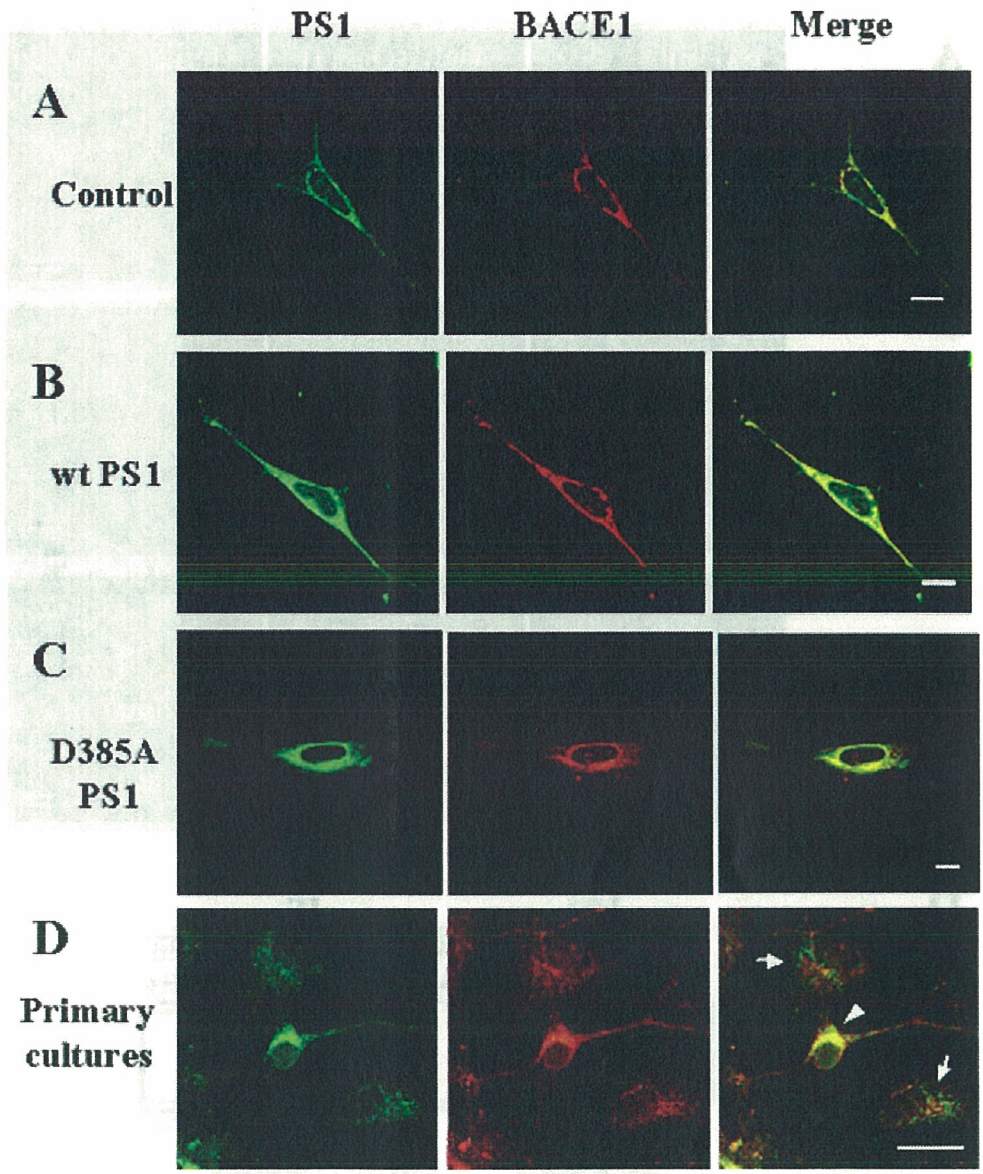

Fig. 5. PS1 and BACE1 colocalization in the SH-SY5Y cells and primary neurons. Each SH-SY5Y cell line (**A–C**) and rat primary cultured cortical neurons (**D**) were doubly stained with anti-BACE1 antibody against the C terminus (MAB5308) and anti-PS1 antibody against the N terminus. In each SH-SY5Y cell line, PS1 was largely colocalized with endogenous BACE1 (A–C, merged images). Note that an increase in wt PS1 immunoreactivity was accompanied by an increase in endogenous BACE1 immunoreactivity (A,B). Endogenous PS1 and BACE1 were considerably colocalized in a primary neuron, especially in a perinuclear area (arrowhead), compared to glial cells (arrows) (D, merged image). Scale bar = 20 μm.

anti-PS1 antibody against the N terminus and the MAB5308 antibody. As shown in Figure 5D, endogenous PS1 and BACE1 were considerably colocalized in a primary neuron, especially in a perinuclear area (arrowhead), compared to glial cells (arrows).

BACE1 in D385A PS1 Cells Fails to be Properly Transported From the ER to the Golgi

As shown in Figures 4 and 5, D385A PS1 is associated with endogenous BACE1 as well as wt PS1 in the immunoprecipitation and immunofluorescent experiments. However, BACE1 maturation is markedly downregulated in D385A PS1 cells in contrast to in wt PS1 cells (Fig. 2C). Because BACE1 undergoes trafficking-dependent maturation through the secretory pathway from the ER to the Golgi, we hypothesized that the transport of endogenous BACE1 from the ER to the Golgi was suppressed significantly in D385A PS1 cells. We first examined the intracellular localization of BACE1 in wt and D385A PS1 cells by immunofluorescent study using markers specific for the ER and the Golgi. Each cell line was double stained with the MAB5308 antibody to visualize BACE1 and either the anti-calnexin antibody to visualize the ER or the anti-mannosidase II antibody to visualize the Golgi. Interestingly, we found that a part of BACE1 in wt PS1 cells was apparently colocalized with mannosidase II in the perinuclear area (Fig. 6A, arrowhead), whereas the colocalization was negligible in D385A PS1 cells (Fig. 6B). Moreover, in D385A PS1 cells, most of BACE1 was colocalized with calnexin, a marker for the ER (Fig. 6C). These results suggest that proper transport of BACE1 from the ER to the

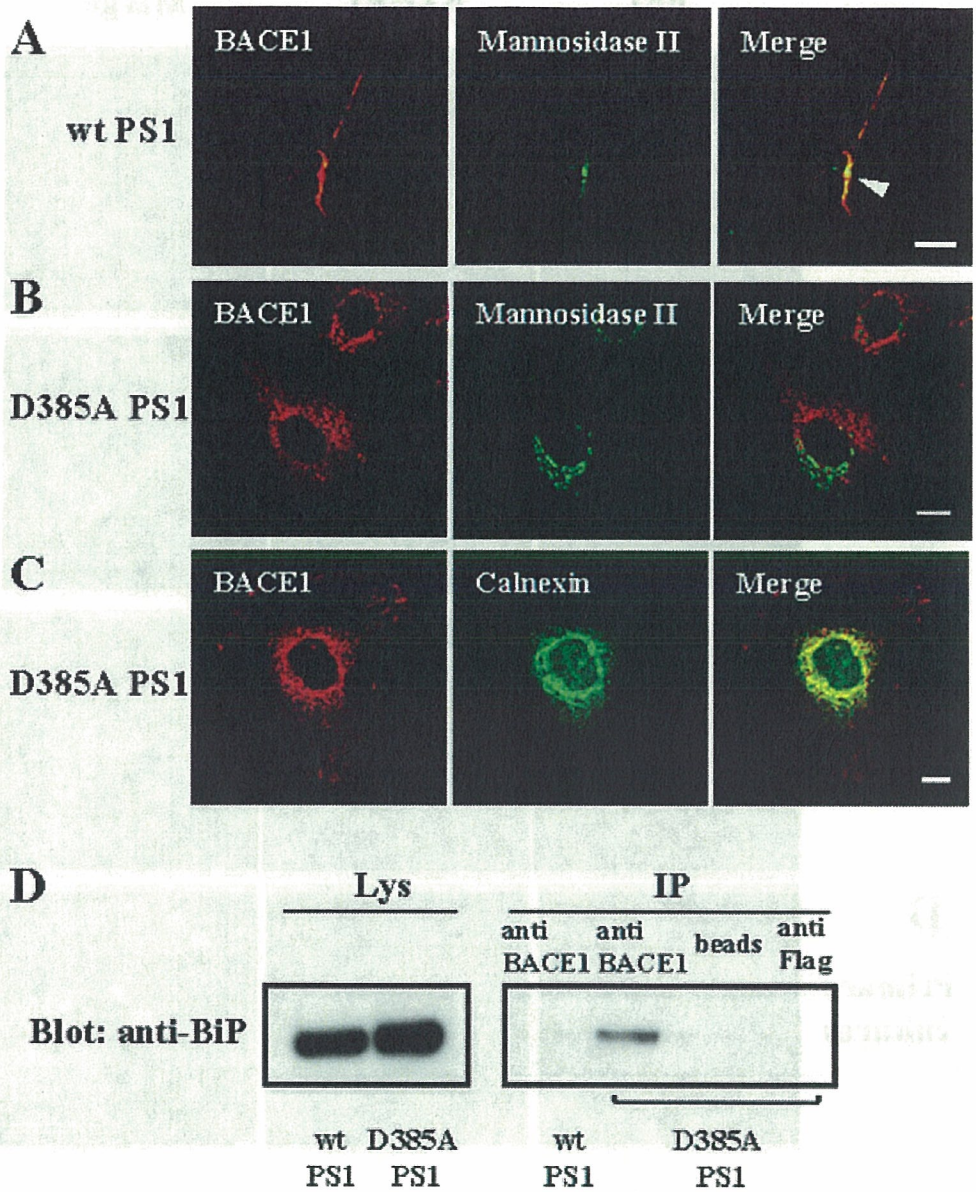

Fig. 6. Endogenous BACE1 in D385A PS1 cells is largely retained in the ER and fails to be transported to the Golgi. **A,B:** Wt PS1 cells and D385A PS1 cells were doubly stained with anti-BACE1 antibody against the C terminus (MAB5308) and anti-mannosidase II antibody. No apparent colocalization of endogenous BACE1 with mannosidase II, a marker for the Golgi, was observed in D385A PS1 cells (B), whereas endogenous BACE1 in wt PS1 cells was partially colocalized with mannosidase II (A, arrowhead). **C:** D385A PS1 cells were doubly stained with anti-BACE1 antibody (MAB5308) and anti-calnexin antibody. Endogenous BACE1 in D385A PS1 cells was colocalized mostly with calnexin, a marker for the ER (C, merged image). Scale bar = 20 μm. **D:** Equal amounts of whole cell lysates from wt PS1 and D385A PS1 cells were immunoprecipitated with anti-BACE1 antibody (MAB5308). Cell lysate (Lys) as well as immunoprecipitates (IP) were subjected to immunoblotting with anti-BiP antibody. The immunoprecipitates from D385A PS1 cell lysate showed BiP immunoreactivity. No BiP immunoreactivity was observed in the immunoprecipitate from the wt PS1 cell lysate, or from the samples of Protein G-Sepharose beads alone (beads) or anti-Flag antibody, used as negative controls.

Golgi is impaired in D385A PS1 cells, and are compatible with the previous observation that BACE1 maturation is downregulated in D385A PS1 (Fig. 2C).

BiP, an ER-resident molecular chaperone, binds to misfolded, underglycosylated, or unassembled proteins and assists with protein folding and retention of misfolded proteins in the ER (Gething, 1999). We examined the possibility that BACE1 in D385A PS1 cells, which might become terminally misfolded after release from the ER folding machinery, was preferentially associated with BiP. Equal amounts of cell lysate obtained from either wt PS1 or D385A PS1 cells were immunoprecipitated using the MAB5308 antibody, and the immunoprecipitate was subjected to Western blotting using an anti-BiP antibody. The immunoreactivity of BiP co-immunoprecipitated with BACE1 was observed in D385A PS1 cells, whereas it was not detected in wt PS1 cells (Fig. 6D).

These results suggest that proper transport of endogenous BACE1 from the ER to the Golgi is impaired in D385A PS1 cells, resulting in aberrant retention of BACE1 within the ER, and its association with BiP as unfolded or misfolded proteins.

DISCUSSION

Despite of extensive research, the pathogenesis of AD is still in an enigma. Several recent reports showed elevated β-secretase expression and enzymatic activity in the absence of the alteration of message expression in AD brains (Fukumoto et al., 2002; Holsinger et al., 2002; Yang et al., 2003), indicating the dysregulation of β-secretase activity may be involved in AD pathogenesis. Interestingly, amino-terminally truncated Aβ peptides, which are known to be β-secretase cleavage products, were more abundant in

the brains of subjects carrying PS1 gene mutations causing FAD than in those with sporadic AD and FAD associated with a point mutation in APP gene (Russo et al., 2000), indicating that FAD-linked mutations in PS1 can somehow affect β-secretase activity. However, the functional link between PS1 and BACE1 has never been elucidated, although they are supposed to be the 'key players' of AD pathogenesis.

In the present study, we showed a solid evidence for a novel function of PS1 in regulating BACE1 maturation. The levels of mature BACE1 either endogenously or exogenously expressed in PS−/− MEFs were reduced significantly, as compared to that in wt MEFs. These results suggest that the presence of presenilins can promote BACE1 maturation. To validate this effect of PS1 in neuronal cells endogenously expressing BACE1, we analyzed stably transfected SH-SY5Y cells with either wt PS1 or dominant-negative (D385A) PS1. Interestingly, the overexpression of D385A PS1 decreased the level of mature BACE1 protein by 50%, accompanied by a 60% reduction in the ratio of mature:proBACE1 as compared to control cells. Conversely, the overexpression of wt PS1 upregulated the level of mature BACE1 protein. Given that the levels of BACE1 mRNA were comparable between wt PS1 and D385A PS1 cells, these results indicate that functional PS1 positively regulates BACE1 maturation post-translationally.

Intriguingly, a recent study showed that targeting BACE1 to lipid rafts, cholesterol- and sphingolipid-enriched microdomains within cellular membranes, upregulated the β-site processing of APP, leading to a drastic increase in Aβ generation in SH-SY5Y cells (Cordy et al., 2003), indicating that proper distribution of BACE1 is crucially important for regulating Aβ generation. Although a growing number of evidence has strongly supported a direct role for PS1 in the γ-secretase cleavage of APP as the catalytic component of the γ-secretase complex (Wolfe et al., 1999a,b), a function of PS1 in protein trafficking has also been shown, especially with regard to APP and nicastrin (Kim et al., 2001; Edbauer et al., 2002; Leem et al., 2002a,b; Cai et al., 2003; Herreman et al., 2003). This function is consistent with a functional analogy to the weakly homologous SPE4 protein of *C. elegans*, which has been implicated in the maintenance of a Golgi-derived membranous organelle and is thought to be important in the partitioning of protein and cell membrane products in maturing spermatocytes (L'Hernault and Arduengo, 1992). These previous findings provide support for the idea that PS1 may regulate BACE1 trafficking, thereby modulating its maturation.

Indeed, we found that BACE1 in D385A PS1 cells was mostly retained within the ER and was negligibly localized within the Golgi (Fig. 6B,C), whereas a part of BACE1 in wt PS1 cells was apparently localized within the Golgi (Fig. 6A). Moreover, we found that BACE1 in D385A PS1 cells was associated significantly with BiP, an ER resident molecular chaperone, in contrast to in wt PS1 cells (Fig. 6D), presumably attributable to its aberrant retention within the ER. Interestingly, a splice variant of BACE1, lacking terminal two-thirds of exon 3, is expressed in pancreas. This isoform of BACE1, lacking β-secretase activity, colocalizes with BiP and its transport along the secretory pathway is blocked at the level of the ER, indicating that misfolded or non-functional BACE1 can associate with BiP in the ER (Bodendorf et al., 2001). These results indicate that wt PS1 upregulates BACE1 maturation, whereas the absence of PS1 or non-functional D385A PS1 downregulates its maturation as a result of its inefficient transport from the ER to the Golgi.

The above data raises the question whether PS1/γ-secretase activity directly affects the trafficking and maturation of BACE1 or not. We observed no significant effect of the γ-secretase inhibitors on BACE1 maturation in primary neurons. Thus, we consider that PS1/γ-secretase activity itself is less likely to be involved in the maturation process of BACE1. Nevertheless, further experiments will be needed to elucidate a relationship between the PS1/γ-secretase activity and the novel function of PS1 in the trafficking and maturation of BACE1.

Finally, how dose PS1 regulate BACE1 trafficking and maturation? Although this question was not clarified in the present study, it was reported recently that PS1 and BACE1 are transported in the same membrane vesicles along the axons *in vivo*, via the direct binding of APP to the kinesin light chain subunit of kinesin-I, a microtubule motor protein (Kamal et al., 2001). Moreover, it was also shown that PS1 and nicastrin, the major components of the γ-secretase complex, interact with BACE1, suggesting that they may regulate β-secretase activity via the interaction with BACE1 (Hattori et al., 2002; Hebert et al., 2003). Consistent with these reports, we showed PS1-BACE1 interaction in the cell lines, primary neurons, and brain tissue and found that PS1 interacted preferably with proBACE1 rather than mature BACE1. Interestingly, we observed the solid binding between D385A PS1 and proBACE1. Although we cannot clarify a mechanism how D385A PS1 downregulates BACE1 maturation despite the solid interaction in the present study, one explanation is that the transport of proBACE1 to the Golgi after the interaction in the ER can be a process required for BACE1 maturation. We think there is a possibility that D385A PS1 is defective in this trafficking function, although the solid interaction with proBACE1 occurs in the ER. Taken together, PS1 may directly be involved in BACE1 maturation via stabilizing proBACE1 or promoting its efficient transport from the ER to the Golgi, although further experiments are needed to elucidate this mechanism.

In summary, our results show that PS1 is involved significantly in BACE1 maturation. This finding, for the first time, provided us the solid link between β- and γ-secretase. From observations obtained from the present study, we would like to extend our view of PS1 function further, and suggest that PS1 contributes to the dual regulation of β- and γ-secretase by regulating the intracellular trafficking of β-secretase and acting as a key component of γ-secretase. Consequently, PS1 may determine the magnitude of amyloidogenic processing of APP, thereby

contributing to the amyloid pathology. Although the regulatory mechanisms remain unknown, aberrant trafficking function of PS1 may lead to increased Aβ generation, and may underlie the pathogenesis of AD. In a future study, it will be significant to investigate effects of the clinical PS1 mutations or lipid raft on the novel function of PS1 in the intracellular trafficking of BACE1.

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AMYLOID β INHIBITS ECTODOMAIN SHEDDING OF N-CADHERIN VIA DOWN-REGULATION OF CELL-SURFACE NMDA RECEPTOR

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Abstract—Dysfunction in the synapse is recognized as an early and the primary pathological process in Alzheimer's disease (AD). N-cadherin, an essential adhesion molecule for excitatory synaptic contact, forms a complex with presenilin 1 (PS1) and β-catenin in the synaptic membrane. N-cadherin is sequentially cleaved by ADAM10 and PS1/γ-secretase, producing a cytoplasmic fragment, N-cadherin C-terminal fragment (Ncad/CTF2) after NMDA receptor stimulation [Marambaud P, Wen PH, Dutt A, Shioi J, Takashima A, Siman R, Robakis NK (2003) A CBP binding transcriptional repressor produced by the PS1/epsilon-cleavage of N-cadherin is inhibited by PS1 FAD mutations. Cell 114:635–645; Reiss K, Maretzky T, Ludwig A, Tousseyn T, de Strooper B, Hartmann D, Saftig P (2005) ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. EMBO J 24:1762]. Ncad/CTF2 translocates to the nucleus together with β-catenin to enhance β-catenin nuclear signaling [Uemura K, Kihara T, Kuzuya A, Okawa K, Nishimoto T, Bito H, Ninomiya H, Sugimoto H, Kinoshita A, Shimohama S (2006a) Activity-dependent regulation of beta-catenin via epsilon-cleavage of N-cadherin. Biochem Biophys Res Commun 345:951–958]. To examine whether an impairment of N-cadherin metabolism is involved in AD pathogenesis, we investigated the effect of amyloid β peptide (Aβ) treatment on sequential N-cadherin cleavage. Here, we demonstrate that both synthetic and cell-derived Aβ species inhibit ectodomain shedding of mouse N-cadherin. Inhibition of N-cadherin cleavage by Aβ treatment was suggested to be mediated by the enhanced endocytosis of NMDA receptor, resulting in reduced turnover of N-cadherin. Since both N-cadherin and β-catenin are essential for synaptic plasticity, impairment of N-cadherin cleavage caused by Aβ may underlie the synapse toxicity involved in AD pathogenesis. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: presenilin 1, N-cadherin, ADAM10, synapse, Alzheimer's disease.

Extensive synapse loss is a pathological hallmark of Alzheimer's disease (AD). Actually, loss of hippocampal synaptophysin immunoreactivity is an early pathological marker in AD (Heinonen et al., 1995) and synaptic loss correlates with the severity of cognitive impairment (Terry et al., 1991). Recently, soluble amyloid β peptide (Aβ) species has been suggested to interfere with synaptic mechanisms (Walsh and Selkoe, 2004). For example, Aβ promotes endocytosis of NMDA receptors in cortical neurons, producing a persistent depression of NMDA-evoked currents (Snyder et al., 2005).

Presenilin 1 (PS1), a causative gene of familial Alzheimer's disease (FAD), is a component of γ-secretase, which cleaves β-amyloid precursor protein (APP) to generate Aβ, a major component of senile plaques (De Strooper et al., 1998). Interestingly, PS1 is located at the synapse and forms complexes with N-cadherin (Georgakopoulos et al., 1999), an essential adhesion molecule for the synaptic contact (Fannon and Colman, 1996). Actually, N-cadherin is another substrate of PS1/γ-secretase (Marambaud et al., 2002).

N-cadherin flexibly modifies synaptic structure and function, constituting a molecular basis for synaptic plasticity (Tanaka et al., 2000; Murase et al., 2002; Togashi et al., 2002) and is cleaved after NMDA receptor stimulation (Marambaud et al., 2003; Reiss et al., 2005). We have previously characterized this N-cadherin cleavage (Uemura et al., 2006b). After NMDA receptor stimulation, N-cadherin is cleaved by ADAM10 in the extracellular domain to produce the N-cadherin C-terminal fragment (Ncad/CTF1). This process is required for the second PS1/γ-secretase-mediated cleavage (Uemura et al., 2006b). Interestingly, this second cleavage is inhibited by FAD-linked PS1 mutants (Marambaud et al., 2003; Uemura et al., 2006a). The cleavage product by PS1/γ-secretase, Ncad/CTF2, translocates to the nucleus and modulate β-catenin nuclear signaling (Uemura et al., 2006a), indicating that N-cadherin metabolism by PS1/γ-secretase is involved in synaptic plasticity. In this study, we wished to determine the effect of soluble Aβ species on N-cadherin metabolism

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Abbreviations: Aβ, amyloid β peptide; AD, Alzheimer's disease; APP, β-amyloid precursor protein; Aβ, amyloid β peptide; CHO, Chinese hamster ovary; DFK, difluoroketone; FAD, familial Alzheimer's disease; FL, full length; Ncad/CTF, N-cadherin C-terminal fragment; PS1, presenilin 1.

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to elucidate the potential mechanism of synapse toxicity by Aβ.

EXPERIMENTAL PROCEDURES

Cell culture

Primary neurons were obtained from the cerebral cortex of fetal mice (14–16 days' gestation) (Uemura et al., 2006b) and cultured in Neurobasal medium supplemented with B27 (Invitrogen, Rockville, MD, USA). Only mature neurons were used for the experiments. Chinese hamster ovary (CHO) cells, stably expressing Swedish (K670/M671→N/L) mutant human APP695 (APPSw-CHO cells) were obtained as follows: the entire coding sequence of human APP695 was subcloned in a mammalian expression vector pME/sf. Swedish mutations (Lys/Met670/Asn/Leu770) (Mullan et al., 1992) were introduced by site-directed mutagenesis. CHO cells were transfected with the APP cDNA together with pSVbsr plasmid and the cells were selected for resistance against 10 μg/ml blasticidin. Cells were maintained in DMEM/F12 (Invitrogen) supplemented with 10% FBS. Control CHO cells were stably transfected with empty vector.

Reagents

Synthetic $A\beta_{1-40}$, $A\beta_{1-42}$ and control peptide, $A\beta_{40-1}$ were obtained from Bachem (Torrance, CA, USA). MK-801, CNQX, L-glutamate and NMDA were from Nakarai Tesque (Kyoto, Japan). γ-Secretase inhibitor, difluoroketone (DFK), mecamylamine and α-bungarotoxin were purchased from Sigma (St. Louis, MO, USA) and DAPT was from Calbiochem (San Diego, CA, USA). Blasticidin was from Invitrogen. Anti-N-cadherin C-terminal antibody was from Transduction Laboratories (Franklin Lakes, NJ, USA). Anti-APP C-terminal polyclonal antibody and anti-β-tubulin monoclonal antibody were purchased from Sigma. Anti-Aβ antibody, 6E10 was from Chemicon (Temecula, CA, USA). Normal mouse IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-NR1, alternative CT polyclonal antibody was from Upstate (Lake Placid, NY, USA). Anti-CREB and anti-phospho(Ser133)-CREB antibodies were from Cell Signaling Technology (Danvers, MA, USA).

Treatment of the cells

Stimulation or inhibition of N-cadherin cleavage was done by exchanging the media into OPTI-MEM (Invitrogen), containing the designated concentration of stimulatory or inhibitory reagents. Control cells were treated by the sham medium exchange by OPTI-MEM. Conditioned media containing Aβ species were obtained as follows: Confluent APPSw-CHO cells or, alternatively, control CHO cells were incubated in φ6 cm dish in 5 ml DMEM (Invitrogen) for 24 h. The media were collected and were centrifuged at 600×*g* for 10 min. The aliquot was used as conditioned media.

Western blot, immunoprecipitation and cell-surface biotinylation assay

The Western blot analysis was carried out as described elsewhere (Uemura et al., 2003). The amount of Aβ species in the medium was examined by immunoprecipitation assay (Uemura et al., 2003). Briefly, 1 ml of conditioned medium obtained above was pre-cleared by 10 μl of protein G-sepharose (Amersham, Uppsala, Sweden) overnight at 4 °C. Pre-cleared medium was then incubated with 3 μg of 6E10 antibody for 1 h, followed by the incubation with 10 μl of protein G-sepharose for 1 h at 4 °C. Immunoprecipitates were washed and subjected to Western blotting, using 6E10 antibody. For immunodepletion of Aβ species from medium, immunoprecipitation using 6E10 antibody was repeated for four times. Control medium was similarly immunoprecipitated using normal mouse IgG. To examine the amount of cell-surface NMDA receptor, cell-surface protein biotinylation assay was performed as described elsewhere (Uemura et al., 2003).

RESULTS

We first analyzed whether the physiological concentration of glutamate can stimulate N-cadherin cleavage to yield

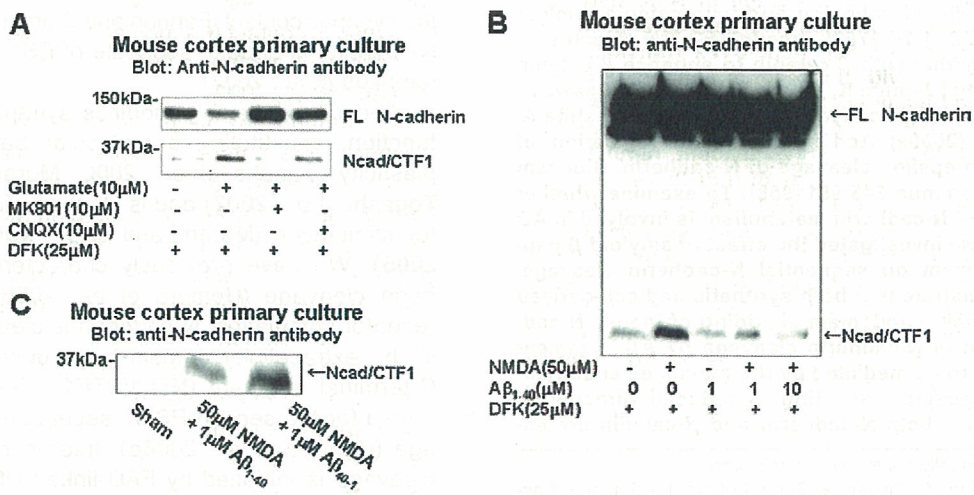

Fig. 1. Synthetic Aβ treatment inhibits ectodomain shedding of N-cadherin. (A) Mouse cortex primary neurons were treated with 10 μM glutamate for 30 min in the presence or absence of glutamate receptor antagonist (10 μM MK801 or 10 μM CNQX). The experiments were performed in the presence of DFK (25 μM). Ncad/CTF1, which was barely seen in control cells (1st), was produced from FL N-cadherin in response to glutamate treatment (2nd). Ncad/CTF1 production was inhibited by MK801 (3rd). CNQX failed to inhibit Ncad/CTF1 production (4th). (B) Primary neurons were pre-incubated by OPTI-MEM in the presence or absence of Aβ1-40 (1 μM or 10 μM) for 15 min. After pre-incubation, the cells were stimulated by NMDA (50 μM) with or without designated concentration of Aβ1-40. The experiments were performed in the presence of DFK (25 μM). NMDA treatment enhanced the production of Ncad/CTF1 (2nd), compared with control (1st). Aβ1-40 dose-dependently inhibited Ncad/CTF1 production after NMDA treatment for 30 min (4th and 5th), compared with treatment by NMDA only (2nd). (C) The inhibitory effect of Aβ1-40 was examined, using reverse peptide, Aβ40-1 as a control. Aβ40-1 failed to inhibit Ncad/CTF1 production (3rd).

Ncad/CTF1 (Fig. 1A). Since Ncad/CTF1 is rapidly degraded by PS1/γ-secretase, the experiments were performed in the presence of γ-secretase inhibitor, DFK (25 μM). Ncad/CTF1 was produced from full-length (FL) N-cadherin in response to 10 μM glutamate treatment for 30 min. Ncad/CTF1 production was inhibited by the NMDA receptor antagonist, MK801 (10 μM), showing that NMDA receptor stimulation is involved in Ncad/CTF1 production, as demonstrated by previous report (Reiss et al., 2005). Conversely, non-NMDA type glutamate receptor antagonist, CNQX, failed to inhibit Ncad/CTF1 production.

Next, primary neurons were stimulated by NMDA (50 μM) with or without designated concentration of synthetic $A\beta_{1\text{-}40}$ for 30 min (Fig. 1B). Aβ treatment dose-dependently inhibited Ncad/CTF1 production, indicating that ectodomain shedding by ADAM10 is impaired. We confirmed this finding by using reverse peptide, $A\beta_{40\text{-}1}$ as a control (Fig. 1C).

Then, we wished to determine whether the inhibitory effect on N-cadherin cleavage is observed by cell-derived Aβ species. To test this, APPSw-CHO cells were established. The expression of APP was confirmed by Western blot, using anti-APP C-terminal antibody (Fig. 2A). The scheme of experimental protocol is presented in the left-side of Fig. 2. Confluent control CHO cells in φ6 cm dish were cultured for 24 h in DMEM. The medium was collected to obtain control conditioned medium. Alternatively, APPSw-CHO cells were cultured in DMEM in the presence or absence of γ-secretase inhibitor, DAPT (1 μM) and the conditioned media were obtained accordingly. Aβ species

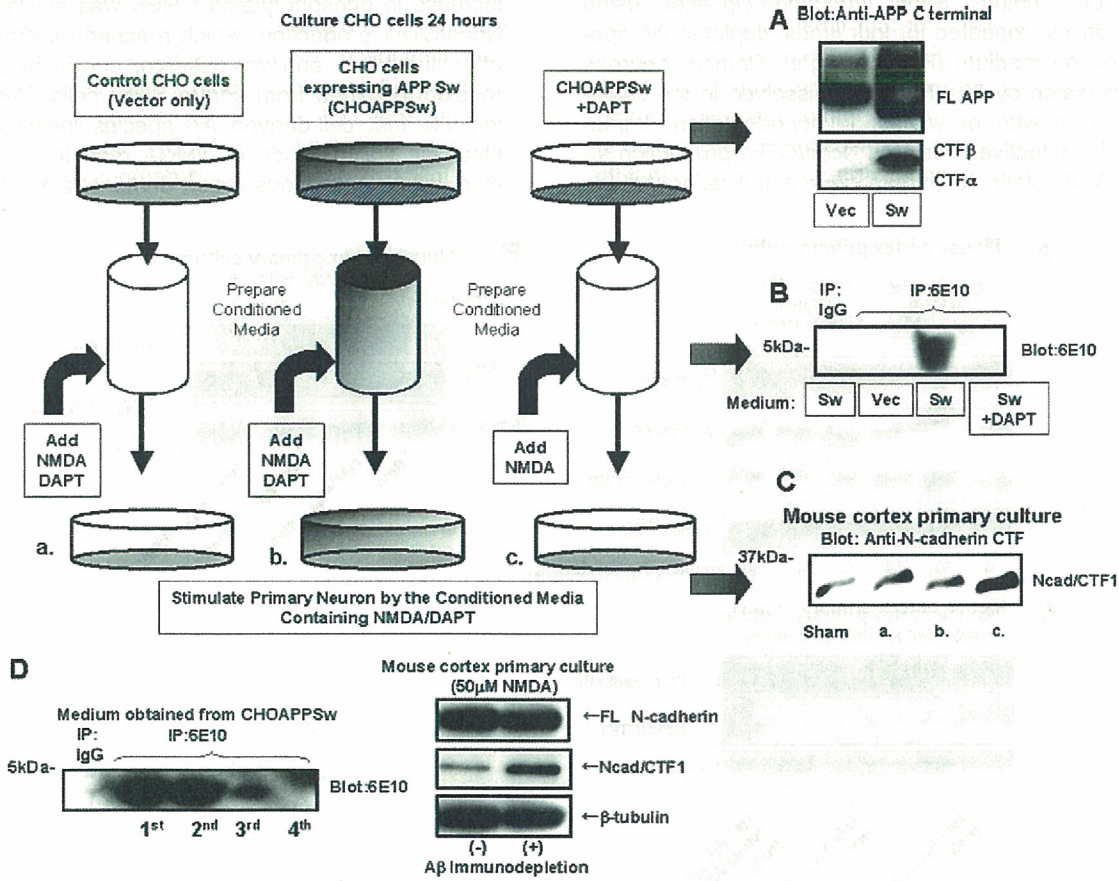

Fig. 2. N-cadherin cleavage is inhibited by cell-derived Aβ species. (A) The characterization of control CHO cells and APPsw-CHO cells, using anti-APP C-terminal antibody. In APPSw-CHO cells, both FL APP and its C-terminal fragment (CTF α&β) were robustly increased (right). (B) Confluent control CHO cells, plated on φ6 cm dish were incubated in 5 ml of DMEM for 24 h. APPSw-CHO cells were incubated in 5 ml of DMEM with or without 1 μM DAPT for 24 h. After incubation, the medium was centrifuged at 600×*g* for 10 min. The aliquot was used as conditioned medium. The amount of Aβ species in the conditioned medium was examined by immunoprecipitation assay, using anti-Aβ antibody (6E10). Aβ was not detected in the immunoprecipitates by control normal mouse IgG (1st) or in the conditioned medium obtained from control CHO cells (2nd). Aβ species (~4 kDa) was detected in the immunoprecipitates from the conditioned medium of APPSw-CHO cells by 6E10 antibody (3rd), which was diminished by the DAPT treatment of the cells (4th). (C) NMDA and DAPT were added to the conditioned medium to give the final concentration of 50 μM for NMDA and 1 μM for DAPT. Each conditioned medium was administered to the primary culture and Ncad/CTF1 production was examined. NMDA treatment stimulated Ncad/CTF1 production (2nd, a), compared with sham treatment in which only 1 μM DAPT was dissolved in the OPTI-MEM (1st, sham). Conditioned medium obtained from APPSw-CHO inhibited Ncad/CTF1 production (3rd, b) after NMDA receptor stimulation. This inhibitory effect of the conditioned medium obtained from APPSw-CHO cells was reversed by the addition of 1 μM DAPT during the incubation period (4th, c). (D) (Right) Aβ was immunoprecipitated using 6E10 antibody from the conditioned media obtained from APPSw-CHO cells. Aβ was depleted after the 4th immunoprecipitation by 6E10 antibody. Normal mouse IgG did not precipitate Aβ species. (Left) Primary neurons obtained from mouse cortex were stimulated by 50 μM NMDA dissolved in the conditioned media with or without immunodepletion of Aβ species for 30 min. Immunodepletion restored Ncad/CTF1 production after NMDA stimulation.

(~4 kDa) were detected only in the immunoprecipitates of conditioned media from APPSw-CHO cells without incubation with DAPT (Fig. 2B). Each conditioned medium was supplemented with final concentrations of 50 μM NMDA and 1 μM DAPT, and was administered to mouse cortex primary neurons for 30 min. Ncad/CTF1 production was reduced when conditioned media obtained from APPSw-CHO cells were used, compared with the treatment using other conditioned medium (Fig. 2C), indicating that cell-derived Aβ species inhibited Ncad/CTF1 production. We then wished to determine whether the inhibitory effect of conditioned media obtained from APPSw-CHO cells on N-cadherin cleavage is actually mediated by Aβ species. To test this, Aβ species were immunodepleted from the conditioned medium. Serial immunoprecipitation using 6E10 antibody, repeated for four times, depleted Aβ species from the medium (Fig. 2D, right). Primary neurons were stimulated by 50 μM NMDA dissolved in the conditioned media with or without immunodepletion. Immunodepletion effectively restored Ncad/CTF1 production after NMDA stimulation of primary neuron, indicating that the inhibitory effect observed is specific for the Aβ species (Fig. 2D, left).

Next, we stimulated primary neurons by 50 μM NMDA dissolved in the conditioned medium obtained from either control CHO or APPSw-CHO cells and analyzed the time course (Fig. 3A). Since NMDA receptor-mediated calcium influx is linked to the phosphorylation of CREB (Deisseroth et al., 1996), we used phosphorylated CREB as a surrogate marker for the NMDA receptor activation. Ncad/CTF1 production was enhanced 30 min after NMDA treatment in the conditioned medium obtained from control CHO cells. Conversely, Ncad/CTF1 production was not stimulated by NMDA treatment in the conditioned media obtained from APPSw-CHO cells. Interestingly, the time course of the increase in phosphorylated CREB was similar to that of Ncad/CTF1 production, which reached maximum 30 min after stimulation, and was observed only in the conditioned medium obtained from control CHO cells. These results indicate that cell-derived Aβ species inhibit N-cadherin cleavage via inhibition of NMDA receptor-mediated calcium influx. A previous report demonstrated that Aβ pro-

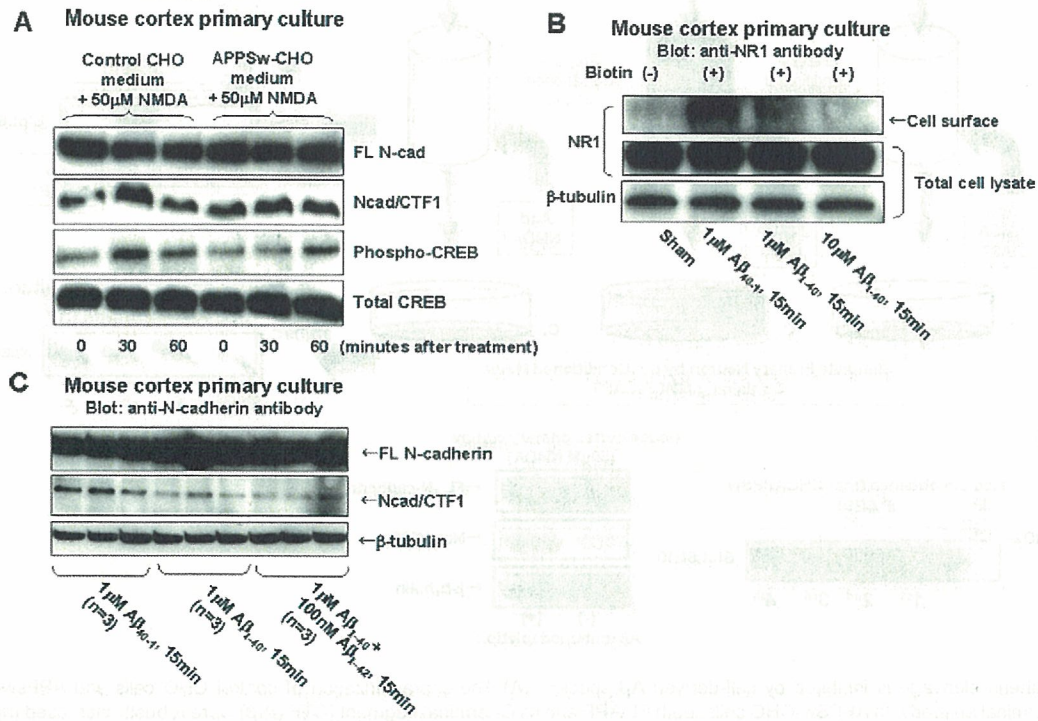

Fig. 3. Aβ treatment enhances endocytosis of NMDA receptor, causing reduced turnover of N-cadherin. (A) Mouse cortex primary neurons were stimulated by 50 μM NMDA dissolved in the conditioned medium obtained from either control CHO cells or APPSw-CHO cells, after 15 min preincubation with each conditioned medium. Ncad/CTF1 production was enhanced 30 min after NMDA treatment in the conditioned medium obtained from control CHO cells, the level of which was restored to the baseline 60 min after treatment. NMDA treatment did not stimulate Ncad/CTF1 production in the conditioned medium obtained from APPSw-CHO cells (Ncad/CTF1). The time course of the increase in phosphorylated CREB reached maximum 30 min after stimulation only in the conditioned medium obtained from control CHO cells (Phospho-CREB). The total level of CREB was unchanged during experiment (Fig. 3A, CREB). (B) Primary cortical neurons were treated with either synthetic $A\beta_{1\text{-}40}$ (1 μM, 10 μM) or $A\beta_{40\text{-}1}$(1 μM) for 15 min. After treatment, cell-surface proteins were biotinylated and analyzed. $A\beta_{1\text{-}40}$ treatment (3rd, 4th) dose-dependently reduced cell-surface NR1, compared with Aβ40-1 treatment (2nd), whereas the total levels of NR1 in the lysates were unchanged. The bottom lane is the immunoreactivities of β-tubulin, used as a loading control. (C) Primary mouse cortical neurons were treated with Aβ40-1(1 μM), Aβ1-40 Aβ1-40 (1 μM) or combination of Aβ1-40 (1 μM) and Aβ1-42 (100 nM) for 15 min. Cells were collected after treatment and analyzed by Western blot. The level of FL N-cadherin was slightly increased after Aβ1-40 treatment (4th–6th) or combined treatment of Aβ1-40 and Aβ1-42 (7th–9th), compared with Aβ40-1 treatment (1st–3rd), whereas Ncad/CTF1 levels were decreased. The bottom lane indicates the loading control by β-tubulin immunoreactivity.

motes endocytosis of NMDA receptors via α7 nicotinic receptor-mediated signaling (Snyder et al., 2005). To test whether this mechanism is involved in impaired N-cadherin cleavage, the amount of cell-surface NMDA receptor was examined after incubation of neurons by $A\beta_{1-40}$ (Fig. 3B). As expected, cell-surface NR1 was dose-dependently reduced after $A\beta_{1-40}$ treatment, which was correlated to the degree of inhibitory effect on N-cadherin cleavage (Fig. 1B), whereas the total level of NR1 was unchanged. We then tested whether α7 nicotinic receptor antagonist can block the effect of Aβ on Ncad/CTF1 production, however, the blocking effect was only partial and not reproducible, whereas the broad acetylcholine receptor antagonist, mecamylamine could block the effect of Aβ, indicating the involvement of acetylcholine receptors other than α7 receptor in the Aβ-mediated NMDA receptor endocytosis (data not shown). Finally, we examined the effect of Aβ treatment on the total level of N-cadherin in primary cortical neurons (Fig. 3C). As shown by triplicate experiments, Ncad/CTF1 was reduced, whereas FL N-cadherin is slightly increased in primary neuron treated with 1 μM $A\beta_{1-40}$ or 1 μM $A\beta_{1-40}$+100 nM $A\beta_{1-42}$, indicating that N-cadherin turnover is impaired immediately after Aβ treatment.

DISCUSSION

In this report, we demonstrated that Aβ inhibits N-cadherin ectodomain shedding after NMDA receptor stimulation. The ectodomain shedding was observed after stimulation by physiological concentration of glutamate (Fig. 1A), indicating it can be observed in the process of synaptic transmission *in vivo*. In addition, the inhibition of N-cadherin metabolism was observed by cell-derived Aβ species, indicating that N-cadherin cleavage is impaired in the AD brain, where large amounts of cell-derived Aβ species are assumed to accumulate at the synapse. The mechanism through which Aβ inhibits N-cadherin metabolism was suggested to be enhanced endocytosis of NMDA receptor.

Fig. 4 illustrates the effect of Aβ on N-cadherin metabolism. Aβ is suggested to interfere with ADAM10-mediated ectodomain shedding through enhanced NMDA receptor endocytosis. Conversely, FAD-linked mutations of PS1 inhibits the second, PS1/γ-secretase-mediated cleavage (Marambaud et al., 2003; Uemura et al., 2006a). Thus, FAD-linked PS1 mutation and Aβ inhibit a different process of N-cadherin cleavage to cause the same consequence, reduction of transcriptionally active molecule, Ncad/CTF2.

Developmentally, nuclear β-catenin signaling promotes the proliferation of neuronal stem cells, neuronal differentiation, axonal remodeling and synaptogenesis (Hall et al., 2000; Chenn and Walsh, 2002; Otero et al., 2004). Moreover, N-cadherin itself flexibly modifies synaptic structure and function, constituting a molecular basis for synaptic plasticity (Tanaka et al., 2000; Murase et al., 2002; Togashi et al., 2002). Taken together, impaired cleavage and reduced turnover of N-cadherin might have negative impact on synaptic plasticity and might constitute

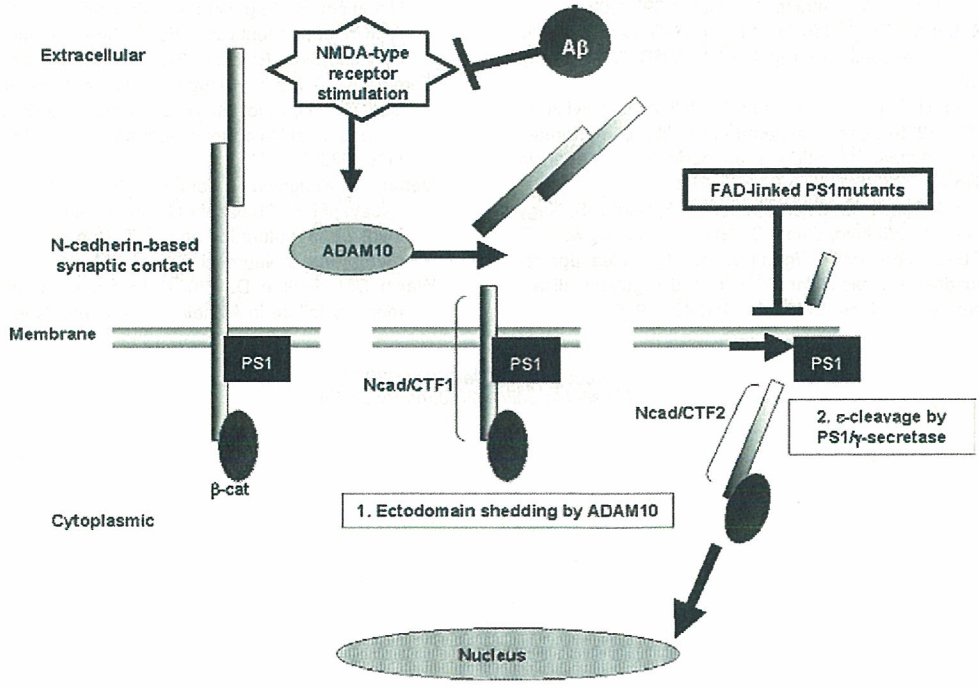

Fig. 4. A scheme of sequential N-cadherin cleavage. N-cadherin is sequentially cleaved by ADAM10 and PS1/γ-secretase. The first step of the cleavage occurs in the extracellular domain, which is mediated by ADAM10. ADAM10-mediated cleavage is inhibited by Aβ species by promoting the endocytosis of NMDA receptor, as suggested in the present study. The second step of the cleavage is mediated by PS1/γ-secretase, which occurs at the membrane–cytosol interface, producing transcriptionally active molecule, Ncad/CTF2. This step is demonstrated to be inhibited by FAD-linked mutations of PS1. Thus, the consequence of FAD-linked mutations of PS1 and Aβ accumulation at the synapse is similar, reducing Ncad/CTF2 production.

a mechanism involved in the synapse toxicity of AD. In the present study, we could not rule out the possibility that NMDA-dependent cleavage of N-cadherin occurs exclusively extrasynaptically. Since extrasynaptic N-cadherin is also observed (Tanaka et al., 2000), whether Aβ species can interfere with the metabolism of synaptic N-cadherin, and are actually involved in synapse toxicity of AD needs to be elucidated in a future study.

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GSK3β ACTIVITY MODIFIES THE LOCALIZATION AND FUNCTION OF PRESENILIN 1

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Running title: GSK3β regulates cell-surface expression of PS1

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Presenilin 1, a causative gene product of familial Alzheimer's disease, has been reported to be localized mainly in the endoplasmic reticulum and Golgi membranes. However, endogenous Presenilin 1 also localizes at the plasma membrane as a biologically active molecule. Presenilin 1 interacts with N-cadherin/β-catenin to form a trimeric complex at the synaptic site through its loop domain, whose serine residues (Serine 353, 357) can be phosphorylated by glycogen synthase kinase 3β. Here, we demonstrate that the cell-surface expression of Presenilin 1/γ-secretase is enhanced by N-cadherin-based cell-cell contact. Physical interaction between Presenilin 1 and N-cadherin/β-catenin plays an important role in this process. Glycogen synthase kinase 3β-mediated phosphorylation of Presenilin 1 reduces its binding to N-cadherin, thereby downregulating its cell-surface expression. Moreover, reduction of Presenilin 1/N-cadherin/β-catenin complex formation leads to an impaired activation of contact-mediated phosphatidylinositol 3-kinase/Akt cell survival signaling. Furthermore, phosphorylation of Presenilin 1 hinders ε-cleavage of N-cadherin, whereas ε-cleavage of APP remained unchanged. This is the first report that clarifies the regulatory mechanism of Presenilin 1/γ-secretase with respect to its subcellular distribution and its differential substrate cleavage. Since the cleavage of various membrane proteins by Presenilin 1/γ-cleavage is involved in cellular signaling, glycogen synthase kinase 3β–mediated phosphorylation of Presenilin 1 should be deeply associated with signaling functions. Our findings indicate that the abnormal activation of glycogen synthase kinase 3β can reduce neuronal viability and synaptic plasticity via modulating Presenilin 1/N-cadherin/β-catenin interaction and thus have important implications in the pathophysiology of Alzheimer's disease.

Pathological features of Alzheimer's disease (AD) are characterized by neurofibrillary tangles (NFT) and amyloid plaques. Amyloid plaques are composed of amyloid β peptide (Aβ), which is derived from the sequential cleavages of amyloid precursor protein (APP), whereas NFTs are characterized by the accumulation of hyperphosphorylated tau. Aβ peptides are yielded by the intramembranous cleavage of the APP C-terminal fragment by Presenilin1 (PS1)/γ-secretase (1). Interestingly, PS1/γ-secretase complex is also involved in the ε-cleavage of various other membrane proteins (2-5).

On the other hand, NFT is composed of phosphorylated tau. Glycogen synthase kinase 3β (GSK3β) plays an important role in tau phosphorylation (6). GSK3β is expressed in a variety of tissues with the highest level in the brain, where it localizes especially in neuron (7). Recent

study showed that PS1 is an unprimed substrate of GSK3β (8) and serine residues in the PS1 loop domain can be phosphorylated (9). Abnormal increases in GSK3β level and activity have been associated with AD pathology (6, 10), suggesting that GSK3β-mediated phosphorylation of PS1 might also be involved in AD pathophysiology.

Classically, PS1 has been reported to be localized mainly in the endoplasmic reticulum and Golgi membranes (11). Recently, it is also demonstrated that endogenous PS1 localizes at the plasma membrane as an active molecule (12). Actually, in neurons, PS1 binds to β-catenin and N- (and E-) cadherin through a large hydrophilic loop region (13) at the synapse. Moreover, N-cadherin is cleaved by PS1/γ-secretase in response to NMDA-type receptor stimulation (14, 15).

N-cadherin is essential for synaptic contact (16) and regulates synaptogenesis and dendritic spine morphology (17, 18). In addition, N-cadherin-based cell-cell adhesion activates PI3K/Akt cell survival signaling by recruiting PI3K to the N-cadherin adhesion complex (19). Actually, PS1 facilitates this process by promoting cadherin/PI3K association in γ-secretase activity-independent manner (20). Collectively, PS1/N-cadhrerin interaction at the synapse seems to be neuroprotective by facilitating PI3K/Akt survival signaling. However, the cellular mechanism which determines the PS1/N-cadherin binding has never been elucidated so far.

Here, we demonstrate that the cell-surface distribution of PS1 is enhanced in the presence of N-cadherin-based cell-cell contact. The physical interaction between PS1 and N-cadherin/β-catenin plays an important role in the cell-surface expression of PS1/γ-secretase. Moreover, GSK3β-mediated phosphorylation of PS1 reduces its binding to N-cadherin, thereby downregulating its cell-surface expression. Reduced PS1/N-cadherin/β-catenin interaction leads to the inhibition of contact-mediated PI3K/Akt cell survival signal activation as well as the differential regulation of substrate cleavage by PS1/γ-secretase. These results demonstrated that the redistribution of PS1 to the plasma membrane is regulated by its phosphorylation and that the distributional change modifies the PI3K/Akt signal as well as substrate cleavage.

Experimental Precedures

Plasmid constructs

A cDNA copy of human N-cadherin (Genbank: No. M34064) gene was amplified from first strand cDNA Human fetal brain (Stratagene) by a forward primer 5'-TTTTTTGCTAGCACCATGGATAAAG AACGCCAGGCC-3', and a reverse primer 5'-TTTTTGGGCCCTCAGGCGTAATCTG GGACGTCGTATGGGTAGTCATCACCTC CACCATACATGTCAGCA-3' for C-terminal HA tagging. The PCR product was cloned into Nhe I–Apa I sites of pcDNA3.1 (+) (Invitrogen). For the expression of human APP, the entire coding sequence of human APP695 was subcloned in a mammalian expression vector pME/sf. The construction of plasmid, expressing wtPS1 was described elsewhere (21). Pseudo-phosphorylation PS1 mutants (S353D, S357D, S353/357D) were obtained by site-directed mutagenesis using Quik Change II Site-Directed Mutagenesis Kit (Stratagene), using following primer sets (5'-GATACACCTGAGTCACGAG-3' and 5'-GCGATGAGGCCCTAGAT-3' for S353D, 5'-GATCGAGCTGCTGTCCA-3' and 5'-CTCAGGTGTAGAGCGATGA-3' for S357D).

Deletion mutants of PS1 were produced as follows. For PS1Δ375-40, PCR products, amplified by two different primer sets from wild-type PS1 construct (5'-TTTTTTGGTACCTTAGCCATGACAG AGTTACCTGCACCG-3' and 5'-TTTTTTGACGTCGTATGGGTATTCCT CACTGAACCCGCCATCATCATTCTC-3': 5'-TTTTTTGGGCCCCTAGATATAAAAT TGATGGAA-3' and 5'-TTTTTTGACGTCCCAGATTACGCCG

AAAGGGGAGTAAAACTTGGATTGGGA -3') were excised by AatII and cloned into Kpn I-Apa I sites of pcDNA 3.1(+). For PS1 Δ350-40, different primer sets (5'-TTTTTGACGTCCCAGATTACGCCCA TCGCTCTACACCTGAGTCACGAGCT-3' and 5'-TTTTTTGGGCCCCTAGATATAAAAT TGATGGAA-3') were used.

For tetracycline-inducible expression of constitutively active (S9A) GSK3β, a cDNA copy of human GSK3β (Genbank: No. M34064) was amplified by PCR, using a forward primer 5'-TTTTTTGGTACCGCCATGTCAGGGC GGCCCAGAA-3' and a reverse primer 5'-TTTTTTGGGCCCTCAGGTGGAGTTG GAAGCTGAT-3'. PCR product was subcloned into Kpn I–Apa I sites of pcDNA 4/TO (Invitrogen). Constitutively active (S9A) GSK3β (22) was produced by site-directed mutagenesis using Quik Change II Site-Directed Mutagenesis Kit (Stratagene), using following primer sets (5'-GGCCCAGAACCACCGCCTTTGCGG AGAGCCTG-3' and 5'-CAGCTCTCCGCAAAGGCGGTGGTTC TGGGCC-3').
Precise cloning of all reading frame was verified by sequencing.

Cell culture and transfection

SH-SY5Y cells were maintained in OPTI-MEM containing 10% fetal bovine serum. HEK293 cells were maintained in DMEM containing 10% fetal bovine serum. For the establishment of cell lines in which S9A GSK3β (S9A-tet cells) expression can be induced by tetracycline (tet-on), SH-SY5Y cells were first transfected with pcDNA6/TR (Invitrogen), using Lipofectamine 2000 (Invitrogen). After selection of cells by 5μg/ml blasticidin, stably transfected clones were then transfected with GSK3β (or S9A GSK3β)/pcDNA4/TO, and selected by 100μg/ml zeocin.

Chinese hamster ovary (CHO) cells were maintained in DMEM/F12 (Invitrogen) supplemented with 10% FBS. PS1/PS2 double knockout mouse embryonic fibroblast (MEF) cells (MEF PS-/- cells) (23)were kindly provided by Dr. De Strooper and were maintained in DMEM. Transient transfection of wtPS1, PS1 mutants, N-cadherin and S9AGSK3β into cells were achieved by lipofection method, using Lipofectamine 2000 (Invitrogen).

Chinese hamster ovary (CHO) cells, stably expressing Swedish (K670/M671->N/L) mutant human APP695 (APPSw-CHO cells) were obtained as follows: the entire coding sequence of human APP695 was subcloned in a mammalian expression vector pME/sf. Swedish mutations (Lys/Met670/Asn/Leu770) (24) were introduced by site-directed mutagenesis. CHO cells were transfected with the APP cDNA together with pSVbsr plasmid and the cells were selected for resistance against blasticidin (10μg/ml). Cells were maintained in DMEM/F12 (Invitrogen) supplemented with 10% FBS. CHO cells stably expressing both Swedish mutant APP and human N-cadherin (APPsw/Ncad-CHO cells) were obtained as follows: APPsw-CHO cells were transfected with N-cadherin/pcDNA3.1(+). Cells were selected by 800μg/ml G418 and the establishment of stably transfected clones was verified by Western blot.

Antibodies and Chemical Reagents

Anti-N-cadherin C-terminus, anti-GSK3β and anti-total β-catenin antibodies are obtained from Transduction Laboratories. Anti-β-actin, anti-nicastrin, anti-APP C-terminus and anti-phosphoserine antibodies are from Sigma. Anti-Phospho-β-catenin (Ser 33/37/Thr 41), anti-total Akt, and anti-Phospho-Akt (Ser 473) antibodies are from Cell Signaling Technology. Rabbit polyclonal anti-PS1 N-terminal fragment (NTF) is from Santa Cruz. Goat polyclonal anti-PS1 C-terminal fragment (CTF) is from R&D systems. Alexa Fluor 546 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG are obtained from Molecular Probes. Anti-mouse and rabbit horseradish peroxidase-conjugated secondary antibodies are from Amersham Biosciences. Anti-goat horseradish

peroxidase-conjugated secondary antibodies are from Santa Cruz. Blasticidin, tetracycline and zeocin are from Invitrogen. G418 is from WAKO chemical industry, Japan. For β-catenin and N-cadherin knock down, pre-designed siRNA constructs were synthesized by Dharmacon (ON-TARGETplus siRNA, CTNNB1, CDH2). Ionomycin was obtained from Calbiochem.

Cell treatment by reagents

For the induction of S9AGSK3β, S9A-tet cells are treated by 1μg/ml tetracycline containing medium. siRNA constructs were transfected into HEK293 cells using Lipofectamine 2000, according to the manufacturer's instruction. Cells were analysed 24hours after siRNA transfection. For calcium switch experiments, confluent MEF PS-/- cells were incubated in serum free medium for 4h, treated with 4mM EGTA for 40mins and then switched to serum-free, calcium-containing medium for the times shown (20). For the sitimulation of N-cadherin cleavage, MEF PS-/- cells were treated by 10μM ionomycin dissolved in OPTI-MEM for 30 mins.

Western Blot and Immunoprecipitation

Preparation of protein samples, the Western blot and immunoprecipitation analysis were carried out as described elsewhere (25). For some experiments, cells were fractionated as previously described (25).

Biotinylation of cell-surface proteins.

Confluent cells grown in ϕ10cm dish were washed three times with ice-cold PBS, and suspended in the solution containing 0.5mg of Sulfo-NHS-LC-Biotin (Pierce) /ml PBS for 30mins. Cells were then washed three times with PBS and biotinylated proteins were precipitated by 30μl of streptoavidin agarose (Invitrogen) from equal amount of cell lysates. Precipitated biotinylated proteins were then subjected to Western blot analysis.

Immunostaining

The samples for immunostaining were prepared as described elsewhere (25). Samples were examined using a laser scanning confocal microscopy, LSM 510 META (Zeiss) or a fluorescence microscopy, Axiovert 200 (Zeiss).

RESULTS

Cell-surface expression of PS1 is enhanced by the cadherin-mediated cell-cell contact

To examine the relationship between the cadherin-based cell-cell contact and subcellular distribution of PS1, we established CHO cell lines stably expressing both Swedish mutation of human APP695 and human N-cadherin (APPsw/Ncad-CHO cells). CHO cells are suitable for the analysis of exogenously introduced N-cadherin, because it barely expresses endogenous cadherin species. APPsw/Ncad-CHO cells were compared with CHO cells stably expressing Swedish mutation of APP695 only (APPSw-CHO cells) by immunostaining (Figure 1). In APPsw/Ncad-CHO cells, N-cadherin immunoreactivity was prominent at the sites of cell contact (Figure 1A). PS1 was also seen at the cell-cell contact sites (Figure 1B), where it co-localized with N-cadherin (Figure 1C). The outline of APPsw/Ncad-CHO cells was clearly visualized by PS1 staining (Figure 1B, C, compare with the perinuclear PS1 staining in Figure 1E), indicating that the N-cadherin expression redistributed the PS1 subcellular localization to the plasma membranes. Next, we compared the PS1 and β-catenin distribution in the presence or absence of N-cadherin. In APPSw-CHO cells, β-catenin and PS1 immunoreactivity was mainly seen at the perinuclear area (Figure 1D-F). Stable expression of N-cadherin recruited both β-catenin (Figure 1G) and PS1 (Figure 1H) to the cell-cell contact sites (compare with the perinuclear PS1 staining in Figure 1E), showing co-localization of both proteins (Figure 1I). Thus, stable expression of N-cadherin led to the redistribution of PS1 to

the plasma membrane, especially to the cell-cell contact sites, where N-cadherin, PS1 and β-catenin all colocalized.

To confirm that the cadherin-based adhesion promotes cell-surface expression of PS1/γ-secretase, we transiently expressed human N-cadherin into CHO cells and examined the level of cell-surface PS1/γ-secretase by biotinylation assay (Figure 1J). Transient expression of N-cadherin enhanced both the cell-surface expression levels of nicastrin and PS1, whereas the total levels of these proteins were comparable, demonstrating that cadherin-based adhesion enhances the cell-surface expression level of PS1/γ-secretase (Figure 1J). We further verified this finding by RNAi (Figure 1K) using HEK293 cells, which endogenously express human N-cadherin, β-catenin and PS1. 24 hours after N-cadherin knockdown, the cell-surface expression levels of both PS1 and nicastrin were reduced, whereas the total levels of these proteins remained unchanged (Figure 1K, 4th lane), compared to control (Figure 1K, 2nd lane). The protein level of β-catenin was reduced in the background of N-cadherin knockdown, indicating that β-catenin lost its stability in the absence of N-cadherin (Figure 1K, 4th lane). We also tested the effect of β-catenin knockdown on cell-surface expression of γ-secretase components. Interestingly, 24 hours after β-catenin knockdown, the cell-surface nicastrin was reduced, without changing the cellular level of N-cadherin (Figure 1K, 3rd lane), although β-catenin knockdown had less impact on the cell-surface PS1 distribution compared to N-cadherin knockdown (compare Figure 1K, 3rd and 4th lanes). Collectively, these experiments indicate that cadherin-based cell-cell adhesion promotes the expression of PS1/γ-secretase at the cell surface.

GSK3β activity modulates the binding of PS1 to N-cadherin/β-catenin

To analyze the effect of the mutual interaction among PS1/N-cadherin/β-catenin on the cell-surface distribution of PS1/γ-secretase, we constructed deletion mutants of PS1, which lack the part of the loop domain (PS1Δ340-75 and Δ340-50, Figure 2A) necessary for the interaction with N-cadherin/β-catenin (26). Transfection into HEK293 cells revealed that wtPS1 binds both N-cadherin and β-catenin (Figure 2B, 3rd lane). The deletion mutants (PS1Δ340-75 and Δ340-50) formed complex with neither N-cadherin nor β-catenin (Figure 2B, 4th and 5th lanes). Therefore, the amino acids around 340-50 in the PS1 loop domain are crucially important for the PS1/N-cadherin/β-catenin complex formation.

PS1 has been reported to be phosphorylated by GSK3β at the serine (353) and serine (357) residues in the loop domain (9) (Illustrated in Figure 2A, bottom). Since the PS1 loop domain around amino acids 340-50 was essential for PS1/N-cadherin/β-catenin interaction (Figure 2B, 5th lane), we assumed that GSK3β-mediated phosphorylation of PS1 would change its binding to N-cadherin/β-catenin. To test this, pseudo-phosphorylation mutants of PS1 (S353D and S357D) were produced by site-directed mutagenesis. Either wtPS1 or pseudo-phosphorylation mutants were transfected into HEK293 cells to investigate the effect of PS1 phosphorylation on PS1/N-cadherin/β-catenin interaction. Immunoprecipitation assay revealed that the association of pseudo-phosphorylation mutants (S353D and S357DPS1) with N-cadherin, as well as β-catenin, was dramatically reduced (Figure 2B, 6th and 7th lanes), compared to wtPS1 (Figure 2B, 3rd lane), suggesting that GSK3β-mediated phosphorylation negatively affects the binding of PS1 to N-cadherin/β-catenin. To examine above findings under PS1-null background, we transiently transfected wild-type, S353D or S357DPS1 into PS1/PS2 double knockout mouse embryonic fibroblast cells (MEF PS-/- cells) and examined the PS1/N-cadherin/β-catenin complex formation (Figure 2C). As expected, both S353D (Figure 2C, 3rd lane) and S357D (Figure 2C, 4th lane) PS1 had reduced ability

to interact with N-cadherin as well as β-catenin, compared to wild-type PS1 (Figure 2C, 2nd lane).

Above experiments suggested that the activation of GSK3β would reduce the PS1/N-cadherin interaction via PS1 phosphorylation in the loop domain. Accordingly, immunoprecipitation assay revealed that the transient transfection of constitutively active GSK3β (S9A mutant) (22)into HEK293 cells reduced the PS1/N-cadherin interaction (Figure 2D). We further wished to confirm the effect of GSK3β activation in neuronal cells. We established human neuroblastoma SH-SY5Y cell lines (S9A-tet cells), in which expression of constitutively active GSK3β (S9AGSK3β) can be induced by tetracycline treatment (tet-on). Tetracycline (1μg/ml) induced S9AGSK3β expression 24 hours after treatment (Figure 2E, left). Phosphorylation of β-catenin, a representative target of GSK3β, was increased whereas the total level of β-catenin was decreased (Figure 2E, left), indicating that the expression of S9AGSK3β enhanced its activity as a phosphokinase, leading to the phosphorylation and degradation of β-catenin. Using these cell lines, we then analyzed the phosphorylation status of PS1 CTF by immunoprecipitation assay. Lysates of S9A-tet cells with or without tetracycline treatment for 24 hours were immunoprecipitaed by anti-phosphoserine antibody, followed by the immunoblotting using anti-PS1 CTF. PS1 CTF, pulled-down by anti-phosphoserine was increased after tet-on, indicating that PS1 phosphorylation by GSK3β was enhanced after tetracycline treatment (Figure 2E, right). Then we examined PS1/N-cadherin interaction, comparing before and 24 hours after tet-on. Tetracycline treatment inhibited the PS1/N-cadherin interaction dramatically (Figure 2F). Collectively, these findings demonstrated that GSK3β activity regulates the binding of PS1 to N-cadherin.

PS1/N-cadherin/β-catenin complex formation is required for the cell-surface expression of PS1/γ-secretase

Since GSK3β activation reduced PS1/N-cadherin interaction, we assumed that the activation would also reduce cadherin-driven cell-surface expression of PS1/γ-secretase. First, we examined the effect of the PS1/N-cadherin/β-catenin complex formation on the cell-surface expression of PS1/γ-secretase. wtPS1, PS1Δ340-75 or Δ340-50 was transfected into MEF PS-/- cells, which endogenously express mouse N-cadherin. Transfected cells were fractionated into the plasma membrane and ER fractions and then subjected to Western blot analysis. wtPS1 was abundantly found in the plasma membrane fraction, whereas PS1Δ340-75 and Δ340-50 were barely detected (Figure 3A), indicating that the lack of PS1/N-cadherin/β-catenin interaction negatively affects the cell-surface expression of PS1/γ-secretase.

To test whether GSK3β-mediated phosphorylation reduces the cell-surface expression of PS1/γ-secretase, either pseudo-phosphorylation PS1 mutant (S353D or S357D) or wtPS1 was transfected into MEF PS-/- cells and the cell surface proteins were examined by biotinylation assay. As expected, the cell-surface expression of both PS1 and nicastrin was reduced in the cells transfected with either S353D (Figure 3B, 4th lane) or S357DPS1 (Figure 3B, 5th lane), compared to wtPS1 (Figure 3B, 3rd lane), whereas the expression levels in the total cell lysates were comparable among the transfected cell lines (Figure 3B). Collectively, the PS1/N-cadherin/β-catenin interaction enhances the cell-surface expression of PS1/γ-secretase and, conversely, GSK3β activation reduces the cell-surface expression of PS1/γ-secretase possibly via inhibiting its binding to N-cadherin/β-catenin.

GSK3β activation downregulates PI3K/Akt cell survival signaling

It has been reported that N-cadherin-based adhesion initiates PI3K-dependent activation of Akt, thereby upregulating anti-apoptotic protein Bcl-2 (19). PS1 plays an important role in this process by

promoting cadherin/PI3K association (20), finally activating cell survival signal and inhibiting GSK3β (Illustarated in Figure 6, left). This function of PS1 is independent of γ-secretase activity (20). Since GSK3β-mediated phosphorylation reduces the PS1/N-cadherin/β-catenin interaction and cell surface expression of PS1/γ-secretase, we assumed that the GSK3β activation inhibits the function of PS1 to stimulate PI3K/Akt signaling.

To test whether GSK3β-mediated phosphorylation affects PI3K/Akt signaling mediated by PS1, either wtPS1 or pseudo-phosphorylation mutant (S353D, S357D or S353/357DPS1) was transfected into MEF PS-/- cells and the amount of activated (phosphorylated) Akt was analyzed by Western blot (Figure 4B). Transfection of wtPS1 (Figure 4B, 2nd lane) promoted Akt phosphorylation compared to control GFP transfection (Figure 4B, 1st lane). The magnitude of Akt phosphorylation after transfection of pseudo-phosphorylation mutants (S353D, S357D and S353/357D) was reduced (Figure 4B, 3rd-5th lanes), compared to wt PS1 (Figure 4B, 2nd lane). Thus, GSK3β-mediated phosphorylation of PS1 inhibits the activation of PI3K/Akt cell survival signaling, possibly by reducing the physical binding of PS1 to N-cadherin.

Next, we wished to determine whether this PS1-mediated activation of PI3K/Akt signaling is affected by the strength of cadherin-based adhesion. To test this, the calcium switch assay was performed using MEF PS-/- cells, since cadherins are known to be calcium-dependent cell-cell adhesion molecules. In the assay, MEF PS-/- cells were cultured in the presence of 4mM EGTA for 40mins for calcium deprivation, after which cells were cultured in the serum free, calcium containing medium (Figure 4B, top). Immunostaining results before calcium deprivation showed that N-cadherin immunoreactivity was present along the outline of the cell-cell contact sites (Figure 4B, bottom, left). After EGTA treatment, N-cadherin immunoreactivity was observed in a granular pattern, indicating the disruption of cadherin-based cell-cell contacts (Figure 4B, bottom, middle). Calcium supplement restored the liner N-cadherin immunoreactivity at the junction (Figure 4B, bottom, right). We utilized the calcium switch assay to examine whether PS1/N-cadherin interaction is necessary for the N-cadherin-mediated transmission of PI3K/Akt survival signaling. Either wtPS1 or PS1Δ340-50 was transiently transfected into MEF PS-/- cells, then followed by the calcium switch assay (Figure 4C). In the presence of wtPS1, Akt phosphorylation before calcium deprivation was prominent (Figure 4C, wtPS1, Pre), which was diminished after calcium deprivation (Figure 4C, wtPS1, time 0) and gradually recovered after calcium supplement (Figure 4C, wtPS1, 30, 90). Conversely, after PS1Δ340-50 transfection, Akt phosphorylation before calcium deprivation was not prominent compared to wtPS1 and remained unchanged throughout the assay (Figure 4C, PS1Δ340-50), indicating that the PS1/N-cadherin interaction is necessary for the contact-mediated transmission of PI3K/Akt survival signaling. Next, we examined the effect of GSK3β activation on the transmission of PI3K/Akt signaling (Figure 4D). In the absence of PS1 (Figure 4D, GFP) or after co-transfection of wtPS1 and S9AGSK3β (Figure 4D, wtPS1+S9AGSK3β), the phosphorylation state of Akt did not change significantly before and throughout the calcium switch assay, which was in contrast to wtPS1 transfection (Figure 4D, wtPS1). Since GSK3β affects many molecules, we cannot conclude that the effect of S9A GSK3β transfection was solely mediated by PS1 phosphorylation, however, above data supports the idea that PS1/N-cadherin interaction plays an important role in the transmission of `contact-mediated' PI3k/Akt signal activation and that GSK3β-mediated phosphorylation of PS1 may inhibit this process by reducing PS1/N-cadherin interaction.

GSK3β-mediated

phosphorylation of PS1 differentially regulates N-cadherin and APP cleavage.

Since GSK3β-mediated phosphorylation of PS1 changes its subcellular distribution and binding to N-cadherin, we assumed that the phosphorylation might also change the substrate specificity of γ-secretase. To test this, PS1 constructs (wtPS1, pseudo-phosphorylation mutant (S353D or S357DPS1) or PS1Δ340-50) were transfected into MEF PS-/- cells (Figure 5A). As we reported previously, N-cadherin is sequentially cleaved by ADAM10 and PS1/γ-secretase (15). Ectodomain shedding by ADAM10 generates a membranous fragment Ncad/CTF1, which is further cleaved by PS1/γ-secretase to produce a cytoplasmic fragment Ncad/CTF2. Immunoblotting by anti-N-cadherin C-terminus antibody revealed that the amount of Ncad/CTF1 is reduced after wtPS1 transfection (Figure 5A, 2nd lane), compared to control GFP transfection (Figure 5A, 1st lane), reflecting degradation of Ncad/CTF1 by PS1/γ-secretase. On the contrary, neither pseudo-phosphorylation mutants (S353D, S357D) nor PS1Δ340-50 transfection effectively reduced Ncad/CTF1 (Figure 5A, 3rd-5th lanes), suggesting that phosphorylation of PS1 inhibits N-cadherin cleavage after ectodomain shedding. We then stimulated ectodomain shedding of N-cadherin in these transfected cells by ionomycin treatment (5). Cells were fractionated after ionomycin treatment for 30 mins and both membrane and cytosolic fractions were subjected to Western blot assay, using anti-N-cadherin C-terminus antibody (Figure 5B). In the membrane fraction, both full-length (FL) N-cadherin and Ncad/CTF1 were observed. The amount of Ncad/CTF1 was reduced in the membrane fraction of the cells transfected with wtPS1, compared to other cell lines (Figure 5B, 2nd lane). Ncad/CTF2 production was observed only in the cytoplasmic fraction of the cells transfected with wtPS1 (Figure 5B, 2nd lane), indicating that the cleavage of Ncad/CTF1 was impaired in the absence of PS1 (Figure 5B, 1st lane) or in the presence of pseudo-phosphorylation mutants (Figure 5B, 3rd and 4th lane). PS1Δ340-50 also failed to produce Ncad/CTF2, indicating that PS1-N-cadherin binding is important for the cleavage of Ncad/CTF1 (Figure 5B, 5th lane).

This led us to ask whether this inhibitory effect of PS1 phosphorylation affects the γ-secretase activity for other substrates. To test this effect on APP cleavage, we introduced human APP into MEF PS-/- cells together with either wtPS1 or PS1 mutant (Figure 5C), followed by the Western blot analysis, using anti-APP C-terminus antibody. Interestingly, all the PS1 constructs equally reduced APP CTFα&β production (Figure 5C, 2nd-5th lanes) compared to control GFP (Figure 5C, 1st lane), indicating that the phosphorylation does not significantly affect APP cleavage by γ-secretase. These results demonstrate that GSK3β-mediated phosphorylation of PS1 differentially affects N-cadherin and APP cleavage by modulating substrate-enzyme binding and subcellular distribution of γ-secretase.

DISCUSSION

PS1 has been reported to be localized mainly in the endoplasmic reticulum and Golgi membranes (11), nevertheless, it has also been demonstrated that endogenous PS1 localizes at the plasma membrane as an active molecule (12). The cell surface localization of PS1 is consistent with the observation that it can process many adhesion and receptor molecules (27, 28). However, how these differential distributions of PS1/γ-secretase are regulated has never been elucidated.

In this report, we demonstrated that the cell-surface expression and its functions of PS1/γ-secretase are regulated by PS1/N-cadherin/β-catenin interaction (Figure 3). N-cadherin is an essential adhesion molecule for synaptic contact (16), indicating that the expression of PS1/γ-secretase at the synaptic membrane is also regulated by cadherin-based synaptic contact. Importantly,

N-cadherin is cleaved by PS1/γ-secretase to disrupt both synaptic contact and PS1/N-cadherin/β-catenin complex in response to NMDA-type receptor stimulation (14, 15). Thus, the subcellular distribution of PS1/γ-secretase in neurons would be altered dynamically in the course of synaptic remodeling, which would also change processing of various adhesion and receptor molecules.

In the present report, we showed that the increase of GSK3β activity reduces PS1/N-cadherin/β-catenin complex formation possibly via direct phosphorylation of the PS1 loop domain (Figure 2). Since activation of GSK3β also enhances the phosphorylation and degradation of β-catenin (Figure 2E, left), reduction of the β-catenin level may also be involved in the process. In accordance with the finding that PS1/N-cadherin/β-catenin complex formation enhances the cell-surface expression of PS1/γ-secretase (Figure 3A), the pseudo-phosphorylated form of PS1, which has less ability to form PS1/N-cadherin/β-catenin complex, is reduced from the cell surface (Figure 3B). To our knowledge, this is the first report that demonstrates the cellular mechanism which modulates the subcellular distribution of PS1/γ-secretase.

Another important finding in this study is that GSK3β activation modulates PS1 functions via two independent ways i) downregulates cadherin -mediated activation of PI3K/Akt signaling (Figure 4), and ii) regulates its cleavage functions (Figure 5). Previous report demonstrated that N-cadherin-based contact recruits PI3K to the cell-surface N-cadherin complex, activating PI3K/Akt cell survival signaling (19). PS1 plays an important role in this process by facilitating PI3K/N-cadherin binding and finally leading to the activation of PI3K/Akt signaling and the inhibition of GSK3β (20) (Illustrated in Figure 6, left). According to our present findings, abnormal activation of GSK3β leads to the phosphorylation of PS1 in the loop domain, reducing its binding to N-cadherin. A decrease of N-cadherin/PS1 interaction leads to the inactivation of PI3K/Akt signaling. At the same time, since PI3K/Akt signaling is an important inhibitory mechanism of GSK3β (20), inactivation of this signaling could lead to further activation of GSK3β, constituting a vicious circle (Figure 6, right). Collectively, PS1 may act as a molecular switch which links cell-cell adhesion to the cell survival signal. GSK3β-mediated phosphorylation of PS1 would separate PS1 from N-cadherin, thereby "switching off" the linkage between cell-cell contact and survival signal.

In addition, we have shown that GSK3β-mediated phosphorylation of PS1 differentially regulates N-cadherin and APP cleavage by γ-secretase (Figure 5). PS1/γ-secretase complex is also involved in the ε-cleavage of various membrane proteins (2-5), however, an important, but an unanwered question is that how PS1/γ-secretase activity is modulated in terms of substrate specificity. Recently, TMP-2 was identified to be a modulator of γ-site (but not ε-site) cleavage, negatively regulating Aβ production (29). As shown in the present study, ε-cleavage of N-cadherin is downregulated by phosphorylation of PS1, which is in contrast to APP cleavage. Our data clearly demonstrated the cellular mechanism involved in substrate specificity of ε-cleavage by PS1/γ-secretase. Whether ε-cleavage of other substrates is affected by the phosphorylation should be investigated in the future.

Recently, a strain of PS1 knock-in mice in which most of the hydrophilic loop sequence was deleted from the endogenous PS1 gene (thus, can not be associated with N-cadherin/β-catenin) was created (30). Surprisingly, the homozygous mice exhibit drastically reduced γ-secretase cleavage at the $A\beta_{40}$, but not the $A\beta_{42}$, site. In addition, it was reported that the inhibition of GSK-3α, blocked the production of Aβ peptides by interfering with APP cleavage at the γ-secretase step, but did not inhibit Notch processing (31). Thus, althrough GSK3β-mediated PS1 phosphorylation seems to have less impact on the ε-cleavage of APP compared to N-cadherin cleavage, whether