

**Effect of GSK3 β -mediated PS1 phosphorylation on A β production
is negatively regulated by IR cleavage**

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Abstract

Presenilin 1 (PS1), a causative molecule of familial Alzheimer's disease (AD), is known to be an unprimed substrate of GSK3 β (Twomey *et al.* 2006) and is phosphorylated at serine 353, 357 residues in its cytoplasmic loop region (Kirschenbaum *et al.* 2001). In this report, we investigated the effect of PS1 phosphorylation on AD pathophysiology and obtained two important results – PS1 phosphorylation increased A β 42/40 ratio, and PS1 phosphorylation was enhanced in the human AD brains. Interestingly, we demonstrated that PS1 phosphorylation promoted Insulin Receptor (IR) cleavage and the IR intracellular domain (IR ICD) generated by γ -secretase led to a marked transactivation of Akt (PKB), which down-regulated GSK3 β activity. Thus, the cleavage of IR by γ -secretase can inhibit PS1 phosphorylation in the long run. Taken together, our findings indicate that PS1 phosphorylation at serine 353, 357 residues can play a pivotal role in the pathology of AD and that the dysregulation of this mechanism may be causally associated with its pathology.

Key words: Alzheimer's disease, Presenilin 1, phosphorylation, regulated intramembrane proteolysis, Insulin Receptor, Akt ,

The abbreviations used are: AD, Alzheimer's disease; APP, amyloid precursor protein; PS1, Presenilin; RIP, regulated intramembrane proteolysis; IR, Insulin Receptor; ICD, Intracellular Domain; GSK3 β , Glycogen synthase kinase 3 β ; MEF, mouse embryonic fibroblast, NTF, N-terminal fragment; CTF, C-terminal fragment

1. Introduction

Alzheimer's disease (AD) is pathologically characterized by amyloid plaques and neurofibrillary tangles (NFTs). Amyloid plaques are composed of amyloid β ($A\beta$) peptides, which are derived from amyloid precursor protein (APP) via proteolytic cleavage by γ -secretase (De Strooper *et al.* 1998). γ -Secretase is an enzymatic complex composed of at least four proteins: Presenilin 1 or Presenilin 2 (PS1 or PS2), Nicastrin, Pen 2, and Aph 1, with Presenilin representing the catalytic core (Yu *et al.* 2000; Francis *et al.* 2002; Goutte *et al.* 2002). PS1 mutations account for the majority of cases of familial AD, and more than 170 pathogenic mutations have been identified within the PS1 coding sequence. On the other hand, NFTs are characterized by the accumulation of hyperphosphorylated Tau in neurons (Grundke-Iqbal *et al.* 1986). One of the candidates which phosphorylate Tau is Glycogen synthase kinase 3 β (GSK3 β) (Hanger *et al.* 1992).

GSK3 β , originally known to inactivate glycogen synthesis, is a serine/threonine protein kinase with a wide variety of substrates, and is expressed in various tissues with the highest level in the brain (Grundke-Iqbal *et al.* 1986). Interestingly, GSK3 β phosphorylates not only Tau but also PS1 at serine 353, 357 residues in its cytoplasmic loop region (Kirschenbaum *et al.* 2001, Twomey *et al.* 2006). Therefore, it is intriguing to examine the function of PS1 phosphorylation at those residues to connect two AD hallmarks – amyloid plaques and NFTs. Although our recent report demonstrated that GSK3 β -mediated PS1 phosphorylation regulates the localization of γ -secretase and inhibits its N-cadherin cleavage (Uemura *et al.* 2007), a direct impact of PS1 phosphorylation on AD pathogenesis has not been elucidated so far.

Here, we demonstrate that GSK3 β -mediated PS1 phosphorylation inhibits APP cleavage by γ -secretase activity and decreases the total $A\beta$ production whereas it increases $A\beta$ 42/40 ratio. Importantly, we found that PS1 phosphorylation was enhanced in the human AD brains. To further elucidate the underlying mechanisms which regulate PS1 phosphorylation, we focused on insulin signaling. We observed that phosphorylated PS1

promotes the cleavage of IR by γ -secretase activity. Intracellular domain of IR (IR ICD), produced by the cleavage, enhances the transcription of Akt (PKB), leading to inhibit GSK3 β activity. Therefore, we propose that PS1, localized downstream of GSK3 β , may play a pivotal role in A β production under the influence of insulin signaling, a mechanism that may be deeply associated with AD pathophysiology.

2. Experimental procedures

2.1 Human brain samples

From the brain tissue collection in Tokyo Institute of Psychiatry (H.A.), which mainly consists of cases from psychogeriatric wards, we employed experiments of six dementia patients' brains with CERAD plaque score 'C' (Mirra *et al.* 1991) and Braak and Braak's neurofibrillary tangles (NFTs) stage IV or higher (Braak and Braak 1991), as those with AD, as well as five control subjects without neurological complications (Table). Fresh-frozen samples were taken in all cases from the mediobasal temporal neocortex. The brain samples were extracted in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X100, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, pH 8.0) with protease inhibitor cocktail (Roche) and sufficiently homogenized on ice. Then the samples were incubated for one night at 4°C and centrifuged at 14,000 g × 20 min. The supernatants were directly used for Western blot analysis. All autopsies were undertaken with written consents and the study was approved by the official ethical committees of Kyoto University as well as of Tokyo Institute of Psychiatry.

2.2 Plasmid constructs

The pcDNA3.1-hIR (human Insulin Receptor) construct was a kind gift from Dr. Ikeuchi (Niigata University, Japan) (Ebina *et al.* 1985). The cDNA encoding the IR ICD was generated by polymerase chain reaction (PCR) using the following primers: IR ICD forward, CGGGTACCCGCCATGAGAAAGAGGCAGCCAGA, and IR ICD reverse, CGGGATCCGGAAGGATTGGACCGAGGCAA. The PCR product was subcloned into the reading frame of the Kpn1/BamH1 sites of the pcDNA3 with HA tag vector. The construction of the plasmids expressing wt PS1, pseudo-phosphorylation PS1 (S353/357D PS1), deletion mutant PS1 (Δ 340-350 PS1), and dominant negative PS1 (D385A PS1) was described previously (Uemura *et al.* 2003; Uemura *et al.* 2007). Precise cloning of all

reading frames was verified by sequencing.

2.3 Cell culture and transfection

SH-SY5Y (derived from human neuroblastoma) cells were maintained in Opti-MEM containing 10 % fetal bovine serum (FBS) (Invitrogen). Establishment of cell lines which stably express hIR was described previously (Maesako *et al.* 2010). PS1/PS2 double knockout mouse embryonic fibroblast (MEF PS^{-/-}) cells were generously donated by Dr. B De Strooper and they were maintained in Dulbecco's modified Eagle's medium (DMEM) (SIGMA) with 10% FBS. CHO cells stably expressing both Swedish mutant APP (K670M/N770L) and human N-cadherin (APP^{sw}/Ncad-CHO cells) were obtained and maintained as described previously (Uemura *et al.* 2007). For transient transfection into SH-SY5Y as well as into MEF PS^{-/-} cells, cells were plated in 6-cm dishes with serum-containing medium. 8 µg of plasmid DNA and 20 µl of TransFectin reagent (Bio-Rad) were mixed into 1 ml of serum-free medium and incubated for 20 min, then added directly to confluent cells in 2 ml of serum-containing medium. 24 to 48 hrs after transfection, reporter gene activity was assayed.

2.4 Antibodies and chemical reagents

Anti-IR, a monoclonal antibody recognizing the C-terminal amino acid residues of human IR, was from Neo Markers. Rabbit polyclonal anti-Akt and phospho-GSK3β (S9) were from Cell Signaling Technology. Mouse monoclonal anti-GSK3β was from BD Transduction Laboratories. Mouse monoclonal anti-β-actin and rabbit polyclonal anti-APP C-terminal antibodies were from SIGMA. Rabbit polyclonal anti-PS1 NTF and goat polyclonal phospho-PS1 (S353/357) antibodies were from Santa Cruz. Mouse monoclonal PS1 CTF antibody was from Calbiochem. Alexa Fluor 546 anti-mouse IgG and Alexa Fluor 488 anti-goat IgG were obtained from Molecular Probes. γ-secretase inhibitor DAPT and L-685458 were from SIGMA. Akt inhibitor IV, TPA, and PKC inhibitor were from Calbiochem. Insulin solution was from SIGMA and DMSO was from Nacalai tesque.

2.5 Western blotting

Cells were washed in phosphate-buffered saline (PBS) two times and scraped off. Cell pellets were suspended in ice-cold TNE buffer (10 mM Tris HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA pH 7.8), supplemented with protease inhibitor cocktail, and briefly subjected to sonication. The samples were centrifuged at 14,000 g × 20 min at 4°C and the supernatants were collected to obtain protein samples. The protein concentration was determined using the Bradford assay (Bradford 1976). Protein samples were diluted with sample buffer (125 mM Tris, 4% SDS, 2% 2-mercaptoethanol, 20% glycerol and 0.01% bromophenol blue, pH 6.8) and denatured at 95°C for 5 min. Samples containing equal amounts of protein were electrophoresed on polyacrylamide gradient gels (5–20%) (Atto, Japan) in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). In this gel, APP CTFs bands were shown as a single band. To detect AICD, samples were electrophoresed on 15% Tricine gels (Atto, Japan) in Tricine SDS Running Buffer. Immunoblotting was carried out by transferring the proteins to polyvinylidene difluoride microporous membrane, blocking this membrane with 5% skimmed milk in 20 mM TBS containing 0.1% Tween 20 (TBS-T), and incubating with the primary antibodies in PBS containing 4% bovine serum albumin (BSA) (Nacalai tesque) overnight at 4°C. The membranes were then washed in TBS-T and incubated with a horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare) in TBS-T for 1 hr at room temperature. The specific reaction was visualized using the enhanced chemiluminescence (ECL) method (GE Healthcare).

2.6 *In vitro* γ -secretase assay

To analyze AICD or IR ICD level by *in vitro* γ -secretase assay (Sastre *et al.* 2001), cells were suspended (0.5 ml/10 cm dish) in homogenization buffer (10 mM MOPS, 10 mM KCl, pH 7.0) and homogenates and the post-nuclear supernatant (PNS) were prepared as described (Steiner *et al.* 1998).

Membranes were pelleted from the PNS by centrifugation for 20 min at 16,000 g at 4°C, washed with homogenization buffer and resuspended (50 µl/10 cm dish) in assay buffer (150 mM sodium citrate, pH 6.4). To allow generation of ICD, samples were incubated at 37°C for 2 hrs in a volume of 25 µl/ assay. Control samples were kept at 4°C. After termination of the assay reactions, samples were separated into pellet fractions (membrane fractions) and supernatant fractions by ultracentrifugation for 1 hr at 100,000 g at 4°C. AICD was detected using polyclonal anti-APP C-terminal antibody and IR ICD was detected using monoclonal anti-IR C-terminal antibody.

2.7 Immunostaining

5.0×10^4 cells were harvested onto 0.1% polyethyleneimine-coated Lab-Tek II Chamber slides (4-well; Nalge Nunc International, Rochester) and maintained in Opti-MEM containing 10% FBS. Cells were cultured for the indicated time and then fixed with 4% paraformaldehyde for 20 min. Fixed cells were blocked with PBS containing 0.2% Triton X-100 for 15 min and incubated overnight at 4°C with each antibody diluted in PBS containing 3% BSA. Immunoreactivity was visualized using the species-specific secondary antibodies mentioned above. The samples were examined using a laser confocal scanning microscope, LSM 510 Pascal (Zeiss).

2.8 Measurement of extracellular A β

The levels of A β 40 and A β 42 were measured by ELISA kits specific to A β 40 and A β 42 (Wako, Japan), as described previously (Uemura. *et al* 2009). Briefly, APPsw/Ncad-CHO cells were cultured in 6 cm dishes at a density of 7.0×10^5 cells /well. 24 hrs after incubation, the cells were treated with reagents for 5 hrs in DMEM/F-12 medium, followed by replacing DMEM/F-12 with 6 ml of Opti-MEM. 1 hr after replacing, 1 ml of medium was collected, centrifuged at 600 g for 5 min, and 100 µl of the aliquot was used for measurement of extracellular A β .

2.9 MTT assay

The viability in APP^{swe}/N-cad-CHO cells was analyzed by MTT assay using MTT Cell Proliferation Assay Kit (Cayman Chemical Company, USA). Briefly, cells were cultured in a 96-well plate at a density of 1.0×10^4 cells /well. 24 hrs after incubation, reagents of indicated concentration were treated for 5 hrs in the presence of DMEM/F-12 medium, followed by replacing DMEM/F-12 to 100 μ l of Opti-MEM with the reagents. 1 hr after replacing, MTT was measured using the Kit and microplate reader.

2.10 Real-time PCR assay

Total RNA was isolated using ISOGEN (NIPPON GENE, Japan), according to the manufacture's protocol. For real-time PCR analysis, 5 μ g total RNA from each sample was used for cDNA synthesis kit (GE Healthcare). Real-time PCR primers were designed as follows: Akt1: fw GACCTCAAGCTGGAGAAC / rv ACTGCACGGCCGTAGTC, Akt2: fw ACCGCCTGTGCTTTGTGATG / rv TCATTGTCCTCCAGCACCTC Akt3: fw CATTATTGCAAAGGATGAAGTGGCAC / rv CCAGCATTAGATTCTCCAACCTTGAG. For the amplification of Akt, 5 μ l of cDNA was added to the SYBR green master mix (Roche) and real-time PCR assay was performed in LightCycler 480 (Roche).

2.11 Statistical analysis

All values are given in means \pm SE. Comparisons were performed using an unpaired Student's t-test. For comparison of multiparametric analysis, one-way factorial ANOVA, followed by the post hoc analysis by Fisher's PLSD was used. $p < 0.05$ was considered to indicate a significant difference. $n = 4$ indicated four independent experiments.

3. Results

3.1 PS1 phosphorylation inhibits APP cleavage but enhances extracellular A β 42/40 ratio

Since PS1 is known to be the essential component of γ -secretase, we examined the effect of its phosphorylation on APP cleavage. To test this, we transfected wt PS1 and pseudo-phosphorylated PS1 mutant S353/357D PS1 (in which serine residues phosphorylated by GSK3 β are substituted with aspartate to mimic phosphorylation state (Uemura *et al.* 2007)) into MEF PS^{-/-} cells. Transfected cells were treated with insulin to promote ectodomain shedding (Linda *et al.* 2007) and to inhibit the phosphorylation of wt PS1 (Maesako *et al.* 2010). APP CTFs band was accumulated in MEF PS^{-/-} cells (Fig. 1A, 1st lane). Interestingly, in the absence of insulin, the level of APP CTFs was slightly higher in S353/357D PS1 cells than that in wt PS1 (Fig. 1A, 2nd and 3rd lanes). Moreover, in the presence of insulin, the level of APP CTFs in S353/357D PS1 was significantly increased compared to that in wt PS1 (Fig. 1A, 4th and 5th lanes, Fig. 1B). Using Tricine gels, we could detect that the level of APP ICD (AICD) in S353/357D PS1 was decreased compared to that in wt PS1 (Fig. 1C, 3rd and 4th lanes). This result was confirmed by in vitro γ -secretase assay (Fig. 1D). Collectively, these results suggested that PS1 phosphorylation inhibited the γ -secretase-mediated cleavage of APP CTFs.

As A β plays a pivotal role in the pathogenesis of AD, we next examined the effect of PS1 phosphorylation on extracellular A β production, as well as A β 42/40 ratio. For this purpose, either Akt inhibitor IV or LiCl, which activates or inhibits GSK3 β respectively, was added to change the phosphorylation status of PS1 in APP^{swe}/N-cad-CHO cells (Fig. 1E). Under this condition, neither of them induced cell death (Fig. 1F). Interestingly, Akt inhibitor significantly decreased the amount of extracellular A β 40 whereas LiCl significantly increased its amount (Fig. 1G). In contrast, the extracellular A β 42 level in the presence of either Akt inhibitor or LiCl remained almost the same (Fig. 1H), thus leading to the increase of A β 42/40 ratio in Akt inhibitor-treated cells or to the decrease of its ratio in

LiCl-treated cells (Fig. 1I). These results collectively indicate that GSK3 β -mediated PS1 phosphorylation down-regulated A β production but enhanced A β 42/40 ratio.

3.2 PS1 phosphorylation is enhanced in AD human brain samples

It was reported that GSK3 β activity was up-regulated in AD brains (Bhat *et al.* 2004). Therefore, we assumed that the phosphorylation ratio of PS1 might be increased in AD brains. Thus, we analyzed the phosphorylation ratio of PS1 in human brains. In order to rule out the post-mortem effect on the phosphorylation state of PS1 and GSK3 β , we examined the levels of PS1 and GSK3 β phosphorylation in mouse brains with different post-mortem intervals and demonstrated that post-mortem intervals did not influence the phosphorylation ratio of either PS1 or GSK3 β up to 36 hrs after sacrifice (data not shown). In accordance with the previous reports, the phosphorylation ratio of GSK3 β S9 was significantly decreased in AD brains compared with that in control brains (Fig. 2A, 3rd and 4th rows, Fig. 2B), indicating the hyperactivity of GSK3 β in AD brains. In contrast, PS1 phosphorylation was significantly increased in AD brains compared with that in control ones (Fig. 2A, 1st and 2nd rows, Fig. 2C). These results suggest that up-regulated GSK3 β activity could enhance PS1 phosphorylation in AD-affected brains.

3.3 PS1 phosphorylation promoted IR cleavage

Since IR is one of key regulators of GSK3 β activity, we analyzed the functional relationship between PS1/ γ -secretase activity and IR. It is now reported that γ -secretase processes IR, a main player of insulin signaling, along with other substrates (Kasuga *et al.* 2007; Marambaud *et al.* 2002; Okamoto *et al.* 2001). As reported previously, ectodomain shedding of IR β -subunit by metalloproteases generates a membranous fragment – IR CTF (Fig. 3A, bottom), which is further cleaved by γ -secretase to produce the intracellular domain – IR ICD (Fig. 4A, bottom). For the analysis of IR cleavage, we co-transfected either wt PS1 or

S353/357D PS1 together with IR into MEF PS^{-/-} cells, followed by insulin treatment. In the absence of insulin, IR CTF level was slightly higher in wt PS1 cells than that in S353/357D PS1 cells (Fig. 3B, 2nd and 3rd lanes). Moreover, insulin treatment led to a robust increase in the level of IR CTF in wt PS1 when compared to that in S353/357D PS1 (Fig. 3B, 4th and 5th lanes, Fig. 3C). In addition, we examined the level of IR ICD using in vitro γ -secretase assay and demonstrated that IR ICD level in S353/357D PS1 cells was higher than that in wt PS1 cells (Fig. 3D). These results suggested that PS1 phosphorylation enhanced the γ -secretase-mediated cleavage of IR CTF.

Since phosphorylation of PS1 reduces the PS1/N-cadherin/ β -catenin interaction (Uemura *et al.* 2007), we asked whether the PS1 interaction with N-cadherin/ β -catenin directly affects IR cleavage. To test this, MEF PS^{-/-} cells were co-transfected with IR and Δ 340-350 PS1 (a deletion mutant lacking the loop domain necessary for the interaction with N-cadherin/ β -catenin (Uemura *et al.* 2007)), followed by insulin treatment. As in S353/357D PS1 transfected cells (Fig. 3E, middle lane), the level of IR CTF in Δ 340-350 PS1 transfected cells was significantly decreased compared to that in wt PS1 cells (Fig. 3E, left and right lanes, Fig. 3F). These findings were also confirmed in neuronal cells (Fig. 3G). Collectively, these results indicated that dissociation of PS1 from N-cadherin/ β -catenin as well as phosphorylation of PS1 promotes the cleavage of IR by γ -secretase.

3.4 IR intracellular domain up-regulates the transcription of Akt

Several lines of evidence suggest that the cleavage of membrane protein by γ -secretase, known as regulated intramembrane proteolysis (RIP), is linked to intracellular signaling events (Wolfe *et al.* 2004; Maetzel *et al.* 2009). γ -secretase generates the intracellular domain (ICD) of the membranous proteins, resulting in the release of ICD from the membrane. Some ICDs translocate to the nucleus and act as transcription regulators. IR ICD also translocates to the nucleus (Kasuga *et al.* 2007), but its exact function remains unknown.

To examine the cellular consequence of IR-RIP, we transfected IR ICD fused with HA-Tag (Fig. 4A, bottom) into SH-SY5Y cells. Immunostaining with anti-HA antibody revealed that IR ICD was located at the

nucleus as in the previous report (Fig. 4B, under panel), whereas full length IR was located at the cell membrane and in the cytoplasmic region (Fig. 4B, top panel). In order to analyze the effect of IR ICD translocation into the nucleus we transiently transfected IR ICD into native SH-SY5Y cells. 36 hrs after transfection, mRNA was extracted and analyzed by real-time PCR assay. In this experiment, we focused on the mRNA level of Akt, because Akt plays important roles in IR signaling. Since Akt has three isoforms – Akt 1, 2 and 3, we evaluated mRNA level of all the isoforms. mRNA levels of Akt 1 and 2 were significantly increased in IR ICD transfected cells, compared with those in control cells (Fig. 4C). The protein level of total Akt was also significantly increased in IR ICD transfected cells 48 hrs after transfection (Fig. 4D and E), suggesting marked transactivation of Akt in IR ICD transfected cells. Moreover, under the same condition, we observed that IR ICD reduced GSK3 β activity, promoted GSK3 β S9 phosphorylation, as well as enhanced Akt levels (Fig. 4F and G). These results collectively indicate that IR ICD can enhance Akt transcription, followed by the reduction in GSK3 β activity.

3.5 PS1/ γ -secretase-mediated IR cleavage changes the expression level of Akt

To confirm that IR-RIP regulates the transcription of Akt, we modulated IR cleavage in several ways. First, we inhibited γ -secretase activity and observed the expression level of Akt. After a 24 hr DAPT treatment, the level of Akt did not change in SH-SY5Y cells which have little endogenous IR (data not shown). On the other hand, the expression level of Akt was dose-dependently decreased in SH-SY5Y wt IR cells (Fig.5A, top row), indicating that inhibition of γ -secretase led to decrease the levels of Akt. These results were confirmed by the treatment with another γ -secretase inhibitor, L-685458 (data not shown), and by non-pharmacological inhibition (Fig. 5B, middle and right lanes), using D385A PS1, which dominant-negatively inhibits γ -secretase activity (Uemura *et al.* 2003).

It was reported that IR might undergo ectodomain shedding by metalloproteases such as ADAM 17 (Kasuga *et al.* 2007). Since protein kinase C activation enhances the metalloprotease-mediated ectodomain

shedding (Ni *et al.* 2001), we treated SH-SY5Y wt IR cells with 500 nM TPA to enhance the ectodomain shedding. In the presence of L-685458, TPA treatment for 24 hrs increased the level of IR CTF, indicating that ectodomain shedding of IR was promoted by TPA (Fig. 5C, 3rd and 5th lanes). To analyze its effect on Akt levels, SH-SY5Y wt IR cells were pre-incubated with either DAPT or vehicle (DMSO) for 18 hrs, followed by TPA treatment with or without DAPT. Interestingly, in the absence of DAPT, the expression level of Akt was increased (Fig. 5D, 5th lane, Fig. 5E). Conversely, TPA treatment in the presence of DAPT significantly decreased the level of Akt (Fig. 5D, 4th and 6th lanes). Similarly, treatment with PKC inhibitor for 24 hrs in different concentrations demonstrated the dose-dependent decrease of Akt levels (data not shown). Taken together, these results indicate that γ -secretase-mediated IR cleavage generates IR ICD, which then transactivates Akt.

4. Discussion

Previous reports demonstrated that GSK3 β activity is up-regulated in AD brains (Bhat *et al.* 2004). On the other hand, conditional transgenic mice overexpressing GSK3 β in the adult brain show clear evidence of neurodegeneration (Lucas *et al.* 2001). Moreover, aberrantly activated GSK3 β phosphorylates Tau, which leads to NFTs (Hanger *et al.* 1992). This evidence clearly indicated that GSK3 β plays essential roles in the pathology of AD. Importantly, we and other groups previously reported that PS1, a causative molecule of familial AD, was also phosphorylated by GSK3 β at serine 353, 357 residues (Kirschenbaum *et al.* 2001, Twomey *et al.* 2006, Uemura *et al.* 2007). Since most of the familial AD-linked PS1 mutations affect APP metabolism, in the present study, we examined the effect of PS1 phosphorylation on APP cleavage by PS1/ γ -secretase. Our analysis using S353/357D mutant PS1 revealed that phosphorylated PS1 significantly inhibited the cleavage of APP CTFs by γ -secretase (Fig. 1A-D). Furthermore, the ELISA results indicated that PS1 phosphorylation reduced extracellular A β 40 levels without changing extracellular A β 42 levels, thereby increasing the A β 42/40 ratio (Fig. 1E-G). Similar phenomena were observed in the familial AD-linked PS1 mutations (Selkoe and Wolfe 2007). Accumulating evidence suggests that partial loss of function in γ -secretase may lead to an increased A β 42/40 ratio as well as to neurodegeneration (Shen 2007, Wolfe 2007). In addition, a recent report suggested that A β 40 may inhibit A β deposition and thus may be physiologically neuroprotective (Kim *et al.* 2007). Thus, PS1 phosphorylation-mediated reduction of A β 40 and increase of A β 42/40 ratio can aggravate the pathology of AD. So far, the significance of PS1 modification including phosphorylation has not yet been fully investigated in AD brains. Importantly, our *in vivo* study clearly showed accelerated PS1 phosphorylation in sporadic AD patient brains (Fig. 2). Considering these results, the disruption of the balance of PS1 phosphorylation may lead to neurodegeneration via aberrant GSK3 β activity.

In addition, we searched for the mechanisms inhibiting PS1 phosphorylation. In the present study, we found a novel role of γ -secretase-mediated IR cleavage as a protective safeguard from aberrant GSK3 β activity. IR is widely distributed in the brain with particularly high concentrations in neurons, both in the cell bodies as well as in synapses (Werther *et al.* 1987, Marks *et al.* 1991). IR is a known substrate for γ -secretase and IR ICD translocates to the nucleus upon γ -secretase-mediated cleavage (Kasuga *et al.* 2007). In this report, we showed that IR ICD activates the transcription of Akt (Fig. 4), a key element in multiple biological processes including glucose metabolism, cell survival, cell growth, cell differentiation, and angiogenesis (Franke *et al.* 2003; Fayard *et al.* 2005; Song *et al.* 2005). Notably, we observed that PS1 phosphorylation promoted the γ -secretase-mediated cleavage of IR (Fig. 3). Thus, GSK3 β activation may promote the phosphorylation of PS1 to enhance cleavage of IR, finally activating the transcription of Akt, which is a strong inhibitor of GSK3 β activity. This phenomenon may constitute a negative feedback mechanism, which up-regulates Akt when GSK3 β is aberrantly activated. Interestingly, this is contrary to the case of the cleavage of N-cadherin, since N-cadherin cleavage is down-regulated by the phosphorylation of PS1 (Uemura *et al.* 2007). Thus, the phosphorylation status of PS1 may explain in part the substrate specificity in various conditions.

In conclusion, we propose that GSK3 β -mediated PS1 phosphorylation, acting as a link between GSK3 β activity and A β generation, may play a pivotal role in AD pathogenesis under the influence of insulin signaling. As our study indicated that GSK3 β -mediated phosphorylation of PS1 increases the A β 42/40 ratio, modulating GSK3 β activity can be a good therapeutic strategy, aiming at both the inhibition of tau phosphorylation and the reduction of A β 42/40 ratio. Thus, IR cleavage could be one of the potential regulatory mechanisms affecting GSK3 β activity as a negative feedback mechanism; disruption of IR cleavage may lead to increased GSK3 β activity, as well as A β 42/40 ratio. Therefore, the *in vivo* regulatory mechanism of controlling PS1 function including phosphorylation should be examined in future studies.

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6. References

- Bhat, R. V., Budd Haeberlein, S. L., and Avila, J. (2004) Glycogen synthase kinase 3: a drug target for CNS therapies. *J Neurochem* **89**, 1313-1317
- Braak, H., and Braak, E. (1991) Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol* **82**, 239-259
- Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54
- De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K., and Van Leuven, F. (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387-390
- Ebina, Y., Edery, M., Ellis, L., Standring, D., Beaudoin, J., Roth, R. A., and Rutter, W. J. (1985) Expression of a functional human insulin receptor from a cloned cDNA in Chinese hamster ovary cells. *Proc Natl Acad Sci U S A* **82**, 8014-8018
- Fayard, E., Tintignac, L. A., Baudry, A., and Hemmings, B. A. (2005) Protein kinase B/Akt at a glance. *J Cell Sci* **118**, 5675-5678
- Francis R, McGrath G, Zhang J, et al. (2002) Aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. *Dev Cell* **3**, 85-97

Franke, T. F., Hornik, C. P., Segev, L., Shostak, G. A., and Sugimoto, C. (2003) PI3K/Akt and apoptosis: size matters. *Oncogene* **22**, 8983-8998

Goutte C, Tsunozaki M, Hale VA, and Priess JR. (2002) APH-1 is a multipass membrane protein essential for the Notch signaling pathway in *Caenorhabditis elegans* embryos. *Proc Natl Acad Sci U S A* **99**, 775–9

Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y. C., Zaidi, M. S., and Wisniewski, H. M. (1986) Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *J Biol Chem* **261**, 6084-6089

Hanger, D. P., Hughes, K., Woodgett, J. R., Brion, J. P., and Anderton, B. H. (1992) Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase. *Neurosci Lett* **147**, 58-62

Kasuga, K., Kaneko, H., Nishizawa, M., Onodera, O., and Ikeuchi, T. (2007) Generation of intracellular domain of insulin receptor tyrosine kinase by gamma-secretase. *Biochem Biophys Res Commun* **360**, 90-96

Kim J., Onstead L., Randle S., Price R., Smithson L., Zwizinski C., Dickson D. W., Golde T. and McGowan E. (2007) Abeta40 inhibits amyloid deposition in vivo. *J. Neurosci.* **27**, 627–633.

Kirschenbaum, F., Hsu, S. C., Cordell, B., and McCarthy, J. V. (2001) Substitution of a glycogen synthase kinase-3beta phosphorylation site in presenilin 1 separates presenilin function from beta-catenin signaling. *J Biol Chem* **276**, 7366-7375

Linda, A., Sofia, H., Gerd, M., Kerstin, I. (2007) IGF-1-induced Processing of the Amyloid Precursor Protein Family Is Mediated by Different Signaling Pathways. *J Biol Chem* **282**, 10203-10209

Lucas, J., Hernández, F., Gómez-Ramos, P., Morán, M., Hen, R., Avila, J. (2001) Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. *EMBO J.* **20**, 27-39

Maesako, M., Uemura, K., Kubota, M., et al (2010) Insulin regulates Presenilin 1 localization via PI3k/Akt signaling. *Neurosci Lett* **483**,157–161

Maetzel, D., Denzel, S., Mack, B., et al. (2009) Nuclear signalling by tumour-associated antigen EpCAM. *Nat Cell Biol* **11**, 162-171

Marambaud, P., Shioi, J., Serban, G., et al. (2002) A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J* **21**, 1948-1956

Marks, J. L., King, M. G., and Baskin, D. G. (1991) Localization of insulin and type 1 IGF receptors in rat brain by in vitro autoradiography and in situ hybridization. *Adv Exp Med Biol* **293**, 459-470

Mirra, S. S., Heyman, A., McKeel, D., Sumi, S. M., Crain, B. J., Brownlee, L. M., Vogel, F. S., Hughes, J. P., van Belle, G., and Berg, L. (1991) The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* **41**, 479-486

Ni, C. Y., Murphy, M. P., Golde, T. E., and Carpenter, G. (2001) gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* **294**, 2179-2181

Okamoto, I., Kawano, Y., Murakami, D., Sasayama, T., Araki, N., Miki, T., Wong, A. J., and Saya H. (2001) Proteolytic release of CD44 intracellular domain and its role in the CD44 signaling pathway. *J Cell Biol* **155**, 755-762

Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condrón, M.M., Teplow, D.B. and Haass, C. (2001) Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. *EMBO Rep* **9**, 835-841

Selkoe, D., and Wolfe, M.S. (2007) Presenilin: running with scissors in the membrane. *Cell* **131**, 215-221

Shen, J. and Kelleher, R. J. (2007) The presenilin hypothesis of Alzheimer's disease: evidence for a loss-of-function pathogenic mechanism. *Proc. Natl Acad. Sci. USA* **104**, 403-409

Song, G., Ouyang, G., and Bao, S. (2005) The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* **9**, 59-71

Steiner, H., Capell, A., Pesold, B., Citron, M., Kloetzel, P.M., Selkoe, D.J., Romig, H., Mendla, K. and Haass, C. (1998) Expression of Alzheimer's disease-associated presenilin-1 is controlled by proteolytic degradation and complex formation. *J. Biol. Chem.* **273**, 32322–32331.

Twomey, C., and McCarthy, J. V. (2006) Presenilin-1 is an unprimed glycogen synthase kinase-3beta substrate. *FEBS Lett* **580**, 4015-4020

Uemura, K., Kitagawa, N., Kohno, R., Kuzuya, A., Kageyama, T., Shibasaki, H., and Shimohama, S. (2003) Presenilin 1 mediates retinoic acid-induced differentiation of SH-SY5Y cells through facilitation of Wnt signaling. *J Neurosci Res* **73**, 166-175

Uemura, K., Kuzuya, A., Shimosono, Y., Aoyagi, N., Ando, K., Shimohama, S., and Kinoshita, A. (2007) GSK3beta activity modifies the localization and function of presenilin 1. *J Biol Chem* **282**, 15823-15832

Uemura, K., Lill, C.M., Banks, M., et al (2009) N-cadherin-based adhesion enhances Abeta release and decrease Abeta 42/40 ratio. *J.Neurochem* **108**, 350-360

Werther, G. A., Hogg, A., Oldfield, B. J., McKinley, M. J., Figdor, R., Allen, A. M., and Mendelsohn, F. A. (1987) Localization and characterization of insulin receptors in rat brain and pituitary gland using in vitro autoradiography and computerized densitometry. *Endocrinology* **121**, 1562-1570

Wolfe M. S. (2007) When loss is gain: reduced presenilin proteolytic function leads to increased Abeta42/Abeta40. Talking Point on the role of presenilin mutations in Alzheimer disease. *EMBO Rep* **8**, 136–140.

Yu G, Nishimura M, Arawaka S, et al. (2000) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. *Nature* **407**, 48–54

7. Figure legends

7.1 Fig. 1. PS1 phosphorylation inhibits APP cleavage but enhances extracellular A β 42/40 ratio

A. Either wt PS1 or pseudo-phosphorylation PS1 mutant (S353/357D PS1) was transfected into MEF PS^{-/-} cells. Control cells were transfected with pcDNA (first lane). 24 hrs after transfection, cells were treated with 50 nM insulin for 30 min (4th and 5th lanes).

B. APP CTFs were normalized by housekeeping gene (β -actin) and relative band density was quantitatively analyzed (n = 3).

C. Either wt PS1 or S353/357D PS1 was transfected into MEF PS^{-/-} cells and APP ICD was detected by Tricin-SDS-PAGE using anti APP-C-terminal antibody. Cells were treated with 500 nM TPA that promoted the processing of APP as a positive control.

D. Membrane preparations from MEF PS^{-/-} cells transfected with pcDNA, wt PS1 or S353/357D PS1 were incubated at 37°C for 2 hrs. Control samples were kept at 4°C. The reaction mix was then separated in pellet fraction as membrane fraction (lower panel) and supernatant fraction (1st and 2nd panels) by ultracentrifugation. These fractions were electrophoresed on polyacrylamide gradient gels or Tricine gels and immunoblotted with anti APP-C-terminal antibody.

E. APP^{sw}/Ncad-CHO cells were incubated in Opti-MEM in the presence of 10 μ M Akt inhibitor IV or 25 mM LiCl for 6 hrs. PS1 (S353/357) and GSK3 β (S9) phosphorylation were analyzed by immunoblotting, using anti-phospho-PS1 S353/357, anti-PS1 CTF, anti-GSK3 β and anti-phospho-GSK3 β S9 antibodies.

F. Cell viability was analyzed by MTT assay. Both 10 μ M of Akt inhibitor IV and 25 mM LiCl (GSK inhibitor) which were used in this study didn't induce cell death (n = 10). 100 μ M of Akt inhibitor was used as positive control.

G and H. APP^{sw}/Ncad-CHO cells were incubated in Opti-MEM in the presence of 10 μ M Akt inhibitor IV or 25 mM LiCl for 6 hrs. DMSO was used as a negative control. After incubation, released extracellular A β 40 or A β 42 for 1 hr were analyzed by ELISA (n = 5).

I. Extracellular A β 42/40 ratio was quantitatively analyzed.

7.2 **Fig. 2.** PS1 phosphorylation is increased in AD brains

A. The lysates of human brain samples, including AD and non-AD brains, were analyzed by immunoblotting, using anti-phospho-PS1 S353/357, anti-PS1 CTF, anti-GSK3 β and anti-phospho-GSK3 β S9 antibodies. Loading control was detected using anti- β -actin antibody (lower panel).

B and C. GSK3 β and PS1 phosphorylation ratio of each sample was quantitatively analyzed and described as a point. The number indicated on the point means average ratio.

7.3 **Fig. 3.** IR cleavage is promoted by PS1 phosphorylation

A. A schematic representation of IR CTF. IR- β -subunit is cleaved by metalloproteases at the extracellular area, producing IR CTF (bottom).

B. Either wt PS1 or S353/357D PS1 was co-transfected with IR into MEF PS^{-/-} cells. Control cells were transfected with pcDNA (first lane). 24 hrs after transfection, cells were treated with 50 nM insulin for 30 min (4th and 5th lanes).

C. IR CTF was normalized by β -actin and relative band density was quantitatively analyzed (n = 3).

D. Membrane preparations from MEF PS^{-/-} cells co-transfected IR with pcDNA, wt PS1 or S353/357D PS1 were incubated at 37°C for 2 hrs. Control samples were kept at 4°C. The reaction mix was then separated in pellet fraction as membrane fraction (lower panel) and supernatant fraction (1st and 2nd panels) by ultracentrifugation. These fractions were electrophoresed on polyacrylamide gradient gels and immunoblotted with anti IR-C-terminal antibody.

E. MEF PS^{-/-} cells were transfected with wt PS1, S353/357D PS1 or Δ 340-350 PS1 and after 24 hrs of transfection, cells were treated with 50 nM insulin for 30 min.

F. IR CTF was normalized by β -actin and relative band density was quantitatively analyzed (n = 3).

G. SH-SY5Y wt IR cells were transfected with either wt PS1, S353/357D PS1 or Δ 340-350 PS1 and then treated with 50 nM insulin for 30 min.

7.4 Fig. 4. IR ICD up-regulates the transcription of Akt

A. A schematic representation of plasmid construction of IR ICD. The HA tag was fused to the C-terminus of IR ICD (bottom). The amino acid of transmembrane domain was indicated (top, gray area).

B. Native SH-SY5Y cells were transfected with IR ICD or full length (FL) IR. Scale bar, 20 μ m.

C. SH-SY5Y cells were transfected with IR ICD. After 36 hrs of transfection, the mRNA levels of Akt were analyzed by real-time PCR assay. The mRNA amount of Akt was normalized by that of β -actin (n = 4).

D. After 48 hrs of transfection, SH-SY5Y cells were analyzed by immunoblotting.

E. The expression level of endogenous Akt was quantitatively analyzed (n = 3). The level of Akt was normalized by that of β -actin.

F. After 48 hrs of transfection of IR ICD, the level of phospho-GSK3 β S9 was analyzed by immunoblotting.

G. The relative phosphorylation ratio of GSK3 β was quantitatively analyzed (n = 3).

7.5 Fig. 5. IR cleavage changes the expression level of Akt

A. SH-SY5Y wt IR cells were treated with DAPT for 24 hrs in different concentrations (0, 0.1, 0.5, 2, 5 μ M).

B. SH-SY5Y wt IR cells were transiently transfected with pcDNA (as a control), wt PS1 and D385A PS1. After 48 hrs, the Akt level was analyzed.

C. SH-SY5Y wt IR cells were treated with 5 μ M of DAPT, L-685458, 500 nM of TPA or L-685458 + TPA for 24 hrs. Control cells were treated with DMSO.

D. After pre-incubation with either DAPT or vehicle (DMSO) for 18 hrs, SH-SY5Y wt IR cells were treated with 500 nM TPA in the presence of DAPT or DMSO for 24 hrs.

E. After TPA treatment, the relative expression level of Akt was quantitatively analyzed ($n = 4$). The level of Akt was normalized by that of β -actin.

7.6 [Fig. 6](#). Schematic presentation of cellular events caused by PS1 phosphorylation

GSK3 β -mediated PS1 phosphorylation enhances the extracellular A β 42/40 ratio and its phosphorylation is promoted in the AD brain. Therefore, PS1 phosphorylation may promote the pathology of AD (left panel). On the other hand, when GSK3 β is aberrantly activated, the phosphorylated PS1 cleaves IR, then activates the transcription of Akt, which finally inhibits GSK3 β activity (right panel). There may be some mechanisms which down-regulate PS1 phosphorylation through inhibition of GSK3 β activity and IR cleavage by γ -secretase can be one of them.

7.7 [Table](#) Characteristics of human brain samples

We employed experiments of six Alzheimer's disease patients' brains, as well as five control subjects without neurological complications. Fresh-frozen samples were taken in all cases from the mediobasal temporal neocortex. There is no statistical difference in the postmortem interval between AD and control cases.