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Are Presenilins Intramembrane-Cleaving Proteases? Implications for the Molecular Mechanism of Alzheimer's Disease[†]

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ABSTRACT: The amyloid-β protein (Aβ) is strongly implicated in the pathogenesis of Alzheimer's disease. The final step in the production of Aβ from the amyloid precursor protein (APP) is proteolysis by the unidentified γ-secretases. This cleavage event is unusual in that it apparently occurs within the transmembrane region of the substrate. Studies with substrate-based inhibitors together with molecular modeling and mutagenesis of the γ-secretase cleavage site of APP suggest that γ-secretases are aspartyl proteases that catalyze a novel intramembranous proteolysis. This proteolysis requires the presenilins, proteins with eight transmembrane domains that are mutated in most cases of autosomal dominant familial Alzheimer's disease. Two conserved transmembrane aspartates in presenilins are essential for γ-secretase activity, suggesting that presenilins themselves are γ-secretases. Moreover, presenilins also mediate the apparently intramembranous cleavage of the Notch receptor, an event critical for Notch signaling and embryonic development. Thus, if presenilins are γ-secretases, then they are also likely the proteases that cleave Notch within its transmembrane domain. Another protease, S2P, involved in the processing of the sterol regulatory element binding protein, is also a multipass integral membrane protein which cleaves within or very close to the transmembrane region of its substrate. Thus, presenilins and S2P appear to be members of a new type of polytopic protease with an intramembranous active site.

The identification of mutant genes associated with human diseases is progressing apace, demonstrating the power and utility of genomics. Often, however, the pinpointing of a disease-causing genetic mutation does not provide clear clues to the normal function of the encoded protein or to the biochemical and cellular processes involved in pathogenesis. This problem has been particularly vexing in the area of neurodegenerative diseases, where genes and their protein products have received names such as huntingtin and parkin because their normal functions are completely unknown.

The amyloid-β precursor protein (APP)[1] and the presenilins (PS), associated with Alzheimer's disease (AD), were so christened for similar reasons. Mutations in these genes cause early-onset (<60 years) autosomal dominant familial AD (FAD) (*1*, *2*), but the biochemical roles of the respective

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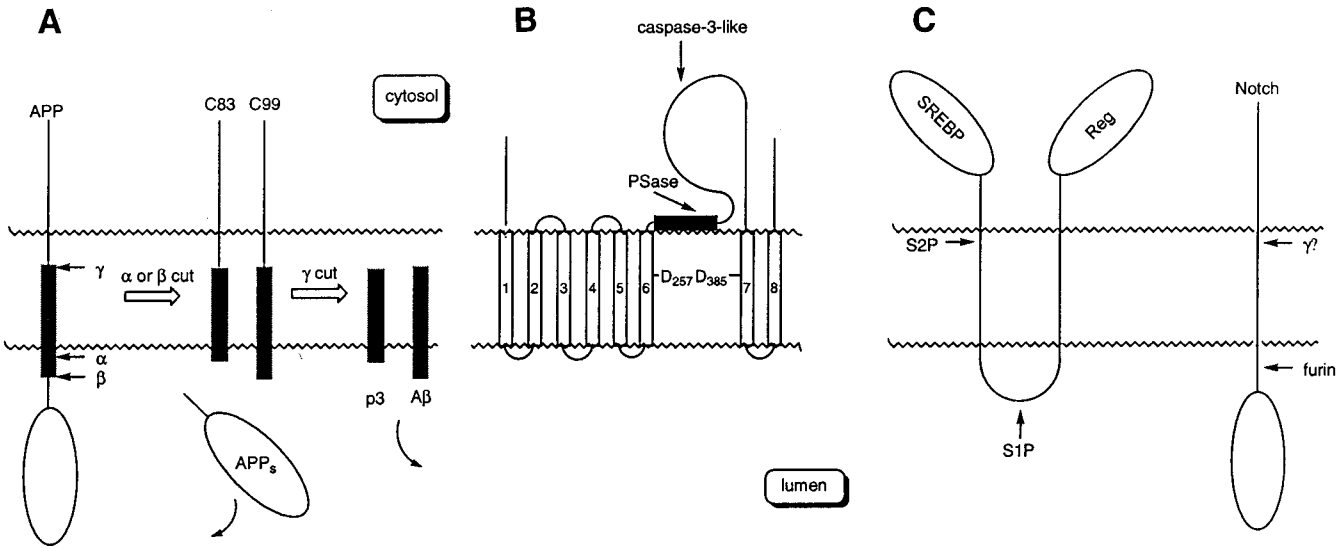

FIGURE 1: Topologies of (A) APP, (B) presenilins, and (C) SREBP and Notch and sites of proteolytic processing. The dark bar in APP represents the Aβ region. The dark bar in presenilins represents the hydrophobic portion of the large cytosolic loop that contains the site of cleavage by a "presenilinase" (PSase).

proteins have been veiled. The APP sequence did, however, offer an immediate hint to how mutations might lead to FAD: as the name indicates, APP is the precursor for the amyloid-β protein (Aβ), the major protein component of amyloid plaques in AD brains. APP, a type I integral membrane glycoprotein, is proteolytically processed by α- and β-secretases, thereby shedding the large ectodomain and producing membrane-associated 83- and 99-residue fragments, respectively (C83 and C99; see Figure 1A) (*3*). These fragments are then cleaved by γ-secretase, an unusual protease that apparently cuts within the transmembrane domain of the substrate, to form the 4 kDa Aβ from C99 and an N-terminally truncated portion of Aβ, p3, from C83.

Mutations Which Cause FAD. AD-causing mutations in APP lie near the β- and γ-secretase cleavage sites and lead to increases in either total Aβ or in a specific Aβ isoform (*3*). Aβ is secreted under normal physiological conditions from a variety of cell types, with roughly 90% as a 40-residue Aβ isoform (Aβ_{40}) and 10% as a 42-residue variant (Aβ_{42}). The latter is more hydrophobic and considerably less soluble than Aβ_{40}, forming Aβ fibrils in vitro via a nucleation-dependent mechanism (*4*). In addition, Aβ_{42} fibrils can seed Aβ_{40} fibril formation in vitro; thus, Aβ_{40} that would otherwise remain soluble can aggregate onto Aβ_{42} template fibrils (*4*). Despite Aβ_{42} being a small fraction of secreted Aβ, this isoform is the major Aβ species in AD plaques (*5*−*7*). Four different FAD-causing point mutations near the γ-secretase cleavage site result in specific increases in Aβ_{42} production, while a double mutation near the β-secretase cleavage site found in a Swedish pedigree makes APP a better substrate for the latter protease, leading to a doubling in total Aβ levels, including Aβ_{42} (*1*, *2*). Two disease causing mutations just beyond the α-secretase cleavage site (i.e., within the Aβ region) affect Aβ production and parenchymal deposition and are associated clinically with hereditary cerebral hemorrhage with amyloidosis, either with or without features of AD (*8*).

Mutations in APP, however, account for only a very small fraction of FAD cases. Most are caused by mutations in presenilins 1 and 2, two closely related integral membrane proteins (*1*, *2*, *9*). All FAD-causing presenilin mutations analyzed to date lead to specific increases in Aβ_{42} formation in transfected cell lines (*10*−*13*) and in transgenic mice (*10*, *11*, *14*). Importantly, living subjects with FAD-linked presenilin 1 (PS1), presenilin 2 (PS2), or APP mutations have elevated plasma Aβ_{42} and increased Aβ_{42} in the media of primary fibroblast cultures (*15*). The discovery that mutations in the presenilin genes account for the majority of FAD cases has generated a flurry of activity to determine the normal cellular roles of the corresponding proteins and how these proteins influence Aβ production. These highly homologous proteins have been localized primarily to the ER and Golgi in transfected mammalian cells (*16*−*19*), and co-immunoprecipitation experiments indicate that small amounts of APP can interact with presenilins in these organelles (*20*, *21*). Evidence strongly supports an eight-transmembrane topology for the presenilins (*1*, *22*−*24*) (Figure 1B), although six- and seven-transmembrane topologies have also been offered (*22*, *25*, *26*).

Remarkably, over 50 different mutations in the presenilins have been found to cause FAD (*27*): all result in missense mutations, although one is a point mutation in the PS1 gene upstream of a splice acceptor site that results in an in-frame deletion of exon 9 (PS1 ΔE9).[2] These mutations are not clustered in one region of the presenilin linear sequence; rather, they are located primarily within or very near to

[1] Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β protein; APP, amyloid precursor protein; APP$_s$, soluble amyloid precursor protein; C83, 83-residue C-terminal proteolytic fragment of amyloid precursor protein; C99, 99-residue C-terminal proteolytic fragment of amyloid precursor protein; CTF, 18 kDa C-terminal proteolytic fragment of presenilin; ER, endoplasmic reticulum; FAD, familial Alzheimer's disease; NTF, 28 kDa N-terminal proteolytic fragment of presenilin; PS, presenilin; S2P, site 2 protease; SCAP, sterol-cleavage activating protein; SREBP, sterol regulatory element binding protein; TM, transmembrane.

[2] This splice alteration also results in a S290C mutation. Interestingly, a recent study has shown that the increase in Aβ_{42} caused by PS1 ΔE9 is due to this point mutation, not the deletion (*28*). Thus, all FAD PS mutants analyzed at this level increase Aβ_{42} as a result of single point mutations.

hydrophobic domains. FAD-causing mutations in PS1 can rescue PS1 knockout mice from otherwise lethal errors in embryonic development, demonstrating that these FAD mutant presenilins are functional (*29*, *30*). This, along with other evidence (see below), suggests that the increased Aβ_{42} production caused by the mutant presenilins is due to a toxic gain of function and not a loss or reduction of function.

Properties of Presenilins and γ-Secretases. PS1 and PS2 both undergo proteolytic processing within the conserved, hydrophobic portion of the cytosolic loop between TM6 and TM7 (dark bar, Figure 1B) to form tightly regulated amounts of heterodimeric complexes of a 28 kDa N-terminal fragment (NTF) and a 18 kDa C-terminal fragment (CTF) (*31*−*35*). Even when overexpressed, the PS holoproteins do not lead to increased cellular levels of NTFs and CTFs, suggesting that presenilins compete for certain limiting cellular factors critical for endoproteolysis and stabilization of their fragments (*36*). The PS holoproteins are unstable, with half-lives of about 1.5 h (*33*, *34*), and their degradation is apparently mediated in part through the proteasome (*37*, *38*). In contrast, the PS fragments produced through normal endoproteolysis of wild-type and FAD-mutant presenilins are quite stable ($t_{1/2} \sim 24$ h) (*33*, *34*), consistent with the hypothesis that the heterodimeric complexes represent the biologically active form of the protein. The normal endoproteolytic site (*34*) is missing in the PS1 ΔE9 variant, which is thus not cleaved in this manner (*31*−*33*, *39*). However, this variant is apparently a functional presenilin (*40*, *41*), consistent with the idea that the exon 9 region is inhibitory and that either cleavage *or* deletion of this region is required for activity. One report claims that certain FAD-causing mutations in PS1 lead to some elevation in the levels of the NTF-CTF heterodimers (*42*), suggesting that accumulation of the mutant heterodimers may contribute to increased Aβ_{42} production. An alternative cleavage mediated by a caspase-3-like cysteine protease occurs within the hydrophilic part of the large loop (Figure 1B), and this proteolysis takes place during apoptosis (*43*−*45*). However, this alternative PS processing leads to unstable fragments that are degraded, in part, by another cysteine protease (*38*).

The presenilins are apparently critical for γ-secretase activity: De Strooper et al. reported that Aβ production is dramatically reduced in primary fibroblasts derived from PS1 knockout mouse embryos compared with corresponding wild-type littermates (*46*). While α- and β-APP$_s$ levels were essentially unchanged, the APP C-terminal fragments that serve as γ-secretase substrates were substantially increased. (Presumably, the residual Aβ production in the PS1 knockout fibroblasts is due to PS2. See below.) These findings have been confirmed directly in brain tissue of PS1 −/− vs +/+ mice (*47*). The biochemical role of presenilins in mediating γ-secretase activity has been widely considered central to the molecular mechanism of AD.

γ-Secretase displays some properties of an aspartyl protease. We have found that substrate-based difluoro ketone peptidomimetics inhibit the production of Aβ at the γ-secretase level: α- and β-secretase-generated APP$_s$ products are not affected, but γ-secretase substrates C83 and C99 (Figure 1B) are dramatically increased in the presence of these inhibitors (*48*). These difluoro ketones readily form hydrates in the presence of small proportions of water, and such hydrates are known to inhibit aspartyl proteases by virtue of their resemblance to the high-energy *gem*-diol intermediate (*49*−*52*). Moreover, the corresponding difluoro alcohols are also inhibitors of Aβ production, albeit weaker than the ketones (*53*). However, the fact that they inhibit at all is inconsistent with γ-secretase working through either a serine or cysteine protease mechanism, which would require the ketone for nucleophilic attack (*54*). In contrast, difluoro alcohol-containing peptidomimetics are known to be capable of inhibiting aspartyl proteases (*55*).

Structure−activity relationships and site-directed mutagenesis studies also indicate that γ-secretase has loose sequence specificity (*53*, *56*−*58*), a finding that might seem inconsistent with the fact that APP is cleaved at specific sites by the enzyme. This apparent paradox can be resolved by considering the distance within the membrane as the key determinant of where γ-secretase cleaves. Thus, the proteolysis within the APP transmembrane domain may actually take place within the membrane. In support of this hypothesis, when the APP transmembrane region is computationally folded into an α-helical conformation, typical of transmembrane domains, the γ-secretase cleavage sites leading to Aβ_{40} and Aβ_{42} formation are on opposite faces of the helix (*53*). Moreover, FAD-causing APP mutations in this region are immediately adjacent to the cleavage site for Aβ_{42} formation. Thus, the helical model, based on the hypothesis that the γ-secretase proteolysis takes place within the membrane, can explain how these mutations can cause specific increases in Aβ_{42}, eventually leading to AD. Results from a recent site-directed mutagenesis study by Lichtenthaler et al., in which numerous residues near the γ-secretase cleavage sites were systematically replaced with phenylalanine, were also consistent with the helical model, and thus with an intramembranous proteolysis (*59*).

Two TM Aspartates in PS Are Critical for Function. If γ-secretase catalyzes an intramembranous proteolysis, then the enzyme would be predicted to have multiple transmembrane domains, suitable to form an active site that allows the entrance of the required catalytic water. Mutations in the protease itself might also be predicted to cause FAD. Surprisingly, the myriad presenilin mutations that raise Aβ_{42} levels and cause FAD are scattered throughout the linear sequence. However, most of these mutations are found either within transmembrane domains or in the hydrophobic portion of the cytoplasmic loop (see Figure 1B) (*1*). These observations suggest a direct role of the presenilins in γ-secretase activity and raise the question: are presenilins γ-secretases? More specifically, are presenilins aspartyl proteases? Provocatively, the presenilins contain two completely conserved aspartates within predicted transmembrane domains (*1*), one in TM6 and one in TM7, regions that flank the large cytoplasmic loop that contains the site of endoproteolysis. These TM aspartates, conserved even in *Caenorhabditis elegans* homologues (*60*, *61*), appear to be aligned in the membrane with each other and with the γ-secretase cleavage sites in APP.

When we mutated either of these two TM aspartates in human PS1 and stably cotransfected them with human APP into Chinese hamster ovary (CHO) cells, we were surprised to find that neither mutant PS1 underwent the normal endoproteolysis in the cytoplasmic loop (*62*). Thus, each of the two TM aspartates is apparently critical for this proteolytic processing of PS1. Introduction of either mutant PS1

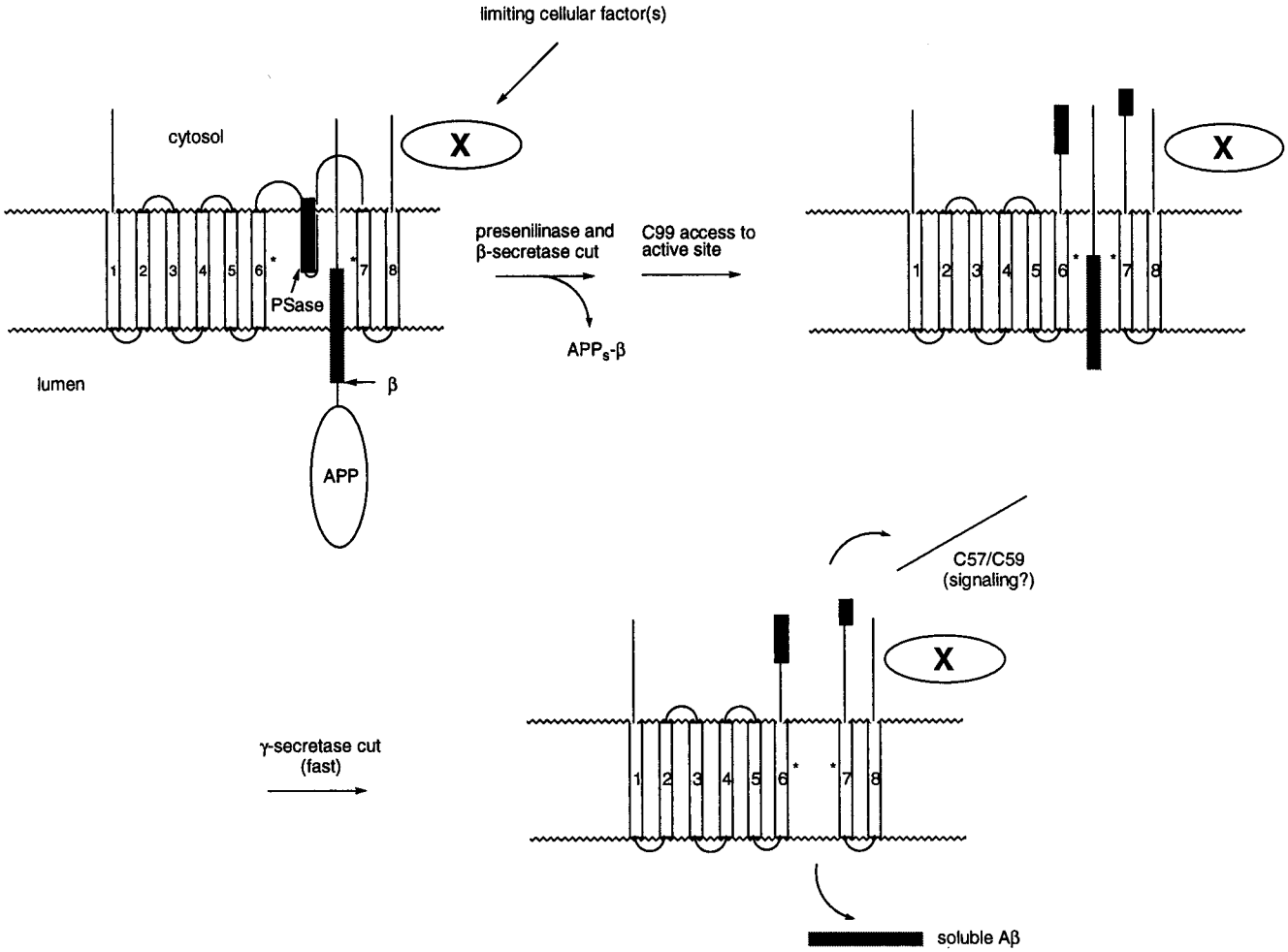

FIGURE 2: Schematic model for the involvement of presenilins in Aβ generation. Presenilins and APP form stable complexes. After presenilin binds to an unidentified limiting cellular factor(s) (protein X), the full-length presenilin undergoes autoproteolysis via the two transmembrane aspartates (asterisks). Shedding of the APP ectodomain by β-secretase allows C99 to bind to the active site of γ-secretase (presenilin) with subsequent generation of Aβ. By analogy with Notch, the other proteolytic product of C99 cleavage (C57/C59) may serve as a signaling molecule.

also led to inhibition of endogenous γ-secretase activity: Aβ levels were reduced and both C83 and C99 levels were increased in rough correlation with the expression of mutant holoprotein (*62*). Apparently, the two mutant PS1 holoproteins act as dominant negatives, blocking the normal role of endogenous PS1 in mediating γ-secretase activity. These results have been observed in several different cell types, including human cell lines, and in transient as well as stable tranfectants. In addition, mutating the aspartates isosterically to asparagine or isoelectronically to glutamate gives similar results, indicating that each of the two TM aspartates is absolutely required and suggesting that the introduced mutations do not simply result in misfolding. Similar effects are observed when either of the two conserved TM aspartates in PS2 are mutated (*81*; W. T. Kimberly et al., unpublished observations).

The possibility remained that the effect of the introduced mutations on γ-secretase activity was only due to the inability of the mutant PS1 holoproteins to undergo proteolytic processing to the stable NTF/CTF complexes that are likely the active form of the protein. We therefore introduced the D385A mutation into PS1 ΔE9, a functional form of PS1 which cannot be processed in this manner (*33*, *40*, *41*). The D385A PS1 ΔE9 did not undergo normal endoproteolysis, as expected; nevertheless, this mutant PS1 also caused increases in C83 and C99 when transiently introduced into either COS-1 monkey kidney cells or human embryonic kidney (HEK) 293 cells (*62*). Thus, this TM aspartate is independently critical for γ-secretase activity, even in a form of PS1 that does not require endoproteolysis for function.

The two TM aspartates in PS1 were also found to be critical for in vitro generation of Aβ (*62*). When C99 was expressed in a cell-free transcription/translation system in the presence of wild-type human PS1-containing microsomes, a new 4 kDa band was immunoprecipitated by an Aβ-specific antibody after incubation under mildly acidic but not neutral conditions, a result expected if γ-secretase were an aspartyl protease. Little or no Aβ was produced when C99 was expressed in the presence of microsomes containing D257A or D385A PS1. Also, Aβ was generated from cell-expressed APP by incubating microsomes containing wild-type but not D$\rightarrow$A mutant PS1. Thus, the results from in vitro experiments mirrored those obtained from whole cells.

The most parsimonious hypothesis consistent with these findings is that the presenilins are γ-secretases, intramembrane-cleaving aspartyl proteases activated through autoproteolysis (Figure 2). APP and presenilins form stable complexes (*20*, *21*), but the APP TM domain cannot access the

active site of PS, which is still in the pro form. Note that, in this model, the hydrophobic portion of the large cytosolic loop is located *within* the boundaries of the membrane, although this portion of the loop may only contact PS TM domains and not lipids. After presenilins bind to a certain as yet unidentified cellular factor(s) (*36*), autoproteolysis takes place to form the active PS heterodimer, each subunit (NTF and CTF) contributing one of the two essential catalytic aspartates. Certain viral aspartyl proteases [e.g., HIV protease (*49*)] also form the active site at the interface between two subunits, each contributing one aspartate, although in these cases the enzymes are homodimeric, not heterodimeric. A necessary correlary to this hypothesis is that the limiting cellular factor(s) must remain bound to PS after facilitating autoproteolysis: the release of the factor(s) would allow the conversion of overproduced PS holoprotein to increased NTF/CTF complexes, which is not observed in transfected cells. The β-secretase cleavage of APP, which sheds the ectodomain, is presumably also a prerequisite for binding of the substrate, C99, to the active site of PS (γ-secretase). In this model, the proteolysis of C99 to Aβ is rapid, explaining why PS/C99 complexes have not yet been detected by co-immunoprecipitation.

That presenilins are merely involved in the trafficking of APP and/or γ-secretase to appropriate subcellular microdomains is not supported by the results from in vitro experiments (*47*, *62*), nor is this notion supported by observations that APP processing by α- and β-secretases is normal in PS1-deficient cells (*46*). However, it is instead possible that the presenilins are critical cofactors for γ-secretases, analogous to the sterol-cleavage activating protein (SCAP) involved in the *extra*membranous processing of the sterol regulatory element binding protein (SREBP) (*63*). SREBP, a transcription factor essential for cholesterol biosynthesis, is expressed as an ER-localized precursor protein with two transmembrane segments (Figure 1C). Reduced cholesterol levels are sensed by the eight-TM SCAP, which then binds to the C-terminal portion of the immature SREBP and allows cleavage of the lumenal loop of SREBP by the membrane-anchored site 1 protease (*64*).

Other Intramembrane-Cleaving Proteases (I-CLiPs). A role for presenilins as proteases would be analogous to the second proteolytic event in SREBP maturation. This second cleavage apparently takes place just within the first TM region and results in release of the transcription factor and translocation to the nucleus (Figure 1C) (*64*, *65*). The selection of CHO cells auxotrophic for cholesterol led to the identification of the site 2 protease (S2P), a multi-TM, ER-resident protein containing a metalloprotease signature sequence (HEXXH) that apparently lies either just within the membrane or at the membrane−cytosol interface (i.e., aligned with the TM cleavage site in SREBP) (*66*). Despite the presence of the HEXXH sequence and the requirement of S2P for the putative transmembrane processing of SREBP, S2P otherwise bears no resemblance to other known metalloproteases, and direct evidence for protease activity of S2P has not yet been obtained. Nevertheless, like the presenilins, S2P is a candidate *i*ntramembrane-*cl*eaving *p*rotease (I-CLiP), that is, a multi-TM protease with an intramembranous active site which cleaves within the transmembrane region of its substrate. Other multi-TM proteases include signal peptidase (*67*) as well as Rce1p and Ste24, which are ER proteins that cleave C-terminal CAAX motifs of proteins after prenylation of the cysteine (*68*). While these proteases do not cleave within transmembrane domains (i.e., they are not I-CLiPs), they are members of a growing class of multi-TM proteases.

Another I-CLiP candidate is an unidentified protease involved in processing the Notch receptor (Figure 1C). Signaling from Notch is critical for cell-fate decisions during development, and this signaling requires cleavage within or immediately adjacent to the single TM segment of Notch (*69*). The intracellular domain of Notch is thus released, whereupon it binds to and activates the CSL transcription factors. PS1 is intimately involved in this apparent TM cleavage of Notch. PS1 knockout mice die during gestation (*70, 71*), and isolation of the defective embryos showed that Notch signaling is dramatically reduced (*71*). Moreover, neurons and fibroblasts isolated from these embryos transfected with a Notch construct showed dramatically reduced release of the Notch intracellular domain, demonstrating that PS1 somehow mediates this TM processing event (*72*); similar results have also been observed in the fly (*73*, *74*). Importantly, the deficiency in PS1 did not affect the maturation of Notch: cleavage by a furin-like convertase within the Golgi, a necessary step for Notch signaling (*75*), was unaffected (*72*). Inhibitors of γ-secretase, including a substrate-based difluoro ketone peptidomimetic designed to mimic the cleavage site within the APP TM, also block the final TM proteolytic event essential to Notch signaling, emphasizing the close similarity between this proteolysis and the γ-secretase cleavage of APP (*72*). If presenilins are indeed γ-secretases, then PS1 would very likely be the I-CLiP responsible for the release of the Notch intracellular domain.

An apparent discrepancy in this hypothesis is that the Notch TM cleavage takes place at or near the cell surface after binding by an extracellular ligand, and most Aβ production apparently requires internalization from the cell surface (*76*). In contrast, presenilins are localized primarily in the ER and Golgi (*16*−*19*). Careful biotinylation studies, though, clearly demonstrate that PS1 NTFs and CTFs are at the cell surface, where they form complexes with Notch (W. J. Ray et al., unpublished observations). The fact that the Notch TM cleavage is sequence specific might also seem inconsistent with the hypothesis that presenilins are γ-secretases, since the APP TM cleavage shows loose specificity. However, the Notch TM domain may be the primary substrate for presenilins, with higher affinity for the protease than the APP substrate. Thus, alterations in the Notch TM domain may have profound effects on its high-affinity binding to presenilins, while mutations in the APP TM might have little impact on this relatively low-affinity binding.

The Origin of I-CLiPs. The presenilins have no obvious sequence homology with known aspartyl proteases. However, other known aspartyl proteases are all cytosolic enzymes, so should sequence homology be expected? From an evolutionary perspective, it seems much less likely that a soluble protease would acquire multiple TM domains than for a multiple TM protein to incidentally acquire proteolytic activity. The latter route might be expected to require fewer mutations, since typically only a few active site residues are essential for catalysis. According to this view, the relationship between presenilins and other aspartyl proteases would be strictly mechanistic, not structural; a limited number of

protease mechanistic motifs may be efficient enough to be useful to a cell or organism, and nature can arrive at essentially the same mechanism from different evolutionary directions.

Catalytic antibodies provide a clear illustration of this type of mechanistic convergence. On a molecular level, the ability of the immune system to respond to a foreign antigen with specific antibodies resembles an accelerated evolutionary process. Antibodies with esterase activity can be elicited with phosphonate transition state analogues as haptens. Remarkably, cocrystallization of one such antibody esterase with its corresponding phosphonate hapten revealed that the active site contained a Ser-His dyad near the phosphorus atom that resembles two of the three components of the classical Ser-His-Asp catalytic triad of serine proteases (*77*). Kinetic data also supported a hydrolytic mechanism for the antibody that is strikingly similar to that of the serine proteases (*78*). This hydrolase originated from antibody genes and is not homologous with natural enzymes. Nevertheless, mutation of the antigen binding region resulted in convergence to a hydrolytic mechanism common to serine proteases.

From this perspective, the lack of sequence homology between S2P and any other known metalloprotease (*66*) is not surprising. The multi-TM S2P likely originated from a multi-TM protein that acquired the HEXXH motif and not from a soluble metalloprotease that acquired multiple TMs over time. The challenge is to identify polytopic proteins that bear some sequence resemblance to S2P or to presenilins as a means of tracing their origins and understanding how they developed their remarkable ability to carry out intramembranous proteolyses. One such protein might be spe-4, a *C. elegans* protein involved in trafficking that shows homology to the presenilins (*79*). Two other *C. elegans* proteins, sel-12 and HOP-1, are much more closely related to human presenilins (*61*). In fact, human presenilins or HOP-1 can rescue mutant sel-12 phenotypes (*40*, *41*, *61*), emphasizing the conservation of function of these proteins from worm to man. Whether spe-4 is a bona fide presenilin is currently not clear.

Molecular Modeling of Presenilin-1. As noted above, the PS TM regions are highly conserved, suggesting that these regions are critical for function. Immunocytochemistry and studies with PS fusion proteins strongly support the eight-TM topology illustrated in Figure 1B (*22*−*24*). Specific regions of PS1 have been predicted to be TMs (*1*), and we have confirmed this prediction using sequence analysis tools available at the Swiss Protein Databank web site. Intriguingly, we find by molecular modeling that almost all the FAD-causing mutations of a given PS1 TM domain would be aligned along a single face if the domain assumes a typical α-helical conformation. Moreover, almost all the mutations in TM6 and TM7 are on the same face of the helix as the critical residues Asp257 and Asp385. It would therefore seem possible to construct a three-dimensional model of PS1[3] in which the various FAD mutations in the predicted TM regions and the critical aspartates are arrayed along the interior of the protein. This interior would represent the active site: that region of PS1 where, according to our hypothesis, the APP TM region binds (*21*) and is cleaved to give Aβ.

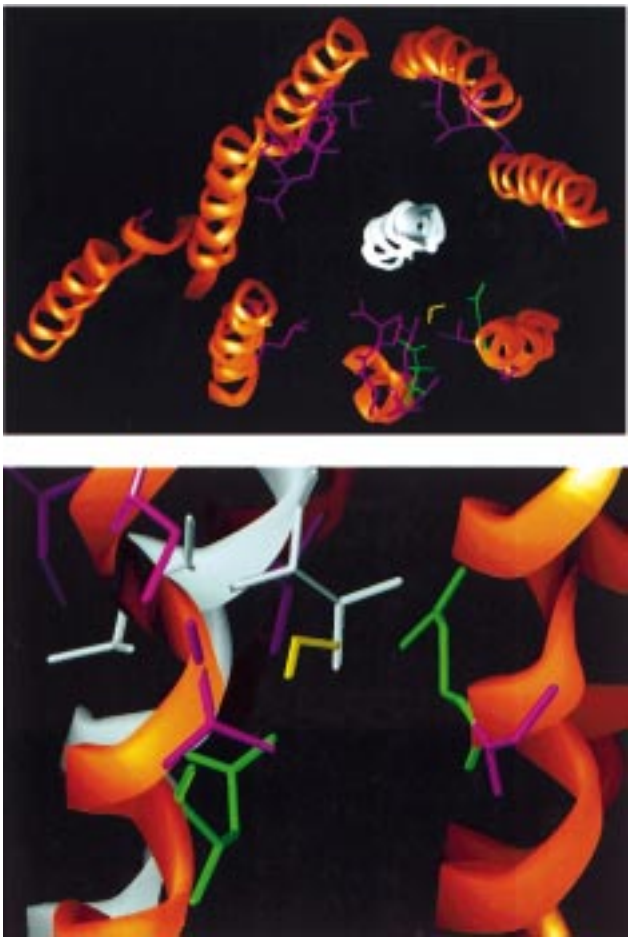

FIGURE 3: Computer-generated model of the TM regions of presenilin-1 (orange) bound to the TM region of APP (white). (Top) From top right, TM regions 1−8 run counterclockwise. In general, sites of mutation in presenilin-1 that cause early-onset familial Alzheimer's disease (magenta) can be arrayed along the same face of a given TM region. The aspartates in TM6 and TM7 (green) were oriented for interaction with water (yellow) in a manner similar to that for aspartyl proteases. For clarity, only side chains of disease-causing PS1 mutations are shown. (Bottom) Close-up view of the TM aspartates coordinated with water, poised to hydrolyze the Val−Ile amide bond and generate Aβ_{40}. Colors are as described in panel A. For clarity, only the side chains of the APP Val−Ile bond (white) and disease-causing PS1 mutations (magenta) are shown.

This would also be the region where the hydrophobic proximal portion of the TM6 → TM7 loop would bind and be cleaved, putatively by autoproteolysis.

The crystal structure of bacteriorhodopsin (one of the few integral membrane proteins for which a crystal structure is available) was used as a template to construct an initial three-dimensional model of the intramembrane regions of PS-1. Each end-capped TM region of PS1 was overlaid, one by one, onto the corresponding TM regions of bacteriorhodopsin, orienting FAD mutation sites toward the center and coordinating the TM6 and TM7 aspartates to a single water molecule. With the addition of each helix, the side chains at the helix−helix interface were relaxed, and the resultant helix bundle was subjected to energy minimization. Other factors considered in arranging helix−helix interactions included the TM hydrophobic moments, identifying nonconserved regions as potentially lipid-interactive, and maximizing the interactions between aromatic residues (Trp, Tyr, Phe) at the membrane interface (*80*). In addition, the PS1 helices were

[3] PS1 contains all but two of the identified PS FAD mutations (*27*).

adjusted to provide space for the APP TM helix. The APP TM region was then docked into the center of the PS1 model, oriented so that the major cleavage site, leading to Aβ_{40}, would be near the catalytic water between the aspartates, and the complex was energy minimized (Figure 3).

Although this three-dimensional model of presenilins is speculative, its construction is nevertheless worthwhile. Given the difficulty of crystallizing integral membrane proteins, it is not likely that the three-dimensional structure of presenilins as they exist in the membrane will be solved soon. In the meantime, a three-dimensional model that is predictive of inhibitor potency would be useful for the design of candidate therapeutic agents for the treatment of AD. Furthermore, the model could guide the design of molecular biology experiments to discern the importance of specific residues in PS function. The results of these inhibitor studies and molecular biology experiments can, in turn, be used to improve the model. In this iterative fashion, a reasonable model of the active site of PS may ultimately be realized.

CONCLUSIONS

The presenilins appear to be among the first examples of I-CLiPs. The finding that two transmembrane aspartates in presenilins are independently critical for two different proteolytic events (PS endoproteolysis and γ-secretase activity) strongly suggests that these polytopic proteins are novel intramembrane-cleaving aspartyl proteases activated through autoproteolysis. Thus, the majority of genetic mutations that cause early-onset FAD would be in the very proteases that produce the Aβ found in the disease-associated amyloid plaques. Exactly how they catalyze intramembranous proteolyses is a fascinating biochemical question, one that will require extensive efforts at reconstitution and identification of essential cofactors or subunits. The question is not strictly academic: the presenilins are prime targets for the development of new therapeutic agents for AD. Finally, these studies on the presenilins offer a glimpse of the new role of the biochemist in the age of genomics. In the past, problems tended to center around identifying key proteins involved in a given biochemical event. The reverse is becoming increasingly more common: the proteins are already largely identified through their corresponding DNA sequences. The problem now is deciphering their biochemical roles in health and in disease.

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