Deducing the Transmembrane Domain Organization of Presenilin-1 in γ-Secretase by Cysteine Disulfide Cross-Linking†

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Received January 18, 2006; Revised Manuscript Received April 27, 2006

ABSTRACT: γ-Secretase is a founding member of membrane-embedded aspartyl proteases that cleave substrates within transmembrane domains, and this enzyme is an important target for the development of therapeutics for Alzheimer's disease. The structure of γ-secretase and its precise catalytic mechanism still remain largely unknown. γ-Secretase is a complex of four integral membrane proteins, with presenilin (PS) as the catalytic component. To gain structural and functional information about the nine-transmembrane domain (TMD) presenilin, we employed a cysteine mutagenesis/disulfide cross-linking approach. Here we report that native Cys92 is close to both Cys410 and Cys419, strongly implying that TMD1 and TMD8 are adjacent to each other. This structural arrangement also suggests that TMD8 is distorted from an ideal helix. Importantly, binding of an active site directed inhibitor, but not a docking site directed inhibitor, reduces the ability of the native cysteine pairs of PS1 to cross-link upon oxidation. These findings suggest that the conserved cysteines of TMD1 and TMD8 contribute to or allosterically interact with the active site of γ-secretase.

Accumulation of the amyloid β-protein (Aβ) is one of the defining pathological features of Alzheimer's disease. Aβ is produced from the amyloid β-protein precursor (APP) as a result of sequential proteolytic cleavages first by β-secretase (*1*) and then by γ-secretase (*2*). γ-Secretase is an aspartyl transmembrane protease that cleaves a number of type I membrane protein substrates, including APP and Notch, in the middle of their transmembrane domains in a poorly understood process of hydrolysis within a hydrophobic environment (*3*). γ-Secretase is composed of four transmembrane proteins, Aph-1, Pen-2, nicastrin (NCT) (*4*−*10*), and presenilin (PS), which is ostensibly the catalytic component of an unusual aspartyl protease (*3*, *11*, *12*). Despite substantial progress in establishing the full identity of γ-secretase (*7*−*9*, *13*, *14*) and the stepwise assembly of the γ-secretase complex (*7*, *15*, *16*), the molecular structure of the protease complex or even any of its individual components remains unknown. PS, as the catalytic component, is of major interest, but only some indirect evidence about its structural arrangement has been reported to date. Indeed, it has only very recently been established that PS1[1] is a 9-TMD protein (*17*, *18*). PS is endoproteolytically processed into an N-terminal fragment (NTF) and C-terminal fragment (CTF) (*19*). These fragments are metabolically stable and remain associated, and their formation is tightly regulated (*20*). Also, it is widely accepted, although not definitively proven, that the conserved aspartates D257 and D385 of presenilin constitute a catalytic dyad, and therefore, these residues are expected to be proximal (*21*). This view implies that TMD6 and TMD7 of PS-1 should be directly adjacent to one another. Studies by Annaert et al. provided evidence for a proposed "ring structure" for PS1, in which TMD1 is proximal to the C-terminus of the protein (*22*). Additionally, we have recently reported evidence that residue L166 in TMD3 may be a part of the active site, proposing that TMD3 is possibly in proximity to TMD6 and TMD7 (*23*). Finally, a report by Brunkan et al. (*24*) provided evidence suggesting that TMD1 contributes to the active site.

An understanding of the mechanism of γ-secretase proteolysis requires a detailed description of the three-dimensional organization of its transmembrane domains. In light of inherent difficulties in obtaining a structure of γ-secretase using methods such NMR spectroscopy, X-ray, or electron diffraction, it is crucial to explore alternative approaches that would allow the determination of transmembrane domain organization and offer hints to how familial Alzheimer-causing mutations in PS, which are scattered along the whole sequence of the protein, cause essentially the same effect on APP processing (increasing the proportion of 42-residue Aβ to its 40-residue form). One very promising strategy, which surprisingly has not been reported in the study

† This work was supported by grants to M.S.W. from the National Institutes of Health (NS41355 and AG17574) and the Alzheimer's Association (IIRG-02-4047) and by a fellowship to H.L. (Wenner-Gren Foundation in Sweden).

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[1] Abbreviations: PS1, presenilin-1; NTF and CTF, N- and C-terminal fragments; TMD, transmembrane domain; ES, embryonic cells; KO, knock out; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; Cu^{2+}Phe, copper phenanthroline; NEM, *N*-ethylmaleimide; βME, β-mercaptoethanol; DTT, dithiothreitol.

10.1021/bi060107k CCC: $33.50
Published on Web 05/26/2006

of γ-secretase, is cysteine mutagenesis/disulfide cross-linking. This method involves the introduction of cysteine residues at specific positions within transmembrane domains by site-directed mutagenesis and subsequent use of chemical reactivity of the introduced sulfhydryl groups. Because transmembrane cysteines are typically not accessible to modifying reagents, the most commonly used approach is cysteine−cysteine disulfide cross-linking, in which an oxidizing agent is used to catalyze the formation of disulfide bonds between proximal transmembrane cysteines and yield unique high molecular weight products. This approach has been widely applied to deduce the oligomeric state of transmembrane proteins (*25*, *26*), the identification of transmembrane domain organization (*27*−*30*), and identification of contact points within the protein (*31*) and even accessing the mechanistic details of drug and substrate interaction with transmembrane targets (*32*).

Here, we report findings about the PS structure obtained by utilizing the powerful approach of cysteine mutagenesis/disulfide cross-linking. To provide direct biochemical evidence for the arrangement of transmembrane domains in presenilin and to establish the means of probing its structure and mechanism, we initiated studies involving oxidative disulfide cross-linking of PS1.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis. Site-directed mutagenesis was performed with the Stratagene Multi-Site QuickChange mutagenesis kit. Mutations were introduced into plasmid pcDNA3.1 (Invitrogen) containing PS1 with a Flag epitope on the N-terminus (*8*). Mutations were confirmed by DNA sequence analysis.

Transfections and Stable Cell Line Generation. We used PS1/PS2 KO ES blastocyst mouse cells, which we obtained as a gift from B. Yankner (Harvard Medical School). ES cells were grown in DMEM supplemented with 15% FBS, nonessential amino acids, penicillin/streptomycin, sodium pyruvate, L-glutamine, 0.01% leukemia inhibitory factor ("ESGRO", Chemicon), and 2-mercaptoethanol (100 μM). Six-well plates of ES cells at 60−70% confluence were transfected with 2 μg of corresponding cDNA and with 6 μL of Lipofectamine (Invitrogen) and 500 μL of OPTIMEM (Invitrogen). Six hours after transfection, 500 μL of ES media was added. Twenty-four hours after transfection, the cells were replated at 12−25% into six-well plates, and 40 μg/mL zeocin was added to select for a stably transfected pool of cells. Single colonies were generated by replating the cells from the pool into 96-well plates at 1.2 cells/well. The protein expression level was analyzed by Western blotting, and the best expressing cell colonies were picked.

Preparation of Microsomes. ES cells (stably expressing the appropriate PS1 construct) or HeLa cells (expressing endogenous PS1) were grown, washed with PBS, and homogenized in buffer A [10 mM HEPES, pH 7.4, 1 mM EDTA, 0.25 M sucrose, and protease inhibitors (Roche)]. The cell homogenate was centrifuged at 20000*g*, and the supernatant was spun at 100000*g* and 4 °C for 1 h to pellet the membranes. The membranes were suspended in HEPES buffer (50 mM HEPES, pH 6.0, 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$) containing 1% CHAPSO and incubated for 1 h at 4 °C. The final suspension was spun at 100000*g* at 4 °C for 1 h to isolate the solubilized membranes.

Disulfide Cross-Linking. Solubilized membranes were diluted to a final concentration of 0.25% CHAPSO by addition of HEPES buffer. For oxidation, 15 μL of this suspension was mixed with 0.5 μL of 30 mM copper phenanthroline (Cu^{2+}Phe) (30 mM $CuSO_4$ and 90 mM 1,-10-phenanthroline in 50 mM PBS, pH 7.3) and incubated at 37 °C for 30 min. To stop the reaction, 4 μL of 4× Laemmli sample buffer [containing no β-mercaptoethanol, but 100 mM EDTA and 100 mM *N*-ethylmaleimide (NEM)] was added. The samples were subjected to nonreducing SDS−PAGE and analyzed by immunoblotting.

Reduction of Cross-linked Products. EDTA (100 mM) was added to oxidized samples before addition of the reducing agents. Oxidized species were reduced in the presence of 1% SDS either with 100 mM β-mercaptoethanol (βME) at pH 6.8 or with 100 mM dithiothreitol (DTT) at pH 9.0 and 60 °C for 30 min.

Effect of Inhibitors on PS1 Oxidation. HeLa cell solubilized membranes were oxidized with Cu^{2+}Phe in the presence of corresponding amounts of γ-secretase inhibitors. The inhibitors were added to the samples and incubated for 20 min prior to addition of oxidizing agent.

Compounds and Antibodies. The syntheses of III-31-C, D-10, and D-13 were accomplished as previously reported (*10*, *33*, *34*). Compound E (*35*) was synthesized according to published procedures. All compounds were HPLC purified and analyzed by MALDI-TOF and dissolved in DMSO to make stock solutions. The following antibodies were used for immunoblotting: 231-f (a-PS1-NTF, polyclonal raised to amino acids 2−20, from B. Yankner), 4627 (a-PS1-CTF, polyclonal raised to amino acids 457−467 of PS1, from Elan), MAB5232 (a-PS1-CTF, monoclonal raised to amino acids 263−378, Chemicon), UD-1 (a-Pen2, polyclonal raised to N-terminal residues ERVSNEEKLNL, from J. Naslund), and a-Aph1 (polyclonal, Zymed).

RESULTS

wt-PS1 Undergoes Oxidative Disulfide Cross-Linking. To test whether wt-PS1 undergoes cross-linking in the presence of oxidizing agents, we incubated CHAPSO-solubilized endogenous γ-secretase isolated from HeLa cells (*36*) with copper phenanthroline (Cu^{2+}Phe), a potent oxidizing agent known to induce disulfide bond formation not only between water exposed cysteines but even between transmembrane cysteines (*25*−*27*, *30*). We observed a decrease in the level of PS1-NTF and the rise of a newly formed ∼45−50 kDa product, a size corresponding to PS1-NTF/PS1-CTF (Figure 1A). We also detected a smaller amount of a newly formed species of ∼35−37 kDa, which was cross-reactive with anti-PS1-NTF antibodies. To establish the identity of the oxidation products, we probed them with two different PS1-CTF antibodies. One CTF antibody (MAB5232) clearly indicated the decrease in PS1-CTF levels and the appearance of a new ∼45−50 kDa species (Figure 1A, second panel). Another antibody (4627, raised against the C-terminus of PS1-CTF), which is less sensitive than MAB5232 but able to detect a caspase-cut 10 kDa alternative PS1-CTF (PS1-*alt*-CTF) (*37*, *38*), detected decreases in levels of both CTF and a putative PS1-*alt*-CTF upon Cu^{2+}Phe oxidation (Figure 1A, third panel). The latter antibody was less sensitive and did not cross-react with ∼45−50 or ∼35−37 kDa species. These

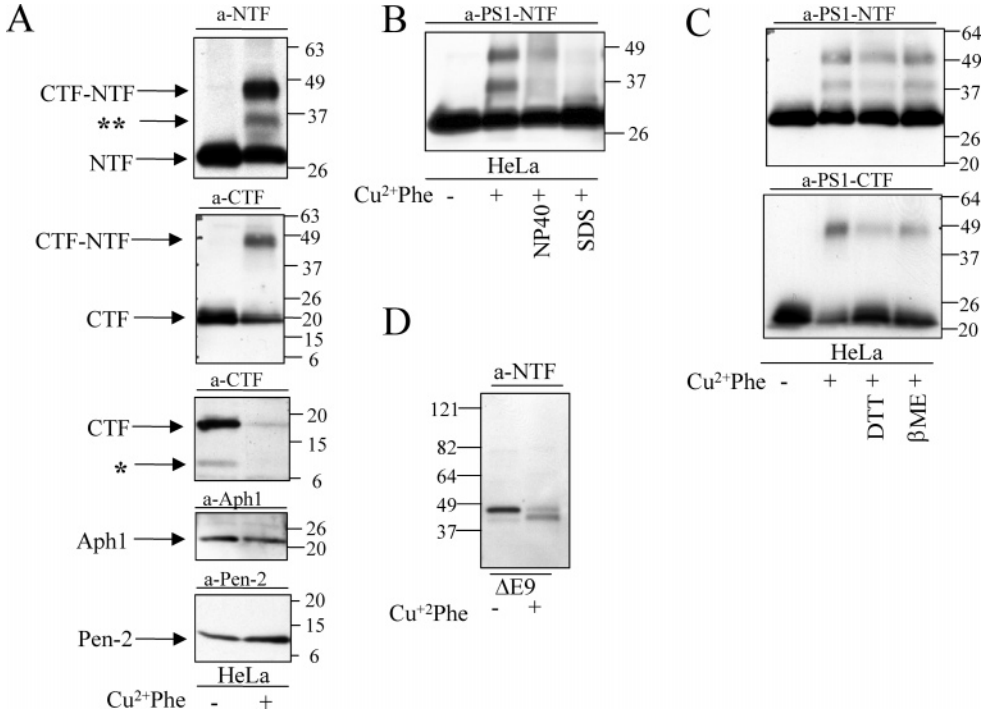

FIGURE 1: Disulfide oxidation of endogenous PS1. (A) PS1 oxidation with Cu^{2+}Phe results in the production of PS1-NTF-CTF cross-linked species in detergent-solubilized HeLa cell membranes (HeLa). Note that PS1-CTF is detected with two different antibodies, MAB5232 (second panel) and MAB4627 (third panel). The identity of bands denoted * and ** could not be confirmed but may be CTF alternatively processed by caspases and its NTF cross-linked counterpart, respectively (see text for details). (B) 1% of NP40 or 1% of SDS prevents disulfide cross-linking of NTF to CTF in the presence of Cu^{2+}Phe. (C) The NTF-CTF oxidized product is reduced in the presence of βME, pH 7, at 60 °C and DTT, pH 9, at 60 °C. Note that not all cross-linked NTF-CTF is being converted back to NTF and CTF. (D) Cross-linking experiment using detergent-solubilized membranes from PS1 KO ES cells stably expressing PS1 ΔE9, a catalytically active PS1 that is not converted to NTF and CTF. PS1 ΔE9 shows slightly altered mobility upon cross-linking but no dimeric form.

results altogether suggest that the observed ~45−50 kDa oxidation product corresponds to NTF being cross-linked to CTF. Reduction in the putative *alt*-CTF level was noted (Figure 1A, third panel) along with the appearance of the 35−37 kDa product (first panel), suggesting that the identity of the latter could be NTF cross-linked to *alt*-CTF. However, while the identity of the 10 kDa band as PS1-*alt*-CTF is consistent with previously reports (*37*, *38*), we cannot say with certainty that it is not another protein with some cross-reactivity to the PS1 C-terminal antibody. Neither is it certain that the ~35−37 kDa oxidation product corresponds to NTF cross-linked to *alt*-CTF, because this product was only detected with anti-NTF antibody. Attempts to increase levels of the putative *alt*-CTF and the cross-linked NTF-*alt*-CTF using staurosporine gave equivocal results. Levels of Pen-2 and Aph-1 did not change upon oxidation, ruling out the possibility of the ~45−50 kDa product being NTF-Aph1 and the 35−37 kDa product being NTF-Pen-2.

Importantly, when 1% NP-40 and 1% SDS were added to the samples before oxidation, reduced levels (in the NP-40 sample) and virtually no oxidized products (inthe SDS sample) were detected (Figure 1B). NP-40 and SDS detergents are known to dissociate the γ-secretase complex and to partially unfold transmembrane proteins (*10*). Therefore, this observation indicates that formation of oxidized wt-PS1 products is specific; that is, they are formed only when PS1 is a part of the assembled γ-secretase complex.

Disulfide bonds are often easily reduced in the presence of an excess of thiol reductant, such as βME and DTT, and SDS. Treatment of the oxidized PS1 products with 100 mM βME (pH 6.8) or with 100 mM DTT (pH 9.0) at 60 °C for 20 min resulted in a partial conversion of the cross-linked NTF-CTF back to NTF and CTF (Figure 1C). Incubation at higher temperature (90 °C) could have improved the conversion efficiency; however, PS1 aggregates at temperatures higher than 60−65 °C (data not shown). Nevertheless, the reducing conversion, although not complete, provides evidence that the PS1 cross-linked products are due to disulfide bond formation.

Our results together indicate that wt-PS1-NTF and wt-PS1-CTF can be cross-linked as a result of disulfide bond formation between proximal cysteines upon oxidation. Therefore, this finding strongly suggests that there are at least two native cysteine residues in the PS1 sequence, one in the NTF and one in the CTF, that are in close proximity (<7 Å). Because some evidence suggests that the γ-secretase complex may contain two PS1 molecules (*39*, *40*), it is possible that the cross-linked NTF-CTF is derived from two different PS1 molecules (i.e., cross-linking in trans). To address this issue, we generated PS1 KO ES cells stably expressing the PS1 ΔE9 mutant (with an N-terminal Flag epitope). This mutant causes Alzheimer's disease and supports γ-secretase activity; however, because the deleted exon 9 encodes the site of PS1 endoproteolysis, this PS1 variant does not form NTF or CTF (*19*, *41*). When solubilized membranes from these cells were subjected to cross-linking, the PS1 ΔE9 band migrated slightly faster (Figure 1D), perhaps due to internal cross-linking within a single PS1 molecule. No sign of dimeric PS1 ΔE9 was seen, suggesting that cysteine cross-linking between NTF and CTF in wt-PS1 does not occur in trans.

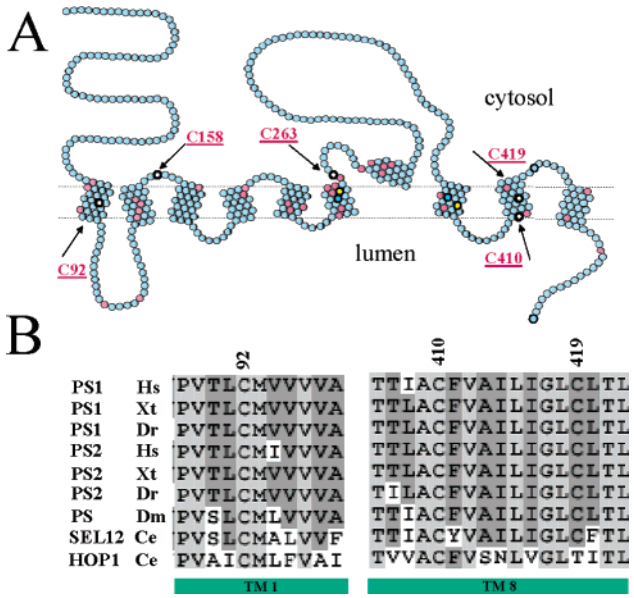

FIGURE 2: Topological model of PS1 and location of its native cysteines. (A) Native cysteines are labeled and shown as white circles. Two conserved transmembrane aspartates are in yellow. Sites of missense mutations associated with familial Alzheimer's disease are in lavender. (B) Multiple sequence alignment of presenilin sequence fragments containing transmembrane cysteines. The alignment was generated by CLUSTALW (at http://workbench.sdsc.edu). Presenilin orthologues from different species are abbreviated as follows: Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio* (zebrafish); Hs, *Homo sapiens*; Xt, *Xenopus tropicalis.*

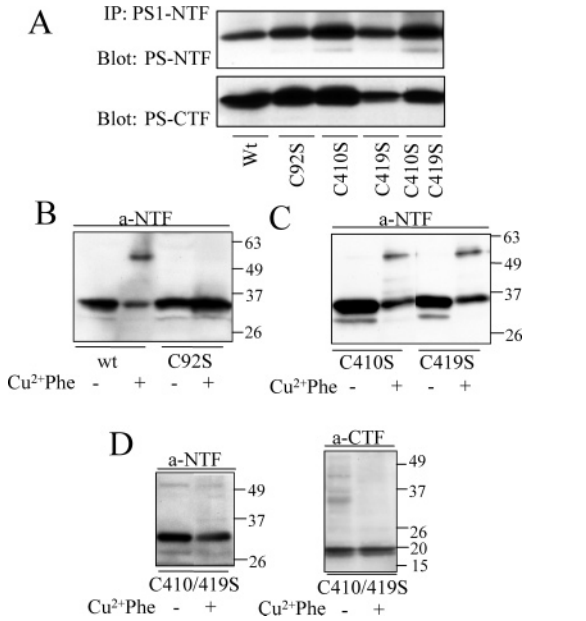

FIGURE 3: Disulfide oxidation of PS1 mutants. (A) CTF-PS1 is co-immunoprecipitated with NTF-PS1-Flag pulled down with anti-Flag beads from lysates of PS1/PS2 KO ES cells stably expressing wt, C92S, C410S, C419S, and C410S/C419S PS1-Flag mutants. (B−D) Oxidation of wt and C92S (B), C410S and C419S (C), and C410/419S (D) PS1 with Cu^{2+}Phe. Oxidation results in the production of NTF-CTF cross-linked species in some cases (wt, C410S, C419S) but not others (C92S, C410/419S). Detergent-solubilized membranes from the corresponding cell lines were incubated with 1 mM Cu^{2+}Phe for 30 min, and the samples were analyzed with PS1-NTF (B−D) and PS1-CTF (D, right panel) antibodies.

Disulfide Oxidation of C92S, C410S, and C419S PS1 Mutants. To establish which native cysteines of PS1 are proximal, we employed site-directed mutagenesis. Human PS1 contains five native cysteines. According to currently accepted PS1 topology (*17*, *18*), three cysteines are in transmembrane domains and two are exposed to cytosol (Figure 2A). Importantly, all three transmembrane cysteines are well conserved (Figure 2B), and Cys92 and Cys410 are sites of FAD mutations (*42*, *43*). Only two of these native cysteines, Cys410 and Cys419, are in the PS1-CTF, and both are located in TMD8. Because the observed product of cross-linking in the γ-secretase complex corresponds to NTF-CTF, apparently either Cys410 or Cys419 forms a disulfide bond with the only other transmembrane Cys92, which is located in TMD1 of PS1-NTF. We aimed to determine which of these pairs are responsible for native PS1 cross-linking and to elucidate whether TMD1 and TMD8 are adjacent to each other.

We generated point mutants C92S, C410S, and C419S of PS1-Flag and raised PS1/PS2 KO ES cells stably expressing each of these mutants. All mutants underwent efficient endoproteolysis (*19*), as indicated by the lack of full-length PS1 (FL-PS1, 50 kDa), suggesting that neither mutation affected PS1 participation in active γ-secretase assembly (Figure 3B,C, nonoxidized samples). In addition, for all mutants we observed efficient co-immunoprecipitation of PS1-CTF with Flag-tagged PS1-NTF using M2 beads (anti-Flag), confirming that these PS1 mutations do not interfere with the ability of PS1 to form γ-secretase complexes (Figure 3A). Upon oxidation with Cu^{2+}Phe, only the C92S-PS1 mutant yielded neither the oxidation product nor the decrease in PS1-NTF level, suggesting that Cys92 (located in PS1-NTF) is responsible for wt-PS1-NTF disulfide cross-linking to wtPS1-CTF (Figure 3B). Surprisingly, both the C410S and C419S PS1 mutants were oxidized similarly to wt-PS1, producing a product corresponding to NTF-CTF (Figure 3C), which suggests that either of these cysteines can contribute to wt-NTF-CTF formation and either one can cross-link to Cys92.

Disulfide Oxidation of a C410S/C419S PS1 Double Mutant. To clarify the role of Cys410 and Cys419 in wt-PS1 cross-linking, we prepared the double mutant C410S/C419S-PS1 (abbreviated as "C410/419S") and generated an ES cell line stably expressing this mutant. Notably, unlike in C92S, C410S, and C419S single mutants, a residual amount of FL-PS1 was observed with the C410/419S-PS1 mutant, although most of the PS1 double mutant did get endoproteolyzed into NTF and CTF (Figure 3D). In addition, we observed efficient co-immunoprecipitation of PS1-CTF with Flag-tagged PS1-NTF using M2 beads (anti-Flag), confirming that these double PS1 mutations do not interfere with the ability of PS1 to form γ-secretase complexes (Figure 3A). Upon oxidation of C410/419S-PS1, we observed no substantial decrease in levels of PS1-NTF and PS1-CTF and no increase in level of FL-PS1 (Figure 3D), indicating that a cysteine-less CTF loses its ability to disulfide cross-link to PS1-NTF. Together with the findings discussed above, these data suggest that Cys92 can cross-link to either Cys410 or Cys419 to yield NTF-CTF cross-linked species. Cys410 and Cys419 are separated by nine amino acids and are expected to be located on almost opposite sides of the same transmembrane helix. Therefore, the proximity arrangement intriguingly implies that TMD8 must have a kink in its helical

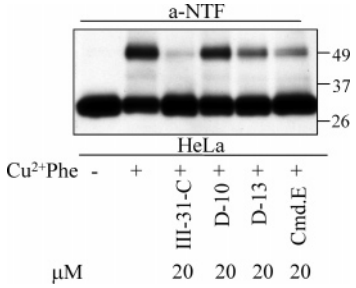

FIGURE 4: The transition-state analogue inhibitor III-31-C, but not the helical peptide inhibitor D-10, prevents disulfide oxidation of the endogenous PS1. Detergent-solubilized HeLa membranes (HeLa) were oxidized with 1 mM Cu^{2+}Phe for 30 min in the presence of 20 μM III-31-C, D-10, D-13, and compound E, and the samples were analyzed by Western blotting using PS1-NTF antibody.

structure in order for both Cys410 and Cys419 to be able to cross-link to Cys92.

Effect of γ-Secretase Inhibitor Binding on wt-PS1 Oxidative Cross-Linking. Because cysteines are required to be in proximity of less that 7 Å in order for a disulfide bond to be formed (*44*), disulfide cross-linking provides a sensitive method to assess conformational changes in protein structure, whether due to ligand or inhibitor binding or due to some other event. We analyzed the ability of known γ-secretase inhibitors to affect the oxidative disulfide cross-linking of wt-PS1. Besides active site directed transition-state analogue inhibitor III-31-C (*10*), we also analyzed non-transition-state analogue compound E (*35*) as well as helical peptides D-10 and D-13, which are docking site directed inhibitors (*23*, *33*). Compounds were added to the samples just before addition of Cu^{2+}Phe oxidant, and after 30 min incubation at 37 °C, the samples were analyzed by SDS−PAGE. Remarkably, active site directed inhibitor III-31-C completely prevented NTF from cross-linking, as almost no oxidized products were detected and the NTF level did not change (Figure 4). In contrast, the docking site directed D-10 helical peptide inhibitor did not affect the formation of cross-linked NTF-CTF. Similar behavior was observed when III-31-C and D-10 were incubated with C410S and C419S PS1 mutants (data not shown). Different effects of III-31-C and D-10 on PS1-NTF-CTF cross-linking independently support our previous findings that III-31-C and D-10 inhibitors bind PS1 at distinct sites, active site and docking site, respectively (*23*, *45*). Compound E and D-13 exhibited an intermediate behavior, as these inhibitors prevented NTF-CTF disulfide cross-linking, but not as efficiently as the transition-state analogue (Figure 4). This is consistent with our previous findings that compound E and D-13 bind PS1 in a mode partially overlapping with the active site of γ-secretase (*23*, *45*). Most importantly, the ability of the active site directed III-31-C to interfere with PS1 oxidative cross-linking suggests that native Cys92, Cys410, and/or Cys419 are close to or within the active site of γ-secretase with the inhibitor binding preventing their interaction or that the occupation of the active site results in a substantial conformational change in the PS1 molecule which in turn translates into a shift in distance between cysteine pairs.

DISCUSSION

γ-Secretase is one of the most important targets in the development of therapeutics for Alzheimer's disease as well as in understanding how mutations in PS1 that cause familial Alzheimer's disease (FAD) shift Aβ production toward the more aggregation-prone Aβ42 peptide. To understand this target, it is crucial to obtain information about its active site organization and the role of FAD residues. Unfortunately, γ-secretase is a difficult protein to study structurally using standard techniques, as it consists of four transmembrane proteins with a total of at least 19 transmembrane domains (an exact protein stoichiometry is still unknown), and it is difficult to express recombinantly. Alternative methods such as disulfide cross-linking should be used to gain structural information about this enigmatic protease. To this end, we initiated the application of cysteine oxidation toward γ-secretase and found that wt-PS1-NTF and wt-PS1-CTF cross-link to each other as a result of disulfide bond formation between native cysteines. Treatment of detergent-solubilized endogenous γ-secretase with Cu^{2+}Phe oxidant results in the formation of PS1-NTF-CTF. This observation, together with the control reactions, strongly suggests that at least two native cysteines in PS1 are in proximity. These native transmembrane cysteines in PS1 and the disulfide cross-linked products exhibit some interesting properties. The newly formed disulfide bonds are extremely stable toward reducing agents. A lack of reactivity is not uncommon for transmembrane cysteines and is considered to be due to a high pK_a of sulfhydryl in the hydrophobic environment. Thus, in aspartate receptors the disulfides are very stable on exposure to low molecular weight thiols, such as DTT and βME, in the presence of SDS and require extreme pH and heat for reduction (*27*).

By employing site-directed mutagenesis, we have determined that highly conserved transmembrane Cys92 in PS1-NTF and Cys410 and Cys419 of CTF are responsible for cross-linking between wt-PS1-NTF and wt-PS1-CTF. Our findings suggest that both Cys410 and Cys419 can cross-link to Cys92. It has been shown that α-carbons of the cross-linked cysteines can be at a maximum distance of 7 Å from each other, with an average of 5−6 Å (*44*). Obviously, the cysteines have to be on facing sides of the two helices for the disulfide bond to form (*27*). Therefore, our data clearly indicate that TMD1 and TMD8 of PS1 are in close proximity. Cys92 in TMD1 is located close to both Cys410 and Cys419 in TMD8. Because Cys410 and Cys419 are separated by nine amino acids, it is puzzling how Cys92 can be close to both of them. This finding raises an intriguing question about the microstructure of TMD8. Presumably, a break in a TMD8 α-helix, such as a kink or a loop, can bring Cys410 and Cys419 closer to each other and Cys92. This "imperfection" in a helix would be consistent with the fact that TMD8 has one of the longest (≥20-residue) hydrophobic stretches in PS1 and, more importantly, that TMD8 has a completely conserved Gly417 residue in a middle of its sequence. Glycine is known to be a transmembrane helix breaker or a hinge, as has been shown in the structure of the Shaker potassium channel (*46*). Overall, our observations provide biochemical evidence for more detailed structural information about the catalytic component of γ-secretase.

Besides providing a better idea of the organization of PS1 TMDs, disulfide cross-linking offered the means to probe changes in PS1 upon inhibitor binding. Thus, we discovered that binding of the transition-state analogue inhibitor III-31-C prevents PS1-NTF and PS1-CTF disulfide cross-

linking. In contrast, binding of docking site directed inhibitor D-10 does not interfere with oxidized product formation. These observations confirm our initial findings that there are two distinct sites on γ-secretase and that III-31-C and D-10 inhibitors bind to the active site and the docking site, respectively (*10*, *23*, *45*). The ability of III-31-C to affect wt-PS1-NTF and wt-PS1-CTF disulfide cross-linking suggests that its binding decreases the proximity of cysteines involved in cross-linking. Such alteration can be induced either by a conformational change of PS1 upon inhibitor binding or by active site directed III-31-C binding directly in proximity to the interacting cysteines. Intriguingly, the latter possibility would indicate that Cys92, Cys410, or Cys419 is located in the active site cavity. In fact, this arrangement is supported by the previously reported finding implicating TMD1, in which Cys92 is located, as part of the active site (*24*). The sophisticated arrangement of three highly conserved cysteines along with the helix-breaking Gly417 suggests that these residues may be crucial for the protease activity and play a major role in its active site. As a result, we believe our findings imply that Cys92, Cys410, and/or Cys419 are located near or are allosterically interacting with the active site of γ-secretase, suggesting that these highly conserved cysteines may have a function in supporting the integrity and hydrophilic environment of the internal active site.

In summary, we have initiated the use of the powerful method of disulfide cross-linking toward probing the three-dimensional structure of γ-secretase. Our observations provide new structural information about the TMD organization of PS1, the catalytic component of γ-secretase. The disulfide cross-linking method may eventually reveal additional structural details about the γ-secretase complex. By incorporating cysteine residues into a cysteine-free PS1 and analyzing the ability to form disulfide cross-links, the complete organization of the TMDs of PS1 and that of its active site could be ultimately determined, and the location within PS1 of contacts with other γ-secretase components could be elucidated as well. These findings will provide important validation in the future, when the long-awaited high-resolution structure of γ-secretase is finally solved.

ACKNOWLEDGMENT

We thank Bruce Yankner for PS1/PS2 KO ES cells and 231-f antibody, J. Näslund for UD-1 antibody, S. Urban for helpful comments on the manuscript, and M. Leissring, J. Fadeeva, and S. Urban for the advice in molecular biology.

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BI060107K