Requirement of PEN-2 for Stabilization of the Presenilin N/C-terminal Fragment Heterodimer within the γ-Secretase Complex*

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γ-Secretase is a protease complex composed of presenilin (PS), nicastrin (NCT), APH-1, and PEN-2, which catalyzes intramembrane cleavage of several type I transmembrane proteins including the Alzheimer’s disease-associated β-amyloid precursor protein. We generated stable RNA interference-mediated PEN-2 knockdown cells to probe mutant PEN-2 variants for functional activity. Knockdown of PEN-2 was associated with impaired NCT maturation and deficient PS1 endoproteolysis, which was efficiently rescued by wild type or N-terminally tagged PEN-2 but not by C-terminally tagged PEN-2 or by the C-terminally truncated PEN-2 ΔC mutant. Although the latter mutants rescued the PS1 holoprotein accumulation associated with the PEN-2 knockdown, they failed to restore normal levels of the PS1 N- and C-terminal fragments and to maturate functional sites (6, 8–18). An exception is APH-1, which is stable in the absence of PS (13, 19, 20). Increased γ-secretase complex formation and activity have been observed when all four γ-secretase complex components are overexpressed in cultured Drosophila and mammalian cells (17, 21, 22). Moreover, reconstitution of γ-secretase complex formation and activity has recently been achieved in yeast, an organism that lacks any endogenous γ-secretase activity (23). This demonstrated that coexpression of all four components is necessary and sufficient for the reconstitution of γ-secretase activity (23).

Overexpression and RNAi-mediated knockdown studies with cultured Drosophila cells have provided a first model of how the γ-secretase complex assembles (17). These studies indicate that the PS holoprotein may assemble first with NCT and/or APH-1 to form a stable assembly intermediate (17). Subsequent assembly of PEN-2 with this intermediate drives the conversion of the PS holoprotein into the active heterodimer (17). These studies indicate that the PS holoprotein may assemble first with NCT and/or APH-1 to form a stable assembly intermediate (17). Subsequent assembly of PEN-2 with this intermediate drives the conversion of the PS holoprotein into the active heterodimer (17). Furthermore, a potential NCT/APH-1 assembly intermediate has been observed recently (24), and NCT has been shown to stably interact with APH-1 in the absence of PS (20, 25). Interestingly, γ-secretase complex assembly is associated with a conformational change of the NCT ectodomain, which adopts a protease-resistant conformation (26). At present, little is known about the subunit organization within the γ-secretase complex organization within the γ-secretase complex.

The Alzheimer’s disease (AD)γ-secretase is a high molecular weight complex with an aspartyl protease ac-

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1 The abbreviations used are: AD, Alzheimer’s disease; APH, anterior pharynx defective; APP, β-amyloid precursor protein; NCT, nicastrin; PEN, presenilin enhancer; PS, presenilin; NTF, N-terminal fragment; CTF, C-terminal fragment; wt, wild type; swAPP, Swedish mutant APP; CHAPSO, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; RNAi, RNA interference; HEK, human embryonic kidney 293.
complex. However, recent studies (27, 28) suggest that PS is a dimer within the γ-secretase complex. In addition, an interaction of the PS1 NTF with PEN-2 has been reported (29).

A large body of evidence suggests that PS carries the catalytic site of the γ-secretase complex. Mutagenesis of conserved aspartates in transmembrane domains 6 and 7 of PS (30, 31) as well as aspartyl protease transition state analogues, which bind to the PS heterodimer (32, 33), inhibit γ-secretase activity. Moreover, PS plays a role in the assembly of other γ-secretase complexes (34-36). Besides their requirement for γ-secretase complex assembly and maturation, the precise functional role of the other γ-secretase complex components is unclear. In particular, apart from its role in facilitation of PS endoproteolysis (14, 17), functional information about the smallest subunit, the 10-kDa PEN-2 protein, is scarce. In order to gain further insight into its functional role, we thus initiated a structure-function analysis of this protein.

EXPERIMENTAL PROCEDURES

Antibodies—The polyclonal antibody 1638 raised to the N terminus of human PEN-2 (11), the polyclonal and monoclonal antibodies against the PS1 C terminus (3067 and BI.3D7) (38), the N terminus (2953) (39), and PSIN (40), and the polyclonal antibody to the C terminus of APP (6697) (36) were described previously. The polyclonal antibodies 453 and 434 were generated against the APH-1αL C terminus (residues 245-265) and affinity-purified using a GST-APH-1αL (residues 207-265) fusion protein. The polyclonal antibody N1660 against the C terminus of NCT and the anti-β-actin antibody were obtained from Sigma, the anti-Xpress antibody was obtained from Invitrogen; the anti-β-catenin antibody was from Transduction Laboratories, and the anti-Myc antibody 9E10 was from Santa Cruz Biotechnology.

cDNA Constructs—To stably knock down endogenous PEN-2 expression by RNAi, short hairpin RNA oligonucleotides (PEN-2-163) corresponding to the PEN-2-160 target region (11) were cloned into the pSUPER vector (41). Silencer mutations that do not alter the encoded amino acid sequence (5'-AAAGAGATACGTGTGGCGATCTGC-3', the mutations are underlined) were introduced in all PEN-2 constructs to escape RNAi. PCR-mediated mutagenesis was used to generate mutant PEN-2 variants. Wild type and mutant PEN-2 variants were cloned into the pcDNA3.1/Zeo(-) vector (Invitrogen). N-terminal hexahistidine-Xpress (H6-HX) epitope-tagged and C-terminal myc-hexahistidine (mH6) epitope-tagged PEN-2 wild type or mutant PEN-2 variants were generated by cloning the respective cDNAs into the pcDNA4/HisC (Invitrogen) or pcDNA4/myc-HisA (Invitrogen) expression vectors. All constructs were verified by DNA sequencing.

Cell Culture and Cell Lines—Stably transfected human embryonic kidney 293 (HEK 293) cells were cultured as described (9). A stable PEN-2 knockdown cell line was generated by cotransfection of HEK 293 cells stably expressing Swedish mutant APP (swAPP) (42) with pSUPER/PEN-2-163 and pcDNA3.1/Hygro(-) (Invitrogen) and selection for hygromycin (150 μg/ml) resistance. The PEN-2 knockdown cell line was subsequently stably transfected with the indicated wt and mutant PEN-2 constructs. Likewise, PEN-2 was stably knocked down in HEK 293 cells stably coexpressing swAPP and PS1 Jlexon9 (38).

Protein Analysis—Membrane fractions of HEK 293 cells were obtained by ultracentrifugation of postnuclear supernatant fractions from cell homogenates that were prepared as described (43). For direct immunoblot analysis, membrane fractions were solubilized with STEN-lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, protease inhibitors [Sigma]). After a clarifying spin, lysates were analyzed for PEN-2 by immunoblotting with antibody 1638, for PS1 by immunoblotting with antibodies PSIN or 3027, for NCT with antibody N1660, for APH-1αL by immunoblotting with antibody 433 or 434, and for APP and APP CTFs by immunoblotting with antibody 6687. For commounprecipitation analysis, membrane fractions were solubilized in CHAPSO-lysis buffer (1% CHAPSO, 150 mM sodium citrate, pH 6.4, protease inhibitors [Sigma]), subjected to a clarifying spin by ultracentrifugation, and incubated with preimmune serum, N1660 or 2953 antibody, and protein G-Sepharose for 2 h at 4°C. Following two washes with CHAPSO-wash buffer (0.5% CHAPSO, 150 mM sodium citrate, pH 6.4), the immunoprecipitates were subjected to immunoblot analysis as above.

RESULTS

In order to identify functional domains of PEN-2 required for γ-secretase complex formation, maturation, and activity, we generated a HEK 293 cell line stably overexpressing Swedish mutant APP (HEK 293/sw) in which PEN-2 expression is stably knocked down by RNAi (Fig. 1A). This PEN-2 knockdown cell line was stably transfected with cDNA constructs encoding RNAi-resistant wt and mutant PEN-2 variants to assess their capability to rescue PEN-2 deficiency. We first transfected the PEN-2 knockdown cells with cDNA constructs encoding untagged wt PEN-2 and N-terminal hexahistidine-Xpress (H6-HX) epitope-tagged and C-terminal myc-hexahistidine (mH6) epitope-tagged wt PEN-2. All three proteins were stably expressed at robust levels, and as expected, the tagged PEN-2 variants migrated at higher molecular weight than untagged PEN-2 (Fig. 1A). Consistent with previous results (6, 17, 24), the PEN-2 knockdown was associated with reduced γ-secretase activity as manifested by the accumulation of APP CTFs, which was rescued by the expression of wt PEN-2 and H6X-PEN-2 (Fig. 1B). APP CTFs also accumulated when PEN-2-mH6 was expressed indicating that the authentic C terminus of PEN-2 is critical for γ-secretase activity.

We next sought to investigate why expression of PEN-2-mH6 caused a defect in γ-secretase activity, and we asked whether a defect in γ-secretase complex maturation was responsible for the observed loss of γ-secretase function. To confirm further the functional importance of the PEN-2 C terminus, we also constructed a PEN-2 variant with a C-terminal truncation of 17 amino acids (PEN-2-ΔC), and we expressed it stably in the PEN-2 knockdown cells (Fig. 1C). We first investigated the maturation of PS. Consistent with previous results (11, 14, 24), down-regulation of PEN-2 was associated with reduced levels of PS fragments and an accumulation of the PS1 holoprotein (Fig. 1D). Expression of untagged PEN-2 allowed efficient recovery of PS1 holoprotein endoproteolysis (Fig. 1D). In contrast, expression of PEN-2-mH6 and of PEN-2-ΔC caused an intriguing, unexpected biochemical phenotype. Although the PS1 holoprotein did not accumulate anymore, the levels of the PS1 NTF and CTF were strongly reduced similar to those observed in the PEN-2 knockdown cells (Fig. 1D). Thus, although the PEN-2 C terminus appears to be dispensable for PS1 endoproteolysis, it is required for the maintenance of normal levels of the PS fragments. As reported previously (11, 24), the PEN-2 knockdown caused impaired NCT maturation, which was efficiently restored by the expression of untagged PEN-2 (Fig. 1E). Interestingly, the levels of APH-1αL were largely unaffected by the knockdown of PEN-2 (Fig. 1E). Neither PEN-2-mH6 nor PEN-2-ΔC rescued the maturation defect of NCT (Fig. 1D). Consequently, as observed for PEN-2-mH6 (Fig. 1B), expression of PEN-2-ΔC was associated with impaired γ-secretase activity as judged from the accumulation of APP CTFs (Fig. 1F). Thus, either addition of an epitope tag to the C terminus or a large deletion of it abrogated PEN-2 function. F. Thus, either addition of an epitope tag to the C terminus or a large deletion of it abrogated PEN-2 function.
NCT, and APH-1aL in these cells (Fig. 2A), consistent with previous results (17). We then analyzed the stability of PEN-2 or PEN-2-ΔC expressed in the background of PEN-2 knockdown cells. wt PEN-2 occurred as a stable protein (Fig. 2B). In contrast, cycloheximide treatment revealed that PEN-2-ΔC was highly unstable and almost completely turned over within 3 h (Fig. 2B). We next investigated the stability of PS1, NCT, and APH-1aL in the background of wt PEN-2 and PEN-2-ΔC expression. The PS1 fragments, NCT and APH-1aL, were stable in the PEN-2 knockdown cells stably expressing wt PEN-2. In contrast, in the PEN-2-ΔC-expressing knockdown cells, the already low levels of PS1 fragments dropped further during the time course of the cycloheximide treatment. This indicates that the PS1 fragments were not stabilized and thus turned over (Fig. 2B). Interestingly, immature NCT and APH-1aL as well as the low levels of residual mature NCT (because of the incomplete PEN-2 knockdown) remained stable (Fig. 2B), consistent with the observation that these two proteins can independently stabilize each other (13, 20, 24, 25). Taken together, these data demonstrate that PEN-2-ΔC is highly unstable. Furthermore, these data indicate that expression of PEN-2-ΔC might cause a selective destabilization of the PS1 NTF/CTF heterodimer within the γ-secretase complex.

We next asked which proteolytic pathway might be involved in the degradation of PEN-2-ΔC and the destabilized PS1 fragments. Because we had shown previously that excess amounts of free PS proteins that have not undergone complex formation are rapidly turned over by proteasomal degradation (44), we treated the above-described cells with the highly potent and specific proteasome inhibitor clasto-lactacystin β-lactone (45). To control the inhibitor treatment, we monitored the appearance of polyubiquitinated β-catenin species (44, 46). As expected from their stable behavior shown above, clasto-lactacystin β-lactone treatment did not result in increased levels of endogenous PEN-2 in control cells or of the residual endogenous PEN-2 in the PEN-2 knockdown cells (Fig. 3A). The levels of wt PEN-2 expressed in the background of PEN-2 knockdown cells were slightly increased indicating degradation of excess free amounts (Fig. 3A). In contrast, clasto-lactacystin β-lactone treatment caused a very strong increase in the levels of PEN-2-ΔC (Fig. 3A) suggesting that the proteasome degrades this unstable protein. The levels of endogenous PS1 fragments in control or of residual endogenous PEN-2 in the knockdown cells were unchanged upon clasto-lactacystin β-lactone treatment (Fig. 3B). A slight increase in the levels of the PS1 holoprotein was observed in the PEN-2 knockdown cells suggesting proteasomal degradation of minor amounts of unstable free holoprotein (Fig. 3B). As expected, no increase in the levels of the PS1
fragments was observed in PEN-2 knockdown cells expressing wt PEN-2 (Fig. 3B). In contrast, the levels of the PS1 fragments were markedly increased in PEN-2 knockdown cells expressing PEN-2-/H9004C, suggesting that following their generation by endoproteolysis, the destabilized fragments were degraded by the proteasome (Fig. 3B). No increase in the levels of the PS1 holoprotein was observed, further supporting that the PS1 holoprotein had undergone endoproteolysis and not simply undergone proteasomal degradation. Consistent with their stable behavior shown above (Fig. 2, A and B), levels of immature and mature forms of NCT as well as of APH-1aL were unaffected upon proteasome inhibition (Fig. 3C). In summary, these results suggest that PEN-2-/H9004C is capable of initiating PS1 endoproteolysis. However, the resulting PS1 NTF and CTF are not stabilized and like PEN-2-/H9004C itself are degraded by the proteasome.

The results described above demonstrate that PEN-2-/H9004C is highly unstable and indicate that the cause of its instability may be due to a defect in stable γ-secretase complex formation. We therefore next asked whether PEN-2-/H9004C might have lost its capability to stably associate with the other γ-secretase complex components. Membrane fractions were isolated from PEN-2 knockdown cells stably transfected with wt PEN-2 or PEN-2-/H9004C and were detergent-solubilized with CHAPSO. The CHAPSO-extracted membrane proteins were subjected to coimmunoprecipitation analysis with antibodies to NCT and PS1. However, the resulting PS1 NTF and CTF are not stabilized and like PEN-2-/H9004C itself are degraded by the proteasome.

The observation by others (14, 17, 24) and us (Fig. 1C) that a PEN-2 knockdown causes an accumulation of the PS holoprotein suggests that PEN-2 is required for PS endoproteolysis. However, as shown above, PEN-2-/mH6 and PEN-2-/H9004C are defective in the stabilization of the PS1 NTF and CTF but not in endoproteolysis. This suggests that following endoproteolysis, PEN-2 also becomes necessary for stabilization of the active site PS heterodimer, and consequently for the further maturation of the γ-secretase complex. In addition, following PS endoproteolysis PEN-2 apparently remains bound to the γ-secretase complex (11, 17, 21, 22), further indicating that PEN-2 may have a function independent of PS endoproteolysis. To directly prove this, we investigated the consequences of PEN-2 down-regulation in the background of PS1-/H9004 exon9 (47), a FAD-associated PS1 variant that undergoes stable γ-secretase complex formation (48) but that does not undergo endoproteolysis because of a deletion of the cleavage site domain (3, 38). Indeed, when we stably knocked down PEN-2 expression by RNAi in
HEK 293/sw cells stably expressing PS1 Δexon9 (38), NCT maturation was impaired (Fig. 5). Consistent with the results shown above, APH-1aL levels were largely unaffected by the knockdown of PEN-2 (Fig. 5). Thus, these data show that PEN-2 has a general function in the maturation of the γ-secretase complex, which is independent from its role in PS endoproteolysis.

**DISCUSSION**

Little functional information is available about PEN-2, the smallest of the four essential subunits of the γ-secretase complex. RNAi-mediated knockdown experiments in Drosophila and mammalian cells demonstrated that a knockdown of PEN-2 is associated with reduced PS fragment levels and ac-

**FIG. 3.** Proteasomal degradation of PEN-2-ΔC and the PS1 NTF and CTF.

A, HEK 293/sw (control), PEN-2 knockdown, and PEN-2 knockdown cells stably expressing RNAi-resistant wt PEN-2 or PEN-2-ΔC were treated with clasto-lactacystin β-lactone (10 μg/ml) for 24 h. Membrane fractions were prepared and analyzed for PEN-2 as in Fig. 1. To control the inhibitor treatment, we monitored the appearance of polyubiquitinated β-catenin species (44, 46) by immunoblotting with an anti-β-catenin antibody. B, membrane fractions of cell lines described in A were analyzed for PS1 holoprotein, NTF, and CTF by immunoblotting as described in Fig. 1E. To control for equal amounts of protein loaded, levels of β-actin were monitored by immunoblotting with an anti-β-actin antibody. The asterisk indicates the phosphorylated form of the PS1 CTF (39). C, membrane fractions of cell lines described in A were analyzed for NCT and APH-1aL by immunoblotting as described in Fig. 1E.

**FIG. 4.** PEN-2-ΔC fails to undergo stable γ-secretase complex formation. Membrane fractions of PEN-2 knockdown cells stably expressing RNAi-resistant wt PEN-2 and PEN-2ΔC were solubilized with CHAPSO and analyzed for γ-secretase complex formation by immunoprecipitation (IP) with antibodies 2953 and N1660 or preimmune serum (PIS) and immunoblotting with antibodies 1638, N1660, PS1N and BI.3D7, and 434 (to the APH-1aL C terminus) as in Fig. 1. The input lanes represent 25% of the material used for the immunoprecipitation.
cumulation of the PS holoprotein (6, 11, 14, 17). Moreover, it has been shown that PEN-2 not only forms stable associates with the PS fragments but can also associate with the PS holoprotein (14). Accordingly, PEN-2 has been suggested to play an important role in γ-secretase complex maturation by conversion of the PS holoprotein into the PS NTF/CTF heterodimer (14, 17). To better understand PEN-2 function and to define its functional domains, we initiated a structure-function analysis of PEN-2 using a stable knockdown cell line in which loss of PEN-2 function can be assayed by functional complementation. Consistent with previous findings, stable knockdown of PEN-2 was associated with impaired NCT maturation, reduced PS fragment levels, and an accumulation of the PS1 holoprotein. This defect in γ-secretase complex maturation was efficiently rescued by expression of untagged or N-terminal-tagged but not by C-terminal tagged PEN-2, suggesting that the C terminus of PEN-2 is a functionally critical domain, which is sensitive to steric alteration. Indeed, expression of the C-terminal truncated PEN-2-ΔC variant also caused loss of PEN-2 function. Both C-terminal loss of function mutants of PEN-2 showed very similar if not identical biochemical behavior. NCT maturation was impaired, and the defective PS1 holoprotein accumulation was rescued without stabilization of the PS1 fragments. Thus, these PEN-2 variants are apparently capable of initiating PS1 endoproteolysis but cannot stabilize the PS fragments generated. A possible reason for the failure of PEN-2 with a C-terminal alteration to stabilize the resulting PS1 NTF and CTF is its own instability within the γ-secretase complex. Indeed, PEN-2-ΔC failed to undergo stable γ-secretase complex formation. As a consequence, PEN-2-ΔC behaved as a highly unstable protein and was rapidly turned over by the proteasome. This is in contrast to correctly assembled γ-secretase complex components, which have been shown to be very stable and have a long half-life time (9, 17, 44, 48). The failure of PEN-2-ΔC to stably assemble with PS, NCT, and APH-1 was associated with a marked selective instability of the PS1 NTF and CTF, which also underwent proteasomal degradation, whereas NCT and APH-1 were stable, consistent with previous findings that these proteins can stabilize each other independently (20, 24, 25). These data suggest that PEN-2 has a dual function as follows: a function in the initiation of PS endoproteolysis as a first maturation step during γ-secretase complex assembly, and a second function in the stabilization of the PS fragment heterodimer within the γ-secretase complex that is required for further maturation of the γ-secretase complex. Finally, when we knocked down PEN-2 in the background of the endoproteolysis-deficient PS1 Δexon9 mutant, NCT accumulated as an immature protein demonstrating that PEN-2 is also required for γ-secretase complex maturation under conditions when PS endoproteolysis cannot occur. Thus, PEN-2 is generally required for γ-secretase complex maturation independent of its role in PS endoproteolysis.

Taken together, our data add important new functional information on PEN-2 in γ-secretase complex assembly, maturation, and activity. We suggest that during γ-secretase complex assembly, PEN-2 interacts with the PS holoprotein that is stabilized by immature NCT and APH-1. Upon interaction with PEN-2, the PS holoprotein may adopt a conformation that allows PS endoproteolysis. Following PS endoproteolysis, the PS NTF and CTF are stabilized by PEN-2 which itself has become stabilized within the complex. Whereas the C-terminal domain of PEN-2 is dispensable for the PS endoproteolysis, the stabilization of the NTF/CTF heterodimer and of PEN-2 itself is only possible when PEN-2 carries an intact and/or accessible C terminus. This strongly suggests that the C terminus carries important sequence information, which may be required to stably interact with other γ-secretase complex components. These stabilizing interactions are necessary for further maturation of the γ-secretase complex by trafficking through the secretory pathway. Future work will aim at elucidating the molecular basis for the critical requirement of the PEN-2 C terminus in γ-secretase complex assembly, maturation, and activity.

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Fig. 5. PEN-2 is required for γ-secretase complex maturation independent of its role in PS endoproteolysis. Membrane fractions of HEK 293/sw cells (control), HEK 293/sw cells stably expressing PS1 Δexon9, and HEK 293/sw cells stably coexpressing PS1 Δexon9 and PEN-2 short interfering RNA were analyzed for PEN-2, PS1, NCT, and APH-1αL as in Fig. 1. The asterisk indicates the phosphorylated form of the PS1 CTF (39).
