GSK3\(\beta\) Activity Modifies the Localization and Function of Presenilin 1*

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Presenilin 1, a causative gene product of familial Alzheimer 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/\(\beta\)-catenin to form a trimeric complex at the synaptic site through its loop domain, whose serine residues (serine 353 and 357) can be phosphorylated by glycogen synthase kinase 3\(\beta\). Here, we demonstrate that cell-surface expression of Presenilin 1/\(\gamma\)-secretase is enhanced by N-cadherin-based cell-cell contact. Physical interaction between Presenilin 1 and N-cadherin/\(\beta\)-catenin plays an important role in this process. Glycogen synthase kinase 3\(\beta\)-mediated phosphorylation of Presenilin 1 reduces its binding to N-cadherin, thereby down-regulating its cell-surface expression. Moreover, reduction of the Presenilin 1-N-cadherin/\(\beta\)-catenin complex formation leads to an impaired activation of contact-mediated phosphatidylinositol 3-kinase/Akt cell survival signaling. Furthermore, phosphorylation of Presenilin 1 inhibits \(\varepsilon\)-cleavage of N-cadherin, whereas \(\varepsilon\)-cleavage of APP remained unchanged. This is the first report that clarifies the regulatory mechanism of Presenilin 1/\(\gamma\)-secretase with respect to its subcellular distribution and its differential substrate cleavage. Because the cleavage of various membrane proteins by Presenilin 1/\(\varepsilon\)-cleavage is involved in cellular signaling, glycogen synthase kinase 3\(\beta\)-mediated phosphorylation of Presenilin 1 should be deeply associated with signaling functions. Our findings indicate that the abnormal activation of glycogen synthase kinase 3\(\beta\) can reduce neuronal viability and synaptic plasticity via modulating Presenilin 1/N-cadherin/\(\beta\)-catenin interaction and thus have important implications in the pathophysiology of Alzheimer disease.

Pathological features of Alzheimer disease (AD)3 are characterized by neurofibrillary tangles and amyloid plaques.

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3 The abbreviations used are: AD, Alzheimer disease; APP, amyloid precursor protein; PS1, Presenilin 1; GSK3\(\beta\), glycogen synthase kinase 3\(\beta\); PI3K, phosphatidylinositol 3-kinase; CHO, Chinese hamster ovary; MEF, mouse embryonic fibroblast; NTF, N-terminal fragment; CTF, C-terminal fragment; GFP, green fluorescent protein; siRNA, small interfering RNA.
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The physical interaction between PS1 and N-cadherin/β-catenin plays an important role in cell-surface expression of PS1/γ-secretase. Moreover, GSK3β-mediated phosphorylation of PS1 reduces its binding to N-cadherin, thereby down-regulating 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 PROCEDURES

Plasmid Constructs—A cDNA copy of human N-cadherin (GenBank accession number M34064) gene was amplified from first strand cDNA human fetal brain (Stratagene) by a forward primer, 5'-TCTTCTTGGTACTGCATGGATATAAGGACGGCAGGCC-3', and a reverse primer, 5'-TCTTCTTGGTACTGCATGGATATAAGACCCAGGGCCCTTCCACAGCTGTT-3'. and a reverse primer, 5'-TCTTCTTGGTACTGCATGGATATAAGACCCAGGGCCCTTCCACAGCTGTT-3'. Precise cloning of all reading frames 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 Dulbecco’s modified Eagle’s medium 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 5 μg/ml blasticidin, stably transfected clones were then transfected with GSK3β (or S9A GSK3β)/pcDNA4/TO, and selected by 100 μg/ml zeocin.

Chinese hamster ovary (CHO) cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen) supplemented with 10% fetal bovine serum. PS1/PS2 double knockout mouse embryonic fibroblast (MEF) cells (MEF PS/−/−) cells (23) were kindly provided by Dr. De Strooper and maintained in Dulbecco’s modified Eagle’s medium. Transient transfection of wtPS1, PS1 mutants, N-cadherin, and S9AGSK3β into cells were achieved by using the lipofection method, with Lipofectamine 2000 (Invitrogen).

CHO cells, stably expressing Swedish (K670N/M671L) 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 (K670M/N770L) (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 (10 μg/ml). Cells were maintained in Dulbecco’s modified Eagle medium/F-12 (Invitrogen) supplemented with 10% fetal bovine serum. 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 800 μg/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 were obtained from Transduction Laboratories. Anti-β-actin, anti-nicastrin, anti-APP C terminus, and anti-phosphoserine antibodies were from Sigma. Anti-phospho-β-catenin (Ser33/37/Thr41), anti-total Akt, and anti-phospho-Akt (Ser473) antibodies were from Cell Signaling Technology. Rabbit polyclonal anti-PS1 N-terminal fragment (NTF) was from Santa Cruz. Goat polyclonal anti-PS1 C-terminal fragment (CTF) was from R&D systems. Alexa Fluor 546 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG were obtained from Molecular Probes. Anti-mouse and rabbit horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences. Anti-goat horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz. Blasticidin, tetracycline, and zeocin were from Invitrogen. G418 was from WAKO Chemicals, Japan. For β-catenin and N-cadherin knockdown, 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 were treated with 1 μg/ml tetracycline containing medium. siRNA constructs were transfected into HEK293 cells using Lipofectamine 2000, according to the manufacturer’s instruction. Cells were analyzed 24 h after siRNA transfection. For calcium switch experiments, confluent MEF PS−/− cells were incubated in serum-free medium for 4 h, treated with 4 mM EGTA for 40 min and then switched to serum-free, calcium-containing medium for the times shown (20). For stimulation of N-cadherin cleavage, MEF PS−/− cells were treated by 10 μM iomronyin dissolved in Opti-MEM for 30 min.

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 10-cm dish were washed three times with ice-cold phosphate-buffered saline, and suspended in the solution containing 0.5 mg of Sulfo-NHS-LC-Biotin (Pierce)/ml of phosphate-buffered saline for 30 min. Cells were then washed three times with phosphate-buffered saline and biotinylated proteins were precipitated by 30 μl of streptavidin-agarose (Invitrogen) from equal amounts 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 mutations 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 (Fig. 1).

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FIGURE 1. Cell-surface expression of PS1 is enhanced by the cadherin-mediated cell-cell contact. A-G, APPsw/Ncad-CHO cells were immunostained with anti-N-cadherin (red) and anti-PS1 antibodies (green). Both N-cadherin (A, arrow) and PS1 (B, arrowhead) immunoreactivities were concentrated at the sites of cell-cell contact, showing co-localization (C). D-I, APPSw-CHO cells (D-F) and APPSw/Ncad-CHO cells (G-I) were immunostained with anti-β-catenin (red) and anti-PS1 (green) antibodies. In APPSw-CHO cells, both β-catenin (D, arrow) and PS1 (E, arrowhead) immunoreactivities were localized in the perinuclear region. Conversely, in APPSw/Ncad-CHO cells, both β-catenin (G, arrow) and PS1 (H, arrowhead) immunoreactivities were concentrated at the site of cell-cell contact, showing co-localization (I). J, native CHO cells were transiently transfected with human N-cadherin. 24 h after transfection, cell-surface proteins were biotinylated. Cells lysates were precipitated by streptavidin-agarose and cell-surface proteins were analyzed by Western blot. The amount of cell-surface PS1 and nicastrin were increased in N-cadherin-transfected cells (third lane), compared with those in control GFP-transfected cells (second lane). The first lane is the negative control without biotinylation. K, HEK293 cells, endogenously expressing human N-cadherin and PS1, were transfected with siRNA construct, targeting N-cadherin for 24 h. In N-cadherin and β-catenin siRNA-transfected cells, the amount of cell-surface nicastrin was reduced (third and fourth lanes), compared with that of control cells (second lane), whereas the total amount of nicastrin remained unchanged. β-Catenin knockdown had less impact on reduction of the cell-surface PS1 (third lane), compared with clear reduction of cell-surface PS1 shown by the N-cadherin knockdown (fourth lane). N-cadherin knockdown reduced the amount of β-catenin (fourth lane), whereas β-catenin knockdown did not change the amount of N-cadherin (third lane). The first lane is the negative control without biotinylation.

Cell-surface expression of PS1 (red) and PS1 (green) were examined by immunostaining (Fig. 1). In APPSw-CHO cells, N-cadherin immunoreactivity was more prominent at the sites of cell contact (Fig. 1A). PS1 was also seen at the cell-cell contact sites (Fig. 1B), where it co-localized with N-cadherin (Fig. 1C). The outline of APPSw/Ncad-CHO cells was clearly visualized by PS1 staining (Fig. 1B and C), compared with the perinuclear PS1 staining in Fig. 1E), indicating that N-cadherin expression redistributed the PS1 subcellular localization to the plasma membranes. Next, we compared PS1 and β-catenin distribution in the presence or absence of N-cadherin. In APPSw-CHO cells, β-catenin and PS1 immunoreactivity were mainly seen at the perinuclear area (Fig. 1, D-F). Stable expression of N-cadherin recruited both β-catenin (Fig. 1G and H) and PS1 (Fig. 1H) to cell-cell contact sites (compare with the perinuclear PS1 staining in Fig. 1E), showing co-localization of both proteins (Fig. 1I). Thus, stable expression of N-cadherin led to the redistribution of PS1 to the plasma membrane, especially to 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...
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human N-cadherin into CHO cells and examined the level of cell-surface PS1/γ-secretase by the biotinylation assay (Fig. 1). Transient expression of N-cadherin enhanced both 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 (Fig. 1). We further verified this finding by RNA interference (Fig. 1K) using HEK293 cells, which endogenously express human N-cadherin, β-catenin, and PS1. 24 h after N-cadherin knockdown, cell-surface expression levels of both PS1 and nicastrin were reduced, whereas the total levels of these proteins remained unchanged (Fig. 1K, fourth lane), compared with control (Fig. 1K, second 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 (Fig. 1K, fourth lane). We also tested the effect of β-catenin knockdown on cell-surface expression of γ-secretase components. Interestingly, 24 h after β-catenin knockdown, cell-surface nicastrin was reduced, without changing the cellular level of N-cadherin (Fig. 1K, third lane), although β-catenin knockdown had less impact on cell-surface PS1 distribution compared with N-cadherin knockdown (compare Fig. 1K, third and fourth 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, we constructed deletion mutants of PS1, which lack part of the loop domain (PS1Δ340–375 and Δ340–350, Fig. 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 (Fig. 2B, third lane). The deletion mutants (PS1Δ340–375 and Δ340–350) formed complexes with neither N-cadherin nor β-catenin (Fig. 2B, fourth and fifth lanes). Therefore, amino acids around 340–350 in the PS1 loop domain are crucially important for 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 Fig. 2A, bottom). Because the PS1 loop domain around amino acids 340–350 was essential for N-cadherin/β-catenin interaction (Fig. 2B, fifth 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 S357D PS1) with N-cadherin, as well as β-catenin, was dramatically reduced (Fig. 2B, sixth and seventh lanes), compared with wtPS1 (Fig. 2B, third lane), suggesting that GSK3β-mediated phosphorylation negatively affects the binding of PS1 to N-cadherin/β-catenin. To examine the above findings under PS1-null background, we transiently transfected wild-type, S353D or S357D PS1 into PS1/PS2 double knock-out mouse embryonic fibroblast cells (MEF PS−/− cells) and examined the PS1/N-cadherin/β-catenin complex formation (Fig. 2C). As expected, both S353D (Fig. 2C, third lane) and S357D (Fig. 2C, fourth lane) PS1 had reduced ability to interact with N-cadherin as well as β-catenin, compared with wild-type PS1 (Fig. 2C, second lane).

The above experiments suggested that activation of GSK3β would reduce the PS1/N-cadherin interaction via PS1 phosphorylation in the loop domain. Accordingly, the immunoprecipitation assay revealed that transient transfection of constitutively active GSK3β (S9A mutant) (22) into HEK293 cells reduced PS1/N-cadherin interaction (Fig. 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 (1 μg/ml) induced S9AGSK3β expression 24 h after treatment (Fig. 2E, left). Phosphorylation of β-catenin, a representative target of GSK3β, was increased, whereas the total level of β-catenin was decreased (Fig. 2E, left), indicating that expression of S9AGSK3β enhanced its activity as a phosphokinase, leading to 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 h were immunoprecipitated by anti-phosphoserine antibody, followed by 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 (Fig. 2E, right). Then we examined PS1/N-cadherin interaction, comparing before and 24 h after tet-on. Tetracycline treatment inhibited the PS1/N-cadherin interaction dramatically (Fig. 2F). Collectively, these findings demonstrated that GSK3β activity regulates binding of PS1 to N-cadherin.

PS1/N-cadherin/β-Catenin Complex Formation Is Required for Cell-surface Expression of PS1/γ-Secretase—Because GSK3β activation reduced PS1/N-cadherin interaction, we assumed that 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, PS1Δ340–375 or Δ340–350 was transfected into MEF PS−/− cells, which endogenously express mouse N-cadherin. Transfected cells were fractionated into the plasma membrane and endoplasmic reticulum fractions and then subjected to Western blot analysis. wtPS1 was abundantly found in the plasma membrane fraction, whereas PS1Δ340–375 and Δ340–350 were barely detected (Fig. 3A), indicating that the lack of PS1/N-cadherin/β-catenin interaction negatively affects cell-surface expression of PS1/γ-secretase.

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

GSK3β Activation Down-regulates PI3K/Akt Cell Survival Signaling—It has been reported that N-cadherin-based adhesion initiates PI3K-dependent activation of Akt, thereby up-
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A

| Plasma Membrane Fraction | MEF PS-/- | PS1 NTF | PS1 A340–350 |
|--------------------------|-----------|---------|--------------|
| Control                  | PS1       | PS1 NTF | PS1 A340–350 |

B

| Biotin | MEF PS-/- | Cell Surface Nicastrin | Total Nicastrin | Cell Surface PS1 NTF | Total PS1 NTF | β-actin |
|--------|-----------|------------------------|-----------------|----------------------|----------------|---------|
| -      | PS1       |                         |                 |                      |                |         |
| +      | PS1       |                         |                 |                      |                |         |
| +      | PS1       |                         |                 |                      |                |         |
| +      | PS1       |                         |                 |                      |                |         |

FIGURE 3. PS1-N-cadherin/β-catenin complex formation is required for the cell-surface expression of PS1/γ-secretase. A, either wtPS1 or one of deletion mutants (PS1A340–375 or A340–350) was transfected into MEF PS-/- cells. 24 h after transfection, cells were fractionated into plasma membrane and endoplasmic reticulum (ER) proteins. Samples were analyzed by Western blot using anti-PS1 NTF antibody. The expression of deletion mutants in the plasma membrane fraction (third and fourth lanes, top) was reduced compared with that of wtPS1 (first lane, top), whereas the expression in the endoplasmic reticulum fraction (bottom) was comparable. B, either wtPS1 or one of the pseudo-phosphorylation mutants (S353D or S357D PS1) was transfected into MEF PS-/- cells. Control cells were transfected with GFP (first lane). 24 h after transfection, cell-surface proteins were biotinylated, precipitated by streptavidin-agarose, and analyzed by Western blot. The cell-surface expression of PS1 as well as that of nicastrin (third and fourth lanes, top) was reduced in the cells transfected with pseudo-phosphorylation mutants, compared with that of wtPS1 (second lane), whereas the expression levels of these proteins in the cell lysate were comparable (Total Nicastrin, Total PS1 NTF). The asterisk designates the glycosylated “mature” Nicastrin. The bottom is the loading control, represented by the β-actin bands.

regulating anti-apoptotic protein Bcl-2 (19). PS1 plays an important role in this process by promoting cadherin/π3K association (20), finally activating cell survival signal and inhibiting GSK3β (illustrated in Fig. 6, left). This function of PS1 is independent of γ-secretase activity (20). Because GSK3β-mediated phosphorylation reduces PS1/N-cadherin/β-catenin interaction and cell-surface expression of PS1/γ-secretase, we assumed that GSK3β activation inhibits the function of PS1 to stimulate π3K/Akt signaling.

To test whether GSK3β-mediated phosphorylation affects π3K/Akt signaling mediated by PS1, either wtPS1 or pseudo-phosphorylation mutants (S353D, S357D, or S353D/S357D PS1) were transfected into MEF PS-/- cells and the amount of activated (phosphorylated) Akt was analyzed by Western blot (Fig. 4B). Transfection of wtPS1 (Fig. 4B, second lane) promoted Akt phosphorylation compared with control GFP transfection (Fig. 4B, first lane). The magnitude of Akt phosphorylation after transfection of pseudo-phosphorylation mutants (S353D, S357D, and S353D/S357D) was reduced (Fig. 4B, third to fifth lanes), compared with wtPS1 (Fig. 4B, second lane). Thus, GSK3β-mediated phosphorylation of PS1 inhibits the activation of π3K/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 π3K/Akt signaling is affected by the strength of the cadherin-based adhesion. To test this, a calcium switch assay was performed using MEF PS-/- cells, because cadherins are known to be calcium-dependent cell-cell adhesion molecules. In the assay, MEF PS-/- cells were cultured in the presence of 4 mM EGTA for 40 min for calcium deprivation, after which cells were cultured in the serum-free, calcium containing medium (Fig. 4B, top). Immunostaining results before calcium deprivation showed that N-cadherin immunoreactivity was present along the outline of the cell-cell contact sites (Fig. 4B, bottom, left). After EGTA treatment, N-cadherin immunoreactivity was observed in a granular pattern, indicating disruption of the cadherin-based cell-cell contacts (Fig. 4B, bottom, middle). Calcium supplement restored the linear N-cadherin immunoreactivity at the junction (Fig. 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 π3K/Akt survival signaling. Either wtPS1 or PS1A340–350 was transiently transfected into MEF PS-/- cells, then followed by the calcium switch assay (Fig. 4C). In the presence of wtPS1, Akt phosphorylation before calcium deprivation was prominent (Fig. 4C, wtPS1, Pre), which was diminished after calcium deprivation (Fig. 4C, wtPS1, time 0) and gradually recovered after calcium supplement (Fig. 4C, wtPS1, 30, and 90). Conversely, after PS1A340–350 transfection, Akt phosphorylation before calcium deprivation was not prominently compared with wtPS1 and remained unchanged throughout the assay (Fig. 4C, PS1A340–350), indicating that the PS1/N-cadherin interaction is necessary for the contact-mediated transmission of π3K/Akt survival signaling. Next, we examined the effect of GSK3β activation on transmission of π3K/Akt signaling (Fig. 4D). In the absence of PS1 (Fig. 4D, GFP) or after co-transfection of wtPS1 and S9AGSK3β (Fig. 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 (Fig. 4D, wtPS1). Because GSK3β affects many molecules, we cannot conclude that the effect of S9A GSK3β transfection was solely mediated by PS1 phosphorylation, however, the above data supports the idea that PS1/N-cadherin interaction plays an important role in the transmission of “contact-mediated” π3K/Akt signal activation and that GSK3β-mediated phosphorylation of PS1 may inhibit this process by reducing PS1/N-cadherin interaction.
FIGURE 4. GSK3β activation down-regulates PS3/K/Akt cell survival signaling. A, the ability of pseudo-phosphorylation mutants to facilitate PI3K/Akt signaling was tested by introducing wtPS1 or one of pseudo-phosphorylation mutants (S353D, S357D, or S353D/S357D double mutant PS1) for 24 h, followed by immunoblotting (bottom). Transfection of wtPS1 enhanced Akt phosphorylation (second lane). All the pseudo-phosphorylation mutants had reduced activity to enhance Akt phosphorylation (third to fifth lanes), compared with wtPS1 (second lane). B, time course of the calcium switch assay (top). MEPS1AR cells were treated with 4 mM EGTA for 40 min for calcium deprivation. After calcium deprivation, cells were cultured in calcium containing serum-free medium. The effect of the calcium switch assay on N-cadherin-based cell-cell contact is shown (bottom). Before ETA treatment, N-cadherin in MEF PS1AR was seen at the sites of cell contacts as linear immunoreactivity (bottom, left, arrows). After ETA treatment, N-cadherin concentration at the junction became weak and showed granular immunoreactivity (bottom, middle, arrowheads). 60 min after calcium supplement, N-cadherin concentration at the cell-cell contact sites was restored and appeared as linear structures (bottom, right, arrows). C, either wtPS1 or PS1Δ340–350 was transfected into MEF PS1AR cells. 24 h after transfection, cells were subjected to the calcium switch assay. Under wtPS1 transfection, Akt phosphorylation before calcium deprivation was prominent (wtPS1, Pre), which was diminished after ETA treatment (wtPS1, 0) and gradually recovered after calcium supplement (wtPS1, 30, 90). Conversely, under PS1Δ340–350 transfection, Akt phosphorylation before calcium deprivation was not prominent and remained unchanged throughout the assay (wtPS1Δ340–350, 0). wtPS1 was transfected into MEF PS1AR cells in the presence or absence of S9A GSK3β. Control cells were transfected with GFP. 24 h after transfection, cells were subjected to the calcium switch assay. In the absence of PS1 (GFP) or after co-transfection of wtPS1 and S9AGSK3β (wtPS1 + S9AGSK3β), the phosphorylation state of Akt did not change significantly before and throughout the calcium switch assay. Conversely, wtPS1 transfection led to prominent Akt phosphorylation before the calcium switch assay (wtPS1, Pre), which is diminished after ETA treatment (wtPS1, 0) and gradually recovered after calcium supplement (wtPS1, 30, 60).

and PS1/γ-secretase. Ectodomain shedding by ADAM10 generates a membranous fragment Ncad/CTF1, which is further cleaved by PS1/γ-secretase to produce the cytoplasmic fragment Ncad/CTF2. Immunoblotting by anti-N-cadherin C terminus antibody revealed that the amount of Ncad/CTF1 is reduced after wtPS1 transfection (Fig. 5A, second lane), compared with control GFP transfection (Fig. 5A, first lane), reflecting degradation of Ncad/CTF1 by PS1/γ-secretase. On the contrary, neither pseudo-phosphorylation mutants (S353D and S357D) nor PS1Δ340–350 transfection effectively reduced Ncad/CTF1 (Fig. 5A, third to fifth 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 min and both membrane and cytosolic fractions were subjected to Western blot assay, using anti-N-cadherin C terminus antibody (Fig. 5B). In the membrane fraction, both full-length N-cadherin and Ncad/CTF1 were observed. The amount of Ncad/CTF1 was reduced in the membrane fraction of cells transfected with wtPS1, compared with other cell lines (Fig. 5B, second lane). Ncad/CTF2 production was observed only in the cytoplasmic fraction of cells transfected with wtPS1 (Fig. 5B, second lane), indicating that cleavage of Ncad/CTF1 was impaired in the absence of PS1 (Fig. 5B, first lane) or in the presence of pseudo-phosphorylation mutants (Fig. 5B, third and fourth lane). PS1Δ340–350 also failed to produce Ncad/CTF2, indicating that PS1-N-cadherin binding is important for cleavage of Ncad/CTF1 (Fig. 5B, fifth 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 PS1AR cells together with either wtPS1 or PS1 mutant (Fig. 5C), followed by Western blot analysis, using the anti-APP C terminus antibody. Interestingly, all PS1 constructs equally reduced APP CTFα and -β production (Fig. 5C, second to fifth lanes) compared with control GFP (Fig. 5C, first 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-enhanced 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 to 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 cell-surface expression and its functions of PS1/γ-secretase are regulated by PS1/N-cadherin/β-catenin interaction (Fig. 3). N-cadherin is an essential adhesion molecule for synaptic contact (16), indicating that 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 N-methyl-D-aspartic acid-type agonists.
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FIGURE 5. GSK3β-mediated phosphorylation of PS1 differentially regulates N-cadherin and APP cleavage. A, PS1 constructs (wtPS1, pseudo-phosphorylation mutant (S353D or S357DPS1) or PS1Δ340–350) were transfected into MEF PS−/− cells, 24 h after transfection. Cells were then collected and subjected to immunoblotting using anti-N-cadherin C terminus antibody. The amount of Ncad/CTF1 was reduced after wtPS1 transfection (second lane), compared with control GFP transfection (first lane). Neither pseudo-phosphorylation mutants (S353D, S357D) nor PS1Δ340–350 reduced Ncad/CTF1 (third to fifth lanes). The bottom lane is the loading control, represented by the β-actin bands. B, MEF PS−/− cells were transfected with the PS1 constructs as indicated. Then the ectodomain shedding of N-cadherin was stimulated by ionomycin treatment (10 μM, 30 min). Cells were fractionated into membrane and cytoplasmatic fractions. Protein samples were subjected to Western blot analysis, using anti-N-cadherin C terminus antibody. Ncad/CTF1 were reduced in the membrane fraction of wtPS1-transfected cells, compared with other transfectants (top, second lane), whereas Ncad/CTF2 production in the cytoplasmatic fraction was observed only in wt PS1-transfected cells (bottom, second lane). C, human APP and one of the PS1 constructs (wtPS1, pseudophosphorylation mutant or PS1Δ340–350) were co-transfected into MEF PS−/− cells. 24 h after transfection, cells were collected and subjected to immunoblotting using anti-APP C terminus antibody. All PS1 constructs equally reduced APP CTF1 and -β (second to fifth lanes), compared with control GFP (first lane). The bottom lane is the loading control, represented by the β-actin bands.

FIGURE 6. Schematic presentation of cellular consequences caused by GSK3β-mediated PS1 phosphorylation. When PS1 is not phosphorylated, PS1 associates with N-cadherin/β-catenin at the cell surface. This PS1/N-cadherin association recruits PI3K to the cell surface and PI3K/Akt signaling is activated, resulting in cell survival and GSK3β suppression (20) (left). After GSK3β activation, PS1 is phosphorylated at its loop domain, which leads to reduced PS1/N-cadherin/β-catenin complex formation, resulting in down-regulation of PI3K/Akt signaling. PS1/γ-secretase is not tethered to the cell surface after phosphorylation, which affects the cell survival signal negatively and further activates GSK3β. Inhibition of PS1/N-cadherin association leads to reduced N-cadherin cleavage as well.
the same time, because PI3K/Akt signaling is an important inhibitory mechanism of GSK3β (20), inactivation of this signal could lead to further activation of GSK3β, constituting a vicious circle (Fig. 6, right). Collectively, PS1 may act as a molecular switch that 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 (Fig. 5). PS1-γ-secretase complex is also involved in γ-secretase of various membrane proteins (2–5), however, an important, but an unanswered 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 e-site) cleavage, negatively regulating Aβ production (29). As shown in the present study, e-cleavage of N-cadherin is down-regulated by phosphorylation of PS1, which is in contrast to APP cleavage. Our data clearly demonstrated the cellular mechanism involved in substrate specificity of e-cleavage by PS1/γ-secretase. Whether e-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, cannot be associated with N-cadherin/β-catenin) was created (30). Surprisingly, the homozygous mice exhibit drastically reduced γ-secretase cleavage at the Aβ40, but not the Aβ42, site. In addition, it was reported that inhibition of GSK3α blocked the production of Aβ peptides by interfering with APP cleavage at the γ-secretase step, but did not inhibit Notch processing (31). Thus, although GSK3β-mediated PS1 phosphorylation seems to have less impact on e-cleavage of APP compared with N-cadherin cleavage, whether the phosphorylation could affect γ-cleavage of APP is an important question to be answered.

AD begins with an impairment of memory, which is caused by a disturbance of hippocampal synaptic function (32). In addition, cognitive decline in AD is correlated to the degree of synaptic loss (33), suggesting that the pathological alteration in the metabolism of synaptic protein is primarily involved in AD pathophysiology. It is known that abnormal increases in GSK3β level and activity have been associated with AD pathophysiology (6, 10). Taken together, our studies propose a causal link that may connect abnormal activation of GSK3β to the synaptic dysfunction in the following two ways. 1) Inhibition of cadherin-mediated PI3K/Akt signal transmission, thereby down-regulating cell-survival signaling leading to neurodegeneration. Because synaptic plasticity should involve the process in which certain synapses survive and some others degenerate, dysregulation of this “contact-mediated survival signal” should hinder synaptic plasticity as well. 2) Inhibition of N-cadherin e-cleavage, thereby reducing the production of Ncad/CTF2. Because Ncad/CTF2 carries various signals transmitted from the cell-surface to the nucleus (14, 34), inhibition of Ncad/CTF2 production under abnormal activation of GSK3β should have negative impact on neuronal plasticity or its viability. Interestingly, FAD-linked mutations of PS1 have been shown to inhibit e-cleavage of N-cadherin (14, 34). Thus, from the viewpoint of Ncad/CTF2 production, FAD-linked mutation and phosphorylation of PS1 act in a similar way. According to the previous report, conditional transgenic mice overexpressing GSK3β in the adult brain show decreased nuclear β-catenin, abnormally phosphorylated Tau and clear evidence of neurodegeneration (35), indicating the possibility that altered N-cadherin metabolism or PS1/N-cadherin interaction could lead to neurodegeneration. Whether these mechanisms are actually involved in neurodegeneration in vivo especially in the case of “sporadic” Alzheimer disease should be elucidated in the future study.
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