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Functional Domains in Presenilin 1

THE TYR-288 RESIDUE CONTROLS γ-SECRETASE ACTIVITY AND ENDOPROTEOLYSIS*

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Hanna Laudon‡, Helena Karlström§, Paul M. Mathews¶, Mark R. Farmery‡, Samuel E. Gandy‖, Johan Lundkvist§, Urban Lendahl§, and Jan Näslund‡**

From the ‡Department of Neurotec, Division of Experimental Geriatrics, Karolinska Institutet, Novum, SE-141 86 Huddinge, Sweden, §Department of Cell and Molecular Biology, Karolinska Institutet, The Medical Nobel Institute, SE-171 77 Stockholm, Sweden, ¶Center for Dementia Research, Nathan Kline Institute, New York University School of Medicine, Orangeburg, New York 10962, and ‖Farber Institute for Neurosciences, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Processing of the Alzheimer amyloid precursor protein (APP) into the amyloid β-protein and the APP intracellular domain is a proteolysis event mediated by the γ-secretase complex where presenilin (PS) proteins are key constituents. PS is subjected to an endoproteolytic cleavage, generating a stable heterodimer composed of an N-terminal and a C-terminal fragment. Here we aimed at further understanding the role of PS in endoproteolysis, in proteolytic processing of APP and Notch, and in assembly of the γ-secretase complex. By using a truncation protocol and alanine scanning, we identified Tyr-288 in the PS1 N-terminal fragment as critical for PS-dependent intramembrane proteolysis. Further mutagenesis of the 288 site identified mutants differentially affecting endoproteolysis and γ-secretase activity. The Y288F mutant was endoproteolyzed to the same extent as wild type PS but increased the amyloid β-protein 42/40 ratio by ~75%. In contrast, the Y288N mutant was also endoproteolytically processed but was inactive in reconstituting γ-secretase in PS null cells. The Y288D mutant was deficient in both endoproteolysis and γ-secretase activity. All three mutant PS1 molecules were incorporated into γ-secretase complexes and stabilized Pen-2 in PS null cells. Thus, mutations at Tyr-288 do not affect γ-secretase complex assembly but can differentially control endoproteolysis and γ-secretase activity.

γ-Secretase catalyzes the final step in the generation of the amyloid β-protein (Aβ)[1] from the transmembrane Alzheimer amyloid precursor protein (APP) in a process known as regulated intramembrane proteolysis (reviewed in Ref. 1). Aβ is the major protein component of the amyloid deposits that neuropathologically characterize Alzheimer's disease. In parallel with γ-secretase-mediated Aβ generation, a cytosolic fragment, the APP intracellular domain (AICD), is formed. The N terminus of AICD is located 8–10 residues C-terminal to the site predicted by the γ-secretase cleavages generating Aβ40 and Aβ42 (2). This cleavage site occurring between Leu-49 and Val-50 (Aβ numbering) has been termed the ϵ site. Competition studies *in vitro* suggest that cleavage at the ϵ site is intimately related to γ-secretase cleavage (3).

Accumulating evidence suggests that the PS proteins are required in order for γ-secretase cleavage to occur (4–6). PS proteins contain 10 hydrophobic domains, 8 of which form transmembrane domains according to most current models (7–9). Endogenously expressed PS protein is endoproteolytically cleaved in the large cytosolic loop, thus generating a 25-kDa N-terminal fragment (NTF) and an 18-kDa C-terminal fragment (CTF). The membrane-bound NTFs and CTFs remain stably associated as heterodimers. The levels of NTFs and CTFs, the form in which PS is presumed to participate in the active γ-secretase complex (10, 11), are tightly regulated in the cell (12, 13). Together with the nicastrin (14), Aph-1 (15), and Pen-2 (16) proteins, the NTF and CTF heterodimers form high molecular weight γ-secretase complexes (17, 18) that predominantly reside in the endoplasmic reticulum (ER) and Golgi apparatus (19). Many substrates in addition to APP have been identified as targets for PS-dependent processing, among them the family of Notch cell surface receptors (4, 20).

Here we used a recently developed luciferase-based reporter system (21, 22) to define structural elements in PS1 that are critical for mediating endoproteolysis and γ-secretase complex assembly and activity. We have previously shown that PS1 NTF and CTF restore γ-secretase cleavage of the two best-known PS-controlled regulated intramembrane proteolysis substrates, APP and Notch, when co-expressed in PS-deficient cells (23). By using progressively truncated NTFs and alanine-scanning mutagenesis, we defined a short motif in the cytoplasmic loop juxtaposed to the endoproteolytic site that is required for γ-secretase activation. The major activity determinant of the motif was the Tyr-288 residue. Extensive mutagenesis revealed the basic biochemical characteristics of the amino acid occupying position 288 critical for restoring PS1 endoproteolysis and PS1-mediated γ-secretase activity. An analysis of the Y288F, Y288N, and Y288D mutants revealed that the capacity for endoproteolysis and γ-secretase activity can be separated,

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** To whom correspondence should be addressed: Karolinska Institutet, Novum Kaspac pl. 5, SE-141 57 Huddinge, Sweden. Tel.: 46-8-585-835-01; Fax: 46-8-585-836-10; E-mail: jan.naslund@neurotec.ki.se.

[1] The abbreviations used are: Aβ, amyloid β-protein; APP, Alzheimer amyloid precursor protein; AICD, APP intracellular domain; PS, presenilin; NTF, N-terminal fragment; CTF, C-terminal fragment; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ER, endoplasmic reticulum; DAPT, *N*-[*N*-(3,5-difluoro-phenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester.

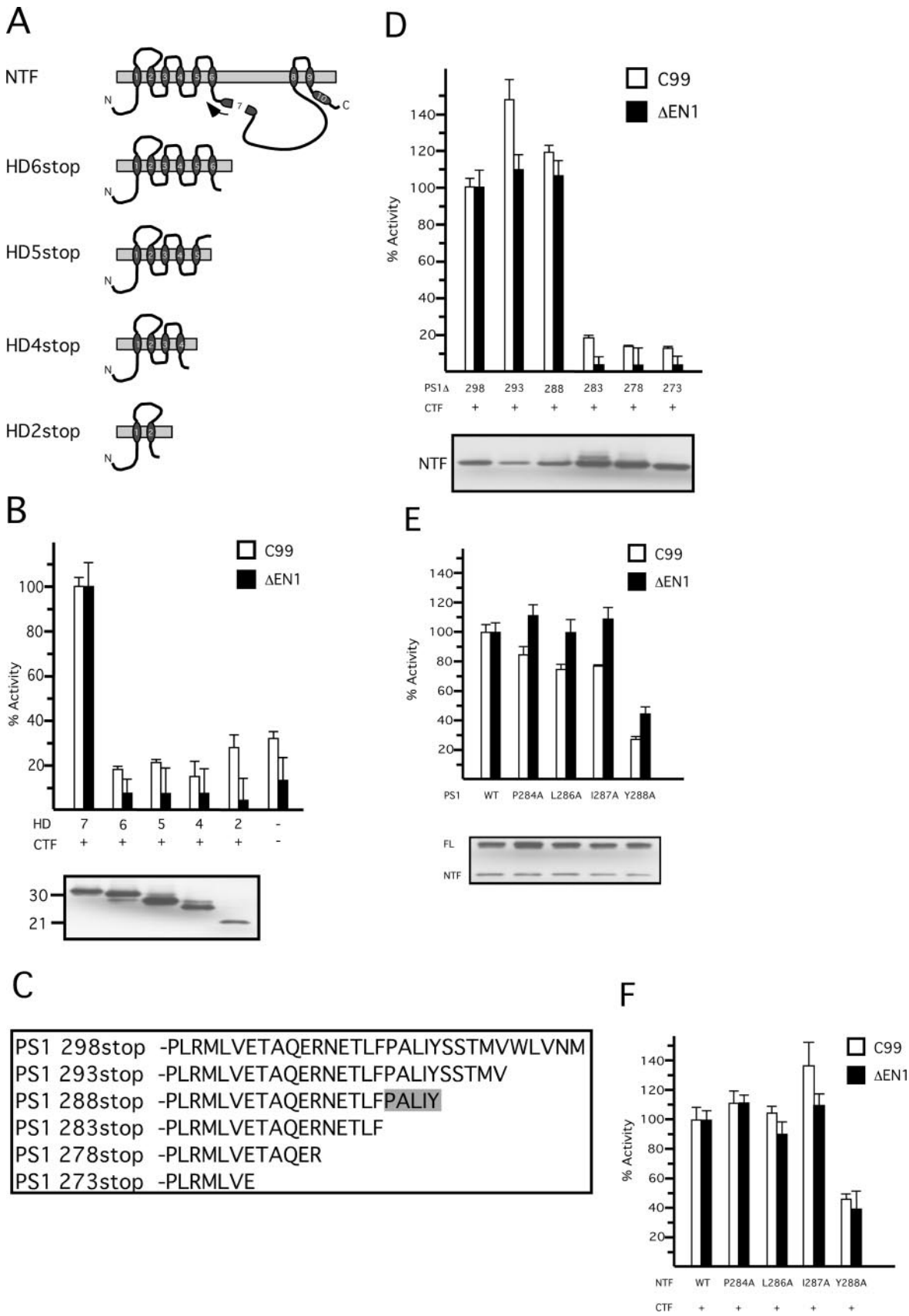

FIG. 1. **Co-expression of C-terminally truncated NTF and native CTF.** *A*, schematic depiction of the PS1 NTF and CTF and a series of C-terminally truncated NTFs. *Numbers* indicate the hydrophobic domains (*HD*) within the PS1 molecule. *B*, monitoring of C99- and ΔEN1-cleavage upon co-expression of the NTF variants and wild-type CTF, using luciferase-based reporter assays. Luciferase-induced bioluminescence, corresponding to intramembrane proteolysis, is presented in the bar graphs as percent activity compared with co-expressed wild type PS fragments. Note that the background luciferase response is higher for the C99 substrate than for the ΔEN1 substrate. Error bars represent the mean ± S.E. for triplicate samples. Expression of the C-terminally truncated NTFs was verified by immunoblotting using antibody NT1 (*lower panel*). *C*, the amino acid sequence of the C-terminal end of an array of C-terminally truncated NTF constructs. The longest construct (298stop) represents wild

whereas the incorporation into the mature γ-secretase complex was retained.

EXPERIMENTAL PROCEDURES

Expression Constructs—The reporter constructs that were used in the luciferase-based reporter system to monitor AICD and notch intracellular domain generation have been described previously (21, 24). Full-length PS1 and PS1 NTFs were in the pcDNA3 backbone, and the CTF was in pcDNA3.1. The NTF constructs illustrated in Fig. 1*A* encode PS1 molecules truncated after amino acids Met-298 (NTF), Gly-266 (HD6stop), Glu-243 (HD5stop), Lys-216 (HD4stop), and Arg-157 (HD2stop), respectively. Mutations were introduced by site-directed mutagenesis according to the QuikChange™ protocol (Stratagene). The DNA sequence of each construct was verified using the DYEnamic ET Terminator cycle sequencing kit (Amersham Biosciences) and the ABI Prism 377 sequencer (PerkinElmer Life Sciences). PS1 constructs used for generating stable cell lines were cloned into the pCAG-IRES-Puro vector using conventional cloning procedures.

Cell Culture and Transfection—Blastocyst-derived embryonic stem cells lacking PS1 and PS2 (BD8 cells) previously had been generated by immortalizing cells from PS1/PS2 double-null mice on embryonic day 3.5 (24, 25). The BD8 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2.4 mM L-glutamine, 0.1 mM β-mercaptoethanol, and non-essential amino acids. Stably transfected BD8 cells were maintained in medium supplemented with 1 μg/ml puromycin (Sigma). All of the transfections were carried out using LipofectAMINE Plus according to the manufacturer's instructions (Invitrogen).

Luciferase-based Reporter Assays and Immunoblotting—Transfections for the luciferase-based reporter assays were carried out in 24-well tissue culture plates essentially as described previously (23). Cells were lysed in 100 μl of lysis buffer/well (10 mM Tris, pH 8, 1 mM EDTA, 150 mM NaCl, and 0.65% Nonidet P-40), and luciferase activity was monitored luminometrically after the addition of luciferin and ATP (BioThema). The β-galactosidase activities of the cell lysates were determined to equalize for differences in transfection efficiencies. Arbitrary β-galactosidase-normalized luminescence units are presented as percent activity compared with wild type PS1 response.

For immunoblotting, cell lysates were briefly sonicated and resolved on 10–20% Tricine gels. Proteins were transferred to nitrocellulose membranes that were subsequently probed with appropriate antibodies. The immunoblots were developed using horseradish peroxidase-conjugated secondary antibodies and ECL substrate (Pierce).

Antibodies—The antibodies used for co-immunoprecipitations of PS1 were polyclonal antibody Ab14 raised against the N terminus of PS1 (18) and monoclonal antibody MAB5232 (Chemicon), which recognizes PS1 CTF. For immunoblotting of PS1, monoclonal antibody NT1 directed toward the N terminus (26) and MAB5232 were used. Nicastrin was immunoprecipitated using polyclonal antibody N1660 (Sigma), and Aph-1 was immunoprecipitated with polyclonal antibody H2D (Calbiochem). Polyclonal antibody 369 (27) and monoclonal antibody C1/6.1 (28), which were both raised against the C-terminal end of APP, were used for immunoprecipitations. Polyclonal antibody UD1 was used for immunoblotting of Pen-2 (29).

Enzyme-linked Immunosorbent Assay—Transient transfections were performed in 6-well plates in triplicates. Per well, cells were transiently transfected with 500 ng of C99-encoding vector. Cells were cultured for 48 h in 0.75 ml of Opti-MEM supplemented with 5% fetal bovine serum. Cell media were flash-frozen in liquid nitrogen and subjected to sandwich enzyme-linked immunosorbent assay measurements essentially as described previously (28).

Co-immunoprecipitations—Co-immunoprecipitations were performed on lysates from stably transfected cells. Cells were cultured to confluency in a 6-well format. Per well, cells were lysed in 0.5 ml of co-immunoprecipitation buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EDTA, protease inhibitor mixture (Roche Applied Science), and 1% CHAPSO) and briefly sonicated. Lysates were subjected to ultracentrifugation (100,000 × *g*, 20 min). The CHAPSO-soluble supernatant was incubated with primary antibody (dilution 1:300) overnight, and proteins were pulled down with a mixture of proteins A- and G-Sepharose (Amersham Biosciences). Pre-immune serum was used as negative control. Immunoprecipitated proteins were resolved on 10–20% Tricine gels (Invitrogen), transferred to nitrocellulose membranes, and detected by immunoblotting as described above.

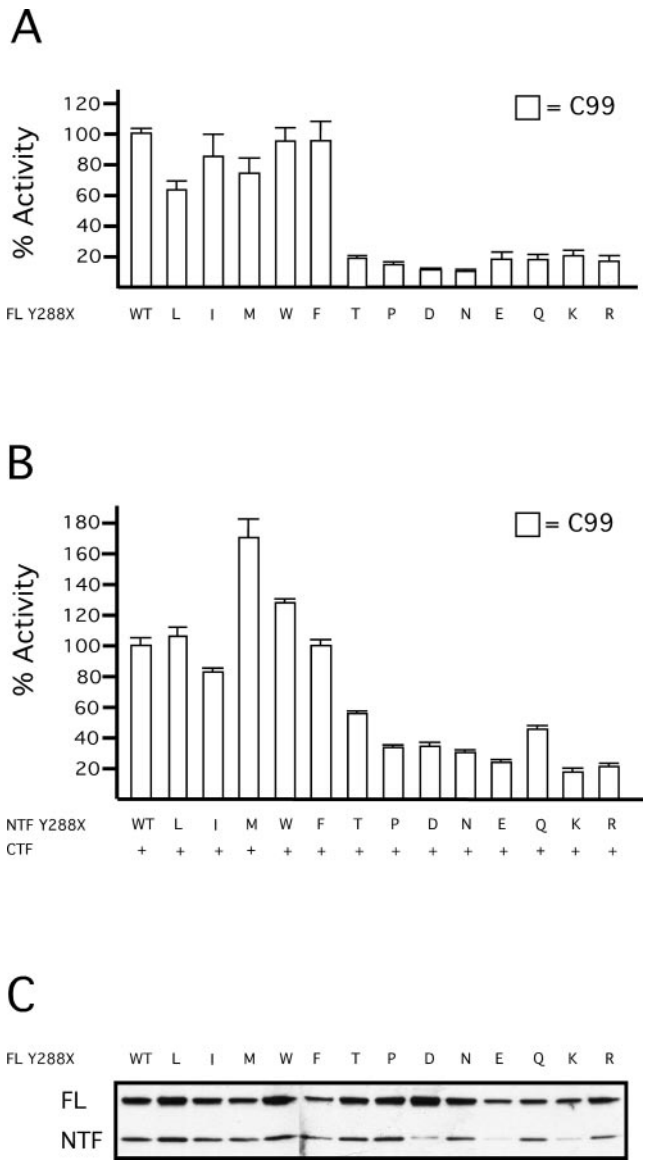

FIG. 2. **Site-directed mutagenesis of Tyr-288**. Effect of amino acid substitutions of Tyr-288 on C99 processing. Results from the luciferase-based reporter assays for mutated full-length constructs (*A*) and co-expressed mutant NTF and wild type CTF (*B*) are given in the bar graphs. The luminescence response for mutant PS1 is presented as percent activity compared with wild type PS1, and the error bars indicate ± S.E. for triplicate transfections. *C*, cell lysates from the full-length PS1 Tyr-288 mutants were subjected to immunoblotting using antibody NT1. Note a pronounced reduction in endoproteolysis compared with wild type PS1 for some of the mutants.

type NTF. *D*, measurements of C99 and ΔEN1 proteolysis upon co-expression of the NTF variants indicated in *C* and wild type CTF, using luciferase based reporter assays. Error bars indicate ± S.E. for triplicate transfections. Expression of the NTFs was confirmed by immunoblotting using antibody NT1 (*lower panel*). *E*, alanine-scanning mutagenesis of the functionally critical sequence of five residues (PALIY) identified in *D*. Substitutions were introduced in full-length PS1, and proteolytic processing of C99 and ΔEN1 was monitored using the luciferase-based reporter assays. Results from triplicate transfections are presented as percent activity compared with wild-type PS1, and error bars represent ± S.E. Immunoblotting using antibody NT1 confirmed expression of the constructs (*lower panel*). *F*, alanine-scanning mutagenesis of the PALIY sequence in the NTF-encoding constructs. C99 and ΔEN1 hydrolysis was measured by the luciferase based reporter systems upon co-expression of alanine-mutated NTFs and wild-type CTF. The luminescence response corresponding to proteolytic processing of the reporter constructs is presented as percent activity compared with the bioluminescence generated upon expression of the wild-type PS1 fragments.

Subcellular Fractionation—PS-deficient BD8 cells and BD8 cells stably expressing wild type or mutant PS1 were cultured in 10-cm plates to near confluency. Per cell line, the cells from two plates were harvested and homogenized in ice-cold homogenization buffer (130 mM KCl, 25 mM NaCl, 1 mM EGTA, 25 mM Tris, pH 7.4) supplemented with protease inhibitor mixture. Lysates were cleared by centrifugation at 1,000 × *g* for 10 min. The supernatants were overlaid onto the top of a step gradient consisting of 1 ml each of 30, 25, 20, 15, 12.5, 10, 7.5, and 5% iodixanol (OptiPrep reagent, Axis-Shield PoC AS) diluted in homogenization buffer. After 90 min of centrifugation at 126,000 × *g* (Beckman), fractions were collected from the top of the gradient. After dilution, membranes were pelleted by centrifugation at 20,000 × *g* for 20 min. Proteins were resolved on 4–20% Tris-glycine gels (Criterion, Bio-Rad), transferred to nitrocellulose membranes, and detected by immunoblotting as described above.

RESULTS

Co-expression of C-terminally Truncated NTF and Native CTF—We have recently shown that co-expression of the PS1 NTF and CTF can functionally substitute for the expression of the full-length molecule in mammalian PS null cells (23). Based on this knowledge, we designed an experimental protocol where truncated NTFs were co-expressed with wild type CTF in a PS-deficient cellular background. We used blastocyst-derived mouse embryonic stem cells lacking PS1 and PS2 (25) and a luciferase-based reporter system that quantifies intramembrane processing (21) to study γ-secretase cleavage of APP and Notch. To this end, we used truncated versions of APP and Notch, which mimic β-secretase-processed APP (C99) and ligand-activated and ectodomain-shedded Notch1 (ΔEN1) (30). C99 and ΔEN1 both represent direct substrates for PS-dependent intramembrane processing.

An array of successively truncated NTFs (Fig. 1*A*) was transfected into PS null cells together with wild type CTF (Fig. 1*B*). Of the fragments tested, only the native NTF was able to mediate γ-secretase processing of C99 and ΔEN1. Expression of the NTFs was confirmed by immunoblotting (Fig. 1*B, lower panel*). Thus, residues 267–298 in the cytoplasmic loop of the NTF are critical for rescuing γ-secretase activity in PS-deficient cells. To demarcate the γ-secretase-activating domain in the 32-residue sequence defined above, an additional array of truncated NTF variants was generated (Fig. 1*C*). These NTFs were co-transfected with native CTF, and γ-secretase cleavage of C99 and ΔEN1 was measured (Fig. 1*D*). PS1-controlled γ-secretase activity was dramatically decreased when PS1 residues 284–288 were deleted, indicating that a key sequence is located in hydrophobic domain 7 between residues Pro-284 and Tyr-288. This PALIY motif is situated just N-terminal to the physiological endoproteolysis site (Fig. 1*A*), which is mapped to a region positioned between residues Thr-291 and Ala-299 (31). Steady-state levels of the expressed NTF variants were corroborated by immunoblotting (Fig. 1*D*, *lower panel*). It is worth noting that this region is important for PS-mediated γ-secretase processing of both C99 and ΔEN1, implying that this motif may be critical not only for APP and Notch but possibly also for most or all of the other PS-controlled regulated intramembrane proteolysis substrates.

To more precisely assess the residues in the PALIY motif critical for mediating the γ-secretase-activating effect, alanine mutants were generated in the PS1 full-length protein. Mutant PS1 proteins were transfected into the PS-deficient cells, and γ-secretase cleavage of C99 and ΔEN1 was recorded. The Y288A mutant exhibited a pronounced reduction in γ-secretase, rescuing activity for both C99 and ΔEN1 (73 and 58% reduction, respectively) (Fig. 1*E*). The effect of exchanging the other residues in the activating element for alanine was less apparent on C99 (16, 26, and 22% reduction for P284A, L286A, and I287A, respectively) (Fig. 1*E*). The results for ΔEN1 were somewhat different compared with C99, and here in fact, there

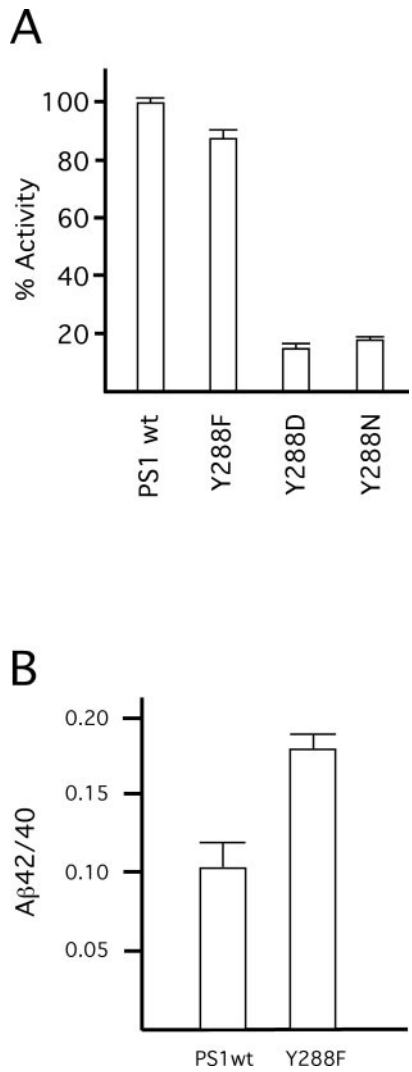

FIG. 3. **Stable expression of PS1 Tyr-288 mutants on a null background.** *A*, assaying of intramembrane processing of C99 for PS-deficient BD8 cells stably expressing wild type PS1 or PS1 Tyr-288 mutants (Y288F, Y288D, and Y288N). Error bars represent ± S.E. for triplicate transfections, and the luciferase response from the reporter assay is presented as percent activity compared with wild type PS1. *B*, Aβ42/40 ratios for Aβ secreted from cells transfected with human C99, and wild type PS1, or PS1 Y288F. Ratios were determined from enzyme-linked immunosorbent assay measurements of Aβ40 and 42 in cell media. The bar graphs represent the mean value for three separate transfections, and the error bars indicate ± S.E.

was a small increase for P284A and I287A (10 and 8%, respectively) (Fig. 1*E*). Importantly, the Ala mutations did not impair endoproteolysis of the mutant PS1 holoprotein (Fig. 1*E*, *lower panel*). However, to exclude the possibility that changes in endoproteolysis too small to be detected by immunoblotting account for the Y288A effect observed above, Ala mutations were also introduced into the NTF. When expressed together with wild type CTF, these NTF mutants showed a pattern similar to the mutant full-length proteins on rescue of γ-secretase processing (Fig. 1*F*). In contrast to the decreased C99 processing for full-length PS1 mutants P284A, L286A, and I287A, when introduced into the NTF, these mutations increased γ-secretase cleavage of C99 with 12, 4, and 38%, respectively. Importantly, the Y288A substitution reduced the γ-secretase-rescuing effect with ~50–60%. Taken together, these data suggest that Tyr-288 is the main determinant of the PALIY motif for sustaining γ-secretase. Apparently, an Ala mutation in position 288 of the activating element disrupts

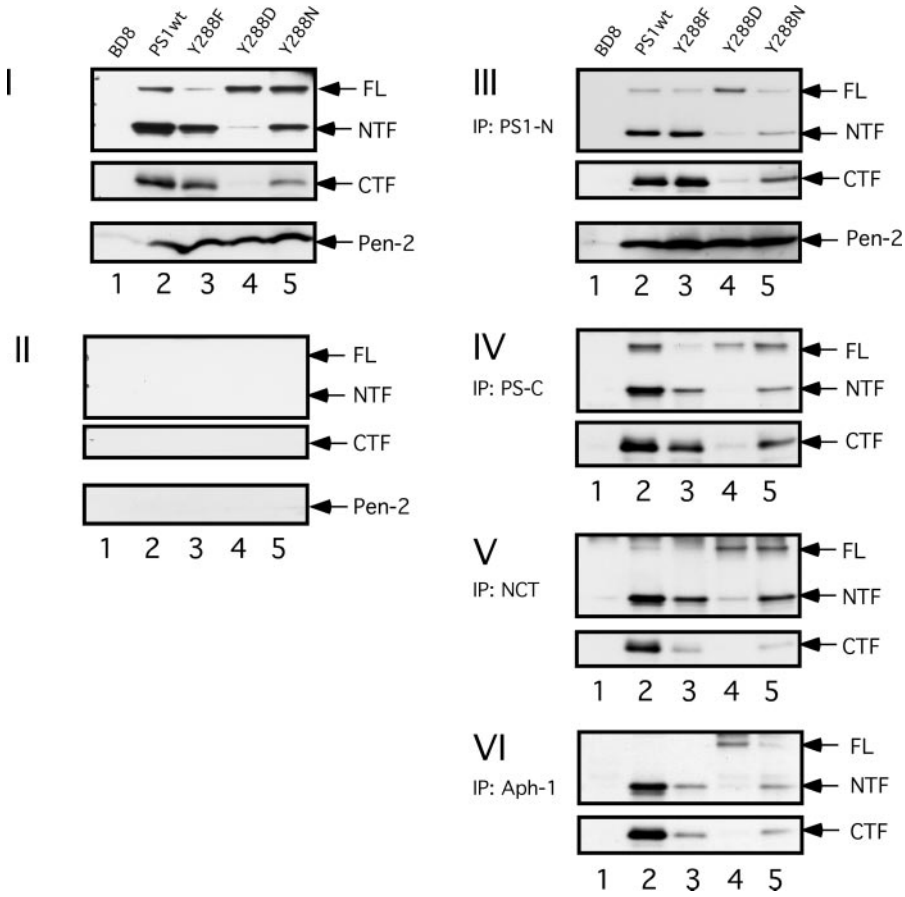

FIG. 4. **Co-immunoprecipitations of the γ-secretase components.** Immunoblotting of γ-secretase components in cell lysates (*panel I*) and after co-immunoprecipitations using antibodies directed toward the known proteins of the γ-secretase complex (*panels III–VI*). Co-immunoprecipitations were performed using the following antibodies: Ab14 recognizing the PS1 NTF (*panel III*); MAB5232 recognizing PS1 CTF (*panel IV*); N1660 recognizing nicastrin (*NCT*; *panel V*) and H2D recognizing Aph-1 (*panel VI*). Lysates (*panel I*) and co-precipitations were subjected to immunoblotting using PS1 antibodies NT1 and MAB5232, and antibody UD1 was raised against Pen-2. Pre-immune serum was used as control for immunoprecipitations (*panel II*).

γ-secretase activity by some mechanism other than inhibition of PS1 endoproteolysis. Moreover, an NTF ending at residue 287, thereby lacking Tyr-288, did not rescue γ-secretase activity when co-expressed with CTF (data not shown). Thus, Tyr-288 in PS1 is crucial for restoring γ-secretase activity in PS null cells.

Site-directed Mutagenesis of Tyr-288—To obtain a more detailed view on the characteristics of the residue occupying position 288, we subjected this residue to mutagenesis in PS1 full-length protein and in the NTF. We used the luciferase assay to study C99 processing when these mutants were transiently expressed in PS-deficient cells. Using this qualitative approach, we could show that hydrophobic and bulky residues in position 288 of the full-length protein or NTF restored γ-secretase processing of C99 (Fig. 2, *A* and *B*). For some mutants, intramembrane proteolysis of C99 was considerably enhanced, in particular when the mutation was introduced in the NTF. For example, the Y288M and Y288W mutations increased γ-secretase activity with 70 and 28%, respectively (Fig. 2*B*). Polar and charged residues were less well tolerated in the 288 position. C99 processing was in some cases dramatically reduced when these mutants (*e.g.* Y288D, Y288N) were expressed (Fig. 2, *A* and *B*). The PALIY motif and the Tyr-288 residue are located close to the PS endoproteolysis site, and because the functional significance of PS endoproteolysis with regard to γ-secretase activity remains unclear, we assayed the Tyr-288 mutants for endoproteolysis. Some of the mutants tested, Y288D, Y288E, and Y288K, also had a profound negative effect on endoproteolysis of PS1 (Fig. 2*C*).

Expression of PS1 Tyr-288 Mutants on a Null Background—We selected three mutants, Y288F, Y288N, and Y288D, for stable expression in the PS-deficient BD8 cells. The Y288F mutant restored C99 processing to the same extent as the PS1 wild type molecule (Fig. 3*A*). In contrast, neither the Y288N nor the Y288D mutant mediated γ-secretase cleavage of the C99 reporter molecule (Fig. 3*A*). Because the reporter assay used here does not differentiate between the γ- and ε-cleavage of the substrates, we wanted to directly analyze γ-secretase cleavage of C99 by measuring Aβ40 and Aβ42 secreted into medium. Consistent with the findings for AICD formation, expression of the Y288N and Y288D mutants did not result in any detectable Aβ40 or Aβ42 secretion (data not shown). Interestingly, expression of the intramembrane proteolysis-active Y288F mutant resulted in an ~75% increase in the Aβ42/40 ratio compared with the wild type PS1 molecule (Fig. 3*B*).

Tyr-288 Mutants and γ-Secretase Complex Assembly—The failure of the Y288N and Y288D mutants to restore PS-mediated intramembrane proteolysis in PS null cells could possibly be related to misincorporation of the mutants into fully assembled and mature γ-secretase complexes. Therefore, we performed immunoblotting experiments of CHAPSO lysates prepared from the stable cell lines (Fig. 4). In lysates, NTF and CTF can be clearly visualized from BD8 cells stably expressing wild type PS1 (*panel I*, *lane 2*). In addition, the expression of PS1 in PS-deficient cells leads to stabilization of Pen-2 as shown previously (29, 32). As suggested from the luciferase experiments, the biochemical phenotype of the Y288F mutant resembled that of the wild type molecule (*panel I*, *lane 3*). As shown above using transient transfections, endoproteolysis of the Y288D mutant is severely impaired also in stably transfected cells (*panel I*, *lane 4*). Interestingly, the Y288N mutant, which cannot restore γ-secretase processing of C99, is endoproteolytically processed to a level almost comparable with wild type PS1. Expression of both Y288D and Y288N leads to the stabilization of Pen-2, indirectly implying that the mutants were incorporated into mature, albeit non-functional, γ-secretase complexes.

Correct complex incorporation of the mutants was further

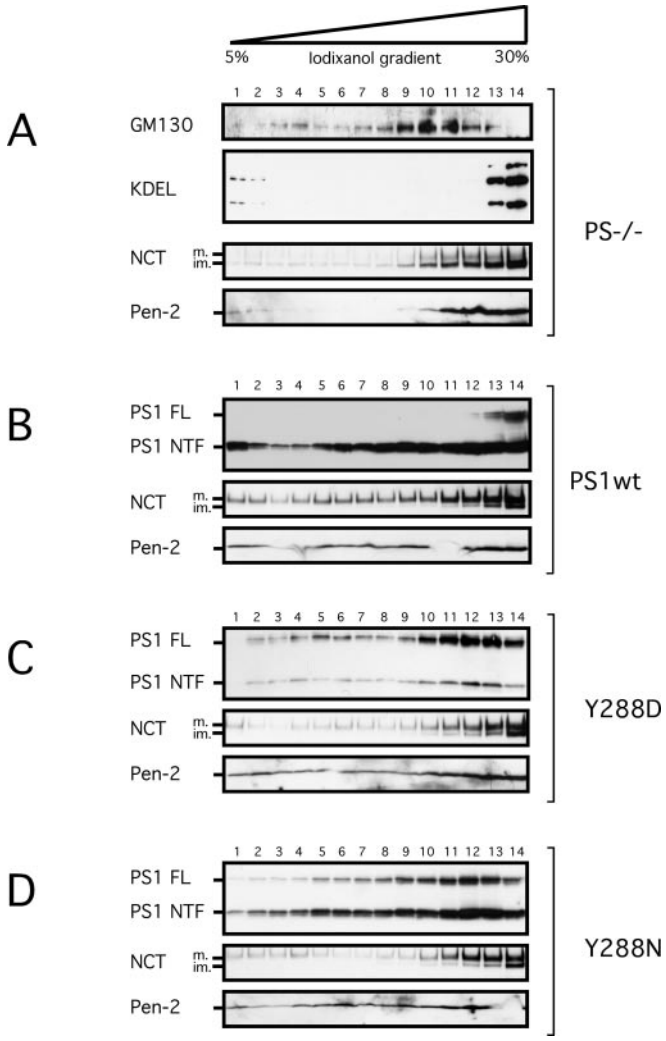

FIG. 5. **Tyr-288 mutants and γ-secretase complex assembly.** Subcellular fractionation of membranes from PS-deficient BD8 cells (*A*) or BD8 cells stably expressing wild type PS1 (*B*), PS1 Y288D (*C*), or PS1 Y288N (*D*) is shown. The distributions of the Golgi marker, GM130, and the ER marker, KDEL, are shown in *A*. Distributions of full-length PS1, PS1 NTF, mature (*m*) and immature (*im*) nicastrin (*NCT*), and Pen-2 were detected by immunoblotting using antibodies NT1, N1660, and UD1, respectively.

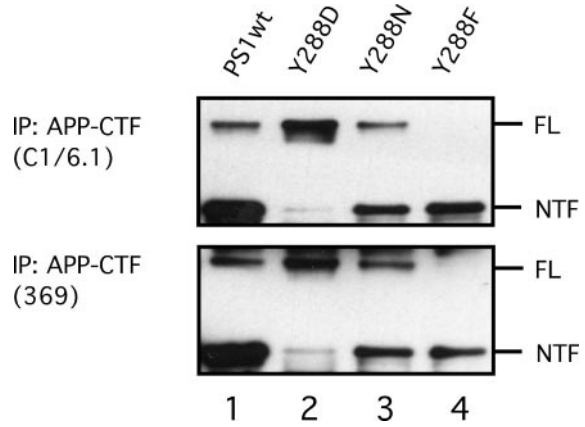

FIG. 6. **Tyr-288 mutants and substrate interaction.** Co-immunoprecipitations of cell lysates from BD8 cells stably expressing wild type PS1 or the PS1 Tyr-288 mutants. Cells were transiently transfected with human C99 and treated with 2 μM DAPT. Co-immunoprecipitations were performed in CHAPSO lysates in the presence of 2 μM DAPT using antibodies 369 and C1/6.1. Antibody NT1 was used for immunoblotting of co-precipitated PS1.

corroborated using co-immunoprecipitations. The CTF was co-precipitated with the NTF for all of the stably expressed PS1 variants, albeit very little for the endoproteolysis-defective Y288D mutant (*panel III*, *lanes 2–5*). It is worth noting that Pen-2 associates and apparently co-precipitates with full-length Y288D (*panel III*, *lane 4*), in agreement with a previous report showing association between Pen-2 and the D257A and D385A PS1 mutants (33). The NTF was associated with the CTF in reciprocal immunoprecipitations (*panel IV*, *lanes 2–5*). Immunoprecipitations against nicastrin and Aph-1 showed very similar immunostaining patterns when probing for the NTF and CTF (*panels V* and *VI*, *lanes 2–5*). The cleavage-compromised Y288D mutant associated with both nicastrin (*panel V*, *lane 4*) and Aph-1 (*panel VI*, *lane 4*) under these conditions.

Complex assembly of the mutants was further investigated by subjecting membranes from the stable cell lines to iodixanol gradient fractionation (Fig. 5). This method allows separation of ER-rich vesicles from Golgi- and post-Golgi-rich vesicles (34). We corroborated the separation between ER and Golgi by using the ER marker KDEL and medial-Golgi marker GM130, respectively. As previously shown by many groups (35–38), nicastrin is not trafficked beyond the early Golgi and does not reach full maturation in PS null cells (*panel A*). Moreover, as reported previously (29), Pen-2 is sequestered in the ER and destined for ubiquitination and proteasomal degradation in the absence of PS (*panel A*). In BD8 cells stably expressing wild type PS1, ER retention of Pen-2 was released and fully glycosylated nicastrin could be found in Golgi-rich fractions (*panel B*). Stable expression of the Y288N and Y288D mutants in BD8 cells resulted in almost identical subcellular distributions of Pen-2 and mature nicastrin for the mutants and wild type PS1 (*panels B–D*). It is notable that the full-length Y288D and Y288N mutants are distributed to Golgi-like fractions that overlap with fractions containing only NTF from the wild type molecule (compare *panels C* and *D* with *B*). In contrast to the wild type PS protein, which is mainly found in its cleaved form in Golgi-like fractions, the mutant full-length molecules are apparently distributed to post-ER compartments. This suggests that blocked endoproteolysis does not prevent the mutant molecules from being trafficked normally. In summary, exchanging Tyr-288 for Asn or Asp, thus rendering the PS1 molecule non-functional, did not in any appreciable way affect the subcellular distribution or ability of the mutant molecule to interact with other known components of the γ-secretase complex.

Mutant PS1 and Substrate Interaction—Because the complex assembly of Y288N and Y288D mutants was not perturbed and the Y288F mutant changed the cleavage preference in favor of Aβ42, we hypothesized that Tyr-288 is located in a domain in PS1 that is important for substrate recognition. Cells stably expressing wild type, Y288F, Y288N, or Y288D PS1 were transfected with C99 and treated with 2 μM of the potent γ-secretase inhibitor DAPT (39). CHAPSO lysates were immunoprecipitated with antibodies 369 and C1/6.1, respectively. The immunoprecipitation buffer and wash buffers also contained 2 μM DAPT. Subsequently, membranes were probed with an antibody against the N-terminal domain of PS1. In Fig. 6*A*, which shows one representative experiment of four, there were no apparent differences in the interaction between the C99/C83 substrate and mutant and wild type PS1.

DISCUSSION

Previous work has documented that co-expression of NTF and CTF in mammalian PS null cells results in authentic γ-secretase complex assembly and reconstitution of intramembrane proteolysis of C99 and ΔEN1 (23). This finding provides an experimental tool for searching the NTF and CTF for do-

mains important for γ-secretase complex assembly and activity when endoproteolysis is bypassed. In this report, we provide data from a screen that focused on the C-terminal region of the NTF. This screen identified the PALIY motif and, in particular, the Tyr-288 residue as critical for PS1 endoproteolysis and PS1-mediated γ-secretase activity. The PALIY motif in hydrophobic domain 7 where Tyr-288 is located is highly conserved between the species. This evolutionary conservation corroborates the data from the mutational analysis and further confirms that this region is important for PS1 function.

Systematic mutational analysis of the Tyr-288 residue revealed several interesting observations regarding the relationship among endoproteolysis, γ-secretase activity, and assembly of the γ-secretase complex. In the qualitative mutagenesis scheme employed, some general themes were apparent (Fig. 2, *A* and *B*). Exchange of Tyr-288 for hydrophobic and bulky residues Leu, Ile, Met, Phe, or Trp in full-length PS1 or NTF did not compromise γ-secretase activity and, in some cases, even enhanced γ-secretase cleavage. Substitution of Tyr-288 for negatively charged Asp or Glu residues or positively charged Lys or Arg residues in position 288 abolished the ability of the NTF or the full-length protein to rescue γ-secretase activity in PS null cells (Fig. 2, *A* and *B*). Exchanging Tyr-288 for the polar but non-charged Asn or Gln residues also resulted in an inefficient C99-processing profile. Substituting Tyr-288 for Thr to assess the importance of a polar hydroxyl group in position 288 reduced in three of four test settings the γ-secretase cleavage of C99 to an intermediate level between the constitutively charged residues and the hydrophobic residues. However, the hydrophobicity of Thr is roughly equal to that of Ala, which also decreased activity to an intermediate level, precluding any conclusion distinguishing the impact of the hydroxyl group *per se*. Finally, introducing the α-helical- and β-sheet-breaking amino acid Pro in the 288 position resulted in a decreased ability of the mutant PS1 to elicit a γ-secretase response.

The relationship between PS endoproteolysis and γ-secretase activity is a central question in PS biology. That endoproteolysis is not an absolute requirement for γ-secretase complex assembly and activity has been shown for the non-cleaved familial Alzheimer's disease-associated PS1 ΔE9 mutant (40) and the artificial M292D mutant (41). Other artificial mutants, most notably the PS1 D257A and D385A mutants, are deficient in both endoproteolysis and in activity (42) but successfully incorporated into mature complexes (43). One of the mutants described here, Y288D, has a similar cellular phenotype (Fig. 4, *panel I*). However, the mechanism of action of this mutant is most probably not related to a mutation affecting the active site of PS1 *per se*, as is the case for the D257A and D385A mutants, but rather reflects the exchange of a bulky polar residue for a constitutively charged residue in HD 7, thereby impeding both endoproteolysis and activity. Interestingly, although the Y288D mutant is very similar to the M292D mutant, in terms of the polar to charged residue change and in the location of the mutation, the Y288D mutant cannot reconstitute intramembrane proteolysis of C99 in PS-null cells. Despite numerous attempts, we failed to detect a phosphorylation of Tyr-288, either by using anti-phosphotyrosine antibodies or ^{32}P labeling (data not shown).

Current topological models of PS1 within the membrane suggest that 8 of the 10 hydrophobic domains form transmembrane domains (7–9). The capacity to affect both endoproteolysis and γ-secretase activity suggests that Tyr-288 is located physically close both to the endoproteolysis site and the active site of the γ-secretase protease activity. This notion is supported by the mapping of endoproteolysis site to a region between residues 291 and 299 (31) and that one of the catalytic Asp residues is located in position 257 in hydrophobic/transmembrane domain 6. In the 8-transmembrane domain model, the 19-residue hydrophobic domain 7 is located in the cytoplasm (Fig. 1*A*). Tyr-288 is located on the N-terminal part of hydrophobic domain 7 that reaches from residue 283 to 301. One can speculate that hydrophobic domain 7 peripherally associates with the membrane bilayer and that it exerts its control of endoproteolysis and γ-secretase activity through this association. The inactivity of the Tyr-288 mutants containing charged residues indirectly supports this notion.

Interestingly, the artificial Tyr-288 mutants tested were all capable of becoming incorporated into mature γ-secretase complexes. This suggests that PS1 functionality, in terms of correct endoproteolysis and/or capacity to conduct γ-secretase activity, is not prescreened before the γ-secretase complex is formed. The data from the Y288N and Y288D mutants are in keeping with previous findings from the PS1 aspartate mutants, which are also complex assembly-competent but non-functional in terms of γ-secretase activity. The fully active Y288F increased Aβ42/Aβ40 by ~75%, indicating that this mutation changes the interaction between enzyme and substrate, thereby shifting the cleavage preference. However, there was no difference in the binding of the C99 substrate between the active Y288F and the inactive Y288D and Y288N mutants, suggesting that Tyr-288 is not located in a domain of PS that directly interacts with substrate.

In conclusion, by co-expressing NTF and CTF, thus bypassing the requirement for endoproteolytic cleavage, we have by mutational analysis identified Tyr-288 as critical for γ-secretase activity and provided further insights into the relationship between γ-secretase activity, PS1 endoproteolysis, and γ-secretase complex assembly.

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