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Expression and analysis of presenilin 1 in a human neuronal system: Localization in cell bodies and dendrites

(Alzheimer disease/presenilins/amyloid precursor protein/amyloid)

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ABSTRACT **Mutations in the recently identified presenilin 1 gene on chromosome 14 cause early onset familial Alzheimer disease (FAD). Herein we describe the expression and analysis of the protein coded by presenilin 1 (PS1) in NT2N neurons, a human neuronal model system. PS1 was expressed using recombinant Semliki Forest virions and detected by introduced antigenic tags or antisera to PS1-derived peptides. Immunoprecipitation revealed two major PS1 bands of approximately 43 and 50 kDa, neither of which were N-glycosylated or O-glycosylated. Immunoreactive PS1 was detected in cell bodies and dendrites of NT2N neurons but not in axons or on the cell surface. PS1 was also detected in BHK cells, where it was also intracellular and colocalized with calnexin, a marker for the rough endoplasmic reticulum. A mutant form of PS1 linked to FAD did not differ from the wild-type protein at the light microscopic level. The model system described here will enable studies of the function of PS1 in human neurons and the role of mutant PS1 in FAD.**

Alzheimer disease (AD) is a progressive neurodegenerative disorder with multiple etiologies (1). Familial forms of AD (FAD) have now been linked to mutations in three genes: the amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2). APP is a type 1 membrane protein from which the 39- to 43-amino acid amyloid peptide (β-amyloid, Aβ) is proteolytically derived (2). Deposition of Aβ in senile plaques and blood vessels in the brain is an important neuropathological feature seen in all forms of AD. Mutations in APP that cause early onset FAD alter proteolytic processing of APP that could accelerate Aβ deposition, either by increasing Aβ production or by generating longer Aβ species that are more amyloidogenic (3, 4). Transgenic mice that overexpress one of these mutant APP genes exhibit AD-like pathology (5), lending further support to the idea that Aβ production and deposition are critical factors in the development of AD.

Nonetheless, only a small fraction of early onset forms of FAD can be attributed to APP mutations (6, 7). Rather, most FAD cases appear to be caused by mutations in the recently identified PS1 and PS2 genes (8, 9). PS1 encodes a novel protein of 467 amino acids that contains seven potential transmembrane domains (8), and at least 24 different missense mutations in PS1 have been identified that cosegregate with the AD phenotype in multiple FAD pedigrees (8, 10–15). PS2 is >70% homologous to PS1 and is predicted to encode a protein of 448 amino acids with a topology identical to that of PS1 (9). At present, two different missense mutations in the PS2 gene have been linked to FAD (9, 16).

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Since mutations in the PS1 and PS2 genes result in early onset FAD that is quite similar to FAD in other pedigrees with APP mutations, the PS mutations may cause AD by altering the metabolism of APP and/or Aβ, thereby accelerating amyloidogenesis. Considerable evidence suggests that neurons are a major source of the Aβ that forms amyloid deposits in AD (17, 18), and a recent study showed that within the brain PS1 and PS2 mRNAs are expressed primarily in neurons (19). For these reasons, it is important to study the biosynthesis and processing of these genes in neurons. To accomplish this goal, we developed a strategy to efficiently express and detect PS1 in a well characterized *in vitro* model of human neurons (NT2N cells) (20). We found that PS1 was localized in the cell body and dendrites of NT2N neurons but not on the cell surface or in axons. In nonneuronal cells, PS1 was largely localized to the rough endoplasmic reticulum (ER) as reported (19). Thus, this experimental system will enable studies of the neurobiology of mutant and wild-type (wt) PS1.

METHODS

The Semliki Forest Virus (SFV) Expression System. The PS1 gene was cloned into a modified pSFV-1 vector containing *Bam*HI and *Cla*I sites in the polylinker. The A(246)E mutation was introduced by PCR techniques. The hemagglutinin (HA) tag was introduced by annealing two oligonucleotides, 5′-CTTGCCTACGATGTTCCGGATTACGCATCT and its complement, and ligating them into the unique *Sty*I site in the PS1 open reading frame (ORF). The herpes simplex virus 1 (HSV-1) gD antigenic tag was introduced at the 3′ end of the PS1 ORF by PCR techniques using the oligonucleotide 5′-GGTCATCGATCTGCAGCTAGCCGCGGAATCGGTTC-GGGTCAGCCATGATATAAAATTGATGG. All pSFV-1 plasmids and the pSFV-helper 1 were linearized with *Spe*I, and runoff transcription was performed using SP6 polymerase. The RNAs were electroporated into BHK cells and the virus-containing supernatant was collected 24 h later (21). Virus was titered by infecting BHK monolayers with 1:10 dilutions of SFV virus stocks. Cells were fixed and incubated with appropriate antibodies to PS1, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Infected cells were then stained with diaminobenzidine and counted to determine infectious viral titer.

Tissue Culture. NTera 2 cells (NT2) were cultured as described (22). Briefly, NT2 cells were differentiated by

Abbreviations: PS1, presenilin 1; PS2, presenilin 2; AD, Alzheimer disease; FAD, familial Alzheimer disease; APP, amyloid precursor protein; Aβ, β-amyloid; SFV, Semliki Forest virus; ER, endoplasmic reticulum; wt, wild type; HA, hemagglutinin; HSV-1, herpes simplex virus 1.

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growth in 10 mM retinoic acid for 5 weeks. The cells were trypsinized, reseeded into flasks, and expanded for 1 week in DMEM-hg (GIBCO/BRL). Neurons were selectively dislodged and grown an additional week in DMEM-hg supplemented with mitotic inhibitors [cytosine arabinofuranonucleoside (Ara-C) (1 μM), 5-fluro-2′-deoxyuridine (10 μM), and uridine (10 μM)]. To achieve neuronal cultures of the highest purity (>99%), the neurons were reseeded one final time into tissue culture dishes coated with poly(D-lysine) (Sigma) and Matrigel (Becton Dickinson). Baby hamster kidney cells were cultured in GMEM (GIBCO/BRL) supplemented with 5% fetal calf serum and 10% tryptose phosphate broth.

Metabolic Labeling, Immunoprecipitation, and Glycanase Digestions. Cells were infected with recombinant SFV for the appropriate time (4 h for low gene expression or overnight for high gene expression) and then incubated in methionine-free medium containing [^{35}S]methionine. Cells were scraped, pelleted, and lysed (0.1 M Tris·HCl/0.5% Triton X-100). Immunoprecipitation was carried out using monoclonal antibodies 12CA5 to influenza HA (Boehringer Mannheim), 1D3 to the HSV-1 gD protein (provided by Gary Cohen, University of Pennsylvania), or with rabbit antisera to PS1-derived peptides. The antibody–antigen complexes were precipitated with protein A-conjugated Sepharose beads (Sigma). Prior to loading for SDS/PAGE, the samples were boiled 5 min in standard reducing sample buffer. Gels were fixed, stained, dried, and visualized using a Molecular Dynamics PhosphorImager. N-Glycanase F digestions were performed as described elsewhere (23). O-Glycanase and neuraminidase were used as in manufacturer's procedures (Boehringer Mannheim).

Immunofluorescence Microscopy. Cells grown on glass coverslips were infected overnight and then processed for immunofluorescence. To stain intracellular proteins, cells were fixed (10 min at −20°C in 95% ethanol/5% acetic acid) and then stained with appropriate antibodies followed by incubation with appropriate fluorescein isothiocyanate- and Texas red-conjugated secondary antibodies (Jackson ImmunoResearch). To stain extracellular epitopes cells were chilled to 4°C, incubated in primary antibodies, and then treated as above. Standard immunofluorescence microscopy was performed using a Nikon Microphot-FXA microscope. Confocal microscopy was performed using a Leica CLSM (Heidelberg, Germany). Some immunofluorescence images were processed using PHOTOSHOP (Adobe Systems, Mountain View, CA). Other antibodies used were anti-MAP2 against microtubule-associated protein 2 (24) and HO14 (an axonal marker) recognizing highly phosphorylated neurofilament protein NF(M) (25).

RESULTS

Expression and Detection of PS1. To express PS1 in neurons and nonneuronal cells, the SFV expression system was used (21). The PS1 gene was cloned into the pSFV-1 expression vector. After *in vitro* transcription, the RNA was electroporated into BHK-21 cells along with a helper RNA encoding viral structural proteins to generate recombinant SFV. To facilitate the detection of PS1, we used antigenic tags and polyclonal sera raised against PS1-derived peptide sequences. To minimize the possibility that the antigenic tags might disrupt protein processing, tags were placed at different locations in PS1. The first, from the influenza virus HA glycoprotein (YDVPDYASL), was inserted into a unique *Sty*I site in the amino-terminal domain of PS1 (between codons 44 and 45). A second antigenic tag (MADPNRFRG) based on the HSV-1 gD glycoprotein was introduced at the carboxyl terminus of PS1. In addition, rabbit antisera were raised against the amino-terminal domain (anti-1398: GHPEPLSNGRPQGNSR) of PS1 and against an epitope (anti-1357: RDSHLGPHRSTPESR) in the loop that connects the sixth and seventh potential transmembrane domains. Thus, we used antibodies to epitopes in three distinct PS1 domains.

To monitor the expression of PS1, NT2N neurons and BHK cells were infected with recombinant viruses that expressed PS1-HA, PS1-gD, or PS1 without modifications. Cells were metabolically labeled, lysed with detergent, and subjected to immunoprecipitation with antibodies to the HA tag or the gD tag or with antisera to the wt protein. PS-1, PS1-HA, and PS1-gD appeared as closely spaced doublets of approximately 43 and 50 kDa in SDS/PAGE in both cell types (Fig. 1, PS1 has an expected molecular weight of 52,716). In addition, smaller bands, most likely proteolytic fragments, were often seen. Also, a variable diffuse band of very high molecular weight material was sometimes observed. The relative intensities of the two major bands varied depending on sample preparation. Thus, while the doublet may be the result of posttranslational modifications, it also may reflect different conformations of the protein since hydrophobic regions (of which PS1 has many) are not always fully denatured by SDS treatment (26). PS1 was not detected in the medium of either NT2N neurons or BHK cells, indicating that it is not secreted (data not shown). Furthermore, endogenous PS1 in NT2N neurons could not be detected by immunoprecipitation with the peptide antisera even after metabolic labeling overnight, suggesting that PS1 is normally expressed at low levels.

Immunolocalization in NT2N Neurons and BHK Cells. NT2N neurons and BHK cells were infected with the recombinant SFV that expresses PS1-HA (Fig. 2). Permeabilized cells were stained with monoclonal antibody 12CA5 to the HA tag (yellow color) and with Evans blue as a fluorescent counterstain (red color). In NT2N cells, PS1 was observed throughout the cell body as well as in some neuronal processes (Fig. 2*A*). In BHK cells, PS1 was distributed throughout the cytoplasm in a lacy reticular pattern characteristic of the ER (Fig. 2*C*). To determine whether PS1 is located on the cell surface, cells were infected with the recombinant SFV that expressed PS1-HA or with influenza virus. The influenza virus HA, from which the HA tag is derived, is efficiently delivered to the cell surface and so served as a positive control (27). Cells were placed at 4°C, incubated with 12CA5, washed, fixed, and then incubated with secondary antibody. As shown in Fig. 2*G*, influenza HA was readily detected on the surface of influenza virus-infected BHK cells. Likewise, APP (a cell surface protein) was detected on the cell surface while two intracellular

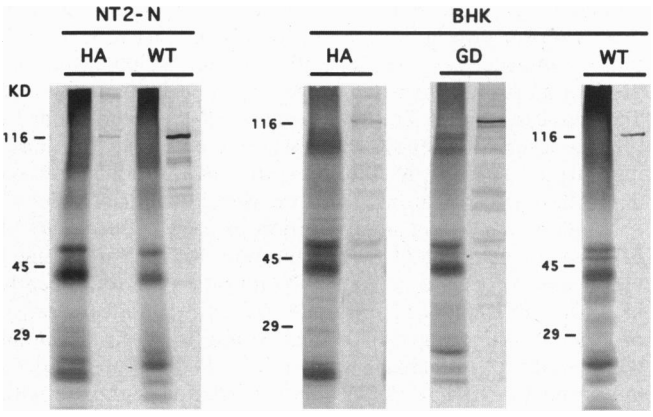

FIG. 1. Immunoprecipitation of PS1 in BHK cells and NT2N neurons. NT2N neurons (*Left*) or BHK cells (*Right*) were infected overnight with recombinant SFV vectors expressing PS1, PS1-HA, PS1-gD, or a control virus expressing LacZ. Cells were radiolabeled for 4 h with [^{35}S]methionine, lysed, and immunoprecipitated with antibodies to the HA tag, gD tag, or the native protein (anti-1398). The precipitated proteins were resolved by SDS/PAGE. The left lanes in each pair represent the PS1 immunoprecipitation, while the right lanes show the results obtained with the LacZ control virus.

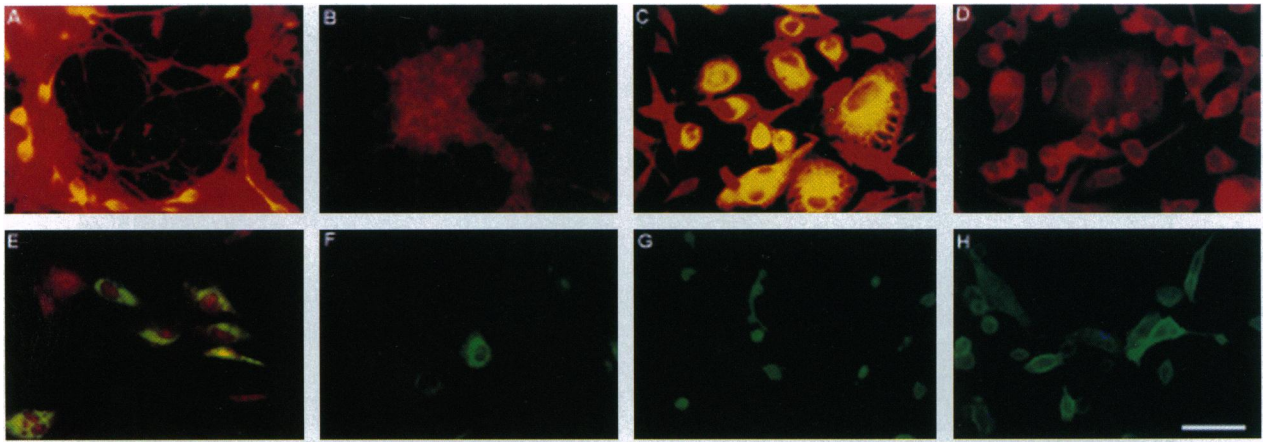

FIG. 2. PS1 is not expressed on the cell surface. For intracellular staining (*A, C, E,* and *F*), cells were fixed, permeabilized, and then incubated with the indicated antibody. For extracellular staining (*B, D, G,* and *H*), antibodies were applied to chilled cells that were then washed, fixed, and permeabilized. All primary antibodies were detected with fluorescein isothiocyanate-conjugated secondary antiserum. The red color is Evans blue (a fluorescent stain to visualize all cells) and yellow is fluorescein isothiocyanate green overlaping with red. (*A–D*) PS1-HA staining was detected only intracellularly in both NT2N neurons (*A* and *B*) and BHK cells (*C* and *D*). Likewise, native PS1 stained with antisera 1357 (*E*) and PS1-gD stained with anti-gD (*F*) were detected intracellularly. Nonpermeabilized BHK cells expressing influenza HA (*G*) or HSV-gD (*H*) were used as controls to demonstrate that both the anti-HA and anti-gD antibodies reacted with their respective epitopes on the cell surface under the conditions used. (Bar = 50 μm.)

markers, vimentin and the low molecular weight neurofilament (NFL) protein, were not (data not shown). These studies indicated that both BHK and NT2N neurons remained impermeable to antibodies under the conditions employed. In contrast to influenza HA and APP, the PS1-HA could not be detected on the surface of NT2N neurons (Fig. 2*B*) or BHK cells (Fig. 2*D*). Only in permeabilized cells could PS1-HA be stained (Fig. 2 *A* and *C*), demonstrating that the amino-terminal domain of PS1 was not on the cell surface or that it is below the detection level of our assay.

Identical experiments were performed with PS1-gD and with untagged PS1. HSV-1-infected cells were used as positive controls. HSV-1 gD was efficiently expressed and detected on the surface of nonpermeabilized cells (Fig. 2*H*), while PS1-gD could only be detected in permeabilized cells (Fig. 3*F*). Similar results were obtained in BHK cells and NT2N neurons (data not shown) expressing wt PS1 and using antisera 1357 directed to the region of PS1 between potential membrane-spanning domains 6 and 7 (Fig. 2*E*). Again, cell surface staining was absent, but intracellular staining was present after permeabilization. Thus, antibodies to epitopes in three topographically distinct PS1 domains failed to detect any cell surface PS1, suggesting that either all or at least the bulk of PS-1 resides in an intracellular location. Furthermore, the staining pattern seen after permeabilization was identical for the three constructs examined: PS1-HA, PS1-gD, and wt PS1, indicating that the antigenic tags did not noticeably affect the distribution of the protein.

Since NT2N neurons are polarized and their processes can be distinguished immunocytochemically by axonal or dendritic markers (Fig. 3*A*), we examined the distribution of PS1 in fully polarized NT2N cells using double-label confocal laser microscopy with axonal and dendritic markers. These studies showed that PS1 was localized in cell bodies and in dendrites (Fig. 3*B*) but not in axons (Fig. 3*C*). Only in rare instances was very faint staining of the proximal portions of axons observed. In cells infected with the recombinant SFV for only 4 h (when PS1 expression levels are low), PS1 was confined to cell bodies and neuritic processes (data not shown), indicating that its presence in dendrites was not an artifact of overexpression.

The reticular distribution of PS1 in BHK cells resembled a typical pattern seen with proteins localized to the rough ER. Indeed, the distribution of PS1 in BHK cells was similar to that of calnexin, a molecular chaperone that is confined to the rough ER (28) (data not shown). In NT2N cells, colocalization of PS1 was observed with calnexin in cell bodies and dendrites (Fig. 3*D*). Thus, we conclude that the bulk of PS1 is localized in the ER. However, we cannot rule out the possibility that some PS1 is targeted to the Golgi or other regions in the cell, since there are examples of proteins that are transported from the ER very inefficiently.

Distribution of a PS1 Mutant Associated with FAD. To assess the effects of missense mutations linked to FAD on the expression of PS1, we introduced one of these mutations [i.e., A(246)E] into the PS1 gene containing the HA tag. A recombinant SFV vector was produced, and the protein was expressed in BHK and NT2N neurons. As shown in Fig. 4, the PS1-A(246)E mutant exhibited an intracellular distribution similar to that seen with the wt protein at the light microscopic level.

PS1 Is Not Glycosylated. Due to its localization in the ER and the presence of two potential N-linked carbohydrate addition sites, we performed experiments to determine whether PS1 is glycosylated. Cells expressing PS1 were metabolically labeled in the presence or absence of tunicamycin, a compound that inhibits addition of N-linked carbohydrates (29). We found that migration of PS1 in SDS/PAGE was unaffected by tunicamycin treatment or by digestion with N-glycosidase F (Fig. 5*A*). In addition, PS1 could not be metabolically labeled with [^{3}H]mannose (data not shown). Finally, digestion of PS1 with a combination of O-glycanase and neuraminidase also failed to alter the protein's migration in SDS/PAGE (Fig. 5*B*). Thus, these data indicate that PS1 is neither N- nor O-glycosylated.

DISCUSSION

Mutations in the PS1 gene lead to the development of early-onset FAD that is similar to forms of AD that arise due to other causes (8). To understand the mechanisms by which these mutations lead to FAD, it will be important to characterize PS1 biosynthesis and processing in neurons because (*i*) neurons are the most severely affected cells in the AD brain, (*ii*) they are likely to be a major source of the Aβ deposited in senile plaques, and (*iii*) PS1 and PS2 gene expression appears to be highest in neuronal populations in the brain (19). NT2N neurons closely mimic postmitotic human neurons (20). Using SFV vectors and antibodies to three distinct PS1 domains, we found that PS1 migrates as a closely spaced doublet under

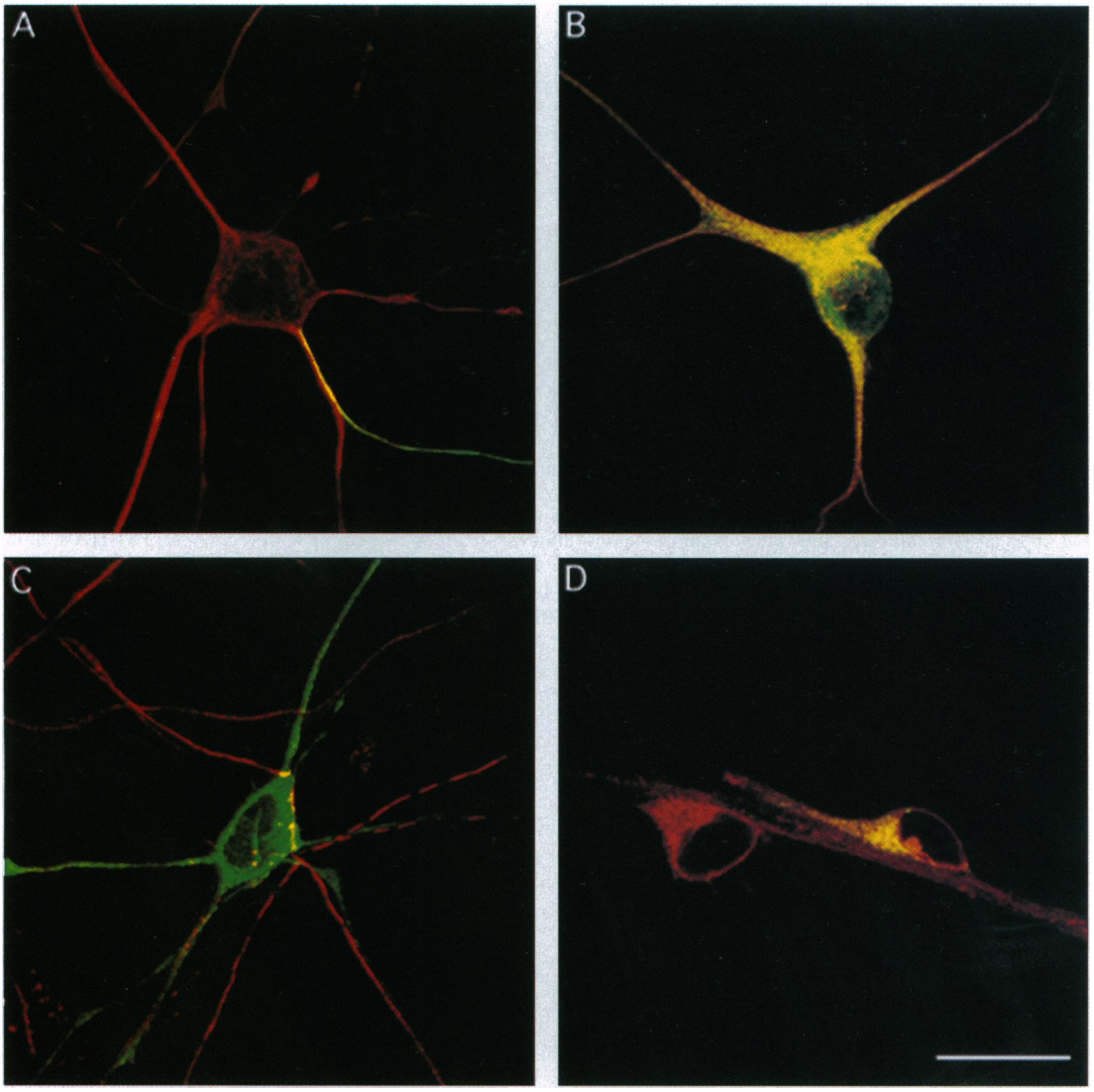

FIG. 3. PS1 is expressed in dendrites but not axons of NT2N cells. (*A*) Confocal scanning laser microscopy of polarized NT2N cells grown for 6 months on rat astrocytes and stained with antibody HO14 to phosphorylated neurofilament NFM axonal proteins (green) and an antibody to the dendritic marker microtubule-associated protein 2 (MAP2) (red). (*B*) PS1-HA (green) and MAP2 (red) colocalize (yellow overlap). (*C*) Identically prepared neurons stained against PS1-HA (green) and HO14 (red) show that PS1 is not located in axons. (*D*) Colocalization (yellow overlap) of PS1-HA (green) and the ER marker calnexin (red). (Bar = 25 μm.)

reducing conditions in SDS/PAGE. This pattern was seen regardless of cell type and with PS1-HA, PS1-gD, and unmodified PS1. Thus, the doublet is not cell-type-dependent or an artifact of the antigenic tags. Several posttranslational modifications may account for the two bands, including glycosylation, phosphorylation, and posttranslational proteolytic cleavages. However, we found that PS1 contains neither N- nor O-linked carbohydrates. In addition, since antibodies to both ends of PS1 (the amino-terminal domain HA tag and the carboxyl-terminal gD tag) immunoprecipitated both forms of the protein, we consider it unlikely that the two bands are the result of proteolysis.

Besides posttranslational modifications, the presence of two distinct PS1 bands in SDS/PAGE could be due to incomplete denaturation of the protein, even after boiling in SDS in the presence of reducing agent. SDS may be relatively inefficient in disrupting hydrophobic helices that can serve to stabilize both inter- and intraprotein interactions (26, 30). Indeed, there are many examples of hydrophobically anchored proteins that display various degrees of resistance to SDS-induced denaturation (31). The seven potential transmembrane domains of PS1 may make this protein difficult to fully denature. In addition, certain cellular proteins localized to the ER and Golgi cisternae form large aggregates that lead to their retention in these organelles (32). Whether the diffuse high molecular weight material seen after PS1 immunoprecipitation reflects such a process remains to be determined, but PS1 is largely localized in the ER and may be present in the Golgi apparatus as well.

The placement of antigenic tags in PS1 along with the development of antisera to the native protein made it possible to monitor the distribution of PS1 in NT2N neurons. Notably, PS1 was not detected on the cell surface by immunocytochemistry using antisera that recognize three topologically distinct domains. While it is possible that all three regions of PS1 seen by our antibodies are oriented toward the cytoplasm, we consider this unlikely since the determinant recognized by antisera 1357 and the gD antigenic tag are on opposite sides of the seventh potential transmembrane domain, a 23-residue stretch of hydrophobic amino acids bounded by charged residues that constitutes a classic membrane-spanning sequence. This, coupled with the colocalization of PS1 with the ER marker calnexin argues that PS1 is not transported to the cell surface. However, we cannot rule out the possibility that PS1 is transported from the ER inefficiently. Several other

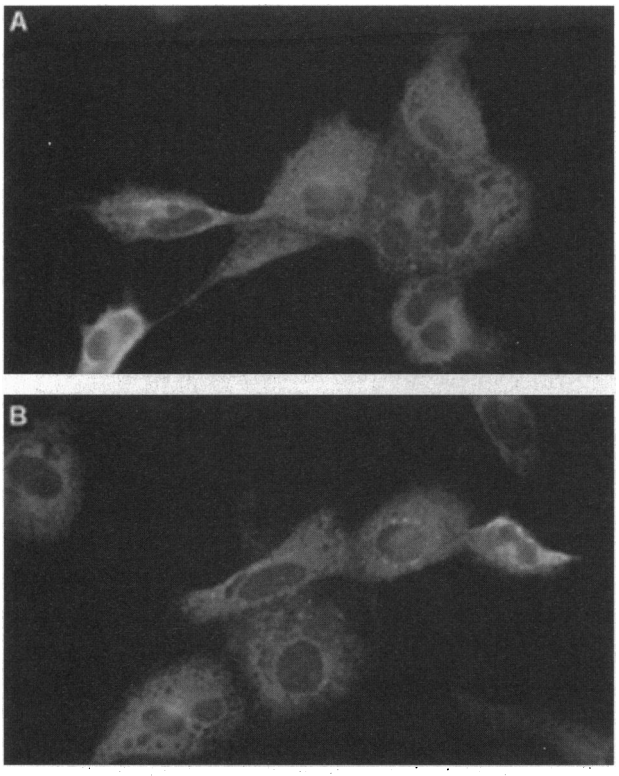

FIG. 4. Distribution of PS1 A(246)E and wt PS1 in BHK cells. BHK cells were infected with recombinant SFV vectors that express PS1-HA (*A*) or PS1-A(246)E (*B*). The cells were fixed and stained with antisera 1398.

proteins exhibit this property, including components of the T-cell receptor (33) and the cystic fibrosis transmembrane conductance regulator protein (34).

Our studies with NT2N neurons show that PS1 is localized primarily to the rough ER in the cell body and to dendrites. Whether this localization reflects association with ER elements that are present in dendrites or is due to the transport of PS1 to other dendritic structures remains to be determined. Interestingly, APP has recently been shown to first be delivered to axons and subsequently to dendrites by a transcytosis process (35). The factors regulating this proposed transcytosis-like mechanism are not known. However, it is interesting to speculate a possible role for the presenilins in this process given recent clues to the possible biological role(s) of these molecules and their strong similarities with the *Caenorhabditis elegans* proteins sel-12 [approximately 50% amino acid identity (36)] and spe-4 [approximately 25% amino acid identity (37)]. The homologies between the presenilins and these nematode proteins have led to the suggestion that the presenilins may function in the intracellular trafficking and localization/recycling of proteins (19). Sel-12, for example, has been suggested to play a role in facilitating the localization and/or recycling of the Notch receptor lin-12 (36). Given these striking homologies along with the localization of both PS1 and APP to neuronal dendrites, it is conceivable that PS1 could play a role in delivering APP to or from dendritic processes.

Younkin and colleagues have recently presented data showing that certain PS1 and PS2 mutations lead to increased production of longer more amyloidogenic forms of Aβ in fibroblasts and plasma from affected and at-risk carriers (S. Younkin, personal communication). It will, therefore, be important to determine whether mutations in PS1 and PS2 ultimately affect the trafficking of APP and production of Aβ. The localization of PS1 in the ER and in dendrites along with APP suggests that if this hypothesis is correct, abnormal processing of APP and increased production of Aβ may likely occur at these intracellular sites.

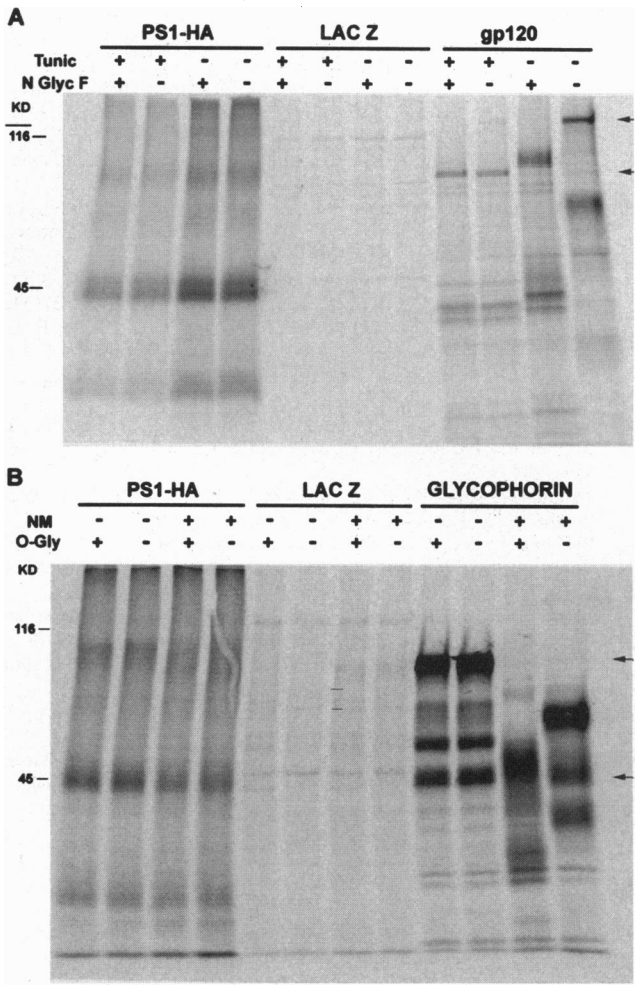

FIG. 5. PSI is not glycosylated. (*A*) BHK cells expressing PS1-HA, LacZ, or the gp120 glycoprotein from human immunodeficiency virus were metabolically labeled in the presence or absence of tunicamycin. The PS1 and gp120 were immunoprecipitated, and portions of the precipitated protein were digested with N-glycosidase F. LacZ-expressing cells were used as a control for the anti-HA antibody used to immunoprecipitate PS1-HA (arrows mark fully and nonglycosylated gp160). (*B*) Immunoprecipitated PS1-HA was digested with neuraminidase and/or O-glycanase as indicated. Glycophorin was used as a positive control (arrows denote dimeric and monomeric glycophorin).

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