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A faster migrating variant masquerades as NICD when performing *in-vitro* γ-secretase assays with bacterially expressed Notch substrates†

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Abstract

Intramembrane proteolysis is a new and rapidly growing field. *In vitro* assays utilizing recombinant substrates for γ-secretase, an intramembrane-cleaving enzyme, are critically important in order to characterize the biochemical properties of this unusual enzyme. Several recombinant Notch proteins of varying length are commonly used as *in vitro* substrates for CHAPSO-solubilized γ-secretase. Here we report that several recombinant Notch construct undergo limited or no proteolysis *in vitro*. Instead, upon incubation with or without γ-secretase, variants of the intact protein migrate during SDS-PAGE at the location expected for the γ-secretase specific cleavage products. In addition, we show that addition of aspartyl- and γ-secretase specific protease inhibitors are able to retard the formation of these variants independent of γ-secretase, which could lead to the erroneous conclusion that Notch cleavage by solubilized γ-secretase was achieved *in vitro* even when no proteolysis occurred. In contrast, substrates produced in mammalian or insect cells are cleaved efficiently *in vitro*. These observations suggest that *in vitro* studies reliant on recombinant, bacterially produced Notch TMD should be performed with the inclusion of additional controls able to differentiate between actual cleavage and this potential artifact.

γ-Secretase, a multi-protein enzymatic complex with aspartyl-protease activity, is a member of a unique class of proteases classified as Intramembrane Cleavage Proteases (I-CliPs), which catalyze cleavage of substrate proteins within the hydrophobic lipid bilayer (1,2). Clinical

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[1]APP; Amyloid Precursor Protein; I-CliP, Intramembrane Cleavage Proteases; ICD, Notch Intracellular Domain; NMR, Nuclear Magnetic Resonance Spectroscopy; TMD, Transmembrane domain. DAPT: N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester. DMSO: Dimethyl sulfoxide CHAPSO: 3-([3-Cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate

interest in γ-secretase arose because it is the enzyme responsible for hydrolysis of the Amyloid Precursor Protein (APP) thus generating the pathogenic Aβ42 peptide, the primary component of amyloid-plaques that are the hallmark pathology and likely cause of Alzheimer's Disease (3,4). γ-Secretase is also responsible for the cleavage of many other proteins, most notably Notch, which is critical not only for early embryonic development and adult immune system function, but is involved in stem cell maintenance and in several diseases including cancer, stroke, and multiple sclerosis (5,6,7,8,9,10). Lastly, despite an intense interest in this enzyme as a therapeutic target (9–14), γ-secretase remains a member of a novel class of proteases whose catalytic properties are largely unknown (15) and thus an increasing number of laboratories will join in the effort to characterize this activity.

Several groups have developed *in vitro* cleavage assays whereby the ability of partially purified γ-secretase, in CHAPSO-solubilized membrane extracts, to cleave recombinant Notch proteins is measured (16). While there are several variations reported for how to enrich membrane fractions with γ-secretase (17,18), the size and complexity of this enzyme may preclude the determination of its high-resolution structure using current techniques. Nonetheless, a structural study of γ-secretase substrates would shed considerable light on the mechanism of cleavage until structural information for the entire enzyme becomes available. It has been established that truncated substrates retain all the properties for cleavage in their TMD (19) and are well suited for structural determination in a micellar environment using nuclear magnetic resonance (NMR) techniques (20,21). A similar approach was previously used to determine the solution structure of the Aβ40 peptide (22). There are essentially two distinct sources of substrate: either the substrate is transfected into the same mammalian or insect cell culture from which the γ-secretase is solubilized (23), or substrate is separately expressed and purified from *E. Coli* and is added to the solubilized enzyme from membrane extract (18,24). All Notch substrates contain the transmembrane domain (TMD) as well as one or more epitope tags to enable detection of the expected N-terminal (Nβ) and C-terminal (NICD) products generated by γ-secretase.

Obtaining a biologically relevant result may depend upon retention of conditions that are compatible with both an *in vitro* γ-secretase assay and NMR analysis. The availability of a bacterially produced γ-secretase substrate opened the door for a variety of biophysical and structural measurements of Notch cleavage that were heretofore unattainable. Notch is a particularly attractive candidate for this analysis because there are several known single amino acid substitutions at the γ-secretase cleavage site (S3) that alter cleavage efficiency (19,25, 26,27). In addition, the structure of γ-secretase substrates is important for recognition and cleavage (19,28,29,30). While other I-CliP (S2P, SPP and Rhomboids) substrates *require* helix-breaking residues for cleavage (31–33), mutations to Valine 1744 in mouse Notch 1 that substitute helix-breaking residues such as Glycine (G) have a surprising deleterious effect (25,34) making them candidates for NMR studies.

To our surprise we discovered that a recombinant Notch protein, HAN100Flag, was unsuitable for NMR studies because it was not structurally stable during incubation at 37°C resulting in the appearance of faster migrating "conformational variants" on SDS-PAGE gels. Because these variants are indistinguishable from the expected cleavage product(s), it became apparent to us that they might have confounding effects on the analysis of Notch cleavage *in vitro*, prompting the analysis reported here. We present alternative assays and controls capable of distinguishing cleavage from conformational variants.

Experimental Procedures

Membrane preparation and detergent solubilization of γ-secretase from mammalian cells

γ-Secretase was isolated as described before (24) with slight modifications (16). Briefly, confluent Hek293 cells grown in T75 flasks were scraped into ice-cold lysis buffer (20mM HEPES, pH 7.0, 1mM EDTA) containing complete protease inhibitor cocktail (Roche) and homogenized. Lysate was centrifuged at 800g for 10 min to pellet unbroken cells and debris. The supernatent was subsequently centrifuged at 100,000g for 60 min. The resultant pellet was resuspended in lysis buffer and the centrifugation was repeated. The pellet was resuspended such that final total protein concentration was 12mg/ml and was stored at −80 C. Isolated membranes were diluted into 1% CHAPSO containing lysis buffer and 2.5mg/ml total protein and incubated for 1 hour at 4 C with rocking. Membranes were centrifuged at 100,000g for 1 hour and the supernatent fraction is considered solubilized γ-secretase. Solubilized γ-secretase was adjusted to 0.25% CHAPSO for cleavage reactions.

Expression and purification of bacterially expressed Notch substrates

HAN100Flag was purified as previously described (35). In short, BL21 (DE3) *E. Coli* bacterial cells (Stratagene) containing the HAN100Flag in the pET21a+ expression vector (Novagen) were grown in 1L LB flasks to an OD of 1.0 at 600nM. Expression was initiated with 1mM (final concentration) IPTG for 2 hours. The cells were pelleted in 1L centrifuge bottles and stored at −80 C. Frozen cell pellets were resuspended in lysis buffer (10mM Tris, pH 7.5, 200mM NaCl, 1%NP-40, 1mM EDTA, Complete Protease Inhibitor Cocktail (Roche) and passed 2X through a French Press. Cell lysates were centrifuged at 17K in a Sorvall SS34 rotor for 30 min and the soluble fraction was passed once over an M2-Flag affinity column (Sigma). Bound substrate was eluted using 100mM Glycine, pH 2.7 and 1% NP-40. Stable HAN100Flag was purified in the same buffers, with the following exceptions: no buffer contained NP-40 and protein was eluted from the M2-Flag column in 100mM Glycine, pH 3.5. Fractions were assayed for purity and stored in elution buffer at −80 C. All protein concentrations were determined using a commercially available BCA assay kit (Sigma). Expression and purification of N102FmH was described previously (36) with the exception that protein used for the *in vitro* reactions was obtained from the soluble fraction of *E. Coli* lysate.

Cleavage reaction and detection for Hek293 cells or buffer only

Purified substrate was added to a final concentration of 1uM in 50ul of solubilized γ-secretase or cleavage reaction buffer only. For 0 hour time points, substrate was added to each reaction and kept at −20 C for the duration of the assay. Cleavage reactions were stopped by addition of laemelli sample buffer and were either incubated at 65 C for 10 min or placed in boiling water for 5 min. No differences were observed between treatment at 65 C or in boiling water prior to loading on the gel. Samples from each reaction were loaded onto 4–20% Tris-Glycine pre-cast gels (BioRad or BioExpress) for SDS-PAGE and subsequently transferred to PVDF membranes (BioRad) for detection by chemiluminescence (Pico, Pierce). Antibodies used for detection are rabbit anti-mNotch1Val1744 (Cell Signaling), mouse anti-Flag M2 (Sigma), rabbit anti-HA (Ha.11; Babco) and mouse anti-c-myc (9B11; Cell Signaling Technology).

Protein Sequencing

Purified, recombinant HAN100Flag and variants were isolated using standard SDS-PAGE and transferred to PVDF membrane. The membrane was stained to detect transferred protein variants. Edman degradation protein sequencing was performed by Midwest Analytical, Inc. (11141 South Towne Square, Suite E, St. Louis, MO 63123).

MALDI-TOF

HAN100Flag was incubated in cleavage buffer (no enzyme) as described. The status of the protein was determined using SDS-PAGE and chemiluminescence. Representative samples were prepared for matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) by directly diluting protein samples in equal volumes of matrix prior to analysis. Samples were analyzed using a Voyager DE Pro spectrometer (Applied Biosystems, Foster City, Calif.).

Baculovirus expression of γ-secretase and substrates

γ-Secretase complex was reconstituted in Sf9 cells by the baculovirus expression system as described by (18) with the following changes. cDNAs that encode PS1-EGFP (PE) (37) or aspartate mutant PE together with HisXpress-Pen-2 (18,38) are cloned into pFastBacDual (Invitrogen) to generate PS1/Pen-2 (PEHP or PEDNHP) recombinant baculovirus. Nct-V5His cDNA with polyhedrin promoter and polyA tail are inserted into pBlueBac4.5 with Aph-1a-mycHis (18) to generate Nct/Aph-1a (NA) recombinant baculovirus. Reconstitution of γ-secretase complex is achieved by coinfection of PEHP and NA viruses, which is confirmed by generation of PE fragments and coimmunoprecipitation analysis (data not shown). Infected Sf9 cells were homogenized and membranes were isolated. Membranes were dispersed in HEPES buffer (10mM HEPES, pH 7.4, 150mM NaCl) containing Complete protease inhibitor mixture (Roche Applied Science) and stored at −80 degrees until use. Membrane fraction containing N100FLAG substrate (39) was isolated from virally infected into Sf9 cells. The ΔICE substrate was stably transfected using retrovirus into mouse embryonic fibroblast double knock-out cells (MEF DKO), which lack presenilin 1 and 2. This construct has a C-terminal read-through error that increases its predicted weight by approximately a factor of 2. However, this does not affect its suitability as a substrate for γ-secretase.

Baculovirus in vitro cleavage reaction and detection

Doubleviruses or mock virus infected-Sf9 dispersed membranes were incubated for 16 hrs with HAN100Flag, ΔICE in MEF DKO membranes, or with N100FLAG (N100ppt) from virally infected Sf9 membranes in HEPES buffer containing Complete protease inhibitor cocktail and 0.25% CHAPSO. Cleavage reactions were performed in a volume of 500ul and terminated by boiling and trichloroacetic acid (TCA) precipitation of protein. Total TCA precipitate was loaded onto 13.5% Tris-Tricine gels for SDS-PAGE. Protein was transferred to PVDF membrane and boiled to enhance detection prior to blocking in 5% Milk. Chemiluminescence was otherwise the same as described (Fempto, Pierce). For the assay using N102FmH, we performed as reported (18), except for adding 0.1% Phosphatidylcholine (from porcine brain, Avanti polar lipids).

Results

The Notch TMD is cleaved twice during hydrolysis by γ-secretase; once near the inner leaflet (S3) that liberates the C-terminal NICD product from the plasma membrane, and a second near the outer leaflet (S4) that releases the amino-terminal Nβ product. HAN100Flag permits detection of both Nβ (with anti-HA) and NICD (with anti-Flag, Figure 1*A*). In addition, an epitope-specific antibody (anti-V1744) can be used to detect NICD following cleavage at the S3 site (V1744). Such antibodies recognize the epitope VLLS only when exposed at the amino terminus of the NICD product following cleavage by γ-secretase, and recognize intact substrate or other proteins very inefficiently (19, 25, 40, 41). It is therefore an extremely sensitive tool for validating the identity of the product generated by γ-secretase and to visualize the NICD product even if it is present with an overwhelming amount of precursor protein (40).

In order to analyze the NMR structure of HAN100Flag, we labored to identify suitable detergent-buffer conditions that are compatible with both an *in vitro* γ-secretase assay and

NMR analysis. We initially set out to replicate the assay conditions that permit hydrolysis of HAN100Flag *in vitro* by γ-secretase (35). Upon addition of HAN100Flag to CHAPSO-solubilized γ-secretase, we observed the expected faster-migrating species corresponding to the expected product (NICD) using anti-Flag (Figure 1*B*, band C). A putative Nβ product (Figure 1*B*, band D) was detected using anti-HA antibody. This appeared to require γ-secretase activity as more "NICD" was generated after incubation with CHAPSO-membrane extracts enriched for γ-secretase by immunoprecipitation with anti-PS1 antibody (Figure 1*B*; PS1-IP). Under all conditions, we also noticed a slower migrating band (Figure 1*B*, band A) with both anti-HA and anti-Flag that we attributed to a dimer or other aggregate of HAN100Flag following incubation for the cleavage assay. While not as effective *in-vitro* (42) as *in vivo*, the γ-secretase specific inhibitors DAPT (Figure 1*C*) and Compound E (Figure 1*D*) were nonetheless able to partially inhibit production of the putative NICD product after 8hr incubation.

Unexpectedly, we noticed that the same faster migrating band believed to represent the C-terminal NICD cleavage product (Figure 1*B*, asterisk) was also detected by anti-HA; this tag should have been lost after proteolysis. Our concern was further enhanced when we failed to identify the NICD cleavage product produced by γ-secretase using the V1744 antibody (data not shown) suggesting that the VLLS epitope was not generated. Presumably, S4 cleavage could still account for the detection of a "NICD"-like fragment with anti-Flag, as would cleavage that does not produce an N-terminal Valine (41). However, neither possibility could explain why "NICD" was still detectable with anti-HA.

One possible explanation for the presence of a faster migrating, HA containing species (Figure 1*B*, asterisk) was that a contaminating bacterial protease, co-eluting with HAN100Flag during purification, cleaved a fraction of HAN100Flag at its C-terminus. This would also explain why the γ-secretase specific inhibitors DAPT (Figure 1*C*) and Compound E (Figure 1*D*) failed to completely block the generation of these faster migrating peptides (35). In support of this possibility, we observed that when HAN100Flag was incubated in CHAPSO cleavage buffer alone, the apparent NICD cleavage products appeared (Figure 1*E*). As was the case with γ-secretase containing assays (Figure 1*C*), the appearance of these faster migrating species was retarded by DAPT (Figure 1*E*) but was unaffected by equivalent concentrations of vehicle (Figure 1*E*, "). If HAN100Flag had indeed undergone cleavage, the N-terminal HA tag should have been separated from the C-terminal cleavage product, however, we could still detect a putative "NICD" product (Figure 1*E*, asterisk) with the HA antibody.

The detection of the putative "NICD" product with N-terminal HA opens the possibility that this faster migrating species was not the product of proteolysis. We noted that the aspartyl-protease inhibitor Pepstatin A could completely prevent the formation of the faster migrating species (Figure 1*A*, band C). We discovered that the addition of Pepstatin A dramatically decreased the pH of the reaction buffer. Indeed, lowering the pH to 4.0 was sufficient to block formation of the faster migrating species and the slower migrating band A was greatly reduced (Figure 2). To eliminate a hypothetical, pH-sensitive bacterial protease(s) that might co-purify with HAN100Flag we pre-incubated the purified protein in buffer at 100°C for 15 minutes, to denature contaminating bacterial proteins, and then chilled to protein and added complete protease inhibitor cocktail (Roche). Surprisingly, the faster migrating species still formed during incubation at 37°C (data not shown).

In an effort to explain these highly unusual observations, we next isolated each of the observed protein bands (Figure 1, bands *A*, *B*, *C*) for N-terminal sequencing. Each produced the identical sequence (MYPYD) corresponding to the amino terminus of the intact HAN100Flag protein, and no "ghost" peaks were detectable on the HPLC trace as would be expected if a mixture of peptides was present as a faster migrating population (data not shown). To rule out the

possibility of carboxyl-terminal cleavage, we determined the molecular weight of these differentially migrating species (Figure 1, bands B, C) by MALDI-TOF mass spectrometry analysis (Figure 3*A*). Both peptides had identical molecular weight of 13.3 kDa (within the experimental error), corresponding to the predicted molecular weight of the intact HAN100Flag. Since these measurements confirmed that the protein was intact, no proteolysis had occurred. The remaining alternative to explain the appearance of this faster migrating species during incubation *in vitro* is the appearance of conformational variants, which mimic the products of protease activity (our experimental evidence is summarized in Figure 3*B*).

We next sought to identify Notch substrates that are cleaved *in vitro*. We established a γ-secretase cleavage assay using the baculovirus system; γ-secretase purified in this way is reported to have a 10-fold greater activity than γ-secretase derived from mammalian cells (18). We assayed the activity of purified Sf9 γ-secretase using a baculovirus produced Notch substrates, N100ppt (43). N-terminal V1744 was readily detected in this assay with anti-V1744, and cleavage of N100ppt was completely inhibited by DAPT (Figure 4*A*). Moreover, no NICD was detected when N100ppt was incubated with solubilized γ-secretase containing a catalytically inactive form of presenilin (DN, Figure 4*B*), demonstrating that product detected is indeed generated by active γ-secretase and not another co-purifying protease.

Next, we wished to determine if γ-secretase from Sf9 membranes could recognize and cleave a mammalian Notch protein produced by cells lacking γ-secretase. To accomplish that, we stably expressed the minimal Notch cleavage construct, ΔICE (19) in mouse embryonic fibroblast cells lacking both presenilin 1 and 2 (MEF PS1/2 DKO). These cells endogenously express APP; simultaneous cleavage of APP was observed in the same reaction (not shown). When CHAPSO-dispersed membranes from these substrate-containing cells are mixed and incubated with CHAPSO-dispersed membranes from γ-secretase producing Sf9 cells, generation of the NICD can be followed with anti-V1744 (Figure 4*C*; *note that NICD made from this ΔICE cell line is longer*). Again, cleavage is inhibited by DAPT, and no cleavage is observed with membranes from Sf9 cells expressing a catalytically inactive form of γ-secretase (DN, Figure 4D). Thus, we definitively demonstrate that purified γ-secretase in CHAPSO-dispersed membranes can recognize and cleave substrates that are not already present in the same membranes.

Though we had been unable to detect any cleavage of HAN100Flag by γ-secretase from mammalian cells, we decided to determine if HAN100Flag could be cleaved using the more robust baculovirus system. We incubated this bacterially produced substrate with Sf9 cell membranes containing γ-secretase. Faster migrating species formed regardless of whether incubation was performed in buffer alone (Figure 4*E*), with membranes containing active enzyme (Figure 4*F*), or with membranes containing catalytically inactive enzyme (Figure 4*G*). Under these conditions, 1μM DAPT failed to inhibit the production of the faster migrating species (band C; note however that it does inhibit with the mammalian membrane prep in Figure 1). Importantly, we were again unable to detect any cleavage product using anti-V1744 even after 16 hours, consistent with lack of HAN100Flag proteolysis in this system (data not shown). Because we show that 1μM DAPT is sufficient to completely inhibit production of NICD in mixed membranes assays, we did not determine if higher concentrations of DAPT could retard the formation of the faster migrating band under these conditions.

We next sought to determine if other recombinant Notch substrates purified from bacteria underwent similar conformational changes, and if any are cleavable by γ-secretase. N102FmH is similar to HAN100Flag, and is reported to undergo cleavage in the baculovirus system (18). When incubated with CHAPSO-dispersed γ-secretase-containing Sf9 membranes, N102FmH does appear to undergo cleavage (Figure 5). However, this is an extremely inefficient reaction: to visualize N102FmH cleavage, extensive sample processing and

extended incubation times are required (see methods for complete description). Interestingly, we also noticed that faster migrating species did form in buffer-only (no enzyme) conditions, and that this faster migrating band was affected by DAPT (Figure 5*A*, arrow). However, unlike HAN100Flag, the appearance of NICD (identified with anti-VLLS) was enhanced upon incubation with γ-secretase, suggesting that some fraction of this substrate is cleaved. Anti Val-1744 interacts with the cleaved Notch species with much higher affinity than with any one of multiple substrate species recognized by other antibodies in these preparations (Figure 4, 5 with anti-Myc, M2 Flag monoclonal, pAb FLAG polyclonal). The one exception (recognition of intact/variant species seen in Figure 5*D*) may be due to the extensive processing required to detect NICD when using bacterial substrates (see methods).

Discussion

The discovery of migration variants casts doubt on conclusions drawn from assays performed thus far. In reexamining previous reports using bacterially produced Notch substrates *in vitro* we noticed that in some, anti-Val1744 could not differentiate the substrate from the cleavage product (35), and that the putative NICD product also is visualized using anti-HA, suggesting that these were variants of the same (uncleaved) protein as described here. It was reported that recombinant Notch based substrates, including HAN100Flag, are cleaved efficiently in small reaction volumes and with relatively short incubation periods (35). Here we show that under such conditions, the “cleavage products” are a faster migrating variant. Cleavage does occur with other bacterial-produced substrates, but so inefficiently that extensive sample processing is required to detect the products and separate them from the faster migrating variants. One possible explanation for the difference observed between substrates that are pre-inserted into membranes (N100ppt, ΔICE) and bacterially produced substrates (HAN100Flag, N102FmH) could be that the long bacterial-produced substrate cannot efficiently integrate into the dispersed lipid membranes containing γ-secretase. Regardless, the presence of a non-cleaved protein that co-migrates with the cleaved “NICD” product (Figure 5) implies that rigorous analysis is required with any bacterial substrate to both characterize its behavior in solution as well as to determine unequivocally if γ-secretase mediated cleavage occurs.

What is the chemical basis of this phenomenon? We were able to establish that inclusion of NP-40 in the buffer enhanced conformational variant formation with HAN100Flag. When NP-40 was excluded from the purification buffers, HAN100Flag was more stable, migrating as a single band when incubated under the common assay conditions described in Figure 1. Unfortunately, as expected in detergent-free purification, yields were substantially reduced and the hydrophobic protein aggregated when incubated for an extended time with CHAPSO-solubilized γ-secretase in the baculovirus system. In contrast, purification of N102FmH in the absence of any detergent did not block conformational variant formation. This substrate was cleaved with g-secretase derived from mammalian or insect cells, though the efficiency is greatly reduced and detection of product requires extensive processing. We also observed that the conformational variants are affected to some degree by the type and location of epitope tags on individual constructs. Based on these observations, we believe that conformational variants are dependent on the Notch sequence, and that buffer/detergent conditions and/or epitope tags only mediate the degree/rate to which they accumulate.

Resolving how conformational variants are generated for any particular construct is perhaps less important then offering solutions to differentiate the artifact from actual cleavage. Future attempts to demonstrate γ-secretase mediated cleavage of Notch substrates *in vitro* must thus rely on definitive characterization of the substrates and their apparent cleavage products. As demonstrated here, bacterially expressed Notch substrates are cleaved much less efficiently than those produced in mammalian or insect cells, perhaps due to their tendency to form

variants and/or aggregate, and one must question whether the residual cleavage observed in some substrates truly reflects the *in vivo* reaction. Despite the appearance of variants that mimic the expected "NICD" in assays conducted with different bacterially-expressed constructs, and the difficulty in properly identifying the actual cleavage product (see antibody comparison, Figure 5), our results suggests that with inclusion of additional controls, detection of cleavage product is possible. These include the following: 1) recombinant protein should be incubated in cleavage buffer alone to determine the potential for forming faster migrating variants, 2) a reaction with a catalytically inactive form of presenilin should be included to rule out involvement of other proteases, 3) Blots with antibodies to both termini should be performed to confirm separation of Tag from the protein, 4) when Val1744-specific antibodies are used, the investigator should be alarmed if they recognize the cleavage product with similar/lower affinity to the precursor (for an example of the higher affinity to NICD see (40)). Whenever possible, definitive determination of protein size and sequence should be confirmed by mass spectrometry or Edman sequencing. Finally, we had the most success performing *in vitro* assays with purified membranes from transiently transfected presenilin-deficient cells (MEF or Sf9) mixed with purified enzyme-containing membranes. While this method introduces additional membrane proteins some of which are substrates and is thus not suitable for kinetic assays, the observed efficiency of cleavage is consistent with that observed in whole cells and no conformational variants are observed.

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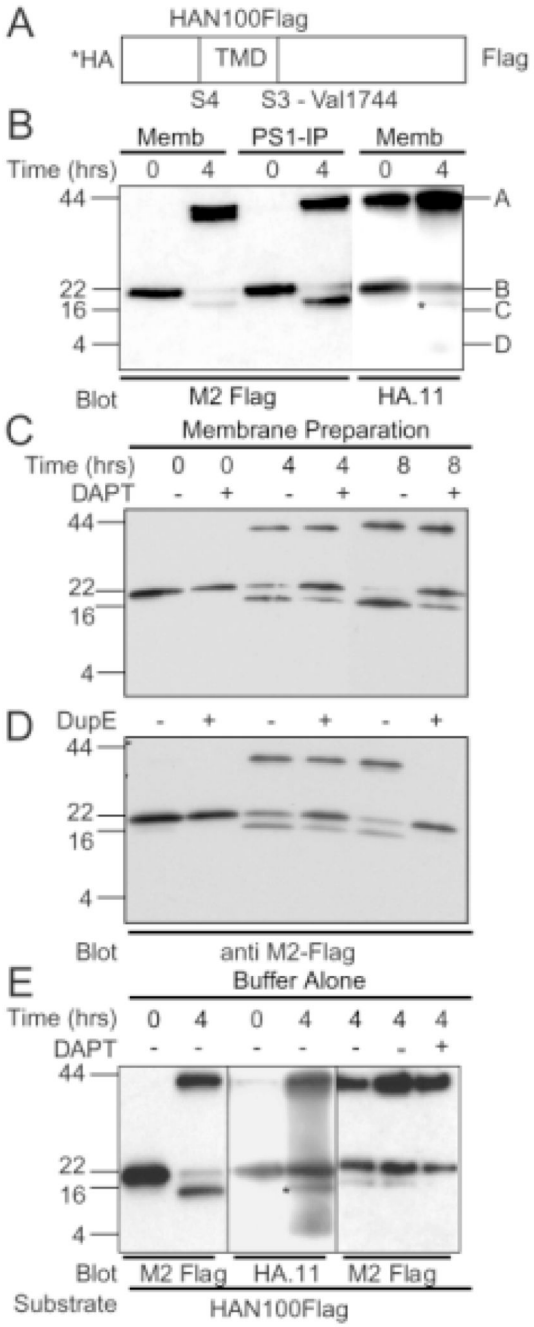


Figure 1. No γ-Secretase cleavage occurs in vitro with the HAN100Flag substrate

(A) Schematic representation of HAN100Flag indicating the γ-secretase cleavage sites within the Notch TMD (S4, S3) and the expected N-terminal (HA-tagged) and C-terminal (Flag-tagged) cleavage products. (B) HAN100Flag was incubated at −20°C or at 37°C (4 hrs) in the presence of either CHAPSO-solubilized Hek293 membranes containing γ-secretase or in extracts enriched for γ-secretase by IP with a PS-1 antibody (9N14 (44)). (C) Cleavage is not completely inhibited by the γ-secretase inhibitors DAPT and DupE (D). (E) HAN100Flag was incubated in cleavage buffer alone (no γ-secretase). Faster protein species, migrating at the expected size for a C-terminal (NICD; anti-Flag) and N-terminal (Nβ; anti-HA) cleavage products are observed. The appearance of the C-terminal (NICD) product was partially blocked

when HAN100Flag was incubated in the presence of 10uM DAPT but not an equivalent volume of vehicle (“). Notice that asterisk (B, E) marks a band detectable with the amino terminal tag (anti-HA) and corresponding to the C-terminal cleavage product NICD.

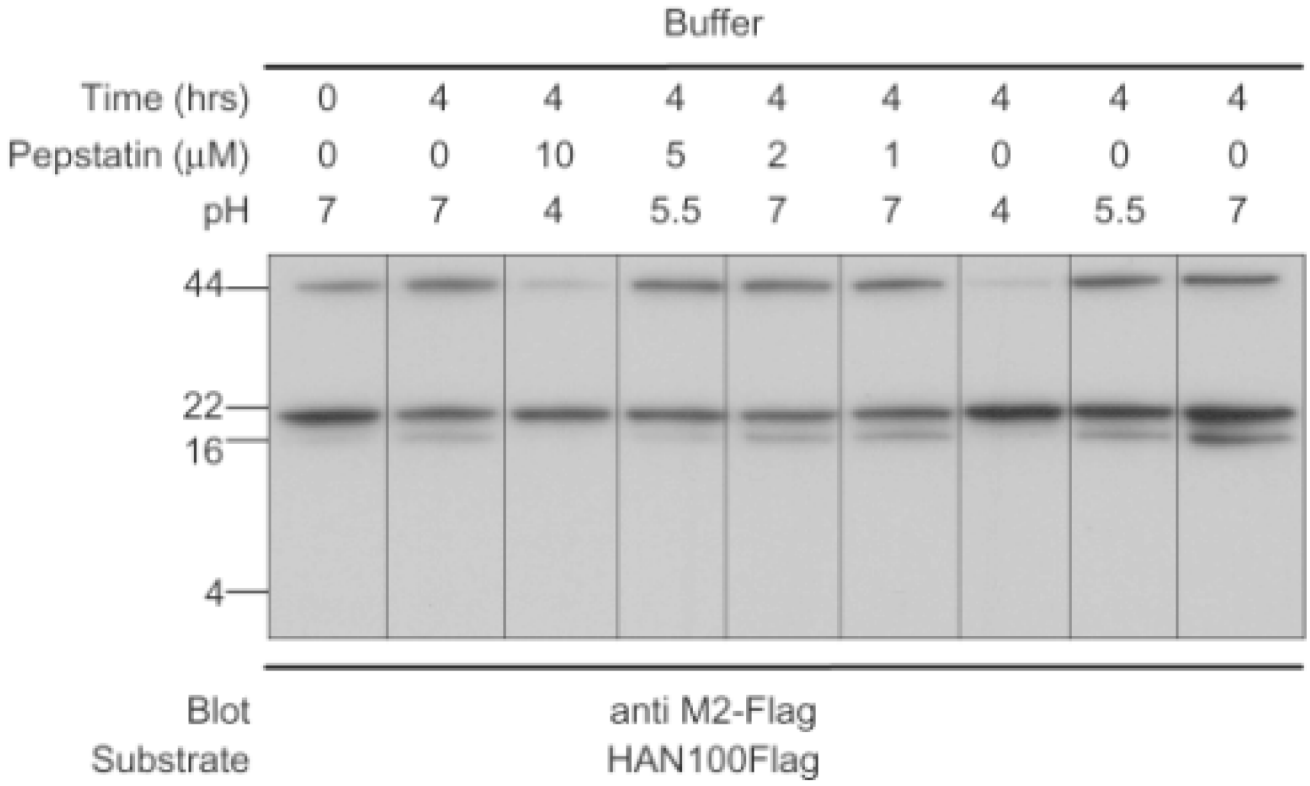
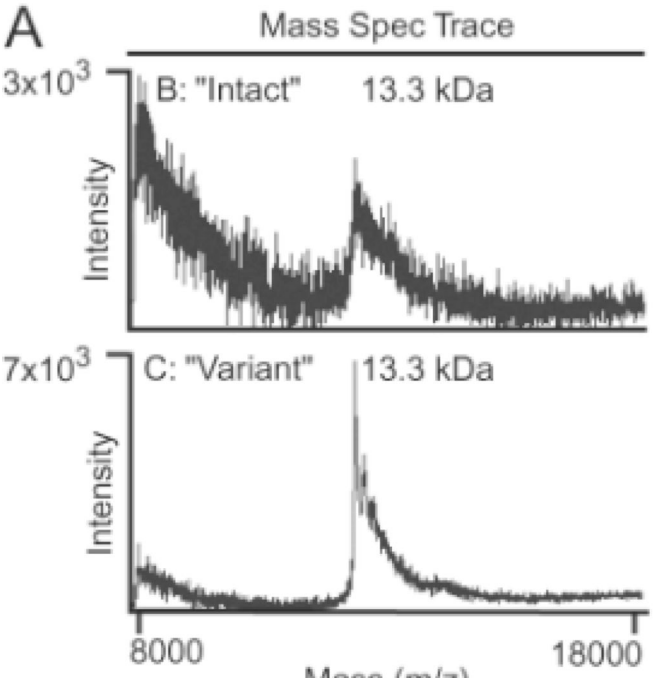


Figure 2. Putative cleavage products are eliminated at low pH

HAN100Flag was incubated in cleavage buffer in the presence of the aspartyl protease inhibitor Pepstatin A at the concentrations indicated. The apparent inhibition of cleavage by Pepstatin A is attributable to the lowering of the cleavage reaction buffer pH by the acetic acid/ethanol solution in which Pepstatin A is dissolved. Appearance of the faster migrating band is inhibited at acidic pH.



B

		Expected	Observed
Flag present		A_B_C	A_B_C
HA present		A_B_D	A_B_C_D
anti Val-1744 +		C only	none
MW (kDa):	A	ND	ND
	B	13.3	13.3
	C	9.2	13.3
	D	4.7	ND
N-term Seq:	A	MYPYD	MYPYD
	B	MYPYD	MYPYD
	C	VLLSR	MYPYD
	D	MYPYD	ND
γ-product without enz		No	Yes

Figure 3. Summary of data demonstrating that HAN100Flag preparations contain a faster migrating conformational variant

(A) The MALDI-TOF traces for the “intact” (band B in Figure 1*A*) and “variant” (band C in Figure 1*A*) forms of HAN100Flag. The trace shown is representative of multiple independent experiments with several preparations of HAN100Flag. (B) Expected (left hand column) and observed (right hand column) results for cleavage of a bacterial substrate by γ-secretase. *ND* indicates not determined.

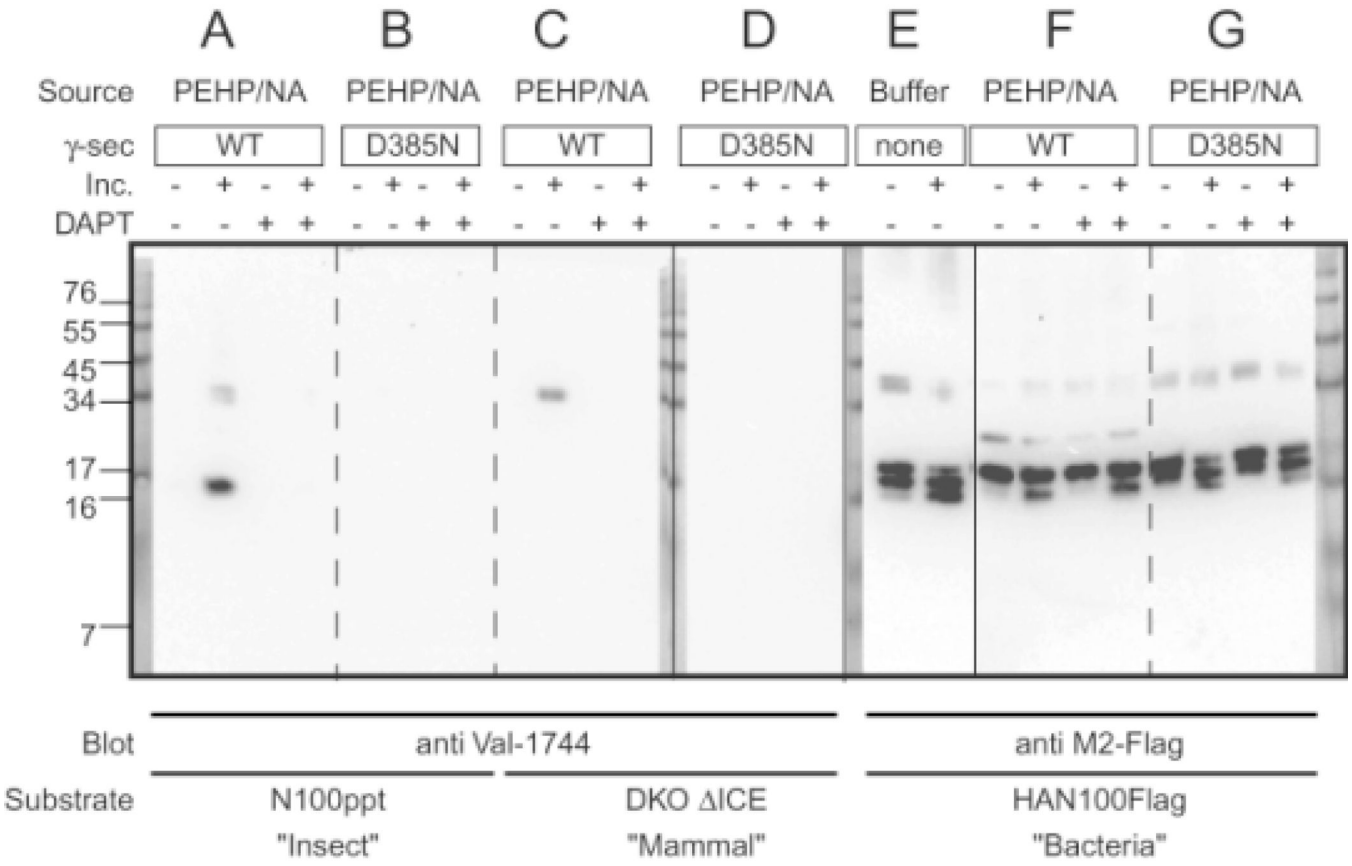


Figure 4. Substrate from non-bacterial sources are cleaved efficiently by γ-secretase

In this figure γ-secretase is produced in Sf9 cells doubly transfected with virus expressing Pen2/PS1 (PEHP) and NCT/Aph (N/A). The identity of the viruses in all experiments is described in the line labeled "source". (A) N100ppt, purified from baculovirus infected Sf9 cells is clearly cleaved when mixed with γ-secretase-containing membranes. The NICD product is detected by the VLLS epitope arising only after cleavage at S3; DAPT blocks NICD formation, (B) No cleavage product is detected when N100P is incubated in membrane preparations containing inactive γ-secretase (D385N) (C, D) Cleavage of Notch also occurs when Sf9 membranes containing active γ-secretase (WT) mixed with a longer substrate derived from a mammalian cells (DKO ΔICE). This demonstrates that γ-secretase can cleave substrate from an exogenous source provided they are pre-inserted into a membrane environment. (E) HAN100Flag was incubated for 16 hours at −20°C ((Inc; −) or 37°C ((Inc; +) in buffer alone, (F) with Sf9 cell membranes containing reconstituted γ-secretase (WT) or (G) in Sf9 cell membranes containing inactive γ-secretase (D385N). A faster migrating species appears at the expected location for NICD in all preparations containg HAN100Flag, and is unaffected by DAPT.

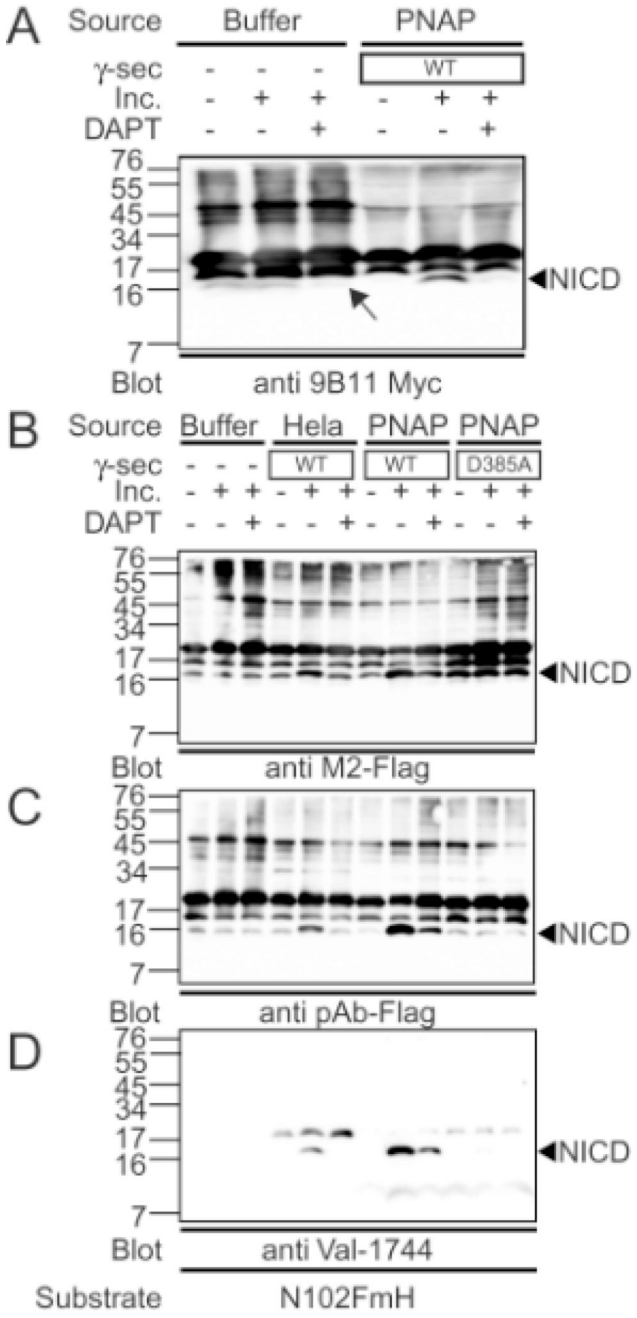


Figure 5. Bacterially produced Notch can undergo limited cleavage by γ-secretase

The bacterially produced Notch substrate N102FmH was incubated with γ-secretase containing Sf9 membranes (PNAP, cells infected with 4 viruses, see methods) for 16 hrs at −20°C (Inc.; −) or 37°C (Inc.; +). Conformational variants were detected with anti-c-myc antibody 9B11. When substrate was incubated at 37°C in buffer alone (Buffer), the appearance of the conformational variant was retard with 1μM DAPT (arrow in A). Incubation with enzyme from Sf9 cells (wt) showed an increase in the faster migrating species relative to the fractions lacking the enzyme. In (B), N102FmH was incubated in the absence of enzyme (Buffer; −), with human HeLa cell membranes containing γ-secretase (HeLa), or with membranes from Sf9 with active (active) or inactive (D385A) enzyme. Using the monoclonal M2-Flag antibody,

conformational variants were observed under all condition. Again, the faster migrating species was increased when incubated with active enzyme from mammalian or insect cells, but not in buffer, inactive (D385A) or inhibited (DAPT; 1μM). (C) Detection of N102FmH with a polyclonal Flag antibody (pAb-Flag) showed increased signal in lanes that contain actual NICD (compared with B and D). (D) The Val 1744 antibody can easily and definitively discriminate between conformational variants and the actual, cleaved NICD product. A slower migrating bend of undetermined identity appears in all Hela extract. The enzyme produced by PNAP is equal in activity to that produced by PEHP/NA cells (not shown).