Direct association of presenilin-1 with β-catenin

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Received 3 June 1998; revised version received 2 July 1998

Abstract Families bearing mutations in the presenilin-1 (PS1) gene develop Alzheimer's disease (AD). However, the mechanism through which PS1 causes AD is unclear. The co-immunoprecipitation with PS1 in transfected COS-7 cells indicates that PS1 directly interacts with endogenous β-catenin, and the interaction requires residues 322–450 of PS1 and 445–676 of β-catenin. Both proteins are co-localized in the endoplasmic reticulum. Over-expression of PS1 reduces the level of cytoplasmic β-catenin, and inhibits β-catenin-T cell factor-regulated transcription. These results indicate that PS1 plays a role as inhibitor of the β-catenin signal, which may be connected with the AD dysfunction.

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Key words: Presenilin-1; β-Catenin; Glycogen synthase kinase 3β; Alzheimer's disease

1. Introduction

Presenilin-1 (PS1) has been cloned as the causal gene in chromosome 14 of familial Alzheimer's disease [1]. Mutations on PS1 cause an early onset form of AD with an autosomal dominant inheritance pattern. Genetic studies show that mutations of PS1 exhibit 100% penetrance in causing AD [2,3]. While the mechanism connecting PS1 with AD is unclear, mutations in presenilins affect amyloid β protein (Aβ) processing. Recent studies indicate that cell lines, transgenic mice or patients expressing mutant forms of PS1 show a selective increase in production of $A\beta_{1-42}$ [4–7]. Mutations in the presenilins also activate apoptotic pathways and render neurons more vulnerable to stressors, such as Aβ neurotoxicity [8–12]. However, the underlying mechanism is still unclear. We recently established that PS1 is associated in vitro and in vivo with GSK-3β and its substrate tau [13]. This suggests that PS1 may act as a molecular tether, connecting GSK-3β with important substrates, much like the kinase binding proteins AKAP and RACK1 modulate activity by controlling the association of protein kinases A and C with their substrates [14,15].

The level of cytosolic β-catenin, which is a component of the Wnt/Wingless signalling pathway, is regulated by GSK-3β, and β-catenin is a substrate for GSK-3β. This makes the connection between PS1 and β-catenin of particular interest.

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Abbreviations: PS1, presenilin 1; AD, Alzheimer's disease; ER, endoplasmic reticulum; Tcf, T cell factor; Aβ, amyloid β protein

2. Materials and methods

2.1. cDNA construct

PS1 mutants (N250, N298, D290–319 and D322–450 and the point mutations) were produced by PCR-based, site-directed mutagenesis using wild type PS1 lacking the VRSQ motif as a template. After subcloning the products into the pCIneo vector (Promega), the sequences were confirmed by sequencing the entire region.

2.2. Antibodies

Polyclonal antibodies N1-12 and hL312 were raised in rabbits against the N-terminal region, amino acid residues 1–12 and the hydrophilic loop domains, amino acids 312–343 of human PS1. The extracted antisera were affinity purified with corresponding peptide-coupled agarose gel columns (Pierce), and the antibodies used for experiments. The monoclonal antibody for β-catenin was obtained from Transduction Lab (Kentucky).

2.3. Transient transfection

COS-7 cells were cultured in DMEM/10% FBS. For transient expression, 5×10^5 cells were plated on 10 cm^2 dishes and grown overnight. Each cDNA (2 μg/ml) was transfected into COS-7 cells using Lipofectamine (4 μl/ml; Gibco BRL), and the cells were harvested after 48 h in TBS (10 mM Tris-HCl (pH 7.4), 150 mM NaCl/1% Triton X-100). The lysates were then centrifuged at 15000 rpm at 4°C. The supernatant was collected and used for immunoprecipitation. For dividing into cytoplasmic and membrane fractions, the cells were homogenized in TBS by teflon homogenizer. The homogenates were then centrifuged at 15000 rpm at 4°C for 30 min, and the supernatant was used as the cytoplasmic fraction. The resulting pellet was lysed in TBS/1% Triton X-100 and the lysate was centrifuged. The supernatant was then used as the membrane fraction.

2.4. Immunoprecipitation

The cell extracts from each transfection were preincubated with protein G Sepharose (Pharmacia) and then incubated with the indicated antibodies at 4°C overnight. The immunocomplexes were precipitated by incubation with protein G Sepharose at room temperature for 2 h, washed 5 times in TBS/1% Triton X-100 and eluted with 0.1 M glycine (pH 2.5). For detection of β-catenin, the immunoprecipitates were eluted by incubation with Laemmli sample buffer at 95°C for 3 min.

2.5. Stable cell line

Each PS1 construct was transfected into human neuroblastoma SH-SY-5Y cells by Lipofectamine (Gibco). The PS1 expressing cells were selected by culture in normal growth medium containing G418. The established cell lines were maintained in normal growth medium within 10 passages and used for experiment.

2.6. Immunocytochemistry

SH-SY cells plated on poly-L-lysine coated slide glasses (Lab Tech) were fixed with 4% paraformaldehyde. The plasma membrane was permeabilized with Tris-buffered saline containing 0.2% Triton X-100 for 5 min. Double immunostaining was performed using anti-PS1 (hL312, 1:500) and anti-β-catenin (1:500) as primary antibodies, and FITC-conjugated rabbit anti-mouse IgG (1:1000) and Cy3-conjugated goat anti-rabbit IgG as secondary antibodies. Dual immunofluorescence images were captured simultaneously using a confocal laser scanning microscope (CLSM, Bio-Rad MRC 1024). Using Voxel

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PII: S0014-5793(98)00886-2

VIew (Vital Image) software on a workstation (SGI, O2), serial optical section images were reconstructed into 3-dimensional (3D) images, where voxel numbers represent relative amounts of different proteins.

2.7. β-Catenin-Tcf-regulated transcription assay

cDNA (2 μg) consisting of 1 μg of hTcf-4-luciferase reporter gene [16] and 1 μg of each PS1 construct or vector was transfected into PC12 cells using Lipofectamine (BRL). After 48 h, cells were lysed in luciferase extraction buffer (40 mM tricine (pH 7.8), 50 mM NaCl, 2 mM EDTA, 1 mM $MgSO_4$, 5 mM DTT, and 1% Triton X-100), and 20 μl of lysate was assayed for luciferase by mixing with assay buffer containing 40 mM tricine (pH 7.8), 10 mM $MgSO_4$, 500 μM EDTA, 10 mM DTT, 500 μM coenzyme A, and 500 μM firefly luciferin. Samples were analyzed on a Turner Designs Luminometer.

3. Results

3.1. Co-localization of PS1 and β-catenin in neuroblastoma cell line SH-SY-5Y cells

To examine the connection between PS1 and β-catenin, we first investigated the localization of both proteins in SH-SY-5Y cells stably overexpressing wild type PS1. Using anti-PS1 and anti-β-catenin antibodies, intracellular 3D co-localization of PS1 and β-catenin was investigated by imaging the two immunostained proteins within a cell. Fig. 1A,B shows the 3D localization of PS1 and β-catenin in SH-SY-5Y cells respectively. As previously indicated [17,18] PS1 is localized in the endoplasmic reticulum (ER)/Golgi apparatus. β-Catenin was detected in peripheral and interior cytoplasm. Co-localization of PS1 and β-catenin was observed in the ER and the proximity of the plasma membrane (Fig. 1C,D). PS1 shared 4.2×10^5 voxels (Fig. 1A), and β-catenin 5.1×10^5 voxels (Fig. 1B). The co-localized area of PS1 and β-catenin was 1.0×10^5 voxels (Fig. 1D). These features of localization were similar in mutant PS1 expressing cells (data not shown).

3.2. Interaction between PS1 and β-catenin in transiently transfected COS-7 cells

To investigate the association of PS1 with β-catenin, we transfected wild type PS1 into COS-7 cells, and examined the distribution of each protein in cell homogenate by classifying the protein populations into cytoplasmic and membrane fractions. In the membrane fraction, the 48 kDa PS1 full length form and the 19 kDa PS1 C-terminal fragment were observed using hL312 (Fig. 2A, lanes 3 and 4), recognizing PS1 residues between 312 and 330. However, no PS1 was observed in the cytoplasmic fraction (Fig. 2A, lanes 1 and 2). PS1 transfection showed an increased level of full length PS1 in the membrane fraction (Fig. 2A, lane 4), which might indicate a lower activity of proteolytic cleavage of full length PS1 in this cell type. Anti-β-catenin antibody indicated the presence of endogenous β-catenin in both cytoplasm and membrane fractions (Fig. 2B, lanes 1–4). We then investigated whether these proteins were associated with each other during immunoprecipitation. β-Catenin was immunoprecipitated using the anti-β-catenin antibody and the subsequent immuno-

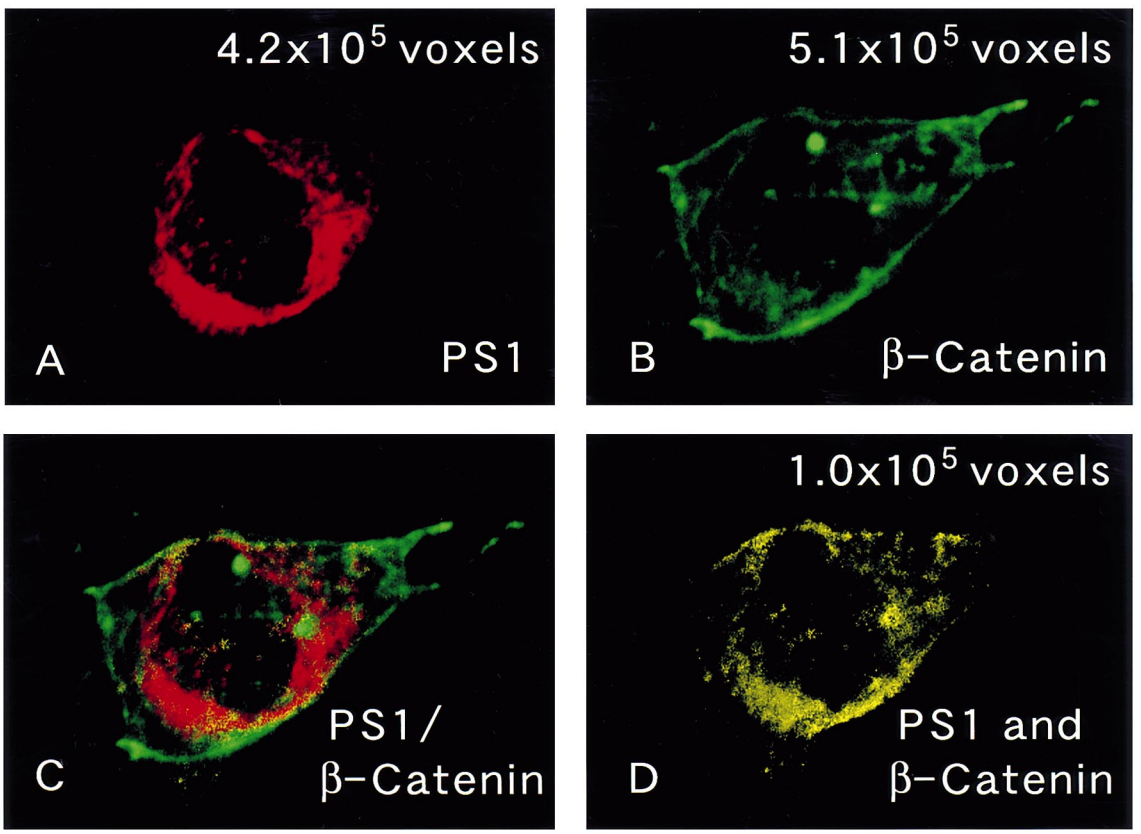

Fig. 1. 3D co-localization of PS1 and β-catenin in the SH-SY-5Y cell. A: PS1, visualized following immunostaining with anti-PS1 and imaging, predominantly localized in endoplasmic reticulum and Golgi apparatus. The PS1 localizing area was 4.2×10^5 voxels as estimated by 3D reconstruction software. B: β-Catenin, visualized following immunostaining with anti-β-catenin and imaging, predominantly localized in peripheral and interior cytoplasm. The localizing area was 5.1×10^5 voxels as estimated by 3D reconstruction software. C: The PS1 image (red) and β-catenin image (green) were merged depicting the co-localizing image (yellow). D: The co-localized area between PS1 and β-catenin was image-processed and estimated to be 1.0×10^5 voxels.

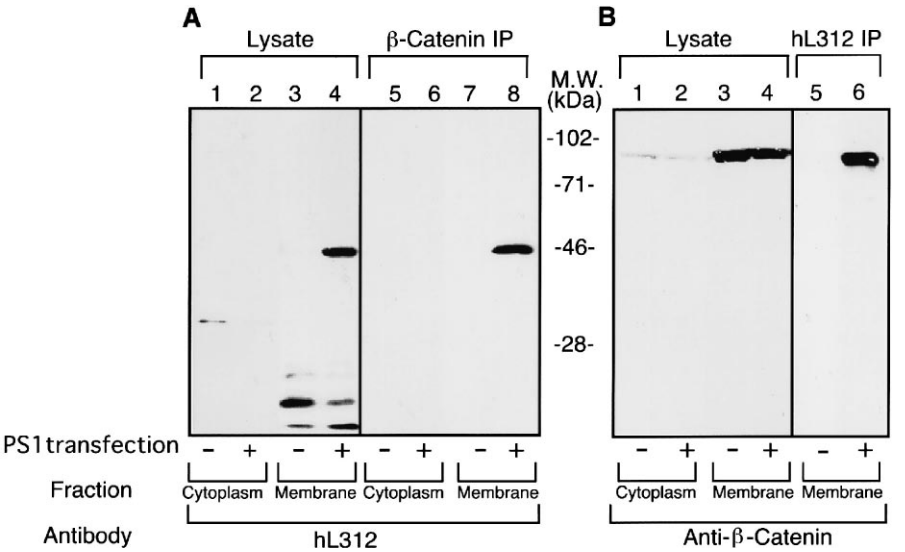

Fig. 2. PS1 associates with β-catenin in COS-7 cells. pCIneo vector and wild human PS1 cDNA construct were transfected into COS-7 cells. Cells were homogenized and separated into cytoplasmic and membrane fractions as described in Section 2. A: PS1 in each lysate (left panel) and immunocomplex using anti-β-catenin antibody (right panel) were probed by anti-PS1 antibody (hL312). PS1 transfection showed the full length form and it co-immunoprecipitated with endogenous β-catenin in the membrane fraction. Cytoplasmic fraction, lanes 1, 2, 5 and 6; membrane fraction, lanes 3, 4, 7 and 8; pCIneo transfection, lanes 1, 3, 5 and 7; wild human PS1 transfection, lane 2, 4, 6 and 8. B: Conversely, β-catenin in each lysate (left panel) and immunocomplex by anti-PS1 antibody (hL312) were probed by anti-β-catenin antibody (right panel). β-Catenin could be detected in both cytoplasmic and membrane fractions, but β-catenin in soluble fraction could not form an immunocomplex with PS1. Cytoplasmic fraction, lanes 1 and 2; membrane fraction, lanes 3, 4, 5 and 6; pCIneo transfection, lanes 1, 3 and 5; wild human PS1 transfection, lanes 2, 4 and 6.

blots were probed using the anti-PS1 antibody, hL312 (Fig. 2A, lanes 5–8). The results indicate the precipitation of 48 kDa full length PS1 in the membrane fraction (Fig. 2A, lanes 7 and 8), but not in the cytoplasmic fraction (Fig. 2A, lanes 5 and 6). Conversely, PS1 was immunoprecipitated using hL312, and then probed by anti-β-catenin antibody (Fig. 2B, lanes 5 and 6). β-Catenin was co-immunoprecipitated with PS1 in the membrane fraction. These results suggest that PS1 and β-catenin are together in the membrane fraction and associate with each other.

3.3. Determination of binding site in PS1 and β-catenin

To determine the binding site in each protein, we generated a number of PS1 and β-catenin cDNA constructs containing varying deletions (Fig. 3A,C). D322–450, N298 and N250 are PS1 constructs that have the C-terminus deleted (Fig. 3A). The D290–319 construct contains a deletion corresponding to exon 9 of the PS1 gene (Fig. 3A). COS-7 cells were transfected with each PS1 construct. The endogenous β-catenin was then immunoprecipitated using the anti-β-catenin antibody, and the subsequent immunoblots were probed using the anti-PS1 antibody, N1-12. Wild type and D290–319 PS1 co-immunoprecipitated with endogenous β-catenin, but D322–450, N298 and N250, with C-terminal deletions, did not. The result indicates that residues 322–450 are necessary for the association of PS1 with β-catenin. Conversely, we produced three β-catenin cDNA constructs containing varying deletions (Fig. 3C). D1–436 is a β-catenin construct in which the first half of β-catenin has been removed. D445–676 is a cDNA construct in which the second half of the repeat has been removed. The wild type PS1 and each of the β-catenin constructs were co-transfected into COS-7 cells. PS1 was then immunoprecipitated using PS1 antibody hL312, and the subsequent immunoblots were probed with the anti-β-catenin antibody. The full length and D1–436 β-catenin co-immunoprecipitated with wild type PS1, but D445–676 did not (Fig. 3D). This finding indicates that the latter half of the repeat in β-catenin (amino acid residues 445–676) is required for the binding of PS1. This suggests that PS1 residues 322–450 are associated directly with β-catenin region 445–676.

3.4. The effects of PS1 on the β-catenin signal

Membrane-associated β-catenin is involved in cell adhesion and its soluble form in Tcf-regulated transcription. The levels of membrane-associated β-catenin were not affected by the expression of PS1 (data not shown). To better understand the physiology of the PS1/β-catenin connection, we investigated the effects of PS1 on β-catenin-Tcf-regulated transcription (CRT) in cells transiently expressing PS1 (Fig. 4A). Wild type PS1 transfection significantly reduced CRT activity by approximately 20% of vector control. In the case of the mutant form of PS1 expression, CRT activity was almost 10% of vector control. Since CRT is regulated by the level of cytoplasmic β-catenin, we investigated the level of cytoplasmic β-catenin after transient transfection of each PS1 in COS-7 cells. The wild type and mutant forms of PS1 expression significantly reduced the level of cytoplasmic β-catenin (Fig. 4B, left panel) without affecting the level of membrane-bound β-catenin (data not shown). This inhibition of cytoplasmic β-catenin by PS1 expression was restored by LiCl treatment (Fig. 4B, right panel), known as an inhibitor of GSK-3β. Our findings suggest that the PS1, GSK-3β, and β-catenin complex inhibits the β-catenin signalling cascade.

4. Discussion

PS1 is known as a causative gene for chromosome 14-linked familial Alzheimer's disease [1], and overexpression of PS1

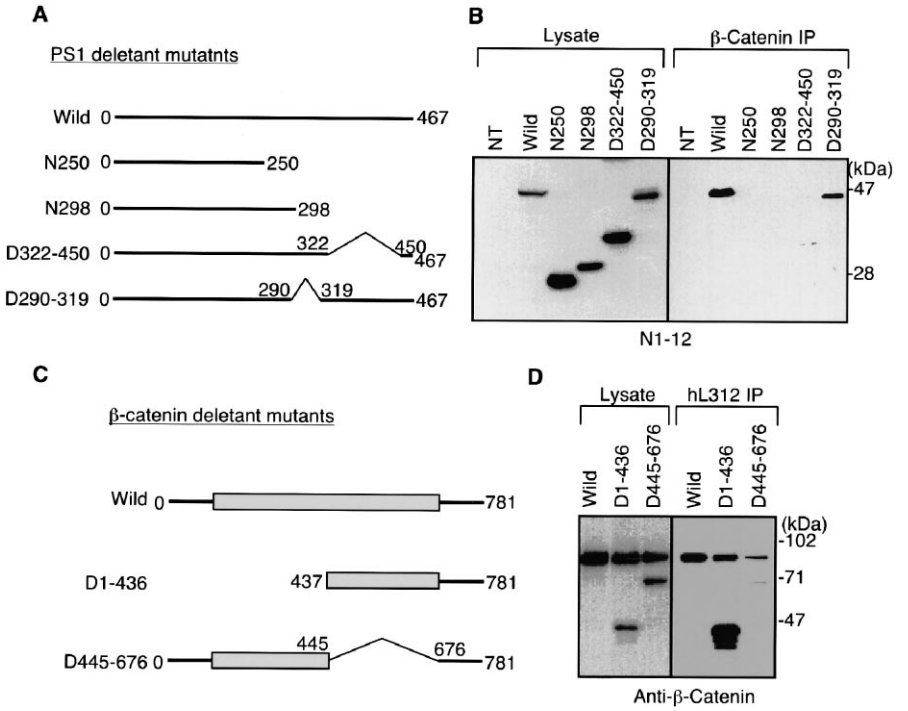

Fig. 3. Analysis of PS1 and β-catenin binding. A: Schematic drawing of PS1 deletion mutants. Wild type PS1 consists of 467 animo acids (wild). The N250 and N298 deletion constructs have the C-terminus of PS1 deleted beyond residues 250 and 298 respectively. The D322–450 and D290–319 constructs contain deletions between residues 322–450 and 290–319, respectively. B: These mutant PS1 constructs were transiently transfected into COS-7 cells. The expression of each PS1 was analyzed using 1/10 volume of total cell lysate (left panel). The rest of each cell lysate was used for immunoprecipitation by the anti-β-catenin antibody, and analyzed for formation of immunocomplexes of β-catenin with PS1 deletant mutants (right panel). Wild and D290–319 co-precipitated with β-catenin, while N250, N298, and D322–450 did not. C: Schematic drawing of β-catenin deletion mutants, lacking residues 1–436 (D1–436) and 445–676 (D445–676) respectively. D: These different forms of β-catenin and human wild type PS1 were co-transfected in COS-7 cells. The expression of each β-catenin was analyzed using 1/10 volume of total cell lysate (left panel). The rest of each cell lysate was used for immunoprecipitation by the anti-PS1 antibody (hL312), and analyzed for the formation of immunocomplexes of PS1 with β-catenin deletion mutants (right panel). D1–436 was recovered in the immunocomplexes with PS1, while D445–676 was not recovered.

mutant enhances Aβ42 secretion in conditioned medium [4–7]. With regard to physiological function, the PS1 null mutant mouse shows loss of normal neurogenesis and axial formation [19,20]. Genetic studies of *Caenorhabditis elegans* suggest that PS1 may be functionally connected with the Notch signalling pathway [21]. Our finding was that PS1 directly binds to β-catenin, and inhibits CRT, which plays an essential role in the Wnt/Wingless signalling pathway [22,23]. The Wingless/Wnt signal interacts with the Notch signal through disheveled (Dsh), suggesting that PS1 may play a role in the signalling pathway of Dsh, which is related to GSK-3β [24], and β-catenin [25]. LiCl treatment restores the inhibitory effect of PS1 on cytoplasmic β-catenin, thus indicating that GSK-3β may account for the inhibitory effect of PS1 on cytoplasmic β-catenin levels. Recently we established what we believe is a direct link between PS1 and GSK-3β. PS1 binds to GSK-3β and its substrate β-catenin, possibly enhancing the phosphorylation of β-catenin, leading to the degradation of β-catenin by the ubiquitin-proteasome cascade [26]. The binding of β-catenin with wild type and mutant PS1 did not show any significant difference (data not shown), but the mutant form of PS1 inhibits CRT more than the wild type. The reason is that mutant PS1 associates more than wild-type with GSK-3β thus increasing kinase activity [13].

Amino acid residues 322–450 of PS1 were required for binding to β-catenin, a region similar to the δ-catenin binding site [27]. GSK-3β associated with PS1 amino acid residues 250–298. In physiological conditions, PS1 is cleaved between 290 and 298, and mainly exists as 28 kDa N- and 19 kDa C-terminal fragments. GSK-3β associates with a region of the N-terminal fragment [13], and β-catenin binds to a region of the C-terminal fragment. Full length PS1 may be required for the interaction of GSK-3β with β-catenin. Some mutant forms of PS1 are resistant to endoproteolytic cleavage [28,29]. However, the possibility cannot be ruled out that the heterodimer of PS1 fragments may induce the interaction of the two proteins.

β-Catenin is known to associate with partner proteins APC [30] or axin [16,31] to regulate its cytoplasm level. The β-catenin signal may be tightly regulated by these partner proteins in response to extracellular stimuli. PS1 is a member of these partner proteins, and is involved in the regulation of the β-catenin signal as well as other partner proteins. Since PS1 is connected with Alzheimer's disease, the β-catenin signal may be connected with the onset of Alzheimer's disease. Alternatively, PS1 may interact with other proteins, and induce neuronal death with formation of amyloid plaques and neurofibrillary tangles.

Acknowledgements: We thank Dr. Michael Miller, RIKEN, for editing the manuscript, Dr. Hans Clevers, University Hospital, Utrecht, for donating the TopFlash (hTCF4-luciferase reporter), and Dr. Akira Nagafuchi, Kyoto University, for the β-catenin cDNA.

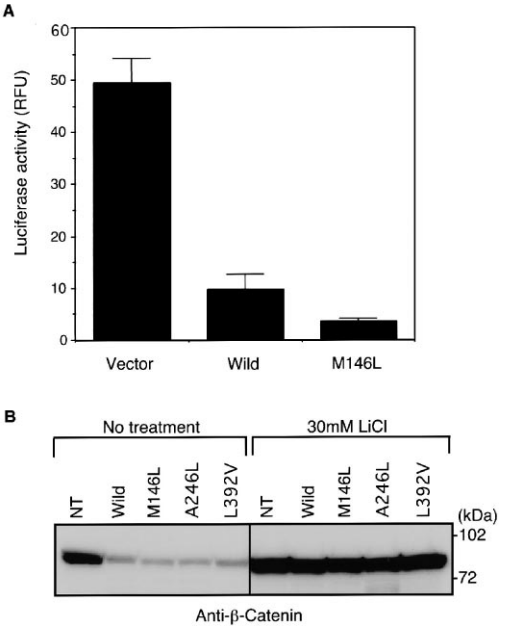

Fig. 4. Effects of PS1 on β-catenin signal. A: β-Catenin-Tcf-regulated transcription (CRT) in transfection assay. Each PS1 construct and pTOPFLASH reporter contained an optimized Tcf-binding site 5′ of a luciferase reporter gene co-transfected into PC12 cells. CRT reporter activity was determined as the luciferase activity by Luminometer, and is expressed as mean ± S.D. ($n = 3$). B: Each PS1 construct was transfected into COS-7 cells and β-catenin in cytoplasmic fractions was examined with and without LiCl (30 mM) treatment. The cytoplasmic β-catenin was reduced by PS1 expression, and restored by LiCl treatment.

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