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A Novel Role for hGas7b in Microtubular Maintenance

POSSIBLE IMPLICATION IN TAU-ASSOCIATED PATHOLOGY IN ALZHEIMER DISEASE[*] [S]

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Here, we report a novel role for hGas7b (human growth arrest specific protein 7b) in the regulation of microtubules. Using a bioinformatic approach, we studied the actin-binding protein hGas7b with a structural similarity to the WW domain of a peptidyl prolyl *cis/trans* isomerase, Pin1, that facilitates microtubule assembly. Thus, we have demonstrated that hGas7b binds Tau at the WW motif and that the hGas7b/Tau protein complex interacts with the microtubules, promoting tubulin polymerization. Tau, in turn, contributes to protein stability of hGas7b. Furthermore, we observed decreased levels of hGas7b in the brains from patients with Alzheimer disease. These results suggest an important role for hGas7b in microtubular maintenance and possible implication in Alzheimer disease.

The microtubule network plays an important role in intracellular trafficking and neurite outgrowth in the differentiated neurons (1), which is mainly regulated by microtubule-associated proteins. Tau, a major microtubule-associated protein in neurons, enhances tubulin polymerization and is required for microtubular maintenance. There are at least two mechanisms that regulate effects of Tau on tubulin polymerization, such as posttranslational modification and association with modifier proteins. First, phosphorylation makes Tau dissociate from tubulin and promotes microtubule disassembly; second, a peptidyl prolyl *cis/trans* isomerase Pin1 selectively binds with the phospho-Tau at the WW domain in Pin1 (2, 3), which, in turn, restores the binding of Tau with tubulin and promotes microtubule assembly (2).

Identification of novel factors that regulate tubulin polymerization is still very important to understand the mechanism of microtubular maintenance. Because the disturbance of this mechanism leads to the pathology of many neurological disorders, such as Alzheimer disease, this effort may have direct implication in clinical neuroscience. Thus, we have searched proteins that contain structurally similar motifs to the WW domain of Pin1 in a human protein data base and focus on a hGas7b (human growth arrest specific protein 7b), which was known as an actin-binding protein (4–6).

Here, we report that hGas7b binds Tau and that the hGas7b facilitates tubulin polymerization in a Tau-dependent manner. In turn, Tau contributes to protein stability of hGas7b, suggesting that the interaction of these two proteins is functionally bidirectional. Furthermore, we obtained evidence that Tau contributes to the levels of hGas7b that are markedly down-regulated in brains from patients with Alzheimer disease (AD).[2]

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Anti-Gas7 polyclonal antibody, Tau5 (BD Biosciences), Tau1 (Chemicon, CA), PHF1 (7, 8), anti-Tau (N-terminal), AT180 polyclonal antibody (Santa Cruz Biotechnology, CA), anti-tubulin monoclonal antibody (Sigma-Aldrich), anti-c-Myc monoclonal antibody (Sigma-Aldrich), and anti-FLAG M2 monoclonal antibody (Sigma-Aldrich). Anti-Gas7 antibody was prepared from the rabbits immunized with a His tag-fused hGas7b. The anti-Gas7 antibody detected hGas7b and hGas7a, as well as mouse Gas7 and Gas7-cb. Enhanced green fluorescent protein (EGFP)-fused Gas7 expression vectors were constructed by insertions of *hGas7b* into the downstream of EGFP in pEGFP-C1 (Clontech).

Protein Data Base—The human protein database “HUGE” (a data base of human unidentified gene-encoded large proteins analyzed by the Kazusa human cDNA project) was used.

Biochemistry—Proteins in the cultured cells and brains were analyzed by Western blotting, pulldown, and immunoprecipitation assays according to our published protocol (9).

Microtubule Polymerization—Crude microtubule proteins were prepared from bovine brains by three cycles of temperature-dependent polymerization and depolymerization as described (10). Tubulins were further purified from microtubule proteins by the PIPES buffer method (11). Tau was purified from microtubule proteins using heat treatment and perchloric acid (12), and recombinant Gas7 protein was prepared from *Escherichia coli.* The bulk polymerization was also measured by turbidim-

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[2] The abbreviations used are: AD, Alzheimer disease; PIPES, 1,4-piperazinediethanesulfonic acid; PHF, paired helical filaments; EGFP, enhanced green fluorescent protein.

etry at 350 nm using a spectrophotometer (U-1500, Hitachi). Microtubule polymerization was observed directly by a dark field microscope (Olympus BX51).

TABLE 1
Normal and AD brain list

Age	Sex	Time after death	Cause of death
		min	
Normal brains			
77	F	60	Mitral regurgitation
75	M	160	Acute ischemic stroke
69	F	100	Amyotrophic lateral sclerosis
70	F	90	Systemic lupus erythematosus
72	M	120	Olivopontocerebellar atrophy
77	M	90	Amyotrophic lateral sclerosis
78	F	80	Polyarteritis nodosa
88	M	340	Congestive heart failure
80	M	720	Aortic dissection
53	F	60	Lung cancer
AD brains			
77	F	300	
89	F	360	
69	F	360	
87	M	420	
90	F	930	
63	F	240	
84	F	390	
81	F	420	
82	M	780	
69	F	360	

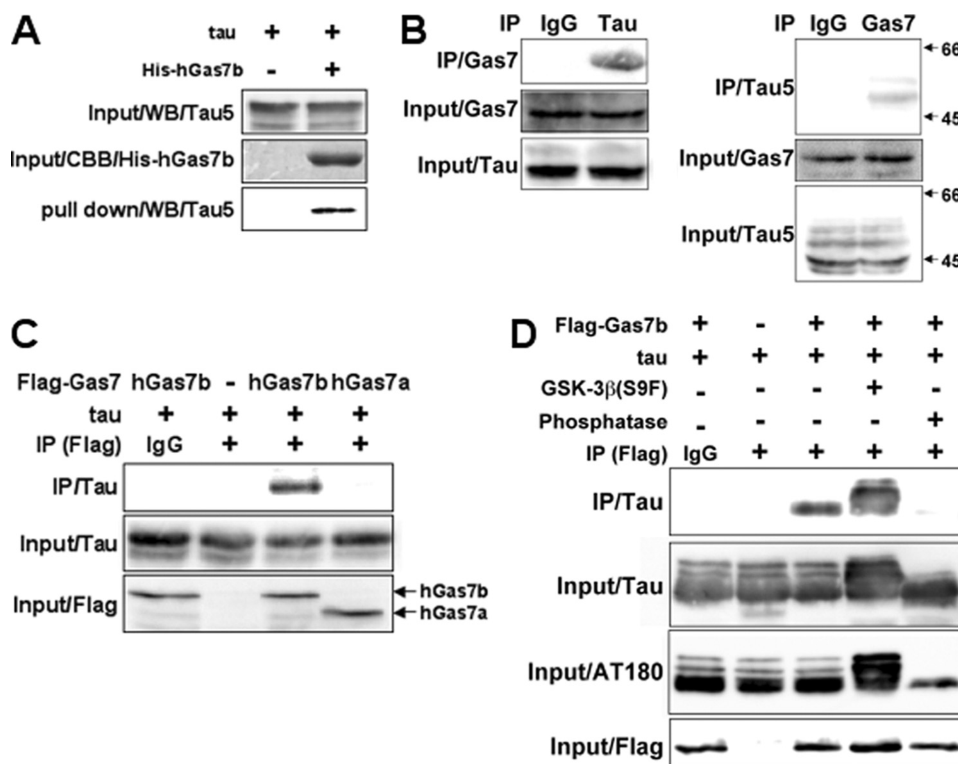

FIGURE 1. **Interaction of hGas7b and phosphorylated Tau.** *A*, a pulldown assay using cell lysates prepared from HEK293 cells transfected with *tau* (the longest isoform) and nickel-chelating beads carrying recombinant His-hGas7b. The captured proteins were analyzed by 10% SDS-PAGE and immunoblotting using FLAG or Tau5 antibodies. *B*, immunoprecipitation (*IP*) assays using polyclonal anti-Tau or anti-Gas7 antibody and lysates prepared from mouse brain. The immunoprecipitants were analyzed by immunoblotting with anti-Gas7 (goat) or monoclonal anti-tau5 antibody. *C*, immunoprecipitation assays using monoclonal anti-FLAG antibody and cell lysates prepared from HEK293 cells cotransfected with *FLAG-hGas7b* or *FLAG-hGas7a* and the longest isoform of *tau*. The immunoprecipitants were analyzed by immunoblotting with anti-Tau (N-terminal) polyclonal antibody. *D*, immunoprecipitation assays using monoclonal anti-FLAG antibody and cell lysates prepared from HEK293 cells cotransfected with FLAG-*hGas7b* and the longest isoform of *tau* as well as either with or without *GSK-3β*. In some experiments, cell lysates of *tau*-transfected HEK293 cells were treated with alkaline phosphatase prior to immunoprecipitation.

Analysis of Binding of hGas7b on Microtubules—In vitro binding assays were conducted in flow chambers (chamber volume, 15 μl). Twenty mg/ml of microtubules was incubated with 1 mM Taxol for 20 min at 33 °C and then introduced in a flow chamber coated with 100 nM anti-α-tubulin antibody and blocked with bovine serum albumin. One nM Tau and 100 pM EGFP or EGFP-hGas7b proteins were subsequently added into the chamber and observed at 25 °C under the fluorescence microscope.

Cell Culture, Transfection, and Immunofluorescent Cell Staining—Transfection of plasmids into COS7 and HEK293 cells was conducted by using Lipofectamine 2000 (Invitrogen), as described previously (9). Forty eight h after the transfection, the cells were lysed, and some aliquots were pretreated with alkaline phosphatase. Neuro2A cells were plated onto glass coverslips and transfected with *pCIneo-tau* and *FLAG-hGas7b* expression vector. After 48 h, cells were fixed with 4% paraformaldehyde, treated with FLAG M2 or PHF1 and Alexa Fluor 488- and 595-conjugated secondary antibodies, and observed under confocal microscopy (Zeiss LSM510; Carl Zeiss, Jena, Germany).

Immunohistochemistry of Human and Mouse Brains—Coronal sections at 5-μm thickness of human (Table 1) and *tau* knockout mouse (13) brains were used. All of these human cases were retrieved from the autopsy specimen files at Tohoku University Hospital, under the approval of the local ethical committee. A series of adjacent sections were immunostained as described (14) using polyclonal anti-Gas7 antibody and PHF1, Tau5, and NeuN monoclonal antibodies (Chemicon).

RESULTS

hGas7b Binds to Tau and Co-localizes with Microtubules—We searched proteins that may contain a motif structurally similar to the WW domain of Pin1 by utilizing a human protein data base and focused on hGas7b (supplemental Fig. 1). Thus, we hypothesized whether hGas7b may interact with Tau and validated this idea by two methods. In a pull-down assay with protein extracts of HEK293 cells that overexpress Tau and recombinant hGas7b-Sepharose beads, we observed the binding of these proteins (Fig. 1*A*). Endogenous protein interaction of Gas7 and Tau was confirmed with extracts of mouse brain by immunoprecipitation (Fig. 1*B*). Tau binding with Gas7 is dependent on the WW domain, as selectively to Gas7b, but not to Gas7a, as we failed to observe

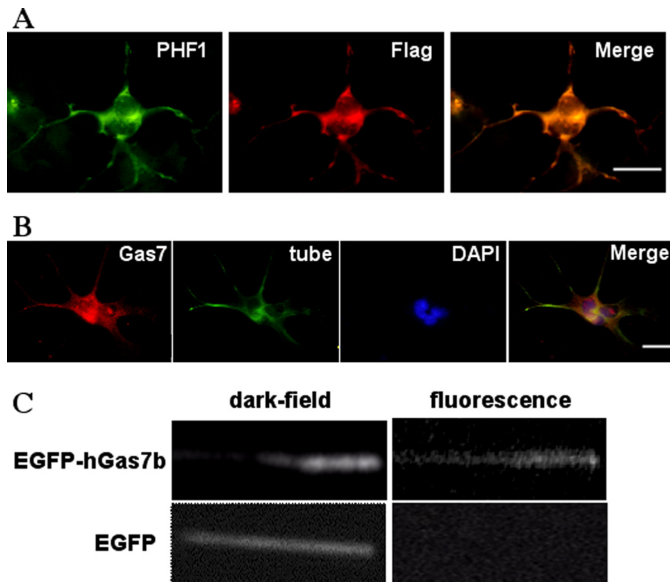

FIGURE 2. **Expression of Gas7 and Tau in Neuro2A cells.** *A*, Neuro2A cells transfected with *pCIneo-tau* and *pFlag-hGas7b*. The phosphorylated Tau detected with PHF-1 (Ser$^{396/404}$ Tau) antibody (*green*) and FLAG-Gas7 detected with anti-FLAG antibody (*red*). Fluorescence was observed under a confocal microscopy. *Scale bars*, 100 μm. *B*, localization of endogenous tubulin detected with anti-tubulin antibody (*green*) and exogenous Gas7 detected with anti-Gas7 antibody (*red*) in Neuro2A cells. *Scale bars*, 200 μm; *DAPI*, 4′,6-diamidino-2-phenylindole. *C*, binding of EGFP-hGas7b/Tau to microtubule; the mixture of recombinant EGFP-hGas7b (100 pM) and Tau (1 nM) prepared from bovine brains were added to the microtubules, stabilized with 1 mM Taxol, and analyzed with a fluorescence and dark field microscope.

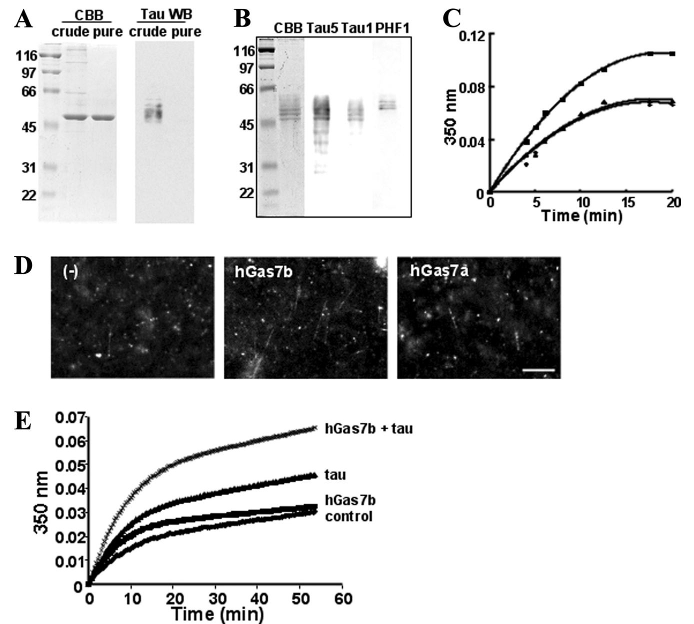

FIGURE 3. **Enhancement of microtubule polymerization by Tau and hGas7b.** Purity of tubulin was tested by Coomassie Brilliant Blue (*CBB*) staining (*A*) and Western blot (*WB*) with anti-Tau antibodies (*B*). Phosphorylation states of Tau was examined by various monoclonal antibodies; Tau5, Tau1, and the PHF-1. Shown are the bulk polymerization of microtubules (*C*). 1.0 mg/ml of crude brain tubulin solution with 2 mM of hGas7a or hGas7b were incubated at 25 °C, and the turbidity was measured at 350 nm. *Closed diamonds*, control; *closed squares*, hGas7b; *closed triangles*, hGas7a. *D*, dark field microscopy of the crude brain tubulin solution in the absence and presence of hGas7b or hGas7a. Microtubules polymerization did not increase from 1.0 mg/ml of crude brain tubulin in the presence of hGas7a. *Scale bars*, 10 μm. *Closed circles*, control; *closed squares*, hGas7b; *closed triangles*, hGas7a. *E*, the bulk polymerization of microtubules. 0.7 mg/ml of purified tubulin solution with 10 nM of Tau or hGas7b were incubated at 37 °C, and the turbidity was measured at 350 nm.

the Gas7/Tau interaction in cells overexpressing Gas7a and Tau (Fig. 1*C*). The binding is dependent on protein phosphorylation, as pretreatment of extracts of HEK293 cells that overexpress Tau and hGas7b with alkaline phosphatase abolishes the protein interaction (Fig. 1*D*).

Next, we addressed subcellular localization of hGas7b and Tau by immunofluorescent cell staining with a confocal microscope. In elongated neurites of Neuro2A cells, overexpressed FLAG-hGas7b staining is observed in association with Tau or tubulin (Fig. 2, *A* and *B* and supplemental Fig. 2). To further validate the association of recombinant EGFP-hGas7b with the microtubules, we used a dark field microscope and confirmed binding (Fig. 2*C*).

hGas7b Is Required for Microtubule Polymerization in a Microtubule-associated Protein-dependent Manner—We reported that Gas7 promotes microtubule polymerization of the crude tubulin preparation containing microtubule-associated proteins *in vitro* by dark field and electron microscopy (15). We hypothesized that hGas7b may cooperate with Tau to promote microtubule polymerization. Thus, we prepared crude and pure tubulin fractions from bovine brains (Fig. 3*A*). Consistent with the previous studies (11), Tau existed in the crude, but not in the pure tubulin fractions (Fig. 3*B*). Addition of recombinant hGas7b, but not hGas7a or mock, increased the turbidity of the tubulin fractions, suggesting that hGas7b, but not hGas7a, may have a unique role in tubulin polymerization (Fig. 3*C*). This observation was confirmed by experiments with a dark field microscope (Fig. 3*D*). The selective influence of hGas7b is required for tubulin-associated proteins, as the effect specifically occurs with crude, but not pure, tubulin fractions (Fig. 3*E*). The critical difference of hGas7b from hGas7a is caused by the WW domain that is for its binding with Tau. Therefore, it is highly probable that Tau may be required for the action of hGas7b on tubulin polymerization.

Tau Increases Stability of hGas7b Protein—In cell culture, decrease in the level of hGas7b in the presence of a protein synthesis inhibitor, cycloheximide, is attenuated by overexpression of Tau (Fig. 4*A*). We examined levels of mouse Gas7 (human hGas7b orthologue) in Tau knock-out mice to validate the idea that Tau may increase the stability of hGas7b. A decreased level of mouse Gas7 was consistently observed in both immunohistochemical analysis and Western blotting (Fig. 4, *B* and *C*).

Decreased Level of hGas7b in Brains from Patients with Alzheimer Disease—It is known that levels of Tau in normal soluble fractions are decreased due to its sequestration into the neurofibrillary tangles in brains from patients with Alzheimer disease. As Tau increases the stability of hGas7b protein, we wondered whether hGas7b might be altered in the patient brains and compared its expression in the hippocampus of normal controls and patients with Alzheimer disease. In normal brains, Tau was localized in neuronal cell bodies and neuropils in a diffuse manner without any sign of neurofibrillary tangles (Fig. 5, *A* and *C*). Substantial levels of

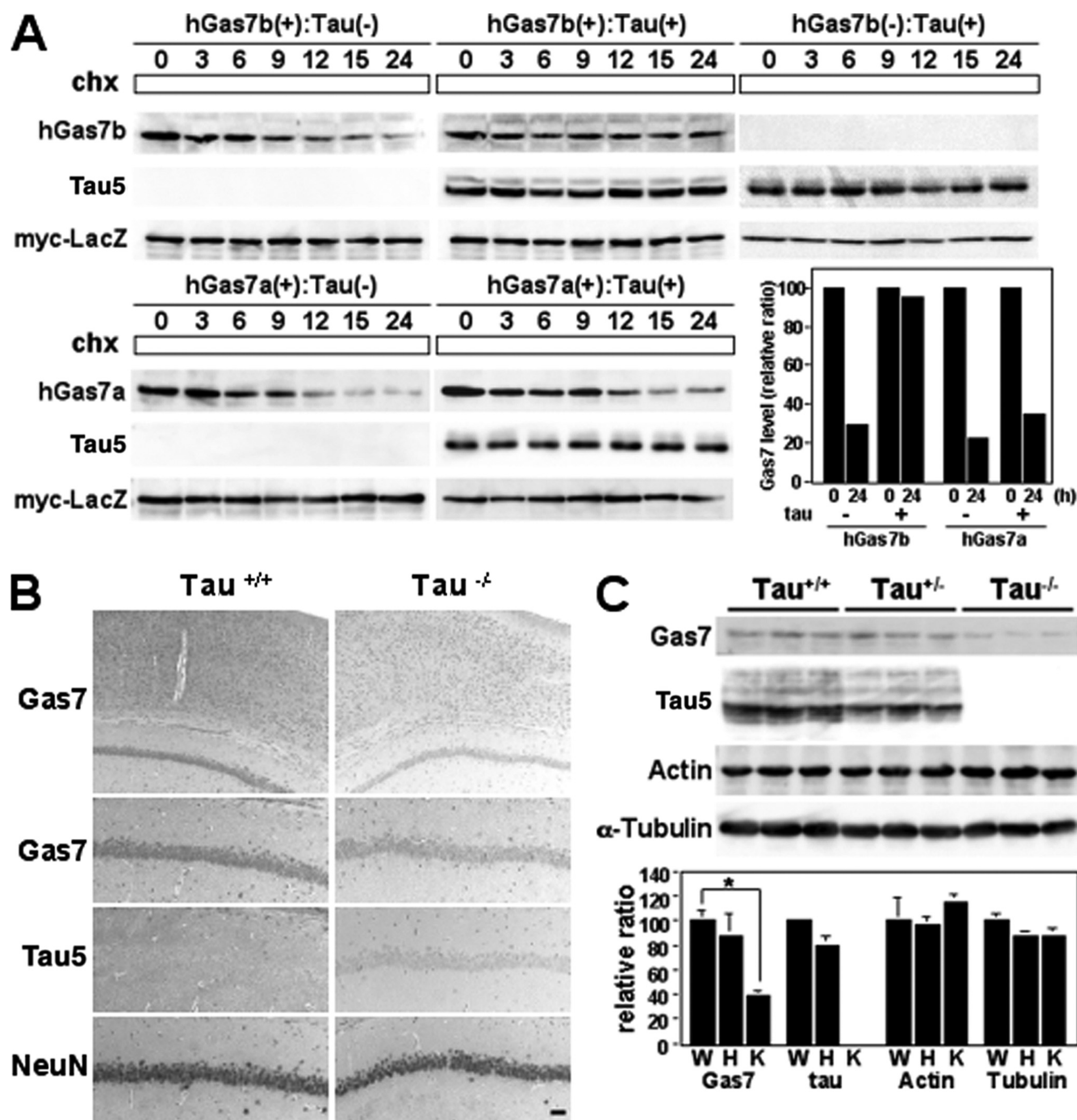

FIGURE 4. **Tau-mediated stabilization of hGas7b against protein degradation *in vitro* and *in vivo*.** *A*, Tau inhibits the degradation of hGas7b *in vitro*. HEK293 cells cotransfected with *hGas7b*, *hGas7a*, *tau*, and *myc-LacZ* were treated with cycloheximide (100 μg/ml) 24 h after transfection and harvested at the indicated time points. The cell lysates were analyzed by 10% SDS-PAGE and immunoblotting using FLAG M2, Tau5, or Myc antibodies. Intensities of the chemiluminescence were quantified with ImageGauge (Fujifilm). *B*, immunohistochemical analysis of the expression of Gas7 (mouse hGas7b) in brains of Tau-deficient mice. Hippocampal sections prepared from Tau-deficient mice (Tau$^{-/-}$) and control littermates (Tau$^{+/+}$) were immunostained with anti-Gas7, Tau5, and NeuN (as an internal control) antibodies. *Scale bars*, 200 μm. *C*, immunoblot analysis of Gas7 expressed in the brains of a Tau-deficient mouse and a control littermate. Data represent means ± S.D. from three individual mouse brains. *, $p < 0.001$. *chx*, cyclohexamide.

hGas7b were also observed in neuronal cells (Fig. 5*B*). In contrast, Tau staining was prominent in the neurofibrillary tangles, and robust reduction in the levels of hGas7 was also observed in neurons in brains from patients with Alzheimer disease (Fig. 5, *D*, *E*, *F*, *G*, and *H*). Marked reduction of hGas7b in Alzheimer disease brains was also confirmed by Western blotting (Fig. 5*I*).

DISCUSSION

The main findings of the present study are as follows: (i) protein interaction of hGas7b with Tau in a protein phosphorylation-dependent manner; (ii) requirement of hGas7b in microtubule polymerization; (iii) stabilization of hGas7b by Tau; and (iv) decreased expression of hGas7b in brains from patients with Alzheimer disease. These novel functions

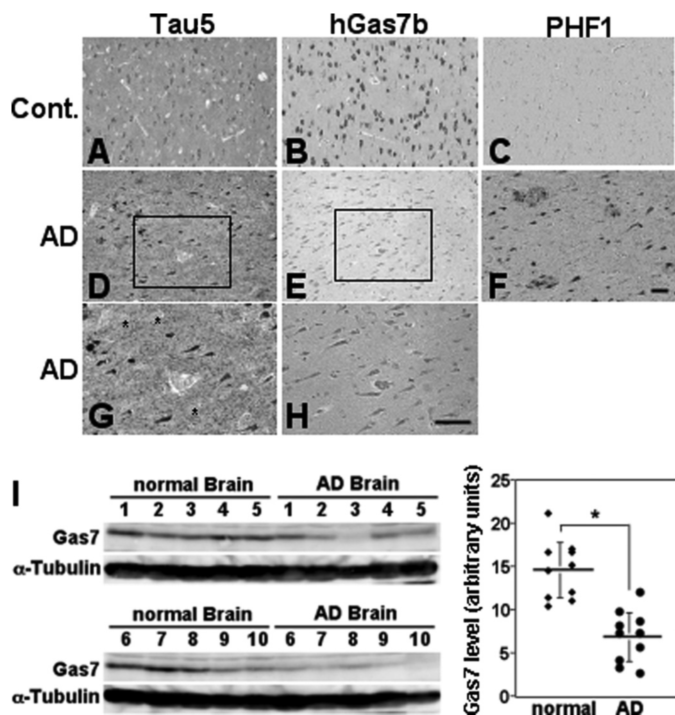

FIGURE 5. **Expression levels of Gas7 and Tau in AD and control brains.** *A–H*, immunohistochemical analysis of tissue sections prepared from control (*A–C*) and AD (*D–H*) brains with Tau5 (*A*, *D*, and *G*), anti-Gas7 (*B*, *E*, and *H*), and PHF1 (*C* and *F*) antibodies. The *boxed areas* in *D* and *E* are magnified in *G* and *H*, respectively. *Scale bars*, 100 μm. *I*, immunoblot analysis of Gas7 in normal and AD brains. Intensities of the chemiluminescence were quantified with Image-Gauge (Fujifilm). Data represent means ± S.D. from 10 individual brains. *, $p < 0.0001$. *Cont.*, control.

are specifically associated with hGas7b, but not with hGas7a. Thus, the WW domain unique to hGas7b is likely to play key roles.

Although we report a novel role for hGas7b in microtubular maintenance, possibly being implicated, at least in part, in the pathology of Alzheimer disease, there are several mechanistic questions to be addressed in future studies. First, we still do not know how the Tau/Gas7b protein interaction is regulated by phosphorylation. As Tau is known as a phospho-protein, it is likely that Tau phosphorylation may participate in this regulation. Nonetheless, a role for possible phosphorylation in hGas7b will also be examined in the future. Second, it remains to be clarified which microtubule-associated proteins mediate the action of hGas7b for tubulin polymerization. We predict that the Tau/Gas7b protein interaction may play a key role. Third, it is important to address how hGas7b functions in association with microtubules in cells. Our preliminary data suggest that knockdown expression of Gas7 inhibits neurite outgrowth in PC12 cells.[3]

The pathological mechanisms for Alzheimer disease are still unclear. Tau-associated pathology, featured by the existence of neurofibrillary tangles, is a clue to address the mechanisms. Normal Tau, which stabilizes hGas7b, is decreased by the pathological sequestration of the protein into the neurofibrillary tangles. Thus, marked reduction of hGas7b in patient brains can be interpreted as a reasonable downstream of the Tau pathology. Because hGas7b plays a key role in microtubular maintenance, the disturbance of hGas7b may be a key executive process to accelerate cellular dysfunction and death in the Tau pathology of Alzheimer disease.

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REFERENCES

1. Drubin, D. G., and Nelson, W. J. (1996) *Cell* **84,** 335–344
2. Lu, P. J., Wulf, G., Zhou, X. Z., Davies, P., and Lu, K. P. (1999) *Nature* **399,** 784–788
3. Smet, C., Sambo, A. V., Wieruszeski, J. M., Leroy, A., Landrieu, I., Buée, L., and Lippens, G. (2004) *Biochemistry* **43,** 2032–2040
4. Ju, Y. T., Chang, A. C., She, B. R., Tsaur, M. L., Hwang, H. M., Chao, C. C., Cohen, S. N., and Lin-Chao, S. (1998) *Pro. Nat. Acad. Sci. U.S.A.* **95,** 11423–11428
5. Chao, C. C., Su, L. J., Sun, N. K., Ju, Y. T., Lih, J. C., and Lin-Chao, S. (2003) *J. Neurosci. Res.* **74,** 248–254
6. Lortie, K., Huang, D., Chakravarthy, B., Comas, T., Hou, S. T., Lin-Chao, S., and Morley, P. (2005) *Brain Res.* **1036,** 27–34
7. Greenberg, S. G., Davies, P., Schein, J. D., and Binder, L. I. (1992) *J. Biol. Chem.* **267,** 564–569
8. Lang, E., Szendrei, G. I., Lee, V. M., and Otvos, L., Jr. (1992) *Biochem. Biophys. Res. Commun.* **187,** 783–790
9. Akiyama, H., Shin, R. W., Uchida, C., Kitamoto, T., and Uchida, T. (2005) *Biochem. Biophys. Res. Commun.* **336,** 521–529
10. Karr, T. L., White, H. D., and Purich, D. L. (1979) *J. Biol. Chem.* **254,** 6107–6111
11. Castoldi, M., and Popov, A. V. (2003) *Protein Expr. Purif.* **32,** 83–88
12. Murphy, D. B. (1991) *Methods Enzymol.* **196,** 235–246
13. Fujio, K., Sato, M., Uemura, T., Sato, T., Sato-Harada, R., and Harada, A. (2007) *Neuroreport.* **18,** 1049–1052
14. Murayama, H., Shin, R. W., Higuchi, J., Shibuya, S., Muramoto, T., and Kitamoto, T. (1999) *Am. J. Pathol.* **155,** 877–885
15. Uchida, T., Akiyama, H., Sakamoto, W., Koga, T., Yan, K., Uchida, C., Hirose, K., and Itoh, T. J. (2009) *J. Mol. Biol.* **391,** 849–857

[3] H. Akiyama and T. Uchida, unpublished observation.