Aspartyl Protease Inhibitor Pepstatin Binds to the Presenilins of Alzheimer's Disease[†]

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ABSTRACT: Mutations in the presenilin genes PS1 and PS2 cause early-onset Alzheimer's disease by altering γ-secretase cleavage of the amyloid precursor protein, the last step in the generation of Aβ peptide. Ablation of presenilin (PS) genes, or mutation of two critical aspartates, abolishes γ-secretase cleavage, suggesting that PS may be the γ-secretases. Independently, inhibition experiments indicate that γ-secretase is an aspartyl protease. To characterize the putative γ-secretase activity associated with presenilins, lysates from human neuroblastoma SH-SY5Y and human brain homogenates were incubated with biotin derivatives of pepstatin, followed by immunoprecipitation of PS and associated proteins, and biotin detection by Western blotting. Precipitation with PS1 antibodies, directed to either N-terminal or loop regions, yielded the same 43 kDa band, of apparent molecular mass consistent with that of full-length PS1, although it may represent an aspartyl protease complexed with PS1. Incubation of cell lysates with pepstatin−biotin, followed by streptavidin precipitation and PS1 Western blotting, revealed PS1 fragments and full-length protein, indicating that pepstatin−biotin bound to both cleaved and uncleaved PS1. Binding could be competed by γ-secretase inhibitor L-685,458 and could not be achieved with a PS1 mutant lacking the two transmembrane aspartates. Pepstatin−biotin was also shown to bind to PS2. PS1 was specifically absorbed to pepstatin−agarose, with an optimal pH of 6. Binding of pepstatin−biotin to PS1 from lymphocytes of a heterozygous carrier of pathologic exon 9 deletion was markedly decreased as compared to control lymphocytes, suggesting that this PS1 mutation altered the pepstatin binding site.

Mutations in the presenilin genes are the most frequent genetic cause of inherited early-onset Alzheimer's disease (AD)[1] (*1*). These alter proteolytic processing of the amyloid precursor protein (APP), the translation product of the other gene directly associated with AD. The type I membrane protein APP undergoes two alternative proteolytic cleavages near its transmembrane domain. Both cleavages result in the shedding of a large ectodomain from the membrane (*2*). The cellular amyloidogenic pathway generates an additional 4 kDa fragment, the Aβ peptide that may accumulate in the extracellular space to form the amyloid plaques characteristic of AD pathology. The generation of Aβ from APP requires at least two proteolytic cleavages. In a first step, β-secretase, identified as the novel membrane-anchored aspartyl protease, BACE (*3*), releases the APP ectodomain sAPPβ and generates a membrane-associated fragment with a free Aβ N-terminus, which is termed CTFβ. In a subsequent step γ-secretase processes CTFβ to free the Aβ C-terminus. γ-Secretase cleaves within the transmembrane domain of APP and generates peptides of various lengths and differing solubility. The most common forms are Aβ40 and Aβ42, the longer being more hydrophobic and prone to aggregate (*4*). All presenilin pathogenic mutations described so far increase the ratio of 42:40, pointing to the involvement of presenilins in the γ-secretase pathway and/or cleavage (*5*). Presenilins interact directly with APP (*6*, *7*) and can modulate its trafficking and metabolism (*8*). Additionally, PS1 gene knock-out disables γ-secretase cleavage of APP (*9*) and the proteolytic processing of the Notch-1 transmembrane domain (*10*), suggesting a direct link between presenilins and a protease activity involved in the cleavage of both proteins. The two aspartates predicted to be contained within transmembrane domains 6 and 7 of PS1 (residues 257 and 385) are critical for γ-secretase activity. Substituting these residues for alanine creates (D-A) mutants that are not proteolytically processed and mimics the effect of PS1 gene knock-out on the processing of APP (*11*) and Notch (*12*). PS1 knock-out, or site-directed mutation, is not sufficient to inhibit totally γ-secretase activity, but expression of PS1 and PS2 double

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[1] Abbreviations: Aβ, Alzheimer's disease amyloid peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; DAN, diazoacetylnorleucine methyl ester; FAD, familial Alzheimer's disease; IP, immunoprecipitation; NAv, neutravidin; Pep-bt, pepstatin−biotin; PS, presenilin; SAvHRP, streptavidin−horseradish peroxidase conjugate; SAv P, streptavidin−agarose precipitation; sta, statine or statinoic acid; WB, Western blot; WT, wild type.

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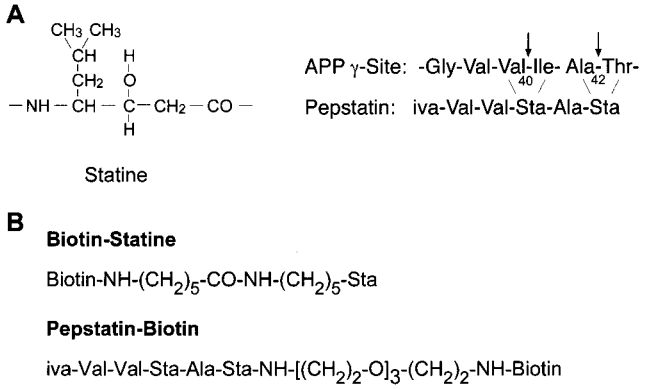

FIGURE 1: Homology between pepstatin and APP γ-secretase cleavage sites and design of the pepstatin−biotin derivatives. Panel A: Alignment of the pepstatin sequence with the two major APP γ-secretase cleavage sites at Aβ positions 40 and 42 (indicated with arrows). Panel B: Chemical structure of biotin−statine and pepstatin−biotin (Sta = statine, iva = isovaleryl).

(D-A) mutants (*13*), or knocking out both PS1 and PS2 genes (*14*, *15*), results in complete loss of γ-secretase cleavage. This suggests that PS2 exerts a complementary proteolytic function to PS1 and that production of Aβ by γ-secretase requires either PS1 or PS2. Whether presenilins are aspartyl proteases themselves, or only protease cofactors, remains controversial. Accumulating evidence indicates that γ-secretase has some characteristics of an aspartyl protease. Our earlier studies pointed to a pepstatin-sensitive activity from human brain as a γ-secretase candidate (*16*). Recent studies have shown aspartyl protease transition-state analogues to inhibit Aβ secretion in cell culture models (*17*, *18*) and that some of their derivatives can be cross-linked to PS1 (*19*, *20*). To further characterize the aspartyl protease activity associated with the presenilins, biotin derivatives of pepstatin and its cognate amino acid statine were tested using an assay that allows direct detection of proteases binding to biotin inhibitors (*21*). Pepstatin was selected for its potency in inhibiting most aspartyl proteases (*22*) and for its hydrophobic structure that resembles the APP sequence at the γ-secretase cleavage site (Figure 1A). Pepstatin A has been shown to inhibit γ-secretase in transfected cells, although it requires high concentrations to be effective due to poor cell penetrance (*23*). Tested in a cell-free assay, its ability to inhibit γ-secretase was further demonstrated, with calculation of an IC_{50} value for γ-secretase in the micromolar range (*18*). Here, the binding of biotin inhibitors to γ-secretase candidates was followed by immunoprecipitation of presenilins and associated proteins and by Western blot detection of biotin. We find that pepstatin−biotin binds to both presenilins 1 and 2.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines. Biotin was chemically coupled to statine and to pepstatin through long spacer arms, as shown in Figure 1B. Statine (0.175 mg, 1 μmol; Sigma) was reacted with 1 mg of EZ-link-NHS-LC-LC-biotin (Pierce) in 0.1 mL of DMSO, at 0 °C, according to the manufacturer's instructions to achieve coupling of biotin to statine's amine function. Biotin was coupled to pepstatin through its carboxyl terminus. Pepstatin (6.86 mg, 10 μmol; Sigma) was activated with BOP-HOBt reagent (Auspep; 6.1 mg) in 0.2 mL of dimethylformamide in the presence of 10 μL of triethylamine, as described before (*24*), and reacted with a solution of biotin-EZ-link-LC-PEO-amine (Pierce; 4.2 mg, 10 μmol in 200 μL of DMF) overnight at room temperature. The pepstatin derivative was precipitated by adding ice-cold water and was recrystallized in absolute ethanol. It was stored at −20 °C as a 1 mg/mL solution in DMSO.

PS1-SY5Y and PS2-SY5Y cell lines have been described (*25*). Full-length wild-type PS1 (463 transcript that lacks the [26−29] VRSQ sequence) was used as a template to introduce the mutations Asp_{253} to Ala and Asp_{381} to Ala by PCR, resulting in the PS1 double mutant PS1(D-A)$_2$. After being cloned into pCEP4 (Invitrogen), the introduction of the mutations was confirmed by sequencing. COS-7 cells were transfected with pCEP4 plasmid containing PS1 wild-type or PS1(D-A)$_2$ sequences. The cells were selected with hygromycin B until stable lines were obtained. Lymphocytes were prepared by the Ficoll−Paque method, transformed with the Epstein−Barr virus, and grown in 20% fetal bovine serum in RPMI (Gibco-BRL), and their characterization will be reported elsewhere. Neural stem cells from embryos of PS1 knock-out (*26*) and wild-type mice were prepared by Dr. R. Rietze (Walter and Eliza Hall Institute, Parkville, Australia) by techniques described before (*27*) and maintained in DMEM/F12 (1:1) medium supplemented with hormone mix and epidermal growth factor.

Preparation of membrane fractions from human brain cortex was as described before (*16*). Cell lines were lysed in (1/10 v/v) 0.05 M Tris-HCl, pH 7.5, 1% Triton X-100, and 1% Nonidet P-40 plus a cocktail of protease inhibitors. Alternatively, 2% CHAPSO was used as a detergent.

Antibodies 98/1 [PS1 1−20] and PS2-3 [PS2 32−45] have been described previously (*25*). Antibody 00/2 was prepared by immunizing New Zealand White rabbits with PS1-loop peptide [301−317-Cys] conjugated to diphtheria toxoid (Chiron, Australia). Rabbit rab-8 antibody was from Santa-Cruz Biotechnologies.

Biotin Inhibitor and Avidin Western Blot Assay. Forty microgram protein aliquots of cell lysates were incubated with 3 μL of biotin inhibitor solution in DMSO (1 mg/mL) in 20 μL of PBS (or 0.1 M citrate−phosphate, pH 6.0) overnight at 25 °C. Then 0.25 mL of STE (150 mM NaCl, 50 mM Tris, 2 mM EDTA, pH 7.5) was added to each incubation, followed with PS antibody-coated protein A−Sepharose suspended in 0.25 mL of STE buffer. One milligram of protein A−Sepharose (Pharmacia) was used per microliter of rabbit antisera. After three washes with STE plus 0.2% Nonidet P-40 (STEN) and one wash with 20 mM Tris, pH 7.5, pellets were resuspended in 30 μL of Laemmli sample buffer and heated at 90 °C for 30 s. The proteins were electrophoresed on 12% acrylamide gels and transferred to PVDF for 45 min at 380 mA. After being blocked with 0.5% hydrolyzed casein, blots were probed with neutravidin−alkaline (NAv) phosphatase conjugate (Pierce) and developed with naphthol−Fast Red, as described before (*16*). Alternatively, the blots were probed with streptavidin conjugated to horseradish peroxidase (SAv-HRP; Pierce) and the bands visualized by chemiluminescence (ECL reagent, Amersham).

Biotin Inhibitor Streptavidin Precipitation Assay. Samples were incubated with the biotin inhibitors as described above. The incubations were then diluted with 0.5 mL of STEN and transferred to microtubes containing 20 μL of streptavidin−agarose slurry (Sigma). After incubation for 1 h at

room temperature, the precipitates were washed twice with STEN plus 0.5% SDS, twice with STEN plus 0.5 M NaCl, once with STEN, and once with 20 mM Tris-HCl, pH 7.4. The precipitates were resuspended in sample buffer containing 2 M urea and 50 mM DTT, heated at 50 °C for 15 min, and analyzed by SDS−PAGE and Western blotting with anti-PS1 antibody 98/1.

Treatment of Cell Membranes with Diazoacetylnorleucine Methyl Ester. PS1-transfected SY5Y cells were lysed on ice, for 10 min, in 10 mM Hepes plus 10 mM KCl, pH 7.3, and resuspended in 20 mM Hepes (pH 7.3) containing 80 mM KCl plus protease inhibitors. Then, they were homogenized by five strokes in a Dounce homogenizer and eight passages through a 26-gauge needle and centrifuged at 1000*g* for 10 min. The postnuclear supernatant was centrifuged at 100000*g* for 1 h at 4 °C. The membrane fraction that sedimented was resuspended in PBS with 5% glycerol, 0.1% bovine serum albumin, and 2.5 mM DTT and stored at −70 °C. Aliquots of the membrane fraction (10 μL, 200 μg of protein) were incubated in acetate buffer (0.1 M, pH 5.3) for 2 h at 37 °C in the presence or absence of 30 mM diazoacetyl-D,L-norleucine methyl ester (Sigma) and 30 mM copper acetate. The reaction was terminated by addition of EDTA (in a 5-fold excess) to chelate copper. The membranes were solubilized with cardiolipin (3 μL of 0.5% ethanol solution; Sigma) and incubated overnight with 3 μL of pepstatin−biotin (1 mg/mL in DMSO). The samples were centrifuged, and the supernatants were immunoprecipitated as described above.

Absorption of Presenilins to Pepstatin A−Agarose. Triton X-100/Nonidet P-40 cell lysates (40 μg of protein) were incubated in the presence of 40 μL of a pepstatin A−agarose slurry (Sigma) overnight at room temperature. The pepstatin−agarose precipitates were centrifuged and washed briefly with STE buffer containing 2% SDS, then once with STEN, twice with STEN plus 0.5 M NaCl, and once with 20 mM Tris-HCl, pH 7.5, before analysis by SDS−PAGE and Western blotting (*25*).

RESULTS

Detection of Pepstatin−Biotin-Bound Proteins in Presenilin Immunoprecipitates. The microbial aspartyl protease inhibitor pepstatin A and its characteristic unusual amino acid statine (or statinoic acid), which represents an aspartyl protease transition-state mimic, were biotinylated by chemical coupling as shown in Figure 1B. To characterize the aspartyl protease/γ-secretase candidates associated with presenilins, the biotinylated inhibitor derivatives were incubated with lysates of SH-SY5Y human neuroblastoma cells, either wild-type or PS1 transfectants. The proteins that immunoprecipitated with presenilin antisera were resolved by SDS−PAGE and analyzed with a neutravidin−alkaline phosphatase conjugate. Bands of 43, 46, and 68 kDa were detected in 98/1 immunoprecipitates of lysates incubated with pepstatin−biotin (Figure 2A, lanes 2 and 5). Similar signals, but less intense, were also detected in lysates incubated with biotin−statine (lanes 1 and 4). The predominant band was the 43 kDa signal, and this was not detected when lysates were immunoprecipitated with a control preimmune serum (Figure 2B, lane 1). Other controls were carried out that included absence of biotin inhibitor, absence of cell lysate (not shown),

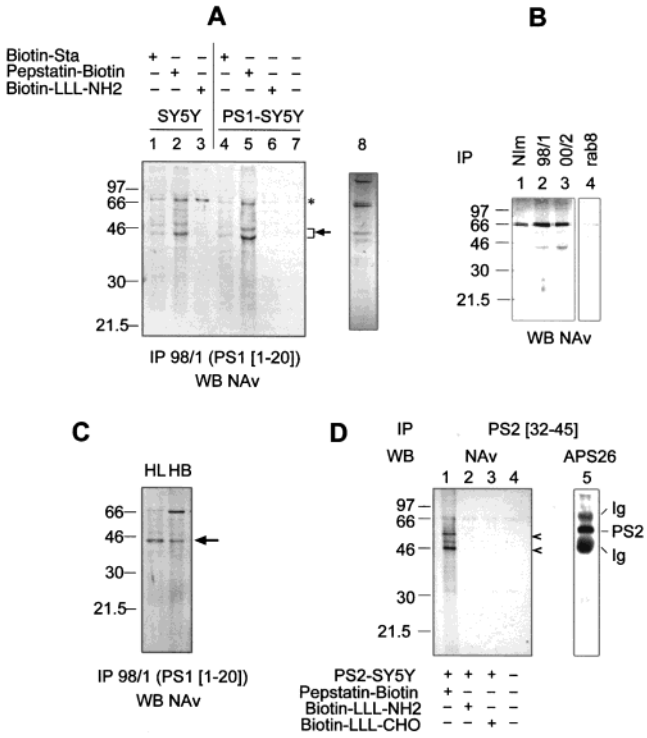

FIGURE 2: Biotinylated derivatives of statine and pepstatin bind to a 43 kDa protein immunoprecipitated with PS1 antibodies. Panel A: Cell lysates (40 μg of protein/lane) were incubated with biotin−statine, pepstatin−biotin, control peptide biotin-Leu-Leu-Leu-amide, or the proteasome inhibitor derivative biotin-Leu-Leu-Leu-aldehyde (*17*). Immunoprecipitation with antibody 98/1 was followed by protein separation on 12% SDS−PAGE gels, electroblotting, and development with neutravidin−alkaline phosphatase (NAv). Prominent bands at 43 and 46 kDa were detected. The asterisk indicates a nonspecific signal. Lane 8 shows the pepstatin−biotin signal obtained with cell lysate that was not immunoprecipitated. Panel B: Conditions were the same as in panel A except that the immunoprecipitation was carried out using a nonimmune serum (lane 1), 98/1 (lane 2), 00/2 (lane 3) or rab-8 antibody (lane 4). Panel C: Lysates from human lymphocytes (HL; 20 μg) and a membrane sample from human brain cortex (HB; 20 μg of 2% Triton X-100 extract of a 20000*g* pellet from total cortex homogenate) were incubated with pepstatin−biotin, followed by immunoprecipitation with 98/1 and Western blotting with NAv. A prominent 43 kDa signal was detected. Panel D: Triton X-100/NP-40 lysates of PS2-transfected SH-SY5Y cells (40 μg/lane) were incubated with pepstatin−biotin, followed by immunoprecipitation with PS2 N-terminal antibody PS2-3 (PS2 [32−45]) and Western blotting with NAv. Prominent bands of 45 and 55 kDa were detected. Lane 5 shows immunoprecipitation of PS2 with N-terminal antibody PS2 [32−45] followed by direct Western blotting with anti-PS2 loop monoclonal antibody APS26.

or incubation with the control peptide biotin-Leu-Leu-Leu-amide (*21*) (Figure 2A, lane 3). All of these controls showed the absence of the 43 kDa signal but often the presence of a 68 kDa band that is presumably nonspecific. The 43 kDa signal was increased in PS1-transfected cells (Figure 2A, lane 5) as compared to nontransfected cells (lane 2), suggesting that it may correspond to PS1 itself. PS1-loop antiserum 00/2 precipitated a similar 43 kDa band (Figure 2B, lane 3), further supporting that it may represent PS1 full length. Pepstatin (Figure 2A, lanes 2 and 5) gave a stronger signal than statine (lanes 1 and 4), suggesting that it bound more tightly. The biotin derivative of MG-132 (Z-Leu-Leu-Leu-CHO), a proteasome inhibitor known to inhibit Aβ secretion in cell culture (*21*), was also tested, and it gave a signal

similar to that of the control, indicating it did not bind to any PS1-associated aspartyl protease activity (data not shown). To test whether pepstatin−biotin would bind nonspecifically to other proteins, we effected immunoprecipitation of rab-8 (Figure 2B, lane 4), α-synuclein, and APP, and we could not detect bands with an electrophoretic mobility similar to that of the corresponding proteins when the blots were probed with neutravidin.

To extend our results to native presenilins, lysates of human lymphocytes and brain cortex homogenates were incubated with pepstatin−biotin and tested using the same assay. A 43 kDa prominent signal (Figure 2C) was observed for the lymphocyte sample (HL), resembling the prominent band obtained with the SY5Y cells. A similar 43 kDa band was also detected in the human brain sample (HB). The result shown is representative of several human brain samples from normal controls and from sporadic AD cases tested.

Binding of the biotinylated inhibitors to PS2-associated species (Figure 2D) was also tested. Prominent bands of 46 and 55 kDa were detected in the PS2 immunoprecipitates of samples incubated with pepstatin−biotin (Figure 2D, lane 1) but not in those of samples incubated with the control peptide (lane 2) or MG-132 derivative (lane 3). We have reported before (*6*) that immunoprecipitation of PS2 from metabolically labeled, transiently transfected, COS-7 cells yielded two bands of 55 and 45 kDa. Immunoprecipitation of PS2 from the SH-SY5Y lysates used in the pepstatin−biotin study followed by Western blotting with mAb APS26 (*28*) yielded a 55 kDa band. The presence of a 45 or 46 kDa band could not be ascertained because of the presence of a prominent immunoglobulin band in the 45−50 kDa detection range.

Detection of Pepstatin−Biotin-Bound Proteins in PS1 Immunoprecipitates from SH-SY5Y CHAPSO Extracts. Presenilins have been shown to associate in high molecular weight complexes containing γ-secretase activity (*18*). This complex can be extracted with CHAPSO and retain its enzymatic activity. Thus, to determine if another aspartyl protease binding to pepstatin was present in the complex, we repeated the previous experiments using CHAPSO lysates in place of Triton X-100/NP-40 lysates of SH-SY5Y cells. Figure 3A shows that a pattern similar to that observed with Triton lysates was obtained with the CHAPSO lysates: presence of two major signals of 43 and 68 kDa. Longer exposure of the blot revealed faint signals of 32 and 20 kDa that may represent the PS1 fragments (Figure 3A, image on the right). The specificity of the 43 kDa signal was demonstrated by competition with a 5-fold excess of unlabeled pepstatin (Figure 3B). By reducing five times the concentration of pepstatin−biotin in this experiment, we were able to eliminate the 68 kDa signal (Figure 3B).

The 43 kDa Species Is Not Detected in PS1 Knock-Out Cells. We tested whether the 43 kDa signal was absent from PS1 gene knock-out cells. Stable lines of neural stem cells prepared from PS1 −/−, PS1 +/−, and PS1 +/+ mouse embryonic brains were used for this study. Direct Western blot analysis of the cell lysates gave the expected results: PS1 was detected in the +/+ cells as a 30 kDa N-terminal fragment (Figure 4A, lane 3). This signal was reduced in the +/− cells (lane 1) and was absent from the homozygous knock-out cells (lane 2). When the cell lysates were incubated with pepstatin−biotin and the blots of PS1 immunoprecipitates developed with neutravidin, the expected 43 kDa signal was obtained with the PS1 +/+ cells (Figure 4B, lane 3) and was absent from the PS1 −/− lysates (Figure 4B, lane 1). It was also markedly reduced in the +/− lysates (lane 2), paralleling the reduction of signal observed in PS1 Western blot.

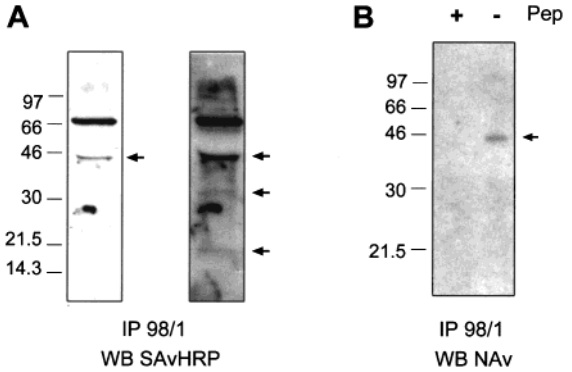

FIGURE 3: Pepstatin−biotin binds to a 43 kDa species immunoprecipitated with PS1 antibody from CHAPSO cell extracts. Panel A: 2% CHAPSO lysates (40 μg of protein) of SH-SY5Y cells were incubated for 18 h with 150 μM pepstatin−biotin and immunoprecipitated with 98/1. The precipitates were resolved by SDS−PAGE, and blots were probed with streptavidin−HRP. 43 and 68 kDa signals were detected. Both images show the same blot, but the image on the right corresponds to a longer exposure to ECL film to highlight the presence of weak signals at 32 and 20 kDa that have the correct size to represent PS1 fragments. Panel B: 2% CHAPSO extracts of SH-SY5Y were incubated for 48 h with 30 μM pepstatin−biotin in the presence (+) or absence (−) of a 5-fold excess of unlabeled pepstatin. The blots were probed with NAv.

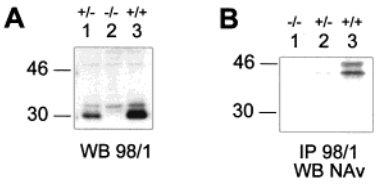

FIGURE 4: The 43 kDa protein that binds to pepstatin−biotin is not immunoprecipitated from PS1 −/− cells. Three cell lines were established from mouse neural stem cells, corresponding to homozygous (−/−) PS1 knock-out, heterozygous (+/−) PS1 knock-out, and wild-type PS1 (+/+). Panel A: Characterization of these cell lines by Western blotting. The expected pattern was observed: no 30 kDa signal was detected in the PS1 (−/−) (lane 2), and the signal was reduced in the PS1 (+/−) (lane 1) as compared to the wild type (lane 3). Panel B: Detection of pepstatin−biotin by Western blotting with NAv after immnuoprecipitation with 98/1. A 43 kDa signal was detected with the wild-type cells (lane 3), a weaker signal was detected with the (+/−) cell lysate (lane 2), and no signal was observed with the (−/−) cell lysate (lane 1).

Binding Specificity. To demonstrate the specificity of pepstatin binding, competition was effected with the γ-secretase inhibitor L-685,458 (*29*). This is the most potent γ-secretase inhibitor described so far, and its photoreactive derivatives have been cross-linked to PS1 (*19*). Preincubation of PS1-SY5Y cell lysate with L-685,458 preceding incubation with pepstatin−biotin reduced the 43 kDa signal almost completely (Figure 5A, lane 2). This result suggests that pepstatin and L-685,458 compete for the same or close presenilin binding sites. As L-685 458 is near 3 orders of magnitude more potent than pepstatin at inhibiting γ-secretase, it was somewhat surprising that it did not block totally pepstatin binding. Since our data suggest that pepstatin−biotin binds to the PS1 holoprotein, a possible explanation

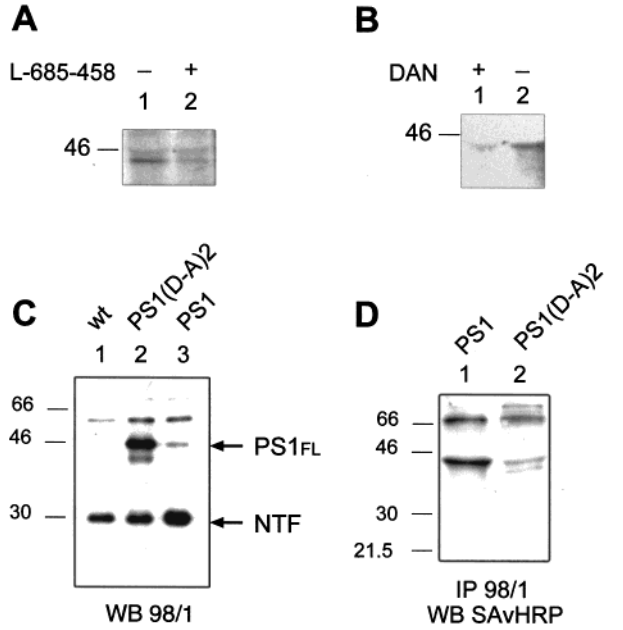

FIGURE 5: Specificity of pepstatin−biotin binding to the 43 kDa species. Panel A: Detection of pepstatin−biotin in immunoprecipitates of PS1-transfected SH-SY5Y. Triton X-100 cell lysates were incubated with pepstatin−biotin (100 μM) as described in Figure 2 or after overnight preincubation with 100 μM L-685,458 (lane 2). Panel B: Detection of pepstatin−biotin in immunoprecipitates of solubilized membranes from PS1-transfected SH-SY5Y with or without treatment by diazoacetylnorleucine methyl ester (DAN). Panel C: Western blotting of lysates of COS-7 cells (20 μg per lane) with anti-PS1 antiserum 98/1. Lane 1 corresponds to wild-type COS-7 cells where endogenous PS1 is detected as a 30 kDa N-terminal fragment. Lane 2 corresponds to the PS1 mutant with Asp_{253} and Asp_{381} (numbering refers to the $PS1_{463}$ sequence) mutated to alanine [PS1(D-A)$_2$]. A strong 48 kDa signal is detected that corresponds to uncleaved expressed mutant protein. Lane 3 corresponds to cells transfected with $PS1_{463}$ wild type. Panel D: Streptavidin detection of pepstatin−biotin labeled proteins precipitated with anti-PS1 antiserum 98/1. Note the marked reduction of the 43 kDa signal observed in the lane corresponding to COS-7 transfected with the PS1(D-A)$_2$ mutant.

is that L-685 458 does not completely displace pepstatin binding because itself binds poorly to the PS1 holoprotein. Indeed, Li et al. reported that their inhibitor binds mostly to the PS1 heterodimer (*19*).

To test if binding of pepstatin−biotin to the 43 kDa species occurred through a reactive aspartate, we used diazoacetylnorleucine methyl ester (DAN), a reagent that alkylates one aspartate of the catalytic site of aspartyl proteases (*30*). Semipurified membranes from PS1-SY5Y cells were solubilized with cardiolipin and treated with DAN. This dramatically reduced binding of pepstatin−biotin to the 43 kDa presenilin associated species (Figure 5B, lane 1).

Mutating the two aspartates of putative transmembrane domains 6 and 7 of PS1 was shown to alter dramatically γ-secretase activity, suggesting that these residues constitute the catalytic site of a new class of transmembrane proteases (*11*). To test the requirement of the transmembrane aspartates for binding of pepstatin−biotin, cells were stably transfected with PS1(D-A)$_2$, a construct in which both aspartates were mutated for alanine. Western blotting for PS1 showed that expression of this mutant in COS-7 cells caused overaccumulation of uncleaved PS1, represented as a 48 kDa band (Figure 5C, lane 2). The level of cleaved PS1, represented as the 30 kDa N-terminal fragment, remained similar to that detected in the nontransfected cells (Figure 5C, compare lanes 1 and 2). Overexpression of PS1 wild type had the expected effect and caused only a modest accumulation of PS1 full length and increased amounts of cleaved PS1 (lane 3). CHAPSO lysates of the cells transfected with PS1 wild type or with the PS1 double mutant were incubated with pepstatin−biotin and analyzed by Western blotting with streptavidin. A 43 kDa signal was observed with the sample of PS1 transfected cells (Figure 5D, lane 1), and this was markedly decreased for the sample of cells expressing the PS1(D-A)$_2$ mutant (lane 2).

Pepstatin−Biotin Binds to Cleaved and Uncleaved PS1. The 43 kDa band detected in the PS1 immunoprecipitates has an electrophoretic mobility consistent with that observed for PS1 uncleaved holoprotein in metabolic labeling/immunoprecipitation experiments. Thus, it may represent either pepstatin−biotin bound to uncleaved presenilin or pepstatin−biotin bound to the PS1 heterodimer with the two fragments migrating together in electrophoresis. To resolve this question, we analyzed by immunoblotting cell lysates that had been incubated with unlabeled pepstatin and heated for 30 s (these are the same conditions that we used to treat the samples incubated with pepstatin−biotin prior to Western blotting with neutravidin). No significant difference was observed between the samples incubated with pepstatin (Figure 6A, lanes 2 and 3) and the control (lane 1). Boiling the samples for 5 min resulted in some loss of the 30 kDa signal and detection of high molecular weight aggregates (Figure 6A, lanes 4−6), but again the control sample gave results similar to those of the samples incubated with pepstatin. Of interest is the detection in all samples briefly heated of a PS1 immunoreactive band of 43 kDa with an electrophoretic mobility similar to that of the 43 kDa species detected in the pepstatin−biotin/neutravidin assay, and that likely represents the PS1 holoprotein.

To detect the eventual association of pepstatin−biotin with the PS1 heterodimer, which would have been unnoticed in the previous assay due to SDS−PAGE denaturing conditions, we designed an alternative experimental paradigm in which the pepstatin−biotin/cell lysate incubations were precipitated with streptavidin−agarose and the precipitates analyzed by Western blotting with PS1 antibodies. Control experiments conducted with protein A−agarose, or with avidin−agarose, indicated that it was necessary to include washing steps with 0.5% SDS to eliminate hydrophobic interactions between PS1 and the gel matrix. Analysis of streptavidin precipitates by Western blotting with 98/1 revealed small amounts of the 30 kDa PS1 N-terminal fragment in samples derived from nontransfected SY5Y (Figure 6B, lane 3) and both the N-terminal fragment and the uncleaved protein (48 kDa band) in the sample derived from PS1-transfected SY5Y (Figure 6B, lane 4). This result demonstrates that pepstatin−biotin binds to the PS1 cleaved form as well as to the uncleaved protein. Under our experimental conditions, only a small fraction of total PS1 immunoreactivity present in the sample was absorbed to streptavidin−agarose (compare lanes 3 and 4 with lanes 1 and 2; in lanes 1 and 2 only one-fifth of the amount of lysate incubated with pepstatin−biotin was loaded on the gel). As it has been established that Triton mostly dissociates the heterodimer constituted of the two fragments of PS1 (*31*), we postulated that pepstatin binding required both fragments to be assembled.

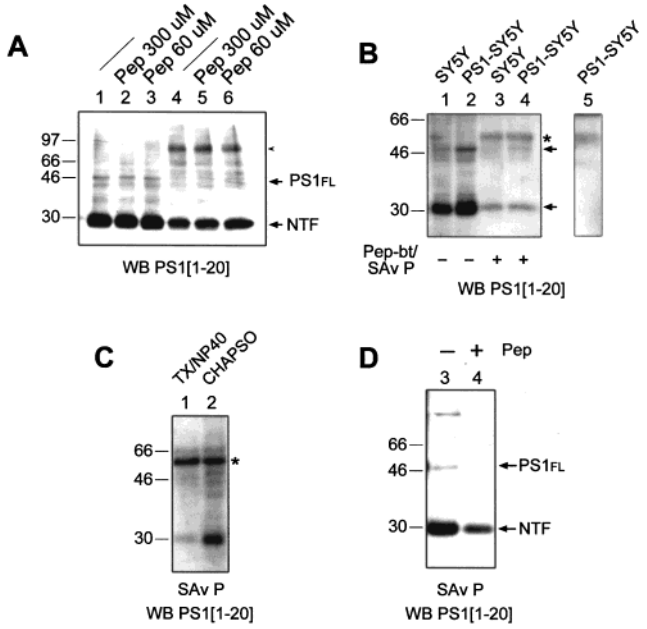

FIGURE 6: Pepstatin−biotin binds to cleaved PS1. Panel A: Western blot of cell lysates (40 μg of protein/lane) from wild-type SH-SY5Y that have been untreated (lanes 1 and 4) or treated with the indicated concentrations of pepstatin (lanes 2, 3, 5, and6) after boiling for 30 s (lanes 1−3) or for 5 min (lanes 4−6) in Laemmli sample buffer. The arrowhead indicates PS aggregates. Pepstatin treatment did not increase a 43 kDa signal. Panel B: Triton X-100/NP-40 lysates (40 μg) of SH-SY5Y cells were incubated with pepstatin−biotin followed by precipitation with streptavidin−agarose and Western blotting with 98/1. The PS1 30 kDa N-terminal fragment was detected in the streptavidin precipitates (lanes 3 and 4). Direct Western blotting of untreated cell lysates (8 μg of protein) is shown (lanes 1 and 2) to illustrate that only a small fraction of PS1 immunoreactivity present in the sample interacted with pepstatin−biotin. Lane 5 shows SAv−agarose absorption of cell lysate untreated with pepstatin−biotin. Panel C: A similar experiment was effected using either 1% Triton X-100 (TX)/1% NP-40 lysate (lane 1) or 2% CHAPSO lysate (lane 2) of PS1-transfected SH-SY5Y. Note that the 30 kDa signal was much more intense in the experiment carried out with CHAPSO lysate. The asterisk indicates the nonspecific signal. Panel D: SH-SY5Y CHAPSO lysate was incubated with pepstatin−biotin in the presence or absence of a 4-fold excess of cold pepstatin and precipitated with streptavidin−DynaBeads prior to Western blot analysis.

To substantiate this point, PS1-SY5Y were lysed in CHAPSO to preserve the integrity of PS1/γ-secretase activity (*18*). Binding of pepstatin to PS1 was dramatically increased in the 2% CHAPSO lysates as compared to 1% Triton X-100/1% NP-40 lysate (Figure 6C) or 0.5% SDS lysate (not shown). This confirms that the integrity of the PS1 heterodimer complex is required for binding to pepstatin. The specificity of the binding of pepstatin−biotin-associated PS1 to streptavidin−agarose was confirmed by showing that it could be competed by a 4-fold excess of unlabeled pepstatin (Figure 6D, lane 4).

Binding of Presenilin to Pepstatin−Agarose. To further demonstrate the binding of PS1 to pepstatin, cell lysates were absorbed to pepstatin−agarose, and the absorbed proteins were analyzed by Western blotting for PS1. Again, extensive washing steps were carried out, including SDS, to reduce nonspecific hydrophobic interactions. PS1 from wild-type SH-SY5Y was retained and detected as a 30 kDa N-terminal fragment that represents the proteolytically processed form (Figure 7A, lanes 3 and 5). The uncleavedPS1 species from the PS1-transfected cell sample may also bind to pepstatin−agarose, as a weak signal corresponding to the PS1 holoprotein was also detected (Figure 7A, lanes 4 and 6). Binding was slightly increased at pH 6.0 as compared to pH 7.4, and this was decreased at pH 5.0. Only a very small fraction of PS1 immunoreactivity bound to pepstatin−agarose, and this was expected as Triton X-100/NP-40 cell lysates had been used for the experiment. Absorption of PS1 from CHAPSO lysates was tested, but no PS1 or fragments were detected (not shown). We presume that PS1 access to immobilized pepstatin is impaired by its association with binding partners since CHAPSO has been shown to extract PS1 combined with other proteins in a high molecular weight complex (*18*).

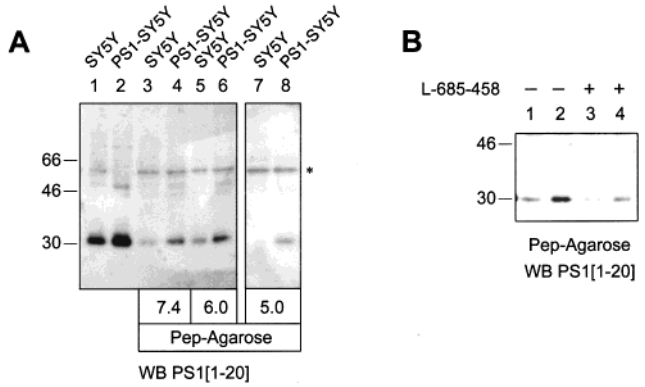

FIGURE 7: PS1 binds to pepstatin−agarose. Cell lysates (40 μg) were adsorbed onto pepstatin−agarose, the matrix was thoroughly washed, and the proteins that remained bound were analyzed by SDS−PAGE and Western blotting with 98/1. Panel A: Lanes 1 and 2 correspond to the total cell lysate prior to pepstatin−agarose adsorption (8 μg/lane). Lanes 3 and 4 show absorption at pH 7.4; lanes 5 and 6, absorption at pH 6.0; and lanes 7 and 8, absorption at pH 5.0. Note that the 30 kDa signal was optimal at pH 6.0, indicating that this is the preferred pH for binding PS1 to pepstatin−agarose. Panel B: Pepstatin−agarose absorption was effected after preincubation of the cell lysates with γ-secretase inhibitor L-685 458 (lanes 3 and 4) and compared with samples with no preincubation (lanes 1 and 2). Lanes 1 and 3 correspond to incubation with pepstatin−agarose at pH 5.0, and lanes 2 and 4 correspond to incubation at pH 6.0.

To confirm the specificity of PS1 binding to pepstatin−agarose, competition was carried out by preincubating the cell lysates with the γ-secretase inhibitor L-685 458 (Figure 7B). Samples preincubated with the inhibitor (lanes 3 and 4) show little binding to pepstatin−agarose as compared to untreated samples (lanes 1 and 2), whenever binding was effected at pH 5.0 (lanes 1 and 3) or pH 6.0 (lanes 2 and 4).

Exon 9 Deletion Alters Binding of PS1 to Pepstatin. The Δ9 mutation, which results in expression of an alternative PS1 isoform, causes a very aggressive form of early-onset AD. Δ9 mutation corresponds to a deletion of exon 9 from the PS1 gene and translation of a protein lacking 30 residues, which encompass the constitutive proteolytic cleavage site of PS1 (*32*−*34*). Thus, expression of PS1-Δ9 is detected as uncleaved protein. Two cell lines derived from a PS1-Δ9 kindred (*35*) were used for this study. The line L-444 derives from a normal homozygous subject and expresses only PS1 wild type, whereas the line L-445 derives from a carrier of the mutation and expresses both mutant and wild-type alleles. Western blot analysis shows detection in the L-444 cell line of PS1 wild type as a 30 kDa N-terminal fragment that represents the PS1 cleaved form (Figure 8A). Minor amounts

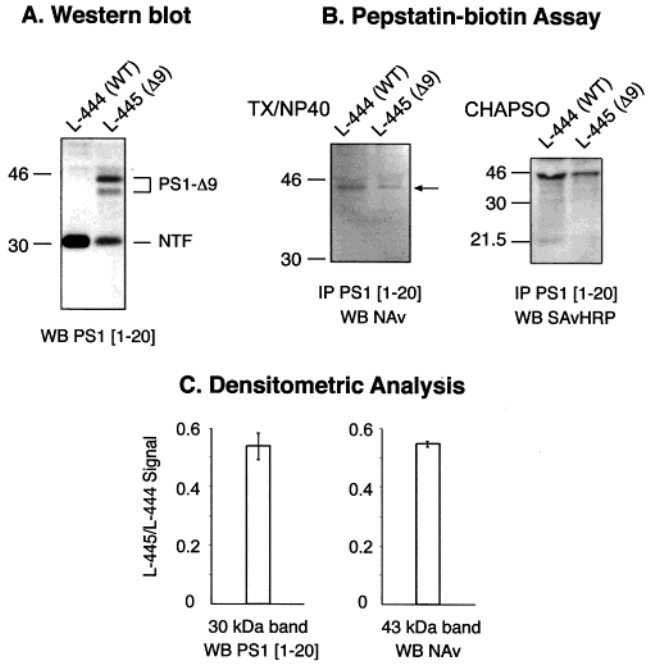

FIGURE 8: Binding of pepstatin to PS1 is altered by exon 9 deletion. Panel A: Western blot of lymphocyte lysates (20 μg/lane) from two members of a PS1-Δ9 kindred. Line L-444 does not carry the mutation; line L-445 carries one wild-type (WT) and one Δ9 allele. The 30 kDa band corresponding to the PS1 N-terminal fragment is markedly decreased in the sample L-445 as compared to L-444. An additional doublet corresponding to uncleaved PS1-Δ9 species is detected in L-445. Panel B: NAv detection of a 43 kDa species in PS1 immunoprecipitates of lymphocyte lysates incubated with pepstatin−biotin. There is a marked decrease of signal observed in the sample L-445 as compared to L-444. Similar results were obtained using Triton and CHAPSO lysates. Panel C: Quantitative analysis of the blots using the NIH Image 1.61 software. Image densities of the Western blot 30 kDa bands were quantitated, and ratios of the signals obtained for L-445/L-444 were calculated. The data shown are representative of three separate experiments and indicate a 45% decrease of the 30 kDa signal for line L-445. Similarly, image densitometry and quantitation of the NAv Western blot showed 46% reduction of the 43 kDa signal in the line L-445.

of holoprotein were also detectable if higher amounts of protein were loaded on the gel (not shown). The intensity of the band corresponding to the PS1 N-terminal fragment was markedly reduced in the line L-445 compared with the control L-444 (Figure 8A), a result consistent with expression of a single cleavable PS1 allele in the line L-445 and expression of two wild-type alleles in the line L-444. A 43−45 kDa doublet was detected in the mutant cell line that corresponds to uncleaved PS1 protein lacking exon 9 due to expression of the PS1-Δ9 allele. Incubation of lymphocyte lysates with pepstatin−biotin resulted in detection of a 43 kDa band, the intensity of which was markedly decreased in the line L-445, when the cells were lysed with Triton or with CHAPSO (Figure 8B). Quantitative densitometry analysis of the bands (Figure 8C) indicates that the mutant cell line displays a 46% reduction in the intensity of 30 kDa band that corresponds to the PS1 N-terminal fragment. A similar reduction of signal (45% decrease) is also observed in the biotin detection assay, suggesting that it represents only wild-type PS1. Additional bands migrating faster and which would correspond to PS1-Δ9 species were not detected. Thus, binding of pepstatin−biotin to PS1-Δ9 appears to be altered in our assay.

DISCUSSION

Presenilins have been implicated in the γ-secretase cleavage of APP since the finding that pathologic mutations caused overproduction of the long Aβ 42 and 43 amyloid peptides (*36*). Studies of PS gene knock-out proved presenilins to be indispensable for γ-secretase cleavage, suggesting that they are γ-secretases or factors required for γ-cleavage to take place (*9*). Evidence has been accumulating recently to support the fact that presenilins are proteases. First, Wolfe et al. (*11*) showed that two aspartate residues located in predicted transmembrane domains 6 and 7 of the eight-transmembrane putative model of PS1 are required for γ-secretase activity and may represent the catalytic site of a novel class of aspartyl proteases. Second, several groups have recently reported cross-linking of γ-secretase inhibitors to PS1 and PS2 (*19*, *20*, *39*). On the basis of our previous studies (*16*), supported by the recent publication that pepstatin is an inhibitor of γ-secretase in a cell-free system (*18*), we used pepstatin derivatives as tools to identify the aspartyl protease associated with PS. We found that pepstatin−biotin bound specifically to a 43 kDa protein immunoprecipitated with PS1 antibodies and to 46 and 55 kDa proteins immunoprecipitated with PS2 antibodies (Figure 2). The 43 kDa species was the major species detected in PS1 immunoprecipitates, suggesting that it does correspond to the γ-secretase activity associated with PS1 that can be inhibited by pepstatin, as reported by Li et al. (*18*). This 43 kDa species can be immunoprecipitated by antibodies directed either to the PS1 N-terminus or to the PS1 C-terminal loop, supporting the premise that it includes both antibody epitopes and corresponds to full-length PS1 (Figure 2B). Alternatively, it may represent a protease associated with the PS1 complex. The observed molecular mass of 43 kDa is close to that of the PS1 holoprotein and would be expected for a PS1−inhibitor complex as binding of pepstatin would cause only a very small shift in the molecular mass. This 43 kDa signal was not obtained with PS1 knock-out cells, proving that it is specifically associated with PS1 (Figure 4B). This result is consistent with the 43 kDa band representing either PS1 itself or a PS1 binding protein that would not be precipitated with 98/1 in absence of PS1. The use of CHAPSO as a detergent to preserve the presenilin complex (Figure 3) did not result in the detection of bands other than those detected with the Triton lysates, suggesting that there was no other aspartyl protease activity associated with the complex.

To further illustrate that pepstatin−biotin interacts specifically with the 43 kDa species through the catalytic site of an aspartyl protease, we showed that treatment with diazoacetylnorleucine methyl ester (DAN) altered the binding of pepstatin−biotin to this species (Figure 5B). DAN reagent has been used to delineate the catalytic site of pepsin (*30*) and other aspartyl proteases. It was also shown recently to inhibit the activity of the prepilin peptidases (*37*), a new class of bacterial transmembrane aspartyl peptidases.

Since two transmembrane aspartates of presenilins were proposed to constitute the catalytic site of an aspartyl protease (*11*), we tested binding of pepstatin to a PS1 analogue in which both transmembrane aspartates had been mutated to alanine. We found that pepstatin did not bind to this PS1 analogue (Figure 5D). If the 43 kDa species represents PS1, our results provide direct evidence for these aspartate

residues, either one or both, to constitute the catalytic site of an aspartyl protease. Alternatively, if the 43 kDa species is part of the PS1/γ-secretase complex, this would not be co-immunoprecipitated with the PS1(D-A)$_2$ mutant since the two transmembrane aspartates are required for the integration of PS1 into the high molecular mass γ-secretase complex (*38*).

Using two alternative experimental protocols, we showed that both cleaved and uncleaved PS1 bind to pepstatin. Again the results are consistent with either direct binding to pepstatin or binding through an aspartyl protease associated with PS1. Western blotting of pepstatin−biotin-bound PS1 precipitated with streptavidin−agarose detected almost uniquely the PS1 30 kDa fragment. This indicates that pepstatin bound mostly to cleaved PS1 (Figure 6B). Direct absorption of cell lysates to pepstatin−agarose showed binding of cleaved PS1 (Figure 7). Binding of pepstatin−biotin to PS1 was markedly improved by using CHAPSO as a detergent rather than Triton (Figure 6C). Triton mostly dissociates the PS1 heterocomplex (*31*), and this is consistent with our finding that only a very small fraction of PS1 total immunoreactivity present in Triton cell lysates could associate in a pepstatin−biotin complex or bind to pepstatin−agarose. Since we showed that free pepstatin−biotin could bind to PS1 in the multimeric complex extracted with CHAPSO, it was somewhat unexpected that the same complex could not be absorbed to pepstatin−agarose. The most likely explanation is that the presence of PS1 binding partners in the complex perturbed access to immobilized pepstatin because of steric hindrance.

Several groups have recently reported cross-linking of γ-secretase inhibitors to PS1 (*19*, *20*, *39*). In these experiments, first the biotinylated inhibitors are cross-linked with the presenilin complex extracted with CHAPSO, and then the samples are denatured in conditions that dissociate the γ-secretase complex and the PS1 heterodimer prior to Western blotting. This results in the detection of PS1 fragments labeled with the inhibitors. Li et al. (*19*) showed that L-685 458, an aspartyl protease transition-state analogue that is a potent inhibitor of γ-secretase (*29*), could be bound to either PS1 N-terminal or C-terminal fragments, depending on the position of the photoreactive group relative to the transition-state mimic. We found that binding of pepstatin to PS1 could be partially competed by L-685,458, suggesting that pepstatin and L-685 458 compete for the same presenilin binding site or two binding sites in close spatial proximity. To support further our data, we used 1-Bt, a difluoro alcohol transition-state analogue inhibitor of γ-secretase (*20*), and 2-Bt, an inactive control compound, in an assay similar to that used for pepstatin−biotin. Samples incubated with 1-Bt produced a 43 kDa signal that was not observed with 2-Bt (data not shown), suggesting that pepstatin and this γ-secretase inhibitor bind to the same PS1 active site. Inhibitors derived from 1-Bt were proved to bind to PS1 in cross-linking experiments (*20*).

We have shown pepstatin to associate with both cleaved and uncleaved PS1 (Figure 7A, lanes 4 and 6). Li et al. (*19*) did not detect binding of L-685 458 derivatives to full-length PS1. Esler et al. (*20*) demonstrated cross-linking of difluoro alcohol transition-state analogues to PS1 fragments but did not exclude the possibility of these binding to full-length uncleaved PS1 as well. Constitutive proteolytic cleavage stabilizes PS1 protein, and the uncleaved form has a much shorter half-life (*40*) and is sensitive to proteolytic degradation (*41*). However, cleavage of PS1 is neither required for its function (*41*−*44*) nor required for its association into the high molecular mass complexes that constitute the active γ-secretase activity (*38*); thus there is no apparent contradiction for γ-secretase inhibitors binding to uncleaved PS1 as well as to the heterodimer.

Our experiments show that pepstatin−biotin bound PS1 from transfected cells and also native PS1 from neuroblastoma SH-SY5Y cells, from lymphocytes, and from human brain homogenates. Interestingly, when testing lysates of lymphocytes from a carrier of a PS1-Δ9 allele, a marked decrease of the 43 kDa signal was observed, and no other signal was detected that may represent pepstatin−biotin bound to a PS1-Δ9 mutant. This result suggests that pepstatin does not bind to a PS1-Δ9 mutant in these experimental conditions. This is in apparent conflict with Li et al. (*19*), showing that L-685 458 derivatives bind to a PS1-Δ9 mutant. Since exon 9 deletion does not alter directly the two transmembrane aspartates, it may be expected that γ-secretase inhibitors bind to a PS1-Δ9 mutant. A possible interpretation to our data may be that binding of pepstatin to PS1-Δ9 is less tight than for wild-type PS1 and dissociates more readily under SDS−PAGE conditions. This difference might only be observed using a reversible inhibitor whereas covalently bound inhibitors would not dissociate from PS1 under boiling and SDS−PAGE denaturing conditions. Deletion of exon 9 could alter binding of the inhibitor either by impairing access to the active site or by modifying the active site itself. PS1-Δ9 has been reported to be less functional than wild-type PS1 at restoring the sel-12 null allele and Notch signaling in *C. elegans* (*33*), and this could be due to a loss of affinity for its substrates paralleling the loss of affinity for pepstatin our data suggest. The expression of the PS1-Δ9 mutant increases dramatically the formation of Aβ 42/43 relative to Aβ 40 by comparison to wild-type PS1 (*34*). Further studies will be required to determine whether exon 9 deletion could alter placement of the substrate relative to the γ-secretase catalytic site and shift cleavage from position 40 to position 42 or 43. Alternatively, if the 43 kDa species represents a protease binding to PS1, exon 9 deletion may alter this binding although this mutation does not prevent the incorporation of PS1 in the high molecular mass complex (*38*).

Taken together, our data indicate binding of the classic aspartyl protease inhibitor pepstatin to presenilins and support the hypothesis that these represent a novel type of aspartyl protease. Although the predicted polytopic membrane structure of the presenilins does not resemble that of any known mammalian aspartyl proteases, it shares some similarities with that of the bacterial prepilin peptidases. These multipass membrane proteins, with eight putative transmembrane domains, require two aspartates for peptidase activity and were recently characterized as a novel family of aspartic acid proteases (*37*). Unlike most aspartyl proteases, but like the presenilins, these lack the consensus triads DSG or DTG within their catalytic site. Sequence alignment shows that similar amino acids surround the transmembrane aspartate residues of presenilins and two aspartyl residues required for prepilin peptidase activity. In particular, the amino acid sequence surrounding Asp$_{385}$ (referring to the PS1$_{467}$ sequence) shows conservation of a GDF triad also present in

the carboxyl-terminal region of prepilin peptidases. This is substantiated by a recent report showing that mutating glycine 384 of PS1 alters γ-secretase cleavage (*45*). Homology surrounding PS1 aspartate 257 and the N-terminal aspartate of these peptidases is more distant and suggests that the catalytic site and specificity of presenilins and prepilin peptidases may differ. This would be illustrated by their different affinity for pepstatin: pepstatin is a very poor inhibitor of prepilin peptidase activity (*37*).

Presenilins appear to orchestrate several cellular proteolytic events. Our results provide more evidence that PS themselves are aspartyl proteases or that they are associated with an aspartyl protease. Clarifying PS mechanism and their possible malfunction in Alzheimer's disease will help the design of new therapeutic strategies.

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