CONTRIBUTION OF PRESENILIN TRANSMEMBRANE DOMAINS 6 AND 7 TO A WATER-CONTAINING CAVITY IN THE γ-SECRETASE COMPLEX

Alexandra Tolia, Lucía Chávez-Gutiérrez and Bart De Strooper*

Neuronal cell biology and gene transfer laboratory, Center for Human Genetics, VIB4 and KULeuven, Herestraat 49, 3000 Leuven.

Running title: A water-containing cavity in the core of γ-secretase

*Address correspondence to: Bart De Strooper, Tel. +32/16346227, Fax. +32/16347181, E-mail: Bart.destrooper@med.kuleuven.be

γ-Secretase is a multi-protein complex responsible for the intramembranous cleavage of the Amyloid Precursor Protein (APP) and other type I transmembrane proteins. Mutations in Presenilin, the catalytic core of this complex, cause Alzheimer’s Disease. Little is known about the protein’s structure and even less about the catalytic mechanism, which involves proteolytic cleavage in the hydrophobic environment of the cell membrane. It is basically unclear how water, needed to perform hydrolysis, is provided to this reaction. Presenilin transmembrane domains 6 and 7 seem critical in this regard since each bears a critical aspartate contributing to catalytic activity. Current models imply that both aspartyl groups should closely oppose each other and have access to water. This has however still to be experimentally verified. Here we performed cysteine scanning mutagenesis of both domains and demonstrate that several of the introduced residues are exposed to water, providing experimental evidence for the existence of a water-filled cavity in the catalytic core of Presenilin. We demonstrate in addition that the two aspartates reside within this cavity and are opposed to each other in the native complex. We, also, identify the conserved tyrosine Y389 as a critical partner in the catalytic mechanism. Several additional amino acid substitutions affect differentially the processing of γ-secretase substrates, implying that they contribute to enzyme specificity. Our data suggest the possibility that more selective γ-secretase inhibitors could be designed.

INTRODUCTION

The Presenilin (PS) proteins are the prototypic members of a group of aspartic proteases involved in Regulated Intramembrane Proteolysis (RIP), a mechanism responsible for cleavage of peptide bonds within the lipid bilayer (1,2). More than 150 mutations in Presenilin 1 and 10 mutations in Presenilin 2 have been associated with Alzheimer’s disease (for a list of the mutations see http://www.molgen.ua.ac.be/ADMutations), demonstrating their pivotal role in the pathogenesis of the disease. Presenilins are critical for the γ-secretase cleavage of the Amyloid Precursor Protein (APP) that generates the amyloid β peptide (Aβ)(3). They are also responsible for the intramembrane proteolysis of several other type I transmembrane proteins (reviewed in (4)), including the S3 cleavage of Notch that releases the Notch Intracellular Domain (NICD), a major regulator of gene transcription (5). Together with Presenilin, three other membrane proteins are necessary and sufficient for processing by γ-secretase (6-8), i.e. Nicastrin, APH-1, and PEN-2. Nicastrin appears to recognize the free amino terminus of potential
substrates (9), whereas the catalytic core resides in Presenilin (reviewed in (10)). Most recent topological studies propose a nine transmembrane domain model for Presenilins, with the N-terminus oriented towards the cytosol and the C-terminus towards the extracellular space (11,12). Mutation of two conserved aspartates, D257 and D385, located in transmembrane domains (TMs) 6 and 7 respectively, abolishes activity, as well as binding to transition state inhibitors of \( \gamma \)-secretase (13-15), supporting the hypothesis that these residues constitute the catalytic site of the protein. Furthermore, inhibitor profiling studies have provided evidence for at least one additional substrate binding site on Presenilin, distinct from, but in close proximity to the catalytic site (16-18).

Structure-function studies of the \( \gamma \)-secretase complex are not easy to perform because of its hydrophobic nature and its sensitivity to membrane lipid composition and detergent extraction procedures. This explains why most of our knowledge of the catalytic activity of the complex is based on indirect evidence and assumptions. For instance, hydrolysis of peptide bonds requires that the active catalytic site of the protease has access to water within the lipid bilayer, but no formal proof for this assumption has been provided in the case of Presenilin. In order to probe experimentally the microenvironment of the catalytic site of Presenilin, we employed cysteine scanning mutagenesis, a method widely used to investigate structural features of polytopic membrane proteins (reviewed in (19,20)). The principle involves substitution of amino acid residues of interest with cysteine, which is average in size, thus normally quite well tolerated, and amenable to highly specific modification with sulfhydryl-directed reagents. Combination of membrane-permeable and impermeable reagents can provide valuable information about the extracellular or cytosolic position of a cysteine (21,22), whereas cysteines embedded in the membrane, unless exposed to a water-containing cavity, are not reactive with these reagents (23-25). This makes the technique a valuable tool for topological studies and even allows detection of conformational changes (26,27). In addition, in combination with disulfide cross-linking strategies, domains can be identified that are remote in the primary structure but in close proximity in the tertiary structure of the protein (28-30).

Taking advantage of these unique properties of cysteine scanning mutagenesis, we studied here the contribution of TMs 6 and 7 to a potential hydrophilic pocket in the \( \gamma \)-secretase complex.

**EXPERIMENTAL PROCEDURES**

**Site-directed mutagenesis and generation of stable cell lines.** All mouse PS1 mutants were constructed by using the Multi site-directed mutagenesis kit (Stratagene). Immortalized mouse embryonic fibroblasts (MEFs) derived from PS1/PS2 deficient mice were cultured in DMEM/F12 containing 10% fetal bovine serum (Sigma). At 30-40% confluency, MEFs were transduced using a replication-defective recombinant retroviral expression system (Clontech) with either wild-type or mutant PS1. Cell lines stably expressing the desired proteins were selected based on their acquired resistance to 5µg/ml puromycin.

**Antibodies.** Polyclonal antibodies against mouse PS1 NTF (B19.3) and CTF (B32.2), APH-1a (B80.2) PEN-2 (B96.2) and APP C-terminus (B63.3) and monoclonal 9C3 against the C-terminus of Nicastrin have been described (16,31). Other antibodies were purchased: anti- N-cadherin from BD Biosciences, anti-NICD (cleaved Notch1 Val 1744) from Cell Signaling, mAb WO2 from Abeta GmbH, (Heidelberg), mAb 9E10 (Sanver Tech) and MAB5232 against PS1 CTF (Chemicon).

**Preparation of cell lysates and immunoblotting.** Total cell extracts were prepared in lysis buffer containing 250mM sucrose, 5mM Tris-HCl (pH 7.4), 1mM EGTA, 1% Triton X-100 and Complete protease inhibitors (Roche). After centrifugation at 13000 x g for 15 minutes at 4°C, 20 \( \mu \)g of protein from the postnuclear extracts were separated on 4-12% Bis-Tris gels (Invitrogen). Proteins were transferred to nitrocellulose membranes, blocked and probed with antibodies as indicated. For detection, HRP-coupled secondary antibodies (BioRad) were used, followed by chemiluminescence detection with Renaissance (Perkin Elmer). Quantifications were performed by means of densitometry.
Labeling with thiol-specific reagents. MEFs in 100mm plates were washed three times in cold PBS (pH 7.4), followed by incubation with 200μM EZ-Link Biotin-HPDP (Pierce) for 45 min at 4°C. After 3 washes with PBS, cells were lysed and 500μg protein were incubated with 35μl of Immobilized NeutrAvidin Protein beads (Pierce) at 4°C overnight. After extensive washing, proteins were eluted from the beads by boiling in Nu-Page sample buffer. For cross-linking experiments, membrane aliquots were treated for 20 minutes at 4°C with 200μM 3,6-dioxaoctane-1,8-diyl bismethanethiosulfonate or 1,2-ethanediyl bismethanethiosulfonate (in 10mM Tris-HCl pH 7.4, 150mM NaCl, Complete EDTA-free protease inhibitors) with or without pretreatment with 10mM NEM/10mM EDTA. The cross-linking reaction was quenched with NEM, and extracts were prepared in Nu-Page sample buffer without β-mercaptoethanol and separated on a 7% Tris-acetate gel.

Statistical analysis. Data from three independent experiments were used for calculations (standard errors (SE) of the mean are indicated), which were then subjected to one-way ANOVA with a Bonferroni correction to determine their significance.

Transduction with APP adenovirus–Urea gel electrophoresis. Subconfluent stable MEF cell lines were transduced with the recombinant adenovirus Ad5/CMV-APP bearing human APP-695 (32) for 7 hours at 37°C, after which they were kept overnight at 37°C in DMEM supplemented with 0.2% fetal bovine serum. 24 hours post-infection, the conditioned medium (1000μl total volume) was collected, cleared by centrifugation and 10μl were assayed immediately for the production of Aβ40 and Aβ42 by specific ELISA (see below). A similar volume sample was used for determination of the amount of secreted APP fragments (APPs) by SDS-PAGE and direct western blotting with the polyclonal antibody 22C11 (Chemicon). The remaining conditioned medium (equal volumes) was immunoprecipitated overnight with mAb B7/8 raised against the N-terminus of the Aβ sequence (33) and 30μl of protein G-Sepharose (Amersham). After extensive washing, the bound material was eluted from the beads by boiling for 10 minutes in sample buffer (0.74M bis-tris, 0.32M bicine, 0.88M sucrose, 2% SDS, 0.015% bromophenol blue) and was loaded on a 12%T/5%C Bicine/Tris SDS-PAGE gel containing 8M urea (34). Separation was allowed to proceed at room temperature for 2 hours at a constant current of 24mA/gel and was followed by western blotting and visualization of the Aβ species with mAb W02.

ELISA. For the measurement of secreted Aβ40 and Aβ42, specific ELISA kits (The Genetics Company) were used, according to the manufacturer’s protocol.

Analysis of Notch processing. Subconfluent MEF cell lines were infected with the Ad5/dE1dE2a/CMV myc-tagged Notch ΔE adenovirus (35) for 24 hours and, after treatment with the proteosomal inhibitor lactacycin (Calbiochem) for 4 hours at 37°C, cell extracts were prepared. Samples of 10μg of total protein were separated on a 7% Tris-Acetate gel, transferred to nitrocellulose membranes and probed with the appropriate antibodies to assess the levels of Notch ΔE infection and NICD production respectively.

Blue native gel electrophoresis. This method was performed as described previously (36), with the exception that the samples (20μg) were separated on a 5-16% polyacrylamide gradient at a constant voltage of 200V for 3.5 hours.

RESULTS

Generation of a cysteine-less PS1
Mouse PS1 contains five endogenous cysteines (Fig.1A), which we replaced with alanine using site-directed mutagenesis. The resulting “cysteine-less” (cys-less) PS1 was able to rescue the maturation of Nicastrin and the stabilization of PEN-2, as well as the accumulation of unprocessed APP C-terminal fragments in PS1-/-PS2-/- fibroblasts (Fig.1B). Further functional characterization of the cys-less PS1 revealed that it has similar rescuing activity to the wild-type PS protein in PS1-/-PS2-/-fibroblasts as far as it concerns the in vivo processing of three major γ-secretase substrates, APP (Fig.1C), N-cadherin and Notch (data not shown).
Transmembrane domains 6 and 7 contain the putative catalytically active aspartates and therefore likely contribute also structurally to the catalytic site of PS1. Alignment of these domains from various species (Fig. S1) revealed, apart from the two catalytic aspartates, several other highly conserved amino acids that could be involved in substrate binding, in catalysis or in delineating a hypothetical interior water-containing chamber in the complex (37). We, therefore, substituted these residues one by one with cysteines and generated stably transfected PS1-/- PS2-/- fibroblast cell lines with the mutants, similarly to the cysteine-less PS1 (Fig.1C). Two mutations (G382C and K395C) resulted in impaired PS1 endoproteolysis, but all 22 mutants were nevertheless able to rescue the stabilization of PEN-2 and the maturation of Nicastrin. In addition we also substituted the catalytic aspartic residues, both individually and in combination, without again perturbing the ability of PS1 to stabilize the other γ-secretase components (Fig.1D).

Water accessibility of introduced cysteines and disulfide cross-linking

Recent EM studies have shown that an electrolucent channel is present in the interior of the γ-secretase complex. Our primary aim was to investigate biochemically whether such a postulated “hydrophilic pocket” (37) indeed exists and to delineate the amino acid residues exposed to it. We used the membrane-permeable reagent EZ-link Biotin-HPDP, which readily reacts via its pyridyldithiol moiety with free sulfhydryl (-SH) groups exposed to water. As shown in Fig.2A and B, out of 10 different residues in TM6 tested, only W247C was labeled. In TM7, in contrast, 6 out of 11 introduced cysteines showed reactivity with Biotin-HPDP. In addition, PS1 D385C in TM7 was modified, while the D257C mutant in TM6, which represents the putative second catalytic aspartate of PS1, was not modified in our assay. However, in order for the aspartic residues to perform substrate cleavage, they should be in close proximity to each other and be exposed to a hydrophilic environment. To investigate this further we used a specific disulfide cross-linking strategy in the cell line expressing the double mutant PS1 D257C/D385C. We chose two homobifunctional alkyliothiosulfonates of the type depicted in Fig.2C (3,6-dioxaoctane-1,8-diyl bismethanethiosulfonate and 1,2-ethanediyl bismethanethiosulfonate, with spacer arm length 13 and 5.2 Å respectively), which react selectively with −SH groups, resulting in the formation of disulfide bridges between two cysteines and the spacer arm of the cross-linker. This reaction can only take place if the cysteines have free sulfhydryl groups accessible to water and are located at a maximum distance from each other equal to the length of the spacer arm of the cross-linker.

Membrane extracts from PS1 D257C/D385C cells treated with either of the two cross-linkers at 4°C (to reduce protein molecular motions) and separated in SDS-PAGE under non-reducing conditions displayed a band running at an apparent molecular weight close to full length PS1 (Fig.2D). Since this band could be stained with antibodies specific for both PS1 NTF and CTF, we propose that it reflects an intermolecular cross-linking product between the two cysteines replacing the aspartates in TMs 6 and 7. The disulfide bond causes an expected shift in mobility compared to the full-length unprocessed protein because it prevents complete unfolding under non-reducing conditions. When the free sulphydryls are blocked with the alkylating agent NEM prior to the cross-linking reaction (Fig.2D, lanes labeled with NEM +) or when reducing conditions are applied (data not shown) this band is not observed, confirming its specificity. Furthermore, no cross-linked products are observed with cys-less PS1, single D257C and D385C PS1 mutants or a control mutant with two cysteines at remote positions in TM1 and 9. Note also that no band derived from intermolecular cross-linking of two different PS1 molecules was observed in any case under our experimental conditions.

This experiment demonstrates that in the tertiary structure of PS1 cysteines introduced at the positions of the catalytic aspartic residues are both accessible to water, facing each other with a maximal distance of ~5.2 Å.

Activity of the mutants on APP processing

We next investigated whether any of the cysteine substitutions influences the γ-secretase processing of APP. As shown in Fig. 3A, APP-CTF fragments generated by α-secretase from endogenously expressed APP (the direct substrate for γ-secretase) accumulate in PS knockout
fibroblasts. This phenotype could be completely rescued by reintroduction of wild-type or cys-less PS1 in these cells, but not, as expected, by PS1 bearing either the D257C and D385C mutations or each of the following substitutions: G382C, G384C, F388C, Y389C or K395C. Therefore, these residues seem to be particularly important for the activity of the protease.

After transduction of the fibroblasts with full length APP695Swe and direct quantification of the Aβ produced by ELISA (Fig. 3B), we observed that indeed 3 of the mutants (G382C, G384C and K395C) were causing a total loss of function with regard to Aβ generation. Interestingly, the Y389C mutation displayed residual activity (~10% Aβ40 production compared to the cys-less PS1), but no detectable Aβ42 production. The remaining mutants can be divided into three categories: 1) the ones that reduce the production of both Aβ40 and Aβ42 (S254C, V261C), 2) those that cause a significant decrease in the levels of Aβ40, with minor effects on Aβ42 production. The remaining mutants were divided into three categories: 1) the ones that reduce the production of both Aβ40 and Aβ42 (S254C, V261C), 2) those that cause a significant decrease in the levels of Aβ40, with minor effects on Aβ42 production, and 3) those that produce amounts of Aβ similar to the wild-type or cys-less PS1 (all the rest). Strikingly, none of these mutations seems to severely affect the ratio of Aβ42/40 produced (Fig. 3B), with the exception of Y256C and F388C, which, due to a dramatic reduction in Aβ40, behave like extreme “clinical” FAD mutations (with 4.5- and 6-fold increase in the ratio, respectively, compared to cys-less PS1). The effect observed for some of the mutations in the individual production of Aβ40 and Aβ42 was also, independently confirmed by urea gel electrophoresis (Fig. S2).

**Effects of the mutations on the processing of other substrates**

Next we analyzed the behavior of the cysteine mutants in the processing of N-cadherin. Similarly to APP, the carboxy-terminal fragment of N-cadherin generated by metalloprotease cleavage accumulates in PS deficient cells. This knockout phenotype was not rescued by the T245C, S254C, Y256C, V261C, G382C, G384C, F388C, Y389C, K395C and the catalytic D257C and D385C mutants (Fig. 4A).

Finally, the production of NICD (Notch Intracellular Domain) from a membrane-tethered form of Notch (myc-tagged Notch ΔE) was investigated (Fig. 4B). Similarly to APP and N-cadherin, PS1 bearing the G382C, G384C, Y389C or K395C mutations were unable to support any NICD production. Minimal activity was, however, also, seen with the T245C, S254C, Y256C and V261C mutants, in contrast to what we observed for APP. In addition, three mutants appeared to increase significantly Notch processing (W247C, L250C, L258C). Finally, and important in the context of the question whether APP and Notch processing can be specifically modulated by γ-secretase, the F388C substitution, which caused a remarkable decrease in the levels of Aβ40, did not affect significantly the production of NICD.

In order to verify that the observed effects on the processing of different substrates was not a consequence of deficient γ-secretase complex formation, we confirmed the integrity of the complexes by blue native gel electrophoresis (Fig. S3).

**DISCUSSION**

In the present study we report our efforts to investigate the catalytic site of PS1 by cysteine scanning mutagenesis. A primary prerequisite for the use of this technique is the generation of a protein lacking all endogenous cysteines but retaining the structure and functional properties of the wild-type molecule. Although clinical mutations have been identified at three out of the five endogenous cysteines in PS1 (C92S, C263F/R, C410Y) (38-40), we found that, under our experimental conditions, their substitution to alanine does not affect γ-secretase cleavage of the three substrates (APP, Notch and N-cadherin) examined here. Thus these mutations seem to be well tolerated and do not affect significantly the structure of PS1, as assessed in our cell biological experiments. Recently, Kornilova et al. (41), suggested that cysteines C92, C410 and C419 may contribute to the active site of PS1. However they did not check to what extent their replacement with serine (or alanine) interferes with this function. This together with significant methodological differences might explain the discrepancies between their findings and the findings in the current manuscript. Although we cannot exclude entirely at this moment that the cysteine substitutions in PS1 might indeed affect activity of the complex in other assays (e.g. cell-free assays), our experiments clearly demonstrate
that the cys-less PS1 protein is able to rescue efficiently many of the normal functions of PS1 in the cell-based assays used in the current work.

When we assessed the accessibility of cysteines introduced in TM7 to a sulfhydryl-specific reagent, several positions, including the catalytic D385, were reactive, implying that these residues are accessible to water. Although this domain does not seem to be a classically amphipathic helix, most of the accessible residues cluster on one side (Fig. 5A, B). Recently, Lazarov et al. (2006) reported the presence of an interior translucent chamber in purified γ-secretase complex as visualized by EM tomography. In accordance with this morphological evidence, our experiments prove biochemically the existence of a water-filled cavity in the intramembranous part of γ-secretase. Surprisingly, of all the mutants analyzed in TM6, including the catalytic D257, only one was readily accessible to our reagent (W247C). This negative data can mean that either these residues are all facing the lipid bilayer or that they are buried in the protein interior and thus inaccessible to the bulky Biotin-HPDP. Since the second aspartic residue (D257) of the catalytic dyad of PS1 should be accessible to water during hydrolysis of the substrates, we reasoned that the second hypothesis was more likely. To discriminate between the two possibilities, we replaced simultaneously both catalytic aspartates with cysteines and showed that they could be cross-linked. This experiment confirms for the first time experimentally that the proposed catalytic aspartates (D257 and D385) are facing each other in an aqueous environment with a distance of maximum 5.2 Å. Thus, we conclude that also D257 is accessible to water, at least in the active conformation when both aspartyl residues should be closely opposed to each other. The fact that the D257C residue did not react with more bulky chemical reagents like Biotin-HPDP might indicate either that TM6 is densely packed (but water exposed) and not accessible to these reagents because of steric hindrance, or that we probe with these chemicals mainly the inactive γ-secretase complex. Indeed, several reports suggest that only part of wild type PS1 in cells is actually associated with the active complex (42-44). Thus, our data are compatible with the possibility that TM6 changes conformation upon binding of the other components of the complex or after binding of the substrates, bringing the active D257 residue in line with the other active D385 residue in the catalytic site of the complex. This possibility is under further investigation and will require discrimination between active and inactive γ-secretase complex.

Furthermore, our scanning approach revealed several new residues within TMs 6 and 7 that are critical for the catalytic function of PS1. Amino acid substitutions G382C, G384C and K395C rendered the complex completely inactive. The two glycines are part of the GxGD motif, highly conserved not only in Presenilins, but also Presenilin-like proteins (45), signal peptide peptidase (SPP) (46) and the type-4 prepeptidases (47). Such transmembrane domain glycines have an essential role in helix flexibility (48) and also, often occur at helix-helix interfaces, facilitating closer packing of the TM helices (49). Especially, G384 faces the water-filled channel and its substitution with alanine constitutes one of the most severe and well-characterized AD clinical mutations, whereas several artificial mutations of this residue render PS1 completely inactive (47). On the other hand, the position and water accessibility of K395 suggest a possible involvement in the catalytic mechanism, although further analysis is needed to confirm this hypothesis.

Interestingly, substitution of F388, which lies on the same face of the helix as K395 (Fig. 5B) results in a remarkable loss of both APP and N-cadherin processing, but with minimal effect on NICD production. Four additional conserved residues were identified in TM6 (T245, S254, Y256, V261), which, when substituted, cause an almost complete loss of Notch and N-cadherin cleavage, while Aβ production is only partially affected. Specifically, the Y256C and T245C mutants are situated on the same side of the helix and decrease only Aβ40, whereas the other two (S254C, V261C), which align with the catalytic D257, decrease both Aβ40 and Aβ42. Taking into account that the active and the substrate binding sites on PS1 are distinct but close to each other, these observations indicate that the amino acids in question affect differentially the binding of the substrates per se or their accommodation in the catalytic site during cleavage.

Finally, particularly revealing was the substitution of the conserved tyrosine Y389 in TM7 with cysteine, causing a severe loss of function of PS1.
According to the most widely accepted acid-base catalytic mechanism for aspartic proteases, one aspartate is deprotonated and acts as a general base, activating a water molecule and forming OH\(^{-}\) in the transition state, and the other aspartate (general acid) donates a proton to the carbonyl of the substrate, facilitating the formation of a tetrahedral intermediate. Very often hydrogen bonds between the carboxylates of the aspartates and neighboring side chains are needed to keep the aspartates coplanar and to assist in the proton transfers during the catalysis (50, 51). Since Y389 is located very close to and aligns with the catalytic D385 within the water-filled cavity, we propose that it is involved in the catalytic mechanism through the formation of a hydrogen bond with the side chain of D385. The substitution of Y389 with cysteine, which is shorter than tyrosine and not as good proton donor, could then affect the catalysis by altering the protonation state of the catalytic residue. Similar interactions underlyng the aspartyl mediated catalytic mechanism have been reported for Cathepsin E (A1 family of aspartic proteases), with Y20 forming a hydrogen bond with D43 (52) and *Trichoderma reesei* cellobiohydrolase II (family B of glycoside hydrolases), where Y179 interacts with deprotonated D175, maintaining it in a charged state (53, 54).

In conclusion, our results provide experimental evidence for the presence of a water-filled cavity within the \(\gamma\)-secretase complex, likely essential for substrate hydrolysis by the two closely opposed aspartic residues in the catalytic core of PS1. Further analysis identified crucial amino acids in TM6 and TM7 that differentially affect substrate proteolysis, providing experimental proof that substrates are differentially handled in the catalytic cleft of Presenilin. Further work is needed to investigate whether these fundamental properties can be translated into the development of more specific drugs blocking the production of the A\(\beta\)42 peptide without affecting other important \(\gamma\)-secretase substrates in significant ways.

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FOOTNOTES

*We wish to thank Katrien Horré for practical assistance and Drs Wim Annaert and Constanze Reinhard for critically reviewing the manuscript. Work in the laboratory is supported by a Freedom to Discover grant from Bristol-Myers-Squibb, a Pioneer award from the Alzheimer’s Association, the Fund for Scientific Research, Flanders; K.U.Leuven (GOA); European Union (APOPIS: LSHM-CT-2003-503330 and NeuroNe); and Federal Office for Scientific Affairs, Belgium (IUAP P5/19).

1The abbreviations used are: APP, amyloid precursor protein; FAD, Familial Alzheimer’s Disease; RIP, regulated intramembrane proteolysis; PS, presenilin; Aβ, amyloid β peptide; NICD, Notch intracellular domain; TM, transmembrane domain; NEM, N-ethylmaleimide.

2Reference: Spacic D., Tolia A., Dillen K., Baert V., De Strooper B., Vrijens S., Annaert W. “Presenilin-1 maintains a nine transmembrane topology throughout the secretory pathway” (2006) J Biol Chem (accepted for publication).

FIGURE LEGENDS

Fig.1: Construction of cysteine-less PS1 and cysteine mutants. (A) Schematic representation of PS1. (B) Expression of the cys-less PS1 on a PS1-/-PS2-/- background rescues the stabilization of γ-secretase components and the accumulation of APP CTFs. (C) Functional comparison of wt and cys-less PS1 in the in vivo processing of APP. Quantifications of both the accumulation of endogenous APP CTFs and the production of Aβ after APPswe overexpression reveal that the activity of cys-less PS1 is similar to the wt protein. (C) Expression levels of PS1, Nicastrin and PEN-2 in knockout fibroblasts expressing PS1 with single amino acid mutations to cysteine.

Fig.2: Accessibility of unique PS1 cysteines to sulfhydryl-specific reagents. (A) Cysteines introduced in TM6 were exposed to Biotin-HPDP and precipitated with neutravidin beads in the presence of Triton X-100. Biotinylated material was revealed by western blotting using the indicated antibodies. PEN-2 (one endogenous extracellular cysteine) and wt PS1 NTF (with three endogenous cysteines) serve as positive controls. PS1 CTF, which does not contain cysteines, and Nicastrin, with all its 12 cysteines clustered in disulfide bridges as predicted with the DISULFIND program (55) are negative controls. (B) Reaction of cysteines in TM7, as well as cysteines substituting the catalytic aspartates (D257C and D385C) with Biotin-HPDP. (C) Chemical structure of the cross-linker 3,6-dioxaoctane-1,8-diyl bismethanethiosulfonate (D) Specific cross-linking of D257C with D385C in the PS1 D257C/D385C mutant. Membrane fractions were treated with cross-linker with and without pre-blocking the free sulfhydryls with NEM and the proteins were separated under non-reducing conditions on a 7% Tris-acetate gel.

Fig.3: Analysis of APP processing. (A) Endogenous APP processing was assessed by western blot. The accumulation of APP CTFs was quantified and normalized to full-length APP. (B) After adenoviral overexpression of APPswe, levels of Aβ40 and Aβ42 in the conditioned medium were measured by ELISA and p values (*p<0.05, **p<0.01, ***p<0.001) were determined for the average from three independent experiments performed in triplicate (n=9).

Fig.4: Processing of N-cadherin and Notch. (A) Accumulation of endogenous N-cadherin CTFs and quantification with respect to the full-length protein (B) Fibroblasts were infected with adenovirus bearing NotchΔE-myc substrate and the levels of NICD produced were visualized with a cleavage specific antibody (cleaved Notch1 Val 1744) and quantified according to the levels of infection (anti-myc staining).
Fig.5: Helical representation of TMs 6 and 7. Amino acids are represented by the single letter code. (A) Helical wheel model of TMs 6 and 7, viewed from the N-terminus (DNASTAR). Arrows and * indicate amino acids reactive and non-reactive to Biotin-HPDP, respectively. (B) Lateral views of helices 6 and 7 (Swiss-PDB Viewer). Side chains the substitution of which with cysteine affected the activity of PS1 are indicated. The water accessibility patterns of the helices are represented as dotted surfaces and the putative hydrogen bond between the catalytic D385 and Y389 is represented as a dashed line.
Figure 1
Figure 2
Figure 3
Figure 4
Contribution of presenilin transmembrane domains 6 and 7 to a water-containing cavity in the γ-secretase complex
Alexandra Tolia, Lucía Chávez-Gutiérrez and Bart De Strooper

J. Biol. Chem. published online July 14, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M604997200

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