focused on the CacyBP/SIP protein, which is highly expressed in brain and was shown to change

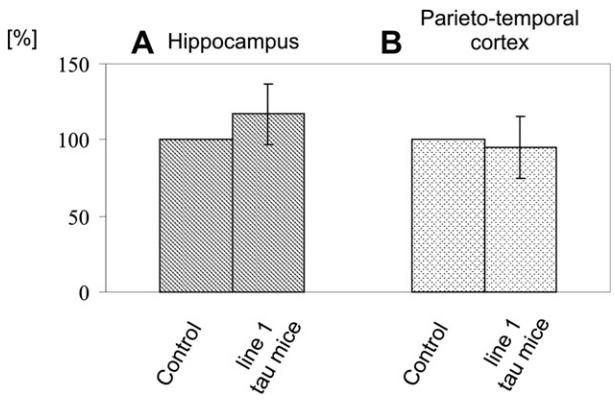

Fig. 5. Level of calcyclin binding protein and Siah-1 interacting protein in the hippocampus (A) and the parietotemporal cortex (B) of 8-month-old control and line 1 tau transgenic mice. In each case proteins (70 μg) from the extract were applied on the gel and analyzed by immunoblotting with anti-calcyclin binding protein and Siah-1 interacting protein antibodies. Results of the densitometric analysis of 4 independent experiments are presented as the mean ± SD.

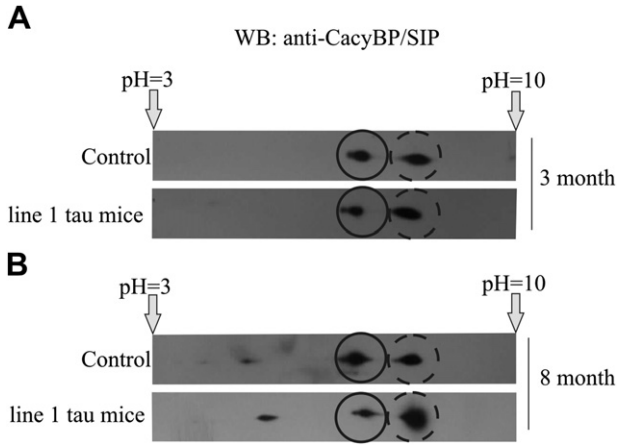

Fig. 6. Calcyclin (S100A6) binding protein and Siah-1 interacting protein (CacyBP/SIP) forms in control and line 1 tau mice brains were analyzed by immunoblotting with anti-CacyBP/SIP antibodies after 2D electrophoresis. Circles and dashed circles show two major CacyBP/SIP forms in brains of (A) 3- or (B) 8-month-old mice. A representative experiment, out of two performed for each age and group, is shown.

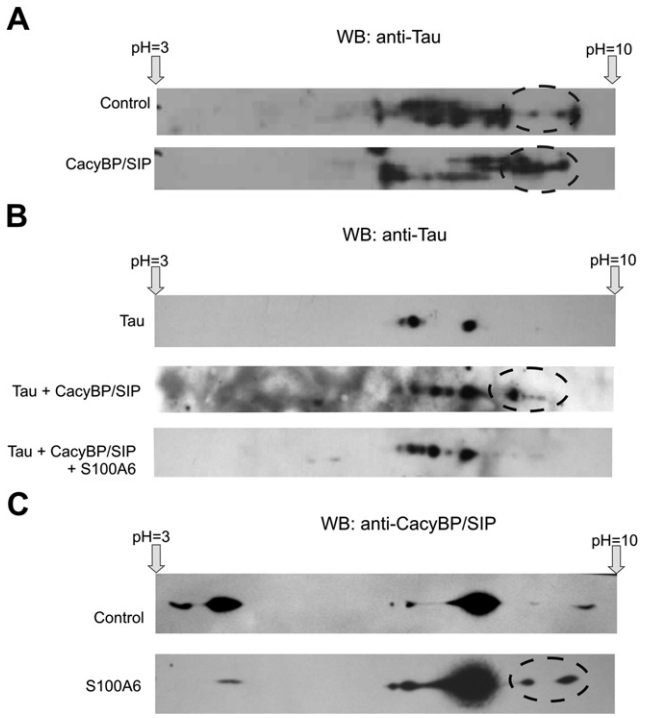

Fig. 7. (A and B) The influence of calcyclin (S100A6) binding protein and Siah-1 interacting protein (CacyBP/SIP) on tau forms analyzed by immunoblotting (WB) with anti-Tau46 antibodies after 2-dimensional electrophoresis. (A) Influence of recombinant CacyBP/SIP on phosphorylated tau isolated from bovine brain, (B) influence of overexpression of CacyBP/SIP (middle panel), and CacyBP/SIP with S100A6 (lower panel) on tau forms in NB2a cell extract. Dashed circles show the appearance of a more basic form of tau. (C) Influence of S100A6 overexpression on the level of CacyBP/SIP forms analyzed by immunoblotting with anti-CacyBP/SIP antibodies after 2-dimensional electrophoresis. Dashed circles show the appearance of a more basic form of CacyBP/SIP. In each case, a representative experiment, out of 3 performed, is shown.

localization in brain neurons in an age-dependent manner. In particular, we examined the characteristics of CacyBP/SIP in neurons affected by AD. We found that in AD brain, CacyBP/SIP was almost exclusively present in neuronal somata, whereas in elderly healthy controls, it was present in somata and neuronal processes. In APP+PS1 transgenic mice at the age of 8 months, in which amyloid-β deposits appeared in the brain, no difference in neuronal subcellular localization of CacyBP/SIP was observed in comparison with control mice. Interestingly, for line 1 tau transgenic mice of the same age, the differences in neuronal localization were similar to those observed for AD patients. These observations are consistent with earlier results of a differential, age-dependent localization of CacyBP/SIP, and the involvement of CacyBP/SIP in organization of microtubules (Filipek et al., 2008; Schneider et al., 2007).

In the present study, we have found that in AD patients and line 1 tau transgenic mice, changes in cellular distribution of CacyBP/SIP were similar to those observed for 2 other microtubule proteins, β-tubulin and tau. Immunohistochemistry of the hippocampal CA1 region and parietotemporal cortex revealed that in line 1 tau transgenic mice, β-tubulin and phosphorylated p-Tau(Ser-404) localized to the soma but were excluded from dendrites, whereas localization of β-tubulin and p-Tau(Ser-404) in control wild type mice was somatodendritic. Similarly, in AD patients, sequestration of both CacyBP/SIP and β-tubulin from the neuronal processes to the soma was accompanied by the presence of strongly labeled PHFs in the neuronal cell bodies. In contrast, in APP+PS1 mice, a model for amyloid pathology, we did not observe any changes in CacyBP/SIP, β-tubulin, and p-Tau(Ser-404) localization in neuronal compartments, when compared with that of control animals. Our results, for the first time, show an altered distribution of the CacyBP/SIP protein in brain neurons of AD patients and line 1 tau transgenic mice. It is interesting that changes of intracellular CacyBP/SIP localization in AD and line 1 tau transgenic mice resemble changes observed for phospho-tau and β-tubulin.

The present finding of altered distribution of the microtubule associated protein, tau, and β-tubulin is in accordance with our previous study in rats (Niewiadomska et al., 2005, 2006), which revealed that during aging the level of glycogen synthase kinase-3 beta (GSK-3β) kinase and of its substrate, tau, was reduced in axons and the proteins were redistributed to the neuronal body compartment. Similar accumulation of neuronal body filamentous inclusions was described earlier when transgenic mice over-expressing the longest human tau isoform carrying 3 mutations, G272V, P301L, and R406W (Lim et al., 2001) or a single R406W mutation were studied (Tatebayashi et al., 2002). In mice carrying the single R406W mutation these inclusions appeared at the age of 18 months. As with human cases, tau inclusions were composed of mutant and endogenous wild type tau and were associated with microtubule disruption and flame-shaped transformations of the affected neurons. Zhang et al. (2004) examined the effect of the R406W mutation on the cellular organization of tau in transgenic mice overexpressing the longest isoform. Immunohistochemical analysis confirmed an age-dependent accumulation of insoluble, filamentous tau aggregates in neuronal perikarya of the cerebral cortex, hippocampus, cerebellum, and spinal cord. The somato-dendritic compartment of neurons in transgenic mice carrying the R406W mutation showed the most intense tau immunostaining relative to a predominantly axonal localization in control animals. Moreover, there was a progressive age-dependent increase in perikaryal accumulation of tau and appearance of NFT-like structures in the hippocampus of the 12-month-old mutation group. These structures resembled NFTs identified in hippocampus of frontotemporal dementia with parkinsonism linked to chromosome 17 patients carrying the same mutation in the tau gene (Hutton et al., 1998). This mutated tau revealed lower binding affinity to microtubules, increased aggregation with insoluble pathological tau, and retarded axonal transport.

In a more recent study, Yan et al. (2010) used the chronic restraint rat model to investigate the effect of chronic restraint stress on the expression and distribution of phosphorylated tau and MAP2. The authors demonstrated that chronic restraint stress induced an increase in the level of soluble phosphorylated tau and altered the distribution patterns of both phosphorylated tau and MAP2. These findings indicate that an emotional stressor results in phosphorylation of tau and in abnormal distribution of both tau and MAP2 in neurons.

Recently, Nizzari et al. (2012) showed that there was also an imbalance between the nuclear and the cytoskeletal pools of phospho-tau in the brains of AD patients. In non-AD subjects, a stable pool of phospho-tau which remained strictly confined to neuronal nuclei was observed, and in neurons of AD patients nuclear localization of phospho-tau was significantly underrepresented. The authors provided evidence that overexpression of amyloid precursor protein modulated tau phosphorylation, altering the ratio between cytoskeletal and nuclear pools, and correlated with cell death. It was suggested that both amyloid precursor protein and amyloid-β modulated the phosphorylation of tau and its subcellular compartmentalization, an event that could lead to the formation of neurofibrillary tangles and to neurodegeneration of postmitotic neurons. These results are not in line with our data obtained for APP+PS1 mice. In neurons of these mice we did not observe a pronounced decrease in dendritic CacyBP/SIP or phospho-tau immunoreactivity in either cortex or hippocampus, although some amyloid-β deposits were present.

To find out the factors responsible for CacyBP/SIP relocalization we applied 2D electrophoresis to analyze CacyBP/SIP forms. We found that, in the case of 8-month-old mice, the ratio between spots representing 2 major CacyBP/SIP forms, differed significantly between line 1 tau and control brains. These 2 forms of CacyBP/SIP are likely to be posttranslationally modified forms of this protein and such modifications might determine CacyBP/SIP localization.

We have found that, as well as having a phosphatase activity toward ERK1/2 kinase, CacyBP/SIP can also affect tau protein phosphorylation status in vitro and in cultured neuroblastoma NB2a cells. Moreover, the effect of CacyBP/SIP on tau forms in cells was reversed by overexpression of a calcium binding protein, S100A6. It is well known that tau protein can be dephosphorylated by protein phosphatase 2A (Tsujio et al., 2005), protein phosphatase 2B (Rahman et al., 2005a), protein phosphatase 1 (Rahman et al., 2005b) or protein phosphatase 5 (PP5) (Liu et al., 2005). Recently, it has been shown that PP5 binds calcium binding proteins such as S100A1, S100A2, S100A6, or S100B and that these S100 proteins activate PP5, when checked using tau as a physiologic substrate. In our experiments, it was shown that S100A6 reversed the effect of CacyBP/SIP. This suggests that S100A6 inhibits CacyBP/SIP activity, probably through the influence on CacyBP/SIP phosphorylation because the amount of the basic CacyBP/SIP form increased after S100A6 overexpression. Thus, a high level of S100A6 might result in a lower rate of tau dephosphorylation. The newly identified association of PP5 with S100 and the interaction between CacyBP/SIP and S100 proteins (Filipek et al., 2002a), suggest a Ca^{2+}-dependent regulatory mechanism of protein dephosphorylation (Yamaguchi et al., 2012). This is not surprising because the level of Ca^{2+} and of calcium binding proteins in AD and other pathological states is deregulated (Fedrizzi and Carafoli, 2011).

Protein kinases and protein phosphatases act on tau and the ability to affect their activities has been considered as a possible therapeutic approach in AD (Gong et al., 2005). The discovery that CacyBP/SIP can influence the phosphorylation status and subcellular localization of tau protein, might shed some light on the molecular mechanism involved in the development of neurofibrillary tangles and on the etiology of AD.

Disclosure statement

The authors declare no conflict of interest.

The experiments on animals were conducted with the approval of the local ethics commission (Polish Law on the Protection of Animals) and carried out in accordance with the Principles of Laboratory Animals Care (NIH publication No 86-23).

The human tissues were obtained following the Austrian legal procedures which state that every patient who died in a hospital could undergo autopsy for diagnostic and/or scientific purposes. Based on the request from the Ethical Committee of the State of Upper Austria, patients sign upon hospital admission a written consent for scientific evaluation of their brain after death.

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