THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 45, Issue of November 5, pp. 46455–46463, 2004

Printed in U.S.A.

Both the Sequence and Length of the C Terminus of PEN-2 Are Critical for Intermolecular Interactions and Function of Presenilin Complexes*

Received for publication, June 7, 2004, and in revised form, August 10, 2004
Published, JBC Papers in Press, August 17, 2004, DOI 10.1074/jbc.M406289200

Hiroshi Hasegawa‡, Nobuo Sanjo‡, Fusheng Chen‡, Yong-Jun Gu‡, Cortney Shier‡, Agnes Petit‡, Toshitaka Kawarai‡, Taiichi Katayama‡, Stephen D. Schmidt§, Paul M. Mathews§, Gerold Schmitt-Ulms‡, Paul E. Fraser‡¶, and Peter St George-Hyslop¶‖****

From the ‡Centre for Research in Neurodegenerative Diseases, University of Toronto, West Toronto, Ontario M5S 3H2, Canada, the §New York University Medical Center/Nathan Kline Institute, New York, New York 10962, the ¶Department of Medicine, Medical Biophysics, University of Toronto, West Toronto, Ontario M5S 3H2, Canada, and the ‖Department of Medicine, Division of Neurology, University Health Network/Toronto Western Hospital, Toronto, Ontario M5S 3H2, Canada

Presenilin 1 or presenilin 2, nicastrin, APH-1, and PEN-2 form high molecular weight complexes that play a pivotal role in the cleavage of various Type I transmembrane proteins, including the β-amyloid precursor protein. The specific function of PEN-2 is unclear. To explore its function and intermolecular interactions, we conducted deletion and mutagenesis studies on a series of conserved residues at the C terminus of PEN-2. These studies suggest that: 1) both the presence and amino acid sequence of the conserved DYLSF domain at the C terminus of PEN-2 (residues 90–94) is critical for binding PEN-2 to other components in the presenilin complex and 2) the overall length of the exposed C terminus is critical for functional γ-secretase activity.

The presenilin proteins (PS1 and PS2) are evolutionarily conserved polytopic transmembrane proteins that are required for the regulated intramembranous proteolysis of several Type 1 transmembrane proteins including the β-amyloid precursor protein (APP),[1] Notch receptor, and p75 (1–4). Several different lines of evidence have revealed that the presenilins form high molecular weight complexes with at least three other proteins (APH-1, PEN-2, and nicastrin) (5–9) and that all of these other proteins are required for functional intramembranous proteolytic activity (10). As a result, null mutations or suppression of the expression of these genes results in loss of regulated intramembranous endoproteolysis of APP (termed γ-secretase cleavage), Notch (termed S3 cleavage), and the other Type 1 transmembrane protein substrates. Conversely, clinical missense mutations in PS1 and PS2 that are associated with familial Alzheimer's disease (11, 12) and selected experimental missense mutations in nicastrin (6) can all alter presenilin-dependent γ-secretase cleavage of APP, leading to overproduction of longer proteolytic derivatives of APP termed amyloid β-peptide (Aβ).

PEN-2 (presenilin enhancer 2) was initially identified during a genetic screen for modifiers of the presenilin homologues in *Caenorhabditis elegans* (SEL-12 and HOP-1) (8). Subsequent studies confirmed that it is a component of the high molecular weight presenilin complexes and that its presence within these complexes is necessary for their maturation and activity (8, 13, 14). Thus, loss of PEN-2 activity is associated with reduced presenilin levels (8, 15, 16), reduced presenilin endoproteolysis (13, 17, 18), and loss of regulated intramembranous proteolytic (γ-/S3 cleavage) activity (8). Analysis of the primary amino acid sequence of PEN-2 provides no clue as to its function. However, the protein is predicted to have two hydrophobic transmembrane domains (19, 20). There are also a series of residues at the hydrophilic C terminus (residues 90–101) that are conserved during evolution and that we hypothesize are functionally important (see Fig. 1*A*). In support of this, one of the three naturally occurring loss-of-function mutants in *C. elegans* is a stop codon mutation at residue Trp^{74}, which truncates the protein near the beginning of this C-terminal hydrophilic loop (8) (see Fig. 1). To explore this hypothesis, we have investigated the functional effects of mutations in this C-terminal domain. Our data indicate that both the overall length of the exposed C terminus of PEN-2 and the sequence of a highly conserved DYLSF motif within this domain are important for binding of PEN-2 to other components of the presenilin complex and for proper function of the complexes during NCT maturation, presenilin endoproteolysis, and Aβ secretion.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids and Stable Cells—A cDNA clone encoding human wild-type PEN-2 was generated by PCR from a human brain cDNA library. Using this cDNA as a template, constructs encoding an N-terminal V5-tagged wild-type and mutant PEN-2 were obtained by PCR using 5′-GAATTCCAGCTATGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGAACCTGGAGCGAGTGTCCAAT-3′ as a forward primer, and 5′-CTCGAGCTCAGGGGGTGCCCAGGGGTAT-3′ for wild-type PEN-2, 5′-CTCGAGCTCACCGGTAGATCTGGAAGAT-3′ for del84–101, 5′-CTCGAGCTCACCCAAGGGCACCCCAGCG-3′ for del90–101, and 5′-CTCGAGCTCAGGTGAAGGAGAGGTAGTC-3′ for del96–101 as a reverse primer. Mutagenesis primers 5′-CGCTGGGGTGCCCTTGGGGCCGCCGCCGCCGCCACCATACCCCTGGGCACCCCCTGACTCGAG-3′ and 5′-CGCTGGCCTGCCCTTGGGACCATACCCCTGGGCACCCCCTGACTCGAG-3′ were used for the generation of constructs encoding AAAAA and del90–94, respectively. In agreement with previous reports (8, 16) preliminary experiments (data not shown) revealed that when PEN-2 was expressed from a vector that included a C-terminal Myc/His tag, the resultant C-terminally tagged PEN-2 protein was not stable and was not fully functionally active. Consequently, all constructs were tagged with V5 at

* This work was supported by grants from the Canadian Institutes of Health Research, the Howard Hughes Medical Institute, the Canadian Genetic Diseases Network, the Scottish Rite Charitable Foundation of Canada, and the Alzheimer Society of Ontario. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed.

[1] The abbreviations used are: APP, β-amyloid precursor protein; PS, presenilin; NCT, nicastrin; Aβ, amyloid β-peptide; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

the N terminus and, following direct sequence verification, were ligated into the expression vector pcDNA4 (Invitrogen). The N-terminal V5 tag partially interferes with the recognition epitope (residues 1–26). However, transient transfection of N-terminal V5-tagged wild-type PEN-2 constructs into cells in which PEN-2 expression had been suppressed by RNAi (see below) confirmed that the V5-tagged protein was expressed and fully complemented the loss of endogenous PEN-2, resulting in restoration of PS1 endoproteolysis, nicastrin maturation, and Aβ production. HEK293 cells stably expressing APP_{swe} were transfected with these plasmids or with empty vector (as a negative control) using LipofectAMINE (Invitrogen). Stable transfectant cells were selected in 150 μg/ml zeocin. For a stable PEN-2 knock-down cell line, the oligonucleotides against PEN-2 160–180 were ligated with pSUP vector (Oligoengine) as per the manufacturer's instruction. After confirming the sequence, pSUP/PEN-2 160–180 and pCDNA6/V5 vector (Invitrogen) were stably transfected into a HEK293 clone stably expressing APP_{swe}, using LipofectAMINE and were selected with blasticidin (10 μg/ml). For rescue analysis, the cDNA sequence of all constructs corresponding to PEN-2 160–180 were mutated without changing the amino acid sequence using mutagenesis primer: 5′-GAACAGAGCCAAATCAAAGGTTACGTATGGCGCTCAGCTGTGGGCTTCCTCTTC-3′. These cDNA constructs were also validated by direct nucleotide sequencing.

Antibodies—The following antibodies were used in this study: anti-PEN-2 antibody (a gift from Dr. Thinakaran) (17), anti-V5 antibody (Invitrogen), anti-PS1 N-terminal antibody (Ab14), anti-Myc antibody (Invitrogen), anti-APP C-terminal antibody against the residues 676–695 of APP_{695} (Sigma), anti-APP N-terminal antibody against the residues 99–126 of APP (Chemicon), anti-APP C-terminal antibody 6E10 (Signet), anti-APH-1–1 antibody (21), anti-nicastrin antibody (Sigma), anti-calnexin antibody (Transduction Laboratories), and anti-GM130 antibody (Transduction Laboratories). The Aβ enzyme-linked immunosorbent assay studies used antibodies JRF/cAβ40/10, JRF/cAβ42/26, and JRF/Aβtot/1, which were gifts from Dr. Marc Mercken (Johnson and Johnson Pharmaceutical Research and Development/Janssen Pharmaceutica, Beerse, Belgium).

Immunoprecipitation—Membrane fractions isolated from HEK293 cells were lysed with 1% CHAPSO (Sigma) in a buffer containing 10% glycerol. After preclearing with protein A-Sepharose CL-4B (Amersham Biosciences) for 1 h, the cell lysates were incubated with the appropriate antibody. The immunoprecipitants were recovered by overnight incubation at 4 °C with protein A-Sepharose CL-4B. The beads were washed three times with 1% CHAPSO in the same buffer.

Cycloheximide Treatment—The stable transfectants of the empty vector, wild-type, and mutant PEN-2 constructs were incubated in the presence of cycloheximide (30 μg/ml) for 6, 12, or 24 h and then analyzed by immunoblotting with anti-V5 or anti-PEN-2 antibodies.

Gradient Centrifugation—For subcellular fractionation, the membrane fractions were isolated from HEK293 cells as described previously (22) and were applied on a step gradient consisting of 1 ml each of 30, 25, 20, 15, 12.5, 10, 7.5, 5, and 2.5% (v/v) iodixanol (Accurate). After centrifugation, 0.8-ml fractions were collected and analyzed by Western blotting. Specific marker proteins of the endoplasmic reticulum (calnexin) and Golgi (GM130) were detected using monoclonal antibodies.

Aβ Assays—$Aβ_{40}$ and $Aβ_{42}$ were measured as described by enzyme-linked immunosorbent assay (23, 24), using conditioned medium collected from PEN-2 knock-down HEK293 cells transiently transfected with either wild-type PEN-2, mutant PEN-2, or an empty vector as a control. The values of both $Aβ_{40}$ and $Aβ_{42}$ were compared by Student's *t* test. Because the DYLSF mutants represent a population of related mutants that are not fully independent of each other, we analyzed the mutations involving the DYLSF domain including the deletion mutants as a single population to avoid the risk of false positive results arising from multiple comparisons. The AAA (IPG), del96–101, KDAS, and del100–101 mutants were individually compared with wild-type PEN-2 because they represent a small number of tests of a different hypothesis (that the length of the C terminus is critical).

RESULTS

Functional Domain Analysis—To explore the function of the conserved residues at the C terminus of PEN-2, we initially created three overlapping deletion mutants: del84–101, del90–101, and del96–101 (Fig. 1*B*). When these cDNAs were stably transfected into HEK293 cells stably expressing APP_{swe}, they produced proteins of the expected molecular weight (Fig. 2*A*, *top panel*). Immunoprecipitation with PS1 revealed that the wild-type exogenous V5-tagged PEN-2 co-precipitated with PS1 (Fig. 2*B*, *panel 1*). The wild-type V5-tagged PEN-2 protein also displaced endogenous PEN-2 (Fig. 2, *A*, *panel 2*, and *B*, *panel 2*). (Note that the N-terminal V5 tag partially interferes with the recognition epitope (residues 1–26), so that the V5-tagged and endogenous PEN-2 peptides can be easily distinguished by their differential affinities for anti-V5 antibodies and the antibody to residues 1–26 of PEN-2. Note also that the interference with the anti-PEN-2 antibody recognition sequence has no functional effect because transient transfection of N-terminal V5-tagged wild-type PEN-2 constructs into cells in which endogenous PEN-2 expression had been suppressed by RNAi (see below) fully complemented the loss of endogenous PEN-2, resulting in restoration of PS1 endoproteolysis, nicastrin maturation, and Aβ production.) In contrast, mutant PEN-2 with a deletion after residue 96 (del96–101) moderately reduced the binding of the exogenous PEN-2 to PS1, nicastrin, and APH-1 and was able to partially displace endogenous PEN-2. However, deletion of residues 84–101 or 90–101 dramatically reduced the amount of exogenous PEN-2 that was co-precipitated with PS1 (Fig. 2*B*, *panel 1*). Furthermore, because these overexpressed deletion mutant PEN-2 molecules did not bind PS1 (Fig. 2*B*, *panel 1*), they also failed to displace the endogenous PEN-2 (Fig. 2*A*, *panel 2*), which therefore continued to interact and co-precipitate with PS1 (Fig. 2*B*, *panel 2*). As would be predicted, the del84–101 and del90–101 did not co-precipitate with either APH-1 or nicastrin (Fig. 2*B*, *panels 4* and *5*). These observations suggest that the C terminus, and in particular residues 90–96, which contain the highly conserved DYLSF motif, may either: 1) be essential for the stability of PEN-2; 2) be essential for the proper trafficking of PEN-2; or 3) be involved in binding of PEN-2 to other components of the presenilin complex.

To explore whether these mutants caused destabilization of PEN-2, we assessed cellular PEN-2 levels following cycloheximide blockade of protein synthesis in HEK293 cells. Endogenous PEN-2, wild-type PEN-2 tagged at the N terminus, and del96–101 mutant constructs had equivalent stability with a half-life of ~9–10 h (Fig. 3). In contrast, both the del84–101 and del90–101 deletion mutants were highly unstable, with a half-life of approximately 4 h. This result is not surprising because the del84–101 and del90–101 molecules are not incorporated into presenilin complexes and are therefore not stabilized. However, it is also possible that these proteins might be intrinsically unstable and that they are simply degraded before they can interact with the other components of the presenilin complexes. To exclude this possibility, we transiently expressed V5-tagged wild-type and mutant PEN-2 peptides in PS1 and PS2 double knock-out ES cells, where the stabilizing influence of presenilin complexes are not present, thereby allowing a direct comparison of the intrinsic stability of the wild-type and deletion mutant peptides. In PS1::PS2 double knock-out ES cells, the stability of transiently expressed wild-type and mutant PEN-2 proteins were equivalently reduced to less than 4 h, confirming that the mutant PEN-2 proteins were not intrinsically unstable (data not shown).

To explore whether these mutants caused mistrafficking of PEN-2, we investigated the subcellular distribution of endogenous and exogenous PEN-2, using biochemical fractionation on iodixanol gradients. This study revealed that endogenous PEN-2 is predominantly located in intracellular membrane structures, especially those of the endoplasmic reticulum (co-localizing with calnexin) and the Golgi network (co-localizing with GM130) (Fig. 4, *panel 3*). The distributions of exogenous V5-tagged wild-type PEN-2 as well as the del84–101, del90–101, and del96–101 mutants were essentially identical and just slightly shifted with regard to the distribution of endogenous

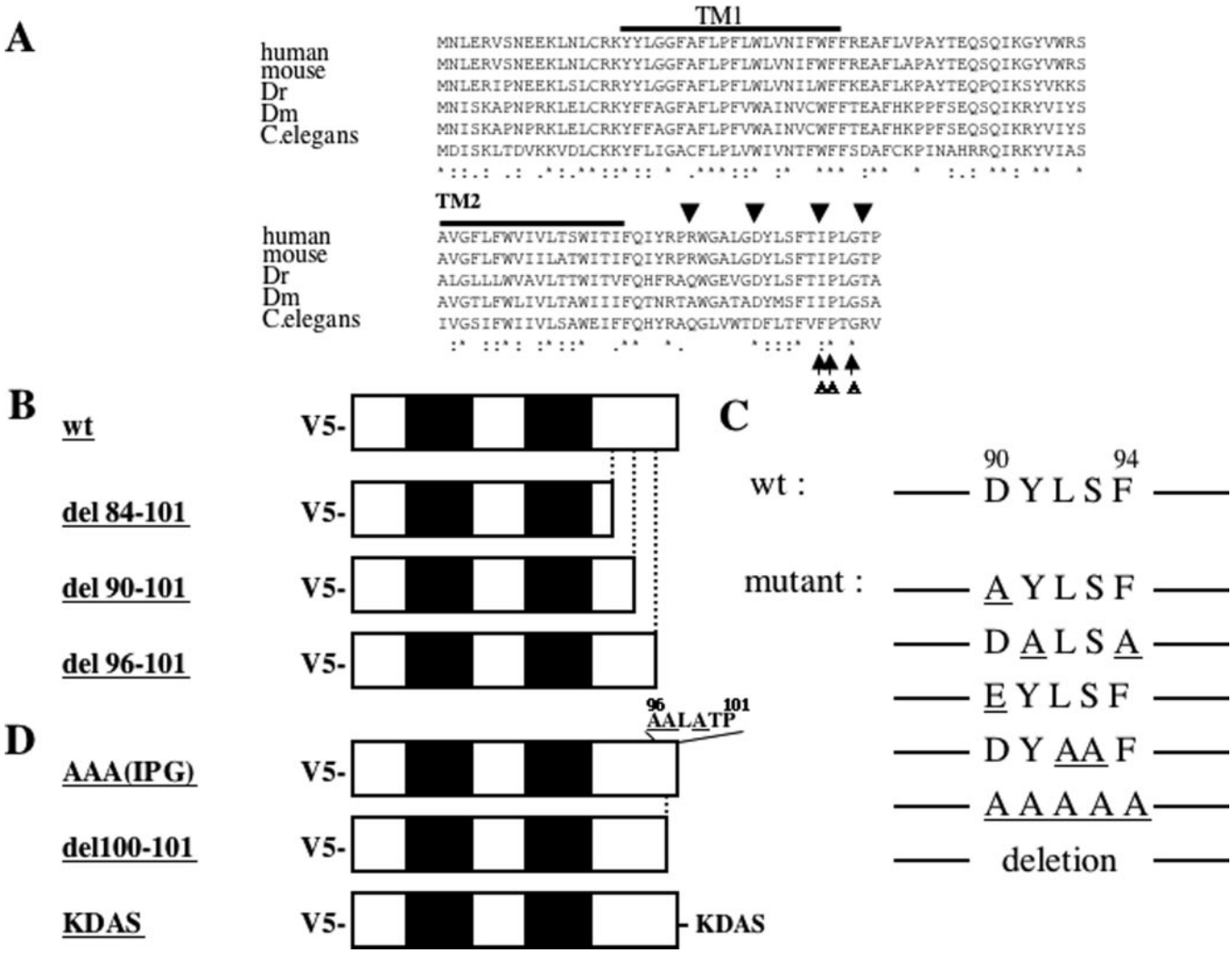

FIG. 1. *A*, alignment of PEN-2 proteins. Transmembrane domains are indicated by *underlining*. The *arrowheads* and *arrows* indicate the truncated positions for a series of deletion mutants and the conserved residues among the last six residues. *B*, the schematic structure of wild-type or a series of large deletion mutants. *C*, the mutants in DYLSF domains. The substituted alanines are *underlined*. *D*, the schematic structure of AAA (IPG), KDAS, and deletion mutant at residues 100 and 101. *wt*, wild type.

wild-type PEN-2 because of the presence of the additional V5 tag and/or because of modest overexpression (Fig. 4, *panels 4–7*).

Previous studies have suggested that PEN-2 plays a role in presenilin endoproteolysis, nicastrin maturation, and Aβ generation (8, 13, 15–18). Several of the mutants described above, even when stably overexpressed, are unable to displace endogenous PEN-2 from functional complexes. As a result, it is impossible to measure the functional properties of these PEN-2 mutants in cells expressing endogenous PEN-2. To circumvent this problem, we created PEN-2 knock-down cells by stable expression of PEN-2 siRNA. In these PEN-2 knock-down cells, endogenous PEN-2 protein is moderately (clone 10) or severely depleted (clones 13, 17, and 19) (Fig. 5, *top panel*). In agreement with previously reported studies using transient siRNA expression of PEN-2 (16, 18), our PEN-2 stable knock-down cells showed significant accumulations of immature glycosylated nicastrin (Fig. 5, *panel 2*), full-length PS1 (Fig. 5, *panel 3*), and APP-CTF (Fig. 5, *panel 6*). These PEN-2 knock-down cells also showed significant reductions in the amount of mature PS1-NTF, and these reductions in PS1-NTF levels were in proportion to the degree of depletion of PEN-2. As expected from previous work showing that APH-1 is a stable initial scaffolding molecule (21), APH-1 (Fig. 5, *panel 4*) and full-length APP (Fig. 5, *panel 5*) were not affected by PEN-2 knock-down. However, Aβ secretion was significantly reduced compared with mock cells (Fig. 5, *panel 7*).

To assess the interaction of these PEN-2 deletion mutants (del84–101, del90–101, and del96–101) with nicastrin, presenilins, and APH-1 in the absence of interference from endogenous PEN-2, we transiently expressed each of these deletion mutants in the PEN-2 knock-down cells. As expected from the initial immunoprecipitation experiments reported above, both the del84–101 and the del90–101 mutants bound poorly to the other presenilin components even in the absence of endogenous PEN-2 (Fig. 6, *lanes 3* and *4*). Again in agreement with the results reported above, the binding of the del96–101 mutant to nicastrin, PS1, and APH-1 was reduced, but less severely than binding of del84–101 and del90–101 mutants. To further explore the sequence specificity of the apparently important conserved DYLSF domain at residues 90–94, we created a deletion mutant (del90–94), and a series of missense mutations in which either single residues, two residues, or five residues within the 90–94 domain were mutated to alanine (Fig. 1*C*). We then repeated the co-immunoprecipitation studies. Deletion of residues 90–94 or missense mutation of all five residues to alanine caused a dramatic reduction in the interaction between presenilin 1, nicastrin, and APH-1 (Fig. 6, *lanes 10* and *11*). In contrast, mutation of a single residue or two residues had variable but generally less severe effects on the interaction

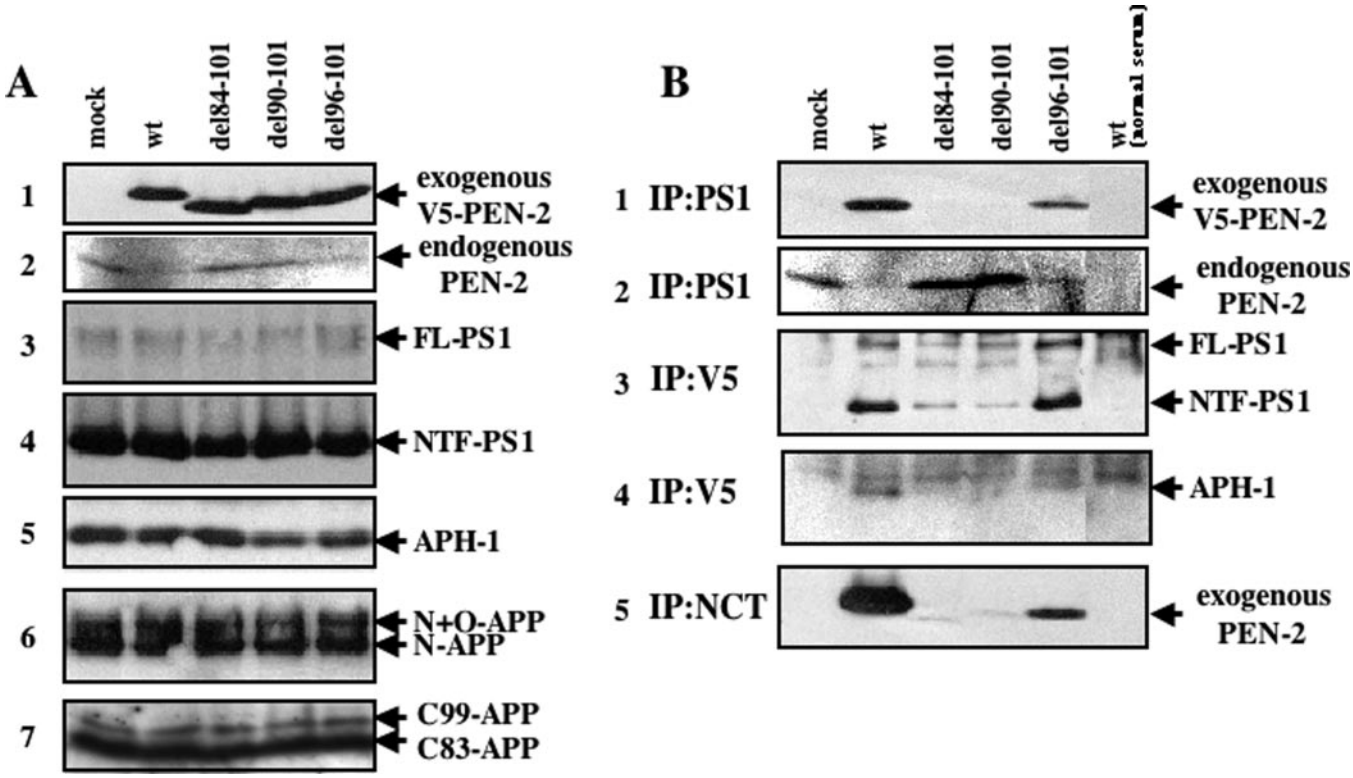

FIG. 2. *A*, the expression levels of components in presenilin complex in wild-type or mutant PEN-2 stable transfectant. *Lane 1*, mock; *lane 2*, wild type; *lane 3*, del84–101; *lane 4*, del90–101; *lane 5*, del96–101. Endogenous PEN-2 in wild-type and del96–101 PEN-2 transfected cells were significantly reduced in the cell lysates (*panel 2*), whereas presenilin (*panels 3* and *4*), APH-1 (*panel 5*), and APP (*panels 6* and *7*) are unchanged. *B*, the binding to presenilin (*panels 1* and *3*), APH-1 (*panel 4*), or nicastrin (*panel 5*) in del84–101 and del90–101 significantly reduced, whereas the binding in del96–101 is not affected much. The binding between PS1 and endogenous PEN-2 (*panel 2*) compensates the failure in the binding to mutant PEN-2 (*panel 1*) in del84–101 and del90–101. *IP*, immunoprecipitation.

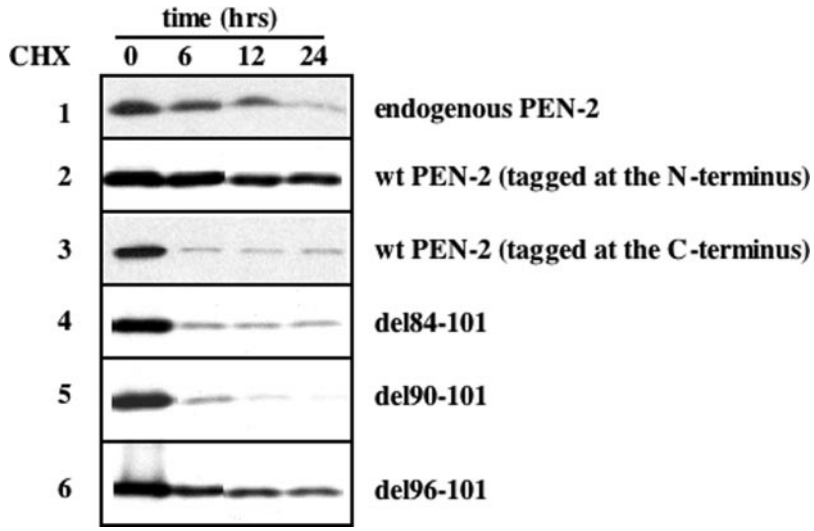

FIG. 3. **The deletion and the tagging at C terminus accelerate the degradation of the PEN-2 proteins.** The stable transfectants expressing mock (*panel 1*), wild-type PEN-2 tagged at N terminus (*panel 2*), wild-type PEN-2 tagged at the C terminus (*panel 3*), del84–101 (*panel 4*), del90–101 (*panel 5*), and del96–101 (*panel 6*) were treated with cycloheximide for indicated time (0, 6, 12, and 24 h), and endogenous or exogenous PEN-2 in total cell lysates were analyzed. *wt*, wild type.

of PEN-2 with the other components as measured by co-immunoprecipitation (Fig. 6, *lanes 6–9*).

To assess the functional properties of these PEN-2 mutants, we next examined their ability to rescue presenilin complex formation and maturation in PEN-2 knock-down cells. Transient transfection of wild-type PEN-2 in PEN-2 knock-down cells significantly rescued nicastrin maturation, PS endoproteolysis, and Aβ secretion (Fig. 7, *panels 1*, *2*, and *5*), and was associated with slight reduction of APP-CTF (Fig. 7, *panel 4*). In contrast, transient expression of the AAAAA poly-substitution of residues 90–94, (Fig. 7) and the del84–101, (Fig. 8) del90–94, (Fig. 7), and del90–101 (Fig. 8) deletion mutants, which do not bind efficiently to other presenilin complex components, had little or no ability to rescue presenilin complex maturation and/or Aβ secretion (Fig. 7, *panels 1*, *2*, and *5*). The sAPP was unchanged in these cells (Fig. 7, *panel 6*), indicating that these mutants had not altered APP maturation, trafficking, or cleavage by α- and β-secretase. These effects were robust, being replicated in all three independent PEN-2 knock-down cells. In contrast to the profound loss-of-function effects of the AAAAA and del90–94 mutants, mutation of only one or two residues of the DYLSF domain caused variable but milder reductions in nicastrin maturation and PS endoproteolysis and Aβ secretion (Figs. 8, *lanes 6–9*, and 9*A*, *lanes 6–9*), and the degree of reduction was commensurate with the effect of each mutant on the interaction with other presenilin complex

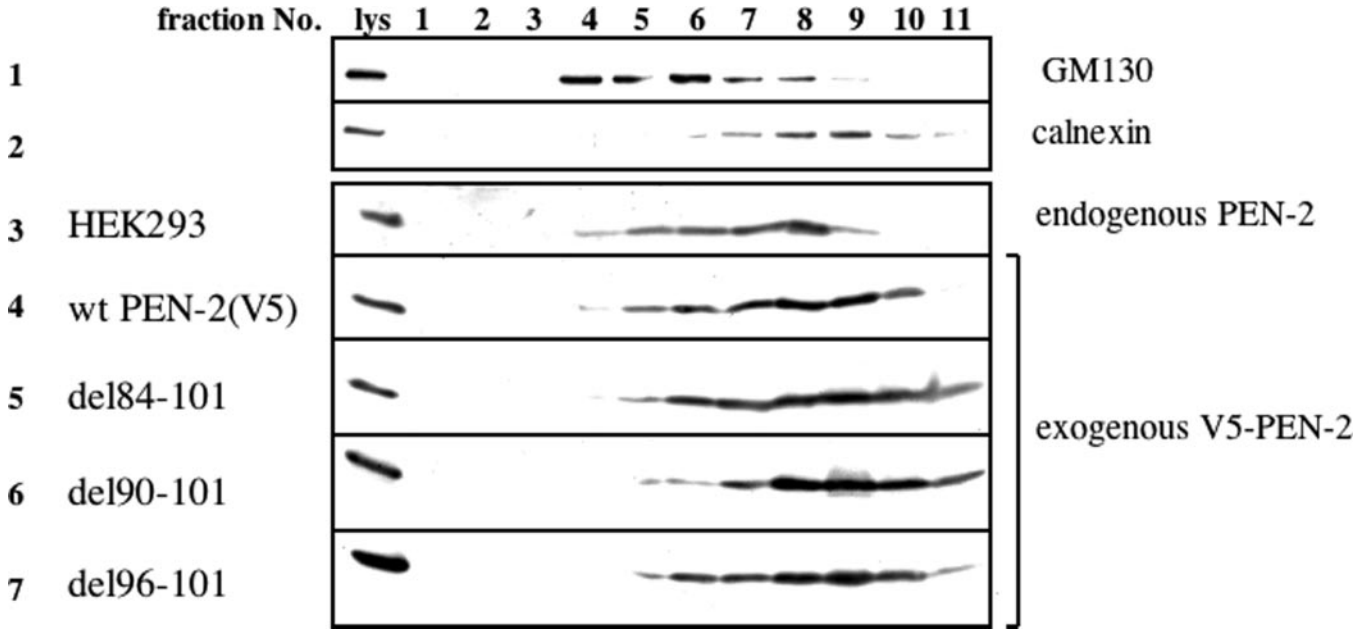

FIG. 4. **The deletion at the C terminus does not change the subcellular distribution.** Total cell lysates from the stable transfectants expressing mock (*panel 3*), wild-type PEN-2 tagged at the N terminus (*panel 4*), del84–101 (*panel 5*), del90–101 (*panel 6*), and del96–101 (*panel 7*) were fractionated with iodixanol gradient fractionation (*lane 1*, 2.5%, to *lane 11*, 30%). GM130 (*panel 1*) and calnexin (*panel 2*) were used as a marker protein of Golgi apparatus and endoplasmic reticulum, respectively.

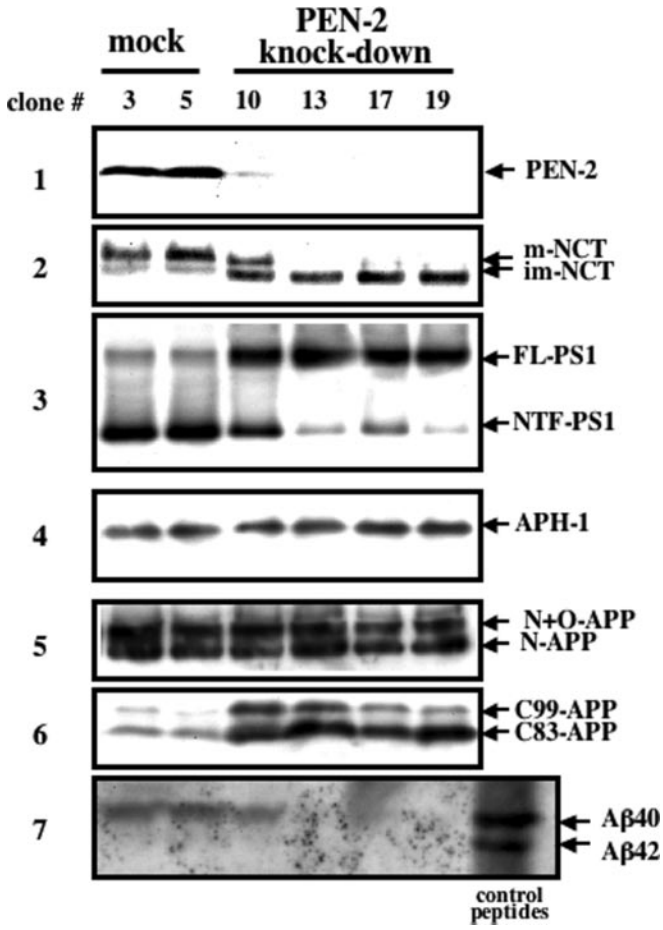

FIG. 5. **PEN-2 knock-down cells fail in NCT maturation, PS endoproteolysis, and Aβ secretion, resulting in the accumulation of APP-CTF.** In mock (clones 3 and 5) and PEN-2 knock-down cells (clones 10, 13, 17, and 19), PEN-2, NCT, PS1, APH-1, APP, and Aβ secretion were analyzed (*panels 1–7*, respectively).

components. For instance the most conservative mutation DYLSF → EYLSF caused modest reductions in binding, complex maturation, and Aβ secretion (Figs. 8, *lane 8*, and 9, *lane 8*).

Despite the fact that the del96–101 mutant binds more efficiently to other presenilin complex components than do the remaining PEN-2 deletion mutations, the del96–101 mutant only partially rescued presenilin complex maturation and function (with activity being equivalent to that of the other deletion mutations). We reasoned that this reduced rescue activity of the del96–101 mutant despite its only mild reduction in binding could occur if there are specific residues in the 96–101 domain that are critical for PEN-2 activity. There are three highly conserved residues in this domain: Ile at codon 96, Pro at codon 97, and Gly at codon 99. An alternate possibility is that the absolute length of the C terminus is critical to the function of PEN-2, and this deletion (and all of the other deletions) has altered this property. This latter possibility is supported by the prior report that exogenous wild-type C-terminally tagged PEN-2 failed to complement loss-of-function alleles in the C. *elegans* PEN-2 gene (8) and by our earlier observation that PEN-2 constructs tagged with C-terminal Myc epitopes were unstable (Fig. 3). To resolve this dichotomy, we made three constructs: one with alanine missense mutations at all three conserved residues, one with a four-residue (KDAS) C-terminal addition, and one with a deletion of the two non-conserved C-terminal residues, AAA (IPG), KDAS, and del100–101, respectively (Fig. 1*D*). Immunoprecipitation experiments following transient expression of these constructs in PEN-2 knock-down HEK293 cells revealed that the AAA (IPG) mutant bound to the other presenilins complex components comparably with that of wild-type PEN-2. However, the KDAS and the del100–101 mutant had a moderate reduction in binding that was similar in magnitude to the reduction in binding of the del96–101 mutant (Fig. 6, *lane 15* for del100–101; not shown for KDAS). As would be expected from these binding assays, transient expression of the AAA (IPG) mutant nearly completely rescued both nicastrin maturation, PS endoproteolysis, and Aβ secretion, whereas the KDAS and the del100–

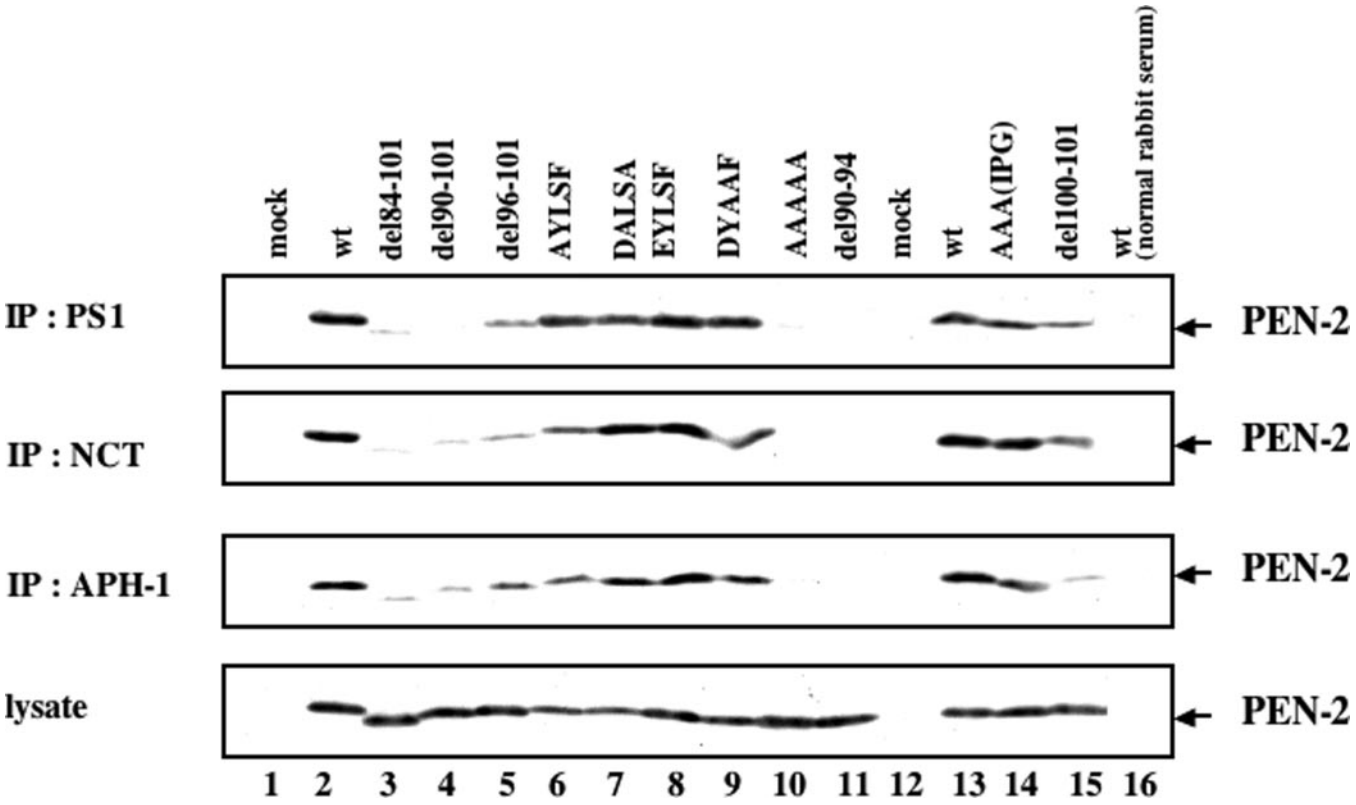

FIG. 6. **The binding of PEN-2 to other components is severely disturbed in del84–101, del90–101, AAAAA, and del90–94 mutants and moderately in del96–101 and del100–101 mutants.** Using PEN-2 knock-down cells transiently transfected with mock, wild-type, del84–101, del90–101, del96–101, AYLSF, DALSA, EYLSF, DYAAF, AAAAA, del90–94, AAA (IPG), or del100–101(*lanes 1–11*, 14, and 15), pulled-down PEN-2 with anti-PS, anti-NCT, or anti-APH-1 antibody was analyzed. *Lane 16* is a negative control using normal rabbit serum. The *bottom panel* shows the expression level of wild-type and mutant PEN-2. *wt*, wild type; *IP*, immunoprecipitation.

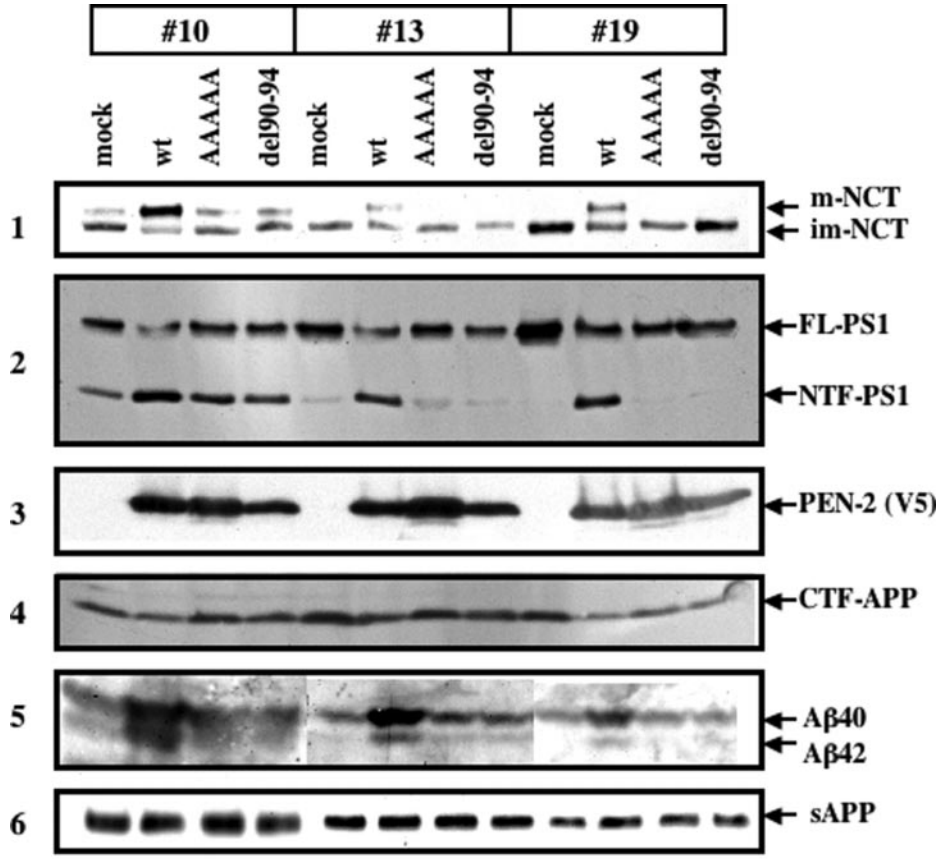

FIG. 7. **Wild-type PEN-2 rescue NCT maturation, PS endoproteolysis, and Aβ secretion but not AAAAA or del90–94 mutant.** Mock (*lane 1*), wild-type (*lane 2*), AAAAA (*lane 3*), or del90–94 (*lane 4*) mutant was transfected into PEN-2 knock-down cells (clones 10, 13, and 19) and then NCT, PS1, PEN-2, APP-CTF, Aβ secretion, and sAPP (panels from *top* to *bottom*) in the conditioned media were analyzed. *wt*, wild type.

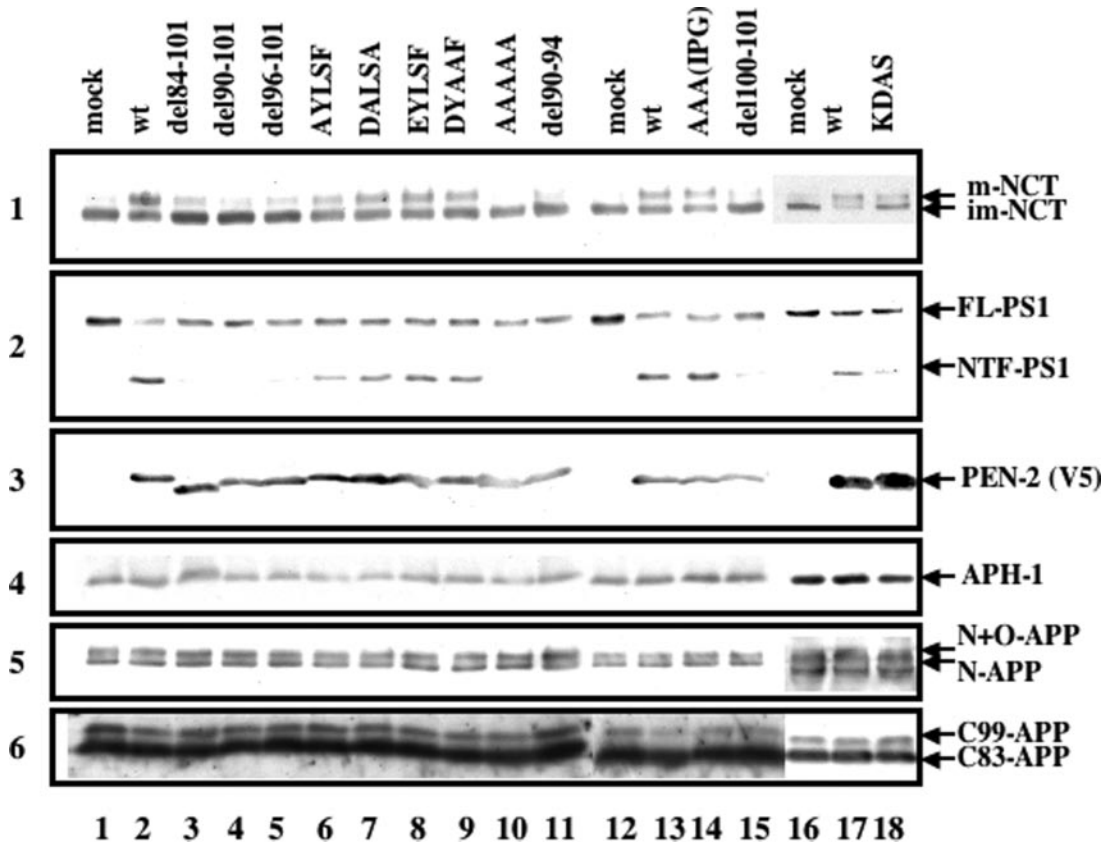

FIG. 8. **NCT maturation and PS proteolysis were rescued by partial substitution mutants in DYLSF domain and AAA (IPG) mutant not by del84–101, del90–101, del96–101, AAAAA, del90–94, del100–101, and KDAS.** NCT, PS1, PEN-2, APH-1, and APP (panels from *top* to *bottom*) were analyzed in PEN-2 knock-down cells transiently transfected with mock, wild-type, del84–101, del90–101, del96–101, AYLSF, DALSA, EYLSF, DYAAF, AAAAA, del90–94, AAA (IPG), KDAS, or del100–101(*lanes 1–11*, *14*, *15*, and *18*). *wt*, wild type.

101 mutant had minimal effect (Figs. 8, *lanes 14*, *15*, and *18*, and 9*A*, *lanes 14*, *15*, and *18*). These data indicate that, in addition to the sequence and size of the conserved DYLSF motif at residues 90–94, another critical factor for nicastrin maturation and PS endoproteolysis is the length of the C terminus (Fig. 9).

DISCUSSION

Our data reveal that PEN-2 plays a role in nicastrin maturation, PS endoproteolysis, and Aβ secretion and are in agreement with several previous reports (8, 15, 16). However, our data also reveal that the C terminus is important in: 1) modulating the interaction of PEN-2 with the other presenilin complex components, 2) supporting nicastrin maturation and PS endoproteolysis, and 3) supporting Aβ secretion. Our intensive mutation analysis suggests that both the sequence of the DYLSF domain and the absolute length of the C terminus are important for these activities.

The deletion mutants, and to a lesser degree the individual missense mutations of the DYLSF domain, affect the binding of PEN-2 to the presenilin complex (and as a result they also affect presenilin complex assembly, maturation, and γ-secretase activity to an equivalent degree). On the other hand, modulation of the length (but not the sequence) of the exposed extreme C terminus of PEN-2 has less impact on PEN-2 binding to the complex but does have a significant affect on presenilin complex maturation and γ-secretase activity. We interpret these findings to mean that: 1) the amino acid sequence of the DYLSF domain is important for the physical binding of PEN-2 to the other presenilin complex components and 2) the absolute length of the C terminus is important for PEN-2 activity within functional presenilin complexes.

The DYLSF domain has no conserved homology by BLAST database search. However, the YLSF sequence matches the tyrosine-based sorting signal (Y*XX*[LMVIF]) in the cytoplasmic tail of several proteins including the mu subunit of the AP (adaptor protein) complex, TGN38, epidermal growth factor receptor, mannose 6-phosphate receptor, and furin (25–28). The tyrosine-based sorting motifs in these proteins are responsible for targeting these proteins to various organelles (25–28). Although the YLSF motifs in these examples work in the cytoplasm, it is conceivable that the DYLSF domain of PEN-2 could also be involved in targeting PEN-2 to nascent presenilin complexes or may simply be a binding motif required for the interaction of PEN-2 with other proteins in the PS1 complex.

The fact that the absolute length (but not the sequence) of the C terminus of PEN-2 is critical for presenilin complex function but does not affect binding of PEN-2 to the complex suggests that the C terminus may be involved in maintaining non-sequence-based spatial interactions (*e.g.* spatial packing) with other components of the presenilin complex. A similar critical sensitivity to the length of the C terminus has been reported for both PS1 and PS2 (29). Because the C termini of both PS1/PS2 and PEN-2 are oriented in the cytoplasm, additional experiments will be needed to discern whether the C termini of these two proteins in fact interact, whether directly with each other or with a third protein. However, taken together these data raise the possibility that PEN-2 might function as an important linker/spacer molecule that maintains spatial interactions between various presenilin complex components.

Acknowledgment—Antibodies JRF/cAβ40/10, JRF/cAβ42/26, and JRF/Aβtot/1 were a gift from Dr. Marc Mercken (Johnson and Johnson

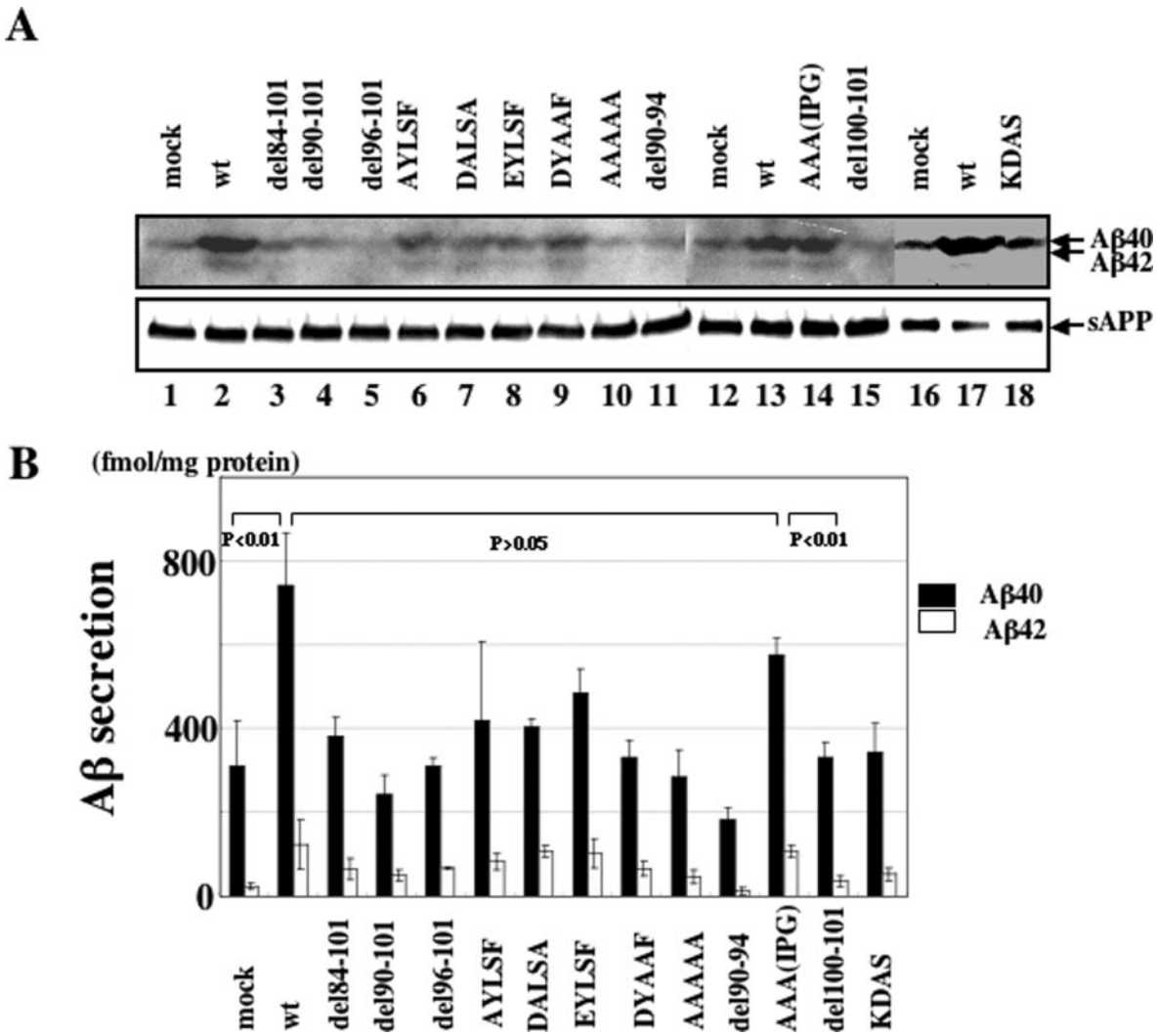

FIG. 9. *A*, Aβ secretion was fully rescued by wild-type PEN-2 and AAA (IPG) and partially rescued by partial substitution mutants in the DYLSF domain. PEN-2 knock-down cells transiently transfected with mock, wild-type, del84–101, del90–101, del96–101, AYLSF, DALSA, EYLSF, DYAAF, AAAAA, del90–94, AAA (IPG), del100–101, or KDAS (*lanes 1–11*, *14*, *15*, and *18*). *B*, secreted Aβ in the condition medium was measured by enzyme-linked immunosorbent assay ($n \geq 3$ independent replications). *wt*, wild type.

Pharmaceutical Research and Development/Janssen Pharmaceutica, Beerse, Belgium).

REFERENCES

1. Sisodia, S. S., and St George-Hyslop, P. (2002) *Nat. Rev. Neurosci.* **3,** 281–290
2. Haass, C., and Steiner, H. (2002) *Trends Cell Biol.* **12,** 556–562
3. Selkoe, D., and Kopan, R. (2003) *Annu. Rev. Neurosci.* **26,** 565–597
4. De Strooper, B. (2003) *Neuron* **38,** 9–12
5. Yu, G., Chen, F., Levesque, G., Nishimura, M., Zhang, D. M., Levesque, L., Rogaeva, E., Xu, D., Liang, Y., Duthie, M., St George-Hyslop, P., and Fraser, P. E. (1998) *J. Biol. Chem.* **273,** 16470–16475
6. Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y. Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D. S., Holmes, E., Milman, P., Liang, Y., Zhang, D. M., Xu, D. M., Sato, C., Rogaev, E., Smith, M., Janus, C., Zhang, Y., Aebersold, R., Farrer, L. S., Sorbi, S., Bruni, A., Fraser, P., and St George-Hyslop, P. (2000) *Nature* **407,** 48–54
7. Goutte, C., Tsunozaki, M., Hale, V. A., and Priess, J. R. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 775–779
8. Francis, R., McGrath, G., Zhang, J., Ruddy, D. A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M. C., Parks, A. L., Xu, W., Li, J., Gurney, M., Myers, R. L., Himes, C. S., Hiebsch, R., Ruble, C., Nye, J. S., and Curtis, D. (2002) *Dev. Cell* **3,** 85–97
9. Gu, Y., Sanjo, N., Chen, F., Hasegawa, H., Petit, A., Ruan, X., Li, W., Shier, C., Kawarai, T., Schmitt-Ulms, G., Westaway, D., St George-Hyslop, P., and Fraser, P. E. (2004) *J. Biol. Chem.* **279,** 31329–31336
10. Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2003) *Nat. Cell Biol.* **5,** 486–488
11. Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Fraser, P. E., Rommens, J. M., and St George-Hyslop, P. (1995) *Nature* **375,** 754–760
12. Rogaev, E., Sherrington, R., Rogaeva, E. A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., Chumakov, I., Cohen, D., Lannfelt, L., Fraser, P. E., Rommens, J. M., and St George-Hyslop, P. (1995) *Nature* **376,** 775–778
13. Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) *Nature* **422,** 438–441
14. Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 6382–6387
15. Steiner, H., Winkler, E., Edbauer, D., Prokop, S., Basset, G., Yamasaki, A., Kostka, M., and Haass, C. (2002) *J. Biol. Chem.* **277,** 39062–39065
16. Prokop, S., Shirotani, K., Edbauer, D., Haass, C., Steiner, H., Crystal, A. S., Morais, V. A., Fortna, R. R., Carlin, D., Pierson, T. C., Wilson, C. A., Lee, V. M., Doms, R. W., Bergman, A., Hansson, E. M., Pursglove, S. E., Farmery, M. R., Lannfelt, L., Lendahl, U., Lundkvist, J., Naslund, J., Fraering, P. C., LaVoie, M. J., Ye, W., Ostaszewski, B. L., Kimberly, W. T., Selkoe, D. J., Wolfe, M. S., Lee, S. F., Shah, S., Yu, C., Wigley, W. C., Li, H., Lim, M., Pedersen, K., Han, W., Thomas, P., Hao, Y. H., Yu, G., Pijak, D. S., Baulac, S., Strahle, J., Xia, W., Warriss, P. D., Brown, S. N., Knowles, T. G., Guo, M., Hong, E. J., Fernandes, J., Zipursky, S. L., Hay, B. A., Costa, J., Nyabi, O., Bentahir, M., Horre, K., Herreman, A., Gottardi-Littell, N., Van Broeckhoven, C., Merchiers, P., Spittaels, K., Annaert, W., De Strooper, B., Kim, S. H., Ikeuchi, T., Sisodia, S. S., Berezovska, O., Ramdya, P., Skoch, J., Bacskai, B. J., Hyman, B. T., Hu, Y., and Fortini, M. E. (2004) *J. Biol. Chem.* **23,** 23
17. Luo, W. J., Wang, H., Li, H., Kim, B. S., Shah, S., Lee, H. J., Thinakaran, G., Kim, T. W., Yu, G., and Xu, H. (2003) *J. Biol. Chem.* **278,** 7850–7854. 8
18. Kim, S. H., Ikeuchi, T., Yu, C., and Sisodia, S. S. (2003) *J. Biol. Chem.* **278,** 33992–34002
19. Crystal, A. S., Morais, V. A., Pierson, T. C., Pijak, D. S., Carlin, D., Lee, V. M., and Doms, R. W. (2003) *J. Biol. Chem.* **278,** 20117–20123. Epub 2003 Mar 14
20. Bergman, A., Hansson, E. M., Pursglove, S. E., Farmery, M. R., Lannfelt, L., Lendahl, U., Lundkvist, J., Naslund, J., Fraering, P. C., LaVoie, M. J., Ye, W., Ostaszewski, B. L., Kimberly, W. T., Selkoe, D. J., and Wolfe, M. S.

(2004) *J. Biol. Chem.* **279,** 16744–16753
21. Gu, Y., Chen. F., Sanjo, N., Kawarai, T., Hasegawa, H., Duthie, M., Li, W., Ruan, X., Luthra, A., Mount, H. T., Tandon, A., Fraser, P. E., and St George-Hyslop, P. (2003) *J. Biol. Chen.* **278,** 7374–7380
22. Chen, F., Yang, D. S., Petanceska, S., Yang, A., Tandon, A., Yu, G., Rozmahel, R., Ghiso, J., Nishimura, M., Zhang, D. M., Kawarai, T., Levesque, G., Mills, J., Levesque, L., Song, Y. Q., Rogaeva, E., Westaway, D., Mount, H., Gandy, S., St George-Hyslop, P., and Fraser, P. E. (2000) *J. Biol. Chem.* **275,** 36794–36802
23. Chishti, M. A., Yang, D. S., Janus, C., Phinney, A. L., Horne, P., Pearson, J., Strome, R., Zuker, N., Loukides, J., French, J., Turner, S., Lozza, G., Grilli, M., Kunicki, S., Morissette, C., Paquette, J., Gervais, F., Bergeron, C., Fraser, P. E., Carlson, G. A., St George-Hyslop, P., and Westaway, D. (2001) *J. Biol. Chem.* **276,** 21562–21570
24. McLaurin, J., Cecal, R., Kierstead, M. E., Tian, X., Phinney, A. L., Manea, M., French, J. E., Lambermon, M. H., Darabie, A. A., Brown, M. E., Janus, C., Chishti, M. A., Horne, P., Westaway, D., Fraser, P. E., Mount, H. T., Przybylski, M., and St George-Hyslop, P. (2002) *Nat. Med.* **8,** 1263–1269
25. Boll, W., Rapoport, I., Brunner, C., Modis, Y., Prehn, S., and Kirchhausen, T. (2002) *Traffic* **3,** 590–600
26. Dell'Angelica, E. C., and Payne, G. S. (2001) *Cell* **106,** 395–398
27. Boll, W., Ohno, H., Songyong, Z., Rapoport, I., Cantley, K. C., Bonifacino, J.S., and Kirchhausen, T. (1996) *EMBO J.* **15,** 5789–5795
28. Ohno, H., Stewart, J., Fourner, M. C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J. S. (1995) *Science* **269,** 1872–1875
29. Tomita, T., Tokuhiro, S., Hashimoto, T., Aiba, K., Saido, T. C., Maruyama, K., and Iwatsubo, T. (1998) *J. Biol. Chem.* **273,** 21153–21160