Endoproteolysis of Presenilin in Vitro: Inhibition by γ-Secretase Inhibitors†

William A. Campbell, Maria-Karnina Iskandar, Megan L. O. Reed, and Weiming Xia*

Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: The final proteolytic step to generate the amyloid β-protein (Aβ) of Alzheimer's disease (AD) from β-amyloid precursor protein (APP) is achieved by presenilin (PS)-dependent γ-secretase cleavage. AD-causing mutations in PS1 and PS2 result in a selective and significant increase in production of the more amyloidogenic Aβ42 peptide. PS1 and PS2 undergo endoproteolysis by an unknown enzyme termed presenilinase to generate the functional complex of N- and C-terminal fragments (NTF/CTF). To investigate the endoproteolytic activity that generates active PS, we used a mammalian cell-free system that allows de novo human PS NTF and CTF generation. PS NTF and CTF generation in vitro was observed in endoplasmic reticulum (ER)-enriched fractions of membrane vesicles and to a lesser extent in Golgi/trans-Golgi-network (TGN)-enriched fractions. AD-causing mutations in PS1 and PS2 did not alter de novo generation of PS fragments. Removal of peripheral membrane-associated and cytosolic proteins did not prevent de novo generation of fragments, indicating that presenilinase activity corresponds to an integral membrane protein. Among several general inhibitors of different protease classes that blocked the presenilinase activity, pepstatin A was the most potent inhibitor. Screening available transition state analogue γ-secretase inhibitors led to the identification of two compounds that were able to prevent the de novo generation of PS fragments, with an expected inhibition of Aβ generation. Our studies provide a biochemical approach to characterize and identify this elusive presenilinase.

Alzheimer's disease (AD)[1] is a progressive neurodegenerative disease characterized by accumulation in the brain of extracellular amyloid fibrils composed of the amyloid β-protein (Aβ) and intracellular neurofibrillary tangles made of aggregated hyperphosphorylated tau protein (*1*). Genetic and neuropathological studies suggest that processing of amyloid precursor protein (APP) to yield Aβ plays an important role in the initiating events leading to AD (*1*). Mutations in two other genes, presenilin 1 (PS1) and presenilin 2 (PS2), account for about 50% of early onset familial Alzheimer's disease cases and lead to increased production and deposition of Aβ42 (*2*−*5*). The molecular mechanism whereby mutations in PS1 or PS2 lead to a selective increase in production of Aβ42 is unknown at this time.

PS1 and PS2 are homologous eight transmembrane (TM) domain spanning proteins that can be found in many locations within a cell including the endoplasmic reticulum (ER) and Golgi (*6*−*9*), in endosomes (*10*), and at the cell surface (*11*, *12*). Compelling evidence for a requirement of PS for γ-secretase cleavage of APP for Aβ generation came from in vivo studies showing decreased Aβ production in neurons derived from PS knockout embryos (*13*, *14*) and in adult brains of conditional PS1 knockout mice (*15*) and from cell culture studies showing that mutations in either of two aspartates in TM domains 6 and 7 of PS1 abolish γ-secretase cleavage of APP (*16*). Similarly, the analogous aspartate residues in PS2 are also required for APP processing by γ-secretase (*17*, *18*). Furthermore, transition-state analogue affinity reagents for γ-secretase prevent Aβ generation and bind directly to PS1 and PS2 (*19*−*21*). We have shown that PS1 and PS2 directly bind to C99/C83, the γ-secretase substrates, at the sites of Aβ generation, i.e., Golgi/trans-Golgi network (TGN)-type vesicles (*22*). We also found that familial AD mutations in PS or APP decrease the effect of γ-secretase inhibitors, providing evidence for a direct involvement of PS1 in the γ-secretase cleavage complex (*23*). Taken together, these results support a role for PS1 and PS2 in the γ-secretase activity as either γ-secretase itself or an intimate and necessary cofactor.

The PS holoproteins undergo constitutive endoproteolysis within a hydrophobic portion of the cytoplasmic loop between the sixth and seventh of the eight putative TM domains to generate functional stable heterodimers of the NH_2-terminal fragment (NTF) and C-terminal fragment (CTF) (*24*). The half-life of full length (FL) PS is less than 1 h; however, the half-life of the heterodimeric complex of NTF and CTF is much longer, i.e., ∼24 h (*25*−*27*). The identity of the enzyme responsible for endoproteolysis of

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* To whom correspondence should be addressed: Weiming Xia, Ph.D., Center for Neurologic Diseases, Harvard Institutes of Medicine, HIM 616, 77 Avenue Louis Pasteur, Boston, MA 02115. Tel: 617-525-5212. Fax: 617-525-5252. Email: wxia@rics.bwh.harvard.edu.

[1] Abbreviations: Aβ, amyloid-β protein; AD, Alzheimer's disease; APP, β-amyloid precursor protein; CHO, Chinese hamster ovary; CTF, C-terminal fragment; DMSO, dimethyl sulfoxide; E-64, trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FAD, familial Alzheimer's disease; FL, full-length; HMW, high molecular weight; kDa, kilodalton; NTF, N-terminal fragment; PMSF, phenylmethanesulfonyl fluoride; PS, presenilin; TGN, trans-Golgi network; TM, transmembrane; WT, wild-type.

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presenilins, nicknamed "presenilinase", is unknown. Nevertheless, we have previously shown that mutation of aspartate residues at TM6 or TM7 blocked endoproteolysis (*16*), suggesting that endoproteolysis of presenilins could occur via autoproteolysis.

The levels of PS1 and PS2 NTF and CTF are known to be highly regulated, because simple overexpression essentially has no effect on the total level of fragments (*24*, *28*). Furthermore, overexpression of human PS1 or PS2 in mouse cell lines or transgenic mice results in replacement of mouse PS fragments with human PS fragments to approximately the same level (*25*). PS NTF and CTF are known to associate in very stable high molecular weight (HMW) complexes (up to ~250 kDa) (*29*, *30*). Although nicastrin has been identified as an important component in the complex (*31*, *32*), the full identity of the complex is unknown. Coimmunoprecipitation experiments have demonstrated that the main PS1 components in the HMW complex are NTF/CTF, but not FL PS1 (*29*, *30*, *33*). The first proline of the PALP motif (amino acid 433 or 414 based on human PS1 or PS2 numbering) in PS CTF is necessary for complex formation (*34*). These reports suggest that the stabilized fragments are the principal functional form of presenilins and that levels of the fragments are saturable. Characterizing and identifying the presenilinase activity responsible for cleaving PS holoprotein, therefore, is critical to understanding the regulation of PS function.

Here, we have used a cell-free system to investigate de novo PS1 and PS2 NTF and CTF generation in vitro. By subcellular fractionation of membrane vesicles, we find that presenilinase is most likely an integral membrane protein that functions to a large extent in ER-enriched vesicles and to a lesser extent in Golgi/TGN-enriched vesicles, where much less FL PS is available. We further demonstrate that two designed γ-secretase inhibitors could block this activity, opening up a venue for characterization and identification of this long sought protease.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. Chinese hamster ovary (CHO) cell lines overexpressing wild-type (WT) human APP together with either WT human PS1 (designated PS1wt-1 and PS1wt-2 cells) (*5*), WT human PS2 (designated PS2wt-1 and PS2wt-2 cells) (*5*), M146L PS1 (*5*), N141I PS2 (*5*), or M239V PS2 (*5*) were maintained in 200 μg/mL G418 (Life Technologies) plus 2.5 μg/mL puromycin (for PS1 expressing lines) or 250 μg/mL of Zeocin (Invitrogen) (for PS2 expressing lines).

Membrane Vesicle Preparation, Sodium Carbonate Wash, and Subcellular Fractionation. Membrane vesicles were prepared from PS expressing CHO cells ($\sim 1 \times 10^8$ cells) as previously described (*8*) and used for subcellular fractionation or de novo PS NTF/CTF and Aβ generation (see below). When needed, vesicles were washed in 0.1 M sodium carbonate (pH 11.3) on ice and centrifuged at 100000*g* for 1 h. More than 50% of the vesicles were recovered after centrifugation. Vesicle precipitate was resuspended in incubation buffer (10 mM KOAc, 1.5 mM $MgCl_2$) and incubated at either 4 °C for basal PS levels or 37 °C for de novo PS NTF/CTF generation (see below). The membrane vesicles were used for preparation of subcellular fractions, as described previously (*8*). The fractionation employs discontinuous Iodixanol gradients, which are used because they effectively separate ER- from Golgi/TGN-rich vesicles in a way that preserves vesicle structure and function, allowing de novo Aβ generation upon incubation at 37 °C (*35*).

De Novo PS NTF/CTF and Aβ Generation. For determination of de novo PS fragment generation in vitro, total membrane vesicles, ER-rich vesicle fractions, or Golgi/TGN-rich vesicle fractions were divided into two aliquots: one aliquot was incubated at 4 °C for determination of basal PS fragment levels. The other aliquot was incubated at 37 °C for 2 h for determination of de novo fragment generation. PS fragment levels in both aliquots were probed by Western blot. Densitometry using the AlphaEase PC software package was used to quantify protein levels from at least three independent blots, and the level of newly generated PS fragment was calculated by subtracting the fragment level observed at 4 °C from that obtained at 37 °C. For determination of de novo Aβ generation in vitro, gradient fractions were divided into two aliquots: one aliquot was used for determination of basal Aβ levels by adding an equal volume of stop solution [2% NP40, 2 mM EDTA, 2× protease inhibitor cocktail, and 1 M guanidine HCl] and storing at −80 °C. The other aliquot was incubated at 37 °C for 2 h followed by addition of an equal volume of stop solution. Aβ levels in both aliquots were then measured by ELISA. The newly generated Aβ levels were calculated by subtracting the Aβ level observed at −80 °C from that obtained at 37 °C. The mean levels of newly generated PS fragment or Aβ in the absence of inhibitor (H_2O, DMSO, and methanol) was used as the denominator (100%), and the relative percentage was obtained by comparing PS fragment or Aβ levels in the presence of inhibitor against this denominator in each experiment. Negative values reflect reduced levels after incubation.

Pharmacological Treatments. For pharmacological inhibition of presenilinase or γ-secretase, gradient fractions were incubated with each protease inhibitor or γ-secretase inhibitor at the specified concentration or with DMSO, methanol, or H_2O vehicle alone, for 2 h at 37 °C. Fractions were then lysed and subjected to Western analysis or ELISA. For the time-course experiment, gradient fractions were incubated with each γ-secretase inhibitor or vehicle alone for variable times at 37 °C. Fractions were lysed after each time point and subjected to Western analysis.

Immunoprecipitation and Western Blotting. Cells were lysed in a buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% BSA, 0.5% Triton X-100, and the protease inhibitor cocktail (Sigma). Cell lysates were precleared with protein A-agarose for 4 h, and supernatants were immunoprecipitated with 4627 and X81 for PS1 or 2C20 for PS2 plus protein A-agarose. Immunoprecipitates were washed and detected as described previously (*36*). 3× sample buffer (10% SDS, 10% β-mercaptoethanol, 50% glycerol) was added to all immunoprecipitates and samples derived from de novo PS fragment generation, followed by SDS−PAGE using 26-well 4−20% Tris-HCl gels (Bio-Rad). Appropriate primary and secondary antibodies and an enhanced chemiluminescence system (Amersham) were used to detect the Western blots.

Antibodies. Polyclonal antibodies X81, Ab14 (gift of S. Gandy), and 4627 were raised against residues 1−81, 3−15,

and 457−467 of PS1, respectively (*24*, *37*). Monoclonal antibody 13A11 was raised against residues 294−309 of PS1 [gift of P. Seubert and D. Schenk (*37*)]. Polyclonal antibodies 2972 (gift of C. Haass) and PS2L (gift of T. Iwatsubo, T. Saido, K. Maruyama, and T. Tomita) were raised against residues 1−75 and 316−339 of PS2, respectively (*38*). Polyclonal antibody 2C20 recognizes the last 20 residues of the PS2 C-terminus (*18*).

ELISA. Aβ sandwich ELISAs were performed as described (*39*). The capture antibody was 2G3 (to Aβ residues 33−40), and the reporter antibody was biotinylated 266 (to Aβ residues 13−28) for Aβ_{X-40} species. These antibodies were kindly provided by P. Seubert and D. Schenk.

RESULTS

De Novo Generation of PS NTF and CTF in a Cell-Free Assay. To begin to characterize presenilinase activity, we first needed a biochemical assay to examine the conversion of substrate to product. We found that we could generate PS NTF and CTF in a cell-free assay using microsomes prepared from CHO cells stably overexpressing either WT PS1 (PS1wt-1 and PS1wt-2) or WT PS2 (PS2wt-1 and PS2wt-2). Total membrane microsomes and also membrane microsomes separated by a previously characterized fractionation on discontinuous Iodixanol sucrose gradients were used to examine PS NTF/CTF generation (*8*); therefore, any cytosolic proteins were removed. Dense fractions were rich in ER-type microsomes, while the less dense portions of the gradient contained Golgi/TGN-type microsomes, just as previously described (*8*). We combined the fractions previously found to be ER-rich into one pool and those previously found to be Golgi/TGN-rich into another pool. To detect basal PS fragment levels in these cells, we measured the amount of PS fragments in microsomes incubated at 4 °C, as further endoproteolysis of full-length (FL) PS was not possible at this temperature. Basal levels of PS1 NTF (~28 kDa) and PS2 NTF (~36 kDa) could be detected in ER pools of microsomes (left lanes in Figure 1A,B); upon incubation at 37 °C for 2 h, more PS fragments were generated from FL PS1 or PS2 (Figure 1A,B, right lanes). Consistent with PS1 NTF generation, we also observed de novo PS1 CTF generation in ER-enriched and Golgi/TGN-enriched microsomes (Figure 1C, lanes 5−8); however, the greatest generation was observed in ER-enriched microsomes, presumably due to the abundance of FL PS substrate (Figure 1C, lanes 5−6). We also used two previously reported nonpeptidic compounds, JLK2 and JLK6 (*40*), to test our de novo PS fragment generation system. Consistent with previous findings that these compounds do not affect PS1 endoproteolysis in live cells (*40*), we failed to detect any effect of these compounds at 0.2 mM on PS1 fragment generation in vitro (Figure 1D, lanes 5−6).

Presenilinase Appears to be an Integral Membrane Protein. To begin to determine the biochemical properties of the presenilinase activity that generated PS NTF and CTF from FL PS in our system, we prepared membrane vesicles, washed the vesicles with sodium carbonate to remove nonintegral membrane proteins, and incubated the washed vesicles at 37 °C for 2 h. We continued to observe PS fragment generation in membrane vesicles after the sodium carbonate wash (Figure 1E), suggesting that presenilinase is

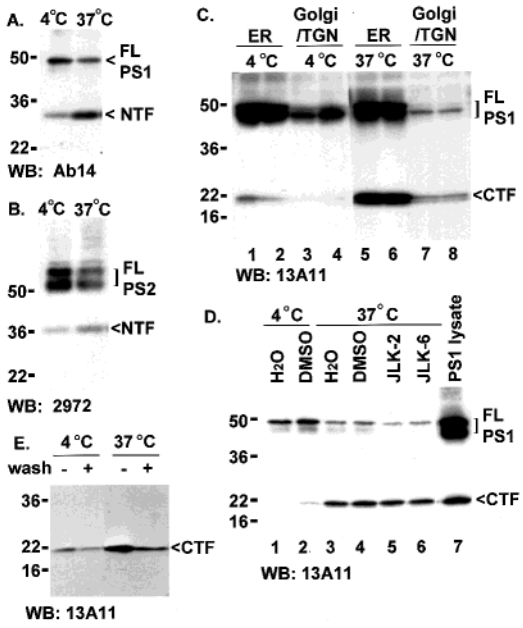

FIGURE 1: De novo generation of PS NTF and CTF in total cell-free microsomes, ER-enriched microsomes, and Golgi/TGN-enriched microsomes. (A and B) ER-enriched membrane vesicles isolated from CHO cells stably expressing WT APP and WT PS1 (PS1wt-1, A) or WT PS2 (PS2wt-1, B) were incubated at 37 °C for 2 h to measure de novo generation of PS fragment. Controls were incubated at 4 °C for measurement of basal PS fragment levels. Vesicles were lysed and probed by Western blotting (WB) for FL PS and PS NTF with PS1 antibody Ab14 (A) or PS2 antibody 2972 (B). (C) Duplicate ER- (lanes 5 and 6) and Golgi/TGN-enriched (lanes 7 and 8) microsomes were incubated at 37 °C for 2 h, lysed, and then probed by WB for FL PS1 and PS1 CTF with PS1 antibody 13A11. (D) ER-enriched microsomes isolated from PS1wt-1 cells were incubated at 37 °C for 2 h in the presence of 0.2 mM JLK2, JLK6, or vehicle. Controls were incubated at 4 °C for detection of basal PS levels (lanes 1 and 2). The microsomes were lysed and probed by WB for FL PS1 and PS1 CTF with antibody 13A11. Lane 7 contains PS1wt-1 cell lysate. (E) Total membrane vesicles isolated from PS1wt-1 cells were washed with sodium carbonate (+), pelleted, resuspended in incubation buffer, and incubated at 37 °C for 2 h. Vesicles were lysed and probed by WB with PS1 antibody 13A11. Consistent with a previous report (*36*), FL PS2 is detected as the doublet above 50 kDa by antibody 2972 (B), and FL PS1 is detected as the doublet below 50 kDa by antibody 13A11 (C and D).

an integral membrane protein. Although we started with the same amount of vesicles, the PS fragment levels were lower in the washed versus unwashed lanes at both 4 and 37 °C. One would expect to have reduced levels of fragments after the washes, as some material was lost during the washing process.

FAD Mutations in PS Do Not Affect de Novo PS NTF/CTF Generation. To determine whether the mechanism whereby FAD mutations in PS1 or PS2 cause an increase in Aβ42 generation is increased generation of PS NTF and CTF, we compared de novo PS fragment generation in CHO cell lines stably expressing WT PS1 (PS1wt-1 and PS1wt-2) or PS2 (PS2wt-1 and PS2wt-2) versus those expressing M146L FAD mutant PS1, or N141I or M239V FAD mutant PS2. ER-enriched microsomes were prepared from these cell lines, and microsomes from each line were incubated at 37 °C for 2 h, lysed, and subjected to Western analysis (Figure 2). There was no consistent alteration in PS1 (Figure 2A) or PS2 (Figure 2B) fragment generation in FAD mutant PS

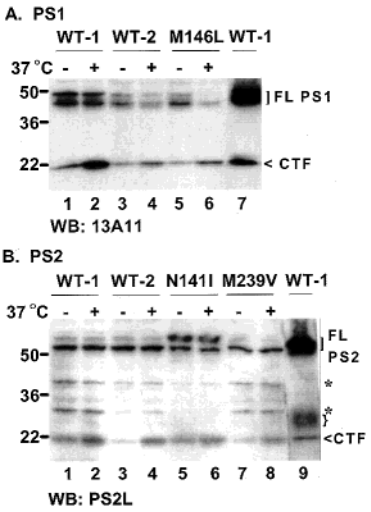

FIGURE 2: De novo fragment generation is not altered in PS carrying FAD mutations. (A) ER-enriched microsomes were isolated from CHO cells stably expressing WT APP and either WT PS1 (PS1wt-1 and PS1wt-2) or M146L FAD mutant PS1 and incubated at 37 °C for 2 h (+). Controls were incubated at 4 °C for measurement of basal PS levels (−). Microsomes were lysed and probed by WB for PS1 with antibody 13A11. Lane 7 shows PS1 immunoprecipitates from PS1wt-1 cell lysates using antibodies X81 and 4627. (B) ER-enriched microsomes were prepared from CHO cells stably expressing WT APP and either WT PS2 (PS2wt-1 and PS2wt-2) or N141I or M239V FAD mutant PS2 and incubated at 37 °C for 2 h. Fractions were lysed and probed by WB for PS2 with antibody PS2L. Lane 9 shows immunoprecipitation of PS2wt-1 cell lysates using PS2 antibody 2C20, and the bracket at 25 kDa denotes IgG light chain. Asterisks denote nonspecific bands.

expressing cells. Thus, we failed to detect an alteration in endoproteolysis of PS carrying FAD mutations.

Blocking Presenilinase Activity by Protease Inhibitors. To determine which type of protease may be responsible for the cleavage of FL PS, we used a variety of protease inhibitors to attempt to inhibit de novo generation of PS fragments in ER- and Golgi/TGN-enriched microsomes from PS1wt-1 cells. Since the conditions for de novo generation of PS NTF and CTF are also appropriate for the de novo generation of Aβ, we measured Aβ generation in Golgi/TGN-enriched microsomes. Our previous studies showed that almost no Aβ generation was detected in ER-enriched microsomes (*22*). We used the ER-enriched microsomes for inhibitor treatment because these microsomes contain the most FL PS substrate and, therefore, generate the most NTF and CTF. ER-enriched microsomes were incubated for 2 h at 37 °C in the presence of varying concentrations of aspartyl (pepstatin A), metallo- (1,10-phenathroline, EDTA, or phosphoramidon), serine (Pefabloc or PMSF), or serine/cysteine (leupeptin) protease inhibitors. The inhibitors were used at or above their reported effective concentrations (Table 1) (*41*). Microsomes were lysed, and Western analysis was performed on the microsomes (Figure 3). The appropriate vehicles (DMSO, methanol, or H_2O, lanes 1−4 and 24) were used as controls, as well as a complete protease inhibitor cocktail (lanes 11 and 12), in which no PS fragment generation would be expected. Pepstatin A at 2 and 1 μM (lanes 5 and 6), 1,10-phenathroline at 4 and 2 mM (lanes 7 and 8), EDTA at 10 and 5 mM (lanes 9 and 10), and Pefabloc at 16 and 8 mM (lanes 13 and 14) consistently inhibited the generation of PS fragments, while phosphoramidon (40, 10, and 1 μM), PMSF (2, 1, and 0.5 mM), and leupeptin (400, 100, and 10 μM) had no effect on fragment generation (Figure 3 and Table 1). The complete protease inhibitor cocktail containing a mixture of inhibitors for serine, cysteine, and aspartic proteases and aminopeptidases (Pefabloc, pepstatin A, E-64, bestatin, leupeptin, and aprotinin) inhibited fragment generation as expected, while the addition of the solvents DMSO, methanol, or H_2O had no effect. Levels of PS1 CTF detected by Western blot in multiple experiments were quantified (see Materials and Methods), and means were normalized against samples in the absence of inhibitors (Table 1). Effects of the protease inhibitor on PS fragment generation in Golgi/TGN microsomes were similar to the results using ER-enriched microsomes (data not shown).

To determine the effect of the protease inhibitors on Aβ generation, parallel Golgi/TGN-enriched microsomes were incubated with protease inhibitors for 2 h at 37 °C, then lysed, and subjected to measurement of Aβ levels by ELISA (Figure 4). Pepstatin A at 1 or 2 μM did not inhibit Aβ generation, consistent with the previous report on inhibition of de novo Aβ generation by pepstatin A with an IC_{50} at 35 μM (*23*). While the metallo-protease inhibitors EDTA and 1,10-phenathroline could inhibit PS fragment generation, they could not completely inhibit de novo Aβ generation (Figure 4), as they each showed a ∼150 and ∼56% generation of Aβ, respectively, relative to samples incubated without inhibitors (Table 1). Likewise, Pefabloc, a serine protease inhibitor, could inhibit PS fragment generation, but these microsomes still showed 56−110% generation of Aβ relative to the vehicle (H_2O). Leupeptin, PMSF, and the complete protease inhibitor cocktail still allowed some Aβ generation (Figure 4 and Table 1).

Inhibition of Presenilinase by γ-Secretase Inhibitors. A series of transition-state analogue inhibitors of γ-secretase have been designed to inhibit Aβ generation (*19*−*21*, *42*−*44*), and some have been shown to bind to PS directly (*19*−*21*). We screened available γ-secretase inhibitors of this type to examine whether any compound could inhibit PS fragment generation. ER- and Golgi/TGN-enriched microsomes were incubated with different γ-secretase inhibitors or vehicle control for 2 h at 37 °C followed by detection of PS fragment and Aβ generation (Figures 5A,B and 4). Among all of these transition-state analogue inhibitors that could inhibit de novo Aβ generation, CM35 (compound 2 in ref *42*), was found to inhibit generation of PS1 fragments (Figure 5A, lanes 7−9). A similar difluoro ketone transition-state analogue inhibitor, MW167, was also shown to inhibit generation of PS1 fragments (Figure 5B, lanes 5−6). Both compounds were able to inhibit PS fragment generation at 0.1 mM. The other γ-secretase inhibitors tested, 31C and 36C (*43*), and 1-Bt (*19*), although inhibiting de novo Aβ generation (Figure 4) failed to block the fragment generation (Figure 5A, lanes 6, 10, and 11). To determine the time-course of PS fragment generation, we incubated ER-enriched microsomes with either an active (CM35) or inactive (36C) presenilinase inhibitor or vehicle control for various lengths of time at 37 °C followed by detection of PS fragment generation. Basal levels of PS1 fragments were detected in microsomes before incubation (Figure 6, lanes 1, 9, and 17). In the absence of active presenilinase inhibitor, additional PS fragments were generated within 15 min of incubation at 37 °C, and levels of fragments were saturated within the first hour of incuba-

Table 1: Vehicles and Inhibitors Used in This Study and Their Effects on de Novo PS Fragment and Aβ Generation

name	class of inhibitor	reported effective [C] (mM)	[C] tested (mM)	normalized PS fragment generation mean ± SEM%	normalized Aβ generation mean ± SEM%
H_2O				88 ± 5	100 ± 12
DMSO				99 ± 11	99 ± 10
methanol				113 ± 17	101 ± 16
pepstatin A	aspartyl	0.001	0.002	23 ± 10	11**1** ± 18
			0.001	21 ± 12	86 ± 14
1,10-phenathroline	metallo	1−10	4	8 ± 15	59 ± 16
			2	23 ± 10	53 ± 21
EDTA	metallo	1−10	10	8 ± 23	152 ± 19
			5	20 ± 15	151 ± 12
protease inhibitor cocktail (Sigma)	all classes	1×	2×	−1 ± 17	41 ± 11
			1×	7 ± 9	52 ± 14
Pefabloc	serine	0.4−4.0	16	−**3** ± 8	56 ± 28
			8	−4 ± 7	72 ± 26
			4	16 ± 5	60 ± 4
			0.4	25 ± 5	110 ± 9
phosphoramidon	metallo	0.001−0.01	0.04	125 ± 3	120 ± 12
			0.01	110 ± 33	121 ± 16
			0.001	132 ± 38	103 ± 10
leupeptin	serine/cysteine	0.01−0.1	0.4	152 ± 24	8 ± 13
			0.1	123 ± 19	24 ± 19
			0.01	136 ± 19	69 ± 14
PMSF	serine	0.1−1.0	2	118 ± 56	32 ± 17
			1	131 ± 35	78 ± 14
			0.5	121 ± 22	68 ± 21
31C	γ-secretase		0.2	109 ± 9	12 ± 10
CM35	γ-secretase		0.4	27 ± 2	29 ± 11
			0.2	48 ± 10	18 ± 8
			0.1	59 ± 23	−20 ± 14
36C	γ-secretase		0.2	90 ± 12	−17 ± 7
1-Bt	γ-secretase		0.2	97 ± 5	5 ± 7
MW167	γ-secretase		0.2	19 ± 3	0 ± 4
			0.1	23 ± 4	−11 ± 1

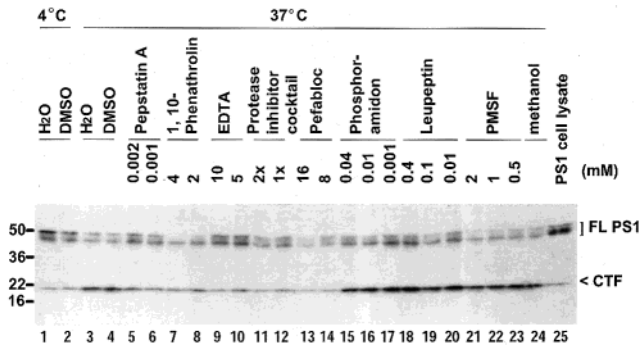

FIGURE 3: De novo PS fragment generation in ER-enriched microsomes can be inhibited by protease inhibitors. ER-enriched microsomes isolated from PS1wt-1 cells were incubated at 37 °C for 2 h in the presence of the indicated protease inhibitors or their vehicles. Controls were incubated at 4 °C for measurement of basal PS levels (lanes 1 and 2). The fractions were then lysed and probed by WB for FL PS1 and PS1 CTF with antibody 13A11. Lane 25 contains PS1wt-1 cell lysate.

tion at 37 °C (Figure 6, lanes 2−8 and 18−24). On the contrary, CM35 immediately blocked presenilinase activity and prevented any new fragment from being generated (Figure 6, lanes 10−16).

DISCUSSION

In vivo manipulation of the levels of the functional PS NTF/CTF relies on the identification of the key enzyme that cleaves FL PS, presenilinase. Ubiquilin, a PS interacting protein that accumulates FL PS (but not fragments) through enhanced PS synthesis apparently is not linked to presenilinase activity (*45*). GSK-3β has been shown to regulate the degradation of PS1 CTF (but not NTF) through the phosphorylation at serine 397 of PS1 (*46*), indicating that GSK-3β does not regulate the endoproteolysis of PS. Recently, Beher et al. reported that a γ-secretase inhibitor can reduce levels of PS NTF/CTF upon treating cells for 7 days (*47*). In this study, we found that our de novo PS NTF/CTF generation could be immediately blocked by several γ-secretase inhibitors. We searched for biochemical properties of the presenilinase and any alteration of NTF/CTF generation from FAD-linked mutant FL PS.

One potential mechanism for the specific increase of Aβ42 production by mutant PS is the enhanced generation of PS NTF and CTF, as suggested by one study showing that FAD-associated mutations in PS1 (A246E, M146L, ΔE9) result in the hyperaccumulation of both NTF and CTF (*48*). For the FAD mutations in PS1 (M146L) and PS2 (N141I, M239V) that we examined, however, we saw no increase in CTF generation versus WT PS expressing cells. This result is in accordance with the assumption that any alteration of PS fragment generation should affect all species of Aβ production, not only Aβ42.

Presenilinase does not appear to be a cytoplasmic protease since de novo generation of PS NTF and CTF occurred in total membrane vesicles, and ER- and Golgi-enriched microsomes after incubation at 37 °C. Although PS fragments were generated in Golgi-rich microsomes, the amount of generation was much less than that observed in ER-rich microsomes, presumably due to the presence of less FL PS substrate. Since FL PS is abundant in the ER, it is not surprising to find most presenilinase activity in this sub-

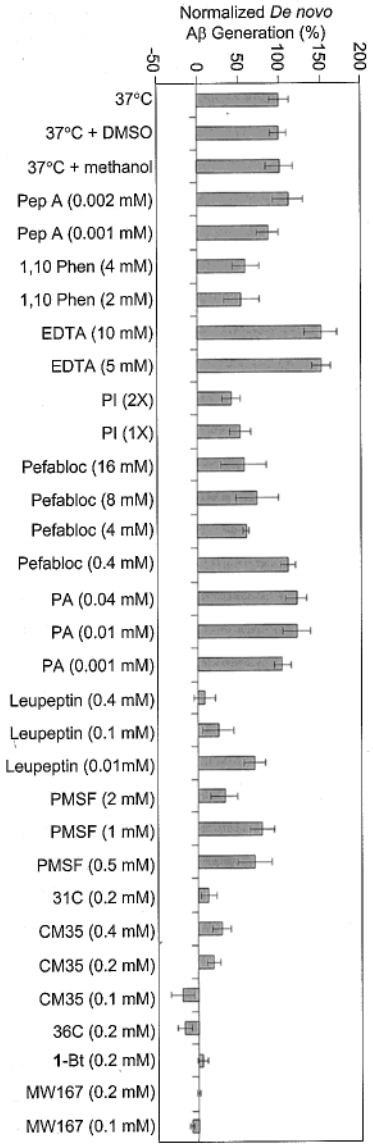

FIGURE 4: Effect of protease and γ-secretase inhibitors on de novo Aβ generation. Golgi/TGN-enriched microsomes isolated from PS1wt-1 cells were incubated at 37 °C for 2 h in the presence of protease inhibitors, γ-secretase inhibitors, or vehicles. Controls were incubated at 4 °C for measurement of basal Aβ levels. These microsomes were lysed and subjected to a sensitive and specific ELISA for Aβ_{X-40}. Levels of newly generated Aβ in the absence of inhibitors are normalized to be 100%, and the relative percentage of Aβ levels in the presence of inhibitors are obtained in each experiment. Negative values denote a reduction of Aβ levels after incubation at 37 °C. Pep A, pepstatin A; 1,10-phen, 1,10-phenathroline; PI, complete protease inhibitor cocktail; PA, phosphoramidon.

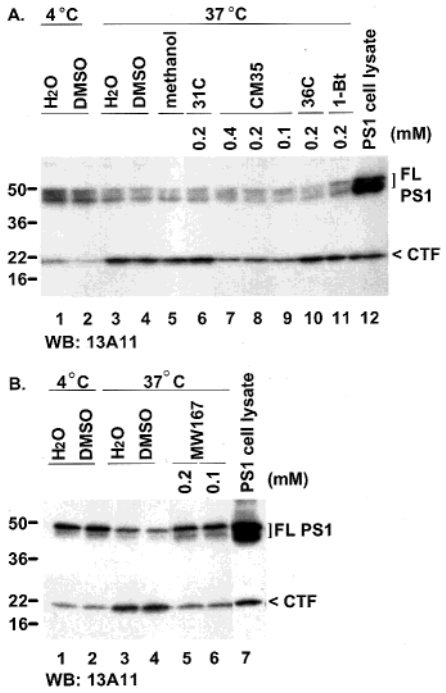

FIGURE 5: De novo PS fragment generation can be inhibited by γ-secretase inhibitors. (A and B) ER-enriched microsomes isolated from PS1wt-1 cells were incubated at 37 °C for 2 h in the presence of γ-secretase inhibitors or vehicles at the indicated concentrations. Controls were incubated at 4 °C for detection of basal PS levels (lanes 1 and 2). The microsomes were lysed and probed by WB for FL PS1 and PS1 CTF with antibody 13A11. Lane 12 (A) and lane 7 (B) contain PS1wt-1 cell lysate.

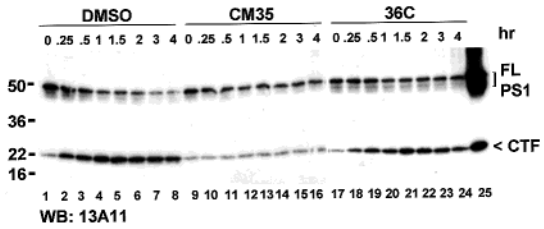

FIGURE 6: Time-course of PS fragment generation. ER-enriched microsomes isolated from PS1wt-1 cells were incubated at 37 °C for the indicated times in the presence of γ-secretase inhibitors or vehicle. The microsomes were lysed and probed by WB for FL PS1 and PS1 CTF with antibody 13A11. Lane 25 contains PS1wt-1 cell lysate.

cellular compartment. NTF/CTF, once generated by presenilinase cleavage, presumably traffics to Golgi/TGN (*11*). Therefore, our data suggest that the majority of presenilinase activity resides in the ER. Presenilinase activity may be present in other compartments, but there is almost no FL PS substrate available to measure the activity.

We further examined the membrane association of presenilinase. Endoproteolytic cleavage sites of PS1 are primarily at residue 292 (*37*, *49*) and also at residue 298 (*37*), which are located in the loop region facing the cytoplasm. It is conceivable that a cytosolic protease or a cytosol-facing peripheral membrane-associated protease could access the loop region and cleave PS. Our results exclude a cytosolic protease(s), because membrane vesicles could still generate PS fragments after removal of cytosol. Peripheral membrane associated proteases were excluded because washing the vesicles with sodium carbonate did not abrogate de novo generation of PS fragment. These results suggest that presenilinase is an integral membrane protein.

Another approach to characterize the presenilinase is to profile different classes of protease inhibitors. Using varying classes of protease inhibitors to inhibit de novo PS NTF and CTF generation did not conclusively demonstrate to which protease class presenilinase belongs. Previous studies indicate that endoproteolysis of PS is evolutionarily conserved, as a mutation of the aspartate residue in zebrafish PS blocks its endoproteolysis (*50*). Human PS1 has also been shown to undergo endoproteolysis in transfected Sf9 insect cells (*51*); however, the homologue of PS in *Caenorhabditis elegans*, SEL-12, undergoes endoproteolysis in *C. elegans* but not in

human cells (*52*). This result suggests that presenilinase may be species-specific, or that it requires other species-specific factors to cleave PS. Some factors, along with the presenilinase, might be different classes of proteases, and may function upstream of presenilinase activity. Nevertheless, among the protease inhibitors that blocked presenilinase activity, the aspartyl protease inhibitor pepstatin A was the most potent compound to inhibit presenilinase.

Pepstatin A was reported to block solubilized γ-secretase activity (*53*). When C100Flag was used as a γ-secretase substrate in an in vitro assay, the IC_{50} for pepstatin A to inhibit Aβ40 and Aβ42 generation was 4.0 and 5.9 μM, respectively (*53*). In a separate study employing a cell-free assay on membrane vesicles derived from C99-transfected cells, the IC_{50} of pepstatin A for inhibition of both Aβ40 and Aβ42 was estimated at $\sim$4 μM (*54*). Because both C100Flag and C99 are immediate substrates for γ-secretase, it is not surprising to see an IC_{50} for pepstatin A at 35 μM when we used microsomes derived from FL APP expressing cells, as previously reported (*23*). At 1 μM, Aβ generation was not affected (*23*). Nevertheless, 1 μM pepstatin A completely blocked new PS fragment generation in vitro (Figure 3). Because we were measuring Aβ generation in Golgi/TGN-enriched microsomes where γ-secretase/PS NTF/CTF complexes have already been assembled, inhibition of additional fragment generation by pepstatin A would not affect existing functional γ-secretase complexes to produce Aβ. Likewise, inhibition of PS fragment generation by 1,10-phenathroline, EDTA, the complete protease inhibitor cocktail, and Pefabloc would not affect Aβ generation. Pepstatin A was shown to bind to PS1 with higher affinity to FL PS1 than to PS1 fragments (*55*). The high efficacy of pepstatin A binding to FL PS1 may lead to efficient inhibition of endoproteolysis compared to the low efficacy of binding to functional NTF/CTF complexes and inhibition of γ-secretase cleavage in the Golgi/TGN.

In addition to pepstatin A, we identified two γ-secretase inhibitors that were able to block presenilinase activity. A previous report has shown that two nonpeptidic compounds, JLK2 and JLK6, reduce levels of Aβ secreted from cultured cells but do not affect PS endoproteolysis in live cells (*40*). Accordingly, we did not see any effect of these inhibitors on PS1 fragment generation in vitro. Among transition-state analogue γ-secretase inhibitors, some failed to block fragment generation, while CM35 and MW167 were able to block PS fragment generation. Therefore, presenilinase and γ-secretase appear to be pharmacologically distinct.

Our current results could not address whether PS1 is the presenilinase and γ-secretase. If another protease is involved in PS endoproteolysis, an effective presenilinase inhibitor might have to bind to this protease or FL PS to block endoproteolysis of PS. Other investigators have shown that transition-state analogue γ-secretase inhibitors (other than MW167 and CM35) specifically bind to PS NTF and CTF in isolated microsomes and living cells, but not to FL PS (*19*, *20*). Therefore, it is not surprising to find an effective inhibition of Aβ generation by these inhibitors without affecting PS endoproteolysis. If PS is presenilinase and γ-secretase, there may well be a difference between blocking an intermolecular interaction (substrate and enzyme) and blocking an intramolecular interaction (enzyme and prodomain), as previously suggested for PS (*56*). The prodomain may sterically prevent access of the inhibitor. Since a close analogue of CM35 directly binds PS (*19*), it will now be interesting to determine whether CM35 and MW167 have higher efficacy for binding to FL PS than to PS NTF and CTF.

Since the most effective inhibitor of presenilinase activity was pepstatin A, and MW167 and CM35 are aspartyl protease transition-state analogue inhibitors, our studies suggest that presenilinase might be an aspartyl protease. The in vitro assay to monitor presenilinase activity will allow us and others to systematically characterize presenilinase and screen compounds to inhibit this activity. Identification of MW167 and CM35 to inhibit both presenilinase and γ-secretase provides a new tool to explore the close relationship between these two key proteases implicated in the pathogenesis of AD.

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