Enhanced Production and Oligomerization of the 42-residue Amyloid β-Protein by Chinese Hamster Ovary Cells Stably Expressing Mutant Presenilins*

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Mutations in the presenilin 1 (PS1) and presenilin 2 (PS2) genes cause the most common and aggressive form of early onset familial Alzheimer’s disease. To elucidate their pathogenic mechanism, wild-type (wt) or mutant (M146L, C410Y) PS1 and wt or mutant (M239V) PS2 genes were stably transfected into Chinese hamster ovary cells that overexpress the β-amyloid precursor protein (APP). The identity of the 43–45-kDa PS1 holoproteins was confirmed by N-terminal radiosequencing. PS1 was rapidly processed (t1/2 = 40 min) in the endoplasmic reticulum into stable fragments. Wild-type and mutant PS2 holoproteins exhibited similar half lives (1.5 h); however, their endoproteolytic fragments showed both mutation-specific and cell type-specific differences. Mutant PS1 or PS2 consistently induced a 1.4–2.5-fold increase (p < 0.001) in the relative production of the highly amyloidogenic 42-residue form of amyloid β-protein (Aβ42) as determined by quantitative immunoprecipitation and by enzyme-linked immunosorbent assay. In mutant PS1 and PS2 cell lines with high increases in Aβ42/Aβtotal ratios, spontaneous formation of low molecular weight oligomers of Aβ42 was observed in media, suggesting enhanced Aβ aggregation from the elevation of Aβ42. We conclude that mutant PS1 and PS2 proteins enhance the proteolysis of β-amyloid precursor protein by the γ-secretase cleaving at Aβ residue 42, thereby promoting amyloidogenesis.

All patients with Alzheimer’s disease (AD) develop extracellular amyloid deposits composed of the 40- and 42-residue amyloid β-proteins (Aβ) in brain areas subserving memory and cognition. Many of the deposits are intimately associated with degenerating axons and dendrites, activated microglial cells, and reactive astrocytes. Mutations or polymorphisms in four genes that strongly predispose individuals to the premature development of AD have been identified to date. First, missense mutations in the APP gene (1–5), which encodes the precursor of Aβ, increase the production of Aβ peptides, particularly Aβ42, in vitro and in vivo (6–10). Second, inheritance of the e4 polymorphism of the apolipoprotein E gene increases the number and density of Aβ deposits in the brain (11–15). The third and fourth genes to be linked to AD, presenilin 1 (PS1) and presenilin 2 (PS2), cause the most common form of early onset familial AD (16–18). These genes encode highly homologous proteins predicted to span the membrane 7–8 times (19). Missense mutations in PS1 and PS2, more than 30 of which have already been identified (20), result in markedly accelerated clinical and neuropathological features of AD.

A clue to the mechanism of the presenilins has come from the recent report of selective elevations in Aβ42 levels in plasma and skin fibroblast media of subjects harboring PS1 or PS2 mutations (10). Because primary fibroblasts expressing different PS1 or PS2 mutations show very low Aβ secretion that cannot be easily studied mechanistically, we examined stably transfected Chinese hamster ovary (CHO) cell lines in which the sole variable is the introduction of normal or mutant presenilin genes, and any other host-derived factors are eliminated (21). Moreover, the formation of Aβ oligomers can be detected in the conditioned media of CHO cells (22). We stably introduced different PS1 and PS2 mutant genes into CHO cells, and characterized the expressed proteins by radiosequencing, pulse-chase experiments and pharmacological treatment. In contrast to PS1-expressing cells, cells expressing mutant PS2 showed presenilin endoproteolytic patterns that differed from wt PS2 cells in both mutation-specific and cell type-specific ways. Using two methods of quantitation, we found that a direct effect of the familial AD-linked presenilin mutations is to increase selectively and significantly the cellular production of the highly amyloidogenic Aβ42 peptide. In mutant PS1 and PS2 cell lines with high increases of Aβ42 secretion, spontaneous Aβ oligomer formation was observed, demonstrating that heightened production of Aβ42 by cells results in enhanced Aβ aggregation.

MATERIALS AND METHODS

Generation of PS1- or PS2-transfected CHO Cells—A cDNA encoding human PS1 was obtained by polymerase chain reaction from a human placental library (Clontech). Mutant PS1 (M146L, C410Y) and PS2 (M239V, N14I) cDNAs were also generated by polymerase chain reaction from wt cDNAs. Wild-type and mutant PS1 or PS2 cDNAs were subcloned into CMV-based mammalian expression vectors PCI-neo (PS1) (Promega) or pZeo (PS2) (InVitrogen) and transfected into CHO cells stably transfected with wt APP751 (7W cells; Ref. 23) using Lipofectin (Life Technologies, Inc.). Cells were maintained in 200 μg/ml 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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The half-life of PS1 and APP proteins in double-transfected CHO cells. PS70 cells stably expressing wt PS1 and APP were pulse-labeled for 5 min and chased for 0–5 h with or without BFA (10 μg/ml). Cell lysates were co-immunoprecipitated with 4627 (to PS1) and C7 (to APP). The half-life of wt PS1 holoprotein was ~40 min, and BFA did not block the turnover of PS1 proteins. Overexpression of PS1 did not change the half-life and the post-translational modification of APP. Maturation of full-length APP and proteolytic formation of its 10- and 12-kDa C-terminal fragments were only detected in the absence of BFA. Note that the lower portion of the gel was overexposed to show the PS1 holoprotein, due to its relatively low abundance compared with APP.

FIG. 1. The half-life of PS1 and APP proteins in double-transfected CHO cells. PS70 cells stably expressing wt PS1 and APP were pulse-labeled for 5 min and chased for 0–5 h with or without BFA (10 μg/ml). Cell lysates were co-immunoprecipitated with 4627 (to PS1) and C7 (to APP). The half-life of wt PS1 holoprotein was ~40 min, and BFA did not block the turnover of PS1 proteins. Overexpression of PS1 did not change the half-life and the post-translational modification of APP. Maturation of full-length APP and proteolytic formation of its 10- and 12-kDa C-terminal fragments were only detected in the absence of BFA. Note that the lower portion of the gel was overexposed to show the PS1 holoprotein, due to its relatively low abundance compared with APP.
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Different Endoproteolysis of Mutant PS2 Proteins in CHO and 293 Cells—The above results (Fig. 2) raised the possibility that the processing of mutant PS2 proteins differs from that of wt PS2. This phenomenon was not observed for the PS1 mutants examined here. As reported previously (32), PS1 holoproteins underwent endoproteolysis to form stable 27–28-kDa N-terminal and 17–18-kDa C-terminal fragments, and the fragment pattern did not differ between wt and mutant PS1 proteins expressed in the CHO cells (data not shown). In the case of the PS2 stable cell lines, we detected both the 35-kDa and 18-kDa N-terminal fragments in wt PS2 transfecants, but not in untransfected CHO cells, as determined under steadystate conditions by combined immunoprecipitation-Western blotting of unlabeled cell lysates with antibody 2972 (Fig. 3). The 35-kDa fragment was the major endoproteolytic product, whereas the 18-kDa fragment occurred at low abundance. The latter result was consistent with that of the pulse-chase experiments, which indicated a quick turnover of the 18-kDa fragment versus considerable stability of the 35-kDa fragment (Fig. 2). In CHO cells expressing the M239V (Italian) or N141I (Volga-German) mutations, only the 35-kDa fragment was detected (Fig. 3), suggesting a different proteolytic processing mechanism for wt and mutant PS2 proteins. To confirm that this difference was not confined to CHO cells, we examined the endoproteolysis of PS2 in human kidney 293 cells stably expressing wt or each of the mutant isoforms (Fig. 3). Here, we observed the 35-kDa fragment in both untransfected and PS2-transfected cells, suggesting a greater level of endogenous PS2 expression in 293 than CHO cells. No 18-kDa fragment was detected in any of the 293 cell lines. Moreover, the Italian PS2 mutation selectively induced a significant reduction of the 35-kDa fragment in both cell types, but this was associated with the formation of an additional 30-kDa N-terminal fragment only in the 293 cells (Fig. 3). Taken together, these results demonstrate a striking variability in the proteolytic processing of PS2 holoproteins as a function both of different mutations (i.e. the Volga-German versus Italian mutation) and of different cell types (e.g. CHO versus 293 cells).

Comparison of the Levels of Total Aβ and Aβ42 in Wild-type and Mutant PS1- or PS2-transfected CHO Cells—To determine whether mutant PS proteins alter APP processing, we examined numerous CHO cell lines stably overexpressing wt and mutant PS genes. A total of 13 clones expressing wt PS1 (clones PS70, PS106, and PS111), mutant PS1 (ML45, ML60, ML86, CY6, CY10, and CY11), wt PS2 (PS2–1 and PS2–2), or mutant PS2 (MV31 and MV42) were used for quantitative analysis of APP processing and Aβ production. The various clones of wt or mutant PS1 or PS2 cell lines expressed substantially different levels of PS protein (Fig. 4, A and B). In the PS70 and PS106 wt lines and the ML45, ML60, CY6, and CY10 mutant lines, the PS doublet at 43–45 kDa and the characteristic PS oligomers at −100–140 kDa (31) were readily seen (Fig. 4A). Cell lines PS111, ML86, and CY11 expressed the same set of proteins but at lower levels (data not shown). The difference between the clones with the lowest and the highest PS1 expression levels was about 10-fold. Two wt PS2 clones and two M239V mutant PS2 clones expressed high levels of PS2 holoproteins at 46–55 kDa and higher molecular weight oligomers at −110–140 kDa that were similar to those of PS1 (Fig. 4B). The higher molecular weight bands that were consistently detected in all immunoprecipitations have been shown to represent aggregates of PS proteins in transfected cells (31).

We quantitated APP expression levels in all of these PS1 and PS2 stable transfecants. Compared with the parental 7W cells that were not transfected with PS genes, the biosynthesis and the steady state levels of full-length APP did not change significantly in any of these 13 clones (data not shown). The levels of Aβ40 and Aβ42 were then compared by immunoprecipitation of conditioned media with antibodies that are highly specific for each derivative. Medium from the same culture dish was aliquoted and precipitated with antibodies R1282 (for Aβ40) or 21F12 (for Aβ42 peptides ending at residue 42). Immunoprecipitation of the Aβ42 and Aβ43 from conditioned media of labeled cells by monoclonal antibody 21F12. Note that the ML60, MV31, and MV42 lines show high Aβ42 levels, accompanied by the appearance of low MW Aβ42 oligomers; such oligomers are also seen in the APP V717F mutant line, as reported previously (22).

PS2 (MV31 and MV42) were used for quantitative analysis of APP processing and Aβ production. The various clones of wt or mutant PS1 or PS2 cell lines expressed substantially different levels of PS protein (Fig. 4, A and B). In the PS70 and PS106 wt lines and the ML45, ML60, CY6, and CY10 mutant lines, the PS doublet at 43–45 kDa and the characteristic PS oligomers at −100–140 kDa (31) were readily seen (Fig. 4A). Cell lines PS111, ML86, and CY11 expressed the same set of proteins but at lower levels (data not shown). The difference between the clones with the lowest and the highest PS1 expression levels was about 10-fold. Two wt PS2 clones and two M239V mutant PS2 clones expressed high levels of PS2 holoproteins at 46–55 kDa and higher molecular weight oligomers at −110–140 kDa that were similar to those of PS1 (Fig. 4B). The higher molecular weight bands that were consistently detected in all immunoprecipitations have been shown to represent aggregates of PS proteins in transfected cells (31).

We quantitated APP expression levels in all of these PS1 and PS2 stable transfecants. Compared with the parental 7W cells that were not transfected with PS genes, the biosynthesis and the steady state levels of full-length APP did not change significantly in any of these 13 clones (data not shown). The levels of Aβ40 and Aβ42 were then compared by immunoprecipitation of conditioned media with antibodies that are highly specific for each derivative. Medium from the same culture dish was aliquoted and precipitated with antibodies R1282 (for Aβ40) or 21F12 (for Aβ42 peptides ending at residue 42). Immunoprecipitation of the Aβ42 and Aβ43 from conditioned media of labeled cells by monoclonal antibody 21F12. Note that the ML60, MV31, and MV42 lines show high Aβ42 levels, accompanied by the appearance of low MW Aβ42 oligomers; such oligomers are also seen in the APP V717F mutant line, as reported previously (22).
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except in MV31, \( p < 0.005 \); two-tailed Student’s \( t \) test. Cell lines ML60, MV31, and MV42 secreted more \( \alpha\beta_{42} \) (Figs. 4C and 5A and Table I) than the other lines. The fold increase in \( \frac{\alpha\beta_{42}/\alpha\beta_{\text{total}}}{\text{mean \ of \ all \ ratios}} \) in the latter three lines was in the same range as that of a cell line expressing the APP V717F mutation (Fig. 4C), which is known to induce substantial overproduction of \( \alpha\beta_{42} \) (8).

To confirm these results, the concentrations of \( \alpha\beta_{42} \) and \( \alpha\beta_{\text{total}} \) in the conditioned media of PS1 and PS2 transfectants were measured by sensitive and specific sandwich ELISAs. A scattergraph of all ELISA results is shown in Fig. 5B. When the mean ratios obtained in all wt PS1 (\( n = 24 \)) or wt PS2 (\( n = 11 \)) determinations were normalized to 1.0, the relative \( \alpha\beta_{42}/\alpha\beta_{\text{total}} \) ratios were increased 1.3–2.2-fold in the mutant PS1 and PS2 lines. The differences between the wt and mutant lines were highly statistically significant (\( p < 0.001 \), except in CY10, \( p < 0.02 \)), and correlated well with the quantitation by immunoprecipitation in the same cell lines.

Oligomerization of Secreted Aβ in PS1 and PS2 Mutant Cell Lines—In the course of the immunoprecipitation studies, we observed the occurrence of low molecular weight oligomers of Aβ in the conditioned media of the APP V717F cell line, as reported previously (22). The V717F line produced more \( \alpha\beta_{42} \) than the APP wild-type 7W cell line, as expected (8), and it showed 42-specific oligomeric Aβ bands by immunoprecipitation with 21F12 and SDS-PAGE (Fig. 4C, lane V717F). Interestingly, the three PS1 or PS2 mutant clones with the highest level of \( \alpha\beta_{42} \) production, ML60, MV31, and MV42 (Fig. 5, A and B), showed low molecular weight oligomeric species indistinguishable from those of APPV717F (Fig. 4C, lanes ML60, MV31, and MV42). Because the presence of two additional hydrophobic residues at the C terminus of \( \alpha\beta_{42} \) is known to increase its fibrillogenic potential in vitro and lead to its seeding the aggregation of the \( \alpha\beta_{42} \) peptide (33), the appearance of \( \alpha\beta_{42} \) containing oligomers in the medium further supports the substantially heightened production of \( \alpha\beta_{42} \) by cell lines expressing mutant PS1 or PS2.

DISCUSSION

The PS1- and PS2-linked cases of AD are distinguishable from common sporadic AD cases principally by their earlier clinical onset and more severe neuropathology (34–36). Therefore, elucidating the genotype-to-phenotype relationship of the presenilin mutations should shed light on factors that are important in the pathogenesis of AD in general. Here, we show that expression of a mutant presenilin gene in cultured cells results in a selective and statistically significant increase in the secretation of the highly amyloidogenic \( \alpha\beta_{42} \) peptide. For most clones, the increase in the \( \frac{\alpha\beta_{42}/\alpha\beta_{\text{total}}}{\text{ratio}} \) obtained by immunoprecipitation was closely similar to that obtained by ELISA (Table I). Thus, the results were internally consistent. Our results are in agreement with the selective elevation of \( \alpha\beta_{42} \) levels in plasma and skin fibroblast media obtained from living PS1 and PS2 patients (10). One of the PS1 mutations examined in the latter studies is at the same codon (146) as one we tested. Moreover, the degree of increase we observed, 1.3–2.5-fold, is the same as that obtained in vivo (10) and in the same range as that resulting from the APP 717 mutation (8).

We observed no obvious relationship between the level of PS1 expression and the degree of increase in \( \frac{\alpha\beta_{42}/\alpha\beta_{\text{total}}}{\text{ratio}} \). For example, the clone with the highest increase (M146L-60) had lower PS1 protein levels than clone C410Y-6, which showed a relatively small but still significant (\( p < 0.001 \)) increase. This result strongly suggests that mutant presenilin proteins confer a dominant negative gain of function, which can be seen even at low levels of expression and in the presence of the endogenous wt presenilins.

The fact that expressing solely a mutant presenilin cDNA in a cultured peripheral cell leads directly to a selective increase in \( \alpha\beta_{42} \) similar to that observed in vivo (i.e. in plasma and primary fibroblasts) indicates that factors unique to the brain or to the AD state are not required for this Aβ overproduction.

**Table I**

| Mutant PS1 or PS2 cell line | \( n \) | \( \frac{\alpha\beta_{42}/\alpha\beta_{\text{total}}}{\text{wt (1.00) (mean ± S.E.)}} \) | \( p \) value |
|-----------------------------|------|---------------------------------|------------|
| PS1: quantitation by gel phosphorimaging (wt: \( n = 40 \)) |      |                                 |            |
| M146L-45                    | 15   | 1.39 ± 0.09                     | <0.001     |
| M146L-60                    | 11   | 1.54 ± 0.23                     | <0.001     |
| M146L-86                    | 14   | 1.54 ± 0.09                     | <0.001     |
| C410Y-6                     | 16   | 1.53 ± 0.07                     | <0.001     |
| C410Y-10                    | 14   | 1.57 ± 0.07                     | <0.001     |
| C410Y-11                    | 12   | 1.57 ± 0.07                     | <0.001     |
| PS1: quantitation by ELISA (wt: \( n = 24 \)) |      |                                 |            |
| M146L-45                    | 12   | 1.34 ± 0.06                     | <0.001     |
| M146L-60                    | 12   | 2.01 ± 0.15                     | <0.001     |
| C410Y-6                     | 12   | 1.50 ± 0.12                     | <0.001     |
| C410Y-10                    | 12   | 1.30 ± 0.09                     | <0.02      |
| PS2: quantitation by gel phosphorimaging (wt: \( n = 10 \)) |      |                                 |            |
| M239V-31                    | 5    | 2.29 ± 0.34                     | <0.005     |
| M239V-42                    | 6    | 2.37 ± 0.25                     | <0.001     |
| PS2: quantitation by ELISA (wt: \( n = 11 \)) |      |                                 |            |
| M239V-31                    | 2    | 2.33 ± 0.06                     | <0.001     |
| M239V-42                    | 2    | 1.61 ± 0.05                     | <0.001     |
Excessive Aβ_{42} production thus appears to be an initial consequence of mutant presenilin expression, a conclusion that is supported by the findings that carriers of PS1 mutations can show elevated Aβ_{42} levels in plasma presymptomatically, while the majority of asymptomatic subjects with sporadic AD show no increase in Aβ_{42} levels despite advanced clinical disease (10). Our results are consistent with the recent demonstration of a significant 2-fold increase in the density of Aβ_{42} plaques in the brains of subjects bearing a PS1 mutation, compared with their density in severe sporadic AD cases (34). Furthermore, Aβ_{42} has been shown to be the initial constituent of plaques in AD and Down’s syndrome, preceding the development of the other cytopathological features of AD by many years or decades (37–39).

Of particular interest in this study was the spontaneous appearance of Aβ_{42} oligomers in the conditioned media of PS1 or PS2 mutant cells with high (>2-fold) elevation in Aβ_{42} secretion. Peptides ending at Aβ_{42} appear to be a major constituent of the diffuse plaques seen initially in AD and Down’s syndrome (34, 36, 40–44), and they have been proposed to serve as a nidus for the aggregation of the more abundant Aβ_{40} peptides (33). In contrast to wt APP expressing cells, cells expressing V717F mutated APP consistently showed Aβ_{42} oligomer formation in the media, and these cells are known to undergo a selective increase in Aβ_{42} secretion. Similar rises in Aβ_{42} secretion caused by mutant PS1 or PS2 also led to Aβ_{42} oligomer formation. The appearance of Aβ_{42} oligomers correlated with the relative increase in Aβ_{42} levels; in cells expressing the same PS1 mutation but showing Aβ_{42} elevations of less than 2-fold, no Aβ_{42} oligomer was detected under the same in vitro condition. Based on these findings, we postulate that PS1 and PS2 mutations, expressed throughout the lifetime of the host, gradually lead to sufficient elevation of extracellular Aβ_{42} levels in the brain to induce Aβ_{42} oligomerization, with subsequent diffuse plaque formation and later accrual of Aβ_{40} peptides. The sequential appearance of first Aβ_{42} and then Aβ_{40} immunoreactive plaques in Down’s patients of increasing age (38, 39) are consistent with this hypothesis.

The mechanism of the selective increase in Aβ_{42} production caused by mutant presenilin remains to be elucidated. It is unlikely that PS proteins generally affect APP synthesis or metabolism, as the posttranslational modification and turnover of total APP proteins were not changed by introduction of wt or mutant PS genes. Rather, we speculate that APP proteins may interact directly with PS1 or PS2 proteins in a presenilin-rich compartment of the cell (e.g. the ER or early Golgi) and that APP interacts with mutant presenilin in a manner that leads to its increased exposure to the γ-secretase cleaving specifically after residue 42 of the Aβ region. Evidence for the existence of 40- and 42-specific γ-secretases in mammalian cells has recently been presented (26). Wild-type PS might, for example, form a complex with APP (or the 12-kDa C-terminal fragment arising from β-secretase cleavage), and this complex might prevent access of APP to a 42-specific γ-secretase in the ER; mutations in PS could prevent the proper formation of such complexes.

Since PS1 is known to undergo constitutive endoproteolysis, and very little intact holoprotein is detectable in cells or tissues expressing this gene (32), we searched for evidence of a change in endoproteolytic patterns in cells bearing mutant presenilins. We did not observe a significant change in fragment formation in the two PS1 missense mutations we examined, despite a consistent and significant increase of Aβ_{42}/Aβ_{total} in all clones expressing these mutations. However, the mutant form of PS1 that has a deletion of exon 9 undergoes no endoproteolysis (32), and yet this mutation also causes a selective increase in Aβ_{42} secretion in transfected cells (45). Therefore, interference with the normal endoproteolysis of PS1 appears not to be an obligatory step in producing the mutant phenotype. In the case of our analyses of PS2 mutations, we searched for PS2 endoproteolytic products and identified a major 35-kDa N-terminal fragment. Its size is larger than the 28-kDa derivative, which is the major N-terminal fragment of PS1, indicating that the size of PS2 endoproteolysis is substantially C-terminal to that of PS1, which occurs at and around residue 298 (46). The proteolytic fragment patterns varied when two distinct mutations were expressed in the same cell type (293) and also varied when the same mutation (M239V) was expressed in two different cell types (CHO and 293). Despite these complex differences in PS2 endoproteolysis, the PS2 M239V mutant cell line still showed increased Aβ_{42} secretion in both CHO and 293 cells (this study). Our findings, therefore, point to the need for caution in interpreting changes in presenilin processing as a result of PS mutations. Any mutation should be analyzed in at least two cell types, and multiple mutations should be examined before any conclusion about the pathogenic role of altered endoproteolysis is reached.

In summary, our results strongly support the hypothesis that familial AD-linked mutations in PS1, PS2, and APP all cause AD by increasing the cellular production of Aβ_{42}, thereby accelerating the polymerization of this and other Aβ peptides and promoting cerebral accumulation of Aβ as an essential early event in AD pathogenesis. These findings provide further impetus for current efforts to identify compounds which inhibit the production or the aggregation of Aβ as therapeutic agents for AD.

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