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Cathepsin L Mediates the Degradation of Novel APP C-Terminal Fragments

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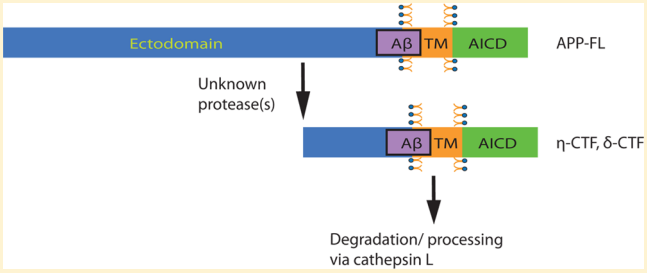
ABSTRACT: Alzheimer's disease (AD) is characterized by the deposition of amyloid β (Aβ), a peptide generated from proteolytic processing of its precursor, amyloid precursor protein (APP). Canonical APP proteolysis occurs via α-, β-, and γ-secretases. APP is also actively degraded by protein degradation systems. By pharmacologically inhibiting protein degradation with ALLN, we observed an accumulation of several novel APP C-terminal fragments (CTFs). The two major novel CTFs migrated around 15 and 25 kDa and can be observed across multiple cell types. The process was independent of cytotoxicity or protein synthesis. We further determine that the accumulation of the novel CTFs is not mediated by proteasome or calpain inhibition, but by cathepsin L inhibition. Moreover, these novel CTFs are not generated by an increased amount of BACE. Here, we name the CTF of 25 kDa as η-CTF (eta-CTF). Our data suggest that under physiological conditions, a subset of APP undergoes alternative processing and the intermediate products, the 15 kDa CTFs, and the η-CTFs aret rapidly degraded and/or processed via the protein degradation machinery, specifically, cathepsin L.



Alzheimer's disease (AD) is a progressive neurodegenerative disease. One of AD's hallmarks is the deposition of extracellular amyloid β (Aβ), which is generated by sequential proteolytic cleavage of its precursor, the amyloid precursor protein (APP). Understanding APP proteolysis is a crucial step in understanding the disease pathogenesis and possibly developing therapeutic intervention.

Canonical APP proteolysis occurs via α-, β-, and γ-secretases. APP can undergo cleavage by α-secretase, releasing the sAPPα ectodomain, and the remaining α-C-terminal fragment (α-CTF) can further be processed by γ-secretase, generating P3 and the APP intracellular domain (AICD). Alternatively, APP can be cleaved by β-secretase [β-site APP-cleaving enzyme (BACE)], releasing the sAPPβ ectodomain,[1] and the remaining β-CTF is then processed by the same γ-secretase, generating Aβ and AICD.[2] Three additional cleavage sites, δ-, ε-, and ζ-cleavages, have been reported[3−5] (Figure 1A). It is thought that the C-terminus of Aβ is generated sequentially by ε-, ζ-, and γ-cleavages.[3] δ-Cleavage occurs 12 residues N-terminal to the Aβ N-terminus, and the resulting δ-CTF has been detected in hippocampal neurons.[5]

Meanwhile, APP and its proteolytic products are actively degraded via protein degradation systems. Cathepsins have been shown to mediate the degradation of α- and β-CTFs and AICD.[6,7] Several studies also suggest that ubiquitin/proteasome is involved in the degradation of APP,[8−10] as well as CTFs.[11−13] In the study presented here, we investigate the role of protein degradation in APP metabolism. By inhibiting protein degradation systems, we detected novel APP CTFs. We further showed that they are normal proteolytic products of APP and that they undergo rapid clearance under physiological conditions via protein degradation, specifically cathepsin L.

MATERIALS AND METHODS

Cell Culture and Treatment. HEK293 and HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin/streptomycin. The SH-SY5Y/APP$_{751}$ cell line was a kind gift from M. Hiltunen (University of Eastern of Finland, Joensuu, Finland). The H4/APP$_{751}$ human neuroglioma cell line has been described previously.[14] The primary bovine brain microvascular endothelial cell was a kind gift from A. Clyne at Drexel University. Rat primary cortical astrocytes were purchased from Gibco. Primary rat cortical neurons (postnatal day 1) were prepared as previously described[15] and treated on day 6 *in vitro* for 24 h before being collected. Cells were treated with pharmacologic agents as indicated for 24 h (6 h for proteasome inhibitors) before being collected. TS20 has been described previously.[16]

Received: March 27, 2015
Published: April 24, 2015

DOI: 10.1021/acs.biochem.5b00329
Biochemistry 2015, 54, 2806−2816

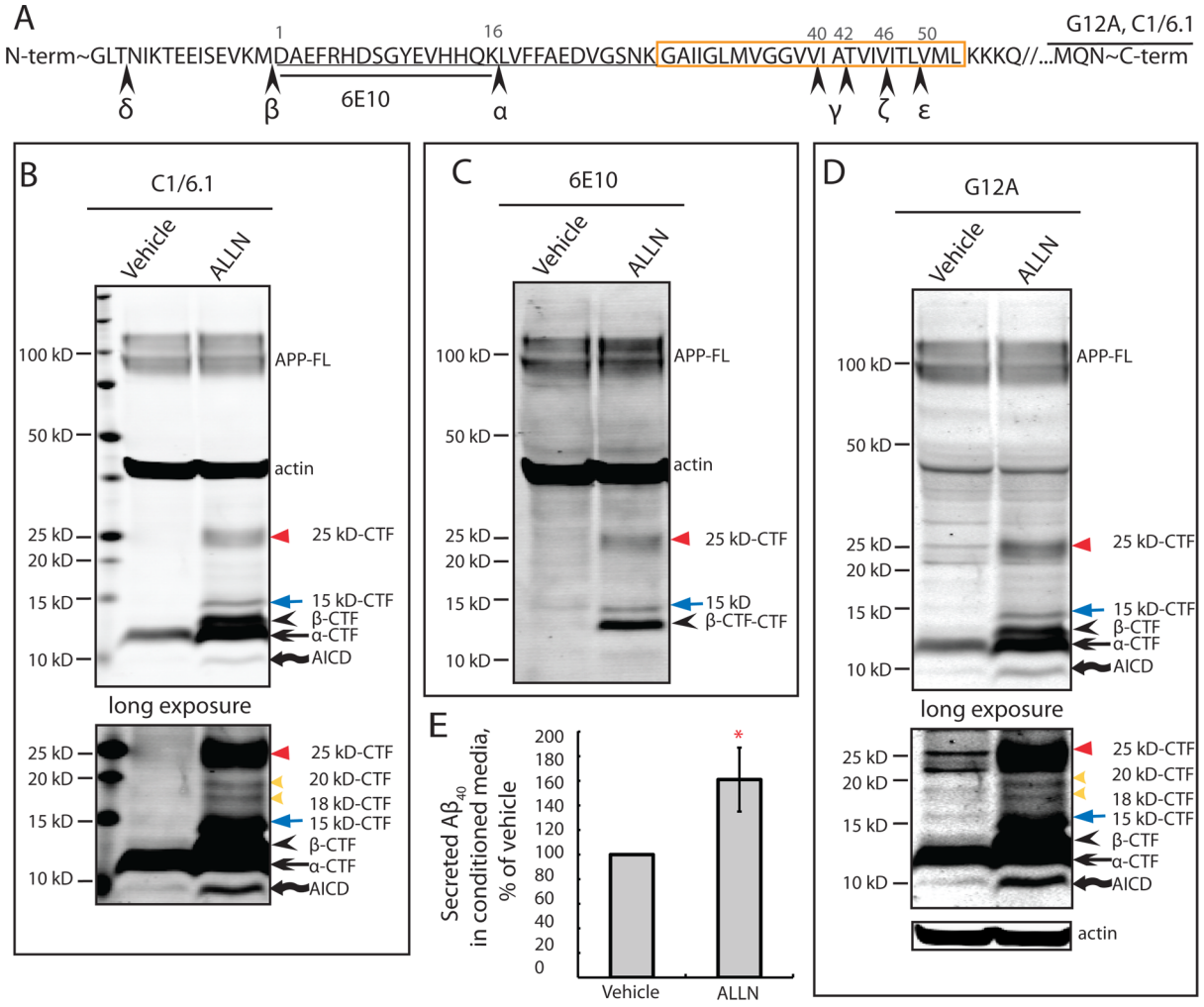


Figure 1. ALLN-induced accumulation of novel APP fragments. (A) Schematic representations of known cleavage sites of APP CTF and the epitopes of C1/6.1, G12A, and 6E10. TM denotes the transmembrane domain. (B–D) HEK293 cells were treated with 5 μM ALLN for 24 h before Western blotting and probed with C1/6.1, 6E10, and G12A, respectively. (E) Aβ_{40} in conditioned medium was measured with an enzyme-linked immunosorbent assay and normalized to the vehicle-treated group.

These cells were maintained at 35 °C and treated with ALLN as indicated while being incubated at either 35 or 39 °C, for 6 h before collection. For transient transfection, HEK293 cells were transiently transfected using Turbofect transfection reagent (Thermo Scientific) according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were treated with ALLN, L-685,458, or vehicle for an additional 24 h before being collected.

Enzyme-Linked Immunosorbent Assay (ELISA). Naïve HEK293 cells were treated with ALLN or vehicle for 24 h, and then conditioned medium was collected, cleared by centrifugation, snap-frozen in liquid nitrogen, and stored in −80 °C. Before being used, medium were thawed on ice, and Aβ_{40} was measured using a human/rat β-amyloid (40) ELISA kit (Wako chemicals) according to the manufacturer's instructions.

Reagents and cDNA Constructs. ALLN (Ac-Leu-Leu-Nle-al), Epoxomicin (Epox), CA-074Me, cathepsin G inhibitor I, cathepsin L inhibitor II, synthetic cell-permeable calpastatin peptide, and scrambled peptide were purchased from Millipore. MDL28170, E-64D, cathepsin L inhibitor III, and proteasome substrate Suc-LLVY-AMC were purchased from Enzo life sciences. MG132, cycloheximide, pepstatin A, and L-685,458 were purchased from Sigma-Aldrich. MK-0822 (Odanacatib) was purchased from ChemieTek. The anti-Myc antibody (9B11) was obtained from Cell Signaling. The anti-APP C-terminal antibody C1/6.1 and anti-β-amyloid (1–16) antibody 6E10 were purchased from Covance. The G12A antibody for APP (rabbit polyclonal, clone C7 targeting amino acid residues 732–751 of APP$_{751}$, custom-manufactured by Thermo Fisher Scientific Inc.) has been previously described.[17] The anti-Talin antibody (TA205) and protease inhibitor cocktail were purchased from Millipore. The mouse anti-β-actin antibody was purchased from Sigma-Aldrich. The anti-BACE1 antibody (PA1–757) was purchased from Thermo Scientific. The APP$_{695}$-Myc construct in pcDNA3.1 was a generous gift from B. T. Hyman at Harvard University.

LDH Cytotoxicity Assay. Conditioned medium was collected after the indicated treatment, and LDH was measured using the Cytotoxicity Detection Kit from Roche Applied Sciences according to the manufacturer's instructions. The 2% Triton X-100 treatment group was used as a 100% cytotoxicity control, and all other conditions were normalized accordingly.

Western Blot Analysis. Cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, and 150 mM NaCl] in the presence of protease inhibitor. The cell lysates were cleared by

DOI: 10.1021/acs.biochem.5b00329
Biochemistry 2015, 54, 2806–2816

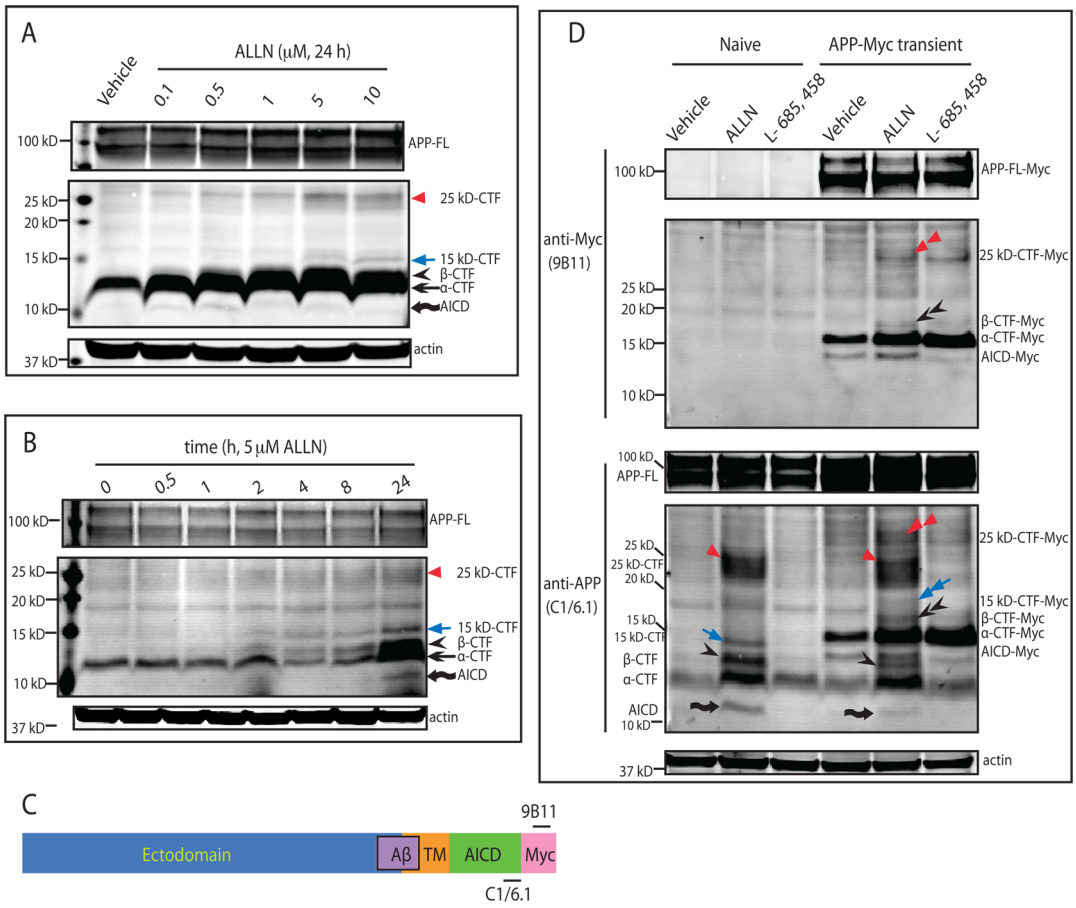


Figure 2. ALLN-induced changes were dependent on dose and time and also observed with exogenous APP. (A) HEK293 cells were treated with different concentrations of ALLN for 24 h before being collected and subjected to a Western blot. (B) HEK293 cells were treated with 5 μM ALLN for different time periods before being collected and subjected to a Western blot. (C) Schematic representation of APP constructs with Myc at the C-terminus. Epitopes of 9B11 and C1/6.1 are also shown here. (D) HEK293 cells were transiently transfected with the APP_{695}-Myc construct. Twenty-four hours post-transfection, cells were treated with 5 μM ALLN, 5 μM L685,458, or vehicle for an additional 24 h before being collected. Naïve HEK293 cells were also treated under the same conditions. The Western blot was probed with anti-Myc antibody 9B11 (top) or C1/6.1 (bottom).

centrifugation. The protein concentration was determined by a BCA assay (Thermo Scientific), and 80−100 μg of protein was used. The cell lysate were heated in Nupage LDS sample buffer (Invitrogen), subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and transferred to PVDF membranes. Membranes were blocked using TBS containing 2% bovine serum albumin (BSA) and incubated with the primary antibody overnight at 4 °C. Blots were washed and incubated with secondary IRDye-conjugated antibody and analyzed with an Odyssey infrared imaging system from LI-COR Biosciences.

***In Vitro* Proteasome Activity Assay.** This assay was described previously.[18] Briefly, after treatment with various proteasome inhibitors for the indicated time, cells were lysed in assay buffer [50 mM HEPES (pH 7.8), 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, and 5 mM DTT]. Lysates were sonicated and centrifuged for 10 min at 4 °C. The supernatant was incubated with proteasome substrate Suc-LLVY-AMC at a final concentration of 100 μM for 1 h at 37 °C with 2 mM ATP. The proteasome activity was then determined by measuring the amount of AMC released using Promega Glomax with a 360 nm/420 nm filter. Fluorescence readings were further normalized to the amount of protein in each sample, determined by BCA assays.

***In Vitro* Cathepsin L Activity Assay.** The cathepsin L activity was measured using the Innozyme cathepsin L acitivity kit (EMD Millipore) and was described previously.[19] Briefly, HEK293 cells were collected in lysis buffer (phosphate-buffered saline containing 1% NP-40, 1 mM EDTA, 5 μg/mL aprotinin, and 0.2 mM phenylmethanesulfonyl fluoride) and diluted in sample buffer to 100 μg/50 μL. Samples were then incubated with activation buffer (containing CA-074) for 15 min at room temperature in the presence of the indicated inhibitor. A cathepsin L inhibitor working solution or the same amount of diluent was then added, and the mixture was incubated for 15 min at room temperature. The substrate working solution was added, and the reaction mixture was incubated for 1 h at 37 °C. The fluorescence was measured using a Promega Glomax multidetection system at an excitation wavelength of 360 nm and an emission wavelength at 460 nm.

Statistical Analysis. Results shown are representative of at least three independent experiments. The standard error is shown, and a Student's t test was used. Values of $p < 0.05$ (one asterisk) and $p < 0.01$ (two asterisks) are considered statistically significant.

DOI: 10.1021/acs.biochem.5b00329
Biochemistry 2015, 54, 2806−2816

RESULTS

Detection of Novel APP Fragments upon Inhibition of Protein Degradation. Cellular protein degradation systems include the ubiquitin/proteasome system, calpain, cathepsins, and other proteases. They are important in maintaining protein quality control. To investigate the role of protein degradation systems in APP metabolism, we utilized ALLN, a general inhibitor of protein degradation.[20−23]

We found that in naïve HEK293 cells, treatment with ALLN led to an accumulation of APP fragments (Figure 1B−D). This accumulation included the APP C-terminal fragments (CTFs), but not the full length APP (FL-APP). Specifically, α-CTF was the only abundant CTF species in the vehicle group and was easily identified by two different APP C-terminal antibodies [C1/6.1 and G12A (Figure 1B,D, black straight arrow)]. β-CTF ran ∼2 kDa higher than α-CTF and was recognized by C1/6.1, G12A, and 6E10, the latter of which recognized the N-terminal sequence of Aβ (Figure 1B−D, black arrowhead). AICD was detected by C1/6.1 and G12A (Figure 1B,D, black curved arrow), but not 6E10, and migrated faster than the α-CTF. The concomitant accumulation of AICD suggested that γ-secretase inhibition was not responsible for the buildup of CTFs upon ALLN treatment. Instead, a common degradation pathway for CTFs and AICD may be affected. This view is supported by previous studies that showed that cathepsins, one of the targets of ALLN, mediate the degradation of both CTFs and AICD.[6,7]

Interestingly, we also observed the appearance of several hitherto undocumented APP fragments, including a 25 kDa CTF (red arrowhead, Figure 1B−D), a 15 kDa CTF (blue arrow, Figure 1B−D), and two less intense fragments migrating at 18 and 20 kDa, which were more visible upon long exposures (orange arrowheads, Figure 1B,D). The novel 25 and 15 kDa CTFs were recognized by C1/6.1, G12A, and 6E10 (Figure 1B−D). These ALLN-induced changes were accompanied by an increase in the level of secreted Aβ_{40}, which has been observed previously[24] (Figure 1E).

We further studied the dose dependence and time course of these changes. As shown in Figure 2A, in naïve HEK293 cells, accumulation of the 25 and 15 kDa CTFs was observed starting at an ALLN concentration of 5 μM treated for 24 h. Using 5 μM ALLN, the accumulation of the 25 and 15 kDa CTFs could be detected starting at 2 h and were prominent at 24 h (Figure 2B).

To further rule out the possibility that these novel CTFs were a result of nonspecific antibody (primary or secondary) binding, we transiently overexpressed the APP$_{695}$-Myc construct in HEK293 cells (Figure 2C) and then treated these cells with ALLN. The C-terminal Myc tag increases the size of exogenous CTFs by approximately 5 kDa. Therefore, if the exogenous APP underwent similar routes of metabolism and responded similarly to ALLN treatment, we would expect the appearance of the 25 kDa CTF-Myc migrating with an apparent molecular mass shift of around 5 kDa. Indeed, we observed the appearance of 30 and 20 kDa APP fragments that were detected by C1/6.1 and anti-Myc antibody (9B11), corresponding to the 25 and 15 kDa novel fragments from endogenous APP, respectively (Figure 2D, double red arrowheads and double blue arrow).

To determine whether this change in APP processing was also present in other cell types, we performed the same experiments in naïve HeLa cells, H4 and SH-SY5Y cells stably overexpressing APP$_{751}$, and bovine brain microvascular endothelial cells. In all of these cells, we observed the accumulation of the 15 kDa (blue arrows) and 25 kDa CTFs (red arrowheads) (Figure 3A) upon ALLN treatment. The conserved APP changes in bovine brain microvascular endothelial cells are especially related to AD, because amyloidosis is not limited to the brain parenchyma, but also

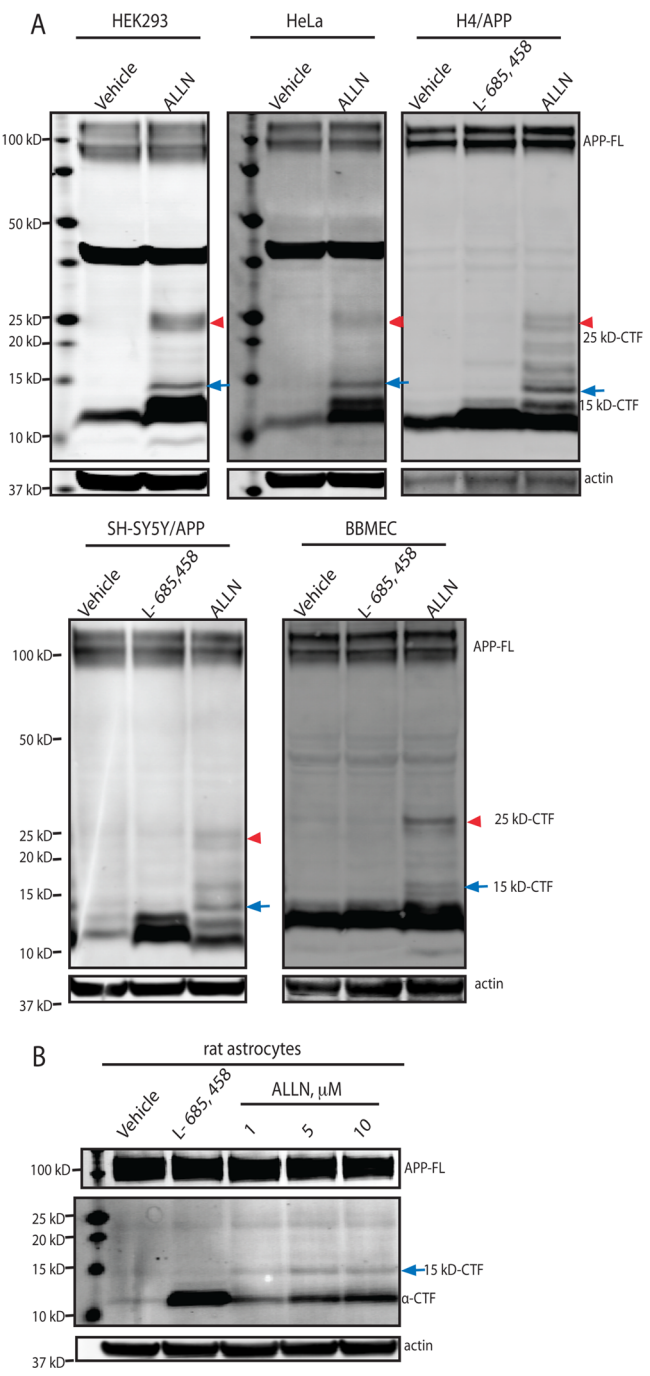


Figure 3. ALLN-induced changes in APP processing were observed across different cell types. (A) Naïve HEK293 cells, naive HeLa cells, H4 cells stably expressing APP$_{751}$, SH-SY5Y cells stably expressing APP$_{751}$, and BBMEC were treated with vehicle, ALLN (1 or 5 μM), or L685,458 (5 μM) for 24 h before being collected. Cell lysates were then subject to a Western blot and probed with C1/6.1 and anti-actin antibody. (B) Primary rat brain astrocytes were treated with ALLN for 24 h before a Western blot.

DOI: 10.1021/acs.biochem.5b00329
Biochemistry 2015, 54, 2806−2816

prominent in the brain vasculature (amyloid angiopathy).[25,26] We also treated primary rat brain astrocytes with ALLN and observed an accumulation of the 15 kDa CTF (blue arrow), but not the 25 kDa CTF (Figure 3B). However, we did not observe the accumulation of novel APP CTFs in rat cortical neurons treated with ALLN (data not shown).

The Accumulation of Novel APP Fragments Is Not Due to Cytotoxicity. Maintaining protein quality control is a key aspect of cellular homeostasis. Inhibiting protein degradation would lead to accumulation of unwanted protein, disrupting normal cellular function and possibly inducing apoptosis.[27] Therefore, it is possible that ALLN-induced accumulation of the novel APP CTFs is due to ALLN-induced cytotoxicity. To test this, we measured the amount of LDH released into the cell medium as a way to assess cytotoxicity. As shown in Figure 4A, there was no significant difference in the amount of LDH release between cells treated with 5 μM ALLN or vehicle for 24 h (p = 0.734). Triton X-100 (2%) treatment served as a positive control and corresponded to 100% cytotoxicity. To test whether the accumulation of novel APP fragments was irreversible, naïve HEK293 cells were treated with 5 μM ALLN for 24 h and then changed into drug-free medium for different periods of time before cell lysates were collected. As shown in Figure 4B, the level of accumulation of novel fragments, particularly the 25 and 15 kDa CTFs (indicated by red and blue arrowheads, respectively), decreased as the time in drug-free medium increased. This suggests ALLN-induced changes were reversible.

The Accumulation of 25 kDa CTF Is Independent of Protein Synthesis. One possibility is that these novel APP fragments come from drug-induced novel transcription products or novel mRNA splice isoforms. If this is the case, the changes we observed should be dependent on protein synthesis. To test this, we treated naïve HEK293 cells with 5 μM ALLN in the presence or absence of cycloheximide (50 μg/mL) for 4 h. As shown in Figure 4C, the level of FL-APP decreased as protein translation was inhibited. The accumulation of novel APP fragments (25 kDa CTF) was still observed, indicating that generation of the 25 kDa CTF was independent of protein synthesis. Therefore, the 25 kDa CTF is unlikely a novel transcription or splicing product of APP mRNA but rather comes from altered protein processing.

Meanwhile, there was no detectable 15 kDa CTF in cells cotreated with ALLN and cycloheximide. This could suggest that the accumulation of the 15 kDa CTF requires protein synthesis. However, it is also possible that the 15 kDa CTF is a proteolytic product of FL-APP, and its absence after cycloheximide treatment is a result of decreased substrate availability.

ALLN-Induced Novel APP CTFs Are Not Ubiquitinated Forms of Canonical CTFs. Because ALLN inhibits proteasome function,[20] and proteasome inhibition leads to buildup of ubiquitinated protein, we speculated that the 25 and 15 kDa CTFs could be ubiquitinated forms of the canonical APP CTFs. Earlier studies have shown that FL-APP undergoes ubiquitin/proteasome-dependent degradation,[8,10,28] and the APP lysine residues that are modified with ubiquitin reside within the CTF region, specifically, K649, K650, and K651.[8−10] Thus, in theory, CTFs can be ubiquitinated. Previous studies have reached the conclusion that APP CTFs undergo degradation through the proteasome, but so far, conclusive evidence demonstrating ubiquitinated CTFs is still lacking.[11−13] Therefore, we set out to determine whether proteasome

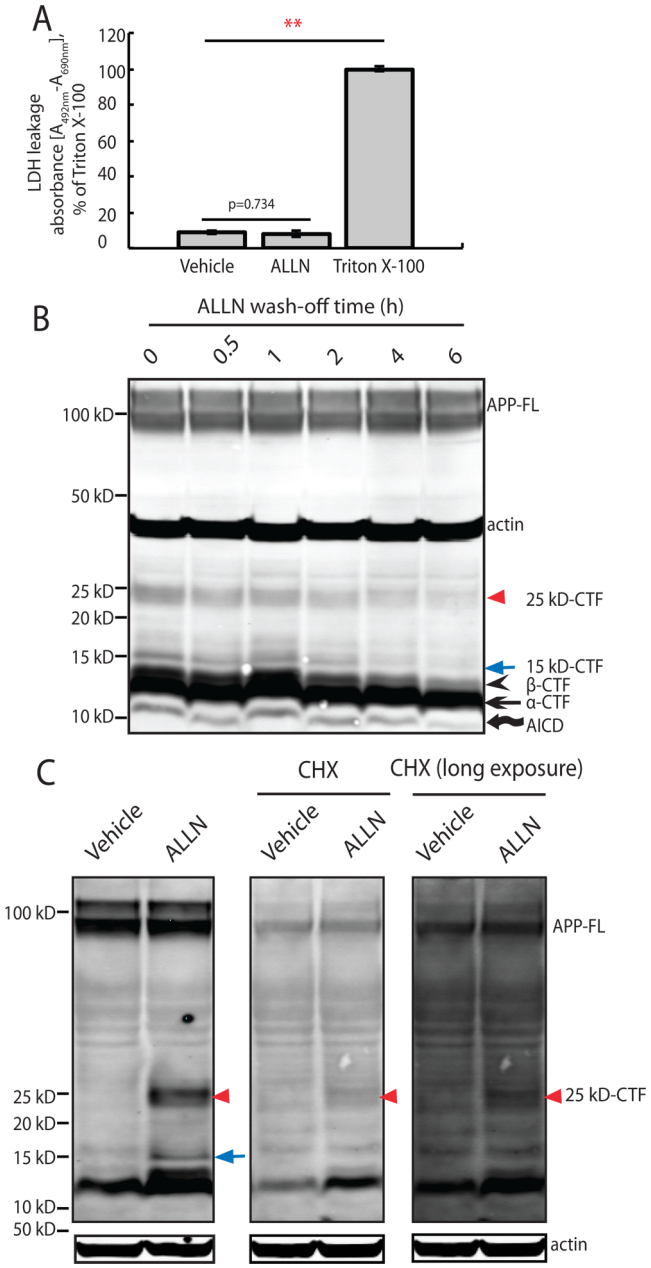


Figure 4. ALLN-induced novel CTF accumulation was independent of cytotoxicity or protein translation. (A) HEK293 cells were treated with vehicle, 5 μM ALLN, or 2% Triton X-100 for 24 h before LDH was measured. (B) HEK293 cells were treated with 5 μM ALLN for 24 h and then washed off for different time periods in drug-free medium before a Western blot. (C) HEK293 cells were treated with vehicle or 5 μM ALLN with or without cycloheximide (50 μg/mL) for 4 h before a Western blot and probed with C1/6.1 and anti-actin antibody.

inhibition was responsible for the accumulation of 15 and 25 kDa APP CTFs.

We used MG132, a commonly used proteasome inhibitor that also inhibits calpain and cathepsin activities.[29] We also used a selective proteasome inhibitor epoxomicin.[30] Inhibition of proteasome activity by MG132 and epoxomicin (Figure 5A, p < 0.01) was confirmed with an *in vitro* 20S proteasome activity assay, as previously described.[18,31] Upon MG132 treatment, we observed accumulation of the 15 and 25 kDa CTFs (Figure 5B). Epoxomicin treatment (1 μM) did not result in the accumulation of novel APP CTFs (Figure 5C).

DOI: 10.1021/acs.biochem.5b00329
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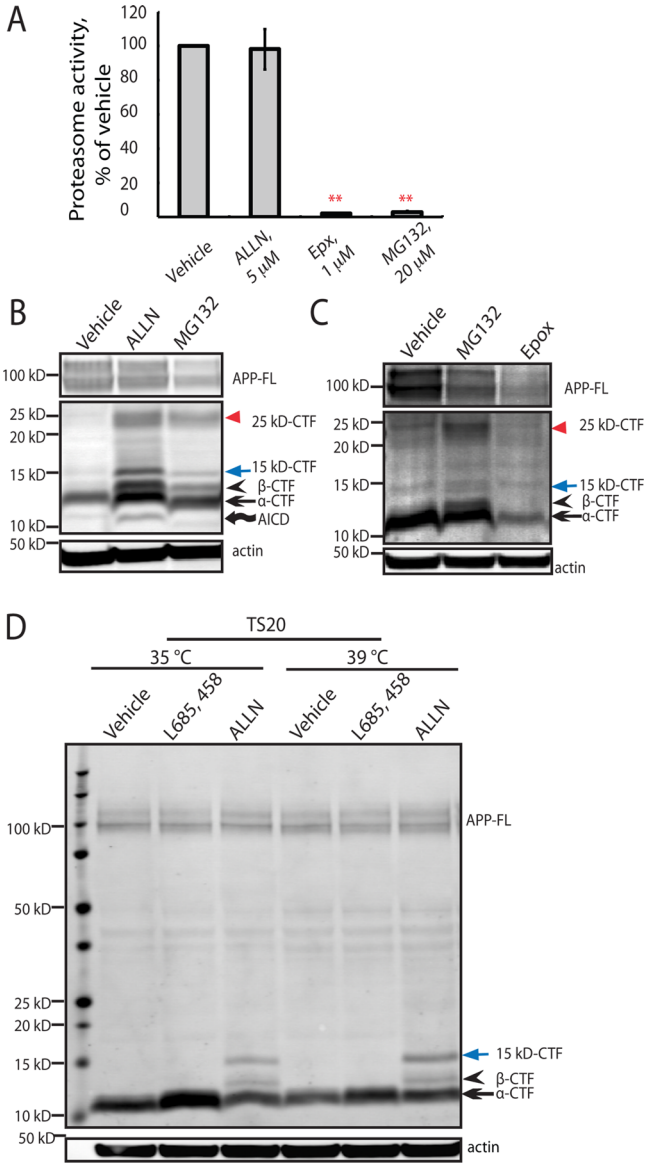

Figure 5. Accumulation of novel APP CTFs was not mediated by proteasome inhibition. (A) *In vitro* 20S proteasome activity was measured after the indicated treatment. (B) Naïve HEK293 cells were treated with vehicle, 5 μM ALLN, or 20 μM MG132 for 6 h before the Western blot and probed with C1/6.1 and anti-actin antibody. (C) HEK293 cells were treated with vehicle, 20 μM MG132, or 1 μM epoxomicin for 6 h before the Western blot and probed with C1/6.1 and anti-actin antibody. (D) TS20 cells were treated with vehicle, 5 μM ALLN, or 5 μM L685,458 at 35 or 39 °C for 6 h before the Western blot and probed with C1/6.1.

This indicates that selective proteasome inhibition does not mediate the accumulation of the novel CTFs, but rather the nonproteasome effects of MG132, possibly cathepsin and/or calpain inhibition.

To confirm this finding, we genetically manipulated the ubiquitin/proteasome system. Specifically, we utilized the mouse fibroblast TS20 cell line, which carries an endogenous mutation in the E1 ubiquitin activating gene, making it temperature-sensitive. At 35 °C, the E1 ubiquitin activating enzyme is functional, but at 39 °C it is inactive, precluding ubiquitination of protein.[16] After treatment of TS20 cells with ALLN at 35 °C, we observed the appearance of the 15 kDa CTF, but not the 25 kDa CTF. This is most likely due to cell type difference. However, this cell line can still be utilized to study the 15 kDa CTF. We reason that if the 15 kDa CTF is ubiquitinated, it should not accumulate if ALLN treatment is performed at 39 °C, when E1 enzyme is inactivated. However, we found that its accumulation was unperturbed at 39 °C (Figure 5D), indicating that the generation of the 15 kDa CTF is independent of ubiquitinaton. Moreover, no novel APP CTFs were observed in cells treated with vehicle at 39 °C, indicating that the degradation of the 15 kDa CTF was also independent of ubiquitination.

Taken together, we conclude that the novel APP CTFs are not proteasome inhibition-induced ubiquitinated CTFs.

The Accumulation of 15 and 25 kDa CTFs Is Not Mediated by Calpain Inhibition, but by Cathepsin Inhibition. MDL28170 is a calpain inhibitor that also cross-reacts with cathepsins.[32,33] Treatment with MDL28170 resulted in the accumulation of both 15 and 25 kDa CTFs, as well as the 18 and 20 kDa CTFs (Figure 6A), suggesting that calpain or cathepsins were potentially responsible for the clearance of novel APP fragments.

To differentiate between these two, we utilized the selective calpain inhibitor, calpastatin peptide (CP, 1 μM).[34] CP is a synthetic cell-permeable peptide corresponding to exon 1B of human calpastatin, an endogenous calpain inhibitor.[35] Efficient calpain inhibition by CP is confirmed by a significant decrease in the level of Talin cleavage, a well-characterized substrate of calpain[36] (Figure 6B,C, $p < 0.05$). However, CP treatment did not result in the accumulation of canonical or novel APP CTFs (Figure 6B). This suggests that selective calpain inhibition is not responsible for the accumulation of novel CTFs.

To investigate the role of cathepsins in the accumulation of these novel CTFs, we utilized a general cathepsin inhibitor, E-64D.[37] Treatment of naïve HEK293 cells with E-64D resulted in the accumulation of the 25 kDa CTF, 15 kDa CTF, and canonical CTFs (Figure 6D).

Cathepsins make up a large protease family and are classified into three categories based on their catalytic types and structures, including aspartyl (D and E), serine (A and G), and cysteine cathepsins (B, C, F, H, K, L, O, S, V, W, and Z).[38] In an effort to narrow down the cathepsin responsible for the clearance of the novel CTFs, we used selective cathepsin inhibitors that are currently available, including pepstatin A for cathepsin D, cathepsin G inhibitor I for cathepsin G,[7] CA-074Me for cathepsin B,[39] MK-0822 (Odanacatib) for cathepsin K,[40] and cathepsin L inhibitors II and III for cathepsin L.[41] Treatments of naïve HEK293 cells with cathepsin L inhibitor II or III induced the accumulation of novel CTFs (Figure 6G,H). However, this was not observed with any of the other selective cathepsin inhibitors (Figure 6E−G). Inhibition of cathepsin L (ALLN, cathepsin L inhibitor II and III) was confirmed using an *in vitro* cathepsin L activity assay (Figure 6I). These data suggest that the clearance of novel CTFs is mediated by cathepsin L, not by cathepsin B, D, G, or K.

BACE Accumulation Did Not Mediate the Generation of the 25 or 15 kDa CTF. The pharmacologically induced accumulation of the 15 and 25 kDa CTFs was always accompanied by an increase in the level of β-CTF (ALLN and MG132 in Figure 5A, MDL28170 in Figure 6A, E-64D in Figure 6D, and cathepsin L inhibitors II and III in Figure 6G,H). BACE is the major β-secretase that cleaves FL-APP and generates β-CTF.[1] BACE is known to undergo degradation via lysosomal[42] and proteasomal pathways.[23] We speculated the

DOI: 10.1021/acs.biochem.5b00329
Biochemistry 2015, 54, 2806−2816

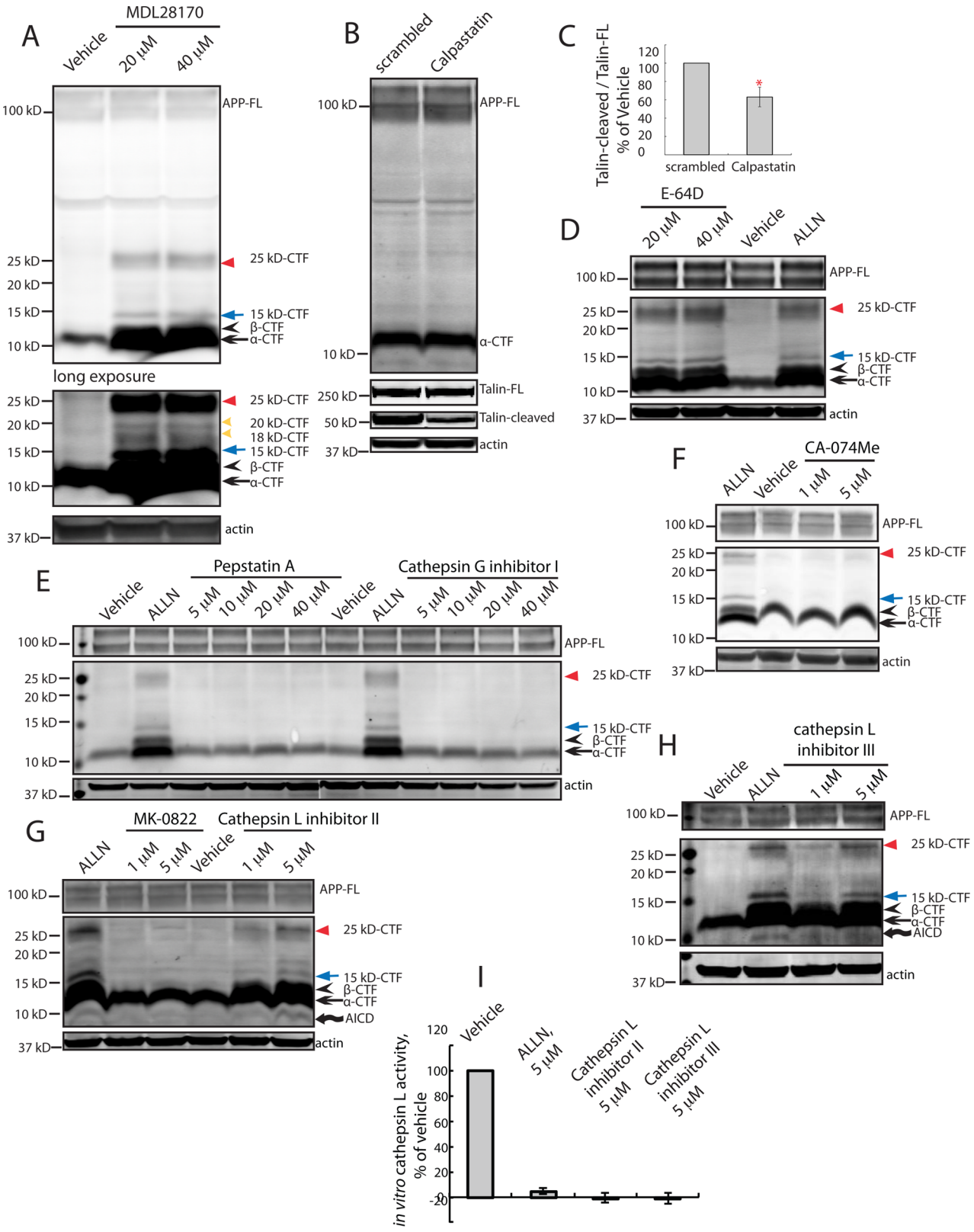

Figure 6. Accumulation of novel APP CTFs was mediated by cathepsin L inhibition. (A) Naïve HEK293 cells were treated with vehicle or MDL28170 (20 or 40 μM) for 24 h before being collected. The Western blot was probed with C1/6.1 and anti-actin antibody. (B) Naïve HEK293 cells were treated with calpastatin peptide (CP, 1 μM) or scrambled peptide (SP, 1 μM) for 24 h before being collected. Cell lysates were subjected to a Western blot and probed with C1/6.1, anti-actin antibody, and anti-Talin antibody TA205. (C) CP induced a significant decrease in the amount of Talin-cleaved product ($p < 0.05$). (D) HEK293 cells were treated with E-64D for 24 h before the Western blot and probed with C1/6.1 and anti-actin antibody. HEK293 cells were treated with selective cathepsin inhibitor as indicated, and Western blots probed with C1/6.1 and anti-actin antibody are shown. (E) Pepstatin A and cathepsin G inhibitor I. (F) CA-074Me. (G) Cathepsin L inhibitor II and MK-0822. (H) Cathepsin L inhibitor III. (I) Inhibition of cathepsin L acitivity is confirmed with an *in vitro* cathepsin L activity assay.

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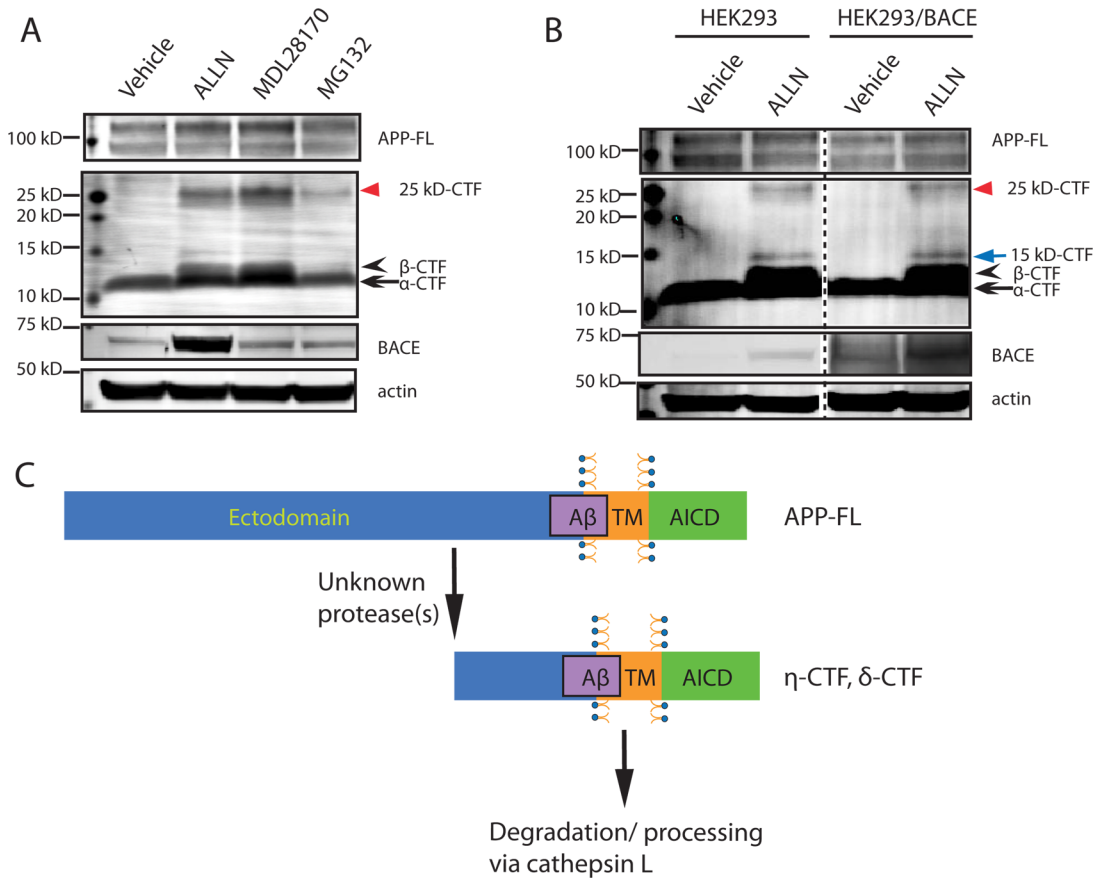

Figure 7. 25 kDa CTF was not generated by an increased amount of BACE. (A) Naïve HEK293 cells were treated with ALLN, MDL28170, or MG132 for 24 h before being collected and subjected to a Western blot. Lysates were probed with C1/6.1 (APP), anti-actin antibody, and anti-BACE antibody. (B) Naïve HEK293 cells or HEK293 cells transiently overexpressing BACE were treated with ALLN or vehicle for 24 h before the Western blot. (C) Schematic representation of our model. Under physiological conditions, APP undergoes proteolysis into *η*-CTF and *δ*-CTF, but these two products undergo rapid clearance/processing via cathepsin L.

inhibition of lysosomal and/or proteasomal pathways that lead to BACE accumulation could result in noncanonical cleavage of FL-APP by BACE to generate the novel 15 and 25 kDa CTFs.

In naïve HEK293 cells, endogenous BACE levels were consistently increased upon ALLN treatment (Figure 7A). However, we did not observe BACE accumulation upon treatment with MG132 or MDL28170 (Figure 7A), both shown to lead to accumulation of novel CTFs. Transient overexpression of BACE (Figure 7B) did not directly result in detectable levels of 15 or 25 kDa CTFs and did not alter the amount of novel CTFs upon ALLN treatment (compared to that in naïve cells treated with ALLN). Together, these data suggest that elevated BACE levels are not responsible for the generation or degradation of the novel 15 or 25 kDa CTFs.

DISCUSSION

In this study, by using a general protein degradation inhibitor, ALLN, we observed an accumulation of novel APP fragments, 15 and 25 kDa CTFs. This process was independent of protein translation and was not associated with cytotoxicity. We showed that the accumulation of these novel APP CTFs was not mediated by proteasome or calpain inhibition, but by cathepsin inhibition, specifically, cathepsin L. Lastly, we showed increased BACE levels do not play a role in the generation or degradation of the 15 and 25 kDa CTFs.

The 15 kDa CTF observed in our study is likely the *δ*-CTF, reported previously in hippocampal neurons with 12 additional residues from the N-terminus of CTFβ[5] (Figure 1A). This is the first evidence that the *δ*-CTFs are also present in other cell types. The 18, 20, or 25 kDa CTFs (Figure 1B,D) may be the same CTFs observed by Haas and colleagues upon leupeptin treatment of HEK293 cells stably overexpressing APP_{751}.[43] We refer to the 25 kDa CTF as *η*-CTF (eta-CTF), and this fragment could contain approximately 230 amino acids of the C-terminus of APP_{751}. It is also important to note that the *η*-CTF could potentially be a cluster of two or more fragments close in size, because in some of our Western blots, two fragments were visible. Attempts to immunoprecipitate the novel fragments for sequencing were not fruitful, because of the following factors. First, the *η*-CTF migrates at the same molecular mass as the immunoglobulin light chain via SDS–PAGE. Second, current commercially available APP C-terminal antibodies recognize the *δ*- and *η*-CTFs, as well as the canonical CTFs and FL-APP that are more abundant, making the enrichment process significantly less efficient for the novel CTFs. Future studies can improve the efficiency of enrichment by using custom antibodies targeting the predicted N-terminal sequence of the novel CTFs.

Our data suggest that the novel CTFs induced by ALLN are proteolytic products of APP, which were not observed under physiological conditions, because of efficient clearance/processing by protein degradation machinery via cathepsin L (Figure 7C).

DOI: 10.1021/acs.biochem.5b00329
Biochemistry 2015, 54, 2806–2816

Aging is a strong risk factor for AD, and protein degradation systems, especially cathepsins, are impaired in aging brains.[44,45] Deficits in protein degradation could serve as a link between aging and neurodegeneration.[46−48] Several lines of evidence have suggested a role of lysosome malfunction in AD pathogenesis. For example, autophagic vacuoles (AV), an early component of the lysosome degradation process, have been found to accumulate extensively in AD brains, suggesting impaired maturation of the lysosome degradation pathway.[49] These changes occur before extracellular plaque formation, and the AV are found to be rich in Aβ, along with β-CTF and γ-secretase components, consistent with a role in Aβ production.[49,50] Interestingly, Glabe and colleague have shown that internalized Aβ_{42} is largely resistant to degradation in the lysosmes.[51] They further demonstrated in HEK293 stably overexpressing APP that intracellular Aβ_{42} can lead to altered APP catabolism and accumulation of a series of APP CTFs in size from 16 to 43 kDa.[52] These connections among aging, Aβ_{42}, lysosome dysfunction, and altered APP metabolism make it intriguing to speculate whether the clearance of η- and δ-CTFs could be impaired in AD and play a potential role in the course of neurodegeneration.

Moreover, the functions of cathepsins are not limited to protein degradation but also include the processing of certain substrates, such as antigen unloading.[38,53] Using selective cathepsin inhibitors, our data in the HEK293 cell model suggest that the clearance of α-CTF is mediated by both cathepsin B (Figure 6F)[7] and cathepsin L (Figure 6G,H), while the clearance of η- and δ-CTFs is selectively mediated by cathepsin L (Figure 6G,H). To the best of our knowledge, this is the first evidence that different CTFs are selectively degraded by specific lysosomal cathepsins, and this selectivity suggests a biological function is associated with the process.

It is also worth noting that, although we have ruled out the possibility that elevated BACE levels mediate the generation of η- or δ-CTF, it is still possible that inhibition of protein degradation leads to the buildup of other protease(s), and that these novel CTFs are novel products of processing of APP by those protease(s).

Our findings warrant follow-up studies to determine the protein sequence of η-CTF, and whether η- and δ-CTFs can be found in aging brain. Future directions should include determining the putative secretases that generate these fragments. It would also be interesting to investigate whether η- and δ-CTFs are subject to cleavage by α- and/or β-secretase. If so, another interesting question is the relationship between the cleavage products and amyloid oligomers.

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Funding

This study is supported by National Institutes of Health Grant R01 NS057295, Drexel University, and the Commonwealth of Pennsylvania.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Mike Akins, Dr. Michal Sharoni, and Ezekiel Crenshaw (Drexel University) for their expertise and helpful discussion. We also thank Dr. Mikko Hiltunen (University of Eastern of Finland), Dr. Bradley T. Hyman (Massachusetts General Hospital/Harvard University), and Dr. Alisa Clyne (Drexel University) for kindly sharing their reagents.

ABBREVIATIONS

AD, Alzheimer's disease; ALLN, Ac-Leu-Leu-Nle-al; APP, amyloid precursor protein; CTF, C-terminal fragment; UPS, ubiquitin/proteasome system.

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