Phosphorylation, Subcellular Localization, and Membrane Orientation of the Alzheimer’s Disease-associated Presenilins*

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Presenilins 1 and 2 are unglycosylated proteins with apparent molecular mass of 45 and 50 kDa, respectively, in transfected COS-1 and Chinese hamster ovary cells. They colocalize with proteins from the endoplasmic reticulum and the Golgi apparatus in transfected and untransfected cells. In COS-1 cells low amounts of intact endogenous presenilin 1 migrating at 45 kDa are detected together with relative larger amounts of presenilin 1 fragments migrating between 18 and 30 kDa. The presenilins have a strong tendency to form aggregates (mass of 100–250 kDa) in SDS-polyacrylamide gel electrophoresis, which can be partially resolved when denatured by SDS at 37 °C instead of 95 °C. Sulfation, glycosaminoglycan modification, or acylation of the presenilins was not observed, but both proteins are posttranslationally phosphorylated on serine residues. The mutations Ala-246 → Glu or Cys-410 → Tyr that cause Alzheimer’s disease do not interfere with the biosynthesis or phosphorylation of presenilin 1. Finally, using low concentrations of digitonin to selectively permeabilize the cell membrane but not the endoplasmic reticulum membrane, it is demonstrated that the two major hydrophilic domains of presenilin 1 are oriented to the cytoplasm. The current investigation documents the posttranslational modifications and subcellular localization of the presenilins and indicates that postulated interactions with amyloid precursor protein metabolism should occur in the early compartments of the biosynthetic pathway.

Alzheimer’s disease is a major health problem. Patients suffer from a progressive dementia caused by massive neuronal loss in cortical and hippocampal areas of the brain (1–6). Neuropathological signs of the disease are tangles and amyloid deposits in the brain parenchyma, and amyloid deposits in the brain vasculature. The cause of the sporadic form of the disease is still unknown, although an increased risk is associated with the presence of apolipoprotein allele E4 (6, 7). On the other hand, familial early onset Alzheimer’s disease is caused by point mutations in the amyloid precursor protein gene on chromosome 21 (8), in the presenilin 2 (PS2) gene on chromosome 1 (9–11), or, most frequently, in the presenilin 1 (PS1) gene on chromosome 14 (12–15). Amyloid precursor protein (APP) is a type I integral membrane protein and is the precursor of the amyloid peptide, the main component of the senile plaques (1–3). Point mutations in exons 16 and 17 of the APP gene cause alterations in the metabolism of APP. This results in an increased production of intracellular βA4 amyloid peptide containing carboxy-terminal APP fragments and in an increased secretion of the potentially neurotoxic βA4 peptide (1–3, 16). 63% of the amino acid residues in the sequences of the two presenilins are conserved, which strongly suggests that both proteins are involved in similar functions and have a similar pathogenic role in Alzheimer’s disease. Based on computer algorithms, seven membrane spanning domains have been defined (9–15), although the possibility of nine transmembrane domains cannot be ruled out at this moment (17). The amino-terminal domain and the acidic loop domain, located between transmembrane domains six and seven, are hydrophilic and can be alternatively spliced (9, 13). The mutations that cause familial Alzheimer’s disease are found all over the protein, but the hydrophilic loop constitutes a “hot spot” with nine different mutations described to date (5, 15). The biological function of the presenilins remains essentially unknown, but, interestingly, the 103 carboxy-terminal amino acid residues of PS2 can inhibit apoptosis in a “deathtrap” assay (18). Based on the homology with Caenorhabditis elegans proteins, roles in intracellular protein sorting and/or intercellular signal transmission have been proposed as well (19, 20).

A hypothetical final common pathway in the pathogenesis of the genetic and sporadic forms of Alzheimer’s disease has been postulated, based on the variable occurrence of the amyloid deposits, the neurofibrillary tangles and the neurodegeneration in all affected brains. The central question is thus how mutations in the presenilin genes can cause this typical neuropathology. Studies indicating an increased production of more amyloidogenic βA4 (1–42) peptide in fibroblasts obtained from patients with presenilin mutations would support the amyloid

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1 The abbreviations used are: PS2, presenilin 2; PS1, presenilin 1; APP, amyloid precursor protein; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TtTC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; NSF, neuroendocrine-specific protein.
hypothesis that postulates abnormal βA4 amyloid peptide production and plaque formation as the pivotal event in the pathogenesis of the disease (2, 21). However, the observed increases in βA4 (1–42) peptide production were relatively small, and the suggested relationship between presenilin mutations and APP metabolism should be further corroborated by experiments showing directly the effect of presenilin mutations on APP processing in transfected cells or in brains of transgenic animals. Moreover, other aspects of APP metabolism such as the production of carboxyl-terminal APP fragments (16) should be investigated in greater detail. The possibility that presenilins interact with the cytoskeleton or exert their effect via apoptotic pathways should not be disregarded at this time (18). In addition to addressing these questions, basic information is needed on the subcellular localization, the posttranslational modifications, and the membrane orientation of the presenilins. We used COS-1 cells and CHO cells to express PS1 and PS2, as well as Myc-tagged PS1 and PS1-containing mutations that cause familial Alzheimer’s disease. The biosynthesis of transfected and untransfected presenilins was studied using immunoblotting or metabolic labeling and immunoprecipitation assays. We demonstrate that transfected presenilins are phosphorylated on serine residues. Using immunofluorescence microscopy, we document the association of presenilin 1 with the early compartments of the biosynthetic pathway and demonstrate the cytoplasmic orientation of the two major hydrophilic domains.

MATERIALS AND METHODS

 Constructs—The cDNA coding for mouse PS1 and human PS1, PS2, and PS1 containing Ala-246 → Glu (FAD1) or Cys-410 → Tyr (NH2) mutations have been described (9, 12). A Myc-tagged PS1 fragment was generated by PCR using primers 5'-GGAGGATCCATGACGTGCACGCGG C-3' and 5'-GATCAGCTTGTGAAGATTAGGACCCGAC-3' (the sequence coding for the Myc tag is underlined). This fragment was used to replace the NarI/BamHI restriction fragment of PS1 in pBSG. The resulting cDNA codes for PS1 with the Myc tag (EQKLISEEDL) immediately after the initiating methionine, as confirmed by cDNA sequencing. Plasmids containing the cDNA for fun (22, 23) or reticulon/NSP (24, 25) were kindly provided by Dr. J. Cremers, Dr. A. Roebroeck, and Dr. W. Van De Ven (Center for Human Genetics, Leuven, Belgium).

 Antibodies—Polyclonal rabbit antiserum B13, B14, and B15 were raised against peptide p45 (NDNRERQEREHNRSLC), which is in the membrane domain of PS1 (12) and is not a substrate for the Myc tag (26), was kindly provided by Dr. J. Creemers. mAbs against the Myc tag (26), was kindly provided by Dr. J. Creemers. mAbs against the Myc tag (26), were purchased from, respectively, StressGen (Victoria, Canada) and Affinity BioReagents (Neuhausen Station, NJ).

 Cell Culture, Protein Labeling, Cell Extraction, and Immunoprecipitation—The fibroblast cell line (designated AG07657, Coriell Institute) from an unaffected individual of the FAD1 lineage was cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum (Life Technologies, Inc.). COS1 cells were cultured as described previously in Dulbecco’s modified Eagle’s medium/Ham’s F-12 with 10% fetal bovine serum (27). CHO cells stably transfected with APP770 were kindly provided by Dr. B. Greenberg (Cephalon, West Chester, PA) and cultured in high glucose Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 0.1 μM minimal essential medium (nonessential amino acids), 3% fetal calf serum, and 250 nM methotrexate.

 COS cells were transfected using DEAE/dextran (27), while CHO cells were transfected using LipofectAMINE according to the instructions of the manufacturer (Life Technologies, Inc.).

 Metabolic labeling was done with 100 μCi of [35S]methionine, 250 μCi of [32P]orthophosphate, 60 μCi of [3H]palmitic acid, 50 μCi of [3H]palmitic acid, or 10 μCi of [3H]glycerol in the appropriate culture medium. All radiolabeled precursors were from Amersham. Cellular labeling was done for 4 h unless otherwise specified, and cell extracts were made as described previously (27). For analysis of phosphorylation, a postnuclear extract was prepared using 0.5% (v/v) Triton X-100 in Tris-buffered saline (TBS) containing protease inhibitors (100 units/ml aprotinin, 1 μg/ml pepstatin) and tyrosine and serine/threonine phosphatase inhibitors (1/100 dilution of ovomucoid, 5 mM EDTA, 5 mM EGTA, 20 mM NaF). The pellets were pelleted by centrifugation at 14,000 rpm (15 min) in a cooled Eppendorf centrifuge. Antiserum were added to the cell extracts at a 1/250 dilution, and antibody-antigen complexes were collected by incubation with immobilized protein G (Pierce) overnight at 4 °C. The immunoprecipitates were washed using Tris-buffered saline containing 1% (v/v) Triton X-100, 0.5% (v/v) deoxycholate, and 0.1% (v/v) SDS (27). For analysis of phosphorylation, phosphatase inhibitors as detailed above, were added to all buffers.

 Phorbol myristic acid, phorbol dibutyric acid, forskolin, okadaic acid and staurosporin (all from Sigma) were added to the cell cultures during the last 30 min of the metabolic labeling at the indicated final concentrations. For in vitro labeling assays, immunoprecipitated PS1 or PS2 bound on protein G beads were incubated with radiolabeled protein A or C in Tris-buffered saline, in the presence of 0.1 mM iBu-32PATP, 2 mM Mg2+ during 60 min at 30 °C. The precipitates were washed and analyzed in SDS-PAGE. Gels were quantitatively analyzed using a PhosphorImager (Molecular Dynamics).

 For immunoblotting experiments, cells were scraped in PBS, centrifuged (10 min, 1000 rpm), and solubilized in Laemmli sample buffer. Blots were stained with mAb 9E10 (Myc tag) as: fixed (1/1000 dilution) or mAb PS1–3 hybridoma supernatant (1/100 dilution) and affinity-purified goat anti-mouse peroxidase-conjugated antibodies (1/10,000; Bio-Rad), using the sensitive ECL system (Amersham).

 Phosphoamino Acid Analysis—Immunoprecipitated 32P-labeled PSs were size-fractionated in SDS-PAGE, and labeled bands were localized by autoradiography. The proteins were hydrolyzed in 6 N HCl (110 °C, 90 min). The hydrolysates were supplemented with phosphoamino acid standards, and analyzed by two-dimensional thin-layer electrophoresis at pH 1.9 and 3.5 in the respective dimensions (28). Phosphoserine, phosphothreonine, and phosphotyrosine were localized using ninhydrin staining, and the radioactive phosphoamino acid residues were visualized by autoradiography.

 Immunocytochemistry—Fibroblasts were grown on coverslips coated with mouse collagen IV (Collaborative Biomedical Products, 1 μg/cm2). Transfected COS-1 or CHO cells were cultured in Lab-TEK chamber slides (Nunc). Cells were washed twice in PBS, fixed in 4% formalde- hyde in PBS for 10 min at room temperature, and washed three times in PBS and once in TBS. Cells were permeabilized with 0.02% (v/v) Triton X-100 in TBS for 20 min or with 0.2% (v/v) saponin for 10 min and washed with 0.1% Tween 20 in TBS. Nonspecific binding was blocked with 0.2% cold water fish gelatin, 2% bovine serum albumin, and 2% fetal calf serum (blocking buffer). Cells were probed with affinity-purified primary antibody 519 (1:25) or with immune serum (1:400). Appropriate FITC- and TRITC-conjugated anti-mouse, anti-rat, and anti-rabbit antibodies (Sigma) were used at 1/400 dilution. Prepara- tions were viewed on a Nikon Diaphot 300 or a Zeiss Axiovert UV microscope. Digitized immunofluorescence images were obtained using an LSM419-inverted laser-scanning confocal microscope (Zeiss Inc.) and processed using NIH Image software.

 Selective permeabilization of the plasma membrane was obtained by incubating fixed cells in 10 mM Pipes buffer (pH 6.8) containing 0.3 M sucrose, 0.1 mM KCl, 2.5 mM MgCl2, 1 mM EDTA, 5 μg/ml digitonin during 15 min at 4 °C (29). Cells were washed in PBS and further processed. A rat monoclonal antibody against the KDEL sequence (30, 31) was used to demonstrate permeabilization of the endoplasmic reticulum membrane.

2 P. Fraser, unpublished results.
**RESULTS**

*Immunocytological Localization of Presenilin 1 in the Endoplasmic Reticulum and the Golgi Apparatus*—The subcellular localization of the presenilins was investigated in permeabilized and fixed fibroblasts using affinity-purified polyclonal antibody 519 raised against the peptide KGQQLYTPFT-EDTE, which is a conserved sequence in the second loop domain of PS1 or antisem B16 against peptide EGDPEAQRRVSKNSKY in the hydrophilic loop domain of PS1 (Fig. 2, a and c). Untransfected COS-1 cells remained negative under the experimental conditions used, probably because of the very low levels of endogenous PS present in these cells (see below). The same pattern of staining was observed with monoclonal antibody IIDD (32) against Serca 2a Ca\(^{2+}\)-ATPase (results not shown), and with antibodies against transfected reticulon/NSP (Fig. 2b). Both proteins are located in the endoplasmic reticulum and the Golgi apparatus (24). The distribution of PS1 and reticulon/NSP remained identical in double transfected cells (compare panels a and b in Fig. 2). Transfected APP (Fig. 2c) and transfected furin (Fig. 2d), in contrast, were mainly found in the Golgi apparatus, as shown previously (33, 34). Similar results were obtained in CHO cells (results not shown).

**Biosynthesis of the Presenilins**—Metabolic labeling of untransfected or “mock” transfected COS-1 or CHO cells using \(^{35}\)S-methionine, followed by immunoprecipitation of the cell extracts using antibodies 519, B14, or B17 and resolution of the immunoprecipitates in SDS-PAGE, yielded no signals (Fig. 3a, lane 4) or, after prolonged exposures (2 weeks and more), only nonspecific signals in autoradiography (results not shown). Immunoprecipitation of detergent extracts of COS-1 cells transfected with plasmids containing the cDNA of wild type PS1, in contrast, yielded strong specific signals of radiolabeled protein migrating with an apparent molecular mass of 45 kDa (Fig. 3a, lanes 1–3). Diffuse protein bands with masses between 100 and 250 kDa were observed to a variable extent (Fig. 3a). Similar results were obtained with PS2 cDNA. The main PS2 species migrated, however, slightly more slowly than PS1, resulting in an apparent mass of 50 kDa (see below). Unrelated polyclonal antibodies, or untransfected cells did not yield these bands, while immunoblots of COS-1 cells transfected with Myc-tagged PS1 and stained with the Myc tag-specific mAb 9E10 revealed again the pronounced smearing (Fig. 3B). This result clearly demonstrated that the 100–250-kDa protein smears consisted of PS protein, either associated or not associated with other proteins. Essential similar patterns were observed when PS1 FAD1 (Ala-246 → Glu) or PS1 NIH2 (Cys-410 → Tyr) were expressed (Fig. 3A). These clinical mutations (12) therefore do not cause major alterations in the biosynthesis of PS1 protein when overexpressed in COS-1 cells (Fig. 3A). Independent experiments performed in CHO cells confirmed completely these results (results not shown).

Since the levels of endogenous PS1 were not detectable using classical immunoprecipitation, a sensitive immunological “sandwich” type assay was developed. PS1 was immunoprecipitated from detergent extracts of 10 \(\times\) 10\(^6\) untransfected COS-1 cells using the polyclonal antisem A against A12 (Fig. 3C, lanes 2 and 4). Endogenous PS1 migrated with the same mobility (45 kDa) as transfected PS1, as is best seen in the experiments using the amino-terminal domain-specific antisem B13 (Fig. 3C, lane 2). Since mAb PS1–3 recognizes an epitope in the carboxyl-terminally located hydrophilic loop, the combination of these antibodies is expected to detect mainly intact PS1 (Fig. 3C, lane 2). With hydrophilic loop-specific antisem B17, in contrast, relatively pronounced 18–30-kDa fragments were visualized together with intact PS1. These fragments represent most likely carboxyl-terminal fragments of presenilin (35). Remarkably, the observed fragments were not, or only marginally, increased in COS-1 cells overexpressing PS1 (compare lanes 3 and lane 4 in Fig. 3C). Pulse-chase experiments on transfected CHO cells (Fig. 4, A and C) and COS-1 cells (results not shown) further showed that the transfected 45-kDa PS1 species has a half-life of about 4 h. No fragments were seen at any time point of this assay, either with amino-terminal (B13) or hydrophilic loop-specific (B17) antisera. APP, immunoprecipitated from the same cell extracts (Fig. 4B), displayed a much faster turnover (half-life: 2 h), indicating that the relative high expression of PS1 did not interfere with the normal turnover of APP.

**Posttranslational Modification and Aggregation of Presenilins**—The problem of the presenilin smears in SDS-PAGE was further investigated. Enzymatic digestion of immunoprecipitated PS1 and PS2 protein with glycosidase F (3 milliunits/\(\mu\)l),
O-glycosidase (60 nanounits/ml), endoglycosidase H (60 nanounits/ml), sialidase (100 nanounits/ml), or combinations of these enzymes did not affect the mobility of the proteins, indicating that no glycosylation of PS1 and PS2 occurred (results not shown). Digestions with heparinase (0.1 milliunits/ml), heparitinase (0.1 milliunits/ml), chondroitinase AC (5 milliunits/ml), and chondroitinase ABC (5 milliunits/ml) also had no effect on the mobility of the protein smears, indicating that PS1 is not modified by glycosaminoglycan chains (Fig. 5). These negative results were independently confirmed by metabolic labeling

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**Fig. 2. Immunofluorescent staining of PS1 in transfected COS cells.** COS-1 cells were double transfected with plasmids coding for PS1 and reticulon/NSP (a and b) or single transfected with plasmids coding for PS1, amyloid precursor protein, or furin (c, d, and e). Cells were fixed with formaldehyde and permeabilized with saponin. Immunostaining was done with antisera B14 against the amino-terminal domain of PS1 (a) or B16 against the loop domain of PS1 (c), with a mix of monoclonal antibodies MON160–162 against reticulon/NSP (b), or MON148 and 152 against furin (d), or mAb 22C11 against amyloid precursor protein (e), followed by the appropriate TRITC-conjugated (red) or FITC-conjugated (green) secondary antibodies. Notice the fine reticular pattern obtained with PS1 antibodies in panels a and c, which distributes with reticulon/NSP (panel b) in double transfected cells. Panel e demonstrates that amyloid precursor protein is located mainly in the Golgi apparatus, similar to furin (panel d).

**Fig. 3. Biosynthesis of PS1 protein in COS-1 cells.** Panel A, COS-1 cells were transfected with constructs containing the cDNA coding for wild type PS1 (PS1 WT), PS1 FAD1 (Ala-246 → Glu), PS1 NIH2 (Cys-410 → Tyr), or with expression vector alone (pSG5 control). Cells were metabolically labeled with [35S]methionine for 4 h. Cells were solubilized, and PS1 was immunoprecipitated with antisera B17 (1/250). Immunoprecipitates were resolved by 4–20% gradient SDS-PAGE. Molecular size markers are indicated at the left in kDa. Panel B, Western blotting of COS-1 cells transfected with PS1 containing amino-terminally inserted Myc epitope. Cells were electrophoresed on a 4–20% gradient acrylamide gel and transferred to a nitrocellulose membrane. mAb 9E10 against the Myc epitope was used to detect PS1. Panel C, combined immunoprecipitation and Western blotting of PS1 in untransfected COS cells. Detergent extracts of 10 × 10⁶ untransfected COS-1 cells were made, and PS1 was immunoprecipitated using antisera B13 (N-term) or B17 (Loop). In lanes 1, 3, 5, and 7, 0.5 × 10⁶ COS-1 cells transfected with wild type PS1 were added to the untransfected cells. Immunoprecipitated material was resolved in 4–20% gradient SDS-PAGE and transferred to a nitrocellulose filter. Filters were reacted with mAb PS1-3 and goat anti-mouse peroxidase-conjugated antibodies (lanes 1–4, indicated by PS1-3) or with goat anti-mouse peroxidase conjugated antibodies alone (lanes 5–8, -Co). The mobility of intact PS1 is indicated by an arrow at the right.
experiments using [3H]glucosamine or [35S]O4. While both radioactive precursors were incorporated in proteins in the cell extracts of the labeled cells, no signal was obtained when transfected PS1 was immunoprecipitated (results not shown). Experiments using [3H]palmitic or [3H]myristic acid demonstrated also that the presenilins did not incorporate fatty acids. Sonication or boiling of the samples in SDS and 6 M urea (data not shown) or extraction of the cells in the presence of 5 mM dithiothreitol did not resolve the aggregates (Fig. 6). Denaturation of the immunoprecipitates at 37 °C instead of at 95 °C reduced the aggregates considerably but not completely (Fig. 6, lane 2). This demonstrated that the aggregates consisted mainly, if not exclusively, of PS1 as the radiolabeled species (Fig. 6) and also suggested that the PS aggregates are at least partially produced during the processing of the samples for electrophoresis. It should be noticed, however, that even when freshly prepared material was used and heating of the samples was avoided, protein smears in the 100–250-kDa region remained visible in SDS-PAGE (Fig. 6).

Phosphorylation of the Presenilins—The high level of serines, threonines, and tyrosines in the primary amino acid sequences of both PS1 and PS2 suggested the possibility that the PS proteins are phosphoproteins (12, 13). Transfected COS-1 cells (Fig. 7) and CHO cells (Fig. 8B) incorporated 32P in presenilins. Acid hydrolysis of immunoprecipitated 32P-phosphorylated PS1 and PS2 yielded mainly phosphoserine (Fig. 7B). Longer exposures revealed very weak signals for phosphothreonine, while phosphotyrosine was never observed. The phosphorylation of PS1 was strongly increased, and that of PS2 slightly or not increased, by treating the cells with the phosphatase inhibitor okadaic acid (300 nM, Fig. 8A). Okadaic acid inhibits the two major classes of serine/threonine phosphatases (49). The protein kinase C inhibitor staurosporin (300 nM) or the agonists phorbol myristic acid (1 μM) and phorbol dibutyric acid (1 μM) and the protein kinase A agonist forskolin (100 μM) had no effect on the phosphorylation extent or pattern (Fig. 8A). Attempts to phosphorylate immunoprecipitated PS1 in vitro using protein kinase A or protein kinase C remained also negative (results not shown).
Cytoplasmic Orientation of the Amino-terminal Domain and Hydrophilic Loop Domain of Presenilin 1

We finally addressed the issue whether the two major hydrophilic domains in presenilin 1, i.e. the amino-terminal domain and the hydrophilic loop domain (12) are oriented toward the cytoplasmic or luminal side of the endoplasmic reticulum. Current models for the orientation of the presenilins in membranes are based on theoretical predictions (see "Discussion"). Both domains are candidate regions for interactions with other proteins, and their orientation determines whether this interaction occurs with cytoplasmic or endoplasmic reticulum proteins. To analyze this question, we used low concentrations of digitonin to selectively permeabilize the plasma membrane (see "Materials and Methods") and antibodies against the amino-terminal domain (antiserum B14), against the Myc tag introduced at the amino terminus (mAb 9E10), against the second loop domain (antibody 519), and, finally, against the hydrophilic loop (antiserum B16) of PS1. To monitor the permeabilization procedure, rat monoclonal antibody against the KDEL sequence (panels a-f). PS1 immunoreactivity is observed as red/yellow staining, while KDEL immunoreactivity is green. Panels a and b show that the amino terminus of PS1 is oriented to the cytoplasmic side of the endoplasmic reticulum membrane. Cells that express PS1Myc stain strongly with the amino-terminal domain directed 9E10 mAb both in digitonin (a) or saponin (b) permeabilized cells. When saponin is used, the untransfected cells are stained with the KDEL antibody (green) since it reacts with endogenous proteins (b). When digitonin is used, no staining is observed (a), indicating that the endoplasmic reticulum membrane is not permeable for antibodies under the conditions used. In panels c and d, similar experiments are displayed but using antiserum 519 against the second loop domain. Staining is only observed when saponin is used, indicating a luminal orientation of this domain (panel d). Panels e and f demonstrate that antiserum B16 against the hydrophilic loop domain of PS1 react both with digitonin (e) or saponin (f) permeabilized cells, indicating a cytoplasmic orientation of the hydrophilic loop.
monoclonal antibody did not result in labeling (Fig. 9, a and e). On the other hand, antibodies against the second hydrophilic loop domain (S19) reacted with PS1 when saponin (Fig. 9d), but not when digitonin (Fig. 9c) was used to permeabilize the cells, suggesting a luminal localization of this domain.

**DISCUSSION**

The current investigation provides a detailed characterization of the biosynthesis of presenilins overexpressed in COS-1 cells and CHO cells. The results show that the PS proteins are unglycosylated phosphoproteins with apparent molecular masses of about 45 and 50 kDa in SDS-PAGE. They have a strong tendency to form SDS-resistant complexes with apparent molecular masses between 100 and 250 kDa. Double immunofluorescence studies showed that PS1 is mainly located in the endoplasmic reticulum and the Golgi apparatus and that its amino-terminal domain and the hydrophilic loop domain are oriented to the cytoplasmic side.

The localization of transfected PS1 in the endoplasmic reticulum of COS-1 cells or CHO cells is not a simple consequence of overexpression of the protein or nonspecific general effects on the biosynthetic pathway in these cells. First, endogeneous presenilins are located in the endoplasmic reticulum of untransfected cells as demonstrated in Fig. 1A using confocal laser microscopy. Second, using identical transfection conditions, APP and furin were found to accumulate in the Golgi apparatus, as demonstrated before (33, 34). Third, the turnover (Fig. 4) and the secretory processing of APP in CHO cells stably expressing human APP770 was not affected by transfection of PS1, ruling out general inhibitory effects on protein transport and processing. On the other hand, the levels of endogeneous presenilin in COS-1 cells are apparently very low. Only the combination of immunoprecipitation to concentrate PS1 from detergent extracts of $10^6$ cells, followed by immunoblotting using mAb PS1–3, allowed us to detect endogeneous PS1 migrating at the same molecular weight as transfected PS1. The disadvantage of this approach is that the antibodies from the immunoprecipitation step interfere with the consecutive detection of possible PS-aggregates in the immunoblotting step (Fig. 3C). This assay also precludes a dynamic analysis of the metabolism of the presenilins using metabolic labeling and pulse-chase experiments. On the other hand, it allowed us to demonstrate the presence or absence of particular antibody epitopes on PS1 fragments. Importantly, the use of hydrophilic loop-specific antibodies in the immunoprecipitation step resulted in the detection of relative large amounts of, presumably proteolytic, fragments of PS1 in the 18–30-kDa range (Fig. 3C). The level of these fragments was not, or only slightly, increased in COS-1 cells that overexpressed PS1, which explains why they were not readily detected in the transfection experiments. The proteolytic process involved is probably easily saturated or even inhibited by PS overexpression (35, 36). Our data therefore do not allow us to speculate any further on the exact nature or the biological significance of this process. We can only conclude that overexpression studies of the type used here are not suitable to study this particular aspect of presenilin metabolism. On the other hand, it is clear that untransfected cells (Fig. 3) and brain tissue in vivo (36, 37) contain detectable amounts of intact presenilin. Since this endogeneously expressed PS1 has the same mobility as transfected PS1, the conclusion that transfected PS1 (and PS2) are not subject to glycosylation, sulfation, glycosaminoglycan modification, palmitoylation, or myristoylation also holds apparently true for the endogeneous protein. These posttranslational modifications are thereby also excluded as contributing to the formation of the high molecular mass, SDS-resistant aggregates (100–250 kDa) in SDS-PAGE electrophoresis. These aggregates were observed to a variable extent in all our experiments, and were noticed by others using other cell types or brain tissue (36, 38, 39). Since the smears were detected with three different antibodies recognizing three different epitopes, and by a Myc-directed mAb using a Myc-tagged PS1 construct, the aggregates must contain presenilin core proteins, alone or associated with unidentified components. Denaturation of immunoprecipitates at 37 °C instead of 95 °C, resulted in less aggregates and increased amounts of the 45-kDa PS1 band (Fig. 3C). The aggregates therefore probably consist mainly of oligomers of presenilins. It is unclear whether this property to form SDS-resistant aggregates in vitro has any physiological significance in vivo, but immuno-electron microscopic observations suggest that clustering of presenilins also occurs in the endoplasmic reticulum of untransfected cells.2 It should be envisaged that in pathological conditions exaggerated aggregation of presenilins could become a problem. In this context, it should be mentioned that antibodies against PS1 stain amyloid plaques in the brains of Alzheimer’s disease patients (40).

Importantly, the two point mutations causing Alzheimer’s

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* B. De Strooper and K. Craessaerts, unpublished results.
disease that were studied here, i.e. PS1 Ala-246 → Glu (FAD1 kindred) and PS1 Cys-410 → Tyr (NIH2 kindred), did not alter the biosynthesis or level of expression of the PS1 protein in COS-1 cells and CHO cells. These mutations must cause therefore more subtle effects. The possibility that they interfere with the fragmentation of PS1, as was shown recently for two other mutations (35, 36), cannot be excluded from the current data. Analysis of brains of transgenic mice expressing the PS1 Ala-246 → Glu mutant indicate, however, that at least this mutation does not interfere with the proteolytic processing of PS1.4 Since all PS mutations known to date act in a dominant way in the pathogenesis of Alzheimer’s disease, a gain of function is most likely. Evidence that fibroblasts derived from patients with presenilin mutations produce more and longer forms of the βA4 amyloid peptide suggests that the mutations exert their effect on cellular metabolism or trafficking of amyloid precursor protein (21). The localization of PS1 in the early compartments of the biosynthetic pathway makes it unlikely that the presenilin mutations directly influence α- and/or β-secretase activity, since these operate in the late-Golgi and transport vesicles, at the cell surface and in endosomes (27, 41–43). The possibility that PS mutants influence the balance between amyloidogenic and non-amyloidogenic processing of APP in an indirect way by changing its intracellular trafficking should now be further explored in polarized Madin-Darby canine kidney cells and neurons (16, 44, 45).

Our study demonstrates furthermore that the presenilins are phosphorylated on serine residues. Phosphorylation of PS1 and PS2 was evident both in COS-1 cells and CHO cells, but the phosphorylation of PS1 was less intense and more variable than of PS2. The observation that okadaic acid enhanced the PS1 phosphorylation suggests that PS1 is more prone to phosphatase activity than PS2 (Fig. 8A). Protein kinase C is not responsible for PS phosphorylation, since neither stimulation of cells by phorbol esters nor purified kinase added to immunoprecipitated PS1 or PS2 resulted in increased labeling. It is therefore unlikely that the presenilins are directly involved in the regulation of APP secretion by protein kinase C (46, 47).

Finally, we showed that the hydrophobic NH2-terminal and the major loop domain of PS1 are exposed to the cytoplasmic side of the endoplasmic reticulum membrane. Digitonin at low concentrations selectively permeabilizes the cell membrane, but not the endoplasmic reticulum membrane (29). Antibodies directed toward the amino-terminal domain of PS1 (Fig. 9) displayed similar immunofluorescent staining patterns in digitonin and saponin permeabilized cells. Control experiments with antibodies against a luminal epitope, i.e. the KDEL endoplasmic reticulum retention signal (30, 31), showed that digitonin did not permeabilize the endoplasmic reticulum membrane. For the major hydrophobic loop domain, essentially identical results were obtained. The conclusion therefore is that the two major hydrophilic domains in PS1 are oriented to the cytoplasmic side of the endoplasmic reticulum, which directs the search for candidate proteins interacting with these domains toward the cytoplasm. Consistent with this conclusion, antibodies against the second loop domain reacted with membranes with a seven-transmembrane domain model with the amino-terminal amino terminus (48). However, several exceptions to this rule are known, mainly of proteins of which the amino-terminal domain contains more than 17 charged amino acid residues (48). In PS1, this domain contains 29 charged amino acids. It would explain its cytoplasmic orientation. The second issue, which has been pointed out by others before (9, 13, 17), is whether two putative hydrophobic stretches in the PS amino acid sequence flanking the hydrophilic loop, can additionally span the membrane, which would make the presenilins nine transmembrane domain proteins. Our data are compatible with a seven-transmembrane domain model with the amino-terminal domain and the hydrophilic loop located in the cytoplasm (Fig. 10; see also Note Added in Proof). In conclusion, the current study has analyzed the biosynthesis and the subcellular localization of the presenilins and provides a basis for the further study of their cell biology and their possible interactions with integral membrane and cytoplasmic proteins such as APP or Tau, both implicated in the pathogenesis of Alzheimer’s disease (4).

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Note Added in Proof—An eight-transmembrane model, with hydrophobic region VIII in the loop domain spanning the endoplasmic reticulum membrane, is also compatible with these data. This would result in a cytoplasmic orientation of the carboxyl-terminal domain of PS1.

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