Presenilin 1 Stabilizes the C-terminal Fragment of the Amyloid Precursor Protein Independently of γ-Secretase Activity*†‡

Didier Pitsi and Jean-Noël Octave‡

From the Laboratoire de Pharmacologie Expérimentale, Université Catholique de Louvain, 1200 Brussels, Belgium

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The cleavage of the transmembrane amyloid precursor protein (APP) by β-secretase leaves the C-terminal fragment of APP, C99, anchored in the plasma membrane. C99 is subsequently processed by γ-secretase, an unusual aspartyl protease activity largely dependent on presenilin (PS), generating the amyloid β-peptide (Aβ) that accumulates in the brain of patients with Alzheimer’s disease. It has been suggested that PS proteins are the catalytic core of this proteolytic activity, but a number of other proteins mandatory for γ-secretase cleavage have also been discovered. The exact role of PS in the γ-secretase activity remains a matter of debate, because cells devoid of PS still produce some forms of Aβ. Here, we used insect cells expressing C99 to demonstrate that the expression of presenilin 1 (PS1), which binds C99, not only increases the production of Aβ by these cells but also increases the intracellular levels of C99 to the same extent. Using pulse-chase experiments, we established that this results from an increased half-life of C99 in cells expressing PS1. In Chinese hamster ovary cells producing C99 from full-length human APP, similar results were observed. Finally, we show that a functional inhibitor of γ-secretase does not alter the ability of PS1 to increase the intracellular levels of C99. This finding suggests that the binding of PS1 to C99 does not necessarily lead to its immediate cleavage by γ-secretase, which could be a spatio-temporally regulated or an induced event, and provides biochemical evidence for the existence of a substrate-docking site on PS1.

The deposition of amyloid-β peptides (Aβ) in the brain is an invariable feature of Alzheimer’s disease. These short 39–42 amino acid peptides result from the sequential cleavage of their precursor, the amyloid precursor protein (APP), by β- and γ-secretase activities. When the β-secretase activity is performed by a well characterized aspartyl protease, β-site APP-cleaving enzyme 1 (BACE 1) (1), the γ-secretase activity has been shown to require the interaction of at least four proteins, namely presenilin (PS), nicastrin (NCT), Pen-2 (presenilin enhancer 2), and Aph-1 (anterior pharynx defective 1) (2, 3). The mutations of the presenilin 1 and 2 genes are the major cause of familial Alzheimer’s disease, and these proteins were, for a long time, the only candidates for γ-secretase activity. As this last cleavage of APP releases a possible causal agent of Alzheimer’s disease, it constitutes a prime target for therapeutic intervention, and studies on the potential proteolytic activity of PS have accumulated over the years. Despite a number of convincing demonstrations, these experiments left a number of unresolved issues, the most striking example of which being the ability of cells lacking PS to still produce intracellular Aβ1–42 (4, 36). The assembly of the γ-secretase complex would first involve the binding of Aph-1 to NCT (5). This first complex, in turn, binds to full-length PS and recruits Pen-2, inducing the cleavage of PS into what is believed to be its active, heterodimeric form (6). The whole complex is then translocated to the Golgi apparatus where NCT is glycosylated, a post-translational modification that could be required for γ-secretase activity (7, 8). Nevertheless, the exact relation between the assembly of this complex and the production of Aβ is still not clear, because the coexpression of all of these partners in cellular models leads to a 10-fold increase of the levels of heterodimeric PS and to the degradation of the substrates of γ-secretase without affecting the secretion of Aβ (9). Moreover, because PS1 is known to influence the trafficking of a number of proteins, it has recently been shown to also regulate the trafficking of APP from the trans-Golgi network and the endoplasmic reticulum, suggesting that it might indirectly regulate the cleavage of APP by controlling its trafficking toward cellular compartments where the γ-secretase activity is located (10). Finally, the endogenous γ-secretase activity observed in most cell types complicates the investigation of the subtle changes that transgenes could trigger. Even in yeast, a model lacking γ-secretase activity in which the coexpression of the four aforementioned proteins allowed the production of Aβ (11), it obviously cannot be excluded that other essential endogenous proteins are present in these cells and are the still undiscovered partners of this activity. To clarify the role of PS1 in the γ-secretase activity, we used Spodoptera frugiperda SF9 insect cells, which are deficient in the amyloidogenic pathway and, like in yeast, have no described orthologue of PS. Here we show that SF9 cells expressing either human APP and BACE 1 or C99, the major substrate of γ-secretase, secrete low levels of Aβ1–40. Examining the effect of the expression of human PS1 in C99 clones, we measured a 2-fold increase in both the production of Aβ and the intracellular levels of C99, which leaves the Aβ/C99 ratio virtually unchanged. This led us to hypothesize that, in our model, PS1 could function as a chaperone protein protecting C99 from degradation. We therefore performed pulse-chase experiments and demonstrated that the...
half-life of C99 doubles in the presence of PS1. Furthermore, we confirmed these results in CHO cells expressing human APP, where C99 is physiologically produced by endogenous β-secretase. Finally, we show that a functional inhibitor of γ-secretase activity, DAPT, inhibits the secretion of Aβ but does not impede the ability of PS1 to increase the intracellular steady states of C99. Taken together, these results show that PS1, independently of its direct involvement in γ-secretase activity, is able to protect C99 from degradation and thus increase its availability for γ-secretase, supporting the idea that the influence of PS1 on γ-secretase activity could also be indirect.

**EXPERIMENTAL PROCEDURES**

**Materials**—The pIZ/V5-His and pB/V5-His plasmids, Insectin-Plus™, Zeocin™, blasticidin, fetal calf serum, cell culture media, and NuPage™ 4–12% bis-Tris gels were purchased from Invitrogen. Protein A-Sepharose CL-4B was purchased from Amersham Biosciences. DAPT was a kind gift from L. Mercken, Aventis, Vitry-sur-Seine, France.

**Antibodies**—The rabbit APPC-ter polyclonal antibody raised against the 17 C-terminal amino acids of APP was a generous gift from A. Delacourte. The mouse WO2 monoclonal antibody raised against the human Aβ5–8 sequence was purchased from Abeta GmbH, Heidelberg, Germany. The rat MAB1563 monoclonal antibody raised against the N terminus (residues 21–80) of human PS1 was purchased from Chemicon International Inc., Temecula, CA. The rabbit non-human specific P7854 polyclonal antibody raised against the C terminus (residues 450–467) of PS1 was purchased from Sigma-Aldrich. The rabbit anti-BACE 1 polyclonal antibody, raised against amino acids 485–501 of BACE 1, was purchased from Affinity Bioreagents, Golden, CO.

**Cell Culture and Treatments**—CHO cells were grown in 80% F12 medium supplemented with 10% fetal serum albumin for 30 min. After the removal of protein A-Sepharose by washing, the immunoreactive bands were visualized by chemiluminescence. Where appropriate, bands were quantified using a Cyclone™ storage phosphor system (Packard Instrument Co.).

**RESULTS**

**Metabolism of APP in Sf9 Cells Coexpressing Human APP and BACE 1**—As demonstrated previously (12), expression of human APP in Sf9 cells leads to the production of the α-secretase cleavage product C83 (Fig. 1, lane 1) but not to the production of the β-secretase cleavage product C99 (Fig. 1, lane 2). This lack of β-secretase activity could explain the inability of these cells to generate Aβ from human APP695 without excluding the possibility that they could also show impaired γ-secretase activity. To establish the amyloidogenic pathway in Sf9 cells, we first transfected the cDNA encoding the human β-secretase, BACE 1, in clones producing APP. This resulted in the production of the immature and mature glycosylated forms of BACE 1 (Fig. 1, lane 4) and induced the production of the expected cleavage product of APP, C99 (Fig. 1, lane 3). In previous experiments, we tried to immunoprecipitate Aβ in the culture medium of Sf9 cells expressing APP and BACE 1. Compared with rat neurons infected with an adenovirus encod-
expression of human APP, which clearly produce amyloid peptide, it was clear that the production of Aβ by Sf9 cells was either non-existent or largely below what is observed in neurons, dropping below our detection limit. Using a much more sensitive ELISA, however, we detected low levels of Aβ1–40 (327 ± 67 pg/ml; n = 3) in the medium of cells expressing APP and BACE 1. As expected, cells expressing APP alone did not produce detectable amounts of Aβ. All assays were performed after 12 h of incubation, in accordance with our previous work in neurons.

Influence of PS1 on the Catabolism of C99, the Substrate of γ-Secretase—Presenilin 1, mutations of which are known to be the cause of most hereditary forms of Alzheimer’s disease, has been demonstrated to be tightly implicated in γ-secretase activity, although its exact function in the γ-secretase complex is unclear. Recent evidence showed that although presenilins bind to the substrates of γ-secretase (12, 15), they are not mandatory for some types of γ-secretase cleavages, indicating a role for presenilins apart from proteolysis, possibly in sorting, transport, or exposure of the substrates to γ-secretase activity (4, 16, 36). Because of the poor characterization of the Sf9 genome so far, it is not known whether these cells contain some PS orthologues, and we wanted to examine whether expression of human PS1 could modulate the production of Aβ in Sf9 cells. Transfection of the PS1 cDNA in clones expressing APP and BACE1 was hampered by the unavailability of an expression plasmid for Sf9 cells encoding an additional antibiotic resistance gene. Moreover, transiently transfected cells only produced low levels of the protein (not shown). Also, we had noticed previously that infection of Sf9 cells by recombinant baculoviruses leads to artifacts as well as to rapid and massive cell death (12). Therefore, we decided to express PS1 in Sf9 cells expressing C99 fused to the signal sequence of human APP, a protein that does not need to be cleaved by BACE1 to become a substrate of γ-secretase. Clones expressing C99 produced large amounts of the protein (Fig. 2, lane 1). When the same C99 clone was transfected with the plasmid encoding human PS1 (Fig. 2, lane 2), we observed a normal endoproteolytic cleavage of PS1 (Fig. 2, lane 3) leading to the formation of the active, heterodimeric form of PS1 (17). Monitoring the production of Aβ1–40 in the culture medium of C99 cells by ELISA, we again observed a low production of Aβ (116 ± 16 pg/ml; n = 5) after 12 h. Interestingly, cells coexpressing C99 and PS1 produced nearly twice (311 ± 18 pg/ml; n = 5) the quantity of Aβ observed in their counterparts devoid of presenilin.

Expression of PS1 Induces an Increase of Both Aβ Production and Intracellular C99—Using the same sensitive ELISA, we then studied the time-dependent production of Aβ in C99 cells (Fig. 3A). These experiments showed that the production of Aβ was maximal after 12 h and that the kinetics of production of Aβ are similar, whether or not PS1 is expressed in the C99 clone. The experiments also confirmed that cells co-expressing C99 and PS1 produce significantly more Aβ than cells expressing C99 alone (Fig. 3B). This finding suggests that γ-secretase activity is increased in cells producing human PS1. Careful analysis of the metabolism of C99 in these cells revealed that this was not the case. On the contrary, cells expressing PS1 produced more than twice the amount of C99 observed in cells expressing C99 alone (Fig 3C). Therefore, the expression of human PS1 in Sf9 cells does not seem to modify their Aβ/C99 ratio or, thus, their γ-secretase activity. It does, however, reproducibly increase the levels of C99 in the cells (n = 5).

PS1 Slows Down the Decay of C99—One hypothesis that explains the increase in C99 levels in the presence of PS1 is that the binding of PS1 to C99 could lead to its stabilization or protect it against degradation. This would increase the steady state of C99 in cells and, hence, its availability for the γ-secretase activity. To assess this possibility, we performed pulse-chase experiments. Labeling the newly synthesized proteins with [35S]methionine for an hour before chasing with cold methionine then indicated that the amounts of C99 recovered after 3 h of chase were approximately doubled upon the coexpression of PS1 (Fig. 4, A and B).

Similar Influence of PS1 on C99 Produced from Full-length Human APP in CHO Cells—Although C99 has the same sequence as the C-terminal fragment of APP generated by BACE1 and is a good substrate for γ-secretase activity in Sf9 cells as well as in primary cultures of rat neurons (12), its subcellular localization is probably partially different. C99 is directly present in the endoplasmic reticulum and travels through the secretory pathway, whereas the cleavage of APP by BACE1 is relatively weak in the endoplasmic reticulum and maximal in the trans-Golgi network (18, 19). We therefore decided to confirm our results in a model containing endogenous β-secretase activity and to study the influence of PS1 on the production of C99 from full-length APP. As it was not possible to obtain stable triply transfected Sf9 cells expressing APP, BACE1, and PS1, we analyzed CHO cells that express an endogenous orthologue of BACE1 and in which the stable expression of human APP alone or together with human PS1 has been established (13). Comparing the amounts of C99 produced in the clone expressing APP alone with the amounts of C99 produced after transfecting the same clone with the PS1 cDNA, we found a 2-fold increase in C99 in the presence of PS1 (n = 4), similar to what we had observed in Sf9 cells expressing C99.
Fig. 4. Influence of PS1 on the turnover of C99. A, typical pulse-chase of C99 in SF9 cells shows the time-dependent decay of C99 in a clone expressing C99 alone (upper lane) and in the same clone also expressing PS1 (lower lane). B, quantification of the pulse-chase experiments on C99 and C99 plus PS1 (C99 + PS1) cells, expressed as percentages of the respective initial (T0) levels, shows that PS1 slows down the decay of C99 (mean of two independent experiments). S.D. values are indicated.

(Fig. 5, A and B). The amounts of C99, the product of the alternative cleavage by BACE1 that is also a substrate for the γ-secretase activity, were also increased in the presence of PS1. Moreover, this effect of PS1 on the steady-state of C99 was not due to overexpression artifacts, because the total levels of the endoproteolytic fragments of PS1 were not increased in cells expressing human PS1, whereas levels of the full-length protein, which is unlikely to be involved in the secretion of Aβ, were only modestly elevated (Fig. 5A, right panel). Cells expressing human APP alone produced 3,624 ± 290 pg of extracellular Aβ1–40 per milliliter (n = 7), whereas cells expressing both APP and PS1 produced 5567 ± 722 pg of extracellular Aβ1–40 per milliliter (n = 9) (Fig. 5B). Because these results were similar to those observed in SF9 cells, pulse-chase experiments were undertaken to measure the decay of C99 produced from APP by CHO cells. Similar to what was observed in SF9 cells, the presence of PS1 slowed down the decay of C99, approximately doubling the amounts of C99 retrieved after 3 h of chase (Fig. 5C). The decay of full-length APP was not significantly modified by the expression of PS1 (not shown).

Stabilization of C99 by PS1 Is Independent of γ-Secretase Activity—Presenilins are often described as multifunctional proteins, as they are involved in both the cleavage of some substrates, like APP or Notch, as well as in the trafficking of other partners such as telencephalin, N-cadherin, or even APP itself (10, 20, 21). The possible relationship between these two functions of PS is, to date, unclear. Therefore, we decided to investigate the possible relation between the levels of C99 and γ-secretase activity in the absence or presence of PS1. To inhibit γ-secretase activity, SF9 cells were treated for 12 h with DAPT, a functional γ-secretase inhibitor that does not affect the levels of PS1 or its endoproteolytic cleavage (not shown). As shown in Fig. 6A, this resulted in a decrease in Aβ1–40 production close to the limit of detection in cells expressing C99 alone (from 176 ± 50 pg to 92 ± 7 pg of Aβ1–40 per milliliter; n = 3) as well as in cells also expressing PS1 (from 369 ± 121 pg to 99 ± 55 pg of Aβ1–40 per milliliter; n = 3). As expected, the inhibition of C99 cleavage by γ-secretase increased the intracellular levels of C99 (Fig. 6B) without modifying the ability of PS1 to increase the steady state of C99. Interestingly, the inhibition of γ-secretase activity also resulted in the appearance of C83, the cleavage product of α-secretase activity (Fig. 6C). This shows that C99 is a substrate of α-secretase in our model and confirms that directly expressed C99 reaches the late secretory compartments and/or plasma membrane where α-secretase activity takes place. The accumulation of C83 in presence of DAPT probably results from both the inhibition of its cleavage by γ-secretase and the increased levels of C99 that cannot be cleaved by the γ-secretase activity late in the secretory pathway, thereby increasing its availability for α-secre-

tase. It can also be noted that, just as for CHO cells (Fig. 5A, left panel), the expression of human PS1 also increased the steady state of C83 in SF9 cells.
Because PS1-null neurons were demonstrated to have a reduced production of Aβ and accumulation of C99 (22), PS proteins have been proposed to be associated with the γ-secretase cleavage of APP. Over the years it has become apparent that these multispan proteins are key components of secretase activity, ultimately releasing the amyloid peptide from its precursor. In addition, PS proteins have been recognized as being part of a larger complex that also contains NCT, Aph-1, and Pen-2 (2, 3). The expression levels of these four proteins influence each other, representing a limiting factor in the generation of functional γ-secretase activity (6). However, it is still not clear whether this 250-kDa complex contains all the partners involved in the last cleavage of APP, because some workers have consistently observed larger complexes containing γ-secretase activity (23). Also, none of the proteins recognized to date resembles a protease. Nonetheless, PS proteins have been suggested as being the unusual catalytic core of the γ-secretase complex, hydrolyzing APP in the lipid-bilayer through their two membrane-embedded, conserved Asp residues (24). Although mutational and pharmacological studies support this contention (24, 25), other experiments argue against this interpretation. For example, inhibitors of the γ-secretase differentially affect the production of Aβ1–40, Aβ1–42, or the cleavage of Notch (25–27). Also, PS proteins are not mandatory for the generation of intracellular Aβ1–42 or extracellular Aβ1–42 (4, 28, 36). Other studies have implicated PS in the trafficking and the post-translational modification of several proteins (10, 16, 29), indicating that if PS proteins indeed possess a proteolytic activity, then they are proteins with a dual role.

Here, we took our initial experiments (12) assessing the function of human PS1 in Sf9 insect cells, a model lacking the amyloidaligenic pathway, a step further. Using a sensitive ELISA, we first demonstrated that the coexpression of APP and BACE1 in Sf9 cells induces the production of extracellular Aβ1–40 that was not detected by immunoprecipitation. As expected, expression of the C-terminal fragment of APP C99 also induced the secretion of Aβ1–40. Interestingly, cells expressing APP and BACE1 produced less β-secretase-cleaved APP than did cells expressing C99 but generated twice as much Aβ1–40. This could be explained by the presence of C99 in the endoplasmic reticulum, as compared with its much later production from APP by BACE1 in the trans-Golgi network or the endosomes. Given that BACE1 has been demonstrated as interacting with NCT (30) and that Sf9 cells express endogenous NCT (data not shown), it is possible that some kind of activation of the β-secretase cleavage occurs when BACE1 is present.

Having assessed a basal γ-secretase activity in Sf9 cells, where the lack of endogenous β-secretase has been bypassed, we tried to modulate this activity. As PS1 is the best candidate for the proteolytic activity of the γ-secretase complex at present, we monitored the effect of its stable expression in Sf9 cells expressing C99. Human PS1 was endoproteolytically processed in insect cells, and its expression increased the production of Aβ1–40, suggesting either a role for PS1 in the cleavage of C99 or the secretion of Aβ. Unexpectedly, the extracellular increase of Aβ was paralleled by an increase of C99 in the cells, leaving the Aβ1–40/C99 ratio unchanged. Therefore, we conclude that the increase in Aβ production was mainly the result of an accumulation of C99 in the presence of PS1. As we have demonstrated previously that PS1 binds to C99 under our experimental conditions (12), we tested whether this association could protect C99 from degradation rather than leading to its cleavage. Pulse-chase experiments established that, in our model, PS1 clearly slows the decay of C99. Although it is not known if an endogenous equivalent of PS is present in Sf9 cells, which could account for their low γ-secretase activity, we show here that the association of human PS1 with C99 does not automatically lead to Aβ generation. On the contrary, PS1 stabilizes C99 and, thus, could play a key role as a chaperone regulating the transport of C99, protecting it from degradation pathways such as the proteasome (31) (data not shown), and increasing its availability for the γ-secretase.

To confirm our results, we investigated whether PS1 exerts similar effects in a model physiologically producing C99 from APP. Directly expressed C99 and C99 generated from the cleavage of APP by BACE1 have the same sequence, and both are substrates of γ-secretase in Sf9 cells as well as in neuronal models (this paper and Ref. 12). However, their intracellular distributions are probably different, with directly expressed C99 being readily produced in the endoplasmic reticulum, while the majority of the β-secretase cleavage of APP occurs later in the secretory pathway (18, 19). In line with previous observations in Sf9 cells directly expressing C99, CHO cells expressing human APP and containing endogenous β-secretase activity showed increased levels of both secreted Aβ1–40 and C99 on the expression of human PS1. This finding was not due to artifacts linked to the overexpression of PS1, as the levels of heterodimeric, active PS1 proteins were not elevated in CHO cells producing human APP, whereas the levels of full-length PS1 proteins in the early secretory pathway were only modestly increased, as reported previously (17). Yet, some effects of PS1 expression are less drastic in the CHO model. These differences in severity could result from the following: (i) the influence of BACE1 on the γ-secretase cleavage mentioned previously; (ii) differences in the levels of transgene expression; (iii) different cellular distributions of C99 and the β-cleaved product of APP; or (iv) the presence of an endogenous orthologue of PS1 in CHO cells. The results obtained in CHO cells nevertheless confirm our observations in a more straightforward Sf9 model.

Finally, we investigated further the relationship between the two potential functions of PS1 in our model, i.e. by testing whether the influence of the expression of PS1 on the production of Aβ came from an upstream increase of the levels of C99. To discriminate between the cleaving and trafficking activities of PS1, we inhibited γ-secretase activity by DAPT for 12 h, a time exceeding by far the half-life of C99. This resulted in a decrease in Aβ production near the detection limits, both in cells expressing C99 alone and in cells expressing C99 and PS1. As the later model produces much higher amounts of Aβ, this result shows that DAPT is able to inhibit the extra production of Aβ induced by the expression of PS1. This finding confirms that the same DAPT-sensitive γ-secretase activity is responsible for the production of Aβ by Sf9 cells, whether they express human PS1 or not. Because it is not known whether DAPT binds to PS1 to exert its effects, two explanations are possible. First, PS1 could be responsible for the proteolytic activity of γ-secretase, which would mean that an orthologue of PS1 exists in Sf9 cells. Alternatively, the effect of PS1 on the γ-secretase activity could be located upstream of the cleavage of C99, and the DAPT-sensitive enzyme producing Aβ would differ from PS1 and would be endogenously present in Sf9 cells. In parallel to decreasing the production of Aβ, DAPT treatments slightly increased the amount of intracellular C99 by blocking a C99 degradation pathway. However, DAPT treatment did not modify the ability of PS1 to increase the steady state of C99. This finding indicates that γ-secretase activity and PS1-mediated stabilization of C99 are independent events, the latter probably being an upstream event.

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DISCUSSION

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Taken together, our results show that the increase in Aβ1–40 production induced by the expression of PS1 reflects an increase in substrate availability for γ-secretase activity rather than a direct increase in secretase activity. Although the results certainly do not rule out a key role for PS1 in Aβ production, they shed new light on its function and ability to influence the steady state of C99. Although PS1 has been shown to regulate the trafficking of full-length APP (10), our results suggest that it can also influence the stability of C99 and thus its accessibility to γ-secretase. Inasmuch as we previously demonstrated an interaction between C99 and PS1 in SF9 cells (12), we now bring forth biochemical evidence to support recent pharmacological reports indicating the presence of a binding site on PS for APP C-terminal fragments that is plex upstream in Aβ. The formation of such a complex upstream in Aβ production could regulate both the trafficking and, along with additional partners, the cleavage of γ-secretase substrates. Fundamentally, this opens the possibility that the association of PS1 with γ-secretase substrates does not necessarily induce their immediate cleavage but could be a spatio-temporally regulated or even an induced event. These advances in understanding the different activities of PS and the relationships between them should help in determining their exact role in the generation of Aβ and might well ultimately reconcile currently discordant data.

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