PEN-2 IS AN INTEGRAL COMPONENT OF THE γ-SECRETASE COMPLEX REQUIRED FOR COORDINATED EXPRESSION OF PRESENILIN AND NICASTRIN

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Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer’s disease; APP, β-amyloid precursor protein; Net, Nicastrin; NICD, Notch intracellular domain; PEN-2, presenilin enhancer 2; PS, presenilin.

Running title: PEN-2 is a γ-secretase complex component
PEN-2 is a $\gamma$-secretase complex component

The Alzheimer's disease (AD) associated presenilin (PS) proteins apparently provide the active site of $\gamma$-secretase, an unusual intramembrane-cleaving aspartylprotease. PSs principally occur as high molecular weight protein complexes that contain Nicastrin (Nct) and additional so far unidentified components. Recently, PEN-2 has been implicated in $\gamma$-secretase function. Here we identify PEN-2 as a critical component of PS1/$\gamma$-secretase and PS2/$\gamma$-secretase complexes. Strikingly, in the absence of PS1 and PS1/PS2, PEN-2 levels are strongly reduced. Similarly, PEN-2 levels are reduced upon RNAi-mediated downregulation of Nct. On the other side, downregulation of PEN-2 by RNAi is associated with reduced PS levels, impaired Nct maturation and deficient $\gamma$-secretase complex formation. We conclude that PEN-2 is an integral $\gamma$-secretase complex component and that $\gamma$-secretase complex components are expressed in a coordinated manner.
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Introduction

The Alzheimer’s disease (AD) associated polytopic membrane proteins presenilin 1 (PS1) and presenilin 2 (PS2) are required for the intramembranous γ-secretase cleavage of the β-Amyloid precursor protein (APP) (1). Following an initial cleavage by β-secretase within the APP ectodomain, γ-secretase cleavage releases the 40-42 amino acid amyloid β-peptide (Aβ) from the membrane (1). The majority of familial AD cases are associated with mutations in the PS1 gene (1). Apparently all PS1 mutations investigated cause an increased generation of the highly amyloidogenic Aβ42 (1). Absence of PS1 reduces γ-secretase activity (2,3) and absence of PS1/PS2 eliminates γ-secretase function completely (4,5). Mounting evidence suggests that PSs are unusual aspartyl proteases with γ-secretase activity (6). All PSs contain two aspartates within transmembrane domains 6 and 7 that are critically required for γ-secretase activity (7,8). Moreover, γ-secretase inhibitors designed to mimic the transition-state of the catalytic mechanism of aspartyl proteases can be covalently cross-linked to PSs (9,10). Finally, PSs are apparently members of a group of polytopic membrane-bound aspartylproteases that are all characterized by a GxGD (x=variable amino acid) signature motif in which the C-terminal active site aspartate is embedded (11). Besides the PSs, the bacterial TFPPs (type 4 prepilin peptidase) (11,12) and SPP (signal peptide peptidase) and its related proteins carry this signature motif (13,14).

The PSs reside in high molecular weight (HMW) complexes (15-18). An integral component of these HMW complexes is the membrane glycoprotein Nicastrin (Nct) (18-20). Downregulation of Nct in cultured mammalian or Drosophila cells inhibits γ-secretase cleavage of APP (18,21), site 3 (S3) cleavage of Notch (21) and reduces PS levels (18,21,22). On the other side, absence of PS1 and PS1/PS2 causes a strong inhibition of Nct maturation (18,23).
$\gamma$-secretase complexes require PSs for the $\gamma$-secretase-mediated processing of APP and intramembrane proteolysis of several other type I transmembrane proteins including the Notch cell surface receptors that are critically required for cell fate decisions (24). Notch signaling depends on the PS-dependent S3 cleavage of Notch that leads to the liberation of the Notch intracellular domain (NICD) from the membrane (24). NICD translocates to the nucleus where it is involved in the transcription of target genes (24). Genetic screening in C. elegans led to the identification of novel components, APH-1 and PEN-2, that are required for $\gamma$-secretase activity in APP and Notch S3 cleavage (21,25). The function of APH-1 and PEN-2 is currently unclear. Apparently, APH-1 and PEN-2 could either be transiently required for the assembly of the $\gamma$-secretase complex or may even be novel $\textit{bona fide}$ complex components required for $\gamma$-secretase activity (21). Here we investigated if PEN-2 is an integral $\gamma$-secretase complex component.

**Results and Discussion**

In order to identify endogenous PEN-2, we raised antibody 1638 to the N-terminus of human PEN-2. To prove the specificity of this antibody, cell lysates of HEK 293 cells were subjected to immunoprecipitation with antibody 1638 or the corresponding preimmune serum. HEK 293 cells are known from numerous previous studies to express a $\gamma$-secretase activity, which has identical functional and biochemical properties as the $\gamma$-secretase activity in neuronal cells or brain tissue (see (26) and citations therein). As shown in Fig. 1a, robust amounts of the ~10 kDa PEN-2 were immunoprecipitated with antibody 1638 but not with preimmune serum.

To address the question whether PEN-2 is a $\textit{bona fide}$ component of PS1 and PS2/$\gamma$-secretase complexes we first performed co-immunoprecipitation analyses using HEK 293 cells stably transfected with N-terminal hexahistidine-Xpress (H$_6$X)-epitope-tagged PS1 or
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PS2. γ-Secretase complexes were isolated from DDM-solubilized membrane fractions by immunoprecipitation with an anti-Xpress antibody. As expected, PS1 and PS2 holoproteins and NTFs were immunoprecipitated and the corresponding CTFs co-immunoprecipitated (Fig. 1b). Under these conditions endogenous PEN-2 was found to co-immunoprecipitate with PS1 and PS2. In addition, Nct also co-immunoprecipitated (Fig. 1b). As shown in Fig. 1c, association of PEN-2 with the γ-secretase complex is independent of endoproteolysis of PS1. Upon stable expression of H6X-PS1 Δexon9, which does not undergo endoproteolysis due to the lack of the cleavage site for PS endoproteolysis (27), PEN-2 was still co-isolated with the PS1 Δexon9 holoprotein and with Nct (Fig. 1c). These results suggest that PEN-2 is a component of individual PS1/γ-secretase and PS2/γ-secretase complexes. Under these conditions we could only immunoprecipitate very minor amounts of PEN-2 with antibody 1638 (data not shown), whereas mild denaturation with 0.1% SDS was sufficient to robustly immunoprecipitate PEN-2 (see Fig. 1a). This suggests that PEN-2 is tightly packed within the γ-secretase complex.

To confirm that PEN-2 is an integral component of the endogenous γ-secretase complex co-immunoprecipitations were carried out with untransfected HEK 293 cells. DDM-solubilized membrane fractions were immunoprecipitated with antibodies to the C-terminus of Nct and the N-terminus of PS1 or PS2. Immunoblotting with anti-PEN-2 antibody 1638 revealed robust amounts of co-immunoprecipitated PEN-2 demonstrating that PEN-2 is a component of endogenous PS/γ-secretase complexes (Fig. 1d). As expected, antibodies to the N-termini of PS1 or PS2 co-immunoprecipitated the corresponding PS1 and PS2 CTFs and mature Nct (Fig. 1d). Taken together, these experiments demonstrate that PEN-2 is an integral bona fide γ-secretase complex component.

Eliminating PS1 or PS1/PS2 causes reduced levels of the γ-secretase complex and inhibits Nct maturation (18,23). Moreover inhibition of Nct expression also strongly reduced
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PS expression and inhibited \(\gamma\)-secretase complex formation (18). This suggested that expression of \(\gamma\)-secretase complex components might be coordinately regulated. We therefore analyzed PEN-2 expression in PS knockout mice (2,4,28). As expected, robust levels of PEN-2 were observed in embryonic fibroblasts derived from littermate control mice (Fig. 2a). Ablation of PS1 caused a strong reduction of PEN-2 levels and absence of PS1/PS2 eliminated PEN-2 expression nearly completely. Compared to the PS1 ablation, ablation of PS2 still allowed significant PEN-2 expression (Fig. 2a). Similar effects of the PS ablations were found on Nct expression. Ablation of PS1 or PS1/PS2 dramatically reduced Nct maturation, whereas ablation of PS2 allowed Nct maturation (Fig. 2a). We next investigated if PEN-2 expression is also dependent on the presence of Nct. HEK 293 cells were transfected with the siRNA duplex Nct-1045 to downregulate Nct expression by RNAi (18). As shown in Fig. 2b, RNAi-mediated downregulation of Nct caused a reduction of PEN-2 levels. Thus, expression of PEN-2 not only requires the presence of PSs (predominantly PS1) but that of Nct as well. To investigate if lowering PEN-2 levels affects PS and Nct levels, we next transfected HEK 293 cells with the siRNA duplex PEN-2-160. PEN-2-RNAi reduced PEN-2 expression, which was accompanied by reduced levels of PS1 and PS2 (Fig. 3a). RNAi-mediated downregulation of PEN-2 levels also impaired the maturation of Nct (Fig. 3a) as had been observed upon inhibition of PS1 or PS1/PS2 (Fig. 2a). As a consequence of reduced PS expression and impaired Nct maturation, inhibition of PEN-2 expression caused a deficiency in the PS1/\(\gamma\)-secretase complex (Fig. 3b) as revealed by BN-PAGE analysis (18).

Taken together our findings demonstrate that at least PS, Nct and PEN-2 are integral components of the \(\gamma\)-secretase complex. Expression of these \(\gamma\)-secretase complex components is coordinately regulated. Removing any of these three components results in reduced amounts of the \(\gamma\)-secretase complex and consequently in a loss of \(\gamma\)-secretase activity (18,21). We propose that any additional so far unknown \(\gamma\)-secretase complex component may affect
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PS, Nct, and PEN-2 expression. Obvious candidates for additional \( \gamma \)-secretase complex components are APH-1a and APH-1b, the two human homologues of \textit{C. elegans} APH-1 (21). Both act directly at or upstream of \( \gamma \)-secretase activity and apparently affect APH-2 (the \textit{C. elegans} homologue of Nct) transport to the cell surface (25). Moreover, like Nct and PEN-2, APH-1a and APH-1b are required for \( \gamma \)-secretase activity (21).
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EXPERIMENTAL PROCEDURES

**Antibodies**

The polyclonal antibody 1638 was raised to the N-terminus (residues 4-15) of human PEN-2 (21). The polyclonal and monoclonal antibodies against the PS1 C-terminus (3027 and BI.3D7) (29) and N-terminus (2953) (30), against the PS2 C-terminus (3711 and BI.HF5c) (29) and N-terminus (2972) (31) were described. The polyclonal antibody N1660 against the C-terminus (residues 693-709) of Nct was obtained from Sigma. The anti-Xpress-antibody was obtained from Invitrogen.

**cDNA constructs and transfections**

N-terminally hexahistidine-Xpress (H\(_6\)X)-epitope-tagged PS1, PS1 \(\Delta\)exon9 and PS2 variants were generated by cloning the respective cDNAs into pcDNA4/HisC expression vector (Invitrogen) and stably transfected into human embryonic kidney 293 (HEK 293) stably expressing Swedish mutant APP (swAPP) (32).

**Cell culture, cell lines, RNA interference (RNAi) and protein analysis**

HEK 293 stably expressing swAPP and mouse embryonic fibroblast cells derived from PS knockout or littermate control mice (28) were cultured as described (18). To inhibit expression of PEN-2 and Nct by RNAi, HEK 293 cells were transiently transfected with siRNA duplexes PEN-2-160 (directed to the target sequence 5´-AAAGGCTATGTCTGGCGCTCA-3´) and Nct-1045 as described (18). Cell lysates of HEK 293 cells were analyzed for PEN-2 by combined immunoprecipitation/immunoblotting with antibody 1638, for PSs by combined immunoprecipitation/immunoblotting as described (29) and for Nct by direct immunoblotting with antibody N1660. Immunoprecipitations of PEN-2 were carried out in the presence of 0.1% SDS. To analyze PEN-2, PS and Nct expression levels in mouse embryonic fibroblast cells, cell homogenates were subjected to ultracentrifugation and the pellet fraction was solubilized in the presence of 1% SDS as
PEN-2 is a γ-secretase complex component described (33). The SDS extracts were diluted 10-fold and analyzed for PEN-2, PSs and Nct by combined immunoprecipitation/immunoblotting or immunoblotting as described above.

**Isolation and analysis of PS complexes**

PS complexes were isolated by detergent-solubilization of membrane preparations with n-dodecyl β-D-maltoside (DDM) (18). Homogenates of HEK 293 cells were extracted with 1% Brij-35/1% Lubrol WX and membranes were isolated by ultracentrifugation from the postnuclear supernatant fraction and solubilized in DDM-lysis buffer [0.7% DDM, 50 mM sodium citrate pH 6.4, 1 mM EDTA, 5% glycerol, protease inhibitors (Sigma)]. Following a clarifying spin by ultracentrifugation, DDM-solubilized membrane fractions were subjected to co-immunoprecipitation analysis or analyzed for the PS1 complex by blue native Polyacrylamid gel electrophoresis (BN-PAGE) as described (18,34). Co-immunoprecipitation analysis was performed with the indicated antibodies for 1-4 h at 4°C followed by two washes in DDM-wash buffer (0.5% DDM, 50 mM sodium citrate pH 6.4, 1 mM EDTA, 5% glycerol, protease inhibitors). Immunoprecipitates were analyzed on 10-20% Tris-Tricine gels (Invitrogen).
FIGURE LEGENDS

**Fig. 1. PEN-2 binds to Nct, PS1 and PS2.** (A) Identification of endogenous PEN-2. Cell lysates from HEK 293 cells expressing endogenous (endog.) PEN-2 were analyzed for PEN-2 by immunoprecipitation with antibody 1638 or with the corresponding preimmune serum (PIS) and analyzed by immunoblotting with antibody 1638. (B) Co-immunoprecipitation of endogenous PEN-2 with H₆X-PS1 and H₆X-PS2. DDM-solubilized membrane fractions from HEK 293 cells stably expressing H₆X-tagged PS1 or PS2 were subjected to immunoprecipitation with an anti-Xpress-antibody (anti-Xpress IP) and analyzed for PEN-2, Nct, PS1 and PS2 by immunoblotting with an anti-Xpress-antibody (anti-Xpress) and with antibodies 1638 (anti-PEN-2-N), N1660 (anti-Nct-C), 3027 (anti-PS1-C) and 3711 (anti-PS2-C). (C) PEN-2 co-immunoprecipitates with uncleavable H₆X-PS1 Δexon9. DDM-solubilized membrane fractions from HEK 293 cells stably expressing H₆X-tagged PS1 Δexon9 (PS1Δ9) were immunoprecipitated with an anti-Xpress-antibody (anti-Xpress IP) and analyzed by immunoblotting with the indicated antibodies as in (B). The asterisk denotes the IgG heavy chain. (D) PEN-2 co-immunoprecipitates with endogenous Nct, PS1, and PS2. Membrane fractions of HEK 293 cells expressing endogenous (endog.) γ-secretase complex components were solubilized with DDM and analyzed for Nct/PEN-2, PS1/PEN-2 and PS2/PEN-2 interactions by immunoprecipitation with preimmune serum and the antibodies N1660 (anti-Nct-C IP), 2953 (anti-PS1-N IP) and 2972 (anti-PS2-N IP) and immunoblotting with antibodies 1638 (anti-PEN-2-N), N1660 (anti-Nct-C), BI.3D7 (anti-PS1-C) and BI.HF5c (anti-PS2-C). The arrow indicates immature Nct.

**Fig. 2. PS1, PS2 and Nct are required for the expression of PEN-2.** (A) Membrane lysates from mouse embryonic fibroblasts (PS1⁺⁺/PS2⁺⁺, PS1⁻⁻/PS2⁺⁺, PS1⁺⁺/PS2⁻⁻, PS1⁻⁻/PS2⁻⁻) were analyzed for levels of PEN-2, PS1 and PS2 by combined immunoprecipitation/immunoblotting with antibody 1638 (to PEN-2), and with antibodies
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3027/BI.3D7 (to PS1) and 3711/BI.HF5c (to PS2). Nct was analyzed by immunoblotting with antibody N1660. Cells lacking PS1 and PS1/PS2 show significantly reduced levels of PEN-2. Note that Nct maturation is strongly reduced in the absence of PS1 and PS1/PS2. The asterisk denotes the phosphorylated form of the PS1 CTF (30). (B) HEK 293 cells were subjected to two consecutive transfections with siRNAi duplex Nct-1045 as described (18). Cell lysates were analyzed for levels of PEN-2 by combined immunoprecipitation/immunoblotting with antibody 1638. Downregulation of Nct was confirmed by immunoblotting with antibody N1660.

Fig. 3. Downregulation of PEN-2 by RNAi is associated with reduced PS levels, impaired Nct maturation and PS1 complex deficiency. (A) HEK 293 cells were subjected to two consecutive transfections with siRNA duplex PEN-2-160 as described (18). PEN-2 and Nct were analyzed as in Fig. 2b and PSs were analyzed by combined immunoprecipitation/immunoblotting with antibodies 3027/BI.3D7 (to PS1) and 3711/BI.HF5c (to PS2). Note the altered ratio of mature/immature Nct in cells subjected to PEN-2-RNAi compared to untreated control cells. (B) HEK 293 cells were subjected to PEN-2-RNAi as in (A). Membrane fractions were solubilized with DDM, subjected to BN-PAGE and analyzed for the PS1 complex by immunoblotting with antibody 2953. Aliquots were subjected to SDS-PAGE to confirm downregulation of PEN-2 by immunoblotting with antibody 1638.
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A

| genotype        | PEN2 | Nct\text{mat.} | Nct\text{int.} | PS1\text{CTF} | PS2\text{CTF} |
|-----------------|------|----------------|----------------|---------------|---------------|
| PS1^{+/+}/PS2^{+/+} |      |               |                |               |               |
| PS1^{+/+}/PS2^{+/+} |      |               |                |               |               |
| PS1^{+/+}/PS2^{-/-} |      |               |                |               |               |
| PS1^{-/-}/PS2^{-/-} |      |               |                |               |               |

B

- Nct\text{-RNAi} + PEN2

- Nct\text{mat.} \ 
- Nct\text{int.}
A

- + PEN-2-RNAi

Net<sub>mat.</sub>  Net<sub>int.</sub>  PS<sub>1</sub><sub>CTF</sub>  PS<sub>2</sub><sub>CTF</sub>  PEN-2

B

kDa  - + PEN-2-RNAi

BN-PAGE  669  PS1 complex

443

SDS-PAGE  PEN-2

Steiner et al., Fig. 3
PEN-2 is an integral component of the γ-secretase complex required for coordinated expression of presenilin and nicastrin
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