Ubiquilin regulates presenilin endoproteolysis and modulates γ -secretase components, Pen-2 and nicastrin

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Mutations in presenilin proteins (PS1 and PS2) lead to early-onset Alzheimer's disease. PS proteins are endoproteolytically cleaved into two main fragments: the NTF (PS N-terminal fragment) and the CTF (PS C-terminal fragment). The two fragments are believed to constitute the core catalytic enzyme activity called γ -secretase, which is responsible for cleaving β -amyloid precursor protein to release A β . Thus, studying factors that modulate PS fragment levels could provide important information about γ -secretase. Previously, we demonstrated that the protein, ubiquilin-1, interacts both *in vivo* and *in vitro* with PS and that overexpression of ubiquilin-1 or -2 leads to increased accumulation of full-length PS proteins. Using wild-type HEK-293 cells (human embryonic kidney 293 cells) and PS-inducible cells, we now show that overexpression of either ubiquilin-1 or -2 decreases the PS NTF and CTF levels. Conversely, siRNA (small interfering RNA)-mediated knockdown of ubiquilin-1 and -2 proteins increased the PS NTF and CTF levels. We considered that ubiquilin might alter PS fragment accumulation by acting as a shuttle factor escorting PS fragments to the proteasome for

degradation. However, through proteasome inhibition studies, we show that this does not occur. Instead, our results suggest that ubiquilin regulates PS fragment production. We also examined whether other components of the γ -secretase complex are affected by ubiquilin expression. Interestingly, overexpression of ubiquilin resulted in a decrease in Pen-2 and nicastrin levels, two essential components of the γ -secretase complex. In contrast, knockdown of ubiquilin-1 and -2 protein expression by RNAi (RNA interference) increased Pen-2 and nicastrin levels. Finally, we show that inhibition of the proteasome results in decreased PS fragment production and that reversal of proteasome inhibition restores PS fragment production, suggesting that the proteasome may be involved in PS endoproteolysis. These studies implicate ubiquilin as an important factor in regulating PS biogenesis and metabolism.

Key words: Alzheimer's disease, presenilin, proteasome, γ -secretase, small interfering RNA, ubiquilin.

INTRODUCTION

The genes encoding presenilin (PS) proteins along with APPs (β -amyloid precursor proteins) are mutated in early-onset Alzheimer's disease (AD) and thus determining their function or dysfunction in normal and diseased states is important. PSs are believed to constitute the catalytic component of γ -secretase, the complex that is implicated in the intramembrane proteolysis of APP to release A β into the extracellular space [1–4]. At least three other components, namely nicastrin, Aph-1 and Pen-2, are required for formation of the γ -secretase complex [1–3,5–13]. Nicastrin is a type 1 transmembrane protein that exists in two forms, an immature unglycosylated form and a mature highly glycosylated form. Only the glycosylated form appears to interact with PS [14,15]. During γ -secretase assembly, nicastrin and Aph-1 form a subcomplex that is involved in stabilizing PS [6,11]. Interestingly, Aph-1 also stabilizes nicastrin. These observations have led to the proposal that Aph-1 functions as a general scaffold for the assembly of the γ -secretase complex [5,6,11,16]. On the other hand, Pen-2 is believed to be required for PS endoproteolysis (see below) because a reduction in Pen-2 levels leads to a build-up of FL (full-length) PS [13]. Conversely, when PS levels are down-regulated, Pen-2 levels decrease [17]. Apart from a role in regulating PS endoproteolysis, recent evidence suggests that Pen-2

might also be involved in stabilization of PS fragments in the γ -secretase complex [18]. Although the exact composition and function of the different γ -secretase components are still being characterized, it appears that PSs, nicastrin, Pen-2 and Aph-1 are necessary and sufficient to reconstitute γ -secretase activity, at least when expressed in yeast [1].

γ -Secretase is responsible for cleaving of not only APP but also many other substrates, including ErbB4, Notch, D- and E-cadherins, LRP (low-density-lipoprotein receptor protein), CD44 and syndecan-3, many of which play critical roles in development [19–21]. Thus identifying inhibitors of γ -secretase to prevent A β build-up in AD is likely to be a difficult target considering that any inhibitor would probably affect processing of the other proteins [3,22–25].

PSs are usually present in cells as either FL proteins or as two cleaved fragments, termed the NTF (PS N-terminal fragment) and the CTF (PS C-terminal fragment). The two PS fragments are derived by endoproteolytic cleavage of the FL protein at a site located within the large loop that spans transmembrane helices 6 and 7 of the protein by an unknown activity, called presenilinase [26–29]. PS endoproteolysis produces fragments with slightly different ends, suggesting that either one protease with processive or lax specificity is involved in PS endoproteolysis or that multiple proteases are involved [30–33]. Although cleaved,

Abbreviations used: AD, Alzheimer's disease; ALLN, *N*-acetyl-Leu-Leu-Nle-CHO; APP, β -amyloid precursor protein; PS, presenilin; CTF, PS C-terminal fragment; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; FL, full-length; GST, glutathione S-transferase; HEK-293 cells, human embryonic kidney 293 cells; NTF, PS N-terminal fragment; PonA, ponasterone A; RNAi, RNA interference; RT, reverse transcriptase; siRNA, small interfering RNA; SEC, siRNA expression cassette.

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NTF and CTF still associate with one another in a heterodimeric complex, and it is this complex that is believed to possess γ -secretase activity, because γ -secretase inhibitors bind specifically to the heterodimeric complex and not to FL PS protein [34,35]. Although there is some speculation that γ -secretase itself might be responsible for PS endoproteolysis, recent evidence suggests that the two activities are in fact separable [30]. Studies of the factor(s) responsible for PS endoproteolysis have revealed that pepstatin A, which is not a γ -secretase inhibitor, but an acidic protease inhibitor, is one of the most potent inhibitors of PS endoproteolysis known to date [36]. Others have reported the involvement of the proteasome in PS cleavage, which is an interesting proposal, considering recent reports that the proteasome has endoproteolytic activity indicating that it has functions other than protein degradation [21,31,37].

Because PS endoproteolysis is required for γ -secretase activity, an understanding of how PS fragments are produced and regulated is important. Normally, PS NTF and CTF are present in cells in roughly 1:1 stoichiometry [26–28]. The amount of PS fragments in cells is tightly regulated by an unknown mechanism, and any excess of the fragments is rapidly degraded [38,39]. Interestingly, fragments generated from either PS1 or PS2 do not interact with fragments of the other proteins, suggesting that the two PS proteins form independent complexes [38,40]. PS fragment production is developmentally regulated [41,42]. Complicating the issue even further is the fact that the production and stability of PS fragments are regulated by both phosphorylation and caspase cleavage [43–45]. Clearly, studies aimed at determining how PS fragments are produced and regulated could be important both in terms of an understanding of how γ -secretase activity is constituted as well as in efforts to design inhibitors to regulate γ -secretase in disease states, such as AD.

We reported previously on the identification and characterization of ubiquilin-1, a PS-interacting protein [46]. We demonstrated that ubiquilin binds PS and that the two proteins partially co-localize in cells. We further demonstrated that overexpression of ubiquilin-1 increases the synthesis of FL PS proteins and inhibits the degradation of ubiquitinated forms of PS proteins [46,47].

There are three ubiquilin genes in humans: ubiquilin-1, -2 and -3. Ubiquilin-1 is expressed in all the cells and tissues examined. Ubiquilin-2, which is 72% homologous with ubiquilin-1, has a more restricted expression pattern, whereas ubiquilin-3 is expressed only in the testis. Ubiquilin proteins contain an N-terminal ubiquitin-like domain, a central more variable domain containing many Asn-Pro repeats and a C-terminal ubiquitin-associated domain. Ubiquilin proteins are somehow linked to the ubiquitin–proteasome system of protein degradation because both the ubiquitin-like domain and the ubiquitin-associated domain of ubiquilin have been shown to bind the S5, a subunit of the proteasomal cap [48,49]. Further supporting its interaction with the proteasome, ubiquilin was found not only to interact with the E3 ubiquitin ligase of E6AP, but also to fractionate partially with the proteasome [50]. Intriguingly, Bertram et al. [51] recently reported that they had found a strong linkage of variants in the ubiquilin-1 gene, in independent families with late-onset AD. This study suggests that, besides APOE ϵ 4 (apolipoprotein E ϵ 4) allele, a known risk factor for late-onset AD, ubiquilin-1 could be another risk factor for late-onset AD.

In the present study, we report that ubiquilin regulates PS endoproteolysis. We demonstrate that overexpression of ubiquilin-1 or -2 reduces the levels of PS NTF and CTF levels in cells, most likely by preventing PS endoproteolysis. We found that the reduction in PS fragments from overexpression of ubiquilin correlates with a decrease in Pen-2 and nicastrin levels. In a converse strategy,

we knocked down ubiquilin levels in cells using RNAi (RNA interference) and found that the levels of PS NTF, PS CTF, nicastrin and Pen-2 levels all increased, suggesting that ubiquilin is capable of regulating the γ -secretase complex. Finally, we demonstrate through the use of proteasome inhibitors that the proteasome might be responsible for PS endoproteolysis.

EXPERIMENTAL

Cell culture

HeLa and HEK-293 (human embryonic kidney 293) cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FBS (fetal bovine serum). PS1- and PS2-inducible cell lines were generated by transfection of the pERV-expressing HEK-293 cell line (Stratagene, La Jolla, CA, U.S.A.) with FL human PS2 cDNA under the control of PonA (ponasterone A)-inducible cassette in plasmid pEGSH (Stratagene). Stable integration of the pEGSH plasmid was achieved by selection for hygromycin resistance. PS expression was induced by adding PonA (final concentration 10 μ M) to the medium. For proteasome inhibition studies, cultures were treated with 40 μ M MG132 (Calbiochem, San Diego, CA, U.S.A.) for indicated time periods. For protein inhibition studies with cycloheximide, two sets of HEK-293 stable cell lines were grown in 100 mM dishes, and one set was transfected with 20 μ g of FL ubiquilin-1 expression construct, and the other set was left untransfected. PonA was then added to half of the transfected and untransfected cultures and, 16 h later, cycloheximide (final concentration 100 μ M) was added to all the cultures. Protein lysates were prepared immediately after the addition of cycloheximide and at 1 h intervals thereafter, as indicated.

Cell staining and immunofluorescence microscopy

HeLa cells were plated on to glass coverslips, fixed with 100% (v/v) methanol at -80°C for 20 min and antibody-stained as described previously [52]. Primary antibodies used were: goat anti-PS2 N-terminus (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.); monoclonal anti-ubiquilin antibody (Zymed Laboratories, San Francisco, CA, U.S.A.), which reacts with ubiquilin-1 and -2 polypeptides; and rat anti-PS1 N-terminus (Chemicon International, Temecula, CA, U.S.A.). Secondary antibodies used were: fluorescein- and rhodamine-conjugated donkey anti-goat, anti-rat and anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.). Fluorescence staining of cells was visualized using an inverted Leica DM IRB microscope and images were captured using a Photometrics SenSys camera and merged using IPLab software.

Pulse–chase studies

Pulse–chase studies of PS2 proteins expressed in PS2-inducible cells were carried out by the method described previously [46]. Equal amounts of protein lysate of the pulse–chase cultures were used to immunoprecipitate PS2 proteins.

Proteasome studies

PS2-inducible cells were treated for 16 h with 10 μ M clasto-lactacystin β -lactone, 10 μ M ALLN (*N*-acetyl-Leu-Leu-Nle-CHO, where CHO stands for carbon, hydrogen, oxygen), 50 μ M epoxomicin, 40 μ M MG132 and 100 μ M MG262. Afterwards, lysates were collected and the fragment levels were analysed by SDS/PAGE and immunoblotting.

PS2-inducible cells were grown in 100 mM dishes. All the cultures were treated with either of the reversible proteasome inhibitors MG132 or MG262 [also called Z-Leu-Leu-Leu-B(OH)₂; Boston Biochem, Cambridge, MA, U.S.A.]. After 7 h, the drugs were washed off from half of the dishes by rinsing twice with warm 1× PBS and replacing with fresh DMEM supplemented with 10% FBS, thereby restoring proteasome activity. Also, at this point, cycloheximide (final concentration 100 μM) was added to all dishes to inhibit new protein synthesis. Lysates were then collected at this point, at 2 h intervals, thereafter, and PS fragment levels were analysed by SDS/PAGE and immunoblotting.

SDS/PAGE and immunoblotting

Preparation of protein lysates, SDS/PAGE and immunoblotting of proteins were described previously [46]. Primary antibodies used were: rabbit anti-PS2 loop and rabbit anti-PS2 N-terminus, both raised against GST (glutathione S-transferase)–PS2 fusion proteins [52]; rabbit anti-ubiquilin [46], which reacts with ubiquilin-1 and -2 polypeptides; rat anti-PS1 N-terminus (Chemicon International); rabbit anti-PS1 loop raised against GST–PS1 fusion proteins; goat anti-p27 and goat anti-actin (Santa Cruz Biotechnology); mouse monoclonal anti-tubulin (Sigma–Aldrich, St. Louis, MO, U.S.A.); rabbit anti-nicastrin (Abcam, Cambridge, MA, U.S.A.); rabbit anti-Pen-2 (Zymed Laboratories); and rabbit anti-Aph-1a (Covance, Berkeley, CA, U.S.A.).

RNAi knockdown of ubiquilin

siRNAs (small interfering RNAs) specific to either ubiquilin-1 (named ubiquilin-1-1 and ubiquilin-1-2) or ubiquilin-2 (named ubiquilin-2-1 and ubiquilin-2-2) or a nonsense sequence were synthesized by Dharmacon RNA Technologies (Lafayette, CO, U.S.A.) and were transfected into HeLa and HEK-293 cells at 10, 15 and 25 nM final concentration using Mirus TKO transfection reagent. siRNA target sequences are as follows: ubiquilin-1-1 (AAGACCCCGAAGGAAAAGGAG), ubiquilin-1-2 (AACUGGACAUCAGCAGUUUA), ubiquilin-2-1 (AACGCUUCAAAUCCCAAACCG), ubiquilin-2-2 (AAACCACGAGUCCUACAUCAG) and a nonsense sequence (AAATGAACGTGAATGCTCAA). Cell lysates were collected and analysed after 48 and 72 h. RT (reverse transcriptase)–PCR was conducted using the Cells-to-cDNA II kit (Ambion, Austin, TX, U.S.A.). Basically, HeLa cells were transfected with the siRNAs and, 48 h later, the RNA was isolated and reverse-transcribed. Next, the cDNA was PCR-amplified using primers specific for either ubiquilin-1 or -2, designed to generate a product of 200 bp. The PCR products were analysed by agarose gel electrophoresis.

Long-term ubiquilin knockdown in the PS-inducible cells was achieved using the Silencer Express kit (Ambion) to generate SECs (siRNA expression cassettes). The SECs were then cloned into Ambion's pSEC (plasmid that expresses SEC) vectors and ubiquilin protein decrease was analysed by immunoblotting. The sequence targeted by the ubiquilin-1 SEC was AACAAATGCAGAATCCTGATA and the sequence targeted by the ubiquilin-2 SEC was AATCATCAAAGTCACGGTGAA.

The effects of decrease in ubiquilin protein expression on the levels of endogenous PS fragments and γ -secretase components were studied by knocking down ubiquilin-1 and -2 individually, and simultaneously, in wild-type HEK-293 cells using the siRNAs, SMARTpools, generated by Dharmacon RNA Technologies. These SMARTpools combine four different siRNAs that are designed to achieve optimal knockdown of each ubiquilin protein. The SMARTpools were transfected into HEK-293 cells using DharmaFECT transfection reagent and, after 48 h, protein

lysates were prepared and analysed for γ -secretase components by immunoblotting.

Statistical analysis

Single-sample Student's *t* test of the data was accomplished using the NCSS (number cruncher statistical system) program (NCSS, Kaysville, UT, U.S.A.).

RESULTS

Overexpression of ubiquilin reduces PS fragment levels

Previous experiments revealed that overexpression of ubiquilin increased the synthesis of PS proteins and decreased the turnover of high molecular mass forms of the proteins, resulting in a net accumulation of FL forms of PS proteins [46]. However, the effects on PS fragments were not examined. To this end, stable HEK-293 cell lines that inducibly express PS1 or PS2 were generated and the effects of overexpression of ubiquilin proteins on PS fragment levels were studied. For these studies, cells were transiently transfected with ubiquilin-1 cDNA plasmids and then induced for PS expression using PonA. Cell lysates were collected the next day and analysed for the various forms of PS proteins by immunoblotting. Contrary to its effects on FL and high molecular mass PS proteins, overexpression of ubiquilin-1 decreased the levels of both the NTF and CTF for both PS1 and PS2, suggesting that ubiquilin either prevents PS endoproteolysis or enhances the degradation of the PS fragments (Figures 1A and 1B, compare lanes 1 and 3 or lanes 2 and 4). To confirm that the effect was not due to differences in antibody detection, different anti-PS2 antibodies were used that were specific to either the N-terminus or the C-terminal loop domain of the PS2 proteins, and similar results were observed (Figure 1B). Densitometric analysis was used to quantify the extent of decrease, which revealed a 30% reduction in PS1 fragments ($P < 0.01$ for PS1 NTF and $P < 0.10$ for PS1 CTF) and an 80% reduction in PS2 fragments ($P < 0.08$ for PS2 NTF and $P < 0.06$ for PS2 CTF), indicating that, although ubiquilin-1 acts on both PS1 and PS2, it exerts a stronger effect on PS2. We presume that the stronger effect produced on PS2 fragments could be a consequence of stronger interaction of ubiquilin with PS2 than with PS1 as determined by yeast two-hybrid studies [46]. To confirm that the reduction in PS fragments was not unique to the PS stable cell lines, we examined the effects of ubiquilin overexpression on endogenous PS fragments in wild-type HEK-293 cells. Similar to the effects seen with the stable PS-inducible cells, ubiquilin-1 overexpression reduced endogenous PS1 NTF levels in normal HEK-293 cells by approx. 40% ($P < 0.04$; Figure 1C). We repeated all of the experiments, overexpressing ubiquilin-2 instead of ubiquilin-1, and found similar results (results not shown). Because we considered it important to illustrate the effects of overexpression of ubiquilin-2 on endogenous PS fragment levels, results for this experiment are the only ones shown (Figure 1C). Like ubiquilin-1, overexpression of ubiquilin-2 reduced endogenous PS1 NTF levels by approx. 40% ($P < 0.001$).

To identify the possible intracellular site where ubiquilin interacts with PS proteins, we double stained a number of neuronal and non-neuronal cells for ubiquilin and PS proteins. Unfortunately, the neuronal cells were non-informative (results not shown), presumably due to the poor resolution and low abundance of the proteins in the cells. However, we obtained good staining of the endogenous PS and ubiquilin proteins in HeLa cells (see Figure 1D). Double-immunofluorescence microscopy revealed that the anti-ubiquilin staining (please note that the monoclonal

Figure 1 Differential modulation of FL and PS protein fragments by ubiquitin-1 in HEK-293 PS-inducible cell lines

(A) PS1 NTF and CTF levels were analysed in PS1 cell lines that were either left uninduced (lanes 1 and 3) or induced with PonA (lanes 2 and 4) and either left untransfected (lanes 1 and 2) or transfected with a ubiquitin-1 expression construct (UBQLN1; lanes 3 and 4). Equivalent amounts of protein lysates were separated by SDS/PAGE (10%, w/v, polyacrylamide) and immunoblotted with anti-PS1 NTF antibody and anti-PS1 loop antibody. The filter was then re-probed with anti-ubiquitin antibody to ensure overexpression of ubiquitin and with an anti-actin antibody to ensure equal protein loading. Note that overexpression of ubiquitin-1 decreases PS1 NTF and CTF levels (lanes 3 and 4). Densitometric analysis was used to quantify the extent of PS fragment reduction. Each experiment was repeated more than three times. (B) Same experiment as described in (A) except that PS2-inducible cells were used to analyse PS2 NTF and CTF levels. Like PS1 fragments, overexpression of ubiquitin-1 decreased PS2 fragment levels. The nature of the 40 kDa band is unknown; however, it appears to be a PS2-related fragment because its levels also decreased upon

Figure 2 Potential mechanisms by which ubiquitin decreases PS fragment levels

(A) FL PS is cleaved into its fragments. The PS fragments then interact with ubiquitin (UBQLN), then escorts the fragments to the proteasome for rapid degradation. (B) Ubiquitin interacts with FL PS, blocking access of the presenilinase responsible for endoproteolysis.

ubiquitin antibody recognizes both ubiquitin-1 and -2) co-localizes with that of PS1 and PS2, most notably in vesicular-like structures in the cells (Figure 1D, panels A–I). The specificity of these stainings was confirmed by lack of staining of the structures with the preimmune sera (results not shown). Moreover, some puncta containing ubiquitin did not stain with PS1 (white arrowhead in Figure 1D, panels A–C), and PS1 staining of the plasma membrane did not co-localize with ubiquitin (red arrow in Figure 1D, panels D–F). Although we do not know the identity of the structures in which PS and ubiquitin co-localized, they appear similar to the vesicular-like structures in which PS has been found [53–55].

Overexpression of ubiquitin inhibits PS endoproteolysis

The above results raised an important question: what is the mechanism by which ubiquitin overexpression decreases PS fragment levels? Does ubiquitin, through its ability to interact with PS polypeptides and proteasomal subunits, escort PS fragments to the proteasome to facilitate their rapid degradation (Figure 2A), or does it prevent production of the PS fragments by inhibiting endoproteolysis (Figure 2B). If ubiquitin acts to increase the rate of degradation of the PS fragments, then it can be expected that by treating cells with a proteasome inhibitor, like MG132, to block proteasome-dependent degradation, PS fragment levels would be restored to the same level as in untransfected lysates. Furthermore, a classical pulse–chase study would also demonstrate whether or not ubiquitin increased the rate of turnover of the PS fragments. On the other hand, if ubiquitin overexpression inhibits endoproteolysis, then cells overexpressing ubiquitin should have a slower rate of production of PS fragments when compared with untransfected cells.

To address whether ubiquitin was decreasing fragment levels by enhancing their turnover, the PS1- and PS2-inducible stable cell lines were either transfected with ubiquitin-1 or -2 expression plasmids or left untransfected, subsequently induced for PS expression with PonA, and on the next day treated with the proteasome inhibitor MG132. Immunoblot analysis of the cell lysates from these MG132-treated cells revealed that both PS1 and PS2 NTF and CTF levels remained reduced after ubiquitin-1 transfection compared with cells that were not transfected with ubiquitin-1 ($P < 0.08$ for PS1 NTF and $P < 0.10$ for PS2 NTF; Figures 3A and 3B, compare lanes 1 and 3 or lanes 2 and 4). Once again, the extent of reduction in PS fragments was greater in the PS2 stable cell lines compared with PS1 cell lines, which is consistent with our earlier findings. In fact, intriguingly, PS2 stable cell lines treated with MG132 had even lower levels of PS fragments compared with cells not treated with MG132, suggesting that the proteasome might be involved in PS endoproteolysis (see below). Again, the experiments were repeated with ubiquitin-2 and similar trends were observed (results not shown), implying that ubiquitin-2, like ubiquitin-1, exerts similar effects on PS endoproteolysis.

Although these results indicated that overexpression of ubiquitin-1 does not enhance PS fragment degradation, it was important to show this through a different method. Hence a classical [35 S]methionine labelling pulse–chase experiment was performed comparing PS fragment turnover in cells in which ubiquitin-1 was overexpressed with cells in which it was not overexpressed. Using this method, we found that there was no significant difference in the turnover of PS2 NTF after a 35 h chase regardless of whether ubiquitin was overexpressed or not (Figure 3C). When taken together with the proteasome inhibitor studies, these results confirm that ubiquitin overexpression does not enhance fragment degradation, but instead could function to block PS endoproteolysis.

To investigate the possibility that ubiquitin overexpression might block PS endoproteolysis, we performed an experiment similar in concept to a pulse–chase study, except that we measured protein production rather than turnover. To this end, protein synthesis was inhibited using cycloheximide, and PS fragment production was monitored over a 6 h time period in cells that were either left untransfected or transfected with a ubiquitin-1 expression construct and then the cells were either induced or not induced for PS expression. Immunoblotting of equal amounts of protein lysate prepared from these cycloheximide-treated cells revealed that ubiquitin-1 was indeed overexpressed by approx. 300 % in the transfected cells, as expected, and an actin immunoblot confirmed equal protein loading (Figures 4A and 4B). Interestingly, cells overexpressing ubiquitin-1 had 20–40 % lower levels of PS2 NTF compared with untransfected cells. Densitometric analysis of PS2 fragment accumulation over time in PS2 induced and uninduced cells treated with cycloheximide indicated that ubiquitin-1 overexpression slows down the rate of PS2 NTF production (Figure 4C). Similar results were obtained with ubiquitin-2 (results not shown). Together, these results are consistent with the idea that ubiquitin-1 and -2 inhibit PS endoproteolysis by a similar mechanism.

ubiquitin overexpression. (C) Endogenous PS fragment levels were analysed in HEK-293 cells that were transfected with increasing amounts of ubiquitin-1 or -2 expression plasmids. Equivalent amounts of protein lysates were separated by SDS/PAGE (10 % polyacrylamide) and immunoblotted with an anti-PS1 NTF antibody. The filter was then reprobbed with anti-ubiquitin antibody to ensure overexpression of ubiquitin and with an anti-actin antibody to ensure equal protein loading. Note that FL PS1 is not visible in these cells. Similar to PS2, overexpression of ubiquitin decreased PS1 NTF levels and the extent of reduction was quantified again by densitometric analysis. The graph shows the quantification of PS1 fragments in cells that were transfected with 8 μ g of ubiquitin expression plasmid compared with untransfected (UT) cells. (D) Endogenous ubiquitin and PS proteins co-localize in vesicular-like structures in HeLa cells. HeLa cells were double stained with rat monoclonal anti-PS1 antibody (A and D) and with a rabbit anti-ubiquitin N-terminal antibody (B and E) or with a goat anti-PS2 antibody (G) and a mouse anti-ubiquitin antibody (H). A merged image of the two primary images is shown to the right of each set. Arrows indicate puncta of unknown origin in which PS and ubiquitin staining was co-localized. Scale bar, 5 μ m (D, A–H).

Figure 3 Overexpression of ubiquitin does not facilitate rapid degradation of PS fragments

(A) PS1 cell lines that were either left uninduced (lanes 1 and 3) or induced with PonA (lanes 2 and 4) and either left untransfected (lanes 1 and 2) or transfected with ubiquitin-1 expression plasmids (lanes 3 and 4) were treated for 7 h with MG132 after which protein lysates were collected and analysed by immunoblotting. Even after MG132 treatment, PS1 NTF and CTF levels remain reduced in transfected cells (compare lanes 3 and 1 or lanes 4 and 2), indicating that ubiquitin does not decrease PS fragment levels by increasing the rate of proteasome-dependent degradation of the fragments. For clarification, if overexpression of ubiquitin was to increase PS fragment degradation via the proteasome, then, on proteasome inhibition, PS fragment turnover would be blocked and PS fragment levels would be similar, or increase, relative to that of untransfected cells. However, this was not the case, suggesting that ubiquitin does not increase PS fragment turnover. Densitometric analysis was used to quantify the extent of reduction. Each experiment was repeated more than three times and similar trends were observed. (B) Same experiment as described in (A) except that PS2-inducible cell lines were used to examine PS2 NTF and CTF levels. (C) Ubiquitin-1 overexpression does not alter the turnover rate of PS2 NTF in PS2-inducible cells. Classical [35 S]methionine pulse-chase study of immunoprecipitated PS2 NTF in cells in which ubiquitin was overexpressed compared with untransfected cells. The rate of PS2 NTF turnover was calculated to be the same irrespective of ubiquitin-1 overexpression.

RNAi-mediated reduction of ubiquitin proteins increase PS fragment levels

All of the initial findings were based on experiments in which we had overexpressed ubiquitin. The next logical step was to

determine if reducing ubiquitin protein levels has the opposite effect of overexpression, namely lowering the ubiquitin levels should increase PS fragments in cells. Four siRNAs were generated, two that were specific for ubiquitin-1 (termed ubiquitin-1-1 and ubiquitin-1-2) and two that were specific for ubiquitin-2

Figure 4 Overexpression of ubiquilin reduces PS fragment production

(A) A time-course experiment using ponA-induced PS2 cell line cultures that were transfected with or without ubiquilin-1 (UBQLN) expression plasmids and treated with 100 μ M cycloheximide (CHX) for 0–6 h. Equal amounts of protein lysate were separated by SDS/PAGE (8.5% polyacrylamide) and subsequently immunoblotted with anti-ubiquilin antibody. These blots illustrated that the transfected cells contained a 300% increase in ubiquilin-1 levels. Next, the protein lysates were immunoblotted using anti-PS2 N-terminal antibody to detect FL and NTF PS2 levels, as indicated. Note the differences in FL PS turnover and NTF production levels in ubiquilin-1-transfected versus untransfected cell lysates. NTF levels of PS2 were significantly reduced in ubiquilin-1-transfected cells and the rate of NTF production was significantly reduced. (B) Same experiment as in (A) except that PS expression was not induced using PonA. Again, overexpression of ubiquilin not only decreased PS2 NTF levels, but also slowed the rate of PS2 NTF production. (C) Relative levels of PS2 polypeptides in PS2-inducible cell lysates after densitometric analysis of the immunoreactive bands shown in (A, B). $\blacktriangle$, UBQLN1-transfected; $\blacksquare$, untransfected. The levels were calculated by normalization of the signals relative to that in lane 1. The Microsoft Excel program was used to produce a best-fit line for each graph. Please note that overexpression of ubiquilin-1 increased FL PS2 levels dramatically. However, this is completely opposite to its effects on PS2 NTF levels. Ubiquilin not only decreased PS2 NTF levels at every time point in the experiment, but it also slowed down the rate of NTF production, as determined by measuring the slope of each line. Similar trends were observed for the CTF (results not shown).

(termed ubiquilin-2-1 and ubiquilin-2-2). The siRNAs were transfected in increasing concentrations into HeLa cells and cell lysates were collected after 48 h, separated by SDS/PAGE, and levels of different proteins were analysed by immunoblotting. Two of the four ubiquilin siRNAs, ubiquilin-1-2 and ubiquilin-2-1, were successful at reducing ubiquilin protein levels as determined by immunoblot analysis (Figure 5A and results not shown). Ubiquilin-1 protein levels were reduced by 72%, whereas ubiquilin-2 protein levels were reduced by 46%. We confirmed that the ubiquilin siRNAs were functioning through the classical RNAi mechanism as revealed by a reduction in the mRNA of the proteins by RT-PCR analysis (results not shown). Having established that ubiquilin-1-2 and ubiquilin-2-1 siRNAs were capable of knocking down ubiquilin-1 and -2 proteins respectively, we transfected the PS2-inducible stable cell line grown in the absence of PonA with increasing amounts of the siRNAs and examined the amount of PS2 CTF that accumulated in the cells by immunoblotting. As shown in Figure 5(A), we observed a dose-dependent increase in PS2 CTF levels with increase in the amount of siRNA transfected, consistent with the idea that a reduction in ubiquilin levels results in increased PS fragment production.

To ensure that the effect produced on PS fragments by the siRNA procedure was not specific to this particular method of ubiquilin knockdown, we utilized two other methods to knock down ubiquilin expression in cells: transfection of plasmids that interfere with ubiquilin RNA expression (pSECs) and transfection of a pool of siRNAs (SMARTpools, see the Experimental section) that combine four target sequences directed against ubiquilin-1 or -2 mRNAs. We found that the RNAi plasmids were more suitable for longer term knockdown of ubiquilin, whereas the SMARTpool siRNAs produced quicker and more notable reduction (>90%) of ubiquilin protein levels compared with transfection of the individual siRNAs. Representative examples of the successful knockdown of ubiquilin-1 and -2 proteins upon transfection of pSEC RNAi plasmids in PS2-inducible HEK-293 cells and transfection of SMARTpools of ubiquilin-1 or -2 siRNAs (see the Experimental section) in wild-type HEK-293 cells are shown in Figures 5(B) and 5(C) respectively. The Figures also show the corresponding blots and histograms of the amounts of PS1 and PS2 NTF fragments in equal amounts of protein in the different transfected cells compared with nonsense or mock transfected cells. By this analysis, we found that ubiquilin-1

Figure 5 RNAi knockdown of ubiquitin protein expression results in increased PS fragment accumulation

(A) The PS2-inducible cell line was transfected with increasing amounts of two siRNA duplexes, ubiquitin-1 and -2, which we found were effective in knockdown expression of ubiquitin-1 and -2 proteins respectively. Equal amounts of protein lysates prepared either from untransfected cells (UT) or from the siRNA-transfected cells were immunoblotted for PS2 CTF, actin and ubiquitin proteins. The graph shows that the two siRNA duplexes induced a dose-dependent increase in PS2 CTF levels. (B) PS fragment levels in PS2-inducible cells transfected with plasmids expressing a nonsense sequence or two RNAi sequences (pSEC) directed against ubiquitin-1 and -2. After 48 h of transfection, protein lysates were collected from mock and pSEC transfected cells, and equal amounts of protein were immunoblotted for PS1 NTF, PS2 NTF, actin and ubiquitin proteins. The graph shows the changes in PS1 and PS2 NTF levels relative to that found in mock (control) transfected cells. (C) Normal HEK-293 cells were transfected either with 30 nM final concentration of a nonsense or individual SMARTpool siRNAs directed against either ubiquitin-1 or -2 proteins or with a mixture of the two SMARTpools against ubiquitin-1 and -2. Cell lysates were collected after 48 h and equal amounts of the proteins were immunoblotted for PS1 NTF, PS NTF, ubiquitin and actin proteins. The graphs show the quantification of PS1 and PS2 NTF levels after transfection of the ubiquitin-specific siRNAs normalized to the levels found in nonsense siRNA-transfected (control) cells.

reduction by siRNA transfection in the PS2 stable cell line resulted in a 60 and 30 % increase in PS1 and PS2 NTF levels respectively, whereas knockdown of ubiquitin-2 increased the two fragments by 30 and 10 % respectively. We presume that, in these experiments, knockdown of ubiquitin-2 protein increased PS fragments to a lesser extent than knockdown of ubiquitin-1 protein because ubiquitin-2 is expressed almost 5-fold lower than ubiquitin-1 and thus any knockdown of ubiquitin-2 would reduce the total pool of ubiquitin (i.e. ubiquitin-1 and -2) in cells only slightly compared with knockdown of ubiquitin-1 protein.

The siRNAs transfections of wild-type HEK-293 cells revealed how a change in ubiquitin proteins affects endogenous PS fragments. In these experiments, we found that knockdown of ubiquitin-1 increased PS1 and PS2 NTF levels by approx. 40 and

25 % respectively ($P < 0.05$), whereas knockdown of ubiquitin-2 increased the two fragments by approx. 20 and 22 % respectively. Interestingly, simultaneous knockdown of ubiquitin-1 and -2 proteins resulted in an even higher increase in PS2 NTF levels (~40 % increase; $P < 0.09$ for PS2 NTF), which is consistent with the notion that regulation of PS fragment accumulation by ubiquitin is sensitive to the total pool of ubiquitin rather than specific to an individual ubiquitin protein. However, for some unknown reason, the increase in PS1 NTF in cells in which ubiquitin-1 and -2 proteins were simultaneously knocked down was intermediate to the levels seen upon knockdown of ubiquitin-1 or -2 alone. Nevertheless, the increases in PS fragment levels upon ubiquitin knockdown were easily reproducible: similar trends were observed in each of six independent experiments. Although

Figure 6 Modulation of Pen-2 and nicastrin levels by ubiquilin

(A) Cultures of the PS1-inducible stable cell line were either left uninduced (lanes 1 and 3) or were induced for PS1 expression with PonA (lanes 2 and 4) and were either left untransfected (lanes 1 and 2) or were transfected with ubiquilin-1 cDNA (lanes 3 and 4). After 24 h of transfection, the cells were lysed and equal amounts of the protein lysates were then immunoblotted for nicastrin, Aph-1a, Pen-2 and actin proteins. A parallel immunoblot of the lysates confirmed that the FL and NTF forms of PS1 changed in the expected manner (PS FL increased and NTF decreased) upon ubiquilin-1 overexpression. (B) The graph shows the normalized levels of Pen-2 in the different cells relative to that of uninduced and untransfected cells after densitometric analysis of the bands of immunoblots shown in (A). The graph also shows the results obtained after a similar experiment in which we used ubiquilin-2 for transfection, instead of ubiquilin-1. Each experiment was repeated more than three times, with similar results. (C) Normal HEK-293 cells were transfected either with 30 nM final concentration of either a nonsense or individual SMARTpool siRNAs directed against either ubiquilin-1 or -2 proteins or with a mixture of the two SMARTpools against ubiquilin-1 and -2. Cell lysates were collected after 48 h and equal amounts of the proteins were immunoblotted for nicastrin, Pen-2, ubiquilin and actin proteins. The graphs show the quantification of nicastrin and Pen-2 protein levels after transfection of the ubiquilin-specific siRNAs normalized to the levels found in nonsense siRNA-transfected (control) cells.

some of these increases appear relatively small, they are likely to be important because levels of endogenous PS fragments are typically invariant and are usually very tightly regulated [38,39].

Reports have suggested that when PS1 fragment levels increase, there is a concomitant decrease in PS2 fragment levels and vice versa, a phenomenon called 'replacement' [26,27]. However, there is debate whether 'replacement' is a general phenomenon as it is not always observed [42]. We therefore examined whether PS1 fragment levels were altered in the same siRNA transfected lysates in which an increase in PS2 fragment levels was observed. Immunoblot analysis indicated that, in fact, levels of both PS1 and PS2 NTF levels increased upon reduction of ubiquilin levels, suggesting that ubiquilin regulates PS1 and PS2 proteins in a similar manner and that this regulation is upstream of the effectors that are responsible for the 'replacement' effect (Figures 5B and 5C).

Ubiquilin modulates components of the γ -secretase complex

Because PS fragments are associated with γ -secretase activity, we examined whether an alteration in PS fragments by ubiquilin affects other components of γ -secretase, namely Aph-1, nicastrin and Pen-2. To this end, PS-inducible cells were transiently transfected with ubiquilin expression plasmids and then induced

for FL PS expression using PonA. Cell lysates were collected the next day and equal amounts of proteins were immunoblotted for the three other γ -secretase components (Figure 6). Aph-1 levels have been demonstrated to be the least affected by either overexpression or knockdown of the other γ -secretase components [3]. Interestingly in our experiments too, Aph-1 levels remain steady irrespective of whether ubiquilin was overexpressed or not (Figure 6A). However, levels of mature nicastrin decreased upon overexpression of ubiquilin-1 (Figure 6A), which is consistent with previous observations, indicating that loss of PS expression correlates with decreased nicastrin expression [56]. Similarly, overexpression of ubiquilin-1 modulated Pen-2 levels in a manner that was directly related to the level of expression of PS fragments in the cell lysates. Thus, when PS1 fragments increased after induction of PS expression by PonA, Pen-2 levels increased concomitantly by approx. 60 % ($P < 0.05$ for Pen-2; Figure 6A, compare lanes 1 and 2). In contrast, overexpression of ubiquilin-1, which was associated with reducing PS levels, resulted in parallel decreases in Pen-2 levels by 30 % ($P < 0.01$ for Pen-2) in the uninduced cell lysates and by 100 % ($P < 0.08$ for Pen-2) in the induced lysates respectively (Figures 6A and 6B). Taken together, these results indicate that overexpression of ubiquilin is associated with a reduction in the levels of three of the essential γ -secretase components (PS fragments, Pen-2 and nicastrin) in cells.

Figure 7 Implication of the proteasome in PS2 endoproteolysis

(A) PS1- and PS2-inducible cells were treated with various classes of proteasome inhibitors for 16 h. Afterwards, cell lysates were collected, separated by SDS/PAGE, and immunoblotted for PS1 and actin proteins. The graphs show that PS1 NTF levels and PS2 NTF levels were reduced by treatment of the cells with all five proteasome inhibitors, albeit to varying extents. FL PS levels are extremely low in these cells and hence are not observable in these immunoblots. (B) PS2-inducible cell cultures were all treated continuously with either MG132 or MG262 and, after 7 h of incubation, the drugs were washed away from half the cultures. Immediately after washing, cycloheximide was added to all the cultures to inhibit new protein synthesis. Lysates were then collected at 2 h time points thereafter and PS fragment levels were analysed by immunoblotting. A significant increase is seen in PS2 NTF levels when the proteasomes inhibition is relieved, suggesting that the proteasome may be involved in PS endoproteolysis (top panel, right). Similar trends were observed for MG262 treatment (second panel, right). Please note that because PS2 expression was not induced with PonA, the FL protein is not detectable in these immunoblots. The filter was reprobed with anti-p27 antibody to monitor recovery of proteasome activity. Please note that p27 has a half-life of approx. 2 h. Therefore once proteasome inhibition is relieved, the level of p27 begins to decrease (third panel, right), whereas, under constant proteasome inhibition, its levels remain steady (third panel, left), serving as an important control. Finally, the filter was reprobed for actin to demonstrate equal protein loading. (C) Densitometric analysis of the immunoblots shown in (B) demonstrating the changes in PS2 NTF levels under conditions of constant proteasome inhibition and after removal of proteasome inhibition. Note that, at zero time, the NTF level in the sample in which MG132 was washed off is equal to that of the MG132-treated sample and that NTF levels quickly rise in the later time points of the former cultures, as proteasome activity recovers, whereas PS2 NTF levels stay steady in the cultures treated continuously with MG132.

We next examined whether a reduction in ubiquitin levels would lead to an increase in the γ -secretase components. Indeed, when ubiquitin levels were knocked down in wild-type HEK-293 cells using SMARTpool siRNAs, we observed a 50% increase in nicastrin levels ($P < 0.05$ for nicastrin; Figure 6C). There was a smaller, but reproducible (seen in six independent experiments), effect of ubiquitin knockdown on Pen-2 levels, with an approx. 20% increase when both ubiquitin-1 and -2 protein levels were reduced by siRNA transfection ($P < 0.05$ for Pen-2; Figure 6C). Together, these results indicate that ubiquitin is not only able to regulate fragment levels but also the entire γ -secretase complex.

Implication of the proteasome in PS2 endoproteolysis

During the course of these studies, we made an interesting observation: cells treated with the proteasome inhibitor MG132 dis-

played reduced PS fragment levels compared with MG132 untreated cells (e.g. compare the reduction in Figures 1B and 3B). We considered two possibilities to account for this effect: either MG132 treatment might inhibit the proteasome, which is responsible for PS endoproteolysis, or MG132 inhibits another factor involved in cleaving PS.

To rule out the possibility that MG132 acts non-specifically to inhibit another factor involved in PS cleavage, PS1- and PS2-inducible cells were incubated for 16 h with five different proteasome inhibitors (clasto-lactacystin β -lactone, ALLN, epoxomicin, MG132 and MG262) and then protein lysates were prepared and equal amounts of protein were probed for PS1 or PS2 NTF by immunoblotting. As shown in Figure 7(A), treatment of cells with all five proteasome inhibitors reduced PS1 and PS2 NTF levels. While each inhibitor had different effects on the extent of reduction, PS1 NTF levels were reduced by an average of 30% ($P < 0.06$ for clasto-lactacystin β -lactone, epoxomicin and

MG132) and PS2 NTF levels were reduced by an average of 40 % ($P < 0.09$ for clasto-lactacystin β -lactone, ALLN, epoxomicin, MG132 and MG262). We consider that the reduction in PS fragments caused by the proteasome inhibitors to be significant, because degradation of the PS fragments by the proteasome has been blocked and because PS fragment levels are normally tightly regulated. In fact, statistical analysis revealed that the reduction in PS NTF by the proteasome inhibitors was significant. Furthermore, our results showing that all five proteasome inhibitors reduce PS fragments lead us to speculate that the proteasome might be involved, either directly or indirectly, in PS endoproteolysis.

To address directly whether the proteasome is responsible for endoproteolysis of PS2, another time-course experiment utilizing proteasome inhibitors was devised. MG132 reversibly inhibits the proteasome providing a method by which the proteasome is first inhibited, then the inhibition is removed, allowing us to observe whether there is an increase in PS fragment production over time. An increase in PS fragment levels after removal of the proteasome inhibitor would suggest that proteasomes are responsible for endoproteolysis. If, by this treatment, no change in PS fragment levels were observed, it would suggest that the proteasome is not involved and instead would imply that PS fragments had already reached saturation.

Thus we devised an experiment in which all cultures of the PS2 stable cell line were treated with MG132 for 7 h, after which MG132 was washed off from half of the cultures and replaced by a fresh medium. Cycloheximide was added to all the cultures at the time MG132 was removed, to inhibit protein translation, and then lysates were collected from those cultures containing MG132 and in which the drug was washed away at 2 h intervals, for 12 h. The protein lysates were then immunoblotted for PS2 proteins. In the cells that were incubated in MG132 for the continuous duration of the experiment, PS2 NTF levels remained relatively constant (Figure 7B, top panel, left). In contrast, cells in which MG132 was washed off displayed increasing accumulation of PS2 NTF with time (Figure 7B, top panel, right). A comparison of certain time points illustrates this difference more clearly (Figure 7C). For example, at 0 h, the NTF levels were approximately the same in the MG132-treated samples versus the samples that were treated and then washed free of inhibitor (compare lanes 1 and 8). Yet, 12 h later, NTF levels rose 80 % in the cells that had been washed free of MG132 (compare lanes 7 and 14), while they remained low and steady in the MG132-treated samples (compare lanes 1 and 7). A similar trend was observed for the PS2 CTF (results not shown). We confirmed that cultures in which MG132 was washed off were capable of recovering proteasome activity by the fact that the cell-cycle protein, p27, remained relatively constant in cultures maintained in MG132, whereas its levels decreased over time after MG132 had been washed off. An anti-actin immunoblot also confirmed that equal amounts of protein were loaded.

Because MG132 has been shown to also inhibit cathepsins and calpains, the experiment was repeated using a more potent and specific proteasome inhibitor, MG262 (Figure 7B, second panel). Again, similar trends were observed, namely that as the proteasomes recover activity after removal of MG262, there is a notable increase in PS2 fragments. These results suggest that the proteasome might function as the 'presenilinase' and at the very least it is somehow involved in the generation of PS NTF and CTF.

DISCUSSION

PS endoproteolysis is required for the maturation of PS proteins from the unstable FL form of the protein to yield the two

stable NTF and CTF fragments, the latter of which constitutes the catalytic core of γ -secretase [27]. In the present study, we demonstrate that overexpression of ubiquilin, a PS interactor, decreases PS NTF and CTF levels, most likely by blocking access to the presenilinase that is responsible for PS endoproteolysis. We further demonstrate that a reduction in ubiquilin protein levels by RNAi leads to increased PS fragment production, supporting ubiquilin's role in regulating PS endoproteolysis. We also demonstrate that overexpression of ubiquilin decreases the levels of both nicastrin and Pen-2, two essential components of the γ -secretase complex, whereas knockdown of ubiquilin protein resulted in an increase in these γ -secretase components. Finally, we implicate the proteasome in PS endoproteolysis providing preliminary evidence that it might function as the presenilinase.

Our results demonstrated that overexpression of ubiquilin induces a greater reduction in PS2 fragments compared with PS1 fragments. One hypothesis in favour of this difference is that ubiquilin proteins might interact and/or localize to different subcellular sites with PS1 and PS2 proteins, thereby differentially affecting PS processing. However, by double-immunofluorescence microscopy, we found that endogenous ubiquilin proteins co-localize with PS1 and PS2 proteins in similar unknown vesicular-like structures in cells. Instead we favour the idea that the strength of interaction between ubiquilin and PS dictates PS fragment production because ubiquilin interacts more strongly with PS2 than with PS1 in yeast two-hybrid assays [46] and because overexpression of ubiquilin-1 or -2 increased FL PS2 levels more so than FL PS1 levels [47]. Therefore there seems to be a direct correlation between strength of ubiquilin-PS interaction, with its effects on increasing FL protein, and decreasing PS fragment production. Despite the slight difference in the regulation of PS1 and PS2 fragment levels, it is nevertheless clear that both ubiquilin-1 and -2 produce similar effects on both the PS proteins, because overexpression of either ubiquilin-1 or -2 decreased both PS1 and PS2 protein fragment levels, whereas knockdown of ubiquilin-1 or -2 increased PS1 and PS2 fragment levels. These results suggest that both ubiquilin-1 and -2 function in a similar manner in cells, at least with respect to their effects on PS fragment regulation.

Since ubiquilin proteins have been linked to the ubiquitin-proteasome pathway, it seemed possible that ubiquilin could decrease PS fragment levels by escorting the fragments to the proteasome for degradation. However, we found that, upon inhibition of the proteasome, PS fragment levels remained low, suggesting that ubiquilin did not function in this manner. Furthermore, classical pulse-chase studies of the turnover of PS fragments in the presence and absence of ubiquilin overexpression revealed no difference in the rates of degradation of the fragments. We therefore considered the alternative possibility that ubiquilin might decrease PS fragments by reducing their production. In agreement with this hypothesis, we found that in cells treated with cycloheximide, to block new protein synthesis, overexpression of ubiquilin decreased PS fragment production. Taken together, our results suggest that overexpression of ubiquilin does not enhance proteasome-dependent degradation of PS fragments but instead inhibits their production.

Finally, while attempting to discern ubiquilin's mechanism of action with respect to PS fragment production, an interesting observation was made. Namely, when proteasome activity was inhibited, PS fragment accumulation decreased, and this occurred in the absence of ubiquilin overexpression. Conversely, when proteasome inhibition was relieved, PS fragment levels increased. These results lead us to speculate that the proteasome is somehow involved in PS endoproteolysis. Either the proteasome directly acts as the presenilinase or it acts indirectly by

regulating the presenilinase. Our experiments do not distinguish between these two possibilities, but it seems more likely that the proteasome is actually involved in the cleavage process because PS fragment production was reduced with five different proteasome inhibitors, making it unlikely that the drugs were all inhibiting an activity other than the proteasome. In addition, the proteasome has been implicated in endoproteolysis of other proteins [37]. Since ubiquitin has domains that are associated with the ubiquitin–proteasome pathway of degradation, it is interesting to speculate that ubiquitin might act as a tether between PSs and presenilinase activity of the proteasome. Cleavage is suggested to occur when PS is embedded in the ER (endoplasmic reticulum) membrane because the large PS loop is orientated towards the cytoplasm where both ubiquitin and proteasomes are found. PS cleavage into its fragments occurs early in its maturation process, further suggesting that this event occurs when PS is in the ER. We envisage the interaction between ubiquitin and PS occurring before γ -secretase assembly mainly because ubiquitin modulates the active form of the PS protein and, in doing so, the levels of downstream interactors of PS are also affected. However, our endogenous cell staining results raise interesting questions. First, what are the vesicles that ubiquitin and PS co-localize to and, secondly, do the vesicles contain cleaved PS? We speculate that the vesicles are either early endosomes or coat protein-coated vesicles and that they contain cleaved PS since this is the predominant form of PS in cells. The co-localization of ubiquitin with PS in the vesicles suggests that ubiquitin guides PS through its entire maturation process, perhaps acting as a molecular chaperone.

Evidence suggests that γ -secretase activity comprises PS NTF and CTF fragments rather than the FL form of PS proteins. In the present study, we have shown that ubiquitin is capable of regulating PS fragments. Our results suggest that high levels of ubiquitin might reduce γ -secretase activity by decreasing the formation of PS fragments. This hypothesis is further supported by our evidence that overexpression of ubiquitin leads to destabilization of both Pen-2 and nicastrin, two components that are essential for γ -secretase activity. If this notion is correct, then it might be possible to regulate γ -secretase activity by manipulating ubiquitin expression.

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