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Structure nor stability of the transmembrane spanning 6/7 domain of Presenilin I correlates with pathogenicity

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Abstract

Since its cloning in 1995, missense point mutations in presenilin I (PS-I) have been shown to be responsible for greater than 70% of the cases of early onset familial Alzheimer's Disease (EOFAD), which can affect individuals as early as age 18. PS-I is known to be a component of γ -secretase, the enzyme responsible for cleavage of the amyloid precursor protein (APP) into 42 amino acid peptides that aggregate to form the plaques found in the neurons of Alzheimer's patients. It has recently been hypothesized that wild type (wt) PS-I contains an autoinhibitory module that prevents γ -secretase cleavage of the APP, while pathogenic PS-I point mutants lack a structure necessary for this inhibition. In this work, spectroscopic data is presented that does not correlate structure or stability of the proposed PS-I autoinhibitory module with pathogenicity.

Keywords

Alzheimer's Disease; presenilin I; early onset familial; autoinhibition; γ -secretase

INTRODUCTION

Alzheimer's Disease (AD) is a tragic neurological disorder that affects more than 4.5 million Americans, with millions more affected worldwide.¹ This disease, characterized by dementia due to vast neuronal loss in the cortical and hippocampal areas of the brain, is the leading cause of dementia and the 8th leading cause of death in America.² This dementia, along with the appearance of amyloid plaques and neurofibrillary tangles was first described by Alois Alzheimer in 1907.³

It is now known that mutations in three genes account for nearly all cases of early onset familial Alzheimer's Disease: APP, PS-II, and PS-I,^{4–25} the latter of which is the focus of our work. Since the cloning of PS-I in 1995,⁵ more than 64 missense mutations resulting from single point mutations and an in-frame deletion of exon 9, due to a single point mutation at a splice acceptor site, have been discovered.^{5, 9–31} Sequence analysis of PS-I identifies ten hydrophobic regions, eight of which have been proposed to function as transmembrane (TM) domains.³² In this model, the N- and C-termini as well as a 145 amino acid hydrophilic loop between TM6 and TM7 are located in the cytosol.^{33–34} The domain between TM6 and TM7

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appears as a hotspot for mutation, with 38% of all mutations being located in exon 8, the N-terminal portion (residues 260–286) of the TM6/7 domain.³⁵

PS-I is part of the γ -secretase complex, which is the enzyme responsible for the final cleavage of the APP to A β peptides (A β ₄₂), which form tightly folded β -sheets that aggregate around neurons.³⁶ γ -Secretase is a heterotetrameric transmembrane spanning enzyme which, in addition to PS-I (or PS-II) also contains Pen-2, Aph-1, and nicastrin.³⁷ Evidence supports that γ -secretase uniquely cleaves peptide bonds through an aspartyl protease mechanism while buried in the hydrophobic lipid membrane of the neuron. The putative catalytic aspartates are located in transmembrane regions 6 and 7 of presenilin.^{38–39}

EOFAD mutations in PS-I enhance A β cleavage, although the detailed mechanism by which this occurs is unclear.^{40–44} It has been shown, however, that an intact exon 9 (residues 290–319 of the TM6/7 domain) in presenilin regulates the activity of γ -secretase through an inhibitory process.⁴⁵ Therefore, wild type PS-I, with an intact exon 9, inhibits the formation of A β ₄₂. Knappenberger *et al.* have hypothesized that specific structure in the TM6/7 domain may bind to a substrate docking site in γ -secretase, interfering with the proteolysis of APP. Further, they suggest that mutations of the TM6/7 domain may be responsible for deformation of this structure necessary for inhibiting γ -secretase; these mutants fail to form the inhibitory region, thus causing the constitutive production of A β ₄₂ as seen in EOFAD PS-I mutations.⁴⁵ To test this hypothesis, we have determined the structure and stability of five pathogenic EOFAD mutants (E280A, L286V, P264L, P267S, and Δ E9) and the wild-type PS-I TM6/7 domain. Results are presented that show neither the structure nor stability of this region of PS-I correlates with pathogenicity.

MATERIALS AND METHODS

Cloning

Wild type, L286V, E280A and Δ E9 human PS-I cDNA was generously donated by Gopal Thinakaran (University of Chicago). The cDNA for all the mutations and wild type were amplified using PCR in the TM6/7 domain, coded by nucleotides 789–1221 in the point mutations and wild type (amino acids 263–407) and nucleotides 789–1131 for the Δ E9 mutant (Δ E9 is the deletion of wt amino acids 290–319). Since mutations P267S and P264L are close to the N-terminal end of the TM6/7 domain, these mutant inserts were derived by PCR amplification of the wild type cDNA with 5' primers that encoded the mutation. The 5' and 3' primers for each of the six constructs encoded restriction sites for *EcoRI* and *HindIII*, respectively, which allowed the PS-I insert to be ligated to the similarly cut pMal-c2g cloning plasmid (New England BioLabs). The resulting construct, encoding an N-terminal maltose binding protein (MBP) fusion tag separated from the PS-I coding sequence by a genease site, was transformed into *E. coli* DH5- α cells. Transformant colonies were sequenced at Joseph Stokes Jr. Research Institute, Children's Hospital of Philadelphia.

Protein over-expression and purification

Colonies containing the wt or mutant MBP-PS-I TM6/7 domains were incubated at 37 °C in 1 L of LB-Amp 100 until an OD₆₀₀ of 0.5–0.6 was reached, and then induced with 1 mM IPTG. Growth was continued for 3 hours at 37 °C, after which time the cells were harvested by centrifuging for 10 minutes at 6000 $\times$ g. The cell pellet was resuspended in 50 mL of column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) and lysed by sonication. Lysed cells were centrifuged at 20,000 $\times$ g for 30 minutes. The high speed supernatant was diluted in 125 mL of column buffer and loaded onto an amylose affinity column (8mL of resin, New England BioLabs) at a flow rate was 1.0 mL/min. The column was washed with 50 mL of column buffer, after which time MBP-PS-I was eluted with 10 mM maltose. Further

purification of MBP-PS-I was achieved by size exclusion chromatography (SEC, Superdex 75 HR 10/30) on a Varian Prostar HPLC at a flow rate of 0.5 mL/min. 150 μ L at approximately 2.5 mg/mL of MBP-PS-I was loaded onto the column, with pure material eluting at 13.8 minutes.

Circular dichroism (CD) spectroscopy

CD measurements were performed on an Olis DSM-10 CD spectrophotometer at 25 °C with an integration time of 1.2 seconds in a 1 mm quartz cuvette. Scans for all the MBP-PS1 constructs were completed in the far UV region, 190–240 nm, at protein concentrations of 9.0 μ M ($\epsilon = 88200 \text{ M}^{-1}\text{cm}^{-1}$) in 20 mM sodium phosphate, 20 mM NaCl, pH 7.4. Each reported spectrum was the average of five scans, from which a buffer blank had been subtracted. The ellipticity in Figure 1 is that of MBP (9.0 μ M in 20 mM sodium phosphate, 20 mM NaCl, pH 7.4) subtracted from the MBP-PS-I fusion proteins. Error is calculated at the 95% confidence level.

Thermal denaturation

To determine thermal stability of the MBP-PS-I constructs, the CD spectroscopic signal was monitored at 221 nm as a function of increasing temperature (25, 40, 50, 60, 65, 70, 75, 80, 84, 88, 90, 95 °C) with a 1.0 second integration time. After reaching each temperature, the protein was equilibrated for 10 minutes prior to monitoring the CD signal for 60 seconds.

Fluorescence spectroscopy

Intrinsic tryptophan fluorescence spectroscopy was performed on a PTI spectrometer with an excitation slit width of 2.5 nm and an emission slit width of 1 nm. All spectra were recorded at a protein concentration of 9.0 μ M (in 20 mM sodium phosphate, 20 mM NaCl, pH 7.4) and were equilibrated at 25 °C for 60 seconds prior to excitation. Five emission scans were collected for each protein from 285 to 450 nm with a 280 nm excitation in a 3 mm quartz cuvette. The intensities in Figure 2 correspond to that of MBP (9.0 μ M in 20 mM sodium phosphate, 20 mM NaCl, pH 7.4) subtracted from the MBP-PS-I fusion proteins. Error is calculated at the 95% confidence level. The maximum wavelengths of emission of the wt and mutant MBP-PS-I TM6/7 domains are reported in Table 1. Standard error of the mean is reported.

RESULTS AND DISCUSSION

PS-I over-expression and purification

The PS-I mutants P264L, P267S, E280A, and L286V were chosen for study to span the range of most (L286V, where a single methylene group is removed in a protein of 467 amino acids converting it to a pathogenic protein) to least (E280A where a carboxylic acid side chain, deprotonated at physiological pH, is converted to a small hydrophobic side chain) conservative. The Δ E9 pathogenic mutant was chosen as it lacks exon 9 (corresponding to the deletion of wt amino acids 290–319), the putative autoinhibitory module of the TM6/7 domain.⁴⁵

The wt and each of the pathogenic mutant PS-I TM6/7 domains were expressed solubly as C-terminal fusions to maltose binding protein (MBP), which is consistent with the reported chaperone-like activity of MBP.⁴⁶ Attempts to directly express the PS-I TM6/7 domains or to express them as glutathione S-transferase fusions resulted in expression in inclusion bodies which proved intractable to folding. Purification of the wt and mutant MBP fusion proteins via amylose affinity and size exclusion chromatography led to isolation of ~34 mg of pure protein per liter of bacterial growth. However, genenase cleavage of the linker between MBP and the PS-I TM6/7 domain did not proceed to completion, which led to an interaction between the PS-I TM6/7 domain and the uncut fusion protein and thus resulted in their co-elution from

multiple chromatography stationary phases. Surprisingly, due to a net charge of -11 of the fusion protein and a single cysteine residue in this PS-I domain, neither the addition of increasing concentrations of NaCl nor β -mercaptoethanol affected this interaction. Inclusion of urea at concentrations up to 5 M also proved ineffective at disrupting this interaction. Incubation with 30% CH₃CN at 37 °C for two hours prior to SEC, and inclusion of 30% CH₃CN in the SEC mobile phase allowed for purification of small quantities of the desired monomeric (as determined by SEC retention time) PS-I TM6/7 domain. Although this result seemed inconsistent with a net charge of -11 for the fusion protein, a hydropathy plot revealed that this charge is clustered in the central portion of the PS-I loop domain, with both ends being hydrophobic. This latter observation is not surprising in that both the N- and C-termini of the TM6/7 domain in the intact PS-I protein are connected to hydrophobic membrane spanning regions. Due to difficulties liberating the PS-I TM6/7 domain, the structure and stability of the wt and mutant fusion proteins are reported.

PS-I secondary and tertiary structure

The secondary structure of the wt and mutant PS-I TM6/7 domains was probed by far UV CD spectroscopy. Figures 1A and 1B show the α -helical content of the wt and mutant PS-I TM6/7 domains (CD signal of MBP-PS-I-TM6/7 minus CD signal of MBP) at 209 and 221 nm, respectively. At 209 nm, only the PS-I P264L mutant has a quantity of structure outside of the 95% confidence interval of the wt domain. At 221 nm, the PS-I E280A, P264L, and P267S mutants have a content of α -helical structure outside of the 95% confidence interval of the wt domain. These excesses in α -helical structure at both wavelengths are minimal and do not support the idea that a common feature of all pathogenic mutants is an alternate secondary structure (inhibitory module) in the TM6/7 region.

The environment of the three tryptophan residues in the wt and mutant PS-I TM6/7 domains (W-294, 340, and 404) was probed by intrinsic fluorescence spectroscopy. Figure 2 shows the intensity of fluorescence emission of the wt and mutant PS-I TM6/7 domains (intensity of MBP-PS-I-TM6/7 minus intensity of MBP) at λ_{max} . Only the L286V mutant has an intensity that is outside of the 95% confidence interval of the wt domain. The combinations of this data with the maximum wavelengths of emission (Table 1) also does not support the idea that a common feature of all PS-I pathogenic mutants is an alternate tertiary structure (inhibitory module) in the TM6/7 region.

PS-I thermal stability

The thermal stability of the wt and mutant MBP-PS-I TM6/7 domains was determined by monitoring the intensity of the α -helical signal at 221 nm by far UV CD spectroscopy. Only the L286V mutant has a melting temperature (57 ± 2) that is outside of the 95% confidence interval of the wt domain (65 ± 3). Interestingly, it is the L286V mutant that also has less fluorescence intensity than the wt and other mutant domains. Although a lack of shift in the λ_{max} of the fluorescence emission of the L286V mutant does not support the tryptophan residues being in an environment of alternate polarity, its decreased fluorescence emission in combination with its decreased τ_m do suggest alterations to the tertiary structure of this mutant causing its decreased stability.

Nonlinearity in the van't Hoff plot for all mutant and the wt MBP-PS-I TM6/7 domains prevented calculations of ΔH° , ΔS° , and ΔG° of the unfolding transition. This nonlinearity suggests that one of the underlying assumptions of the van't Hoff approach, that of temperature independence of ΔH° over the temperature range studied, is invalid for both the wt and mutant domains.⁴⁷ Overall, this data does not support the idea that a common feature of all PS-I pathogenic mutants is an alternate stability in the TM6/7 region.

In summary, the findings reported here are important for three reasons. The first is the demonstrated ability of MBP, as a fusion partner, to keep otherwise insoluble proteins soluble during over-expression. The second is that neither the secondary nor tertiary structure of the TM6/7 domain of PS-I correlates with pathogenicity. This is inconsistent with the hypothesis that the exon 9 region of the TM6/7 domain has a structure in the wt protein that inhibits proteolysis of the APP, while the mutants lack this structure and thus lack the ability to inhibit this proteolysis. Finally, this data does not support a correlation between stability of the PS-I TM6/7 domain and pathogenicity. Thus, there must be factors others than structure and stability in the PS-I TM6/7 domain that affect the production of the plaque forming 42 amino acid A β peptides. One possibility, due to the inclusion of presenilin in the heteromeric high molecular weight γ -secretase complex, is an altered interaction of the mutant presenilin with other components present in γ -secretase (Pen-2, Aph-1, and nicastrin) that would affect its ability to cleave the APP.

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Figure 1. α -helical ellipticity of the wt and PS-I TM6/7 domain mutants at (a) 209 nm and (b) 221 nm. E280A: diagonal stripes, Δ E9: horizontal stripes, L286V: light grey, P264L: dark grey, P267S: black, wt: white. Proteins are at 9.0 μ M in 20 mM sodium phosphate, 20 mM NaCl, pH 7.4. Error is reported at the 95% confidence interval.

Figure 2.

Fluorescence emission of the wt and PS-I TM6/7 domain mutants at $\lambda_{\max}$. E280A: diagonal stripes, Δ E9: horizontal stripes, L286V: light grey, P264L: dark grey, P267S: black, wt: white. Proteins are at 9.0 μ M in 20 mM sodium phosphate, 20 mM NaCl, pH 7.4. Error is reported at the 95% confidence interval.

Table 1

Maximum wavelength of emission of wt and mutant MBP-PS-I TM6/7 domains. Proteins are at 9.0 μ M in 20 mM sodium phosphate, 20 mM NaCl, pH 7.4. Standard error of the mean is reported.

Protein	Maximum Wavelength (nm)
MBP-PS-I E280A	341 $\pm$ 0
MBP-PS-I Δ E9	339 $\pm$ 1
MBP-PS-I L286V	340 $\pm$ 1
MBP-PS-I P264L	340 $\pm$ 1
MBP-PS-I P267S	341 $\pm$ 1
MBP-PS-I WT	341 $\pm$ 1

Table 2

τ_m of the wt and mutant MBP-PS-I TM6/7 domains. Proteins are at 9.0 μ M in 20 mM sodium phosphate, 20 mM NaCl, pH 7.4. Error is reported at the 95% confidence interval.

Protein	τ_m (°C)
MBP-PS-I E280A	61 $\pm$ 3
MBP-PS-I Δ E9	64 $\pm$ 2
MBP-PS-I L286V	57 $\pm$ 2 [*]
MBP-PS-I P264L	63 $\pm$ 3
MBP-PS-I P267S	70 $\pm$ 3 [*]
MBP-PS-I WT	65 $\pm$ 3 [*]

* : Error estimated based on the average 4.3% error in the three mutants for which error was experimentally determined.