Novel Role of Presenilins in Maturation and Transport of Integrin $\beta1$[†]

Kun Zou,*[,‡,§,||] Takashi Hosono,[‡] Toshiyuki Nakamura,[‡] Hirohisa Shiraishi,[‡] Tomoji Maeda,[||] Hiroto Komano,[‡,||] Katsuhiko Yanagisawa,[‡] and Makoto Michikawa*[,‡]

Department of Alzheimer's Disease Research, National Institute for Longevity Sciences, NCGG, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan, Japan Society for the Promotion of Science (JSPS), Tokyo 102-8471, Japan, and Department of Neuroscience, Faculty of Pharmaceutical Sciences, Iwate Medical University, 2-1-1 Nishitokuda, Yahaba, Iwate 028-3694, Japan

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ABSTRACT: Presenilins (PSs) play important roles in modulating the trafficking and maturation of several membrane proteins. However, the target membrane proteins whose trafficking and maturation are regulated by PS are largely unknown. By characterizing PS-deficient fibroblasts, we found that integrin $\beta1$ maturation is promoted markedly in PS1 and PS2 double-deficient fibroblasts and moderately in PS1- or PS2-deficient fibroblasts; in contrast, nicastrin maturation is completely inhibited in PS1 and PS2 double-deficient fibroblasts. Subcellular fractionation analysis demonstrated that integrin $\beta1$ maturation is promoted in the Golgi apparatus. The mature integrin $\beta1$ with an increased expression level was delivered to the cell surface, which resulted in an increased cell surface expression level of mature integrin $\beta1$ in PS1 and PS2 double-deficient fibroblasts. PS1 and PS2 double-deficient fibroblasts exhibited an enhanced ability to adhere to culture dishes coated with integrin $\beta1$ ligands, namely, fibronectin and laminin. The inhibition of γ-secretase activity enhances neither integrin $\beta1$ maturation nor the adhesion of wild-type cells. Moreover, PS deficiency also promoted the maturation of integrins $\alpha3$ and $\alpha5$ and the cell surface expression of integrin $\alpha3$. Integrins $\alpha3$ and $\alpha5$ were coimmunoprecipitated with integrin $\beta1$, suggesting the formation of the functional heterodimers integrins $\alpha3\beta1$ and $\alpha5\beta1$. Note that integrin $\beta1$ exhibited features opposite those of nicastrin in terms of maturation and trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus in PS1 and PS2 double-deficient fibroblasts. Our results therefore suggest that PS regulates the maturation of membrane proteins in opposite directions and cell adhesion by modulating integrin maturation.

Mutations in the genes encoding presenilin-1 (PS1[1]) and PS2 account for most cases of familial early onset Alzheimer's disease (FAD) (*1, 2*). PS1 and PS2 most likely provide the catalytic subunit of the γ-secretase complex (*3*). FAD-linked mutant PS proteins increase the level of highly amyloidogenic Aβ42, which is generated by the proteolytic processing of the amyloid precursor protein (APP) and deposited early as senile plaques in the brains of aged individuals and AD patients (*4–6*). PS-mediated cleavage occurs within the transmembrane domain of several type I membrane proteins such as Notch, APP, the APP homologues APLP1 and APLP2, ErbB-4, CD44, N- and E-cadherins, the low-density lipoprotein receptor-related protein (LRP), Syndecan, Delta, Jagged, and Nectin1α (*7*).

PS1 and PS2 may also have other functions, in addition to their central role as catalytic subunits of the γ-secretase complex. Previous studies have shown their involvement in β-catenin turnover, apoptosis, Ca^{2+} homeostasis, and protein trafficking (*8, 9*). PS proteins have also been shown to function as endoplasmic-reticulum (ER)-resident chaperones affecting the maturation of nicastrin (*10–12*), APP (*13–15*), TrkB (*16*), N-cadherin (*17*), and the neurotrophin receptor-like death domain (NRADD) protein (*18*). Nicastrin maturation and cell-surface delivery are completely inhibited in the absence of PS1 and PS2 (*10–12*). PS1 aspartic acid mutants expressed in a PS-null background restore nicastrin maturation but not γ-secretase activity, suggesting a γ-secretase-independent function of PS in the maturation and trafficking of nicastrin (*19*). PS1-null neurons exhibit compromised TrkB maturation (*16*). The transfection of dominant-negative PS1 D385A in SH-SY5Y cells leads to disrupted maturation and a decreased cell-surface expression level of N-cadherin (*17*). In addition, the absence of PS1 and PS2 results in the intracellular retention of caveolin 1, the loss of caveolae (*20*),

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* To whom correspondence should be addressed. Tel: +81 562 46 2311. Fax: +81 562 46 8569. E-mail: kunzou@iwate-med.ac.jp (K.Z.); michi@nils.go.jp (M.M.).

[‡] National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology.

[§] Japan Society for the Promotion of Science (JSPS).

[||] Iwate Medical University.

[1] Abbreviations: PS, presenilin; PS1, presenilin-1; PS2, presenilin-2; wt, wild-type; PS-ko, presenilin-1 and -2 double knockout; Aβ, amyloid β-protein; APP, amyloid precursor protein; PNGase F, peptide: *N*-glycosidase F; ER, endoplasmic reticulum; NTF, N-terminal fragment; CTF, C-terminal fragment.

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and an abnormal accumulation of telencephalin/ICAM in intracellular compartments (*21*). These suggest that PS deficiency disrupts the ER-to-Golgi apparatus trafficking of a set of membrane proteins. In contrast to these membrane proteins, APP exhibits enhanced expression and cell surface accumulation in PS1- and PS2-deficient cells. The expression of dominant-negative PS1 D385A or treatment with a γ-secretase inhibitor, DAPT, also leads to an enhanced cell surface accumulation of APP, via the acceleration of APP trafficking (*13*) or the delay of APP endocytosis (*14*).

We determined whether PS deficiency affects the maturation of other membrane proteins and whether ER-to-Golgi apparatus trafficking is generally disrupted in PS-deficient cells. We examined several type I membrane proteins in PS1 and PS2 double-deficient cells and found that the loss of PS1 and PS2 results in an enhanced maturation of integrin β1 and an enhanced cell-surface delivery of mature integrin β1.

MATERIALS AND METHODS

Cell Culture and Antibodies. Wild-type (wt), PS1- and PS2 double-knockout (PS-ko), PS1-deficient (PS1 −/−), and PS2-deficient (PS2 −/−) mouse embryonic fibroblast (MEF) cell lines were kindly provided by Dr. Bart De Strooper (*22*). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS). The cells were lysed in RIPA buffer [10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 0.2% sodium deoxycholate, containing a protease inhibitor cocktail (Roche, Mannheim, Germany)] at a point before or after confluence. Monoclonal antibodies against integrins β1, α3, αV, syntaxin 6, BiP/GRP78, and calnexin were obtained from BD Biosciences (San Jose, CA). Polyclonal antibodies against integrins β1, α1, α2, α4, α5, α6, α7, αL, and the N-terminus of PS1 (H-70) were from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody against the loop of PS1 (MAB5232), which recognizes the C-terminal fragment of PS1, was purchased from Chemicon (Temecula, CA). A polyclonal antinicastrin antibody raised against the C-terminus of nicastrin (amino acids 693–709) was purchased from Sigma (Saint Louis, MO).

Electrophoresis, Immunoblotting, Deglycosylation, and Immunoprecipitation. Total protein (50 μg) from cell lysates was dissolved in SDS sample buffer, separated on 4–20% gradient gels, and transferred to nitrocellulose membranes (equal loading was confirmed by Western blotting for Bip/GRP78 or α-tubulin). The target proteins were visualized using SuperSignal (Pierce, Rockford, IL) with antibodies to integrins, nicastrin, Bip/GRP78, calnexin, syntaxin 6 and PS1. To assess integrin β1 maturation and nicastrin glycosylation, lysates from the wt and PS-ko cells were treated with PNGase F, *O*-glycanase, or sialidase A using an enzymatic deglycosylation kit according to the manufacturer's instructions (PROzyme, San Leandro, CA). For immunoprecipitation, the cells were homogenized in a solution of 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, and 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) containing a protease inhibitor cocktail, and the homogenate was centrifuged at 10,000*g* and 4 °C for 10 min. The supernatant was immunoprecipitated with a polyclonal antibody to integrin β1 or PS1 and protein G sepharose (Amersham Biosciences, Uppsala, Sweden). Coimmunoprecipitated PS1, nicastrin, integrin β1, and integrin α subunits were detected by Western blotting.

Subcellular Fractionation on Iodixanol Gradient. The wt and PS-ko cells were grown in eight 10-cm tissue culture dishes, and subcellular fractionation was performed as previously described (*23*). They were homogenized in an ice-cold homogenization buffer [10 mM HEPES (pH 7.4), 1 mM EDTA, and 0.25 M sucrose containing a protease inhibitor cocktail]. The postnuclear supernatant was centrifuged for 1 h at 4 °C and 65,000*g*. The resultant vesicle pellets were rehomogenized in 0.8 mL of the homogenization buffer and layered on a step gradient consisting of 1 mL of 2.5%, 2 mL of 5%, 2 mL of 7.5%, 2 mL of 10%, 0.5 mL of 12.5%, 2 mL of 15%, 0.5 mL of 17.5%, 0.5 mL of 20%, and 0.3 mL of 30% (v/v) iodixanol (GIBCO). After centrifugation at 90,000*g* (SW41 rotor, Beckman) for 2.5 h at 4 °C, 11 fractions were collected from the top of the gradient.

Transfection, γ-Secretase Inhibitors Treatment and Aβ ELISA. The retrovirus-mediated gene expression of human APP695, PS1, PS2, PS1D257A, PS1D385A, PS1Δ*E* 9, PS1I143F, PS1R278K, and PS1L392V was carried out as previously described (*24*). The fibroblasts were transfected at 10% confluence and maintained in DMEM containing 10% fetal calf serum. The transfection efficiency was nearly 100% in this study, as estimated by the control transfection of the pMX-green fluorescent protein (pMX-GFP). γ-secretase inhibitors, namely, DAPT and L-685,458, were added to the wt cells stably expressing hAPP695 immediately after passage. The culture medium was collected two days after confluence, and the level of Aβ1-40 secreted was measured using an Aβ ELISA kit (Wako Pure Chemical, Osaka, Japan).

Cell Surface Biotinylation and Cell Surface Uptake of Integrin β1. Cell surface biotinylation was carried out using a Pinpoint cell surface protein isolation kit (Pierce). The wt and PS-ko cells were grown in four 10-cm tissue culture dishes, and washed twice with ice-cold PBS (GIBCO). The cells were incubated in 10 mL of ice-cold 0.25 mg/mL sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-Biotin) (Pierce) in ice-cold PBS for 30 min at 4 °C. Then, 500 μL of the quenching solution was added to each dish to quench the reaction. The cells were scraped and washed twice with Tris-buffered saline (TBS) [10 mM Tris/HCl (pH 7.5) and 150 mM NaCl] and lysed in the lysis buffer containing protease inhibitors. Each lysate was incubated with streptavidin-agarose beads (Pierce) at 4 °C for 60 min, and captured proteins were eluted with 50 mM DTT in Laemmli's SDS sample buffer. To assess cell surface integrin β1 internalization, immunostaining was performed as previously reported (*14*). The cells plated on a fibronectin-coated culture slide were washed in ice-cold PBS, and incubated on ice with a monoclonal antibody against integrin β1 at 1:200 dilution in PBS containing 0.1% BSA. After 20 min, the cells were washed with ice-cold PBS, and then incubated in prewarmed culture medium for various durations at 37 °C. After the indicated durations, the cells in the culture slides were fixed with 4% paraformadehyde in PBS for 20 min. After rinsing three times in PBS, permeabilization was achieved in 0.1% Triton X-100/PBS for 5 min, and the slides were incubated with rhodamine-coupled goat antimouse IgG

(Chemicon) for 20 min. Confocal images were taken with a Zeiss LSM 510 confocal system (Carl Zeiss, Jena, Germany).

Cell Attachment Assay. Ninety-six- and 6-well plates (Corning Inc., Corning, NY) were coated with 10 μg/mL fibronectin or laminin (Sigma) for 8 h at 4 °C. After aspirating the coating reagent, 0.2 or 1.5 mL of 10 mg/mL filtered, heat-denatured bovine serum albumin (BSA) (Sigma) was dispensed into the wells, and the plates were incubated at 4 °C for 16 h. The cell attachment assay using single resuspended cells was carried out in 96-well plates as previously reported (*25*). Subconfluent cells were washed with HEPES-buffered saline [HBS, 150 mM NaCl, and 25 mM HEPES (pH 7.5)] and resuspended at a density of 0.2 to 1 $\times$ 10^6 cells/mL. A 50-μL aliquot of the cell suspension was then added to each well. The plates were incubated for 30 min at 37 °C in 5% (v/v) CO_2. Unbound or loosely bound cells were removed by aspiration and gentle washing with HBS. To assess the total number of cells added, 100%, 75%, 50%, 25%, and 0% cells were added to the wells and fixed by adding 1/10 vol of 50% (v/v) glutaraldehyde. To assess the attachment strength of the wt and PS-ko cells after the cells reached confluence, the cell attachment assay was performed in 6-well plates. After aspirating the conditioning medium, the cells were incubated in 1 mM EDTA for 30 min at room temperature. Detached cells were washed out with HBS. The cells were fixed in the wells by adding 5% (v/v) glutaraldehyde in HBS and stained with 0.1% (w/v) crystal violet in 200 mM MES (pH 6.0) for 60 min. The solution in the wells was then aspirated, and the wells were washed with water. Acetic acid [10% (v/v)] was dispensed into the wells of the 96-well plates, and the absorbance at 570 nm of each well was measured with a multiscan plate reader. Images of the 6-well plates were taken when the wells were dried, and the area of the wells occupied by adherent cells was measured using Image J 1.36b software (NIH, Bethesda, MD).

RESULTS

Integrin β1 is synthesized as an 87-kDa polypeptide that undergoes glycosylation in ER and the Golgi apparatus. In ER, the most prevalent, incompletely glycosylated immature integrin β1 has a mass of 105 kDa. The mature form of this 105 kDa integrin β1 has a mass of 125 kD (*26, 27*). The immature form is not found on the cell surface and has no role in cell adhesion or cell signaling (*26, 28*). These features of integrin β1 in terms of maturation are similar to those of nicastrin, a member of the γ-secretase complex. To determine how the absence of PS affects integrin β1 maturation, we performed a Western blotting of cell lysates prepared from wt, PS-ko, PS1(−/−), and PS2(−/−) fibroblasts. In the wt cells, integrin β1 was detected as a nonglycosylated core protein (87 kDa), a poorly glycosylated immature protein (105 kDa), and a highly glycosylated mature protein (125 kDa). Most of these integrin β1 isoforms are of the immature form. Interestingly, a marked increase in the expression level of mature integrin β1 and a decrease in that of ER-localized immature integrin β1 were observed in the PS-ko cells, suggesting that PS proteins exert an inhibitory effect on the post-translational maturation of integrin β1 (Figure 1A). The PS1(−/−) or PS2(−/−) cells exhibited an intermediate increase in the expression level of mature integrin β1 and a decrease in that of immature integrin β1, suggesting that the regulation of the post-translational maturation of integrin β1 is PS-dependent, not cell-line-dependent (Figure 1A). The PS-ko cells showed a level ratio of mature integrin β1 to immature integrin β1 18-fold higher than that of the wt cells. The PS1(−/−) cells showed a 4-fold increase in the level ratio of mature integrin β1 to immature integrin β1 (Figure 1C). In contrast to integrin β1, the 125-kDa mature nicastrin species showed a significant decrease in expression level (Figure 1B), in agreement with previous reports (*10–12*). These results suggest that PS regulates the maturation of membrane proteins in opposite directions.

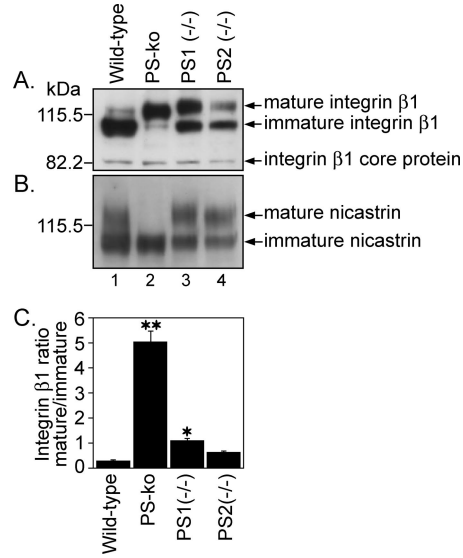

FIGURE 1: Presenilin deficiency promotes integrin β1 maturation and inhibits nicastrin maturation. The wt, PS-ko, PS1(−/−), and PS2(−/−) fibroblasts were lysed in RIPA buffer 2 days after reaching confluence. Western blots of 50 μg of total protein from the cells were probed with an anti-integrin β1 monoclonal antibody (A) or antinicastrin polyclonal antibody (B). Three isoforms of integrin β1 were observed in the cells: the ~87-kDa core protein, ~105-kDa immature glycosylated form, and ~125-kDa mature glycosylated form (indicated by arrows). Note that the predominant isoform of integrin β1 in the wt fibroblasts was the immature form and that in the PS-ko fibroblasts was the mature form. For nicastrin, the immature form of ~105 kDa and the mature form of ~125 kDa were observed in the wt fibroblasts. The mature form of nicastrin was absent in PS-ko fibroblasts. The expression level ratio of mature integrin β1 to immature integrin β1 was determined by densitometry (C). Data represent the means ± SEM; $n = 3$, $*p < 0.05$, $**p < 0.001$, PS-ko or PS1(−/−) vs wt, Bonferroni/Dunn test.

To determine whether the mature integrin β1 with an increased expression level is glycosylated in PS-ko cells as it is in wt cells, we experimentally examined the glycosylation of mature integrin β1. Similar to nicastrin, both immature and mature integrin β1s were sensitive to PNGase F. Digestion with PNGase F decreased the apparent size of mature and immature integrin β1s to 95 kDa as a major species in the wt cells (Figure 2A, lane 5). Integrin β1 in the PS-ko cells was partially resistant to PNGase F. In addition to the 95-kDa species, a 105-kDa species was detected after digestion with PNGase F. This PNGase F-resistant 105-kDa integrin β1 was likely generated from the 125-kDa mature integrin β1 because of its abundance in the PS-ko cells and scarcity in the wt cells (Figure 2A, lane 6). However, the wt cells showed a 105-kDa nicastrin PNGase F-resistant species in addition to the 70-kDa

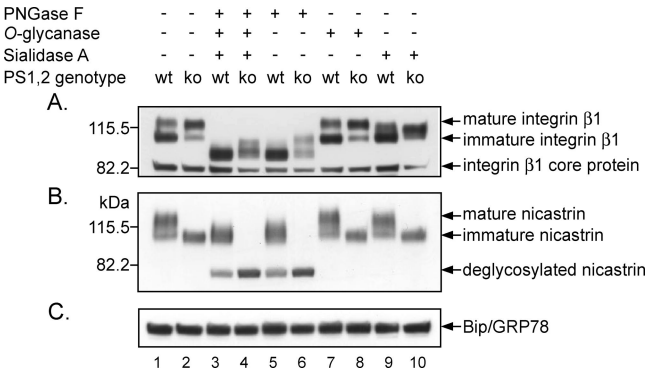

FIGURE 2: Deglycosylation of integrin β1 and nicastrin. Total protein (50 μg) from the wt or PS-ko fibroblast lysate was digested with PNGase F (0.1 U/mL), *O*-glycanase (0.025 U/mL), or sialidase A (0.1 U/mL) and analyzed by SDS−PAGE and immunoblotting. Western blots were probed with an anti-integrin β1 monoclonal (A), antinicastrin polyclonal (B), or anti-Bip/GRP78 (C) antibody. Lanes 1 and 2, no treatment; lanes 3 and 4, PNGase F, *O*-glycanase, and sialidase A treatment; lanes 5 and 6, PNGase F treatment; lanes 7 and 8, *O*-glycanase treatment; and lanes 9 and 10, sialidase A treatment. The mature form of integrin β1 in the PS-ko cells and the mature form of nicastrin in the wt cells are partially PNGase F-resistant. Equal amounts of protein loaded are shown by the Western blot of Bip/GRP78.

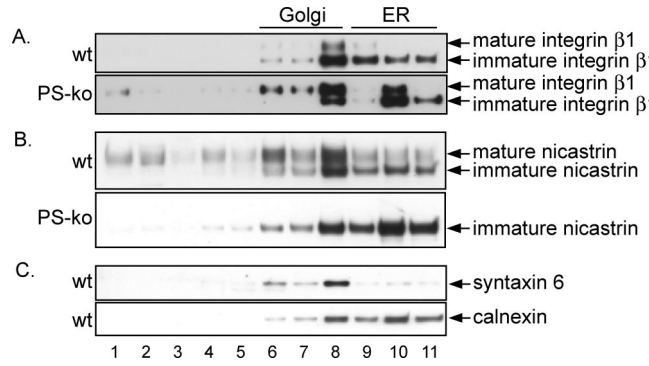

FIGURE 3: Absence of PS1 and PS2 enhances integrin β1 maturation in the Golgi apparatus and has no effect on the intracellular distribution of integrin β1. The wt and PS-ko fibroblasts were harvested and fractionated on iodixanol gradients. Fractions rich in ER (lanes 9–11) are at the bottom, and 11 fractions were collected from top to bottom. Golgi-apparatus-rich fractions are shown in lanes 6–8. The fractions were analyzed by immublotting with an anti-integrin β1 antibody (A), an antinicastrin antibody (B), and antibodies to the Golgi apparatus marker protein syntaxin-6 and ER marker protein calnexin (C). Note that nicastrin was retained in the ER fractions of the PS-ko cells, but not integrin β1. The maturation of integrin β1 in PS-ko fibroblasts in the Golgi apparatus was markedly accelerated compared with that in wt fibroblasts.

deglycosylated species; the PS-ko cells showed that the apparent size of the 105-kDa immature nicastrin as a single species decreased to 70 kDa (Figure 2B, lanes 1, 2, 5, and 6). Treatment with *O*-glycanase had no effect on the SDS−PAGE mobility of either integrin β1 or nicastrin, indicating the absence of an *O*-linked glycosylation of these two proteins (Figure 2A and B, lanes 7 and 8). The mature forms, not the immature forms of integrin β1 and nicastrin were sensitive to sialidase A digestion, indicating the sialylation of the mature forms of both proteins (Figure 2A and B, lanes 9 and 10). The ER protein Bip/GRP78 served as the internal control protein, which indicated the same amount of protein loaded in each lane (Figure 2C). These results show that (i) in contrast to that of nicastrin, whose mature form is absent in PS-ko cells, the maturation of integrin β1 in PS-ko cells is enhanced compared with that in wt cells and that (ii) mature integrin β1 in PS-ko cells is normally glycosylated by *N*-glycans, the characteristics of which are similar to those of *N*-glycans in mature nicastrin in wt cells.

The results described above demonstrate that the absence of PS1 and PS2 promotes integrin β1 maturation. We also confirmed that the absence of PS1 and PS2 inhibits nicastrin maturation, as shown by previous studies. To investigate how the absence of PS1 and PS2 disrupts the processing and intracellular distribution of integrin β1 and nicastrin, we carried out iodixanol gradient fractionation to separate the Golgi apparatus and ER-derived membranes (*23*). Syntaxin 6 and calnexin in the wt cells served as the Golgi apparatus and ER markers, respectively (Figure 3C). The distributions of syntaxin 6 and calnexin in PS-ko cells did not differ from those in wt cells (data not shown). In the wt cells, integrin β1 and nicastrin underwent normal maturation and ER-to-Golgi apparatus trafficking, with most of their immature forms localizing in the ER, and most of their mature forms localizing in the Golgi apparatus. The mature and immature forms of nicastrin showed patterns similar to those of the mature and immature forms of integrin β1. In contrast, in the PS-ko cells, mature forms of nicastrin were absent, and most of the immature forms of nicastrin were restricted in the ER fractions, suggesting the disrupted exit of nicastrin from ER. Interestingly, in the PS-ko cells, the expression level of mature integrin β1 increased predominantly in the Golgi apparatus, whereas that of immature integrin β1 significantly decreased in the Golgi apparatus, as compared with those in the wt cells, indicating that the maturation of integrin β1 in the Golgi apparatus is enhanced. In addition, there was no selective retention of integrin β1 in the ER in the PS-ko cells. These results suggest that the trafficking of immature integrin β1 from ER to the Golgi apparatus is accelerated in PS-ko cells (Figure 3A and B).

Because PS1 and PS2 can regulate cell signaling pathways via γ-secretase activity (*29*), we determined whether the blockade of γ-cleavage leads to an enhanced maturation of integrin β1. We treated hAPP-transfected wt cells with two major γ-secretase-specific inhibitors, namely, DAPT and L-685,458, to inhibit γ-secretase activity and monitored the level of Aβ1-40 secreted in the culture medium to evaluate γ-secretase activity (Figure 4). γ-secretase inhibitors at a concentration higher than 2.5 μM completely inhibited γ-secretase activity but did not facilitate integrin β1 maturation, indicating that the inhibition of γ-secretase activity is not sufficient to facilitate integrin β1 maturation (Figure 4 A and B). In Figure 1, we show that the expression level of mature integrin β1 was enhanced in three independent PS-deficient cell lines, namely, PS-ko, PS1(−/−), and PS2(−/−). The expression level of mature integrin β1 inversely correlated with the expression level of PS in these three independent cell lines. The transfection of PS-ko cells with human PS1 and PS2 restored the normal expression of mature nicastrin and inhibited the maturation of integrin β1, suggesting that PS is essential for post-translational maturation of nicastrin and for inhibiting the maturation of integrin β1 (Figure 4C). The transfection with PS1 aspartate mutants lacking γ-secretase activity, namely, PS1 D257A and PS1 D385A, did not restore the expression of mature integrin β1, whereas it restored the normal expression of mature

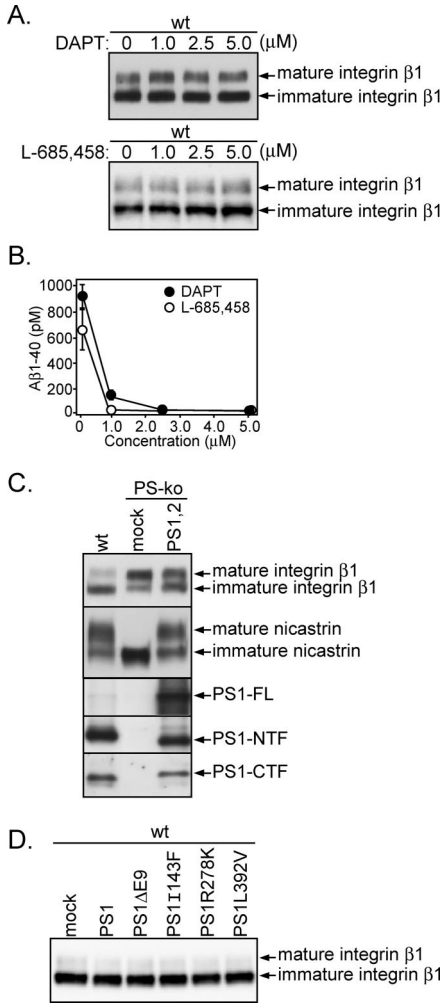

FIGURE 4: Effects of γ-secretase inhibitors and FAD PS mutants on integrin β1 maturation and restoration of integrin β1 and nicastrin maturation by transfection with PS1 and PS2. The wt fibroblasts stably expressing human APP695 were treated with or without DAPT or L-685,458 immediately after passage. The cells were lysed after reaching confluence, and the lysate was analyzed by immunoblotting with an anti-integrin β1 antibody (A). The level of Aβ1-40 secreted to the culture medium was measured using an Aβ1-40 ELISA kit (Wako) (B). γ-secretase inhibitors at a concentration greater than 2.5 μM completely inhibited γ-secretase activity, which was monitored by analyzing Aβ1-40 secretion; however, integrin β1 maturation remained unchanged. The PS-ko fibroblasts were transfected with human PS1 and PS2. Western blots of 50 μg of total protein from the transfected PS-ko fibroblasts were probed with an anti-integrin β1 antibody, an antinicastrin antibody, and anti-PS1 antibodies (C). Double transfection of PS-ko fibroblasts with human PS1 and PS2 restored the maturation of integrin β1 and nicastrin. The wt fibroblasts were transfected with human PS1 and FAD PS1 mutants, and integrin β1 maturation remained unchanged (D). FL, full-length; NTF, N-terminal fragment; CTF, C-terminal fragment.

nicastrin (data not shown). Because PS1 aspartate mutants do not form the mature, high molecular weight PS complexes (*30*), these results suggest that the formation of the high molecular weight PS complex may be required to inhibit integrin β1 maturation. The expression of transfected PS1 was confirmed by Western blotting. The exogenous human PS1 in PS-ko cells was maintained in larger amounts of the full-length form and smaller amounts of the N-terminal fragment (NTF) and C-terminal fragment (CTF) than those of endogenous mouse PS1 in wt cells. Interestingly, the human PS1-NTF in the PS-ko cells showed a lower molecular weight than the mouse PS1-NTF in the wt cells, whereas the full-length human and mouse PS1s showed the same molecular weight. In agreement with this result, the human PS1-CTF in the PS-ko cells showed a higher molecular weight than the mouse PS1-CTF in the wt cells (Figure 4C, bottom three panels). These results suggest that mouse and human PS1s may undergo principal endoproteolytic cleavage at different sites or that presenilinase cleaves PS1 at different sites in wt and PS-ko cells. We also examined whether the overexpression of the PS1 mutants of familial Alzheimer's disease (FAD) alters integrin β1 maturation in wt cells. The overexpression of human wt PS1 and FAD PS1 mutants, namely, PS1ΔE 9, PS1I143F, PS1R278K, and PS1L392V, did not affect integrin β1 maturation in the wt cells, suggesting that the loss of PS function, probably the loss of both the γ-secretase and the chaperone protein functions of PS, may facilitate integrin β1 maturation (Figure 4D).

Integrin β1 associates with multiple integrin α-subunits to form transmembrane receptors of extracellular matrix proteins, including fibronectin, collagen, and laminin (*31–33*). To determine whether mature integrin β1 with an increased expression level in PS-ko cells is delivered to the cell surface, we determined the expression level of integrin β1 on the cell surface by surface biotinylation. Neither immature integrin β1 nor immature nicastrin was biotinylated in the wt or PS-ko cells, indicating that no immature forms of the two proteins localize on the cell surface (Figure 5A and B). The expression level of surface-biotinylated mature integrin β1 in the PS-ko cells significantly increased compared with that in the wt cells, indicating that the cell-surface delivery of integrin β1 is enhanced in PS-ko cells (Figure 5A, lanes 3 and 6). The expression level of integrin β1 on the surface of the PS-ko cells was 2.5-fold that on the surface of the wt cells (Figure 5C). In contrast to integrin β1, surface-biotinylated nicastrin was detected in the wt cells; however, no apparent signal of this protein was detected in the PS-ko cells, indicating that the cell-surface delivery of nicastrin is impaired in PS-ko cells (Figure 5B, lanes 3 and 6). Because the increased surface expression level of mature integrin β1 can be induced by delayed internalization and accelerated trafficking to the cell surface, we investigated the internalization of mature integrin β1 in living wt and PS-ko cells. The cells were labeled on ice with an antibody that recognizes integrin β1, washed, and incubated at 37 °C to initiate internalization. At the indicated time points, the cells were fixed, permeabilized and processed for immunofluorescence staining. The PS-ko cells had a large amount of surface-labeled integrin β1 in the absence of incubation at 37 °C (Figure 5D), which was consistent with the results of biotinylation. After 10 min of incubation at 37 °C, surface integrin β1 was partially internalized, and after 30 min, surface integrin β1 disappeared and was completely internalized in both the wt and PS-ko cells, indicating that PS deficiency has no effect on integrin β1 internalization. Combined with the results of the iodixanol gradient fractionation, these results suggest that the increased cell-surface expression level of mature integrin β1 is induced by the accelerated trafficking of integrin β1 from ER to the Golgi apparatus and then to the cell surface.

To determine whether an increased cell-surface expression level of mature integrin β1 has the same effect in PS-ko cells as in wt cells, we performed a cell attachment assay to

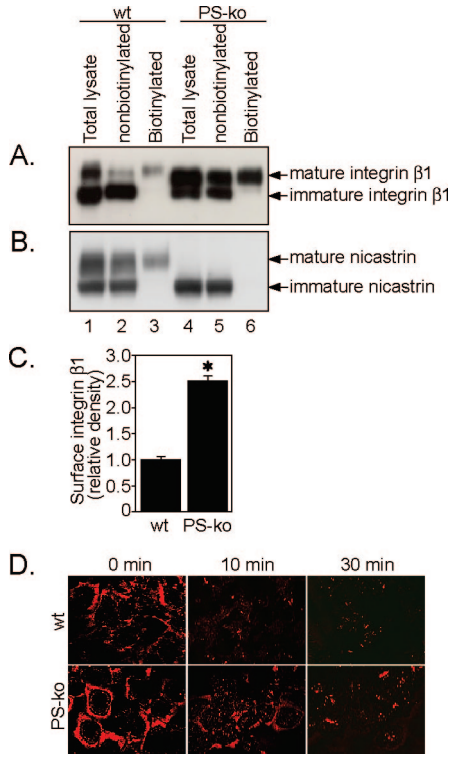

FIGURE 5: Presenilin deficiency promotes integrin β1 cell surface delivery and has no effect on integrin β1 internalization. Lysates of surface-biotinylated wt and PS-ko cells were incubated with streptavidin-agarose. Total lysate (lanes 1 and 4), nonbiotinylated (streptavidin-agarose nonbound, lanes 2 and 5), and biotinylated proteins (streptavidin-agarose bound, lanes 3 and 6) were analyzed by immunoblotting with antibodies against integrin β1 (A) and nicastrin (B). Only mature integrin β1 and nicastrin were delivered to the cell surface. The cell surface expression level of integrin β1 significantly increased in the PS-ko cells, and the relative density of integrin β1 on the cell surface was calculated (C). Data represent the means ± SEM; $n = 3$, $^*p < 0.001$, Bonferroni/Dunn test. Living wt and PS-ko cells were labeled on ice with an antibody to integrin β1, washed, and incubated at 37 °C to initiate internalization. After 0, 10, or 30 min of incubation at 37 °C, the cells were permeabilized and stained with rhodamine-coupled goat antimouse IgG. Confocal images were taken with a Zeiss LSM 510 confocal system (D).

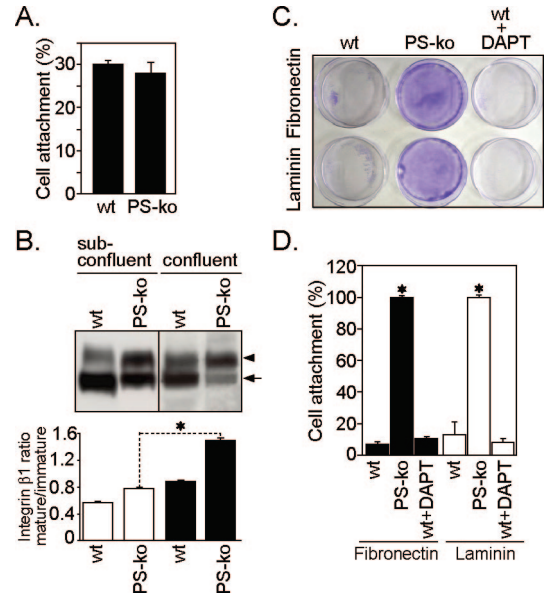

FIGURE 6: Enhanced cell adhesion to integrin β1 ligands, fibronectin and laminin in confluent PS-ko cells. The single wt and PS-ko fibroblasts prepared from subconfluent cultures were plated on fibronectin-coated 96-well plates. The plates were incubated for 30 min at 37 °C in 5% (v/v) CO_2. Unbound or loosely bound cells were removed by aspiration and gentle washing in HBS. The bound cells were fixed and stained. The percentage of bound cells was measured and calculated (A). Lysates of the subconfluent or confluent wt and PS-ko fibroblasts were subjected to SDS−PAGE and Western blotting. The confluent PS-ko fibroblasts showed enhanced integrin β1 maturation compared with the subconfluent PS-ko fibroblasts. Arrowhead, mature integrin β1; arrow, immature integrin β1; □, subconfluent fibroblasts; ■, confluent fibroblasts. Data represent the means ± SEM; $n = 3$, $^*p < 0.001$, confluent PS-ko cells vs subconfluent PS-ko cells, Bonferroni/Dunn test (B). The wt and PS-ko fibroblasts were plated on fibronectin- or laminin-coated 6-well plates with or without 5 μM DAPT after reaching confluence, and adhesion ability was estimated. The cells were incubated with 1 mM EDTA for 30 min at room temperature. Detached cells were washed out with HBS buffer, and attached cells were stained with 0.1% (w/v) crystal violet (C). The percentage area occupied by the attached cells was measured. ■, fibronectin-coated; □, laminin-coated. Data represent the means ± SEM; $n = 3$, $^*p < 0.001$, PS-ko cells vs wt or wt + DAPT cells, Bonferroni/Dunn test (D).

measure the ability of cell adhesion to integrin-β1-ligand-coated dishes. Resuspended wt and PS-ko single cells prepared from subconfluent cultures showed similar attachment strengths to fibronectin-coated dishes (Figure 6A). In their subconfluent states, the PS-ko cells showed a moderate increase in the expression level of mature integrin β1 compared with the wt cells. However, in their confluent states, the new PS-ko cells showed a significantly increased expression level of mature integrin β1, with the expression level ratio of mature integrin β1/immature integrin β1 increasing 2-fold that in the subconfluent PS-ko cells (Figure 6B). We then examined whether PS-ko cells exhibit stronger attachment in their confluent states. The wt and PS-ko cells were plated on fibronectin- or laminin-coated 6-well dishes and cultured until the cells reached confluence. After treatment with EDTA, detached cells were washed out, and adhering cells were stained with crystal violet. The PS-ko cells exhibited a significantly stronger attachment to the fibronectin- or laminin-coated dishes than the wt cells. DAPT, a γ-secretase inhibitor, did not enhance the adhesion of the wt cells, indicating that the stronger attachment of the PS-ko cells to fibronectin- and laminin-coated dishes was a γ-secretase-independent effect (Figure 6C and D). These results suggest that cell-surface-mature integrin β1 with an increased expression level in PS-ko cells works as a receptor of fibronectin and laminin and that this increased expression level induces a strong adhesion of the cells to the ligands of integrin β1.

Because the increased expression level of integrin β1 on the surface of PS-ko cells seems to generate multiple functional integrin heterodimers, which enhance cell adhesion, we determined whether PS regulates the maturation of integrins α1-α7, αV, and αL and whether PS1 associates with integrin β1. No integrin α1, α4, α6, α7, or αL was detected in these cells by Western blotting (data not shown). The total expression level of integrin α2 was downregulated by PS deficiency (Figure 7A). Integrin αV expression level remained unchanged in the PS-ko cells and increased in the PS1(−/−) and PS2(−/−) cells; however, PS deficiency had no effect on the maturation of integrins α2 and αV (Figure 7A). Interestingly, similar to integrin β1, integrins α3 and α5 in the PS-ko, PS1(−/−), and PS2(−/−) cells showed higher molecular weights than those in the wt cells, indicating

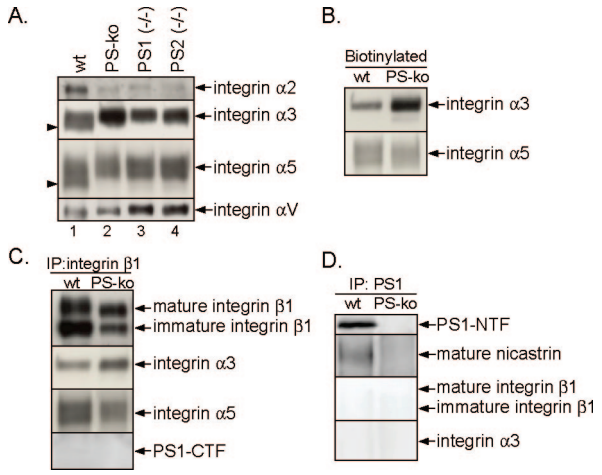

FIGURE 7: Presenilin regulates the expression, maturation and cell surface delivery of integrin α subunits. The wt, PS-ko, PS1(−/−), and PS2(−/−) cells were lysed in RIPA buffer after reaching confluence. Western blots of 50 μg of total protein from the cells were probed with anti-α2, anti-α3, anti-α5, and anti-αV integrin antibodies. Arrowheads indicate immature integrins α3 and α5; arrows indicate mature integrins α2, α3, α5, and αV (A). Biotinylated cell surface proteins of the wt and PS-ko cells were probed with anti-integrin α3 and α5 antibodies. Arrows indicate cell surface mature integrins α3 and α5 (B). Total cell lysates of wt and PS-ko cells were immunoprecipitated with a polyclonal anti-integrin β1 antibody and probed with monoclonal antibodies to integrin β1, α3, and PS1 and with a polyclonal antibody to integrin α5. Mature integrins α3 and α5 were coimmunoprecipitated with integrin β1 (C). Total cell lysates of wt and PS-ko cells were immunoprecipitated with a polyclonal anti-PS1 antibody and probed with polyclonal antibodies to PS1 and nicastrin and with monoclonal antibodies to integrin β1 and α3 (D). Mature nicastrin was coimmunoprecipitated with PS1, whereas integrins β1 and α3 were not.

that their maturation was enhanced (Figure 7A). We used cell-surface biotinylation to examine the cell-surface delivery of integrins α3 and α5. As expected, only mature integrins α3 and α5 with high molecular weights were delivered to the cell surface; moreover, the expression level of integrin α3 on the cell surface increased in the PS-ko cells, whereas that of integrin α5 remained unchanged (Figure 7B). We determined whether integrin β1 forms heterodimers with integrins α3 and α5 using immunoprecipitation analysis. Integrins α3 and α5 were coimmunoprecipitated with integrin β1, indicating the formation of functional heterodimers of integrins α3β1 and α5β1 (Figure 7C). We also found that the level of coimmunoprecipitated integrin α3 in the PS-ko cells increased compared with that in the wt cells, suggesting the enhanced maturation and cell-surface delivery, and the heterodimer formation of integrins β1 and α3 in PS-ko cells (Figure 7C). PS1 was not coimmunoprecipitated by the anti-integrin β1 antibody, indicating that PS1 was not associated with integrin β1 (Figure 7C). This was also confirmed by immunoprecipitation using the anti-PS1 antibody; mature nicastrin was coimmunoprecipitated with PS1, whereas neither integrin β1 nor integrin α3 was coimmunoprecipitated with PS1 (Figure 7D). These results suggest that PS may exert an inhibitory effect on the maturation of integrins β1 and α3 via a less direct pathway.

DISCUSSION

In this study, we demonstrated that PS deficiency leads to the enhanced maturation and cell-surface delivery of integrin β1. A marked decrease in the expression level of immature integrin β1, which is partially glycosylated and localized in ER, and an increase in that of mature integrin β1 in the PS-ko cells indicate the accelerated trafficking of integrin β1 from the ER to the Golgi apparatus. These data suggest that PS proteins also exert an inhibitory effect on the trafficking of a membrane protein from the ER to the Golgi apparatus in addition to serving as an ER-resident chaperone, which was consistent with the results of previous studies showing that the loss of function of PS results in a disrupted maturation or an enhanced intracellular retardation of nicastrin, TrkB, N-cadherin, caveolin 1, and telencephalin/ICAM (*12, 16, 17, 20, 21*). Therefore, we conclude that PS regulates the trafficking of membrane proteins from the ER to the Golgi apparatus in opposite directions, that is, PS promotes or inhibits the trafficking of membrane proteins in a protein-specific manner. In agreement with our results, results of previous studies showed that APP maturation is enhanced in PS-ko cells (*15*) and that PS1 deficiency or the loss of the PS function enhances APP maturation or causes cell-surface APP accumulation (*13, 34*). Moreover, a PS1 deletion mutant (ΔM1,2) exhibits an increased cell-surface expression level of nicotinic acetylcholine receptors (AChRs) (*15*). The post-translational maturation of integrin β1 is strictly regulated by the expression of PS. The PS-ko cells showed a marked increase in the expression level of mature integrin β1, whereas the PS1(−/−) or PS2(−/−) cells exhibited an intermediate increase in that of mature integrin β1 (Figure 1).

It is reasonable to speculate that the cell surface accumulation of integrin β1 is a result of attenuated internalization or an increased expression level of integrin β1 because a previous study showed that the loss of PS1 or PS2 function induced by the mutation of one of the critical aspartate residues or by γ-secretase inhibitors results in delayed APP reinternalization and APP accumulation on the cell surface (*14*). Another study showed that an enhanced cell-surface expression level of mature APP is not accompanied by a decrease in the expression level of immature APP in PS-ko or ΔM1,2 cells, suggesting that the total expression level of APP increases in these cells (*15*). We also found that the expression levels of both mature and immature APPs are higher in PS-ko cells than in wt cells (data not shown), in agreement with the finding of a previous study. This is also the case of acetylcholine receptors (AChRs), that is, the cell-surface and total expression levels of AChRs increase in PS1-ΔM1,2 cells (*15*). In our study, the total expression level of integrin β1 in the PS-ko cells was not altered compared with that in the wt cells because the increase in the expression level of mature integrin β1 was always accompanied by a decrease in that of immature integrin β1. The localization or trafficking of integrin β1 strictly depends on the glycosylation state of integrin β1, and partially glycosylated immature integrin β1 forms a stable pool within the ER (*26, 35, 36*). Subcellular fractionation studies showed an enhanced maturation of integrin β1 in Golgi apparatus fractions (Figure 3). Moreover, mature integrin β1 was modified by sialic acid (Figure 2). Note that the modification of complex N-linked oligosaccharides by sialic acid occurs within the Golgi apparatus (*37*). Finally, we demonstrated that the internalization of mature integrin β1 in PS-ko cells is unchanged compared with that in wt cells. Thus, these

lines of evidence suggest that an increase in the expression level of mature integrin β1 in PS-ko cells is specifically caused by the accelerated trafficking of integrin β1 from ER to the Golgi apparatus, not by an increase in integrin β1 total expression level or the delayed internalization of mature integrin β1.

PS can regulate cell adhesion via its γ-secretase substrates, such as E-cadherin, N-cadherin, CD44, and the voltage-gated sodium channel β2-subunit, and via the regulation of β-catenin and telencephalin turnover (*9, 29, 38*). Here, we demonstrated that PS regulates cell adhesion to integrin β1 ligands by modulating integrin β1 maturation and cell-surface delivery, although whether integrin β1 is a substrate of PS remains to be elucidated. We also provided evidence that the inhibition of γ-secretase activity does not enhance the expression level of mature integrin β1 or the adhesion of wt cells to fibronectin or laminin. The results of our study and previous studies show that PS1 D257A or PS1 D385A lacking γ-secretase activity expressed in a PS-null background restores nicastrin maturation; however, these mutants did not restore the expression of mature integrin β1. Because PS1 aspartate mutants do not form the mature, high molecular weight PS complexes (*30*), these results suggest that the formation of a high molecular weight PS complex is necessary for its inhibitory effect on integrin β1 maturation. Another possibility is that the loss of both the γ-secretase activity and the chaperone protein function of PS may be required to facilitate integrin β1 maturation because enhanced integrin β1 maturation was only found in PS-deficient cells.

Integrin β1 associates with multiple α-subunits to form integrin heterodimers that show cell adhesion activity (*31–33*). Our results suggest that an increase in the expression level of integrin β1 results in enhanced cell adhesion to integrin β1 ligands, fibronectin and laminin. We postulated that some integrin α-subunits are also regulated by PS deficiency. We estimated the maturation of some integrin α-subunits and observed increases in the cell-surface expression level of mature α3 subunits in PS-ko cells. Mature integrin α3 coimmunoprecipitated with integrin β1 also showed an increase in expression level in the PS-ko cells. However, previous studies have consistently shown that integrin α3β1 is a strong receptor for laminin-5, laminin-10, and laminin-11 but that it mediates cell adhesion to fibronectin or laminin-1 either very poorly or not at all (*39, 40*). Some studies showed that integrin α3β1 inhibits the activities of receptors for these ligands through transdominant inhibition (*41, 42*). Therefore, although the integrin α3β1 heterodimer showed an increase in expression level in the PS-ko cells, the stronger adhesion of the PS-ko cells may have been due to changes in the expression levels of other integrin heterodimers. In agreement with the results of previous studies, it was found that mature nicastrin associates with PS1 and that immature nicastrin is impeded in the ER of PS-ko cells, suggesting that nicastrin maturation is directly mediated by PS. We did not find a physical interaction between PS1 and integrins β1 or α3. These results suggest that the maturation of integrins β1 and α3 is inhibited by PS via a less direct pathway, which may be activated by the absence of PS. The maturation of integrins β1 and α3 is likely regulated via the same pathway through their interaction.

PS mutants play an important role in the neurodegeneration of familial Alzheimer's disease; however, the mechanism of such neurodegeneration is not yet fully understood. Although the overexpression of human FAD PS1 mutants did not affect integrin β1 maturation in the wt cells, the chronic effects of PS1 mutants on the maturation of integrins need to be investigated in vivo. Recently, some roles of integrin β1 in the nervous system have been identified. Conditional integrin β1 gene deletion in neural crest cells leads to a delayed migration of Schwann cells and induces multiple defects in spinal nerve arborization and morphology (*43*). Interestingly, a postnatal forebrain and excitatory neuron-specific knockout of the integrin β1 mouse model shows impaired hippocampal AMPA receptor-dependent synaptic transmission, synaptic plasticity, and working memory (*44*). These studies suggest that integrin β1 is important for nervous system development and serves as a regulator of synaptic glutamate receptor functions and working memory. Thus, our results suggest that modulation of integrin β1 maturation by PS plays a role in nervous system development and memory.

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