Subcellular Distribution and Turnover of Presenilins in Transfected Cells

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The mechanisms by which mutations in presenilin-1 (PS1) and presenilin-2 (PS2) result in the Alzheimer’s disease phenotype are unclear. Full-length PS1 and PS2 are each processed into stable proteolytic fragments after their biosynthesis in transfected cells. PS1 and PS2 have been localized by immunocytochemistry to the endoplasmic reticulum (ER) and Golgi compartments, but previous studies could not differentiate between the full-length presenilin proteins and their fragments. We carried out subcellular fractionation of cells stably transfected with PS1 or PS2 to determine the localization of full-length presenilins and their fragments. Full-length PS1 and PS2 were principally distributed in ER fractions, whereas the N- and C-terminal fragments were localized predominantly to the Golgi fractions. In cells expressing the PS1 mutant lacking exon 9 (ΔE9), we observed only full-length molecules that were present in the ER and Golgi fractions. The turnover rate was considerably slower for the ΔE9 holoprotein, apparently due to decreased degradation within the ER. Our results suggest that full-length presenilins are primarily ER resident molecules and undergo endoproteolytic cleavage within the ER. The fragments are subsequently transported to the Golgi compartment, where their turnover rate is much slower than that of the full-length presenilin in the ER.

Alzheimer’s disease (AD), the major age-related dementia disorder, usually occurs sporadically in the elderly population. In a subset of cases, familial AD (FAD) occurs as an inherited autosomal dominant disease. Mutations in three different genes are associated with the familial form of AD (reviewed in Ref. 1). The first FAD gene is the APP, amyloid precursor protein; WT, wild type; ΔE9, PS1 mutant lacking exon 9; Aβ, amyloid β-peptide; NTF, N-terminal endoproteolytic fragment; CTF, C-terminal endoproteolytic fragment; CHO, Chinese hamster ovary.

presenilin 2 (PS2) on chromosome 1 (3, 4), account for the majority of the early onset cases of FAD.

PS1 and PS2, which are functionally homologous to the C. elegans Sel-12 molecule (5, 6), are hydrophobic proteins that cross the membrane 6–8 times (7, 8). In transfected cells, both PS1 and PS2 holoprotein are rapidly turned over with a half-life of under 1 h (9, 10). However, in transfected cells and in brain tissue, stable N- (NTF) and C-terminal fragments (CTF) are apparently generated by endoproteolysis from full-length presenilin molecules (9, 11, 12). Immunolocalization studies of presenilins in transfected cells have demonstrated a predominant endoplasmic reticulum (ER) and Golgi distribution (13, 14). The localization pattern is similar regardless of whether the antibodies are generated to either N- or C-terminal epitopes, raising the possibility that the NTFs and CTFs remain in proximity to each other. However, these studies cannot distinguish the full-length presenilin molecules from the stable fragments. APP is also located in the ER and Golgi, where direct or indirect interaction with the presenilins may contribute to the production of longer forms of amyloid β-peptide (Aβ) (15–17). Thus, knowledge of the processing and trafficking of presenilin molecules is important to our understanding of the pathways of Aβ production.

The role of endoproteolysis in presenilin maturation and function remains to be defined. Interestingly, a PS1 FAD mutation lacking exon 9 (ΔE9) that results in the absence of conventional endoproteolysis (12) can nevertheless functionally replace an egg-laying defect resulting from a C. elegans sel-12 mutant (5). To date, neither the subcellular distribution of the holoprotein and its fragments nor the compartment where endoproteolytic cleavage occurs is known. To define the distribution of full-length forms and stable fragments of PS1 and PS2, we carried out subcellular fractionation of stably transfected cells. Our results indicate that full-length PS1 and PS2 are located mainly in the ER, while their N- and C-terminal fragments are located principally in the Golgi. Turnover rate of the ΔE9 mutant PS1 protein was slower than wild type (WT) PS1 holoprotein. Concomitantly, the steady state subcellular distribution of ΔE9 PS1 extends to both ER and Golgi fractions. Finally, pulse-chase experiments combined with subcellular fractionation suggested that the endoproteolytic cleavage of PS1 takes place in the ER.

MATERIALS AND METHODS

PS1 and PS2 Stably Transfected Cells—Chinese hamster ovary (CHO) cells stably transfected with WT or mutant (M146L or C410Y) PS1, or with WT or mutant (N141D) PS2 were described previously (10, 17). cDNAs encoding WT and ΔE9 PS1 were subcloned into an ecdysone-inducible mammalian expression vector (pNiD, Invitrogen) and transfected into human embryonic kidney 293 cells previously transfected with the PvrXR construct that encodes the regulatory ecdysone

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† The abbreviations used are: AD, Alzheimer’s disease; FAD, familial Alzheimer’s disease; PS1, presenilin-1; PS2, presenilin-2; ER, endoplasmic reticulum; APP, amyloid precursor protein; WT, wild type; ΔE9, PS1 mutant lacking exon 9; Aβ, amyloid β-peptide; NTF, N-terminal endoproteolytic fragment; CTF, C-terminal endoproteolytic fragment; CHO, Chinese hamster ovary.

1 The abbreviations used are: AD, Alzheimer’s disease; FAD, familial Alzheimer’s disease (AD), the major age-related dementing disorder, usually occurs sporadically in the elderly population. In a subset of cases, familial AD (FAD) occurs as an inherited autosomal dominant disease. Mutations in three different genes are associated with the familial form of AD (reviewed in Ref. 1). The first FAD gene is the APP, amyloid precursor protein (APP), the precursor to amyloid β-protein, the major component of senile plaques seen in brains of AD individuals. The other genes, presenilin 1 (PS1) on chromosome 14 (2) and
receptor (EcR293, Invivogen). Stable cell lines were selected for Zeocin and G418 resistance. PS1 expression was induced by overnight treatment with 0.0625–1.0 μM muristerone A.

**Antibodies**—PS1 polyclonal antibodies J27 and 4627 were raised against residues 27–42 and 457–467 of PS1, respectively, and have been previously characterized (9, 10). The PS1 monoclonal antibody, PSM2, was generated against a synthetic peptide corresponding to residues 31–56 of PS1 (18). A PS2 polyclonal antibody, 1209, was raised against a glutathione S-transferase bacterial fusion protein encompassing residues 1–70 of PS2. Polyclonal antibody CT15 was raised against the last 15 residues at the C terminus of the APP (19). Additional polyclonal antibodies included calnexin (StressGen) and rat Na⁺/K⁺-ATPase (Upstate Biotechnology, Inc., Lake Placid, NY), plus a monoclonal antibody to Na⁺/K⁺-ATPase (America BioResearch Co.).

**Pulse-Chase Experiments**—Confluent CHO and 293 cells were incubated in methionine-deficient medium for 1 h, followed by pulse labeling with 200 μCi/ml [³H]methionine for 20 min. The cells were then either lysed immediately or chased in regular medium for 1–8 h. The cells were then collected and fractionated as described below.

**Subcellular Fractionation**—Cultured CHO and 293 cells were detached from confluent cultures grown in 15-cm dishes with 20 mM EDTA in ice-cold phosphate-buffered saline. Cells were pelleted and resuspended in homogenization buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.5% sucrose, supplemented with a protease inhibitor mixture). The cells were disrupted using 10 strokes in a Dounce homogenizer followed by four passages through a 25-gauge needle. Nuclei and unbroken cells were pelleted by centrifugation at 3000 × g for 10 min. The pellets were resuspended in 1.5 ml of homogenization buffer and centrifuged at 80,000 × g for 1 h. The vesicle pellet was resuspended in 0.8 ml of homogenization buffer. All operations were carried out at 4 °C. Each cell line was analyzed 2–5 times, and representative experiments are shown under “Results.”

The viscosity of the gradient medium is a major determinant of the sedimentation rate. In addition, because subcellular organelles are osmotically sensitive, the osmotic activity of the gradient medium is particularly important. Thus, although sucrose, glycerol, and Ficoll are used, they do not ideal in osmolality and viscosity. OptiPrep (60% (w/v) Life Technologies) and Nycodenz (Manese and Staubli (20), in which the addition of [³H]galactose onto the oligosaccharides of an acceptor protein, ovomucoid, was measured.

**RESULTS**

**Subcellular Fractionation by Iodixanol Gradient**—CHO, stably transfected with PS1 (WT, M146L, or C410Y) or PS2 (WT or N141I), and 293 cells, stably transfected with PS1 (WT or ΔE9), under the ecdysone-inducible system were analyzed by subcellular fractionation. Subcellular vesicles were separated on 1–20% continuous Iodixanol gradients as described under “Materials and Methods.” ER-rich fractions were found, as expected, at the bottom of the gradient, using an antibody against the well characterized ER marker protein, calnexin (21) (Fig. 1, A and B). Calnexin-reactive ER vesicles were most enriched in fractions 1–4. Golgi-containing fractions were identified by assaying for β-1,4-galactosyltransferase, a trans-Golgi enzyme (20), and this was principally found in...
fractions 4–7 (Fig. 1, A and B). Immunoreactivity against Na\(^+\)/K\(^+\)-ATPase, a marker for plasma membrane, was found on the top of the gradient, i.e. fractions 9–12, although lesser amounts of immunoreactivity were also present in the mid-density region (fractions 5–8) (not shown). The latter reactivity was derived from molecules transiting the Golgi. As an additional marker, CHO cells stably expressing WT APP751 were fractionated and analyzed by blotting with antibody CT15 (to the C terminus of APP). As expected, the immature (primarily N-glycosylated) forms of APP appeared in the ER-rich fractions (fractions 1–4) (Fig. 1C), whereas the mature (N- plus O-glycosylated) forms of APP appeared only in Golgi-rich fractions 4–7. These data indicate that ER and Golgi plasma membrane fractions are effectively separated on these 1–20% Iodixanol gradients.

Subcellular Distribution of PS1 and PS2—To determine the distribution of PS1, we performed Western blot analysis of the gradient fractions from PS1 stably transfected CHO cells. Antibody J27 raised to the N terminus of PS1 recognized a ∼45-kDa PS1 holoprotein derived from the transgene mainly in gradient fractions 1–4, where it co-localized with the ER marker calnexin (Fig. 2, A and B). In contrast, the major N-terminal endoproteolytic fragment (∼29 kDa) was consistently localized to the lighter fractions toward the middle of the gradient, principally in fractions 4–7 (Fig. 2, A and B). Similarly, the major C-terminal fragment (∼18 kDa) was distributed in fractions 3–8 (Fig. 2, C and D). Both PS1 NTF and CTF colocalized with the Golgi marker, β-1,4-galactosyltransferase. Fractionation of stable transfectants expressing the PS1 missense mutations M146L or C410Y showed no significant differences from WT PS1 in subcellular distribution of the holoprotein and its N- and C-terminal fragments (not shown).

We next examined CHO cells stably transfected with PS2 cDNA using the same gradient fractionation and blotting with a PS2 N-terminal antibody (antibody 1209). Full-length PS2 molecules were detected as a characteristic ∼50-kDa band principally in ER-rich fractions 3 and 4 (Fig. 3, A and B), whereas the N-terminal fragment was localized primarily in Golgi-rich fractions 5–8. Moreover, as seen above with PS1 mutations, the distribution of the N141I mutant form of PS2 was identical to that of WT PS2 (data not shown). Therefore, the subcellular distribution of PS2 and its proteolytic fragment was very similar to that obtained for PS1 and its fragments.

293 cells expressing WT PS1 showed a pattern of distribution of holoprotein and stable fragments very similar to that seen in the CHO-transfected cells, although there was a tendency for the Golgi fractions to be distributed over a larger number of lighter fractions. However, ΔE9 mutant protein, which does not undergo the conventional endoproteolytic cleavage (12), was widely distributed throughout the gradient fractions (Fig. 4, A and B). The extension of ΔE9 PS1 into the lighter Golgi fractions was not a result of overexpression, because the same result was seen when muristerone A was reduced in this cell line to match the level of expression seen in WT PS1-transfected 293 cells (Fig. 4, A and B). As expected, we observed no conventional proteolytic fragments from ΔE9 PS1 protein. Furthermore, as reported (22), the level of N-terminal fragment derived from endogenous PS1 decreased as the ΔE9 PS1 expression increased with higher muristerone A induction (Fig. 4A). The distribution of the NTF in the WT PS1 and ΔE9-transfected cell lines, the latter with or without induction, was identical; in the case of the latter cells, the NTF was derived only from endogenous PS1. Therefore, the subcellular distribution of transgene-derived PS1 fragments was not altered by the transfection or overexpression.

ΔE9 Mutant PS1 Has a Longer Half-life—It has been re-
results showed that ΔE9 protein, extending from ER to Golgi-rich and lighter fractions, had a distinctly different subcellular distribution as compared with WT PS1 holoprotein. To determine whether the ΔE9 mutant has a different turnover rate, WT and ΔE9 PS1 stably transfected 293 cells were pulse-labeled for 20 min and chased for 0, 1, 2, 4, or 8 h. The half-life of WT PS1 was under 1 h (average of approximately 50 min from multiple experiments) for the holoprotein and more than 8 h for the NTF (Fig. 5, A and B). On the other hand, the half-life of ΔE9 was consistently twice as long as WT PS1 holoprotein (Fig. 5, A and B). At steady state conditions as documented in the experiments described above, ΔE9 protein was also present in the Golgi fractions, where stable proteolytic fragments derived from endogenous PS1 were located. Surprisingly, the distribution of the ΔE9 mutant PS1 was not substantially altered during the chase period (Fig. 5C). After 4 h, the profile of ΔE9 protein was similar to that seen at earlier time points, i.e. predominantly in ER fractions, whereas there was virtually no labeled PS1 WT holoprotein remaining in the cell (Fig. 5C). Therefore, diminished degradation within the ER appears to account in large part for the prolonged half-life of ΔE9 rather than a shift of the proteins into the Golgi fractions, where the turnover rate may be slowed.

Closer inspection of the NTF generated during the pulse-chase period showed that at time 0, small amounts of labeled NTF can be detected in the PS1 WT cell line (Fig. 5C). Surprisingly, at this time point, the distribution of NTF was virtually identical to the full-length protein, i.e. with the peak signal in fractions 3 and 4. After 1 h of chase, the levels of labeled NTF were increased and were distributed more widely, extending into the lighter fractions. Finally, after 4 h of chase, labeled NTF was predominantly within the Golgi-enriched fractions, similar to that seen at the steady state conditions. These findings indicated that the initial cleavage of PS1 occurred in the ER and the proteolytic fragments were subsequently trans-
hypothesized that the selective increase in the production and release of the amyloidogenic A\(_{\beta}42\) as a result of the presenilin mutations is a key factor in the pathogenesis of FAD (10, 23–26). At present, the intracellular compartment where presenilin interacts directly or indirectly with APP is unknown. In this study, we analyzed the subcellular distribution of presenilins in transfected cells. Our results showed that PS1 and PS2 are similarly distributed, with holoproteins being principally located in the ER, while the stable PS1 and PS2 fragments were principally located in the Golgi. The \(\Delta E9\) PS1 mutation results in a broader distribution of the protein in both ER and Golgi-rich fractions as well as a prolonged turnover rate due to diminished degradation within the ER. Our results also indicate that endoproteolysis to generate stable N- and C-terminal fragments occurs in the ER.

Previous immunolocalization studies of transfected cells demonstrated that both PS1 and PS2 are located in the ER and Golgi (13, 14). These studies, however, could not distinguish the signals derived from full-length protein versus stable NTFs and CTFs. We therefore carried out subcellular fractionations of CHO and 293 cells stably transfected with PS1 or PS2 to determine the localization of full-length presenilin and its fragments. Our results showed that full-length PS1 and PS2 were distributed in dense fractions that colocalized with the ER marker, calnexin. Both stable NTF and CTF were distributed predominantly in the lighter fractions that colocalized with the

**FIG. 5. Half-life of WT and \(\Delta E9\) mutant PS1.** After overnight induction with 1 \(\mu M\) (WT PS1) and 0.125 \(\mu M\) (\(\Delta E9\)) muristerone A, the cells were pulse-labeled and chased for 0–8 h. An aliquot of cells at each time point was lysed and immunoprecipitated with the N-terminal antibody, J27 (A). The signal at each time point of the chase period shown in A was quantitated and expressed as a percentage of time 0 (B). In this experiment, the half-life of WT PS1 and \(\Delta E9\) was 40 and 80 min, respectively. In C, immunoprecipitation with J27 antibody was performed from each fraction after Iodixanol separation at time 0 and after 1 and 4 h of chase. Note that the distributions of both WT PS1 and \(\Delta E9\) holoprotein were not substantially changed during 0–4 h of chase. The gradient fractions showed that the longer half-life of \(\Delta E9\) was due primarily to diminished turnover of protein in the ER fractions. The distribution of NTF (arrowhead) in PS1 WT cells was shifted from the dense to lighter fractions during the chase period (top). NTF derived from endogenous PS1 in \(\Delta E9\) cells did not appear until the 4 h time point (bottom).
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Therefore, most mutations do not overtly alter presenilin trafficking. In fact, the noncleavable ΔE9 PS1 mutant was localized to a larger number of gradient fractions, being distributed to both ER and Golgi fractions. In addition, as was shown recently (22), pulse-chase studies showed that the turnover rate of ΔE9 mutant protein was considerably slower than WT PS1 holoprotein. We initially speculated that the transport of ΔE9 to the Golgi compartment where the stable presenilin fragments are located may underlie the decrease in turnover rate of this mutant protein species. However, pulse-chase labeling combined with subcellular fractionation showed that a significant pool of pulse-labeled ΔE9 protein remained intact within the ER at a time when WT PS1 holoprotein was completely degraded. This result suggests that reduced proteolytic degradation of ΔE9 within the ER is responsible for the increased half-life of this molecule. In time, a small fraction of undegraded protein, together with the endogenous NTF and CTF, are found in the Golgi compartment.

Previous studies have consistently shown an inverse relationship between ΔE9 expression and endogenous NTF levels (22, 26), suggesting that mutant holoprotein competes with the normal cleavage of endogenous PS1. In this study, increasing expression of ΔE9 mutant protein by muristerone A induction similarly led to progressively lower levels of endogenous PS1 stable fragments. Interestingly, in the pulse-chase/fractionation studies, the appearance of labeled endogenous fragments was delayed. Therefore, production of NTF from proteolysis of endogenous PS1 is affected both quantitatively and kinetically in the presence of ΔE9 mutant protein. This competition is likely to occur in the ER, because ΔE9 appeared to be largely degraded in this compartment.

Our study also provides evidence that constitutive endoproteolysis of PS1 to generate stable NTF and CTF occurs in the ER. Pulse-chase experiments combined with subcellular fractionation showed that the initial pool of labeled NTF, albeit in very low levels, was located predominantly in the ER fractions, similar to labeled holoprotein. During the chase period, however, the amount of labeled NTF increased, consistent with a precursor-product relationship between full-length protein species and their fragments within the ER. Importantly, the NTF was seen to distribute to lighter fractions during the chase. By 4 h of chase, labeled NTF was distributed predominantly to the Golgi-enriched fractions, similar to that seen at steady state. Taken together, these observations provide compelling evidence that constitutive endoproteolysis of PS1 occurs initially in the ER compartment.

With the notable exception of the ΔE9 mutation, the subcellular distribution of all of the other PS1 (M146L, C410Y) and PS2 (N141I) mutations was indistinguishable from WT PS1 and PS2, respectively. Since all of these presenilin mutations have been reported to show a selective increase in Δβ42 production (10, 17), our results suggest that perturbations in the ER to Golgi processing at this level cannot explain how mutants alter Δβ42 generation. Like the presenilins, maturation of APP in the exocytic pathway occurs in the ER and Golgi compartments. Thus, colocalization of both APP and presenilins in the same subcellular compartments leads to the possibility of an interaction between these two molecules, as has been reported in transfected cells (15, 17). Using subcellular fractionation, we have recently detected increased levels of Δβ42 in ER and Golgi fractions of cells expressing mutant presenilins (16). These observations suggest that understanding the molecular interactions between APP and presenilins as well as other proteins within these cellular compartments will be important in resolving the mechanisms of AD pathogenesis.

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