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Protease digestion indicates that endogenous presenilin 1 is present in at least two physical forms ☆

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Abstract

The membrane-bound protein complex γ-secretase is an intramembranous protease whose substrates are a number of type I transmembrane proteins including the β-amyloid precursor protein (APP). A presenilin molecule is thought to be the catalytic unit of γ-secretase and either of two presenilin homologues, PS1 or PS2, can play this role. Mutations in the presenilins, apparently leading to aberrant processing of APP, have been genetically linked to early-onset familial Alzheimer's disease. To look for possible molecular heterogeneity in presenilin/γ-secretase we examined the ability of proteinase K (PK) to digest endogenously expressed presenilins in intact endoplasmic reticulum vesicles. We demonstrate the existence of two physically different forms of γ-secretase-associated PS1, one that is relatively PK-sensitive and one that is significantly more PK-resistant. A similarly PK-resistant form of PS2 was not observed. We speculate that the structural heterogeneity we observe may underlie, at least in part, previous observations indicating the physical and functional heterogeneity of γ-secretase. In particular, our results suggest that there are significant differences between γ-secretase complexes incorporating PS1 and PS2. This difference may underlie the more dominant role of PS1 in the generation of β-amyloid peptides and in familial Alzheimer's disease.
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Keywords: Presenilin; Alzheimer's disease; γ-Secretase; β-Amyloid; Intramembranous protease; Proteinase K

The brains of patients suffering from Alzheimer's disease (AD) contain characteristic 'senile' plaques, a major constituent of which are aggregated β-amyloid peptides [1–3]. The final step in the generation of these peptides is the proteolytic cleavage of the β-amyloid precursor protein (APP) by γ-secretase, a member of a recently discovered novel class of intramembranous proteases [4,5]. The substrates of γ-secretase are a family of type I transmembrane proteins that it cleaves within their membrane spanning domains [4]. γ-Secretase is a complex consisting of four integral membrane proteins: presenilin, nicastrin, Aph-1, and PEN-2, all of which are required for proteolytic activity [5]. Two presenilin (PS) homologues, PS1 and PS2, exist in mammals and either can be incorporated into the γ-secretase complex. The presenilin molecule is thought to be the proteolytic component of γ-secretase and two aspartate residues within its transmembrane region have been shown to be required for activity and are thought to form a part of the catalytic site [1,2,4,5]. Mutations in the presenilins that result in increased production of longer forms of β-amyloid that are more prone to aggregation have been shown to be associated with many cases of early-onset familial AD [1–3]. While the over-production of this more aggregation-prone β-amyloid is thought to lead to plaque formation in these cases of inherited AD, the reasons for plaque formation in spontaneous AD are still unclear. Because of its role in β-amyloid production

☆ *Abbreviations*: AD, Alzheimer's disease; APP, β-amyloid precursor protein; PS, presenilin; NTF, N-terminal fragment; CTF, C-terminal fragment; ER, endoplasmic reticulum.

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the γ-secretase complex and its components have been a major focus of research into the pathology and treatment of AD.

Recent evidence indicates that the assembly of the γ-secretase complex occurs in the early compartments of the secretory pathway [6–8]. Immature nicastrin and Aph-1 first form a stable precursor complex with which the ~50 kDa presenilin holoprotein and then PEN-2 associate [9–11]. The incorporation of PEN-2 into the complex leads to the endoproteolysis of presenilin [11] by an as yet unknown mechanism, resulting a ~30 kDa N-terminal fragment (NTF) and a ~20 kDa C-terminal fragment (CTF). These events in turn lead to the stabilization and activation of the γ-secretase complex [9,11]. Endogenous presenilin holoprotein that is not associated with γ-secretase is rapidly degraded by the proteosome [12,13] and is typically observed at very low levels (if at all) relative to the NTF and CTF.

In the literature PS1 has received considerably more experimental attention than PS2. In this regard hereditary AD is much more commonly associated with mutations in PS1 than in PS2 (www.alzforum.org/res/com/mut/pre/default.asp) and, perhaps not surprisingly, PS1 has been found to play a more significant role in the generation of β-amyloid peptides [14,15]. Although the stoichiometry of the γ-secretase complex is still uncertain, it has been shown that PS1 and PS2 do not co-assemble [16,17] and thus that they form physically distinct γ-secretases. Furthermore, studies with PS1/PS2-deficient cells [18] and presenilin transgenic mice [14] indicate that PS1 and PS2 are associated with functionally distinct but overlapping γ-secretase activities. More generally, aside from differences associated with presenilin homologues it has been speculated that γ-secretase itself may not be a single enzyme but rather occurs as a number of functionally distinct variants [14]. In this regard γ-secretase complexes ranging in size from 250 to 2000 kDa have been described [19–25]. The smallest of these corresponds to the combined molecular masses of presenilin, nicastrin, Aph-1, and PEN-2, but most of the γ-secretase activity is associated with higher molecular weight complexes, suggesting that some components are present in multiple copies in the functional enzyme. Whether some of these various molecular weight species occur as a result of dissociation or aggregation of others during the experimental maneuvers associated with sample preparation remains to be determined.

In the present paper, we have employed a different approach to look for possible molecular heterogeneity in presenilin/γ-secretase. Here, we examine the ability of proteinase K to digest endogenously expressed presenilins in intact endoplasmic reticulum (ER) vesicles. Consistent with the notion of γ-secretase structural heterogeneity we find direct evidence for two pools of endogenous endoproteolysed PS1, one that is readily digested by the protease and the other that is much more protease resistant. Interestingly, there is no pool of similarly protease-resistant PS2. Since endoproteolysed PS1 occurs only as a component of the γ-secretase complex these results indicate that PS1-containing γ-secretase is present in at least two physical forms.

Materials and methods

Cell culture and transfection. HEK-293 cells were cultured as previously described [26]. Transfections were carried out using FuGENE (Roche) according to the manufacturer's instructions. The human PS1 cDNA clone was generously provided by Dr. Todd E. Golde (Mayo Clinic Jacksonville).

Antibodies. We used commercial polyclonal antibodies against calregulin (Santa Cruz Biotechnology, sc-11398), the N-terminus of calnexin (Santa Cruz Biotechnology, sc-11397), the N-terminus of human PS1 (Santa Cruz Biotechnology, sc-7860), and the C-terminus of signal peptide peptidase (Abcam, ab16080). Antibodies against the 'loop' regions of human PS1 and PS2, which recognize their respective CTFs, were purchased from Chemicon (MAB5232; raised against amino acids 263–378 of human PS1) and Cell Signaling Technology (#2192; raised against the residues surrounding amino acid 330 of human PS2).

Preparation of endoplasmic reticulum (ER) vesicles and proteinase K digestion. The procedures for preparing intact ER vesicles and their treatment with proteinase K were essentially as previously described [27]. Briefly, HEK-293 cells were collected in buffer A (10 M Hepes–KOH, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM Na-EDTA, 5 mM Na-EGTA, and 250 mM sucrose; all steps at 4 °C), homogenized by passing through a 22 gauge needle 15 times, and centrifuged at 3000*g* for 10 min. The supernatant was centrifuged at 20,000*g* for 15 min and the resulting pellet containing sealed cytosolic-side-out ER vesicles (see Results and discussion and Ref. [27]) was resuspended in buffer A plus 100 mM NaCl. Aliquots of this preparation (~10 μg of protein) were treated with 0.1 mg/ml proteinase K (Roche) in the presence or absence of 1% Triton X-100 in a total volume of 12 μl for 30 min on ice unless otherwise noted. The reaction was stopped by adding PMSF at a final concentration of 20–30 mM and samples were subjected to SDS–PAGE and immunoblot analysis (see below).

In some experiments ER vesicles were subjected to treatment with urea or high salt before proteinase K treatment. In these cases freshly prepared ER vesicles were resuspended in 4 M urea (4 M urea, 5 mM EDTA, 5 mM EGTA, and 20 mM Hepes, pH 7.4) or 0.5 M NaCl (0.5 M NaCl, 5 mM EDTA, 5 mM EGTA, and 20 mM Hepes, pH 7.4), incubated for 10 min on ice with gentle vortexing every 2 min, then centrifuged at 20,000*g* for 15 min and resuspended in buffer A plus 100 mM NaCl before proteinase K addition.

SDS–PAGE, Western blotting and data analysis. SDS–PAGE and Western blotting were carried out as previously described [28]. Secondary antibodies (Pierce) conjugated to horseradish peroxidase were used at a dilution of 1:10,000. Detection was carried out using the ECL kit (Amersham) and X-Omat AR film (Kodak). Quantitation of Western blots was done using ImageQuant 5.2 software (Molecular Dynamics).

Results and discussion

Proteinase K is a highly active, non-specific protease capable of cleaving all peptide bonds. As a consequence proteins that are resistant to proteinase K digestion are rare and this resistance is generally thought to be an indication of a tightly packed highly stable structure [29]. In a previous publication [27], we examined the accessibility of PS1 antibody epitopes to proteinase K in intact cytosolic-side-out ER vesicles in order to obtain information about the PS1 topology. In these experiments, we demonstrated that epitopes in the N-terminus and 'loop' regions of PS1 were susceptible to digestion by proteinase K and thus were located on the cytosolic side of the membrane.

In the preliminary experiments for those studies we also noted that, relative to most proteins, rather high concentrations of proteinase K (20 mg/ml; incubation for 30 min on ice) were required to digest these sites. In Fig. 1 we examine the ability of a much lower concentration of proteinase K (0.1 mg/ml; see Materials and methods) to digest these same and other antibody epitopes after a 5 or 30 min period of incubation.

As controls we first assayed our vesicle preparation via Western blotting for calregulin, an ER luminal protein, and calnexin, an ER membrane protein (Fig. 1A). In the case of calnexin we used an antibody directed against a luminal (N-terminal) epitope. In both cases we found that virtually all of the immunoreactive signal was preserved after proteinase K treatment (Fig. 1B), but that the apparent molecular weight of calnexin was shifted downward ~10 kDa (Fig. 1A) consistent with the digestion of its cytosolic ~90 amino acid C-terminus by the protease. Both the calregulin and calnexin signals were lost when digestion was carried out in the presence of 1% Triton X-100 (Fig. 1A) which solubilizes the vesicle membrane. These results confirm that virtually all of the ER membranes in this preparation are in the form of sealed cytosolic-side-out vesicles. We next probed these vesicles with the antibodies raised against the N-terminus and 'loop' region of PS1, which recognize the PS1 NTF and PS1 CTF, respectively (Fig. 1A). In contrast to calnexin whose cytosolic sequence is completely digested within 5 min, both of these cytosolic epitopes were only partially digested by proteinase K after 5 min and there was no significant difference between the degree of digestion observed at 5 and 30 min (Fig. 1B).

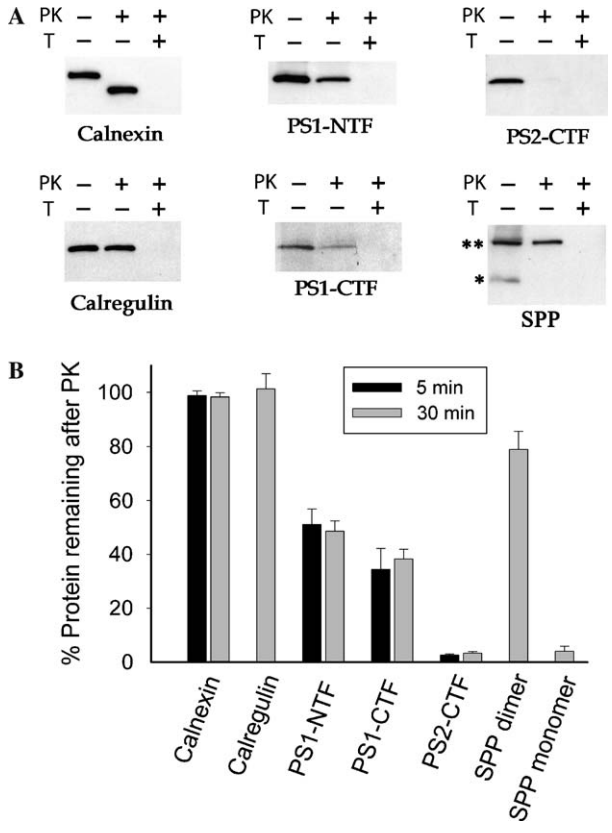

Fig. 1. Proteinase K digestion of endogenously expressed proteins in intact ER vesicles from HEK-293 cells. (A) Representative Western blots of ER vesicles before (−) and after (+) treatment with proteinase K (PK, 0.1 mg/ml on ice for 30 min) in the presence (+) or absence (−) of 1% Triton X-100 (T); see Materials and methods for details. Blots were probed with antibodies against calnexin, calregulin, PS2-CTF, PS1-CTF, PS1-NTF, and the C-terminus of SPP, as indicated. Two bands are seen on the SPP blot [32], one representing an SPP monomer (*) and the other an SPP dimer (**). (B) Percentage of protein remaining after proteinase K digestion for 5 (black bars) or 30 (grey bars) min on ice ($n \geqslant 3$ for all points). Except in the case of calnexin no detectable shift in the apparent molecular mass of the immunoreactive signal was seen after proteinase K treatment.

In our previous studies, we demonstrated that both of the PS1 epitopes examined in Fig. 1 could be essentially completely digested (~95%) by higher concentrations of proteinase K under similar experimental conditions to those used here and without disrupting vesicle integrity [27]. Taken together with the present results these observations indicate the existence of at least two physically different forms of endogenous endoproteolysed PS1, one that is relatively rapidly digested by proteinase K and one that is markedly more protease resistant.

For comparison purposes we also assayed proteinase K treated vesicles with antibodies directed against the 'loop' region of PS2 (recognizing the PS2 CTF) and the C-terminus of signal peptide peptidase (SPP). In contrast to our observations with PS1, the PS2 CTF immunoreactive signal is virtually completely lost after 5 min of digestion with proteinase K (Fig. 1B). SPP is a presenilin homologue that has also been shown to be an intramembranous protease [30]. Topology studies have shown that the SPP C-terminus is cytosolic [31]. In cells SPP is predominantly present as an SDS-stable ~95 kDa homodimer that is thought to be (a part of) the functional unit [32]. In Fig. 1 we demonstrate that although the SPP monomer signal is quite sensitive to proteinase K, the homodimer, like PS1, is partially resistant to digestion.

Since endoproteolysed PS1 is found in the membrane in a large protein complex it seems reasonable to hypothesize that the stable folding of this complex may act to protect PS1 cytosolic epitopes from proteinase K digestion. Accordingly, we wondered if treatments that were known to disrupt protein–protein interactions might result in the exposure of PS1 sites. In the experiments illustrated in Fig. 2 we incubated our ER vesicle preparation in the presence of 4 M urea or 0.5 M NaCl before proteinase K digestion. In both cases, however, we found that ~50% of the PS1 NTF signal was resistant to proteinase K as observed in samples not treated with urea or high salt (Fig. 1B).

As already discussed, the assembly of the four components of γ-secretase results in the endoproteolysis of PS1 and the stabilization and activation of the γ-secretase complex [9,11]. Excess endogenous PS1 holoprotein that is not associated with γ-secretase is rapidly degraded [12,13]. However, in cells over-expressing recombinant PS1,

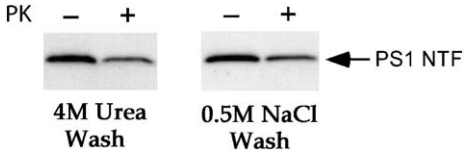

Fig. 2. Proteinase K digestion of endogenously expressed PS1-NTF in intact ER vesicles after treatment with urea or high salt. Representative Western blots of ER vesicles before (−) and after (+) digestion with proteinase K (PK, 0.1 mg/ml on ice for 30 min). Vesicles were pre-treated by incubation in 4 M urea or 0.5 M NaCl before proteinase K digestion as indicted (see Materials and methods). The PS1 NTF signals remaining after proteinase K were 50.5 ± 1.1% and 48.3 ± 5.1% of those of similarly pre-treated but undigested controls (average ± range, $n = 2$ in both cases).

significant amounts of PS1 holoprotein are often observed, presumably because of overloading of the cellular degradation pathways. In order to determine whether resistance to proteinase K is an intrinsic property of PS1 or a property of PS1 within the γ-secretase complex we examined the ability of proteinase K to digest the PS1 holoprotein in HEK-293 cells transiently over-expressing human PS1. As illustrated in Fig. 3, easily detectable levels of PS1 holoprotein are seen in these cells, but in contrast to its endoproteolysed fragments (Fig. 1), the holoprotein is completely digested by 0.1 mg/ml proteinase K after 30 min of incubation.

As detailed in Introduction, there is considerable experimental evidence suggesting that γ-secretase may be structurally and functionally heterogeneous and that γ-secretase complexes incorporating PS1 and PS2 are functionally distinct. The results presented here provide direct evidence for the existence of at least two physically different forms of endoproteolysed PS1, one that is relatively rapidly digested by proteinase K and one that is significantly more protease resistant. A similarly protease-resistant form of PS2 was not observed. Since PS1 endoproteolysis occurs during the maturation of the γ-secretase complex, these

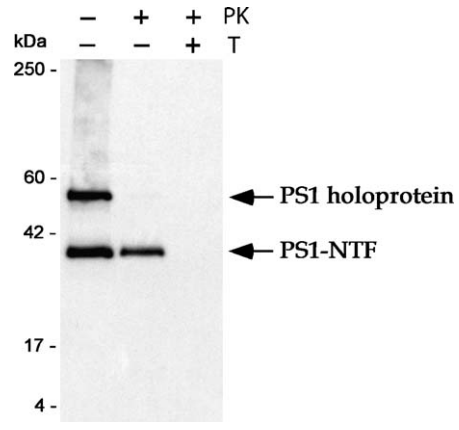

Fig. 3. Proteinase K digestion of the PS1 holoprotein in transiently transfected HEK-293 cells. ER vesicles were prepared from HEK-293 cells transfected overnight with human PS1. The vesicle preparation was then treated with proteinase K (0.1 mg/ml on ice for 30 min) and Triton X-100 as in Fig. 1 and probed with the antibody against the N-terminus of PS1. The experiment was repeated 3 times on independent preparations with similar results. A representative experiment is shown.

results indicate that PS1-containing γ-secretase is present in at least two physical forms. Our results do not address the nature of the transition of PS1 within the γ-secretase complex between the protease-sensitive and protease-resistant forms, however, we speculate that the structural heterogeneity we observe may underlie, at least in part, previous observations indicating the physical and functional heterogeneity of γ-secretase. In particular our results suggest that there are significant differences between γ-secretase complexes incorporating PS1 and PS2 in that the latter do not exhibit a proteinase K resistant form. This difference may underlie the functional differences previously documented between PS1 and PS2 [14,15,18] and, in particular, the more dominant role of PS1 in the generation of β-amyloid peptides.

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