Presenilin/ γ -secretase and α -secretase-like peptidases cleave human MHC Class I proteins

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HLA (human leucocyte antigen)-A2 is an MHC Class I protein with primary functions in T-cell development and initiation of immune cell responses. MHC I proteins also play roles in intercellular adhesion, apoptosis, cell proliferation and neuronal plasticity. By utilizing a sequence comparison analysis, we recently identified HLA-A2 as a potential substrate for the Alzheimer's disease-associated PS1 (presenilin 1)/ γ -secretase. α -Secretase-like membrane metalloproteinases are responsible for an initial shedding event, partially mediated by ADAM (a disintegrin and metalloproteinase)-10. Accordingly, activation or inhibition of α -secretase-like membrane metalloproteinases directly modulated levels of a 14 kDa HLA-A2 CTF (C-terminal fragment) in CHO (Chinese-hamster ovary) cells. To show that the HLA-A2 CTF is subsequently cleaved by PS1/ γ -secretase, we reduced its activity in cell lines stably expressing HLA-A2 and in

Jurkat T-cells expressing endogenous MHC I. Treatment with specific PS1/ γ -secretase inhibitors or expression of a dominant-negative construct led to a significant accumulation of HLA-A2 CTFs. We also identified the PS1/ γ -secretase cleavage product of HLA-A2 CTF, termed HLA-A2 intracellular domain, in cell-free and cell-based experiments. In the absence of proteasome inhibitors, HLA-A2 intracellular domain underwent rapid degradation. These data indicate that MHC I proteins undergo extracellular domain cleavage mediated by α -secretases and the cleavage product is subsequently cleaved by PS1/ γ -secretase.

Key words: α - and γ -secretases, a disintegrin and metalloproteinase (ADAM), human leucocyte antigen, matrix metalloproteinase (MMP), MHC Class I, presenilin.

INTRODUCTION

Along with at least three additional proteins, namely Aph-1, Nicastrin, and Pen-2, the presenilins [PS1 (presenilin 1) and PS2] form a proteolytic complex termed γ -secretase. γ -Secretase is responsible for cleavage of type I membrane proteins in a process termed regulated intramembrane proteolysis. Prior to regulated intramembrane proteolysis, shedding of ectodomain regions occurs by MMPs (matrix metalloproteinases). Once MMPs such as TNF α (tumour necrosis factor α)-converting enzyme or α -secretases ADAM (a disintegrin and metalloproteinase)-10 and -17 cleave their substrates, a catalytic aspartate residue in the presenilins allows γ -secretase complexes to cleave within the transmembrane domain. γ -Secretase cleavages liberate ICDs (intracellular domains) and several substrates [Notch-1, APP (amyloid precursor protein), CD44, E-cadherin and ErbB4] have the ability to transduce nuclear signals and affect gene activation [1–7]. γ -Secretase cleavage within the transmembrane domain does not appear to require sequence specificity; however, one site in particular, the APP- ϵ /Notch S3 site near membrane–cytosol interface, has been used to identify novel substrates [8,9].

MHC I (MHC Class I) proteins are type I membrane proteins found on the cell surface of almost all nucleated cells. They largely form non-covalent heterodimeric complexes with β_2 -microglobulin and present small cytosolic peptides of 9–11 amino acids to T-cells. During T-cell development, MHC I complexes are found on antigen-presenting cells where they play a central role in maturation of CD8⁺ cytotoxic T-lymphocytes. Additionally,

when cells are invaded by foreign microbes such as viruses, MHC I complexes signal immune surveillance cells to initiate proper immune system responses. In both cases MHC I complexes are primary components of a highly dynamic immunological synapse where they ligate with TCRs (T-cell receptors) and CD8 molecules [10]. The immunological synapse involves additional proteins for proper stabilization between cells and communication: co-stimulatory molecules (CD28, CD80 and CD86), adhesion proteins [LFA-1 (lymphocyte function-associated antigen-1) and ICAM-1 (intercellular adhesion molecule 1)] and additional large glycoproteins to bridge contacts between cells (CD43, CD44 and CD45) [11]. It is interesting that both CD43 and CD44 have previously been identified as γ -secretase substrates [12,13].

In recent years it has been discovered that MHC I proteins are expressed in neurons throughout the CNS (central nervous system). MHC I expression was found to be regulated by neuronal activity during development, with peak expression occurring in the perinatal period [14,15]. Moreover, it has been shown that neuronal MHC I functions as a mediator of synaptic plasticity and activity-dependent refinement during development of the visual system [14,15]. Neuronal MHC I expression is regulated similarly to immune system MHC I, whereupon exposure to cytokines induces up-regulation of MHC I [16]. Additional studies suggest a role for neuronal MHC I during treatments such as axotomy where they may regulate the ability of neurons to maintain synapses [16,17].

The present study identifies a specific protein of the HLA (human leucocyte antigen)-A locus, HLA-A2, as a substrate of both

Abbreviations used: ADAM, a disintegrin and metalloproteinase; APP, amyloid precursor protein; CHO, Chinese-hamster ovary; CNS, central nervous system; CTF, C-terminal fragment; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester; HLA, human leucocyte antigen; ICD, intracellular domain; MHC I, MHC Class I; MMP, matrix metalloproteinase; PS1, presenilin 1; TNF, tumour necrosis factor; TAPI-1, TNF α processing inhibitor-1; TCR, T-cell receptor.

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α - and γ -secretases. Cell-free and cell-based experiments show that HLA-A2 first undergoes an α -secretase-like shedding event, followed by a PS1/ γ -secretase-mediated event that releases an ICD. Since MHC I proteins are important components of the immune system, the developing CNS and during activity-dependent synaptic remodelling, the present study provides new insights into the function of PS1/ γ -secretase.

MATERIALS AND METHODS

Plasmids and transfections

An expression construct encoding full-length human HLA-A*0201 heavy chain (HLA-A2) was obtained from Dr Hidde Ploegh (Harvard Medical School). The construct contained a signal peptide from the mouse H-2K^b haplotype that enhances *in vitro* translation [18]. The primers used for PCR amplification were 5'-ACCATGGTACCGTGACGCTGCTCCT-3' and 5'-CACTTTACAAGCTGTGAGAGACACAT-3'. Subsequent cloning and transformation were performed using TOPO cloning vector (pcDNA3.1 containing a C-terminal V5/His tag) in One Shot TOP10 Chem competent *Escherichia coli* (Invitrogen). Sequencing was later confirmed at Massachusetts General Hospital facilities. Effectene (Qiagen) was used for transfecting cell lines. We produced stably transfected CHO (Chinese-hamster ovary) cells as well as B104 rat neuroblastoma cells (Dr David Schubert, The Salk Institute, La Jolla, CA, U.S.A.). Jurkat cell line E6.1 was purchased from A.T.C.C.

Western-blot analysis, immunoprecipitation, antibodies and inhibitors

Cell extracts were prepared by directly lysing cells in a buffer containing 10 mM Tris/HCl (pH 6.8), 1 mM EDTA, 150 mM NaCl, 0.25 % Nonidet P40, 1 % Triton X-100 and a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.), followed by centrifugation at 16 000 g (4 °C, 15 min). Samples were quantified using the BCA (bicinchoninic acid) protein assay kit (Pierce). Protein (20–100 μ g) was resolved on 4–12 % gradient Bis-Tris gels. Immunoprecipitations were done as described in [8]. Primary antibodies V5 (1:5000 dilution, Invitrogen), anti-HLA (1:250), W6/32 were purchased from Biotrend Chemicals. HC10 antibodies were obtained from Dr Hidde Ploegh, and anti-HLA-A and -B (TA-17) were obtained from Dr Tanigaki Nobuyuki (Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY, U.S.A.). The blots were developed using ECL[®] (enhanced chemiluminescence) with SuperSignal CL-HRP substrate (Pierce) according to the manufacturer's instructions. The γ -secretase inhibitors DAPT {N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester} and L-685,458 were obtained from Calbiochem, and WPE31C was a gift from Dr Michael S. Wolfe (Department of Neurology, Brigham and Women's Hospital, Boston, MA, U.S.A.). The α -secretase inhibitor, TAPI-1 (TNF α processing inhibitor-1; IC-2), was from Biomol.

Cell-free generation of HLA-A2 ICD

Membrane preparation and cell-free generation of HLA-A2 proteolytic products were performed as described in [8]. The P2 and P3 fractions were resuspended in Buffer H (20 mM Hepes, 150 mM NaCl, 10 % glycerol and 5 mM EDTA, pH 7.4) with protease inhibitors. *In vitro* cleavage experiments were performed by incubating the membrane fractions at 37 °C for 1 h in the presence or absence of indicated amounts of DAPT. After incubation, the

soluble and membrane-associated fragments were separated by centrifugation of the reaction mixture at 120 000 g for 45 min.

Immunohistochemistry

Cells were treated and fixed in 4 % (w/v) paraformaldehyde for 10–20 min at room temperature (25 °C). Cells were permeabilized with Triton X-100, and primary and secondary antibodies were incubated for 1 h at room temperature. Anti-V5 (1:200) stained cells were visualized using confocal microscopy (Olympus).

RESULTS AND DISCUSSION

HLA-A2 forms functional MHC I complexes in CHO and B104 cells

HLA-A2 is one of the most commonly expressed MHC I proteins. To analyse proteolytic processing of HLA-A2, we stably transfected CHO and B104 cells with an HLA-A2 cDNA C-terminally tagged with V5/His. Two commonly used antibodies were chosen to characterize the overexpressed HLA-A2 protein. These antibodies distinguish between MHC I complexes containing β_2 -microglobulin (W6/32) and MHC I proteins lacking β_2 -microglobulin, termed β_2 -free MHC I (HC10) [19,20] (Figure 1a). Endogenous MHC I proteins are known to be found in complexes with β_2 -microglobulin. β_2 -free MHC I arise occasionally during biogenesis of MHC I complexes and after both β_2 -microglobulin and peptide dissociate from MHC I at the cell surface [21–23]. β_2 -free MHC I occur at low levels in naïve cells, but are known to accumulate during T-cell activation [21]. It was previously reported that expression of HLA-A2 in murine cells leads to easily detectable amounts of W6/32-reactive complexes [24].

In stably transfected CHO cells we observed HLA-A2 protein at or near the cell surface by anti-V5 immunostaining (Figure 1b). Using the aforementioned antibodies, W6/32 and HC10, we tested whether HLA-A2 was complexed with β_2 -microglobulin in these cells. After immunoprecipitation, most HLA-A2 was found in W6/32-positive complexes in CHO-HLA-A2 cells (Figure 1c). This suggests that MHC I complexes containing β_2 -microglobulin were formed. We also tested the B104 rat neuroblastoma cell line stably expressing HLA-A2. In these cells we found an equal distribution of MHC I complexes and β_2 -free MHC I after similar immunoprecipitations (Figure 1c). These results show that in hamster and rat cell lines, expression of HLA-A2 protein can form MHC I complexes containing β_2 -microglobulin.

HLA-A2 is processed by α -secretases

Prior to γ -secretase cleavage, all substrates undergo ectodomain shedding where they are cleaved by α -secretase-like MMPs at or near the cell surface. To establish a similar role for MMP activity in the generation of HLA-A2 CTFs (C-terminal fragments), we tested whether the α -secretase inhibitor TAPI-1 could affect HLA-A2 CTF generation in CHO-HLA-A2 stable cells. Stably transfected cells differed from transient HLA-A2 expression in that they showed constitutive HLA-A2 CTF generation at 14 kDa under untreated conditions. Constitutive HLA-A2 CTF production was significantly reduced by TAPI-1 treatment alone for 6 h (Figure 2a). Conversely, PS1/ γ -secretase inhibition by DAPT treatment alone for 6 h increased HLA-A2 CTF levels. However, the increase due to γ -secretase inhibition was abolished during co-treatment of DAPT with TAPI-1 (Figure 2a). These data suggest that α -secretase activity is required for generation of HLA-A2 CTFs prior to γ -secretase cleavage.

To test whether a specific α -secretase, ADAM-10, contributes to HLA-A2 CTF generation, we used B104 neuroblastoma cells

Figure 1 Stably expressed HLA-A2 in CHO and B104 cells

(a) Expression construct of HLA-A2 containing a C-terminal V5/His tag. W6/32 and HC10 antibodies recognize dimerized and free forms of HLA-A2 respectively. (b) Anti-V5 immunostaining of CHO-HLA-A2 cells shows surface/early endosome localization of HLA-A2. (c) Immunoprecipitation (IP) of HLA-A2 from CHO and B104 cell lines stably expressing the protein indicates that HLA-A2 forms hybrid complexes with β_2 -microglobulin. Heavy chain of W6/32 antibody is not readily detected because of low concentrations used in the immunoprecipitations (0.2 μ g for W6/32; 6.8 μ g for HC10).

stably expressing HLA-A2. Transient expression of ADAM-10 in these cells significantly increased HLA-A2 CTF generation. DAPT treatment of cells expressing ADAM-10 resulted in increased HLA-A2 CTF accumulation when compared with cells with normal ADAM-10 levels (Figure 2b). Together, our results indicate that HLA-A2 CTFs are generated by MMP activity prior to PS1/ γ -secretase cleavage and the α -secretase ADAM-10 contributes to the extracellular-domain shedding event.

HLA-A2 is cleaved by a PS1-dependent γ -secretase-like activity

Next, we characterized PS1/ γ -secretase-mediated processing of HLA-A2. Our sequence comparison search using the APP- ϵ /Notch S3-like domain previously revealed γ -secretase substrates including nectin-1 and a voltage-gated sodium channel subunit (β_2) [8,9]. Using the same method, we now found that HLA-A, -B and -C proteins also contain this site homology (Figure 3a). Western-blot analysis of CHO cells transiently transfected with HLA-A2 showed that the full-length HLA-A2 protein migrated at approx. 44 kDa (Figure 3c). In transiently transfected CHO cells treated for 3 h with PMA ('TPA'), an activator for α -secretases such as ADAM-family metalloproteases, we did not

see a consistent and significant accumulation of HLA-A2 CTFs (Figures 3c–3e). However, co-treatment of PMA with the γ -secretase inhibitor DAPT resulted in the accumulation of a C-terminally tagged HLA-A2 fragment at 14 kDa, suggesting that γ -secretase activity may be responsible for cleaving this fragment. In addition, a 6 h treatment of DAPT alone was sufficient to significantly increase HLA-A2 CTFs (Figure 3c). We confirmed these results using two additional γ -secretase inhibitors, L-685,458 and WPE31C. Each inhibitor independently increased HLA-A2 CTFs, which were augmented upon co-treatment with PMA in 3 h treatments (Figure 3d). We also stably expressed HLA-A2 in B104 rat neuroblastoma cells and found accumulation of HLA-A2 CTFs after PS1/ γ -secretase inhibition. This effect was potentiated by co-treatment with PMA (Figure 3e). To further confirm that PS1/ γ -secretase activity was responsible for cleaving HLA-A2, we tested whether a similar accumulation of the 14 kDa fragment occurs in cells stably expressing dominant-negative mutations in PS1 (D385A). Figure 3(f) shows that lack of γ -secretase activity in PS1 (D385A) cells resulted in accumulation of the HLA-A2 CTF. These results show that PS1/ γ -secretase activity is responsible for cleavage of the HLA-A2 CTF detected at 14 kDa.

Figure 2 HLA-A2 is cleaved by α -secretase-like proteases

(a) Treatment with TAPI-1 (50 μ M for 3 h), an α -secretase inhibitor, attenuates HLA-A2 CTF production compared with untreated cells (CTL). Accumulation of HLA-A2 CTF with the PS1/ γ -secretase inhibitor DAPT alone is significantly reduced upon co-treatment with TAPI-1 and DAPT. HLA-A2 is stained with an anti-V5 antibody. Transferrin receptor is shown as control. (b) HLA-A2 is cleaved by the α -secretase ADAM-10. Overexpression of ADAM-10 in B104 rat neuroblastoma cells stably expressing HLA-A2 shows that HLA-A2 CTF accumulation by PS1/ γ -secretase inhibition is further enhanced in ADAM-10-expressing cells. HLA-A2 is stained with an anti-V5 antibody. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is used as a loading control. FL, full-length.

Next we tested whether endogenous MHC I in human Jurkat T-cells exhibited a processing pattern similar to overexpressed HLA-A2. For these experiments we used a C-terminally directed antibody known to immunoprecipitate HLA-A and -B [25]. Endogenous MHC I CTF accumulated at approx. 12 kDa after a 24 h incubation with DAPT (Figure 3g). These data indicate that endogenous MHC I proteins are cleaved in cultured human T-cells.

γ -Secretase activity is required for generation of HLA-A2 ICD *in vivo* and *in vitro*

For additional confirmation that γ -secretase cleaves HLA-A2 CTFs, we performed a cell-free γ -secretase cleavage assay on CHO cells stably expressing high levels of HLA-A2. Cell-free assays have previously been successful for detection of ICDs, such as nectin-ICD [8]. CHO-HLA-A2 cell membranes were incubated with or without DAPT for 1 h and treated with various protease and proteasome inhibitors. Control samples, incubated at 0°C, showed significant amounts of HLA-A2 CTF (Figure 4a). After incubation at 37°C, a fragment of approx. 12 kDa was generated in cells with γ -secretase activity. This band was not observed in DAPT-treated cell extracts, indicating that the HLA-A2 ICD is generated by γ -secretase activity (Figure 4a).

To investigate whether HLA-A2 ICD can be detected *in vivo*, we utilized lactacystin β -lactone to inhibit potential proteasomal degradation of the ICD. CHO-HLA-A2 stable cells were first treated with PMA to activate α -secretases and increase both HLA-A2 CTF production and cleavage. Upon co-incubation with PMA and lactacystin β -lactone for 3 h, an HLA-A2 ICD was detected at approx. 12 kDa. The production of this fragment was blocked when similarly treated cells were also incubated with one of two γ -secretase inhibitors, DAPT and L-685,458 respectively (Figure 4b). Our results indicate that γ -secretase activity is required for generation of the HLA-A2 ICD fragment, which is highly unstable and quickly degraded by the proteasome system *in vivo*. We also observed an approx. 21 kDa band which was increased with PMA and lactacystin β -lactone treatment for 3 h, previously observed in PS1(D385A) cells transiently expressing HLA-A2. This band did not appear consistently in other experiments; perhaps it is a very unstable dimer of HLA-A2 CTFs or an intermediate degradation product. Additional studies would be required for further characterization.

Our study shows that MHC I proteins, such as HLA-A2, are substrates for both α - and γ -secretases in three different cell lines. Metalloprotease or α -secretase activities mediate ectodomain shedding, producing an HLA-A2 CTF, followed by sequential

Figure 3 HLA-A2 is cleaved by PS1/ γ -secretase

(a) Sequence comparison of the APP- ϵ /Notch-S3 domains in ten known human PS1/ γ -secretase-like substrates, and in HLA-A2. (b) Predicted fragment sizes before and after PS1/ γ -secretase-mediated cleavage of HLA-A2 CTF. (c) CHO cells expressing HLA-A2 were resolved by SDS/12% PAGE and stained with anti-V5. Upon co-treatment with PMA (TPA) and DAPT, an HLA-A2 CTF accumulates around 14 kDa. (d) Anti-V5 immunostaining shows that HLA-A2 CTFs are increased by two additional γ -secretase inhibitor treatments, L-685,458 and WPE31. Transferrin receptor is shown as loading control. (e) Anti-V5 immunostaining of B104 rat neuroblastoma cells stably expressing HLA-A2 shows accumulation of HLA-A2 CTFs after treatment with PMA or DAPT, and further accumulation in co-treatments. Transferrin receptor is shown as loading control. (f) The dominant-negative form of PS1, D385A, elevates HLA-A2 CTF levels. Expression of PS1(D385A) results in the accumulation of the 14 kDa HLA-A2 CTF (arrowhead). The asterisk indicates an unidentified band that is not seen with PS1/ γ -secretase inhibitor treatments. (g) Jurkat T-cells show accumulation of an endogenous MHC I CTF. A 24 h incubation of DAPT leads to accumulation of an approx. 12 kDa MHC I CTF. Immunostaining was with anti-HLA-A and -B (TA-17). Transferrin receptor is shown as a control.

Figure 4 HLA-A2 ICD is produced under cell-free and *in vivo* conditions

(a) Cell-free generation of a 12 kDa HLA-A2 ICD. Partially purified membrane fractions of CHO-HLA-A2 were subjected to a γ -secretase cleavage assay [8]. Membranes were incubated with protease inhibitors at 0 and 37°C in the presence or absence of DAPT. Samples incubated at 37°C generate a HLA-A2 ICD fragment. Blots were stained with an anti-V5 antibody. (b) *In vivo* generation of HLA-A2 ICD in CHO-HLA-A2 cells after incubation with a proteasomal inhibitor lactacystin. HLA-A2-ICD accumulation was blocked upon inhibition of PS1/ γ -secretase activity with DAPT and L-685,458 respectively. The asterisk indicates an unidentified band that is not seen with PS1/ γ -secretase inhibitor treatments.

PS1/ γ -secretase cleavage. We found that the PS1/ γ -secretase cleavage product, HLA-A2 ICD, undergoes rapid proteasomal degradation upon generation by PS1/ γ -secretase. The present study adds MHC I proteins to the growing list of PS1/ γ -secretase substrates and provides further insight on PS1/ γ -secretase function in various tissues.

MHC I functions as a central protein involved in cell–cell interactions between antigen-presenting cells and cytotoxic T-lymphocytes (CD8+) of the immune system. Interestingly, two additional PS1/ γ -secretase substrate proteins, CD43 and CD44, are also involved in cell–cell interactions proximal to MHC I proteins [13,26]. Additionally, a novel function for MHC I in the CNS has been suggested in recent years. Several reports support a role for MHC-dependent signalling in development of the mammalian visual system, specifically activity-dependent remodelling and retraction of inappropriate synapses [14,27,28]. Mice deficient in surface MHC I complexes show abnormal development of retinal projections in the visual system pathways [15]. In addition, these neurons also show perturbations in long-term potentiation and long-term depression in the adult hippocampus [15]. These studies suggest that PS1/ γ -secretase cleavage of MHC I may occur not only in immunological cells but also in neuronal cells during synapse formation.

It is well established that the PS1/ γ -secretase cleavage product of Notch, NICD (Notch intracellular domain), translocates to the nucleus to initiate transcription [1]. Recent work has shown that α - and PS1/ γ -secretase-mediated cleavages of p75 neurotrophin receptors activate Rho and subsequent signals, resulting in axonal growth inhibition [29]. While MHC I-mediated signalling in the CNS is not known, it may play a role in T-cell activation. MHC I-mediated signalling in T-cells has been studied largely using antibody cross-linking, or ligation, of MHC I molecules. MHC I signalling pathways have been suggested to include induction of tyrosine kinase and PLC γ (phospholipase C γ) activity as well as

JAK (Janus kinase) Tyk2 and Stat-3 activation [33,34]. Furthermore, MHC I-mediated signalling can activate ZAP-70 [ζ -chain (TCR)-associated protein kinase of 70 kDa] and Src kinase p56^{Lck}, leading to induction of apoptosis in Jurkat T-cells [35]. Using a different cell type, it was suggested that MHC I-mediated signalling can prevent apoptosis and is mediated by intact p56^{Lck} activity [36]. These data suggest that MHC I-mediated signalling is involved in both T-cell survival and death. However, previous work on MHC I proteins has revealed that all but four proximal amino acids from the ICD can be deleted with no effect on T-cell activation [30,31]. Similarly, membrane-associated MHC I cytoplasmic domains did not appear to affect cellular functions, including cytoskeletal association, aggregation and internalization [32].

Our results suggest that α -secretase and PS1/ γ -secretase activities are involved in degradation of MHC I proteins under physiological conditions. β_2 -free MHC I molecules have been reported as selectively cleaved and released from the plasma membrane by membrane-bound metalloproteinases, similar to α -secretase-mediated cleavages described in our study [37,38]. PS1/ γ -secretase-like activity has also been described in virus-mediated down-regulation of MHC I proteins [39]. As previously suggested, PS1/ γ -secretase may function as a proteasomal complex for a group of plasma membrane proteins [40]. Characterization of novel substrate proteins such as MHC I contributes to a full understanding of PS1/ γ -secretase function under physiological and pathological conditions.

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