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Presenilin endoproteolysis is an intramolecular cleavage

Anne L. Brunkan, Maribel Martinez, Emily S. Walker, and Alison M. Goate*

Departments of Psychiatry, Neurology, and Genetics, Washington University School of Medicine, St. Louis, MO 63110, USA

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Mutations in the presenilin genes (PS) account for most cases of familial Alzheimer's disease. PS contain the active site of the γ-secretase complex that cleaves within the transmembrane domain of β-amyloid precursor protein (APP). Full-length PS undergoes regulated endoproteolysis to produce fragments that comprise the active form of PS. The "presenilinase" responsible for endoproteolysis is unknown but may be the same presenilin-dependent γ-secretase activity that cleaves APP. To investigate the mechanism of endoproteolysis, we examined sequence specificity at the cleavage site and tested whether PS dimers are important for endoproteolysis as well as γ-secretase activity. No single point mutation, or a double mutation M292D/V293K, was able to completely abolish endoproteolysis and all mutants supported γ-secretase activity. When wtPS1 was co-expressed with either M292D/V293K or D257A, it was unable to restore normal endoproteolysis to either mutant. Lack of transcleavage by wtPS1 suggests that PS1 endoproteolysis occurs via intramolecular cleavage and does not require dimerization.

Introduction

Alzheimer's disease is the most common cause of progressive neurodegeneration and is characterized pathologically by the accumulation of senile plaques and neurofibrillary tangles in the brain. The major component of senile plaques, amyloid-β peptide (Aβ), is produced by proteolytic processing of the β-amyloid precursor protein (APP). APP is first cleaved by either α-secretase (e.g., TACE, ADAM-10) or β-secretase (BACE) to release a large ectodomain and generate membrane-embedded C-terminal fragments of 83 (C83) or 99 (C99) residues (Esch et al., 1990; Seubert et al., 1993). C83 and C99 are subsequently cleaved near the intracellular side of the membrane (Murphy et al., 1999) by γ-secretase to release an intracellular C-terminal fragment (CTFγ) that enters the nucleus and interacts with transcription factors (Cao and Sudhof, 2001; Cupers et al., 2001; Gao and Pimplikar, 2001; Kimberly et al., 2001). γ-secretase also cleaves midway through the membrane domain to generate the p3 (from C83) and Aβ (from C99) fragments (Busciglio et al., 1993; Haass and Selkoe, 1993; Haass et al., 1992; Shoji et al., 1992). Several lines of evidence suggest that these CTFγ and p3/Aβ fragments are not produced by a single cleavage event (Hecimovic et al., 2004). Aβ species produced in this manner range in length from 37 to 43 residues (Wang et al., 1996). The major Aβ species is comprised of 40 residues ($A\beta_{40}$); however, the longer 42–43 residue species ($A\beta_{42}$, $A\beta_{43}$) are associated with Aβ deposition (Jarrett et al., 1993).

Notch, a protein that plays an important role in cell fate determination, undergoes processing similar to that of APP. Notch is first cleaved by furin at the S1 site during transport to the plasma membrane (Blaumueller et al., 1997; Logeat et al., 1998). When activated by ligand at the cell surface, Notch then undergoes cleavage by a metalloprotease (such as TACE (Brou et al., 2000)) at the S2 site to release the large ectodomain (Mumm et al., 2000). The remaining membrane-embedded fragment is a substrate for γ-secretase cleavage at the S3 site near the intracellular edge of the membrane. Proteolysis at the S3 site releases the Notch intracellular domain (NICD) that travels to the nucleus to modulate transcription (Schroeter et al., 1998).

Mutations in APP and in the Presenilin 1 and 2 genes (PS1 and PS2) account for approximately 50% of inherited early-onset familial Alzheimer's disease and increase the production of the highly fibrillogenic $A\beta_{42}$ species (Scheuner et al., 1996). PS is hypothesized to contain the active site of γ-secretase, a multiprotein complex that also contains Nicastrin (NCT), APH-1, and PEN-2 (Edbauer et al., 2003; Francis et al., 2002; Goutte et al., 2002; Kimberly et al., 2003; Steiner et al., 2002; Yu et al., 2000b). γ-secretase has characteristics of an aspartyl protease and mutation of either of two aspartic acid residues in PS1 (D257 and D385) inactivates γ-secretase (Kimberly et al., 2000; Steiner et al., 1999b; Wolfe et al., 1999a,b). PS1 is a putative 8-transmembrane (TM) protein that undergoes endoproteolytic processing to generate a 6-TM N-terminal fragment (NTF) and a 2-TM C-terminal fragment (CTF) (Thinakaran et al., 1996). The NTF and CTF remain

Abbreviations: Aβ, amyloid-β peptide; APP, β-amyloid precursor protein; CTF, C-terminal fragment; ELISA, enzyme-linked immunosorbent assay; FL, full-length; HEK, human embryonic kdney 293; IP, immunoprecipitation; MEF, mouse embryonic fibroblasts; NΔE, Notch ΔE; NCT, Nicastrin; NICD, Notch intracellular domain; NTF, N-terminal fragment; PS, presenilin; TM, transmembrane; wt, wild-type.

* Corresponding author. Fax: +1 314 747 2983.
E-mail address: goate@icarus.wustl.edu (A.M. Goate).
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associated to form the active PS molecule. The active site aspartate residues are each located in one fragment, D257 in the NTF (TM6) and D385 in the CTF (TM7). Mutation of either of these aspartic acid residues not only abrogates γ-secretase activity but also abolishes PS1 endoproteolysis (Kimberly et al., 2000; Steiner et al., 1999b; Wolfe et al., 1999a; Yu et al., 2000a).

It has been proposed that full-length PS1 (FL-PS1) is a zymogen that is activated by autoproteolysis to form the active NTF/CTF heterodimer (Li et al., 2000; Wolfe et al., 1999a; Yu et al., 2000a). The NTF and CTF are present in a 1:1 ratio and protein levels remain constant, suggesting that endoproteolysis is a highly regulated event (Thinakaran et al., 1996). Indeed, overexpression of PS1 leads to an accumulation of FL-PS1 rather than an increase in the NTF/CTF levels (Thinakaran et al., 1997). FL-PS1 is rapidly degraded and is found in low molecular weight inactive γ-secretase complexes, while the NTF/CTF remain stable for over 24 h and are present in high molecular weight active γ-secretase complexes (Capell et al., 1998; Ratovitski et al., 1997; Thinakaran et al., 1997; Yu et al., 1998; Zhang et al., 1998). The major PS species in vivo is the cleaved NTF/CTF heterodimer (Thinakaran et al., 1996), and it is these fragments that bind to transition state analogue inhibitors of γ-secretase (Li et al., 2000), again suggesting that the cleaved NTF/CTF PS complex is the active form of the molecule. Finally, although the "presenilinase" that cleaves the FL-PS1 molecule is unknown, it has the characteristics of an aspartyl protease and some, but not all γ-secretase inhibitors block PS1 endoproteolysis (Beher et al., 2001; Campbell et al., 2002, 2003), supporting the hypothesis that PS endoproteolysis is an autocatalytic event (Wolfe et al., 1999a) such that the same γ-secretase active site within PS that cleaves APP and Notch may also cleave and activate PS itself.

It has been suggested that γ-secretase activity might require PS1 dimerization. We and others have shown that two PS1 molecules can interact through co-immunoprecipitation (Schroeter et al., 2003), cross-linking between NTF:CTF and NTF:NTF (Schroeter et al., 2003), and yeast two-hybrid studies showing NTF:NTF and CTF:CTF homodimerization (Cervantes et al., 2001). Furthermore, the PS homologue SPP exists primarily in a homodimer and γ-secretase inhibitors bind the dimeric form of SPP (Nyborg et al., 2004), supporting the hypothesis that dimerization may be important for γ-secretase activity. If the same γ-secretase activity functions as the "presenilinase" that cleaves PS as well as the γ-secretase that cleaves APP and Notch, dimerization would be important for presenilin endoproteolysis. This model would predict that autoproteolysis by PS could occur by two possible mechanisms: intermolecular cleavage, in which one PS molecule contains the active site that cleaves a second PS molecule, or intramolecular cleavage in which a single PS molecule contains the active site to cleave itself. This mechanism has not been directly addressed although some observations have been made with PS1 mutants such as the active site mutant D257A that does not undergo presenilinase cleavage. When expressed at high levels in cells that contain endogenous PS, the D257A transgene "replaces" the endogenous PS, making it impossible to determine whether or not the endogenous wtPS would be able to endoproteolyse D257A. However, when D257A is expressed at low levels in these cells, the endogenous wtPS NTF is indistinguishable from possible D257A fragments.

In this study, we further characterize the sequence specificity of PS1 endoproteolysis and demonstrate that endoproteolysis is not an intermolecular cleavage event in which two PS1 molecules cleave and activate each other. Rather our results indicate that dimerization is not important for presenilinase activity and that endoproteolysis is an intramolecular event dependent upon the activity present within a single PS1 molecule.

Results

Mutations at the site of PS1 endoproteolysis do not eliminate PS1 processing

We introduced point mutations at the site of endoproteolysis to further characterize the sequence specificity at this site and to determine whether subtle mutations that block endoproteolysis would also block presenilin activity. The P1′ residue at the Notch S3 site, which is cleaved by γ-secretase, is a valine (V1744), as is the P1′ residue at the APP S3-like site (V721). Mutation of Notch V1744 to lysine, leucine, or glycine diminishes production of NICD from the Notch ΔE precursor (Huppert et al., 2000; Schroeter et al., 1998), and mutation of APP V721 to lysine or alanine results in decreased steady-state levels of the CTFγ produced from the C99 precursor, while V721G and V721L mutations have minimal effects (Hecimovic et al., 2004). Interestingly, the P1′ residue at the PS cleavage site is also a valine (V293). Previous work (Steiner et al., 1999a) suggested that an M292D mutation at the P1 site of cleavage created a molecule that does not undergo endoproteolytic processing or affect PS1′s amyloidogenic capability. We further analyzed the M292D mutation as well as another non-conservative mutation (M292E) at the P1 site. We also introduced conservative and non-conservative mutations at the P1′ site similar to those previously tested in Notch and APP: V293G, V293L, V293A, and V293K.

We first tested the ability of point mutations to block PS1 processing in primary fibroblasts from PS1/PS2 double knock-out mice (Herreman et al., 2000) (PS1/2KO cells) (Fig. 1A). These cells contain no endogenous PS1, allowing assessment of endoproteolysis without the need to distinguish between the transgene and endogenous fragments. Vector alone (-PS1) or PS1 constructs (PS1, D257A, or PS1 endoproteolysis mutants) were transiently co-transfected into the PS1/2KO cells with either Notch ΔE (NΔE) or APP-C99 (C99) and lysates were examined for endoproteolysis or γ-secretase production of Notch NICD (NICD) or APP-CTFγ (CTFγ) (Figs. 1B, C). The NΔE and APP-C99 constructs each contain a 6-myc tag at the C-terminus for detection with commercial antibodies. In the absence of PS1 (lane 1), neither NICD nor CTFγ was produced by these cells. As expected, the wtPS1 (PS1) molecule underwent endoproteolysis and was able to support both NICD and CTFγ production, while the aspartic acid mutant D257A was not cleaved and was unable to rescue γ-secretase activity. The P1 mutations M292D and M292E both substantially inhibit endoproteolysis but long exposures of another western blot reveal low levels of endoproteolysis for both mutants (Fig. 1A lower panel). The conservative P1′ mutations V293G, V293L, and V293A all support wild-type levels of the PS1-NTF, while the non-conservative V293K mutation shows an intermediate endoproteolysis phenotype. The double mutant M292D/V293K was cleaved at low levels, similar to the P1 site mutants (Fig. 1A). All endoproteolysis mutants supported similar levels of CTFγ production (Fig. 1C) and restored NICD production (Fig. 1B), however, the V293K and M292D/V293K mutations resulted in lower steady state levels of NICD. This reduction was confirmed using an antibody specific to NICD (data not shown). Thus, the