The Role of Presenilin-1 in the \( \gamma \)-Secretase Cleavage of the Amyloid Precursor Protein of Alzheimer's Disease*

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Presenilin-1 (PS1) is required for the release of the intracellular domain of Notch from the plasma membrane as well as for the cleavage of the amyloid precursor protein (APP) at the \( \gamma \)-secretase cleavage site. It remains to be demonstrated whether PS1 acts by facilitating the activity of the protease concerned or is the protease itself. PS1 could have a \( \gamma \)-secretase activity by itself or could traffic APP and Notch to the appropriate cellular compartment for processing. Human APP 695 and PS1 were coexpressed in SF9 insect cells, in which endogenous \( \gamma \)-secretase activity is not detected. In baculovirus-infected SF9 cells, PS1 undergoes endoproteolysis and interacts with APP. However, PS1 does not cleave APP in SF9 cells. In CHO cells, endocytosis of APP is required for \( \beta \)-secretion. Deletion of the cytoplasmic sequence of APP (APPΔC) inhibits both APP endocytosis and \( \beta \) production. When APPΔC and PS1 are coexpressed in CHO cells, \( \beta \) is secreted without endocytosis of APP. Taken together, these results conclusively show that, although PS1 does not cleave APP in SF9 cells, PS1 allows the secretion of \( \beta \) without endocytosis of APP by CHO cells.

The production and accumulation of the amyloid peptide (\( \beta \)) in the cerebral cortex is a central event in the pathogenesis of Alzheimer's disease. \( \beta \) is produced in the course of normal cellular metabolism of the \( \beta \)-amyloid precursor protein (APP) (1). A portion of APP is cleaved by \( \alpha \)-secretase within the \( \beta \) region. This non-amyloidogenic pathway, which precludes the formation of full-length \( \beta \), occurs during the processing of APP to the plasma membrane. This cleavage releases the soluble N-terminal domain of APP containing the first 17 amino acids of \( \beta \). Another fraction of newly synthesized APP appears at the plasma membrane. Following endocytosis of this transmembrane APP, the cleavage by \( \beta \)-secretase at the N terminus of \( \beta \) generates a C-terminal fragment of APP that contains the entire \( \beta \) sequence. The final step in the generation of \( \beta \) is an apparently intramembranous cleavage of this C-terminal fragment by \( \gamma \)-secretase.

The most common causes of familial Alzheimer's disease are mutations in genes encoding presenilins (PS) 1 and 2. These mutations alter APP processing and cause increased production of the high amyloidogenic \( \beta \) 42 (2). Moreover, PS1-deficient mice show decreased \( \gamma \)-secretase processing of APP (3). PS are hydrophobic proteins that cross 6–8 times the membrane of the endoplasmic reticulum. A limited portion of PS undergoes endoproteolysis, and the resulting N- and C-terminal fragments are localized predominantly in the Golgi (4). Presenilins are homologous to proteins involved in vesicle transport or in the Notch developmental pathway in the nematode Caenorhabditis elegans (5), and PS1-deficient mice show developmental abnormalities consistent with altered Notch signaling (6).

Signaling through the receptor protein Notch requires ligand-induced cleavage of Notch (7). The recent demonstration that PS1 deficiency reduces the proteolytic release of the Notch intracellular domain indicates that PS1 regulates both APP processing and Notch signaling by influencing protein cleavage events (8–11). Because PS1 is required for both the release of the intracellular domain of Notch and the \( \gamma \)-secretase cleavage of APP, it has been proposed that PS1 acts by facilitating the activity of the protease involved or is the protease itself. Mutagenesis of two aspartate residues completely abolishes PS1 endoproteolysis and \( \gamma \)-secretase activity, suggesting that PS1 could be an autoactivated membranous aspartyl protease (8, 12).

To study whether PS1 indeed displays a \( \gamma \)-secretase activity, human APP 695 and PS1 were coexpressed in SF9 cells using recombinant baculoviruses. The insect cells have been demonstrated to transport human APP at the cell surface in the correct transmembrane orientation (13). Moreover, human APP expressed by insect cells is cleaved by an \( \alpha \)-secretase activity generating a C-terminal fragment of APP identical to that produced in mammalian cells (14). However, SF9 cells are unable to produce \( \beta \), although they accumulate intracellular C-terminal fragments of APP that contain the full-length \( \beta \) sequence (15). Here, we show that PS1 expressed in SF9 cells undergoes endoproteolysis and interacts with APP. However, this interaction does not result in the \( \gamma \)-secretase cleavage of APP. We also demonstrate that, in CHO cells, PS1 allows the secretion of \( \beta \) without endocytosis of APP.

**Experimental Procedures**

**Baculovirus-infected Cells**—The cDNA encoding human APP 695 or human PS1 was cloned in the pACL29 vector. Spodoptera frugiperda SF9 insect cells were cotransfected with 1 \( \mu \)g of linear Autographa california viral DNA and 5 \( \mu \)g of the recombinant plasmid as previously described (15). Recombinant viruses were harvested 5 days post-transfection, purified, and titrated as described (15). SF9 cell cultures and infections were subsequently carried out in IPL41 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum.

The W02 monoclonal antibody has been described (16). The Jonas anti-APP monoclonal antibody, specific for APP 643–695, was from...
Roche Molecular Biochemicals. MAB1563, a rat monoclonal antibody specific for human PS1 21–80, was from Chemicon International, Inc. The co-immunoprecipitation experiments were performed exactly as described previously (17), using the B14 anti-PS1 polyclonal serum (18) or a preimmune rabbit serum. Proteins were resolved by SDS-PAGE on 7.5, 12, or 4–12% gels. Immunoblots were developed with peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Transfected CHO Cells—The CHO cell lines expressing human APP 695 or APPΔC have been characterized (19). These cells were stably transfected with the cDNA encoding wild type PS1, together with a second plasmid containing the hygromycin resistance gene in a 10/1 ratio. Cells coexpressing PS1 and APP were maintained in culture in the presence of 250 μg/ml each of G418 and hygromycin. The amount of APP at the plasma membrane was quantified by the specific binding of 125I-radiolabeled antibody raised to the extracellular domain of APP (19). This specific binding was completely removed by acidic (pH 2.5) washes of the cells at 0 °C. The radioactivity internalized following a 15-min reincubation at 37 °C was resistant to these acidic washes, and the internalization was expressed as a percentage of the specific binding. The amount of soluble Aβ was normalized for total APP in each cell line.

RESULTS

Sf9 cells were infected at a multiplicity of infection of 10 with a human APP 695 recombinant baculovirus. Under these experimental conditions, we previously demonstrated that, by 48 h post-infection, virtually all cells become infected and express APP at the maximal level (15). Following expression of human APP, the C-terminal fragments were analyzed by Western blot using the WO2 monoclonal antibody (16), which recognizes the Aβ5–8-amino acid sequence (Fig. 1a). In addition to APP, the WO2 antibody detected several C-terminal fragments (from ~15–30 kDa, Fig. 1b). The same C-terminal fragments were recognized by the Jonas monoclonal antibody raised against the APP 643–695-amino acid sequence (Fig. 1c). However, an additional ~10 kDa C-terminal fragment was detected, which does not contain the Aβ5–8 sequence (Fig. 1b). This C-terminal fragment has been previously demonstrated to start at the α-cleavage site of APP (14) and is a substrate of γ-secretase (21). PS1 was coexpressed with APP using an additional recombinant baculovirus at a multiplicity of infection of 10. Using the MAB1563 monoclonal antibody raised against the 21–80 N-terminal residues of human PS1, holoproteins together with N-terminal fragments and aggregates were detected in Sf9 cells (Fig. 1c), clearly indicating that part of PS1 undergoes endoproteolysis in intact cells. The formation of stable complexes between PS1 and APP in intact living cells has been demonstrated previously by precipitating these complexes with anti-PS1 and anti-APP antibodies (17). Sf9 cells expressing human APP 695 were infected with a wild type baculovirus or the recombinant baculovirus encoding human PS1.

Cell lysates were immunoprecipitated with the anti-PS1 polyclonal serum B14(18) or a preimmune rabbit serum, and the immunoprecipitates were analyzed in Western blot using the WO2 antibody.

Fig. 1. Processing of APP and PS1 in baculovirus-infected cells. a, schematic diagram of APP 695, in which the cleavage sites of α-, β-, and γ-secretases as well as the approximate epitopes of the WO2 and the Jonas antibodies are indicated. b, Sf9 cellular extracts (4–12% SDS-PAGE, 100 μg/lane) were analyzed in Western blot using the WO2 monoclonal antibody specific for human Aβ5–8 or the Jonas monoclonal antibody specific for the C terminus of APP (APP 643–695). Arrows identify human APP holoprotein (APP) as well as a C-terminal fragment generated by α-secretase-mediated processing (α-stubs). Several C-terminal fragments (CTF, from 15 to 30 kDa (K)) are also indicated. c, Sf9 cellular extracts (12% SDS-PAGE, 50 μg/lane) were analyzed in Western blots using the MAB1563 monoclonal antibody specific for human PS1 21–80. PS1 holoprotein (PS1) and N-terminal fragments (NTF) are indicated. d, Sf9 cellular extracts (50 μg of protein) were immunoprecipitated (I.P.) with a preimmune serum (pre) or the B14 anti-PS1 serum (B14). The immunoprecipitates (4–12% SDS-PAGE) were analyzed in Western blot (W.B.) using the WO2 monoclonal antibody. The WO2 reveals APP in the APP-PS1 complexes (APP). IgG bands (IgG) represent the cross-reaction of the sheep anti-mouse secondary antibody used for Western blot with the rabbit serum used for immunoprecipitation.

Fig. 2. APP endocytosis and Aβ secretion by CHO cells. a, endocytosis of APP and of APP deleted from its internalization signal (APPΔC) was quantified in CHO cells following a 15-min incubation at 37 °C, and expressed as the percent of the transmembrane APP measured by the specific binding of radiolabeled antibody specific for the extracellular domain of human APP. b, 1 ml of culture medium from CHO cells, which stably express APP or APPΔC, was immunoprecipitated with an anti-Aβ polyclonal serum, and the immunoprecipitates were analyzed in Western blot using the WO2 antibody.
immunoprecipitates were analyzed in Western blot using the WO2 anti-APP monoclonal antibody. APP was specifically detected in the immunoprecipitate of Sf9 cells expressing both human APP and PS1, and a preimmune serum was not able to precipitate PS-APP complexes (Fig. 1d). Expression of PS1 did not modify the electrophoretic profile of the APP C-terminal fragments detected by the WO2 or the Jonas antibody (Fig. 1b), indicating that coexpression of APP and PS1 does not result in the production of additional C-terminal fragments generated by γ-secretase-mediated processing of APP.

Because PS1 does not cleave APP in Sf9 cells, we investigated the role of PS1 in the cellular trafficking of APP. In CHO cells, endocytosis of transmembrane APP has been characterized to be required for Aβ secretion (19, 22). In the C-terminal cytoplasmic domain, APP contains a tetrapeptide motif, Tyr-Glu-Asn-Pro, which is the endocytic signal of the protein (23). Deletion of this signal was performed by introducing a stop codon after Tyr-653 of APP 695, generating APPΔC. Although 56\% of APP was endocytosed by CHO cells during a 15-min incubation at 37 °C, only 1.4\% of APPΔC was internalized during the same period of incubation (Fig. 2a). In agreement with our previous study (19), this reduction of APP endocytosis was concomitant with inhibition of Aβ secretion (Fig. 2b). Stable CHO cell lines expressing human APP and PS1 were established. Both PS1 holoprotein and N-terminal fragments were detected in the cellular extract by the MA1563 monoclonal antibody (Fig. 3b), and the WO2 monoclonal antibody recognized human APP in both cellular extracts and culture medium (Fig. 3a). Coexpression of APP and PS1 in CHO cells does not significantly increase the total Aβ secretion (Fig. 3, c and d). Stable CHO cell lines expressing human APPΔC and PS1 were also established. Both full-length PS1 and N-terminal fragments were detected in cellular extracts (Fig. 4b), and soluble APP was detected in the culture medium (Fig. 4a).

As previously observed (19), expression of APPΔC did not induce any secretion of extracellular Aβ. However, when PS1 was produced in CHO cells expressing APPΔC, a significant Aβ secretion was measured (Fig. 4, c and d). This Aβ production did not result from APPΔC internalization, because PS1 did not induce endocytosis of APPΔC (Fig. 2a). Although this extracellular Aβ production was lower than that measured in CHO cells expressing the full-length APP, these results clearly demonstrate that PS1 allows secretion of Aβ without endocytosis of APP by CHO cells.

**DISCUSSION**

The identification of the enzymes that make Aβ from APP is very important, not only for drug development but also for the further understanding of APP catabolism. The recent cloning of the β-site APP-cleaving enzyme (BACE) cDNA indicates that it encodes a protein with all the properties of a β-secretase (24). As for the γ-secretase, the second enzyme needed to release Aβ from APP, it has been suggested that PS1 may be in fact be that enzyme (8, 12). However, PS1 may also help in the transport of APP or the secretases to the cell site where APP cleavage occurs (25). To address this question, APP and PS1 were coexpressed in Sf9 cells. In Sf9 cells, human PS1 is found as a holoprotein together with N-terminal fragments. We conclude, therefore, that PS1 undergoes endoproteolysis in insect cells. This endoproteolysis is important for PS1 activity, because PS1 holoprotein is primarily localized in the endoplasmic reticulum, whereas Golgi-type vesicles are known to contain principally the endoproteolytic fragments of PS1 that are thought to be the biologically active form (4, 26). In Sf9 cells expressing human APP, several C-terminal fragments accumulate and contain the full-length Aβ sequence. The length of these fragments...
suggests that they are not cleaved by β-secretase. An additional C-terminal fragment is generated by α-secretase, as demonstrated by its N-terminal sequence (14), and is a substrate for γ-secretase (21). In Si9 cells expressing both human APP and PS1, a polyclonal anti-PS1 serum was able to precipitate PS1-APP complexes. A polyclonal anti-APP serum also immunoprecipitated APP-PS1 complexes (not shown), but a preimmune serum did not. These results indicate that PS1 interacts with APP in insect cells. However, this physical interaction does not result in the cleavage of APP at the γ-secretase cleavage site, suggesting that PS1 does not have a γ-secretase activity. Our results are more consistent with a more direct role for PS1 in the cellular trafficking of APP. In CHO cells, an APP protein lacking the C-terminal domain is not endocytosed and is not transformed into Aβ. The C-terminal domain of APP is dispensable for the interaction of the protein with PS1 (17), and we demonstrate here that this interaction allows the production of Aβ without endocytosis of the transmembrane APP. In CHO cells, this PS1-mediated amyloidogenic catabolic pathway of APP produces 10 times less Aβ as compared with the endocytosis-mediated amyloidogenic catabolic pathway. However, the contribution of PS1 in the production of Aβ without the endocytosis of APP could be much more important in other cell types. In the brain, PS1 shows a neuronal distribution (27), and the PS1-mediated production of Aβ could be much more important in neurons, in which large amounts of intracellular Aβ are detected (20, 28, 29).

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