Presenilin Modulates Pen-2 Levels Posttranslationally by Protecting It from Proteasomal Degradation†

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ABSTRACT: The γ-secretase complex functions to cleave several type I transmembrane proteins within their transmembrane domains. These include the amyloid precursor protein, which is central to Alzheimer's disease pathogenesis, as well as N-cadherin and Notch, which regulate transcription. This complex is composed of four requisite integral membrane proteins: presenilin 1 (PS1) or presenilin 2 (PS2), nicastrin, Pen-2, and Aph-1. How these proteins coordinately regulate one another and assemble to form a functional complex is not well understood. In this report we demonstrate that PS1 selectively enhances the stability of Pen-2 protein but not that of nicastrin or Aph-1. In the absence of PS1, Pen-2 was rapidly degraded by the proteasome. As PS1 levels increased, so too did the half-life of Pen-2 and therefore its steady-state levels. In addition, Pen-2 protein levels correlated with PS1 levels not only in cell culture but in transgenic mouse models as well. The genetic absence of PS1 and PS2, and therefore of γ-secretase-dependent mediation of transcriptional activity, did not affect Pen-2 mRNA levels. Rather, presenilin (PS) regulates Pen-2 levels posttranslationally by preventing its degradation by the proteasome. Thus, the amount of Pen-2 protein is effectively titrated by its PS binding partner, and the rapidity with which Pen-2 is degraded in the absence of PS interactions could provide a mechanism to tightly regulate γ-secretase complex assembly.

The γ-secretase complex is composed of four integral membrane proteins: presenilin 1 (PS1)[1] or presenilin 2 (PS2), nicastrin (Nct), Pen-2, and Aph-1 (*1*−*5*). This protein complex, once assembled, mediates the regulated intramembranous cleavage of a growing list of substrates including amyloid precursor protein (APP), Notch (*6*), and E-cadherin (*7*). Of these substrates, the cleavage of APP by the γ-secretase complex is of particular interest as it represents the final step in the production of the amyloid β peptide, the major component of senile plaques which are a pathological hallmark of Alzheimer's disease (AD) (*8*). Mutations in PS1 (*9*), PS2 (*10*), and APP (*11*), and their resulting perturbations in γ-secretase activity (*12*, *13*), have been linked to familial forms of AD. γ-Secretase-mediated cleavage of Notch guides normal development through the generation of the Notch intracellular domain (*14*, *15*). Similar processing of E-cadherin releases a soluble intracellular domain termed N-Cad/CTF2, resulting in the downregulation of CBP/CREB-mediated transcription (*16*) which controls numerous gene pathways critical in processes as diverse as cell growth, differentiation, and long-term memory (*17*). Thus, understanding how the γ-secretase assembles and how its activity is regulated is of obvious importance.

While each protein in the γ-secretase complex is required for its enzymatic activity, the functions of each in promoting complex assembly, maturation, and regulation are not well understood (*1*, *3*, *5*, *18*−*21*). Several observations indicate that these proteins can at some level coregulate the expression, processing, and trafficking of other complex members. For example, each protein is required for the endoproteolysis of presenilin (PS) into the N- and C-terminal PS fragments present in the active γ-secretase complex (*1*, *22*). Similarly, Nct promotes the generation of an Aph-1 C-terminal fragment (*23*). Additionally, in the absence of PS, human Nct fails to exit the endoplasmic reticulum (*24*, *25*). Several studies suggest that Aph-1, which interacts directly with Nct (*26*−*28*), may stabilize the PS holoprotein while Pen-2 functions to promote presenilinase activity (*4*, *18*, *19*), but how they do so is unknown. Most relevant for this study, Pen-2 levels are significantly reduced in the absence of PS1, and more so in the absence of both PS1 and PS2 (*18*, *19*), while the levels of Nct or Aph-1 protein appear to be unchanged. Levels of Pen-2 in PS-deficient cells can be rescued by stable transfection of PS1 (*29*). The genetic absence of PS1 and PS2 could result in dramatic reduction

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[1] Abbreviations: PS1, presenilin 1; PS2, presenilin 2; Nct, nicastrin; APP, amyloid precursor protein; AD, Alzheimer's disease; PS, presenilin; GFP, green fluorescent protein; mAb, monoclonal antibody; pAb, polyclonal antibody; HEK, human embryonic kidney; RIPA buffer, radioimmunoprecipitation assay buffer; BFA, brefeldin A.

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in Pen-2 protein levels through either of two mechanisms or through a combination of the two. First, Pen-2 protein could be destabilized in the absence of its normal binding partner, PS. Alternatively, Pen-2 transcription could be downregulated as a consequence of the absence of γ-secretase-mediated regulation of transcription.

In this study, we show that Pen-2 protein levels are closely linked to PS1 protein levels. In the absence of PS1, Pen-2 levels are low, and when PS1 levels are high, Pen-2 amounts are likewise increased. Importantly, Pen-2 was the only γ-secretase component whose levels were affected by PS1. Pen-2 levels were affected by PS1 levels in several cell systems as well as in a mouse model that accumulates PS1 holoprotein. Regulation of Pen-2 by PS is largely if not exclusively at the posttranslational level as Pen-2 mRNA levels were unaffected by the genetic absence of PS1 and PS2. Thus, the role of the γ-secretase in transcriptional control does not alter expression levels of Pen-2. Finally, we show that, in the absence of PS, Pen-2 was rapidly degraded by the proteasome, while overexpression of PS1 resulted in a dramatic extension in Pen-2 protein half-life. Rapid proteasomal degradation of this component of the γ-secretase complex could provide a way to regulate γ-secretase activity in vivo.

MATERIALS AND METHODS

Plasmids. Construction of Aph-1aS, Pen-2, Nct, PS1, green fluorescent protein (GFP), and DC-SIGN plasmids have been described previously (*28*, *30*−*32*). CD4 cDNA was PCR amplified and cloned into pcDNA3.1D-V5/His TOPO (Invitrogen, Carlsbad, CA) to generate C-terminally V5 epitope-tagged CD4. Pen-2 constructs with a 5′ HA epitope tag and a 3′ 6-methionine tag (Pen-2 5′HA, 3′6Met) were generated by PCR essentially as described (*32*), with the amplified products being cloned into pcDNA3.1D-V5/HisTOPO (Invitrogen). Plasmid DNA was purified using Qiagen plasmid kits (Qiagen, Valencia, CA). The identity of all plasmids was confirmed by sequencing.

Antibodies. HA eptiope-tagged Pen-2 was detected by Western blot with monoclonal antibody (mAb) HA11 (Covance, Princeton, NJ). PS1 was detected with anti-PS1 mouse mAb MAB5232 (Chemicon, Temecula, CA). V5 epitope-tagged proteins were detected with anti-V5 mAb purchased from Invitrogen. The anti-nicastrin rabbit polyclonal antibody (pAb) was purchased from Cell Signaling Technology (Beverly, MA). The anti-β-tubulin Ab was purchased from Sigma-Aldrich (St. Louis, MO). The anti-Pen-2 rabbit polyclonal antibody, UD-1, was generously provided by Dr. Jan Näslund of the Karolinska Institutet. UD-1 was raised against the N-terminus (ERVSNEEKLNL) of Pen-2 and was affinity purified using the peptide immunogen bound to a SulfoLink column (Pierce, Rockford, IL) using the manufacturer's protocol.

Cell Culture and Western Blot. Human embryonic kidney (HEK) 293T, quail QT6, wild-type mouse embryonic stem cells (ES PS +/+), and mouse embryonic stem cells deficient in PS1 and PS2 (ES PS −/−) (*33*) were grown on six well plates and transfected using Lipofectamine 2000 transfection reagent (Invitrogen) at approximately 50% confluency. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 50 mM Tris, pH 8, 150 mM NaCl) with Complete protease inhibitor (Roche Molecular Biochemicals, Indianapolis, IN), sonicated briefly, and clarified of cellular debris by centrifugation (10000*g*, 10 min, 4 °C). All samples were electrophoresed on precast 10−20% or 4−15% Tris-HCl Chemicon gels (Bio-Rad Laboratories, Hercules, CA) and transferred to PVDF membranes, and the antigens were detected by Western blot with the appropriate antibodies followed by imaging with an ECL+plus Western blotting detection system (Amersham Biosciences) on a Fujifilm (Stamford, CT) LAS-1000 camera.

Metabolic Labeling. HEK 293T cells were transfected with either 5′HA Pen-2 or 5′HA Pen2 and PS1. Cells were also transfected with GFP or GFP and PS1 as a negative control. Twenty-four hours after transfection the cells were incubated in methionine- and cysteine-deficient media for 30 min and then metabolically labeled with [^{35}S]methionine or [^{35}S]-cysteine at 150 μCi/well for 2 h. Following the labeling period, the cells were washed with PBS and then placed in complete media with 10% FBS for the duration of the chase period (0, 10, 30, 60, 120, or 240 min). After the chase period the cells were washed with PBS and then lysed in 500 μL of RIPA buffer. All samples were frozen and thawed once, then sonicated, and centrifuged at 13000 rpm in a tabletop microfuge for 10 min at 4 °C. The lysates were precleared with protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) and then immunoprecipitated with an anti-HA mouse mAb (Covance) prebound to protein A/G beads (Santa Cruz Biotechnology). The immunoprecipitates were washed three times with RIPA buffer and then eluted in 20 μL of sample buffer. The samples were electrophoresed on precast 10−20% Tris-HCl Chemicon gels for 2 h at 120 V, stained with Coomassie blue, and incubated in destain buffer (50% methanol, 10% acetic acid) until the antibody was clearly visible. The fixed gels were incubated in Amplify (Amersham Biosciences) for 20 min before being vacuum dried onto Whatman paper, then placed on a Phosphor screen (Amersham, Piscataway, NJ) for 24 h, and imaged on a Storm phosphoimager (Molecular Dynamics, Piscataway, NJ).

Semiquantitative rtPCR and Southern Blotting. Each cell line [ES +/+, ES−/−, QT6 (quail)] was grown to near confluency on 10 cm dishes, at which time the RNA was harvested using the RNeasy RNA purification kit (Qiagen). Total RNA was quantified by UV spectroscopy. The RNA from ES cells was diluted to 0.1 μg/μL and treated with amplification grade DNase I (Invitrogen) using the manufacturer's protocol. The DNase was then inactivated by adding EDTA to 2.5 mM and incubating at 65 °C for 15 min. This DNase-treated RNA was then used, following titration into RNA prepared from QT6 cells, to prepare cDNA using the Super Script First Strand Synthesis kit for rtPCR (Invitrogen). cDNA was prepared from 10 ng, 2 ng, 400 pg, 80 pg, 16 pg, or 3.2 pg of total ES cell, and the RNA was brought to 10 ng of total RNA with RNA prepared from QT6 cells. Twenty percent of the prepared cDNA (2 μL) was used to perform a 50 μL, 35 cycle PCR using Expand High Fidelity Polymerase (Roche). Ten microliters of this reaction was loaded on a 1% agarose gel with ethidium bromide and then transferred to a nylon membrane and Southern-blotted using a ^{32}P-end-labeled oligonucleotide designed to complement the expected PCR product. Fol-

lowing incubation and washing, the blot was exposed to a Phosphor screen (Amersham) for 24 h and imaged on a Storm phosphoimager (Molecular Dynamics).

Proteasome Inhibition. Twenty-four hours following transfection, ES PS −/− or HEK 293T cells were treated with concentrations of lactacystin ranging from 1.25 to 10 μM in complete media for 6 h, at which time the cells were washed in PBS and lysed in RIPA buffer for SDS−PAGE and Western blot analysis. To inhibit lysosomal degradation, HEK 293T were similarly treated with concentrations of brefeldin A (BFA) ranging from 1.25 to 10 μg/mL.

Transgenic Mice. Human PS1 harboring the D385A mutation was generated by site-directed mutagenesis of the wild-type cDNA (QuikChange site-directed mutagenesis kit; Stratagene, La Jolla, CA) and subsequently cloned into the mouse PrP.Xho expression vector (*34*) at the *Xho*I restriction site. DNA was linearized by digestion with *Not*I and microinjected into C57Bl/C3H mouse eggs (Transgenic and Chimeric Mouse Facility, University of Pennsylvania). Genomic DNA was isolated from mouse tails (PureGene genomic DNA purification kit; Gentra Systems, Minneapolis, MN), and founders were identified by Southern blot and PCR analysis. Three separate mouse lines demonstrating stable integration of the transgene were maintained. Following an overdose injection of ketamine (Abbott Laboratories, Chicago, IL) and xylazine (Vedco, Inc., St. Joseph, MO) and transcardial perfusion with PBS containing 1 unit/mL heparin sodium (American Pharmaceutical Partners, Inc., Schaumberg, IL), mouse brain cortices were dissected and solubilized to a final concentration of 150 μg/mL in RIPA buffer containing a protease inhibitor cocktail (1 μg/mL each of pepstatin A, leupeptin, TPCK, TLCK, soybean trypsin inhibitor, and 0.5 mM PMSF). Animal studies were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

RESULTS

Presenilin Modulates Pen-2 Levels. We and others have observed that Pen-2 is present at low levels in mouse embryonic stem (MES) cells lacking both PS1 or PS2 (*19*). In contrast, endogenous Pen-2 is readily detected in embryonic stem cells that are wild type for both PS1 and PS2 (Figure 1). Thus, endogenous Pen-2 is present at very low levels in the absence of PS1 and PS2 in comparison to steady-state levels in wild-type cells.

To determine if PS1 modulates the levels of other members of the γ-secretase complex, we transfected 293T cells with constructs driving the expression of V5 epitope-tagged Nct, Aph-1, Pen-2, or CD4, a T-cell marker irrelevant to the γ-secretase pathway. Appropriate cells were also transfected with plasmids expressing either GFP or PS1 in order to monitor the effects of PS1 overexpression. In addition, all cells were transfected with a plasmid expressing Au1 epitope-tagged DC-SIGN, an irrelevant type II transmembrane protein (*35*), to monitor transfection efficiency. The cells were lysed and aliquots analyzed by SDS−PAGE and Western blot with detection of the V5 epitope tag. We also monitored PS1, β-tubulin, and DC-SIGN-Au1 levels by Western blot to control for PS1 transfection, total protein, and transfection efficiency (Figure 2). We found that

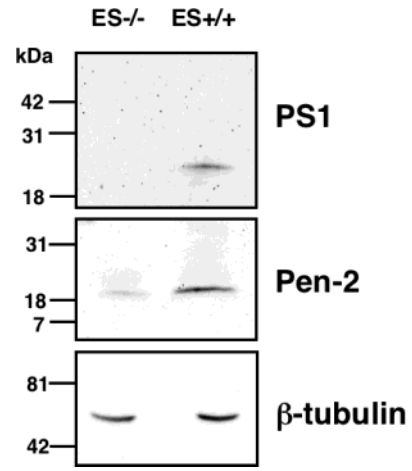

FIGURE 1: Endogenous Pen-2 levels are modulated by presenilin levels. Analysis of cell lysates from ES PS −/− and ES PS +/+ cells. Cell lysates were subjected to SDS−PAGE and Western blotting with anti-PS1 mAb, anti-Pen-2 pAb, or anti-β-tubulin mAb as indicated.

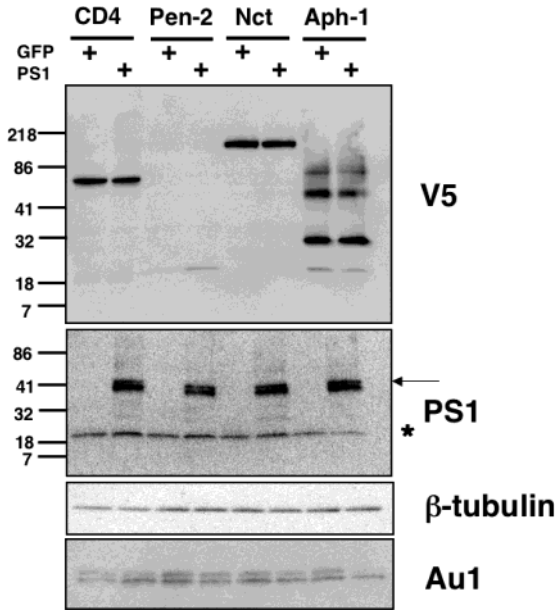

FIGURE 2: PS1 specifically increases steady-state levels of Pen-2. Human 293T cells were transfected with either V5-tagged CD4, Pen-2, Aph-1, or Nct together with Au1 epitope-tagged DC-SIGN as indicated. In addition, cells were transfected with either PS1 or GFP as shown at the top of the figure. Cell lysates were subjected to SDS−PAGE and Western blotting with anti-V5 mAb, anti-PS1 mAb, anti-β-tubulin mAb, or anti-Au1 mAb to detect DC-SIGN as shown. The arrow indicates the PS1 holoprotein, while the asterisk indicates the PS1 C-terminal fragment.

transfected Pen-2 levels were upregulated significantly in the presence of PS1 but not GFP. This effect of PS1 on Pen-2 was unique, as PS1 did not alter the levels of Nct, Aph-1, or CD4. Notably, because the four proteins examined contained identical C-terminal epitope tags, it was unlikely that this alteration accounted for the unique PS1-dependent upregulation. In addition, we, and others, have previously shown that the addition of a C-terminal epitope tag has no effect on the ability of Pen-2 to associate with other members of the γ-secretase complex (*3*, *18*, *19*, *26*, *32*). The epitope tag does, however, cause Pen-2 to migrate more slowly than endogenous Pen-2 in SDS−PAGE. It should be noted that overexpression of PS1 resulted in increased levels of PS1

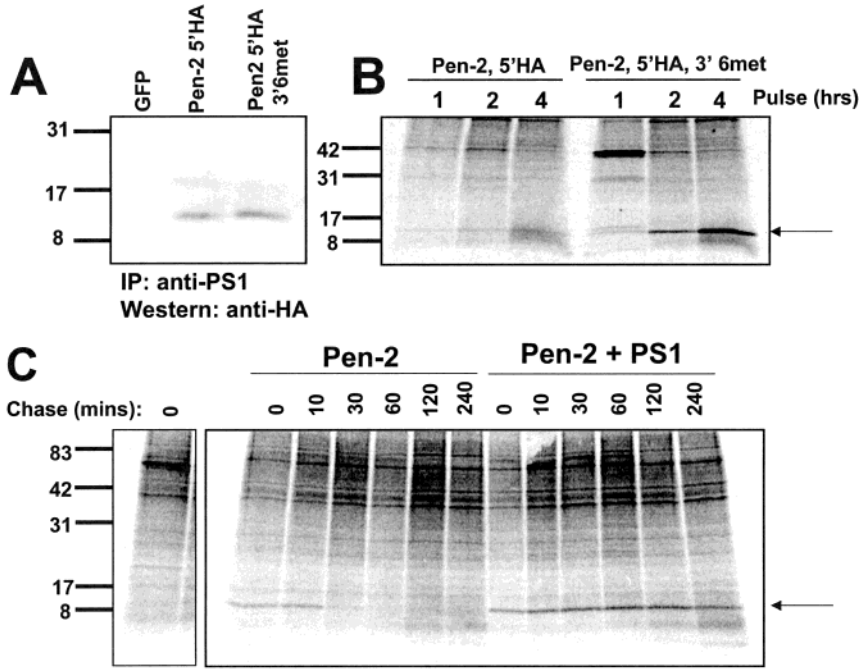

FIGURE 3: Presenilin extends the half-life of Pen-2 protein. (A) Analysis of the Pen-2 protein with an N-terminal HA epitope tag and a C-terminal 6-Met tag demonstrating that the alteration does not affect the interaction of Pen-2 with PS1. Lysates of 293T cells cotransfected with PS1 and either GFP, Pen-2 5′HA, or Pen-2 5′HA, 3′6Met as shown were immunoprecipitated with anti-PS1 mAb, subjected to SDS−PAGE, and immunoblotted with anti-HA antibody. (B) The 6-Met tag allows better detection of radiolabeled Pen-2. 293T cells transfected with PS1 and either Pen-2 5′HA or Pen-2 5′HA, 3′6Met were radiolabeled for 1, 2, or 4 h, then lysed, immunoprecipitated with anti-HA mAb, and subjected to SDS−PAGE. The gels were exposed to a phosphorimaging screen for 24 h. (C) PS1 extends the half-life of Pen-2. 293T cells transfected with Pen-2 5′HA, 3′6Met with or without the coexpression of PS1 were radiolabeled for 2 h and then chased for up to 240 min in complete media. Cells were transfected with GFP to control for the specificity of the identified band (left panel). Following immmunoprecipitation with anti-HA mAb the samples were subjected to SDS−PAGE and then exposed to a phosphorimaging screen for 24 h.

holoprotein and not of the N- and C-terminal fragments, perhaps because levels of Aph-1 and Nct were limiting. Thus, enhanced expression of PS1 holoprotein was sufficient to increase Pen-2 protein levels. Together, our results show that both endogenous and transiently expressed PS1 increase the levels of both endogenous and transiently expressed Pen-2.

Interactions with PS1 Stabilize Pen-2 Protein. The low levels of Pen-2 observed when PS1 expression was absent could result from reduced Pen-2 expression, enhanced Pen-2 degradation, or some combination of the two. To distinguish between these possibilities, we performed pulse−chase experiments to measure the half-life of Pen-2 in the presence of endogenous or overexpressed PS1. It was not possible to incorporate sufficient [^{35}S]methionine or [^{35}S]cysteine into Pen-2 due to the presence of only one methionine residue and one cysteine residue in this 101 amino acid long protein. Therefore, to increase the labeling efficiency of Pen-2, we introduced a 6-methionine tag to the C-terminus of N-terminally HA-tagged Pen-2. We reasoned that because epitope tags can be introduced at either the C- or N-termini of Pen-2 without affecting its ability to associate with γ-secretase components (*3*, *18*, *19*, *26*, *32*), this modification would not alter the interaction of Pen-2 with PS1. To confirm this, we cotransfected 293T cells with PS1 and either the Pen-2 5′HA, 3′6Met construct or the Pen-2 5′HA construct, lysed the cells in CHAPSO buffer, and immunoprecipitated with anti-PS1 antibody. The immunoprecipitates were then analyzed by Western blot with an antibody to the HA epitope tag in order to detect coimmunoprecipitated Pen-2. We found that the 6-methionine tag did not affect the association between Pen-2 and PS1 (Figure 3A). Furthermore, as predicted, the 6-methionine tag significantly improved the efficiency of Pen-2 metabolic labeling (Figure 3B). The Pen-2 5′HA, 3′6Met construct was therefore a useful tool to study the effect of PS1 levels on Pen-2 stability.

To monitor the effects of PS1 levels on the half-life of Pen-2, 293T cells were transfected with Pen-2 5′HA, 3′6Met with or without the addition of PS1. After 24 h, the cells were radiolabeled with [^{35}S]methionine and cysteine for 2 h and then chased for 0, 10, 30, 60, 120, or 240 min in complete media. Following lysis, immunoprecipitation with a monoclonal anti-HA antibody was performed under conditions that resulted in near quantitative recovery of Pen-2. In the absence of exogenous PS1, the transfected, epitope-tagged Pen-2 was rapidly degraded with a half-life of less than 30 min (Figure 3C). The addition of PS1 resulted in a dramatic extension in the half-life of Pen-2 such that nearly all the material present at time 0 was still present 4 h later. Thus, the Pen-2 protein was stabilized to a significant degree by the presence of PS1 protein.

Presenilin Levels Correlate with Endogenous Pen-2 Levels in Transgenic Mice. To determine if Pen-2 levels are affected by PS1 levels in a more physiologically relevant setting, we examined Pen-2 levels in brain lysates obtained from wild-type mice and from mice expressing PS1 D385A, a form of PS1 which lacks activity due to the ablation of the catalytic aspartic acid residue in the seventh transmembrane domain and tends to accumulate as holoprotein rather than being cleaved into N- and C-terminal fragments (*29*, *36*). Mice that overexpress PS1 D385A exhibit high levels of PS1 D385A holoprotein. Wild-type control mice and age-matched transgenic mice were sacrificed, and the cortex of the right brain

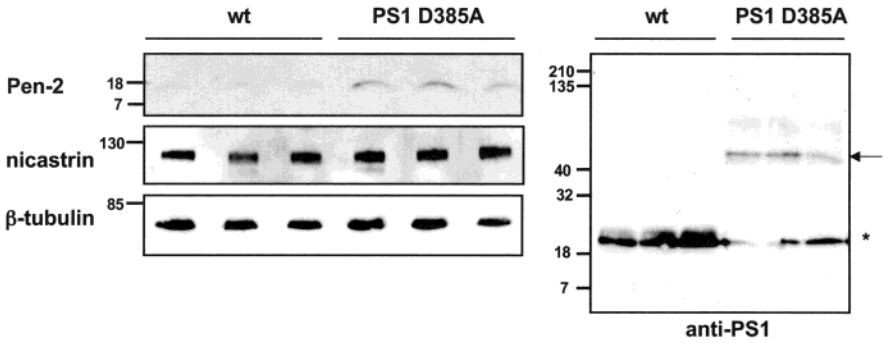

FIGURE 4: Mice overexpressing PS1 D385A accumulate endogenous Pen-2 in the brain cortex. Brain lysates from wild-type mice and mice overexpressing PS1 D385A were subjected to SDS−PAGE and Western blotting with anti-Pen-2 pAb, anti-Nct pAb, or anti-β-tubulin mAb as shown. In addition, levels of PS1 were detected by Western blot with anti-PS1 mAb (right panel). The asterisk indicates the presenilin C-terminal fragment, and the arrow indicates the PS1 holoprotein.

hemisphere of each mouse was solubilized in RIPA buffer. The samples were balanced for total protein and analyzed by SDS−PAGE and Western blot with antibodies to Pen-2, PS1, Nct, and β-tubulin. As shown in Figure 4, the D385A mice accumulated significantly more Pen-2 that the wild-type mice, though Nct levels were unchanged. β-Tubulin levels were also equivalent. A second line of mice overexpressing D385A was also analyzed and determined to accumulate endogenous Pen-2 as well (data not shown). Thus, increased levels of PS1 result in increased levels of Pen-2 both in vitro and in vivo. Furthermore, PS1 holoprotein levels appear to be sufficient to upregulate Pen-2 protein levels in vitro and in vivo since the D385A mice accumulated primarily PS1 holoprotein (Figure 4, right panel). This is similar to the observed effect of overexpressed PS1 in cell culture (Figure 2) in which changes in PS1 holoprotein levels were sufficient to upregulate Pen-2.

Pen-2 Transcription Is Unaltered by the Presence or Absence of PS1. Our results indicate that Pen-2 protein is rapidly degraded unless it interacts with PS1. To determine if Pen-2 transcription might be altered due to the genetic absence of PS and therefore the absence of γ-secretase-mediated transcriptional regulation, we examined Pen-2 mRNA levels in mouse ES cells derived from wild-type mouse embryos or from embryos that lack both PS1 and PS2. RNA was prepared from these two cell lines, and the amounts were normalized by spectroscopic analysis. Semi-quantitative rtPCR was then performed in order to compare the levels of Pen-2 RNA (Figure 5). The starting RNA template was serially diluted, and cDNA was prepared using a gene-specific primer. The cDNA was then used to perform a 35 cycle PCR using primers that flanked an exon so that amplification resulting from Pen-2 mRNA could be distinguished from products amplified from genomic DNA. The PCR product was then analyzed by both ethidium bromide staining (data not shown) and Southern blot with a ^{32}P-end-labeled hybridization probe designed to flank an excised intron. We found that the amount of Pen-2 mRNA was the same in wt ES cells and ES cells that lack PS1 and PS2. No PCR product was observed in the control lane that excluded reverse transcriptase in the cDNA synthesis. Because Pen-2 expression was not downregulated at the transcriptional level, even in the face of prolonged absence of PS1 and PS2, it is clear that the role of the γ-secretase as a regulator of transcriptional activity is not a contributor to the dramatic

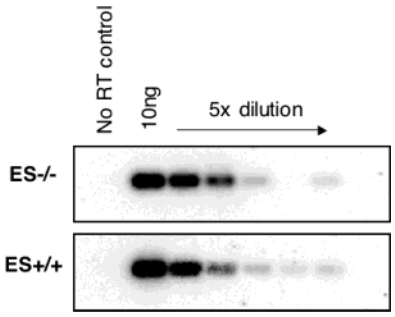

FIGURE 5: Pen-2 mRNA levels are equivalent in wild-type and PS1/PS2 double-knockout cells. Southern blot analysis of semi-quantitative PCR. cDNA template was prepared from serially titrated RNA prepared from either ES PS −/− or ES PS +/+ using a gene-specific primer. The prepared cDNA was used as a template in a 35-cycle PCR. This product was electrophoresed and detected with a ^{32}P-labeled oligonucleotide specific for product amplified from the expected cDNA sequence.

downregulation of Pen-2 protein levels observed in cells which lack PS1 and PS2.

Pen-2 Is Degraded by the Proteasome. Having demonstrated that Pen-2 protein is stabilized by the presence of PS1, we sought to understand the mechanism of Pen-2 degradation. Since Pen-2 is rapidly degraded in the absence of PS1, we thought it most likely that Pen-2 is degraded by the proteasome following reverse translocation from the ER, as this is the fate of other proteins that either fail to fold correctly or that fail to associate with their normal assembly partners (*37*, *38*). To test this hypothesis, we treated 293T cells transfected with V5 epitope-tagged Pen-2 with increasing concentrations of lactacystin, a specific proteasome inhibitor. We found that addition of lactacystin resulted in increased levels of epitope-tagged Pen-2 but no buildup of higher molecular weight species which might represent polyubiquitinated Pen-2 (Figure 6A). In contrast, treatment with brefeldin A (BFA), which blocks transport of proteins from the ER (*39*), had no effect on Pen-2 levels, suggesting that lysosomal degradation plays little or no role in modulating Pen-2 levels. These data are consistent with the hypothesis that PS1 stabilizes Pen-2 protein and that in the absence of PS1 Pen-2 is degraded via the proteasome. We reasoned that if this hypothesis were true, proteasome inhibition would have little or no effect on Pen-2 levels in cells in which PS1 was overexpressed. To test this hypothesis, we cotransfected 293T cells with Pen-2 and PS1 and treated the cells with increasing concentrations of lactacystin or BFA (Figure 6B).

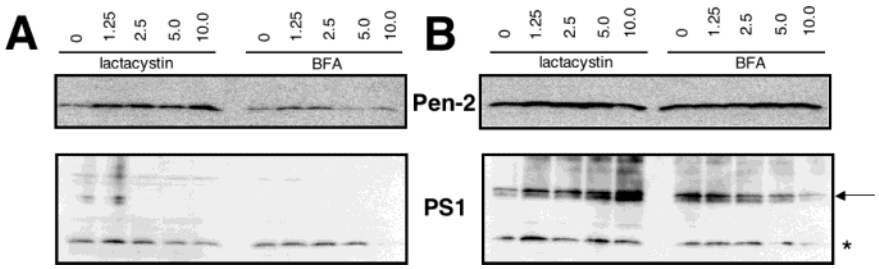

FIGURE 6: Pen-2 protein levels are increased by either proteasome inhibition or PS1 overexpression. 293T cells were transfected with either Pen-2-V5 (A) or Pen-2-V5 and PS1 (B). 24 h after transfection the cells were treated with either lactacystin (0, 1.25, 2.5, 5, or 10 μM) or BFA (0, 1.25, 2.5, 5, or 10 μg/μL) for 6 h. The cells were then lysed and subjected to SDS−PAGE and Western blot with anti-V5 mAb to detect Pen-2 or with anti-PS1 mAb to detect PS1 holoprotein and CTF. The asterisk indicates the presenilin C-terminal fragment, and the arrow indicates the PS1 holoprotein.

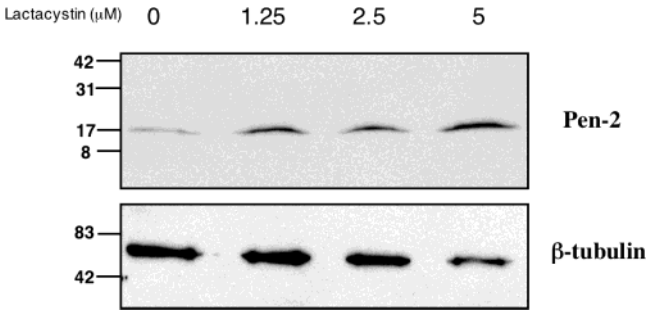

FIGURE 7: The effect of proteasome inhibition on Pen-2 is independent of presenilin levels. ES PS −/− cells transfected with Pen-2-V5 were treated, 24 h after transfection, with 0, 1.25, 2.5, or 5 μM lactacystin for 6 h, then lysed, and subjected to SDS−PAGE and Western blot with anti-V5 mAb or anti-β-tubulin mAb as indicated.

As we had seen earlier, the addition of PS1 resulted in dramatic upregulation of steady-state levels of Pen-2. However, in the presence of overexpressed PS1, the effect of lactacystin on steady-state levels of Pen-2 was ameliorated. Neither lactacystin nor BFA altered steady-state levels of Pen-2 in the presence of PS1 overexpression, suggesting that PS1 is capable of rescuing Pen-2 from proteasome-mediated degradation.

The effects of lactacystin on Pen-2 levels in 293T cells could result from inhibited degradation of PS1 or PS2 holoprotein, which is known to occur in cells treated with proteasome inhibitors (*40*−*43*). When PS1 was overexpressed in 293T cells, PS1 holoprotein accumulated with lactacystin treatment in a dose-dependent manner (Figure 6B). To eliminate the possibility that PS1 or PS2 accumulation might indirectly regulate the lactacystin-mediated increase in steady-state Pen-2 levels, we monitored the effects of proteasome inhibition on Pen-2 levels in ES cells deficient of both PS1 and PS2. To do so, ES PS −/− cells were transfected with V5 epitope-tagged Pen-2 and 24 h later were treated with increasing concentrations of lactacystin for 6 h. As was the case in 293T cells, ES PS −/− cells treated with lactacystin accumulated Pen-2 in a dose-dependent manner (Figure 7). The decrease in β-tubulin levels observed in the cells treated with 5 μM lactacystin is consistent with the change in morphology we regularly noted in ES cells due to lactacystin toxicity at this concentration. Higher doses could not be included as a consequence.

DISCUSSION

PS, Nct, Pen-2, and Aph-1 have each been shown to be required for γ-secretase activity (*1*, *44*, *45*). It is likely that together these four proteins constitute the complete complex as they are sufficient to reconstitute γ-secretase activity in *Saccharomyces cerevesiae*, which does not express known γ-secretase orthologues (*22*). Having identified what appears to be the complete γ-secretase, it now becomes important to understand the interactions between these proteins and the role each plays in regulating γ-secretase activity.

An important observation made soon after the discovery of PS was that both PS1 and PS2 are endoproteolytically cleaved into the C- and N-terminal fragments present in the active complex. However, overexpression of PS by itself results in the accumulation of holoprotein and little change in the terminal fragments, suggesting that PS endoproteolysis is limited by one or more cofactors (*46*). Given their necessity for γ-secretase function and PS endoproteolysis, it would appear that Pen-2, Aph-1, and Nct are the cofactors that regulate PS cleavage and enzymatic activity. In addition, the presence of multiple subunits, each necessary for function, provides additional opportunities for both transcriptional and posttranscriptional regulation of γ-secretase assembly and activity.

The precise roles that Pen-2, Aph-1, and Nct play in γ-secretase complex structure, activity, and regulation are not known. Data exist that support a role for Aph-1 as a stabilizer of the PS1 holoprotein while Pen-2 may serve to promote PS1 cleavage (*4*, *18*, *19*, *42*, *47*). The role of Nct is obscure, though it can directly interact with Aph-1 in the absence of PS1 (*27*, *28*) and therefore may represent the first step in complex assembly. PS, Nct, and Aph-1 all appear to exhibit relatively long half-lives and so once synthesized may be incorporated into functional γ-secretase complexes over a prolonged period of time depending on subunit availability. In contrast, Pen-2 protein expression is in some manner dependent upon PS. Our study investigated this relationship and suggests the Pen-2 may prove to be rate-limiting for complex assembly and may thus be a key regulatory component of this multisubunit enzyme.

To accomplish this, we investigated the relationship between Pen-2 protein levels and PS expression in several contexts, including the genetic absence of PS and transient overexpression of PS, as well as the constitutive overexpression of PS in vivo. In all cases, both endogenous and transiently expressed Pen-2 protein levels were highly and uniquely sensitive to PS levels. Interestingly, levels of Pen-2 mRNA were not impacted to an obvious extent even in the face of genetic ablation of the PS1 and PS2 genes and the resulting absence of γ-secretase-mediated transcriptional regulation. Rather, in the systems examined here the regula-

tion of Pen-2 by PS appeared to be entirely posttranslational. We found that newly synthesized Pen-2 is destined for rapid degradation by a proteasome-dependent mechanism and thus most likely involves reverse translocation of Pen-2 from the ER membrane to the cytosol.

The mechanism of Pen-2 degradation is proteasomal but may or may not depend on the addition of ubiquitin to Pen-2. Pen-2 contains one cytosolic and two luminal lysine residues, any of which could serve as conjugation sites for ubiquitin. However, we have seen no direct evidence that Pen-2 is ubiquitinated. Therefore, the proteasome-mediated degradation of Pen-2 may be polyubiquitin independent, as has been observed with several other proteins (*48*−*51*). Alternatively, the levels of ubiquitinated Pen-2 species may simply have been below the limit of detection in our assays. Importantly, the accumulation of PS that occurred as a result of proteasome inhibition was not responsible for the accumulation of Pen-2 that occurred under these conditions, since Pen-2 also accumulated in lactacystin-treated cells devoid of PS. While this suggests that Pen-2 is directly degraded by the proteasome, it does not eliminate the possibility that the accumulation results indirectly from lactacystin-mediated accumulation of another, as yet unidentified, cofactor responsible for stabilizing Pen-2. Since we have no direct evidence for such a cofactor, and because the downregulation of Pen-2 observed in the absence of PS was ameliorated by proteasome inhibition, we favor a model in which Pen-2 is directly degraded by the proteasome when it fails to bind PS.

In the presence of PS, Pen-2 is stabilized and exhibits a much longer half-life. Since we can routinely coimmunoprecipitate all or nearly all Pen-2 from cells by the use of antibodies to PS1 when PS1 is overexpressed, it appears that stabilization of Pen-2 by PS1 entails direct interactions between these molecules. Thus, the amount of Pen-2 protein is effectively titrated by its PS binding partner, most commonly PS1. To what extent, if any, Aph-1 or Nct play a role in this process is not yet clear. Finally, Pen-2 stabilization can be mediated by the PS holoprotein, since increased expression of PS1 D385A is capable of rescuing Pen-2 in vitro (*29*) and in vivo. This is consistent with Pen-2 playing a role in PS endoproteolysis and indicates that the ability of PS1 to rescue Pen-2 protein is separate from its function as an aspartyl protease.

The rapid turnover of Pen-2 in the absence of PS stands in marked contrast to Aph-1 and Nct, both of which are long-lived when overexpressed in PS-negative cells. It is not clear why Pen-2 is degraded so rapidly when it fails to associate with PS. Perhaps the most obvious possibility is that Pen-2 fails to fold correctly in the absence of PS and that the misfolded molecule is recognized as such and rapidly removed from the ER membrane for degradation, a common fate to many other integral membrane proteins that fail to fold correctly (*38*, *52*). Because of its rapid turnover, Pen-2 is an attractive candidate for the regulation of γ-secretase complex formation. Downregulation of Pen-2 transcription would lead almost immediately to the cessation of γ-secretase complex assembly, since preexisting Pen-2 which had yet to complex with PS would be rapidly degraded by the proteasome. In contrast, the other members of the γ-secretase complex are more long-lived and so have the potential to represent a pool of subunits that can assemble into functional complexes even hours after their initial synthesis.

The redirection of a labile subunit from proteasomal degradation through interaction with a binding partner is not without precedent. The retinoic acid related orphan receptor (RORα) is a member of the ROR subfamily which regulates physiologic processes ranging from thymopoesis to adipogenesis (*53*). It has been shown to be rapidly degraded by the proteasome unless if it binds to its corepressor, Hairless (*54*). Perhaps more analogous to the assembly of the quartet of γ-secretase proteins, the T cell antigen receptor is a heptamer containing six different proteins that, like the γ-secretase complex, assemble in the endoplasmic reticulum (ER) (*55*). In immature CD4 + CD8 + T cells, the expression of this receptor complex is severely limited by the α subunit, which has a half-life of only 15 min in immature thymocytes (*56*). In contrast, the α subunit has a half-life of greater than 75 min in mature T cells, resulting in far more efficient antigen receptor assembly. We propose that like the α subunit of the T cell antigen receptor, Pen-2 may limit complex assembly and be stable only upon association with its binding partner or partners.

It will be interesting to identify conditions that regulate not only Pen-2 expression but its half-life as well, as changes in either could be expected to alter γ-secretase complex assembly and, as a result, its activity. To do so efficiently, it will be necessary to identify the functional domains of PS and Pen-2 that mediate their interaction and stabilization. Another potential factor that could impact Pen-2 half-life is the dysregulation of the ubiquitin−proteasome system that has been described in AD as well as other neurodegenerative disorders (*57*). This could impair the ability of the cell to degrade Pen-2, perhaps resulting in the undesirable upregulation of γ-secretase activity with the subsequent generation of excessive Aβ.

In conclusion, we find that Pen-2 is a proteasome substrate uniquely regulated by PS at the posttranslational level, with Pen-2 levels being effectively titrated by PS protein. PS-dependent transcriptional regulation of Pen-2 was not evident under the conditions examined in our study. Given its highly labile nature and the potential Pen-2 has for regulating the efficiency of γ-secretase complex assembly, it will be of considerable interest to identify conditions that result in enhanced or reduced Pen-2 expression.

NOTE ADDED IN PROOF

While this study was in press, Bergman et al. (*58*) reported similar results demonstrating that Pen-2 is degraded by the proteasome in the absence of presenilin and that Pen-2 is a target for polyubiquitination.

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