Distinct Mechanisms by Mutant Presenilin 1 and 2 Leading to Increased Intracellular Levels of Amyloid β-Protein 42 in Chinese Hamster Ovary Cells[†]

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ABSTRACT: To characterize the properties of presenilin (PS) 1- and PS2-associated γ-secretases, we established stable transfectants overexpressing amyloid precursor protein and wild-type (wt) or a number of mutant (mt) PS1 or PS2. Quantification of the intracellular amyloid β-protein (Aβ) levels in mtPS1 and mtPS2 cell lines revealed the presence of two subtypes. One group consists of N141I, M239V, and T122P mutations of the *PS2* gene and homologous mutations of PS1, N135D and M233T. These mutations led to an increase in the intracellular Aβ42 levels and a concomitant decrease in the intracellular Aβ40 levels. A cell-free assay for Aβ production using the membranes prepared from these transfectants exhibited predominant cleavage at position Aβ42 with marginal production of Aβ40. The other group consists of M146L, H163R, and G384A mutations of PS1, leading only to an increase in the intracellular Aβ42 levels. While the intracellular Aβ levels in M146L cells were consistent with the results from cell-free Aβ production, H163R and G384A cells showed significant discrepancies between the intracellular Aβ levels and cell-free Aβ production. Thus, all the mtPS1/2 examined here result in increases in the intracellular Aβ42 levels. This suggests that the underlying mechanisms for this shared phenotype may be diverse.

Amyloid β-protein (Aβ)[1] plays a central role in the pathogenesis of Alzheimer's disease (AD) (*1*). This view has been substantiated by genetic studies, which made great strides in the past decade. Thus far, three causative genes for familial AD (FAD), *β-amyloid precursor protein* (*APP*), *presenilin* (*PS*)*1*, and *PS2*, have been identified. All of their mutations appear to have the same effect on the Aβ production: to increase Aβ42 production (*1*). In addition, a recently found susceptibility gene on chromosome 10, which is associated with increased Aβ42 levels in the plasma, may also be linked with increased Aβ42 production (*2*).

There is tight coupling between PS1/2 and γ-secretase (*3*, *4*), a postulated protease that cleaves the carboxy terminus of Aβ either at Val-40 or Ala-42, following β-cleavage of APP by the β-site APP-cleaving enzyme (BACE) (*5*). Ablation of PS1 resulted in a marked reduction in Aβ production, which was accompanied by marked accumulation of carboxy terminal fragments (CTFs) of APP designated as CTFα and CTFβ, the immediate substrates of γ-secretase (*6*). Near complete suppression of Aβ production in the PS1/PS2 double-knockout mouse indicates a critical role of PS1/PS2 for γ-secretase activities (*7*, *8*). On the basis of the effects of γ-secretase inhibitors, it was proposed that PS1/2 themselves might be unusual membrane aspartyl proteases cleaving APP, a type 1 membrane protein, in the middle of the membrane (*9*, *10*). This was supported by the observations that mutation of one or two highly conserved Asp residues in the transmembrane domains (TM6 and TM7) of PS1 and PS2 causes a profound reduction in the γ-secretase activity (*4*, *11*). Furthermore, transition-state analogue γ-inhibitors can affinity-label the PS1 and PS2 fragments (*4*, *12*). These results indicate direct involvement of PS1 and PS2 in γ-cleavage, although it remains unclear whether PS1/PS2 themselves are or are not γ-secretases.

Thus, one of the most important directions in current AD research is to clarify how the specificity of γ-cleavage may be governed or influenced by FAD mutations of PS1/2 and APP. The Swedish double mutation greatly enhances β-cleavage by BACE (*5*), thereby increasing the levels of CTFβ, leading to increased production of both Aβ40 and Aβ42 (*13*, *14*). Several mutations of APP clustered in the carboxy terminus of Aβ close to the γ-cleavage sites, result in increased production of Aβ42, possibly through preferential cleavage at Ala-42 (*15*). A resulting subtle increase in the levels of Aβ42 eventually leads to the development of AD in humans after several decades. In contrast to APP, many mutations in PS1 and PS2, especially in PS1, are distributed along the entire molecule (see http://www.alzforum.org/members/resources/pres_mutations/index.html), which makes the mechanisms of their effects difficult to understand. We have originally been concerned about why there are only a few FAD mutations of PS2, whereas there are a great number for PS1. Thus, to clarify how the missense mutations of PS1 and PS2 influence the specificity of γ-cleavage, we trans-

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[1] Abbreviations: Aβ, amyloid β-protein; APP, β-amyloid precursor protein; AD, Alzheimer's disease; BACE, β-site APP-cleaving enzyme; CTF, carboxy terminal fragment; CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; FAD, familiar Alzheimer's disease; mt, mutant; NTF, amino terminal fragment; PS, presenilin; PBS, phosphate-buffered saline; wt, wild-type.

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fected numerous pathogenic PS1/2 mutations to a Chinese hamster ovary (CHO) cell line overexpressing APP751 and investigated their effects on the production of Aβ40 and Aβ42.

EXPERIMENTAL PROCEDURES

Cell Culture. Chinese hamster ovary (CHO) cells, 7WD10, stably transfected with wild-type (wt) human APP751 cDNA (a gift of Dr. E. H. Koo, University of California) (*16*) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 200 μg/mL G418.

Generation of Stable CHO Cell Lines Coexpressing APP and PS2 or PS1, and Other Cell Lines. Plasmids containing mutant (mt) PS1 (N135D, M233T, H163R, and G384A) and mtPS2 (T122P) were generated with Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using plasmids containing wtPS1 (*17*) or wtPS2 (*18*) cDNA as template and oligonucleotides carrying desired mutations. Wt and mtPS1 (N135D, M233T, H163R, and G384A) or wt and mtPS2 (N141I (*18*) and T122P) cDNAs were subcloned into a mammalian expression vector pcDNA3.1 containing a Zeocin-resistant gene (Invitrogen, The Netherlands). The identity of these constructs was confirmed by DNA sequencing. Each expression vector was transfected into 7WD10 cells using Lipofectamine (Life Technologies, Rockville, MD) according to the manufacturer's instruction, and stable cell lines were selected by culturing in the presence of 500 μg/mL Zeocin. The expression levels of transgene-derived wt or N141I mtPS2 were assessed by quantitative Western blotting within a linear range: the expression levels of CTFs and amino terminal fragments (NTFs) of PS2 in a particular cell line (1F9) was assumed to be 1. Cell culture was maintained in the presence of 250 μg/mL Zeocin and 200 μg/mL G418. The 7WD10 cells stably overexpressing M239V mtPS2 or M146L mtPS1 (*19*) were kindly provided by Dr. Koo (see above) and maintained in the presence of 5 μg/mL puromycin (Sigma-Aldrich, St. Louis, MO) and 200 μg/mL G418. M239V mtPS2 cDNA was transfected into the 7WD10 cells using retrovirally mediated infection, as described elsewhere (*20*).

Antibodies. The monoclonal antibodies against Aβ used here were BA27 (raised against Aβ1−40; specific for Aβ40), BC05 (raised against Aβ35−43; specific for Aβ42), and BNT77 (raised against Aβ11−28). The specificities of these antibodies were described previously in detail (*15*, *21*). Antibody 4G8 (specific for Aβ17−24) was obtained from Senetek PLC (Maryland Heights, MO). The polyclonal antibodies used against PS2 were 2972N (raised against residues 1−75 of human PS2) (*22*) and Ab-2 (raised against residues 324−335 of PS2) (Oncogene, Cambridge, MA). The polyclonal antibodies to PS1, anti-G1Nr2 (raised against glutathione S-transferase (GST) fused to residues 2−70 of human PS1) (*23*) and anti-G1L3 (raised against GST fused to residues 297−379 of PS1) (*24*), were kindly provided by Dr. T. Iwatsubo. The antibodies against APP were 5A3/1G7 (a gift of Dr. Koo) (*16*) and C4 (raised against the 30-residue carboxy terminal cytoplasmic domain of APP) (*25*).

Enzyme-Linked Immunosorbent Assay (ELISA). Conditioned media of cells cultured for 8 h were collected and subjected, after dilution at 1:20, to a well-characterized two-site ELISA (*26*). In short, a microtiter plate was precoated with BNT77 as a capture antibody, and the captured Aβ was detected with horseradish peroxidase-labeled BA27 and BC05 for quantification of AβX-40 and AβX-42, respectively.

Western Blotting and Quantification. For Western blot analysis of cell lysates, harvested cells were homogenized in four volumes of 1% Triton X-100 in 150 mM NaCl, 50 mM Tris-HCl, (pH 7.6). The homogenates were cleared by centrifugation at 540 000*g* for 20 min, and the supernatants were subjected to Western blotting. The proteins separated on an SDS−polyacrylamide gel were transferred onto a poly(vinylidene difluoride) membrane (Immobilon; Nihon Millipore Ltd, Yonezawa, Japan). The bound antibodies were detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). In the case of blotting for PS1 and PS2, the sample was supplemented with a final concentration of 8 M urea and applied onto a gel without heating to avoid aggregation. ECL bands of interest were quantified with a model GS-700 imaging densitometer using Molecular Analyst Software (Bio-Rad Laboratories, Hercules, CA).

For the detection of Aβ, the proteins were separated on a 16.5% Tris/tricine gel and transferred to a nitrocellulose membrane (pore size, 0.2 μm, Schleicher & Schnell, Keene, NH), as described previously (*27*). The membrane was immersed in boiling phosphate-buffered saline (PBS) for 5 min to enhance protein detectability, followed by labeling with Aβ antibodies. The blots were developed using an ECL system and detected using LAS-1000plus luminescent image analyzer (Fuji Film, Tokyo, Japan). Scanned images were quantified using Image Gauge software (Fuji Film) with defined amounts of synthetic Aβ1−40 or 1−42 as an authentic control. In addition to a band at 4 kDa, a band at 3 kDa was usually visible on the blot, probably representing Aβ11−40 or Aβ11−42 (*28*). This is consistent with the finding that BAN50, the epitope of which is located in Aβ1−10, did not label the 3-kDa band. Only the full-length Aβ1−40 or Aβ1−42 at 4 kDa was quantified throughout the present work. It should be noted that the levels of truncated Aβ at 3 kDa correlated well with full-length Aβ.

Detection of Aβ in the Cell Lysate. Cells grown on a 10 cm dish were washed twice with ice-cold PBS and collected by centrifugation. The cell pellets were suspended in 150 μL of ice-cold methanol and 300 μL of chloroform, and then ice-cold methanol was added drop by drop, altering the specific gravity to precipitate proteins at the bottom of the tube. After brief sonication, the samples were incubated for 20 min at room temperature and centrifuged for 5 min. The pellets were resuspended in chloroform/methanol/water (1:2:0.8) by sonication, incubated for 20 min, and recentrifuged. The delipidated residues were extracted with 70% formic acid at room temperature for 1 h. After centrifugation at 265 000*g* for 20 min, the supernatants were dried up using a Speed Vac (Savant instruments, Farmingdale, NY). The proteins solubilized by the SDS sample buffer were subjected to Western blotting.

Membrane Preparation and Cell-Free Aβ Production Assay. Cells were washed twice with ice-cold PBS, scraped into PBS, and collected by centrifugation. The pellets were homogenized in four volumes of buffer A (20 mM Pipes, pH 7.0, 140 mM KCl, 0.25 M sucrose, 5 mM EGTA, 0.1 mM diisopropyl fluorophosphate, 0.1 mM phenylmethylsul-

fonyl fluoride, 1 μg/mL N$^{\alpha}$-p-tosyl-L-lysine chloromethyl ketone, 1 μg/mL antipain, 0.1 μg/mL pepstatin, 1 μg/mL leupeptin, and 10 μg/mL aprotinin) using a glass/Teflon homogenizer. The homogenates were centrifuged at 800*g* for 10 min to remove nuclei and cell debris. The pellets were rehomogenized in buffer A and centrifuged again. The resulting postnuclear supernatants were combined and centrifuged at 100 000*g* for 1 h. The pellets were resuspended in buffer A and centrifuged again. The obtained membrane pellets were stored at −80 °C until use.

For cell-free Aβ production, the membrane fractions were suspended in buffer B (20 mM Pipes, pH 7.0, 140 mM KCl, 0.25 M sucrose, 5 mM EGTA, 5 mM 1,10-phenanthroline, 0.1 mM diisopropyl fluorophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/mL N$^{\alpha}$-p-tosyl-L-lysine chloromethyl ketone, 10 μg/mL antipain, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) to give a final protein concentration of 2.5 mg/mL. The reaction mixtures were incubated at 37 °C for the various times indicated and terminated by placing the tube containing the sample into liquid nitrogen. After extraction of lipids with chloroform/methanol (2:1), the protein residues were extracted with formic acid and subjected to quantitative Western blotting for Aβ as described above. For the experiments using DFK-167 (*9*), the membrane fractions were preincubated with the inhibitor on ice for 15 min, with the final concentrations of dimethyl sulfoxide (DMSO, solvent for the inhibitor) being kept at 1%, and then incubated at 37 °C for 20 min. The sample including 1% DMSO alone was used as a control. In this assay system, Aβ production appeared to depend exclusively on γ-secretase and preexisting CTFβ, but not on β-secretase, because the addition of specific β-secretase (BACE) inhibitors did not suppress Aβ production.[2] For unknown reasons we could not detect the production of p3 beginning at Leu-17 (Aβ17−40 and 17−42) using this cell-free system.[2]

Other Methods. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL) in the presence of 1% SDS.

RESULTS

Characterization of 7WD10 Cells Overexpressing wt or N141I mtPS2. To investigate the characteristics of PS2-associated γ-secretase, we established several CHO cell lines expressing both APP and wt or N141I mtPS2. The CHO cells overexpressing human APP751 (7WD10 cells) were stably transfected with cDNA encoding either wt or N141I mtPS2. Three pairs of the double transfectants were selected by assessing the expression of exogenous PS2. Western blot analysis of the cell lysates showed that each paired cell line expressed similar levels of full-length wt or N141I mtPS2 as well as a 20 kDa CTF and a 33 kDa NTF, which were generated through endoproteolysis of the full-length PS2 (Figure 1A,B). According to quantitative Western blotting, the relative mean levels of CTFs in WT1, NI1, WT2, NI2, WT3, and NI3 were 1.1, 1.1, 0.72, 0.88, 0.72, and 0.70 (n = 2 each), whereas those of NTFs in corresponding cell lines were 0.92, 1.3, 0.87, 1.1, 0.87, and 0.77 (n = 2 each). Consistent with a previous report (*29*), overexpression of exogenous PS2 displaced the endogenous PS1 fragments to varying extents. The extents of displacement appeared to correlate with the expression levels of exogenous PS2 (Figure 1C,D). Displacement of the endogenous PS2 fragments was not confirmed, because murine PS2 was barely detectable in CHO cells and its fragments had almost the same electrophoretic mobilities as exogenous human PS2 fragments. The expression levels of APP did not differ significantly among these six PS2-transfected cell lines, control mock transfectant, and parental 7WD10 cells (Figure 1E). These results strongly suggest that significant alterations in intracellular Aβ levels observed in these cells (see below) are caused primarily by wt or N141I mtPS2 transgene.

Marked Predominance of Aβ42 over Aβ40 in the Culture Medium and Lysate of N141I mtPS2 Cells. The levels of secreted Aβ40 and Aβ42 in the media were quantified using a two-site ELISA (Figure 1F). Aβ42 secretion from the N141I mtPS2 cells was greatly increased (2.8−3.4-fold), whereas the Aβ40 secretion was decreased by ∼40−80%, as compared with wtPS2 cells (Figure 1F). These observations are consistent with a previous report (*30*). Consequently, the total Aβ secretion from N141I mtPS2 cells was not so discrepant from that from wtPS2 cells, while the percentage of Aβ42 (%Aβ42) relative to the total Aβ (sum of Aβ40 and Aβ42) in the media was remarkably increased. These ELISA data on secreted Aβ were confirmed by immunoprecipitation with 4G8, followed by quantitative Western blotting (data not shown). These characteristics were observed consistently among three pairs of wt and N141I mtPS2 cell lines, and the increased percentage of Aβ42 in the media correlated well with the displacement extents of endogenous PS1 fragments. Conversely, the proportion of Aβ40 was inversely related to the displacement extent of PS1 (Figure 1C,D). This indicates that a decrease in the secretion of Aβ40 and an increase in the secretion of Aβ42 are caused by the overexpression of N141 mtPS2. A decrease in the secretion of Aβ40 was unexpectedly, but consistently associated with this.

To exclude the possibility that the overexpression of N141I mtPS2 affects the secretion of Aβ, we examined the steady-state concentrations of intracellular Aβ in these transfectants using Western blotting. On the blot, BA27 or BC05 labeled a major band at ∼4 kDa, likely representing Aβ1−40 or Aβ1−42, and an additional band at ∼3 kDa, presumably a truncated species beginning at Glu-11, Aβ11−40 or Aβ11−42 (Figure 1G). Overexpression of wtPS2 resulted in a definite increase in the intracellular Aβ40 level and a small increase in the Aβ42 level, compared with 7WD10 and mock cells. In contrast, overexpression of N141I mtPS2 caused a marked increase in the Aβ42 level, and unexpectedly, a moderate decrease in the Aβ40 level, an observation compatible with the proportion of secreted Aβ. The extents of increases in the levels of intracellular Aβ42 in N141I mtPS2 cells also correlated with the displacement extents of endogenous PS1 fragments (see Figure 1C,D). Greater displacement of PS1 led to a larger increase in the Aβ42 level.

Taken together, alterations in the levels of intracellular Aβ were in parallel with those in the levels of the secreted Aβ, suggesting that overexpression of N141I mtPS2 does not alter the secretion of Aβ, but alters processing of APP (CTFβ) at positions Aβ40 and Aβ42 in a way in which the production of Aβ42 is increased.

[2] Sato, T., and Ihara, Y., manuscript in preparation.

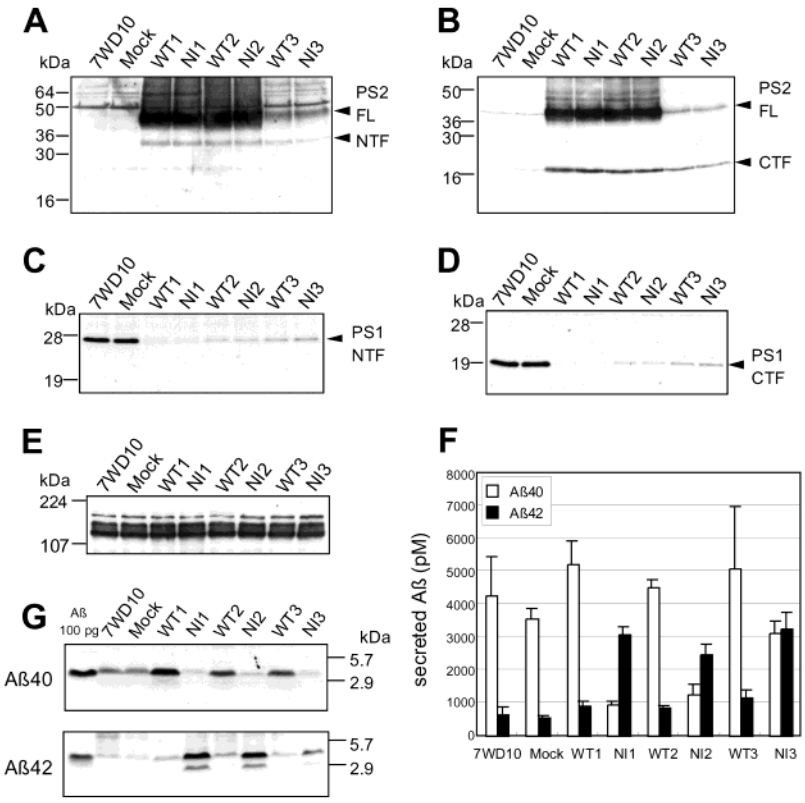

FIGURE 1: Effects of the levels of wt and N141I mtPS2 on secreted and intracellular Aβ. From 7WD10 cells (parental cell overexpressing APP751), mock transfectant (empty vector), and three pairs of the transfectants stably overexpressing wt (WT1−3) or N141I mt (NI1−3) PS2, lysates were prepared and subjected to Western blotting with 2972N (A), Ab-2 (B), anti-G1Nr2 (C), anti-G1L3 (D), and 5A3/1G7 (E). The three pairs of the transfectants (WT1/NI1, WT2/NI2, and WT3/NI3) are placed in the order of decreasing expression of PS2 (A and B), which parallels the displacement extent of endogenous PS1 fragments (C and D). Full-length form (FL), NTF, and CTF of PS2 or PS1 are indicated by arrowheads. 5A3/1G7 labeled two major bands representing mature and immature APP (E). The top band was not labeled by C4, and was probably nonspecific. (F) Effects of wt or N141I mtPS2 on secreted Aβ. The levels of Aβ40 (open bars) and Aβ42 (closed bars) in the conditioned media of these stable transfectants were quantified using two-site ELISA. The data represent the means ± SD (n = 3). (G) Effects on intracellular Aβ levels. The cell extracts were prepared as described in "Experimental Procedures" and then subjected to Western blotting with BA27 (upper panel) and BC05 (lower panel). A major Aβ band at ∼4 kDa represents Aβ1−40 or Aβ1−42, and an additional faint band at ∼3 kDa may represent a truncated species, Aβ11−40 or Aβ11−42. Synthetic Aβ1−40 or Aβ1−42 (100 pg) was loaded in the left-most lane as a control.

Other PS2 Mutation, M239V and their Homologous PS1 Mutations, N135D and M233T, Cause Similar Effects on the Intracellular Aβ Levels. We next asked whether the effects on positions of Aβ40 and Aβ42 are specific for this particular mutation, or whether similar effects can also be observed in the cells overexpressing other mtPS2 or mtPS1 that are homologous to N141I or M239V. We chose a pair of wt and N141I mtPS2 cell lines (W1 and NI1, respectively), M239V mtPS2-transfected 7WD10 cell line and wtPS1, N135D, and M233T mtPS1-transfected 7WD10 cell lines. The steady-state levels of intracellular Aβ40 and Aβ42 in all of these PS1/2 stable transfectants were quantified by Western blotting. This is because we were concerned about cross-reaction of the BNT77/BC05 ELISA with full-length APP, as reported previously (*31*).

The levels of the PS2 NTFs and CTFs were similar among wt, N141I, and M239V mtPS2 cell lines, in which displacement of endogenous PS1 fragments was almost complete (data not shown). The levels of PS1 NTFs and CTFs were also similar among wt, N135D, and M233T mtPS1 cells, in which the endogenous PS1 fragments were nearly completely displaced (data not shown) and PS2 fragments that were barely detectable in the parental 7WD10 cells were undetectable (data not shown). These observations indicate that in these cell lines the effects on the intracellular Aβ levels can be attributed exclusively to the exogenous PS1/2 transgenes.

Quantitative Western blotting for Aβ showed almost equivalent levels of intracellular Aβ40 and Aβ42 in parental 7WD10 cells (Figure 2B,D,F), which sharply contrasts with a high level of Aβ40 or a low level of Aβ42 in its culture medium (Figure 1F). Overexpression of wtPS2 resulted in an increase in the intracellular Aβ40 levels and a very small, but significant, increase in the intracellular Aβ42 levels (student's t-tests; $P = 0.003$ and 0.002, respectively; Figure 2B). In contrast to wtPS2, the levels of both intracellular Aβ40 and Aβ42 in wtPS1 cells did not significantly differ from those in 7WD10 cells ($P > 0.05$) (Figure 2D,F).

When N141I or M239V mtPS2 was overexpressed, the levels of intracellular Aβ42 increased greatly to similar levels (3.2-fold) ($P < 0.0001$ for N141I and $P = 0.01$ for M239V), while those of intracellular Aβ40 decreased substantially (2.4- or 2.2-fold, respectively) ($P = 0.0007$ for N141I and $P = 0.007$ for M239V), as compared with wtPS2 cells (Figure 2B). In consequence, the proportion of Aβ42 (%Aβ42) against total Aβ (the sum of Aβ40 and Aβ42) increased to 87% in N141I mtPS2 cells and to 86% in M239V mtPS2 cells, whereas it was ∼50% in wtPS2 cells (see Figure 5B).

Similar effects on the intracellular Aβ levels were also observed in the cells overexpressing N135D and M233T

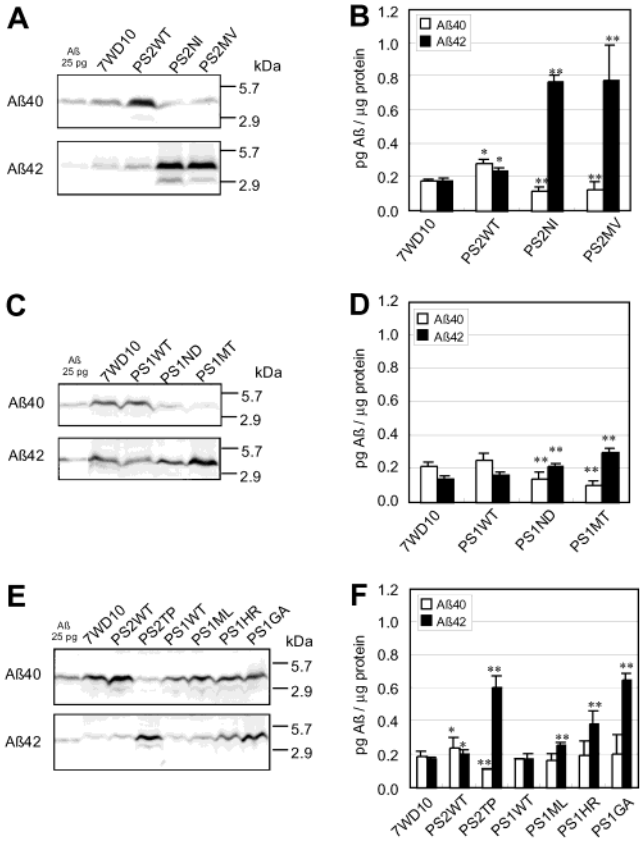

FIGURE 2: Alterations in the intracellular Aβ levels caused by mtPS1 and mtPS2. Quantitative analysis of intracellular Aβ levels in 7WD10, wt (WT) and N141I (NI) and M239V (MV) mtPS2 cells (A and B), in N135D (ND) and M233T (MT) mtPS1 cells, the mutations of which are homologous to the former PS2 mutations, respectively (C and D), and in other mtPS1/2 cells (E and F). The cell extracts prepared from those cells were subjected to Western blotting with BA27 (upper panel) and BC05 (lower panel; see Experimental Procedures) (A, C, and E). The bands at ~4 kDa probably represent Aβ1−40 and Aβ1−42. In the left-most lane, synthetic Aβ1−40 or Aβ1−42 (25 pg) was loaded. The intracellular levels of Aβ40 (open bars) and Aβ42 (closed bars) in each of the cell extracts were quantified using LAS-1000plus luminescent image analyzer, and are expressed as pg per μg of total cellular protein (B, D, and F). The data shown are the means ± SD ($n = 3$). *$P < 0.05$ as compared with the levels of Aβ1−40 or Aβ1−42 in 7WD10 cells. **$P < 0.05$ as compared with those in wtPS1 or wtPS2 cells.

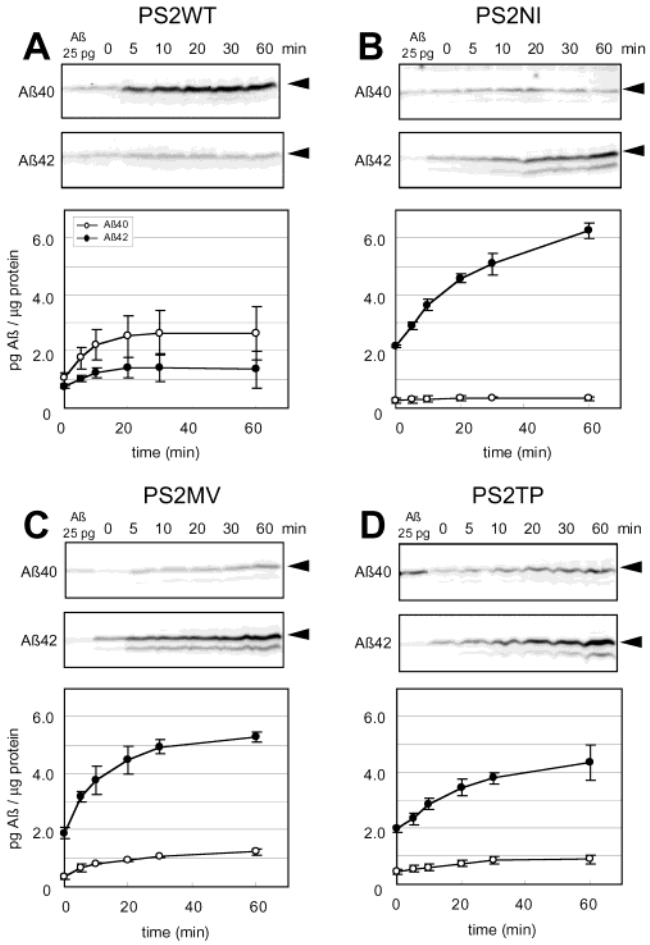

FIGURE 3: Cell-free Aβ production in the membranes prepared from wt or mtPS2 cells. The membrane fraction was prepared from wt (WT) PS2 (A), N141I (NI) mtPS2 (B), M239V (MV) mtPS2 (C), or T122P (TP) mtPS2 (D) cells and incubated at 37 °C for indicated times as described in "Experimental Procedures". After delipidation, the protein extracts were subjected to Western blotting with BA27 (upper panel) and BC05 (middle panel). Arrowheads at ~4 kDa in the each upper panel indicate Aβ1−40 and Aβ1−42 bands for quantification. In the left-most lane, synthetic Aβ1−40 or Aβ1−42 (each 25 pg) was loaded. The levels of Aβ40 (open circles) and Aβ42 (closed circles) in the reaction mixture at each time point were quantified using LAS-1000plus luminescent image analyzer and are expressed as pg per μg of membrane protein. The data shown are the means ± SD from triplicate measurements in using independent membrane preparations.

mtPS1 which are homologous with N141I and M239V mtPS2, respectively (Figure 2C,D). The levels of intracellular Aβ42 increased (1.3- and 1.8-fold, respectively) ($P = 0.0037$ for N135D, and $P < 0.0001$ for M233T), whereas those of intracellular Aβ40 decreased (by 1.7- or 2.4-fold, respectively; $P = 0.01$ for N135D, and $P = 0.002$ for M233T), in comparison with those found in wtPS1 cells (Figure 2C,D). In consequence, the proportion of Aβ42 against total Aβ increased to 60% in N135D mtPS1 cells and to 75% in M233T mtPS1 cells, whereas it was ~50% in wtPS1 cells (see Figure 5B).

Thus, these homologous mutations of PS1 and PS2 cause similar effects on intracellular Aβ levels: an increase in the intracellular Aβ42 levels and a concomitant decrease in the intracellular Aβ40 levels. Similar effects on secreted Aβ40 and Aβ42 were also confirmed by immunoprecipitation of conditioned media with 4G8 (data not shown), suggesting that these mtPS1/2 do not affect the secretion of Aβ but alter the production of Aβ40 and Aβ42.

Three other PS1 Mutations Examined Caused Only an Increase in the Intracellular Level of Aβ42. To see whether similar effects on the intracellular Aβ levels can also be induced by other mutations of PS2 and PS1, we established and examined several other stably transfected 7WD10 cell lines. We chose the following mutations: (1) T122P mtPS2, the mutation of which is located on a large loop in the luminal side between transmembrane (TM) 1 and TM2; (2) M146L mtPS1, the mutation of which is located on an α-helix domain of TM2; (3) H163R mtPS1, the mutation of which is located adjacent to TM3 in a small loop in the cytosolic side (or in the peripheral portion of TM3); and (4) G384A mtPS1, the mutation of which is located immediately adjacent to the critical Asp-385 of TM7, which was previously shown to be essential for the activity of γ-secretase (*32*). The levels of the PS1 NTFs and CTFs were similar

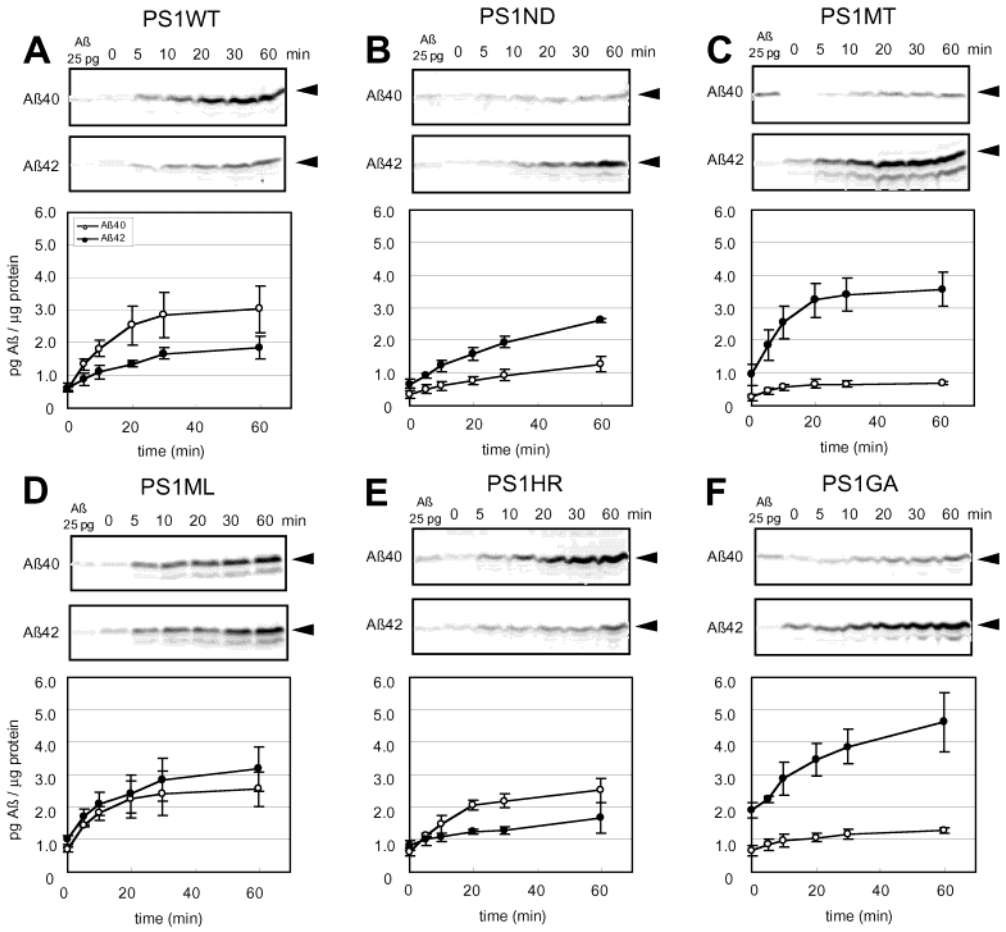

FIGURE 4: Cell-free Aβ production in the membranes prepared from wt and mtPS1 cells. The membrane fraction was prepared from wt (WT) PS1 (A), N135D (ND) mtPS1 (B), M233T (MT) mtPS1 (C), M146L (ML) mtPS1 (D), H163R (HR) mtPS1 (E), or G384A (GA) mtPS1 (F) cells and incubated at 37 °C for indicated times as described in "Experimental Procedures". After delipidation, the protein extracts were subjected to Western blotting with BA27 (upper panel) and BC05 (middle panel). Arrowheads at ~4 kDa indicate Aβ1−40 and Aβ1−42 bands for quantification. In the left-most lane, synthetic Aβ1−40 or Aβ1−42 (each 25 pg) was loaded. The levels of Aβ40 (open circles) and Aβ42 (closed circles) in the reaction mixture at each time point were quantified using LAS-1000plus luminescent image analyzer and are expressed as pg per μg of membrane protein. The data shown are the means ± SD from triplicate measurements using independent membrane preparations.

among wt, M146L, H163R, and G384A mtPS1 cell lines, in which displacement of endogenous PS1 fragments was almost complete (data not shown). The levels of PS2 NTFs and CTFs were also very similar among wt and T122P mtPS2 cells, in which the endogenous PS1 fragments was nearly completely displaced (data not shown).

These stably transfected cell lines were subjected to quantitative analysis for the intracellular levels of Aβ40 and Aβ42. In all of these mtPS1 and mtPS2 cells, the levels of intracellular Aβ42 were increased to varying extents, compared with those in wtPS1 and wtPS2 cells, respectively. The increases were 3.1-fold in T122P mtPS2 cells (vs wtPS2 cells), 1.5-fold in M146L mtPS1 cells, 2.3-fold in H163R mtPS1 cells, and 3.8-fold in G384A mtPS1 cells (vs wtPS1 cells; see ref *32*; Figure 2E,F). In T122P mtPS2 cells, the level of intracellular Aβ40 was significantly decreased (2-fold; $P = 0.02$), as compared with wtPS2 cells, an effect which was observed in the other three mtPS2 cells examined above. However, there was no significant alteration in the levels of intracellular Aβ40 between those mtPS1 cells and wtPS1 cells ($P > 0.05$; Figure 2E,F). The %Aβ42 reached 61%, 67%, and 77% in M146L, H163R, and G384A mtPS1 cells, respectively (see Figure 5B). In all of these cell lines, the secreted Aβ levels faithfully reflected the changes in the intracellular Aβ levels, as shown by immunoprecipitation of conditioned media with 4G8 (data not shown). Thus, these results suggest that M146L, H163R, or G384A mutation of PS1 enhances the cleavage efficiency only at position Aβ42, but not at position Aβ40.

Cell-Free Assay for wt or mtPS1/2-Associated γ-Secretase. The intracellular Aβ level simply represents its steady-state level within the cell, a net effect of its production, degradation, secretion, and/or uptake. Thus, to further characterize the properties of mtPS2-associated γ-secretase in these cells, we employed a cell-free assay for Aβ production. The membrane fractions were prepared from the 7WD10 cell overexpressing wt or mtPS1/2, and incubated in the presence of various protease inhibitors at 37 °C. The amount of Aβ produced in this membrane preparation was quantified by Western blotting. Both Aβ40 and Aβ42 were produced in a time-dependent manner (Figures 3 and 4), and CTFγ, the other product of γ−cleavage, was concomitantly produced in this system (data not shown; see ref *33*). The Aβ production proceeded linearly at least until 10 min and then in many cases its rate declined moderately (Figures 3 and 4). Both Aβ40 and Aβ42 production showed similar temporal profiles in each cell line, but there was some variability between the cell lines. For example, in the N141I mtPS2

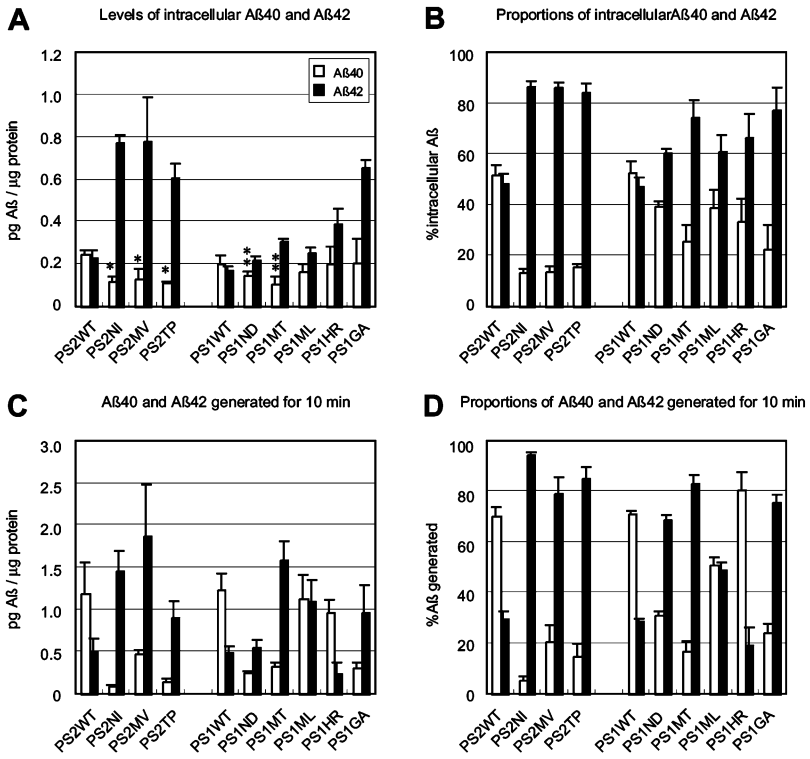

FIGURE 5: Intracellular Aβ levels and cell-free Aβ production among wt and mtPS1/2 cells. (A) Steady-state intracellular Aβ levels in wtPS1/2 and mtPS1/2 cells. * and ** represent significant (P <0.05) decrease in the levels of Aβ40, as compared with wtPS2 or wtPS1cells, respectively. (B) Proportion of Aβ40 and Aβ42 in total intracellular Aβ levels (the sum of intracellular Aβ40 and Aβ42). (C) The absolute yields of cell-free production of Aβ for 10 min. The Aβ levels in the membrane at 0 min were subtracted from the Aβ levels at 10 min. (D) The production of Aβ40 and Aβ42 are expressed as percentages of the total Aβ level (the sum of the produced Aβ40 and Aβ42). Aβ40 is indicated as open bars, and Aβ42 is indicated as closed bars. The data shown are the means ± SD (n = 3).

membrane, Aβ42 production proceeded almost linearly until 40−60 min (Figure 3B), which may be explained in part by the presence of a larger amount of CTFβ (data not shown).

In the membrane from wtPS2 cells, the proportion of Aβ40 was found to be ~70% of the produced Aβ (Figure 3A and 5D). The amounts of Aβ40 and Aβ42 produced per initial 10 min in the wtPS2 membrane were roughly equivalent to those in the wtPS1 line (Figure 4A and 5C), and thus %Aβ42 in the former was also very similar to that in the latter (Figure 5D).

On the other hand, predominant production of Aβ42 was observed in all the three mtPS2 membranes, which accompanied profoundly decreased production of Aβ40 (Figure 3B-D). This resulted in %Aβ42 being 94%, 79%, and 85% in the membranes from N141I, M239V, and T122P mtPS2 cells, respectively (Figure 3B-D and 5D). Thus, N141I, M239V, and T122P-associated γ-secretases share a novel characteristic: dramatically increased Aβ42 production and remarkably suppressed Aβ40 production. Likewise, the membranes from N135D or M233T mtPS1 cells produced Aβ42 predominantly (Figure 4B, C), with %Aβ42 being 69% or 83%, respectively (Figure 3B-D and 5D). For unknown reason(s), the N135D mtPS1 cell membrane produced Aβ with very low efficiency per μg of protein (Figure 4B and 5C), and the amount of Aβ42 produced in the initial 10 min is almost equal to that in the wtPS1 membrane (Figure 4A, B, and 5C). These data strongly suggest that all PS2 mutations and their homologous PS1 mutations have similar effects on Aβ production, although their extents differ slightly from each another.

In contrast to mtPS2 cells in which resulting alterations were uniform, mtPS1 cells showed various profiles in Aβ production according to the site of mutation (Figure 4B-F). Except for H163R mtPS1 cells, all of those mtPS1 cells examined showed increased proportions of Aβ42 in the cell-free assay, as compared with wtPS1 cells (Figure 5D). The M146L mtPS1 membrane produced almost equal amounts of Aβ40 and Aβ42 (Figure 4D), and thus %Aβ42 increased substantially (Figure 5D). The amounts of Aβ40 produced per initial 10 min in the M146L mtPS1 membranes were almost the same as those in the wtPS1 ones (Figure 5C). On the other hand, the G384A mtPS1 membranes produced a large amount of Aβ42 (Figure 4F), but much smaller amounts of Aβ40 than did the wtPS1 membranes (Figure 5C), with %Aβ42 being 75% (Figure 5D). Most unexpectedly, the H163R mtPS1 membranes produced Aβ with a profile very similar to the wtPS1 membranes (Figure 4E).

Taken together, the above data indicate that all of mtPS2 membranes examined here share uniform characteristics for Aβ production, whereas the mtPS1 membranes have differing characteristics. The intracellular levels of Aβ40 and Aβ42 largely reflect the efficiency in Aβ40 and Aβ42 production as revealed by the cell-free assay, except for H163R mtPS1 cells (Figure 5A, C). Thus, the major determinant of intracellular Aβ levels is mostly the production of Aβ40 and Aβ42, and this reflects the activity of γ-secretase on positions Aβ40 and Aβ42 (Figure 5C, D).

The Effect of γ-Secretase Inhibitor on Aβ Production. We next examined the effect of γ-secretase inhibitors on Aβ production using the cell-free system prepared from wt or

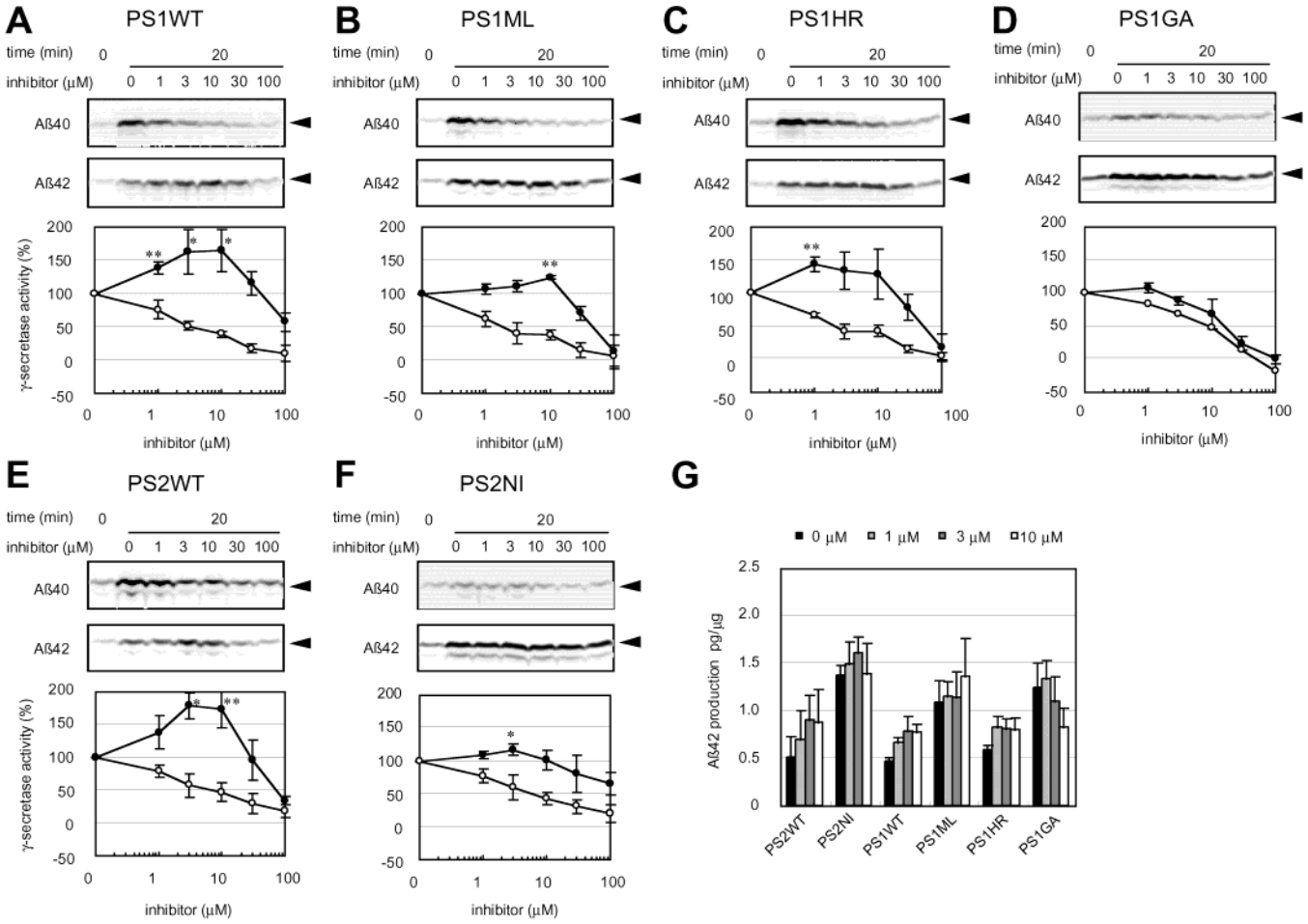

FIGURE 6: Effects of DFK-167 on Aβ production in the membranes from wt and mtPS1/2 cells. The membranes were prepared from wt (WT) PS1 (A), M146L (ML) mtPS1 (B), H163R (HR) mtPS1 (C), G384A (GA) mtPS1 (D), wt (WT) PS2 (E), and N141I (NI) mtPS2 cells (F). The membranes were incubated at 37 °C for 20 min in the presence of various concentrations of DFK167. After delipidation, the protein extracts were subjected to Western blotting with BA27 (upper panel) and BC05 (middle panel). The control membranes without incubation were loaded on the left-most lane on each panel to give basal Aβ levels. Arrowheads indicate the bands representing Aβ1−40 and Aβ1−42. The levels of Aβ40 (open circles) and Aβ42 (closed circles) in these cell membranes were quantified using LAS-1000plus luminescent image analyzer. The produced Aβ levels in the presence of indicated concentrations of DFK-167 are expressed as percentages of the Aβ production in the absence of DFK-167. The data shown are the means ± SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ (G). The amount of Aβ42 produced in the absence or presence of indicated low concentrations of DFK-167. Production of Aβ in the membrane at 20 min was measured after subtracting the Aβ levels at 0 min. The data shown are the means ± SD ($n = 3$).

mtPS1/2 membranes. Specific γ-secretase inhibitors, L685,458 (*34*; data not shown) and DFK-167 (*9*), suppressed Aβ generation in the membrane fractions prepared from these cells, indicating the validity of this assay system for assessing the acitivity of γ-secretase (Figure 6A−F). When the wtPS1/2 membranes were incubated in the presence of DFK-167, Aβ40 production was reduced in a dose-dependent manner (Figure 6A,E). In contrast, Aβ42 production in these membranes was enhanced significantly at low concentrations of DFK-167 (∼1.67−1.71-fold) and then suppressed at higher concentrations (Figure 6A,E). Similar effects of DFK-167 were observed on the membranes from mtPS1 or mtPS2 cells. Aβ40 production was suppressed in a dose-dependent manner, and to a similar extent at every concentration of the inhibitor in all wt and mtPS1/2 membranes (Figure 6A−F). The membranes from mtPS1/2 cells showed various profiles in Aβ42 production in the presence of DFK-167, but all of them appeared to have an enhanced phase at its low concentrations and then a suppressed phase at its high concentrations (Figure 6B−D,F). When the extent of Aβ42 production was high, this paradoxical effect was unremarkable (Figure 6D for G384A mtPS1 and Figure 6F for N141I mtPS2). This resulted in a more robust Aβ42 production, a less remarkable enhancement (8% increase—not significant—in G384A mtPS1 and 17% increase in N141I mtPS2), and less Aβ42 production and more remarkable enhancements in wtPS1 (64% increase), in H163R mtPS1 (41%), and in wtPS2 (71%; Figure 6A−F).

DISCUSSION

We have been primarily concerned with the specificity of γ-secretase in wt or mtPS1/2-overexpressing 7WD10 cells: namely, how the specificity of the γ-secretase is altered by mtPS1/2. It is reasonable to postulate that the observed γ-cleavage specificities are primarily determined by the wt or mtPS1/2-transgene, and thus represent those of wt or mt transgene-associated γ-secretase. In every cell line, endogenous PS1/2 were almost completely displaced by overexpressed exogenous wt or mtPS1/2 genes.

Here, we concentrated on the quantification of the intracellular levels of Aβ40 and Aβ42, because we thought that

these parameters should reflect γ-secretase activities more closely than the secreted levels of Aβ40 and Aβ42, which are widely used parameters. In this context, 7WD10 is a suitable cell line that secretes a large amount of Aβ, and contains high levels of intracellular Aβ that can be readily quantified by Western blotting. When the intracellular levels of Aβ40 and Aβ42 were quantified, a discrepancy between their intracellular and secreted Aβ levels became evident. Despite severalfold higher concentrations of Aβ40 in the culture media, its intracellular levels were similar to those of Aβ42. This suggests that the Aβ40 and Aβ42 molecules are on distinct pathways to constitutive secretion: once produced, Aβ40 would be quickly secreted, while Aβ42 would stay longer within the cell, and only a fraction of it would be secreted.

A marked increase in the intracellular Aβ42 levels and a substantial decrease in the intracellular Aβ40 levels that were originally found in N141I mtPS2 cells were also observed in other mtPS2 cells, M239V and T122P cells. We were initially puzzled with such decreased intracellular Aβ40 levels, but the relationship of the secreted levels of Aβ40 with the displacement extent of PS1 in those cell lines led us to postulate that these intracellular Aβ concentrations should reflect the unusual specificity of mtPS2-associated γ-secretase. These data led us to hypothesize that the unusual specificity toward cleavage at the position of Aβ42 is an important feature of mtPS2-associated γ-secretase. This view was indeed substantiated by the cell-free Aβ production system using the membranes prepared from mtPS2 cells, which clearly showed markedly predominant Aβ42 production and almost marginal Aβ40 production. Differences in the temporal profiles of the γ-secretase activities would be due to the amounts of the available immediate substrate (CTFβ; Figure 3A−D).

Because we were not certain about whether the above unusual characteristics are specific for mtPS2, several transfectants overexpressing mtPS1 were established and subjected to quantification of the intracellular Aβ levels and to cell-free Aβ production assay. The same characteristics were found for N135D and M233T mtPS1, which are homologous with N141I and M239V in PS2, respectively. However, for unknown reason(s), the extents of the productions appeared to be slightly smaller in N135D mtPS1, as compared with all of the mtPS2 examined. Affected family members carrying the PS1 mutations have the earliest age of onset at 35 years, whereas the mtPS2-carriers have ages of onset mostly at 40−70 years. One possible explanation would be that the specific activity of PS2-associated γ-secretase is much lower than the PS1-associated one, thereby not significantly contributing to Aβ42 production as a whole. However, this is not the case. Overexpression of PS1 or PS2 in individual CHO cell lines should lead to similar levels of active forms of PS1 or PS2 complexes, respectively, which are determined by the concentration of the shared cellular limiting factor (*29*). In addition, the membranes prepared from PS1 and PS2-transfected cells produced comparable amounts of total Aβ per initial 10 min per μg protein (see Figures 3, 4, and 5C). Thus, PS1- and PS2-associated γ-secretases should have comparable specific activities.

An alternative explanation is that the expression levels of PS2 may be much lower than those of PS1 in brain cells. There has been some confusion about whether PS1 is expressed more abundantly than PS2 (*35*, *36*). The levels of PS2 (fragments) in brain cells may be severalfold to 10-fold lower than those of PS1. If so, only such mtPS2 as produces predominantly Aβ42 can contribute significantly to an increase in the relative proportion of Aβ42 in total secreted Aβ, and so lead to development of FAD. However, mtPS2 that shows only some preference for cleavage at Aβ42 to the extent that can be observed with M146L mtPS1 would never cause FAD, because the expression levels of mtPS2 are too low to have obvious effects on %Aβ42 in vivo. In contrast, PS1 mutations resulting in only some preference for the cleavage at Aβ42 can cause FAD because PS1 is abundantly expressed and its associated γ-serectase activity is much higher than that associated with PS2. In this context, it is of interest to note that N135D in PS1, homologous to N141I in PS2, causes a very aggressive form of AD with an age of onset of only 34−38 years of age (*37*), whereas the latter PS2 mutation is characterized by rather a later onset 44−75 years. M233T in PS1 is characterized also by a very early age of onset before 35 years (*38*), whereas the homologous PS2 mutation, M239V, produces a rather later onset ∼50 years.

Some other PS1 mutations, M146L, H163R, and G384A, result in a distinct alteration in the intracellular Aβ levels: an increase in the intracellular levels of Aβ42 without an accompanying decrease in those of Aβ40. Thus, these three PS1 mutations likely have distinct mechanisms. In M146L mtPS1 cells, alterations in the intracellular Aβ levels are in parallel with the results obtained by the cell-free Aβ production assay. This suggests that a substantial increase in the production of Aβ42 is a cause of a moderate increase in the intracellular and extracellular Aβ42 levels, which eventually leads to development of FAD.

Likewise, one can speculate from the intracellular Aβ levels in G384A mtPS1 cells that the activity of mtPS1-associated γ-secretase producing Aβ40 is similar to that of wtPS1-associated γ-secretase, whereas the activity of the mtPS1-associated γ-secretase producing Aβ42 is much higher than that of wtPS1-associated γ-secretase (Figure 2E, F). The cell-free assay for the activity of γ-secretase in G384A mtPS1 cells showed predominant Aβ42 production, which is consistent with higher intracellular Aβ42 levels, but only marginal Aβ40 production, which cannot directly account for the intracellular levels of Aβ40 (Figure 2F, 4F, and 5A, C). Thus, for the G384A mutation, there is a discrepancy between intracellular levels of Aβ40 and cell-free production of Aβ40.

H163R mtPS1 exhibited almost marginal production of Aβ42 in the cell-free Aβ production assay, despite a great increase in the intracellular levels of Aβ42 (Figure 2F and 4E). Thus, one explanation would be that the membranes from H163R mtPS1 cells are unable to produce Aβ42 as efficiently as the whole cell. A certain cytosolic factor(s), which may interact with H163R mtPS1 and enhance Aβ42 production within the cell, might have been lost during membrane preparation. The same point could be applied to the Aβ40 production in G384A mutation: Aβ40 production may have been greater within the cell. However, further studies are required to test whether this is the case.

Previous reports suggest that Aβ40 and Aβ42 are generated by distinct γ-secretases, Aβ40-γ-secretrase and Aβ42-γ-secretase (*39*), because γ-secretase inhibitors can affect

Aβ40 and Aβ42 production differentially. This assumption is compatible with the present data that show that the activities of Aβ40- and/or Aβ42-γ-secretase can be modified independently by mtPS1/2. In N141I, M239V, and T122P mtPS2, and N135D, M233T mtPS1, the activity of Aβ40-γ-secretase is significantly suppressed, while the activity of Aβ42-γ-secretase is markedly enhanced. In contrast, in M146L mtPS1, only Aβ42-γ-secretase is enhanced. Thus, one can consider that the activity of Aβ40- or Aβ42-γ-secretase would be regulated by mtPS1 or mtPS2 in an independent manner. This assumption is also consistent with the recent screening data for γ-secretase modifiers showing that Herp, an ER-resident protein, can enhance only Aβ40-γ-secretase, leading to an increase in the intracellular Aβ40 levels.

It has been reported that peptide aldehyde and difluoroketone inhibiors increase Aβ42 production at low concentrations and decrease Aβ42 production at high concentrations, whereas their effects on Aβ40 production are monophasic (*10*,*40*). It is likely that the enhancement of Aβ42 production at low concentrations of DFK-167 comes from a defined level of activation, irrespective of the extent of Aβ42 production (Figure 6G). This enhancement effect cannot be explained by the uniform activation of γ-secretase itself, but presumably by involvement of other factor(s). One possible explanation would be that the substrate that would have been normally cleaved by Aβ40-γ-secretase was overflowed and delivered to Aβ42-γ-secretase which is still active at low concentrations of inhibitor. It is of note that these phenomena with Aβ42-γ-secretase are observed not only in wtPS1/2 membranes, but also in mtPS1/2 membranes. Thus, the properties of Aβ40-γ-secretase and Aβ42-γ-secretase may not be modified even in mtPS1/2 membranes, but their proportions in the total γ-secretase molecules may become altered according to the mutations.

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