Calpain activity regulates the cell surface distribution of amyloid precursor protein: inhibition of calpains enhances endosomal generation of C-terminal APP fragments.

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running title: redistribution of APP with calpain inhibitors
Summary

In murine L cells, treatment with calpeptin or calpain inhibitor III increased Aβ42 secretion, but not Aβ40, in a dose-dependent fashion. This correlated with an increase in the levels of amyloid precursor protein (APP) carboxy-terminal fragments (CTFs). Immunoprecipitation with novel mAbs directed against the carboxy-terminus of APP or specific for the β-cleaved CTF showed that generation of both α- and β-cleaved CTFs increase proportionately following inhibition of calpains. Pulse-chase metabolic labeling confirmed that inhibiting calpains increases the production of α- and β-cleaved APP metabolites. Immunolabeling showed greater βCTF signal in calpeptin-treated cells, primarily in small vesicular compartments that were shown to be predominantly endosomal by colocalization with early endosomal antigen 1. A second mAb, which recognizes an extracellular/lumenal epitope found on both APP and βCTFs, gave more cell surface labeling of calpeptin-treated cells than control cells. Quantitative binding of this antibody confirmed that inhibiting calpains caused a partial redistribution of APP to the cell surface. These results demonstrate that (1) calpain inhibition results in a partial redistribution of APP to the cell surface, (2) this redistribution leads to an increase in both α- and β-cleavage without changing the ratio of αCTFs/βCTFs, and (3) the bulk of the βCTFs in the cell are within early endosomes, confirming the importance of this compartment in APP processing.
Introduction

The deposition of the small – approximately 40 to 42 residues – Aβ peptide as insoluble β-amyloid plaque in the brain parenchyma is an invariant feature of Alzheimer’s disease. Aβ is generated by proteolysis of the ~100 kDa amyloid precursor protein (APP), a broadly expressed type-1 transmembrane protein that is found primarily in the trans-Golgi network (TGN) and at the cell surface (reviewed in (1)). The β-cleavage of APP occurs within the lumenal/extracellular domain of APP and generates two APP fragments: a large, soluble amino-terminal fragment (sAPPβ) that is secreted from the cell, and a transmembrane, carboxy-terminal fragment (CTF) that remains associated with the cell. This βCTF consists of 99 amino acids, contains the whole Aβ peptide, and has a molecular weight of ~10 kDa. An alternative pathway involves the cleavage of APP sixteen residues downstream of this site at the α-cleavage site. Like β-cleavage, α-cleavage generates a sAPP fragment (sAPPα) that is secreted from the cell and an αCTF (of 84 residues and ~8 kDa) that remains membrane associated. α-cleavage occurs within the Aβ peptide sequence, and as such prevents the generation of Aβ from a given APP molecule. Aβ is generated from the βCTF by an intramembrane cleavage (γ-cleavage) that occurs primarily at 40, and to a lesser extent 42, residues downstream from the β-cleavage site, releasing Aβ1-40 or Aβ1-42.

Much progress has been made recently in identifying the major proteases/protease complexes responsible for α-, β- and γ-cleavage (the α-, β- and γ-secretases). α-cleavage appears to be due to the activity of two metalloproteases (TACE and ADAM10) and is thought to occur primarily at the cell surface (2-7). The BACE proteases, a recently identified family of transmembrane aspartyl proteases, appear to account for much of the β-secretase activity within normal cells (8,9). BACE has an
endosomal-lysosomal pattern of distribution as well as an acidic pH optimum (9-11); BACE-mediated cleavage of APP in the endocytic system is consistent with prior work that has identified, through various trafficking mutants of APP, the early endosome as an important site for Aβ generation (12-14) and as a compartment showing functional alterations at the earliest stages of AD (15). The presenilin (PS) proteins play an intimate role in γ-cleavage: expression of familial AD-causing mutant presenilin increases the production of Aβ terminating at residue 42 (16-18), the PS-null phenotype includes the inability of the cell to generate Aβ and the intracellular accumulation of CTFs (19,20), and recent work has directly implicated PS itself as the γ-secretase (21), although other proteins within the PS complex such as nicastrin (22) may well be directly involved in γ-cleavage.

The calpain system consists of two Ca^{2+}-activated, cytosolic, neutral pH, cysteine proteases, which are typically distinguished by their different affinities for Ca^{2+} in vitro (m-calpain and μ-calpain), as well as an endogenous inhibitor, calpastatin (23-25). Calpains play a central role in cytoskeleton remodeling (26), have been suggested to play a regulatory role in some vesicular transport events (27), and are the primary mediators of cell death following an unregulated increase in intracellular Ca^{2+} concentration, as occurs with excitotoxicity (28,29). The calpain system appears to be upregulated during AD, with evidence that both m-calpain and μ-calpain are abnormally activated in neurons (30-32). These changes in the calpain system are most often thought to impact tau metabolism, due in part to their known roles in tau turnover (33,34) and regulation of kinases thought to be involved in tau hyperphosphorylation and proteolysis (32,33,35).

Calpain activity may also modulate Aβ production. Inhibiting calpain activity increases Aβ production by cells in culture, with Aβ42 increasing more than Aβ40 (36-39). In addition, calpain inhibitors have been shown to increase the levels of CTFs in cells (39,40). Zhang et al. (39) examined this effect in cells overexpressing an APP βCTF construct and concluded that the increase in CTFs was
due to their stabilization, but the mechanism(s) underlying such an increase in CTFs derived from APP holoprotein has yet to be examined in detail.

To this end, we have examined the metabolism and intracellular distribution of APP in cells following treatment with calpain inhibitors. Our results confirm that calpain inhibitors greatly increase Aβ42 generation, while having a smaller effect on Aβ40. We found that inhibiting calpains resulted in a substantial and proportionate increase in the generation of both α- and β-cleaved CTFs and that this increase in the production of CTFs is likely to be the result of a partial redistribution of APP from the TGN to the plasma membrane. Using a monoclonal antibody that specifically recognizes the βCTF, we show that βCTFs colocalize with a marker of the early endosome, both without treatment and following calpain inhibitor treatment. These data suggest a model in which inhibiting the calpain system drives a greater proportion of APP to the cell surface and early endosomes, where α-cleavage and β-cleavage, respectively, can then occur. The additional βCTFs generated are then substrate for γ-secretase activity, further linking the generation of this highly pathogenic species of Aβ to the early endosome.
Methods

Cell lines, cDNA constructs, and transfections. Ltk- cells (a murine fibroblast-like cell line; (41)) were maintained at 37°C and 5% CO₂ in high glucose DMEM (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (Gibco/BRL, Grand City, NY), 2mM glutamax I (Gibco/BRL), and penicillin/streptomycin (Cellgro). cDNAs encoding human APP_{695} (42) or PS1 with the P_{117}L mutation (43) were inserted into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). Following transfection using lipofectin (manufacturer’s protocol; Gibco/BRL), stable L cell lines were selected in 400µg/ml G418 (Gibco/BRL) and screened for expression (42,44). N2a (17) and IMR-32 (45) neuroblastomas were obtained from the American Type Culture Collection (Gaithersburg, MD), and maintained as recommended by the ATCC.

Antibodies. Table I describes the six monoclonal antibodies directed against APP and/or APP metabolites used in this study; Figure 1 is a cartoon showing the use of these antibodies to detect APP metabolites. C1/6.1 was raised against the conserved carboxy-terminal 20 residues of APP (residues 676-695 of APP_{695}), and is useful for immunolabeling, immunoprecipitation, and Western blot analysis (42). JRF/AβN/25 was raised against a synthetic peptide encompassing residues 1 to 7 of human Aβ. Evidence that JRF/AβN/25 requires β-cleavage at residue 1 of Aβ has been presented by Vandermeeren et al. (46) and is substantiated by this study. JRF/Aβtot/17 was raised against human Aβ1-40; mapping of its epitope against synthetic peptides has demonstrated that JRF/Aβtot/17 binds within residues 1 to 15 and is specific for human Aβ. JRF/rAβ1-15/2 was raised against a synthetic peptide encompassing residues 1 to 15 of murine Aβ, and does not recognize human Aβ or human APP (42,46). JRF/cAβ40/10 was raised against the carboxy-terminal 5 residues of Aβ1-40; and JRF/cAβ42/26, the carboxy-terminal 10 residues of Aβ1-42. These Aβ carboxy-terminal antibodies, their specificity and their use in our sandwich ELISA has been recently described (42,47). The anti-APP-lumenal domain
monoclonal antibody P2-1 was the generous gift of Dr. Maria Kounnas at Sibia (48); monoclonal antibody 6E10 was purchased from Senetek. Purified anti-early endosomal antigen 1 (EEA1) rabbit serum was the kind gift of Dr. S. Corvera of the Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA (49,50).

**Sandwich ELISAs.** Sandwich ELISAs using these antibodies were done essentially as previously reported (42,47,51), with modifications to detect either human or murine Aβ secreted into the growth media. Nunc-Immuno Plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C using 10 μg/ml of either JRF/cAβ40/10 in 10mM Tris, 10mM NaCl, pH 8.5 or JRF/cAβ42/26 in 100mM bicarbonate buffer, pH 9.6 and remaining protein binding sites blocked by incubating with 1% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) in PBS for 4 hours at room temperature. Cells were seeded onto 6-well dishes and allowed to settle overnight. Human APP or PS1P117L expression in L cells was induced with 20 mM butyrate for ~40 hours (41) while neuroblastoma lines were allowed to grow to near confluence. Growth media was then replaced with 0.8 ml fresh media containing calpeptin or calpain inhibitor III (Calbiochem, San Diego, CA), and, for transfected L cells, butyrate. After 6 hours, this conditioned media was collected, HEPES (pH 7.4) added to 25mM, and loaded neat into duplicate wells for ELISA. Human and murine Aβ1-40 and Aβ1-42 peptide standards were purchased from American Peptide Co. (Sunnyvale, CA), stock solutions stored at -70°C, and further diluted immediately prior to use. ELISA plates were incubated overnight at 4°C with samples and standards. Aβ was detected by incubating for 4 hours at room temperature with HRP-conjugated JRF/Aβtot/17 (human Aβ) or JRF/rAβ1-15/2 (murine Aβ) diluted in 20 mM Na phosphate, 2 mM EDTA, 400 mM NaCl, 1% BSA pH 7.0. ELISA plates were developed using a color reaction (TMB Microwell Peroxidase Substrate System, Kirkegaard & Perry, Gaithersburg, MD) and the OD<sub>450</sub> read. ELISA signals are reported as the mean ± SE of two or more wells in femtomoles Aβ per ml of media.
Metabolic labeling, immunoprecipitation and Western blot analysis. To detect APP and CTFs, 5 \( \times 10^5 \) cells were seeded onto 35-mm diameter tissue culture dishes followed by induction with 20 mM butyrate for 24 hours (41). Cultures were methionine/cysteine starved for 20 min., pulse-labeled for 15 min. with 100 \( \mu \text{Ci/ml} \) TRANS\(^{35}\)S-LABEL (DuPont-NEN, Boston, MA), washed, and chased in complete media containing 2 mM unlabeled methionine (41). Calpain inhibitors, as described in Results, were added to the growth media 3 hours prior to methionine/cysteine starvation and then throughout pulse-labeling and chase. Cell lysates (prepared in 1% Triton X-100, 140 mM NaCl, 25 mM Tris pH 7.4, 0.5 mM EDTA, 10 mM methionine, and protease inhibitors; (41,52)) were subjected to immunoprecipitation with one of several monoclonal antibodies as described in Results. Sequential immunoprecipitation of the growth media to isolate metabolically labeled sAPP\(\alpha\) and sAPP\(\beta\) has been described (42). Immunoprecipitated proteins were sized by SDS-PAGE and labeled proteins visualized by exposure to x-ray film and analyzed quantitatively using a Storm 840 phosphorimager (Molecular Dynamics, Sunnyvale, CA) and/or by scan analysis (NIH Image).

For Western blot analysis, protein concentration in cell lysates was determined (BioRad DC Protein Assay, BioRad, Hercules, CA), equal amounts of proteins were separated by SDS-PAGE gels and transferred to PVDF membrane. Membranes were incubated in C1/6.1 (2 \( \mu \text{g/ml} \)) overnight, washed, and incubated with HRP-conjugated goat anti-mouse IgG for 1.5 hours. Membranes were washed and ECL substrate (Amersham, Piscataway, NJ) added before exposure to x-ray film.

Quantitative \(^{125}\)I-mAb binding. Sodium \(^{125}\)iodide was purchased from NEN (Boston, MA). Monoclonal antibodies JRF/A\(\beta\)tot/17 and JRF/A\(\beta\)N/25 were iodinated using iodogen tubes (Pierce, Rockford, IL; manufacturer’s protocol) and \(^{125}\)I-mAb recovered (41). APP overexpressing L cells grown in 6-well dishes were butyrate induced for ~40 hours as described above, fixed at room temperature (as for immunolabeling) and incubated for 2 hours with \(10^6\) cpm \(^{125}\)I-mAb (adjusted to 2
μg/ml total mAb with unlabeled mAb) in Hank’s BSS, 10% horse serum, 20 mM HEPES (pH 7.4) as previously described (41). Following washes, bound \(^{125}\text{I}\)-mAb was recovered in 2 ml 1N NaOH and counted in a gamma counter. 0.1% saponin (wt/vol; Sigma) was added to the binding solution to permeabilize cells. Non-specific binding was estimated using identical cultures of nontransfected L cells and a 50-fold excess of unlabeled mAb in the binding solution; all data points represent non-specific binding subtracted from specific binding, each the mean of triplicate measurements ± SE.

**Immunolabeling.** For indirect immunofluorescence labeling, L cells were seeded onto glass coverslips and expression of APP induced. Cells were fixed in 4% paraformaldehyde, 5% sucrose in PBS (pH 7.4) at room temperature for 15 min, and immunolabeled as previously described (41). Fluorescein- and Texas Red- coupled secondary antibodies were purchased from Cappel (Durham, NC). Cells were permeabilized by the addition of 0.1% saponin to the antibody-containing solutions. Cells were mounted in Vectashield (Vector Labs, Burlingame, CA) and examined by epifluorescence and laser confocal microscopy.
Results

*Calpain inhibitors increase Aβ42 secretion selectively while increasing CTF levels.* Initially, we sought to confirm previous findings (36-38), which have showed that inhibition of calpains predominantly affected Aβ by increasing Aβ42 production, using a murine fibroblast-like line (L cells) as well as murine and human neuroblastoma lines (Table II). By sandwich ELISA, we determined the amount of Aβ40 and Aβ42 secreted into the growth media over a six hour period by control cells or by cells treated with the calpain inhibitor calpeptin. In L cells, calpeptin treatment consistently increased the amount of secreted Aβ42, typically 2- to 3-fold over that secreted by control cells, while increasing Aβ40 secretion less than 1.5-fold. In an L cell line expressing an FAD-mutant PS1 (L/PS1P117L; (43)), again we found that Aβ42 increased more than Aβ40, although the initial levels of Aβ42 were higher due to expression of the mutant PS1 (see Fig. 2). When we examined Aβ secretion in an L cell line expressing human APP695 (L/APP; (42)) using an ELISA specific for human Aβ, we obtained similar results, with Aβ40 essentially unchanged and Aβ42 again increased 3.5-fold by calpeptin treatment. Similar increases with calpeptin treatment were also seen in the murine neuroblastoma cell line N2a and the human hippocampal neuroblastoma line IMR-32 (45), with Aβ42 increasing approximately 3-fold and Aβ40 remaining nearly unchanged. These data emphasize that calpain inhibition with calpeptin has a much great effect on Aβ42 than on Aβ40 and, importantly, that this effect is seen in non-neuronal as well as neuron-like cells.

Previous reports have shown that, in addition to increasing Aβ42 production, inhibiting calpains increases the steady-state level of APP CTFs detected within cells (39,40). In the experiment shown in Figure 2, we examined the interrelationship between Aβ production and the steady-state CTF levels following calpain inhibition in L cells. Panel A shows ELISA data for Aβ generated from the endogenous murine APP in L cells and in L cells expressing a mutant presenilin 1 (PS1P117L; (43)) at
increasing concentrations of calpain inhibitor III. Aβ40 levels in the parental and the PS1<sub>P117L</sub> expressing L cells were found to be similar and did not increase significantly with calpain inhibitor III at any concentration. Baseline Aβ42 production was greater in the mutant PS1 expressing L cells. Five to 25 µM calpain inhibitor III treatment resulted in a nearly parallel increase in Aβ42 produced by either the control or PS1<sub>P117L</sub> expressing L cells. Both Aβ40 and Aβ42 levels were found to dramatically decrease to below detectibility at the highest calpain inhibitor III concentrations (100 and 250 µM). Additional experiments, including pulse-metabolic labeling and immunoprecipitation of APP, demonstrated that this was the result of calpain inhibitor III blocking protein biosynthesis at these high concentrations (data not shown). This is consistent with the observations of Zhang et al. (39) that high concentrations of calpain inhibitors decreased both Aβ40 and Aβ42 generation and may explain an apparent decrease in CTF degradation at these toxic concentrations.

We initially examined the relationship between CTF levels and calpain inhibition by Western blot analysis using the monoclonal antibody C1/6.1 (42), which was raised against the carboxy-terminal 20 amino acids of APP and recognizes the holoprotein as well as multiple CTFs (Fig. 2B). Quantitation of this Western blot demonstrates a substantial increase in CTF levels with increasing calpain inhibitor III concentrations. At the highest concentration, however, both APP holoprotein, and to a lesser extent, CTFs decline precipitously, which is consistent with these high doses of the inhibitor blocking protein biosynthesis. These results demonstrate that CTF levels and Aβ42 production following calpain inhibition correlate strongly.

*Calpain inhibitors increase the generation of both α- and β-cleaved APP metabolites.* To determine what species of CTFs were appearing following calpain inhibitor treatment, we immunoprecipitated APP holoprotein and CTFs using a set of monoclonal antibodies that recognize different epitopes within the carboxy-terminal 99 residues of APP (see Table I). In addition to the
monoclonal antibody C1/6.1, we used two monoclonal antibodies raised against Aβ epitopes: 

JRF/AβN/25, which was raised against a peptide consisting of the amino-terminal 7 residues of Aβ, and 

JRF/Aβtot/17, which was raised against Aβ1-40 and has been shown by peptide-epitope mapping to recognize a sequence contained within residues 1-16 of human Aβ. In the experiment shown in Figure 3, L cells overexpressing human APP_{695} (L/APP cells) were metabolically labeled for 15 min. followed by a chase for 1 hour. Cells were treated for 3 hours prior to as well as during the pulse and chase periods with the indicated calpain inhibitors. Equal volumes of detergent lysates prepared from the pulse and chase periods were subjected to immunoprecipitation with each monoclonal antibody and labeled APP species were resolved by 4-20% gradient SDS-PAGE. In untreated L/APP cells (lanes 1-6), C1/6.1 immunoprecipitated labeled holoprotein from the pulse period (lane 1). Following 1 hour chase, C1/6.1 immunoprecipitation also revealed two rapidly migrating APP fragments that migrated at ~10 and 8 kDa, consistent in size with the αCTF and βCTF (lane 4; arrows indicating αCTF and βCTF). JRF/AβN/25 immunoprecipitation failed to bring down APP holoprotein (lanes 2 and 5), confirming peptide mapping data (not shown) that the epitope for JRF/AβN/25 requires a cleaved amino-terminus at residue 1 of Aβ. That JRF/AβN/25 immunoprecipitated, following the 1 hour chase, a protein that co-migrated with one of the CTFs revealed by C1/6.1 immunoprecipitation (compare lanes 4 and 5), conclusively identifies this as the β-cleaved CTF of APP. JRF/Aβtot/17 immunoprecipitated APP holoprotein in the pulse and chase periods (lanes 3 and 6 respectively), as well as the βCTF following chase. Given the specificities of these three monoclonal antibodies and the mobility on SDS-PAGE of the CTFs they immunoprecipitate, we conclude that the most rapidly migrating species identified by C1/6.1 is the α-cleaved CTF.

In cells treated with either 10 µM calpeptin or 10 µM calpain inhibitor III, the immunoprecipitation pattern from the pulse-labeled lysates was similar to that seen from untreated cells
(compare lanes 1-3 with 7-10 and 13-15), although calpain inhibitor III treatment increased the relative signal from a number of bands detected by C1/6.1 and JRF/Aβtot/17 that migrated more rapidly than APP holoprotein but more slowly than the CTFs. With calpain inhibition, striking differences in the abundance of CTFs, however, were seen following 1 hour chase. C1/6.1 immunoprecipitation demonstrated a substantial and apparently proportionate increase in the levels of both αCTFs and βCTFs with either calpain inhibitor (compare lane 4 to lanes 10 and 16). This increase in the βCTF was confirmed by immunoprecipitation with JRF/AβN/25 and JRF/Aβtot/17 (lanes 11, 12, 17 and 18). Densitometric quantitation of these bands from the C1/6.1 immunoprecipitation confirmed our observation that the ratio of αCTF to βCTF appears not to be affected by calpain inhibition (remaining at ~2:1) in spite of an approximate 4-fold increase in both CTFs. Finally, it should be noted that the APP holoprotein contains 5 times more methionines than do the CTFs, so that metabolic labeling substantially underestimates the relative abundance of the CTFs.

The 1 hour chase shown in Figure 3 suggests that calpain inhibition increases the production of CTFs, although a slowing of their turnover might have the same effect. We directly addressed the generation and turnover of the CTFs as well as the production of sAPP in the pulse-chase experiment shown in Figures 4 and 5. In Figure 4, L/APP cells were pulse-labeled for 15 min. and chased for the indicated times up to 6 hours prior to immunoprecipitation of lysates with C1/6.1. In Figure 4A, the top panel shows that the turnover of APP holoprotein in control (lanes 1-6) and 10 μM calpeptin-treated cells (lanes 7-12) is similar. The bottom panel is a longer exposure of the same immunoprecipitation showing the generation and turnover of the CTFs. In agreement with the data in Figure 3, 10 μM calpeptin treatment substantially increased the generation of both α and β CTFs during the initial 1 hour of chase (compare lanes 1-3 with 7-9); in contrast to a previous interpretation (39), however, this calpeptin concentration did not appear to reduce the turnover of CTFs. Quantitation of these data
confirmed that the turnover of APP holoprotein is unchanged by calpeptin treatment (Fig. 4B). Additionally, the rate of CTF degradation, like the turnover of APP, does not appear to be affected by calpeptin treatment. These findings also support the hypothesis that the increase in Aβ42 seen following calpain inhibition is due to increased production of βCTFs. Indeed, with calpeptin treatment and at 1 hour chase, the combined αCTF and βCTF signal accounts for approximately 40% of the metabolically labeled APP present in the pulse period, indicating the importance of these metabolic pathways following calpain inhibition.

In order to demonstrate that calpain inhibition promotes the α- and β-cleavage of APP, we immunoprecipitated sAPPα and sAPPβ from the growth media of untreated L/APP cells (Fig. 5, lanes 1-4) and calpeptin treated cells (lanes 5-8) at various chase times following a 15 min. pulse labeling. In general, greater levels of sAPPα and sAPPβ were seen in the calpeptin treated cells when compared to the control cells. In both conditions, sAPPα and sAPPβ were detectable after a 1 hour chase, with their levels increasing significantly by 2 hours of chase time. While sAPP levels remained constant from 2 to 4 hours chase time in the untreated cells, a small increase in both sAPPα and sAPPβ levels were seen in the calpeptin treated cells between 2 and 3 hours (compare lanes 6 and 7). As sAPP levels were found to be nearly maximal after a 2 hour chase period, we did a series of four pulse-labeling experiments in which sAPPα and sAPPβ were immunoprecipitated following a 2 hour chase period from untreated and 10 µM calpeptin treated cells. Quantitation of these experiments showed over a doubling of both sAPPα and sAPPβ levels in the media of calpeptin treated cells vs. untreated cells (sAPPα: 2.2 ± 0.8, sAPPβ: 2.4 ± 0.6; mean increase of calpeptin treated cells relative to untreated cells ± SE). Given that both sAPP species appear to be quite stable in the growth media in both control conditions as well as following calpain inhibition, the likely explanation for an increase in sAPPα and sAPPβ levels following calpeptin treatment is an increase in α- and β-cleavage of APP. This result argues that calpain
inhibition results in an increase in these cleavage events and, therefore, an increase in the production of sAPPα, sAPPβ, αCTF, and βCTF. It should be noted, however, that the increase in maximal CTF levels detected by pulse-chase labeling was found to be greater (approximately 4-fold; see Fig. 4) than the increase in sAPP levels shown above, suggesting that part of the calpain inhibitor effect may be a slowing in the turnover of the CTFs after their generation. While we cannot rule out this possibility, our pulse-chase data (Fig. 4) show that the stability of the CTFs following calpain inhibition is not extended greatly.

βCTFs colocalize with an early endosomal marker, EEA1. That JRF/AβN/25 specifically recognizes the βCTF of APP allowed us to determine the levels and intracellular distribution of this APP proteolytic fragment by immunolabeling. Figure 6 shows the JRF/AβN/25 labeling pattern in control L/APP cells (panels A-C) and L/APP cells treated for 6 hours with 10 µM calpeptin (D-F). JRF/AβN/25 primarily decorated small, punctate structures distributed throughout the cell (A). Consistent with our Western blot and metabolic labeling data, the intensity of JRF/AβN/25 immunolabeling increased following calpeptin treatment, although the pattern of labeling was similar (compare A to D). Given that this pattern suggests a small vesicular compartment and previous work that has suggested early endosomes as a likely site of APP proteolysis leading to Aβ generation (12,13,15), we co-immunolabeled with a polyclonal antibody directed against early endosomal antigen 1 (panels B and E), a protein that interacts with the early endosomal membrane and associated regulatory proteins (49,50,53,54). JRF/AβN/25 labeled βCTFs showed striking coincidence with EEA1, both in the control cells (C) and the calpeptin-treated cells (F). This co-immunolabeling result suggests that much of the βCTFs within these cells is located in early endocytic compartments, and that calpain inhibition does not substantially alter this distribution.
Calpain inhibitors cause a partial redistribution of APP to the cell surface. We did similar immunolabeling experiments using JRF/Aβtot/17, which recognizes APP holoprotein as well as the βCTF, and C1/6.1, which recognizes APP holoprotein and all CTFs. Figure 7 shows co-immunolabeling with JRF/Aβtot/17 and the EEA1 antiserum; results with C1/6.1 were similar. In control L/APP cells, JRF/Aβtot/17 showed strong perinuclear labeling, consistent with an important TGN localization for APP, as well as lesser labeling of peripheral structures (A). With calpeptin treatment, however, JRF/Aβtot/17 showed similar perinuclear labeling yet much greater labeling of the cell’s periphery (E). This peripheral labeling included demarcation of the cell’s surface, which we confirmed to be plasma membrane localization by demonstrating immunolabeling of non-permeabilized cells with JRF/Aβtot/17 (compare panel D, untreated cells, with panel H, calpeptin treated). A consistent finding by immunolabeling was greater JRF/Aβtot/17 binding at the cell surface following calpain inhibition. The EEA1 localization (panels B and F) and the overlay of JRF/Aβtot/17 and EEA1 immunolabeling in these cells (C and G), demonstrated that, unlike the JRF/AβN/25 immunolabeling (Fig. 6), the majority of the APP detected by JRF/Aβtot/17 is not within early endosomes.

While these results suggest a partial redistribution of APP to the cell surface following calpain inhibition, we further confirmed this finding by quantitative 125I-mAb binding to intact and permeabilized cells (41). Table III shows the results of binding 125I-JRF/Aβtot/17 to control L/APP cells as well as cells treated for 3 hours with 10 µM calpeptin or calpain inhibitor III. Treatment with either inhibitor resulted in a small increase in the number of JRF/Aβtot/17 binding sites in permeabilized cells (~1.2-fold with both treatments), consistent with the slight increase in APP levels seen by Western blot analysis following calpain inhibition (Fig. 2). JRF/Aβtot/17 binding at the cell surface increased approximately 1.6-fold with inhibitor treatment, with the JRF/Aβtot/17 binding at the cell surface as a percentage of total binding increasing from 8.6% to approximately 11%. Thus, inhibiting calpains
results in a partial redistribution of APP (and possibly βCTFs as JRF/Aβtot/17 recognizes both species), so that the steady-state levels in the plasma membrane are increased. In an additional experiment, we did a similar analysis of surface versus total JRF/AβN/25 binding sites at multiple time points following the addition of 10 µM calpeptin (data not shown). We found that the increase in JRF/AβN/25 binding at the cell surface was extremely rapid (1.8-fold increase after 15 min. treatment compared to a maximal 2.3-fold after 6 hours). As previously seen, JRF/AβN/25 binding to permeabilized cells increased as well (remaining at 1.2-fold from 1.5 to 6 hours), although the kinetics were somewhat slower than the increase in surface JRF/AβN/25 binding sites.

To determine the effect of calpain inhibition on βCTF levels and its cell surface distribution, we performed a similar quantitative 125I-JRF/AβN/25 binding experiment (Table IV). Calpeptin and calpain inhibitor III increased the binding of JRF/AβN/25 to permeabilized cells by 2-fold and 2.5-fold, respectively, confirming the dramatic increase in the levels of this APP metabolite following calpain inhibition. Calpain inhibition also dramatically increased JRF/AβN/25 binding at the cell surface (nearly 5-fold). As a percentage of total JRF/AβN/25 binding, cell surface binding increased 2.8-fold with calpeptin treatment and 1.6-fold with calpain inhibitor III treatment. The roughly proportionate increase in total (average of 2.3-fold for both inhibitors) and cell surface (average 2.2-fold) JRF/AβN/25 binding suggests that βCTFs are in equilibrium between the intracellular pool and the plasma membrane population following calpain inhibition.

Taken together, these 125I-mAb binding findings and the immunolabeling patterns seen in Figures 5 and 6 indicate greater levels of APP holoprotein in the plasma membrane following calpain inhibition. Importantly, JRF/Aβtot/17 does not detect the αCTF, an important APP species seen with calpain inhibition (see Figs. 3 and 4) and an APP metabolite thought to be generated at the cell surface (7,55). Therefore, the increase in JRF/Aβtot/17 binding to intact cells following calpain inhibition is
likely to substantially underestimate the total pool of APP cell-associated metabolites (APP holoprotein, αCTF, and βCTF) found at cell surface.
Discussion

Our results confirm previous reports that calpain inhibition increases the secretion of Aβ42 relative to Aβ40 (36,37,39); in fact, we find that treating cells with calpain inhibitors increases Aβ42 production far beyond the increase due to the expression of FAD-mutant PS1 in the same cell (Fig. 2) and beyond that generally reported by others following mutant PS1 expression in either cell culture or transgenic mice (16-18). A number of lines of evidence suggests that this effect results specifically from a reduction of calpain activity within the cell. In addition to the two inhibitors used in this study (calpeptin, calpain inhibitor III), others have shown similar effects using these and other calpain-selective peptidyl aldehyde protease inhibitors (38,39). While many of these inhibitors can also inhibit the proteasome at high concentrations, Yamazaki et al. (36) demonstrated that the proteosome-selective inhibitor lactacystin did not increase Aβ42 secretion selectively, as did calpain-selective inhibitors. Additionally, in this study it was shown that cells treated with an inhibitory domain of calpastatin showed an increase in Aβ42 secretion relative to Aβ40, arguing for a direct role of calpain activity in this effect. Similarly, we have transiently overexpressed a single inhibitory domain of calpastatin in L/APP4 cells and seen increased APP immunolabeling at the cell surface in transfected cells (Schmidt and Mathews, unpublished data). The manipulation of calpain activity in a cell by calpastatin overexpression is likely to be the best mechanism to directly implicate calpain activity in APP trafficking and metabolism, and is something we are currently pursuing.

Inhibiting calpains redistributes APP to the cell surface, resulting in an increase in α- and β-cleavage. The distribution of APP between the TGN and the cell surface is highly regulated. Phosphorylation of APP by protein kinase C, for example, results in a redistribution of the protein to the plasma membrane, increasing α-cleavage while reducing Aβ generation (56,57). Multiple lines of evidence suggest that much α-cleavage occurs at the cell surface, including shedding of cell-surface
iodinated APP as sAPPα (7), the localization of the proteolytically active form of ADAM 10 to the plasma membrane (5), and the significant reduction of α-cleavage by a cell-impermeant metalloproteinase inhibitor in a human neuroblastoma cell line (6). The picture may be more complex, however, as Lee and colleagues have proposed that the protein kinase C-regulated increase in α-cleavage occurs within the TGN, in contrast to constitutive α-cleavage, which occurs at the cell surface (58). Given that we detect a greater proportion of APP at the cell surface following calpain inhibition, the simplest explanation for the increase in α-cleavage seen in these cells would be the increased availability of APP for cell surface TACE and/or ADAM 10.

Moreover, the increase in both α- and β-cleavage is likely to be due to a rapid equilibrium between APP at the cell surface – where α-cleavage is likely to occur – and APP within early endosomes – where growing evidence points towards this compartment as being a major β-cleavage site. The evidence that cell surface APP is likely to be in rapid communication with early endosomes is substantial (59) and includes the findings that removal (14) or replacement (12) of the cytoplasmic-tail of APP results in more APP at the cell surface by preventing its internalization. Koo and collaborators have gone further by mapping discrete internalization motifs within this domain (13). Interestingly, those mutations that prevent or reduce the endocytosis of APP also reduce Aβ generation, one line of evidence that endosomes are critical sites for either β- or γ-cleavage. Most reports have now placed BACE within early endosomes and/or throughout the endosomal-lysosomal system (9-11), although some disagreement remains as to other intracellular compartments where this protease might be located (60). Coupled with our data showing βCTFs within early endosomes (Fig. 5), this strongly suggests that early endosomes are a major, if not the major, site for β-cleavage of APP.

Our findings support a model in which the intracellular distribution of APP is altered by calpain inhibition. Our data directly support increased levels of APP at the cell surface following calpain
inhibition, and rapid communication between the plasma membrane and early endosome is likely (14). Were these two compartments in equilibrium, such an increase in APP levels would result in a similar increase in both α-cleavage at the cell surface and β-cleavage within early endosomes, as we found by metabolic labeling and immunoprecipitation. This redistribution of APP toward the plasma membrane/early endosome from the TGN could result from either of two, not mutually exclusive, mechanisms: (1) APP egress from the TGN is increased and/or (2) recycling of APP from the cell surface/early endosome to the TGN is inhibited. Such a TGN recycling pathway plays a critical role in maintaining the distribution of some proteins that are primarily found within the TGN but also at the cell surface (e.g. furin (61) and TGN38 (62)). Additional experimentation will be required to determine whether the TGN recycling pathway contributes to the subcellular distribution of APP. Nevertheless, if this were the case, it would raise the possibility that other metabolites of APP, such as the βCTF, may also recycle, permitting APP fragments generated in early endosomes to return to the Golgi apparatus, perhaps to undergo further proteolytic processing (e.g. γ-cleavage).

Relationship of calpain inhibition and γ-42-secretase activity. While the changes in the intracellular distribution of APP following calpain inhibition appear to lead to increased βCTF production and then to drive Aβ42 generation, we do not yet know why these additional βCTFs are substrates primarily for γ-42-secretase activity. One possibility is that inhibiting calpains has a direct impact on the γ-secretase activity of a cell, favoring γ-42-secretase activity over γ-40-secretase activity. The available evidence suggests that this is, at best, only a partial explanation of the calpain-inhibition effect. We and others (36,37,39) have shown that Aβ40 production remains the same or is slightly increased following calpain inhibition, suggesting that calpain inhibition does not prevent γ-40-secretase activity, thereby driving all γ-cleavage toward a γ-42-secretase pathway. Additionally, we were unable to saturate Aβ42 production with increasing concentrations of calpain inhibitors in either control or...
mutant PS1 expressing L cells. While these experiments are limited by the toxicity of these inhibitors at the highest concentration, the increase in Aβ42 appeared to closely parallel the increase in CTFs. This suggests two distinct mechanisms by which Aβ42 production can be increased: by modified activity of the presenilin complex independent of an increase in βCTFs (e.g. mutant PS (16-18)) or in a βCTF-dependent fashion following calpain inhibition. Interestingly, an interaction between the large cytoplasmic loop domain of PS2 and μ-calpain has been reported, suggesting that calpains may directly regulate PS function (63), potentially linking calpain activity with γ-secretase function. A second possibility is that the subcellular compartment in which γ-cleavage occurs can play a critical role in determining whether Aβ40- or Aβ42 is generated (64). Some experiments have shown early endosomes to have the capacity to generate Aβ42 (13), with others suggesting that compartments within the secretory pathway may (65) or may not (66) generate Aβ42. If calpain inhibition affects the intracellular trafficking of the βCTF as well as APP, it may well be that the βCTFs are directed to a compartment(s) within the cell that is primarily γ42-cleavage competent.

**Relationship between the calpain system and neurodegeneration.** Generally, increased calpain activity is thought to play a role in the development of pathological cytoskeletal changes (32-35) or to place a neuron at greater risk following an additional insult. At higher levels of activation, calpains may directly precipitate cell death, particularly in ischemia and excitotoxicity (28,29). If reduced calpain activity were to promote AD pathology by increasing Aβ generation, particularly Aβ42, this would contrast with increased calpain activity promoting pathological tau changes. It may be that during the extending time course of AD in humans, the calpain system plays multiple roles, initially promoting Aβ generation while later in the disease, responding to and exacerbating tau pathology. Both a greater understanding of the role of the calpain system in APP metabolism and a better assessment of endogenous calpain activity in neurons (67) will be necessary to dissect the relationship between either
decreased or increased calpain activity and developing pathology. Nevertheless, as our understanding of the complex relationships between calpain activity and the regulation of seemingly disparate cellular events grows, the probability that calpains are intimately involved in the disease process appears likely. Finally, this study and previous studies (36,37,39) should raise concern as calpain inhibitors are aggressively pursued for their neuroprotective effects. Chronically inhibiting calpain activity and, therefore, inducing a dramatic increase in Aβ42 generation, may have negative consequences.
Acknowledgements

We would like to thank Stephen P. Jacobsen for help with figures and Dr. Suzana Petanceska for helpful discussions. We would also like to thank Dr. Silvia Corvera, University of Massachusetts Medical School, Worcester, MA for the kinds gift of the anti-EEA1 antibody and Nicole Terrio in our group for antibody production. This work was supported by the National Institute of Aging, the Alzheimer’s Association, and the New York State Office for Mental Hygiene.
Table I: Monoclonal antibodies.

<table>
<thead>
<tr>
<th>name</th>
<th>epitope specificity</th>
<th>APP-species detected</th>
<th>species specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1/6.1</td>
<td>carboxy-terminal 20 residues of APP</td>
<td>APP holoprotein all CTFs</td>
<td>mouse/human</td>
</tr>
<tr>
<td>JRF/AβN/25</td>
<td>first 7 residues of Aβ; requires β- cleavage</td>
<td>βCTF Aβ1-X by ELISA</td>
<td>mouse/human</td>
</tr>
<tr>
<td>JRF/Aβtot/17</td>
<td>within residues 1-16 of human Aβ</td>
<td>APP holoprotein βCTF Aβ by ELISA</td>
<td>human</td>
</tr>
<tr>
<td>JRF/rAβ1-15/2</td>
<td>within residues 1-15 of mouse Aβ</td>
<td>Aβ by ELISA</td>
<td>mouse</td>
</tr>
<tr>
<td>JRF/cAβ40/10</td>
<td>carboxy-terminus of Aβ40</td>
<td>Aβ40 by ELISA</td>
<td>mouse/human</td>
</tr>
<tr>
<td>JRF/cAβ42/26</td>
<td>carboxy-terminus of Aβ42</td>
<td>Aβ42 by ELISA</td>
<td>mouse/human</td>
</tr>
</tbody>
</table>

The monoclonal antibodies used in this study are listed, as well as the known epitope specificities based upon the antigen used for immunization and the antibody’s binding by ELISA to synthetic peptides. The binding of these antibodies to various APP proteolytic species is as characterized in this study and in similar unpublished results (Mathews and Jiang). The species specificity for each antibody has been determined empirically.
Table II: Treatment with calpeptin preferentially increases Aβ42 production.

<table>
<thead>
<tr>
<th>cell line</th>
<th>percentage of control (+ SE) following 10 µM calpeptin treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>L cell</td>
<td></td>
</tr>
<tr>
<td>murine Aβ40</td>
<td>116 ± 3%</td>
</tr>
<tr>
<td>murine Aβ42</td>
<td>200 ± 11%</td>
</tr>
<tr>
<td>L/PS1P_{117L}</td>
<td></td>
</tr>
<tr>
<td>murine Aβ40</td>
<td>135 ± 1%</td>
</tr>
<tr>
<td>murine Aβ42</td>
<td>281 ± 19%</td>
</tr>
<tr>
<td>L/APP</td>
<td></td>
</tr>
<tr>
<td>human Aβ40</td>
<td>103 ± 2%</td>
</tr>
<tr>
<td>human Aβ42</td>
<td>339 ± 37%</td>
</tr>
<tr>
<td>N2a</td>
<td></td>
</tr>
<tr>
<td>murine Aβ40</td>
<td>110 ± 8%</td>
</tr>
<tr>
<td>murine Aβ42</td>
<td>278 ± 17%</td>
</tr>
<tr>
<td>IMR-32</td>
<td></td>
</tr>
<tr>
<td>human Aβ40</td>
<td>106 ± 2%</td>
</tr>
<tr>
<td>human Aβ42</td>
<td>289 ± 46%</td>
</tr>
</tbody>
</table>

The growth media for the indicated cells was replaced with control media or media containing 10 µM calpeptin and the Aβ secreted in 6 hours measured by sandwich ELISA as described in Methods.
Table III: Calpain inhibition results in a partial redistribution of APP to the cell surface.

<table>
<thead>
<tr>
<th></th>
<th>no treatment (cpm)</th>
<th>10 μM calpeptin (cpm + SE)</th>
<th>10 μM calpain inhibitor III (cpm + SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>total JRF/Aβtot/17 binding</td>
<td>9,219 ± 481</td>
<td>10,786 ± 339 (p &lt; 0.05)</td>
<td>11,257 ± 239 (p &lt; 0.05)</td>
</tr>
<tr>
<td>surface</td>
<td>796 ± 29</td>
<td>1,274 ± 88 (p &lt; 0.05)</td>
<td>1,236 ± 39 (p &lt; 0.001)</td>
</tr>
<tr>
<td>percentage</td>
<td>8.6 ± 0.3%</td>
<td>11.8 ± 0.8%</td>
<td>11.0 ± 0.3%</td>
</tr>
<tr>
<td>JRF/Aβtot/17 binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>no treatment (cpm)</td>
<td>10 µM calpeptin (cpm + SE)</td>
<td>10 µM calpain inhibitor III (cpm + SE)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------</td>
<td>-----------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>total JRF/AβN/25 binding</td>
<td>983 + 32</td>
<td>1,928 + 58</td>
<td>2,496 + 60</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>surface JRF/AβN/25 binding</td>
<td>41 + 3</td>
<td>225 + 22</td>
<td>174 + 9</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.01)</td>
<td></td>
</tr>
<tr>
<td>percentage JRF/AβN/25 binding at cell surface</td>
<td>4.2 + 0.2%</td>
<td>11.7 + 1.2%</td>
<td>6.7 + 0.4%</td>
</tr>
</tbody>
</table>
**Figure legends**

**Figure 1:** Cartoon showing monoclonal antibody binding to APP and APP metabolites. The Aβ domain is hatched, showing alternative γ40- or 42-cleavage. Binding specificities for the monoclonal antibodies described in Table I are illustrated.

**Figure 2:** Calpain inhibitor III increases Aβ42 in a dose-dependent fashion while increasing the levels of APP CTFs. L cells or L cells expressing a familial AD-causing presenilin 1 mutation (PS1P117L; (43)) were treated with the indicated concentration of calpain inhibitor III for 6 hours. Conditioned media were collected and analyzed by sandwich ELISA for murine Aβ40 and Aβ42 (A). In B, cell lysates were prepared and proteins sized by SDS-PAGE prior to Western blot analysis using C1/6.1; full-length APP (APPfl) and CTFs are indicated in the Western blot and the quantitation of this blot. Similar results were obtained with calpeptin (data not shown).

**Figure 3:** Calpain inhibitors increase the generation of both α- and β-cleaved CTFs. An L cell line overexpressing human APP was metabolically labeled for 15 min. and chased for 1 hour as indicated. Cells were pretreated with the indicated calpain inhibitors for 3 hours prior to metabolic labeling , as well as during labeling and chase. Cell lysates were prepared, and equal volumes immunoprecipitated with one of three monoclonal antibodies: C1/6.1, which recognizes an epitope within the 20 carboxy-terminal-most residues of APP; JRF/AβN/25, which recognizes residues 1-7 of Aβ; and JRF/Aβtot/17, which recognizes an epitope within residues 1-16 of Aβ. Labeled, immunoprecipitated proteins were
sized on SDS-PAGE and detected as described in Methods. Arrows indicate the APP holoprotein (APP<sub>f</sub>) and the α- and β-cleaved CTFs (αCTF and βCTF, respectively).

**Figure 4: Inhibition of calpains increases proportionately αCTF and βCTF levels without substantially affecting their turnover rate.** The L cell line overexpressing APP was metabolically labeled and chased for the indicated times. Calpeptin treatment was done as in Figure 3. Cell lysates were immunoprecipitated with C1/6.1. The top panel in A is a short exposure showing the turnover of the APP holoprotein (APP<sub>f</sub>). The bottom panel is a longer exposure showing the APP holoprotein and the α- and β-cleaved CTFs (αCTF and βCTF). In B, quantitation of this pulse-chase experiment is shown, illustrating the rapid turnover of the APP holoprotein, which is unaffected by calpeptin treatment, and the dramatic and proportionate increase in αCTF and βCTF generation with calpeptin treatment.

**Figure 5: sAPP<sub>α</sub> and sAPP<sub>β</sub> production increases following calpain inhibition.** L/APP cells were metabolically labeled for 15 min. and chased for the indicated times. sAPP<sub>α</sub> and sAPP<sub>β</sub> were isolated from the chase media by sequential immunoprecipitation using 6E10 to first capture sAPP<sub>α</sub> followed by P2-1 (48) immunoprecipitation to isolate the remaining sAPP<sub>β</sub> (42). Cells were treated with 10 μM calpeptin as described in Figure 3.
**Figure 6: Intracellular localization of βCTFs.** L/APP cells were seeded onto glass coverslips and APP expression was induced. Calpeptin at 10 µM was added for the final 6 hours of incubation as indicated. Panels A and D are indirect immunolabeling using JRF/AβN/25 and detected by confocal microscopy; B and E, with anti-EEA1 antiserum, and C and F, the overlay of the two immunolabeling patterns. Some cell-to-cell variability in the levels of human APP following butyrate induction is typical in these cells.

**Figure 7: Cellular distribution of APP detected with JRF/Aβtot/17 following calpeptin treatment.** L/APP cells were grown, treated and examined as in Figure 6. Panels A, D, E and H are indirect immunolabeling with JRF/Aβtot/17; B and F, with anti-EEA1 antiserum, and C and G, the overlay of the two immunolabeling patterns. The cells shown in panels A, B, C, E, F, and G were permeabilized prior to immunolabeling to show the complete distribution of APP within the cell. The cells in panels D and H were not permeabilized prior to immunolabeling to show the cell surface distribution of APP using JRF/Aβtot/17.
References


Figure 1: Mathews, et al.
Figure 2: Mathews, et al.
Figure 3: Mathews, et al.
Figure 4: Mathews, et al.
Figure 5: Mathews, et al.
Figure 6: Mathews, et al.
Figure 7: Mathews, et al.
Calpain activity regulates the cell surface distribution of amyloid precursor protein: inhibition of calpains enhances endosomal generation of β-cleaved C-terminal APP fragments
Paul M. Mathews, Ying Jiang, Stephen D. Schmidt, Olivera M. Grbovic, Marc Mercken and Ralph A. Nixon

*J. Biol. Chem.* published online June 26, 2002

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