Published in final edited form as:

Biochim Biophys Acta Mol Cell Res. 2021 June ; 1868(7): 119019. doi:10.1016/j.bbamcr.2021.119019.

The C2 domain of calpain 5 contributes to enzyme activation and membrane localization

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Abstract

The enzymatic characteristics of the ubiquitous calpain 5 (CAPN5) remain undescribed despite its high expression in the central nervous system and links to eye development and disease. CAPN5 contains the typical protease core domains but lacks the C terminal penta-EF hand domain of classical calpains, and instead contains a putative C2 domain. This study used the SH-SY5Y neuroblastoma cell line stably transfected with CAPN5-3xFLAG variants to assess the potential roles of the CAPN5 C2 domain in Ca^{2+} regulated enzyme activity and intracellular localization. Calcium dependent autoproteolysis of CAPN5 was documented and characterized. Mutation of the catalytic Cys81 to Ala or addition of EGTA prevented autolysis. Eighty μM Ca^{2+} was sufficient to stimulate half-maximal CAPN5 autolysis in cellular lysates. CAPN5 autolysis was inhibited by tri-leucine peptidyl aldehydes, but less effectively by di-Leu aldehydes, consistent with a more open conformation of the protease core relative to classical calpains. In silico modeling revealed a type II topology C2 domain including loops with the potential to bind calcium. Mutation of the acidic amino acid residues predicted to participate in Ca^{2+} binding, particularly Asp531 and Asp589, resulted in a decrease of CAPN5 membrane association. These residues were also

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

found to be invariant in several genomes. The autolytic fragment of CAPN5 was prevalent in membrane-enriched fractions, but not in cytosolic fractions, suggesting that membrane association facilitates the autoproteolytic activity of CAPN5. Together, these results demonstrate that CAPN5 undergoes Ca^{2+}-activated autoproteolytic processing and suggest that CAPN5 association with membranes enhances CAPN5 autolysis.

Keywords

calpain; calcium; cell culture; immunofluorescence; membrane; plasma membrane; protease; protein-lipid interaction

1. Introduction

Calpains are a family of Ca^{2+}-activated neutral proteases first discovered over 50 years ago as Ca^{2+}-activated proteolytic activity at neutral pH in the soluble fraction of rat brain and skeletal muscle [1, 2]. The originally observed Ca^{2+}-dependent proteolytic activity is now ascribed to calpain 2, one of 15 calpain isoforms present in mammals [3]. Calpain 5 (CAPN5) was discovered by cloning in 1997 and is the mammalian orthologue of *C. elegans* gene *tra-3* [4].

The term “calpain” refers to the active enzyme(s), subunits are named using CAPN followed by the appropriate number (http://calpain.net/structure/index.html). For example, calpain 1 is composed of subunits CAPN1 and CAPNS1 and can also be referred to as CAPN1/CAPNS1. Calpain 5 is not known to require other subunits or proteins for activity. Thus, the terms calpain 5 and CAPN5 may be used interchangeably, and this enzyme was also previously referred to as hTra-3 [5]. For consistency, we use CAPN5 throughout.

Classical calpains, such as calpains 1 and 2, are hetero- or homo-dimers that include penta-EF hand type calcium-binding domains in each subunit. CAPN5 lacks a penta-EF hand domain and is therefore considered a non-classical, atypical, or non-conventional calpain [6, 7]. CAPN5 contains the protease core domain of classical calpains, and also has a putative calcium-binding C2 domain at the C-terminus (Fig. 1). CAPN5 has recently become the target of more intensive investigations due to its high expression levels in the CNS [8], discovery of its role in a rare inherited eye disease [9], its relevance to retinal development and regeneration [10], and evidence linking it to hepatitis C viral entry [11]. Although CAPN5 was discovered more than 20 years ago, a basic characterization of the enzyme awaits description.

Efforts to obtain active full-length recombinant CAPN5 have been unsuccessful to date (DE Croall & AJ Bolduc, unpublished; [12]). Analysis of CAPN5 has therefore relied on structural modeling [12, 13], expression of CAPN5 constructs in mammalian systems [8, 9, 14, 15], and analysis of the recombinant, catalytically active protease core [12, 16], a strategy which has been successful with other calpains [17, 18]. The protease core domain of CAPN5 (PC1 & PC2) (Fig. 1) contains two conserved Ca^{2+} binding sites that enable activation in the absence of other domains [13, 16], as with classical calpains [17]. However, the proteolytic activity of the recombinant protease core domains of CAPN1 and CAPN2 is

very weak, with full catalytic activity requiring the binding of 8 additional Ca^{2+} ions to the penta-EF hand domains [17, 19, 20] that are lacking in CAPN5. It remains to be determined if Ca^{2+} binding to additional domains is required for the full activity of CAPN5.

Like many other calpains, CAPN5 has a calpain-type β-sandwich (CBSW) domain (also known as Domain III or C2-like domain) adjacent to the catalytic protease core domains [20]. Although some data suggested that the CBSW domain was a Ca^{2+}-regulated phospholipid binding domain [21], calcium binding to this region was not observed in crystal structures of calpain 2 [22, 23] and mutations in the putative phospholipid-binding domain did not alter lipid affinity [24]. The function of the CBSW domain of CAPN5 is not known and was not investigated in the present study.

A key distinction of CAPN5 is its C terminal domain, initially referred to as domain „T" for the nematode enzyme tra-3 [4] but now annotated as a C2 domain [6]. This is in contrast to the C terminal penta-EF hand domain of classical calpains that not only mediates Ca^{2+} binding, but also hetero- or homo- dimerization [20]. Although it has been more than 10 years since the CAPN5 domain was designated as a putative C2 domain, no functional significance has yet been demonstrated for it. C2 domains are typically about 130 residues organized as a pair of four stranded β-sheets, each arranged in an anti-parallel fashion as first identified in Protein Kinase C [25, 26]. Ca^{2+} binding typically occurs via clusters of acidic amino acid residues (Asp/Glu) at one side of the loops [26–28]. C2 domains are involved in binding to cell membranes, vesicular trafficking, protein-protein interactions, and cell signaling in response to changes in Ca^{2+} concentrations and lipids [29]. In general, proteins with a single C2 domain, such as protein kinase C, are involved in signal transduction, while proteins with two or more C2 domains, such as synaptotagmin 1, are membrane trafficking proteins [30]. However, Ca^{2+}-binding and non-Ca^{2+} binding C2 domains have been identified, and C2 domains are found in both enzymatic and non-enzymatic proteins [25, 26, 31–33]. The only other mammalian calpain with a putative C2 domain is CAPN6, which is non-catalytic [4].

Autoproteolytic processing of one or more subunits of several calpains, including calpains 1 & 2 [34, 35], calpain 3 [3, 36, 37], calpain 7 [38] and defective kernel 1 (DEK1) [39, 40], is often observable after exposure to calcium. Although the functional significance of most of these cleavage events has been debated, autoproteolysis has proven to be a useful indicator of enzyme activity or activation [36, 41–44]. Currently there is no known substrate or activity assay to measure enzymatic activity of CAPN5. We hypothesized that autoproteolytic processing might be observable and useful as tool to assess basic enzymatic properties of CAPN5. The aims of this study were to characterize the autoproteolytic processing of CAPN5 and to provide evidence as to the function of its C2 domain in enzyme activity and/or localization.

2. Materials and Methods

2.1 Generation of wild-type (WT) and mutant CAPN5 stable cell lines

Human full-length CAPN5 (Thermo Fisher Scientific/Open Biosystems, Clone ID 3904914) was subcloned into p3xFLAG-CMV-14 (Sigma-Aldrich, E4901). The D589N mutation was

introduced with the GeneArt Site-Directed Mutagenesis System (Thermo Fisher Scientific/Invitrogen, A13282) using primers designed with Invitrogen‟s online primer design tool. The C81A, D531N and E590Q mutations were introduced with the QuikChange II Site-Directed Mutagenesis Kit (Agilent, 200523) using primers designed with the Agilent QuikChange Primer Design program. The tag-less CAPN5 expression construct was generated by inserting the CAPN5 coding sequence immediately followed by two stop codons into p3xFLAG-CMV-14.

For some studies, the human full-length CAPN5 cDNA was subcloned into pTRE3G-mCherry (Takara/Clontech, 631171), and the C81A mutation was introduced with the GeneArt System as above. The C81A mutated DNA was then subcloned into pIRESpuro3 (Takara/Clontech, 631619) with a 3xFLAG tag added to the 3-prime end of the coding sequence.

All constructs were confirmed with sequencing. Care was also taken to confirm that no additional mutations were present in the DNA. The plasmids were transfected into the SH-SY5Y human neuroblastoma cells (ATCC, CRL-2266) using LipoJet (Signagen SL100468) as per the manufacturer‟s instructions. Stable cell lines of the C81A mutant were generated using puromycin (Gold Biotechnology, P-600-100) at a final concentration of 3 μg/ml, utilizing the puromycin resistance gene in the pIRESpuro3 vector. Other stable cell lines were generated using G418 (Gold Biotechnology, G-418-25) at a final concentration of 2.5 mg/ml, utilizing the neomycin resistance gene in the p3xFLAG-CMV-14 vector.

2.2 Cell culture, immunofluorescence, and confocal microscopy

SH-SY5Y human neuroblastoma stable cells expressing wild-type CAPN5, CAPN5-3xFLAG or $CAPN5_{D531N}$, $CAPN5_{D589N}$, $CAPN5_{E590Q}$, $CAPN5_{C81A}$, or $CAPN5_{C81A/D589N}$ (all with C terminus 3x-FLAG) were cultured and maintained in Minimum Essential Medium (MEM) with Earle’s salts and L-glutamine (Corning 10-010-CV) supplemented with 10% Fetal Bovine Serum (R&D Systems, S11150H), 1% Penicillin-Streptomycin (Corning, 30-002-CI) and 2.5mg/ml G418 or 3 μg/ml puromycin for $CAPN5_{C81A}$ in the pIRESpuro3 vector, at 37°C in 5% CO_2.

For immunofluorescence cells were grown in 35-mm glass bottom dishes (Mattek, P35G-0-14-C) for 24 hrs. Cells were then fixed with 4% paraformaldehyde in Phosphate Buffered Saline (PBS, Na_2HPO_4 0.072 M, NaH_2PO_4 0.028M and NaCl 0.9%, pH 7.0) for 30 minutes at room temperature, followed by three PBS washes. Cells were permeabilized in PBS with 0.25% Triton-X100 (Fisher Scientific, BP151-100) for 10 minutes at room temperature followed by three PBS washes and were blocked for 30 minutes with PBS supplemented with 0.05% Tween 20 and 1.5% normal goat serum (Life Technologies, 10000C). The primary and secondary antibodies were also diluted in this buffer. The cells were then incubated with mouse anti-FLAG antibody (Sigma, F3165) for 24 hours with gentle shaking at 4°C. After three PBS washes, the cells were incubated with Alexa Fluor 594 Goat anti Mouse IgG (Life Technologies, A11005) for one hour at room temperature. After three PBS washes in the dark, cells were incubated with Hoechst 33258 (Molecular Probes, H-3569) in PBS for 10 minutes for nuclear visualization. The cells were washed

three more times with PBS and stored in dark in PBS until visualization on a Nikon C2 confocal microscope using NIS-Elements AR4.6.0.00 software.

2.3 Maitotoxin treatment

Cells (2×10^6) stably expressing WT CAPN5 or $CAPN5_{C81A}$ were plated in 60-mm dishes for 24 hours. The cell media was changed to MEM without serum prior to treatment with 0.8 nM maitotoxin (WAKO, 134-17161) for 60 minutes, in the presence or absence of 5 mM EGTA. Maitotoxin treatment, in the absence of EGTA, resulted in the cells lifting from the plate and the cells were collected by centrifugation. Cells without maitotoxin treatment, or treated with maitotoxin plus EGTA, were lifted from the plate and collected with a P1000 Pipetman. The cells were washed once with cold PBS supplemented with protease inhibitors (cOmplete Mini, EDTA-free Protease Inhibitor Cocktail, Roche-Sigma, 11836170001), centrifuged and were lysed with RIPA buffer (1% Nonidet P40 Substitute, Sigma, 74385), 0.5% deoxycholic acid (Sigma, D6750) and 0.1% sodium dodecyl sulfate (SDS) (Sigma, L4390) in PBS) plus protease inhibitors, sonicated, centrifuged at 12,000 x g for 10 minutes and the supernatants were collected. Protein concentrations were measured followed by immunoblotting.

2.4 Calcium activation and calpain 5 inhibition

Cells (3×10^6) stably expressing WT or mutant CAPN5 were plated in 60-mm dishes for 24hrs. For calcium activation studies, the cells were washed two times with cold HEPES buffer (20 mM HEPES pH7.4 supplemented with 130 mM NaCl, 4.5 mM KCl, 22 mM D-glucose, 1 mM sodium pyruvate and 0.001 mM glycine, and 1 mM serine protease inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (EMD Millipore, 101500). The cells were scraped into 2-mL tubes, centrifuged at 1,200 x g for 10 minutes at 4°C. The cells were then resuspended in HEPES buffer +1% NP-40 + calcium chloride (various concentrations), vortexed briefly and incubated at 37°C for 30 minutes. They were then lysed with the addition of an equal volume of lysis buffer (1% deoxycholate + 0.2% SDS in HEPES buffer +1% NP-40), sonicated briefly and centrifuged at 12,000 x g for 10 minutes at 4°C. Supernatants were collected and protein concentration was measured using Thermo Fisher Scientific Pierce BCA protein assay (Fisher Scientific, PI23225).

For calpain 5 inhibition experiments, 3×10^6 cells stably expressing WT or mutant CAPN5 were plated in 60-mm dishes. Four hours later, the cells were treated with the inhibitors overnight. The inhibitors were MG132 (Sigma-Aldrich, M7449, 10 μM), PD150606 (Sigma-Aldrich, D5946, 100 μM)), calpain inhibitor IV, Z-LLY-FMK (Millipore, 208724, 20 μM), bortezomib (Selleckchem, S1013, 0.05 and 1 μM), calpeptin (Sigma-Aldrich, 03-04-0051, 10 μM), calpain inhibitor III-MDL28170 (Calbiochem, 208722, 20 μM), acetyl calpastatin 184–210 (Sigma-Aldrich, CH6H11E4F558, 1, 10 and 50 μM) leupeptin (APExBIO, A2570, 1, 20, and 100 μM) and calpain inhibitor I-ALLN (APExBIO, A2602, 1, 20, and 100 μM). After overnight treatment with inhibitors, the cells were washed as described above, resuspended in HEPES buffer +1% NP-40 + 1 mM calcium chloride along with inhibitors at the same concentrations as indicated above, vortexed briefly and incubated at 37°C for 30 minutes. The cells were then lysed, sonicated, and centrifuged as described above.

2.5 Denaturing gel electrophoresis and immunoblotting

SDS-PAGE and immunoblotting were performed as previously described [8]. Briefly, samples were separated on Bis-Tris 4–12% gradient protein gels (Thermo Fisher Scientific, NP0335BOX), transferred onto a 0.2μm nitrocellulose membrane (Bio-Rad,1704158), blocked with 5% non-fat dry milk in TTBS (Tris-Buffered Saline – 50 mM Tris, 150 mM NaCl, pH7.5 + 0.05% Tween 20) incubated at room temperature for one hour. All antibodies were applied in 5% milk/TTBS, and washes were done in TTBS. The blocked membranes were incubated with primary antibodies rabbit anti-Calpain 5 (Genetex, GTX103264), rabbit anti-Calpain 1 (Abcam, ab28257), mouse anti-β-actin (Sigma, A2228), mouse anti-FLAG (Sigma, F3165), mouse anti-alpha 1 Na^+/K^+-ATPase (Abcam, ab7671), rabbit anti-translocase of outer mitochondrial membrane 20 (TOMM20, Abcam, ab186735) and rabbit anti-extracellular-signal-related kinase 1 (ERK 1, Santa Cruz Biotechnology, sc-94) overnight at 4°C with gentle shaking. For the Genetex anti-CAPN5 antibody, membranes were incubated for 48 to 72h. After the incubation, the blots were rinsed three times, 20 minutes each. The corresponding IRDye 800CW conjugated goat anti-rabbit IgG (Li-Cor, 926-32211) and/or Alexa Fluor 680 conjugated goat anti-mouse IgG (Thermo Fisher Scientific, A21058), were added in 5% milk in TTBS and incubated in the dark for one hour at room temperature, followed by three washes with TTBS. The blots were scanned using a LiCor Odyssey CLx instrument (Li-COR Biosciences, Lincoln, NE). Intensity of the signal was measured using Odyssey Application Software V5.2.5 (Image Studio) with appropriate background subtraction.

2.6 Enrichment of cytosolic and membrane fractions

Cell lines stably expressing CAPN5 or its mutants were grown to 100% confluence in 150-mm dishes. The cells were trypsinized with 0.25% Trypsin and 2.21 mM EDTA (Corning, 25-053-CI) and centrifuged at 1,200 x g for 10 minutes at 4°C. The cytosolic and membrane associated proteins were separated using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, 89842) with slight modifications. All incubations and washes were at 4°C. The trypsinized cells were washed two times with cell wash solution from the kit by centrifugation at 500 x g for 6 minutes. The cell pellets were resuspended in 500 μl of permeabilization buffer supplemented with and without 2 mM $CaCl_2$, and cOmplete Mini, EDTA-free Protease Inhibitor Cocktail (Roche-Sigma, 11836170001), vortexed briefly and incubated for 30 minutes with rotation. After incubation, the permeabilized cells were centrifuged at 16,000 x g for 15 minutes. The supernatants consisting of the enriched cytosolic fractions were carefully collected. To eliminate any traces of cytosol, the pellets were incubated two times with cold PBS supplemented with protease inhibitor for 5 minutes with rotation, and centrifuged again at 16,000 x g for 10 minutes. The washed pellets were incubated in 500 μl Solubilization buffer supplemented with protease inhibitor for 30 minutes and centrifuged at 16,000 x g for 15 minutes. The enriched membrane-associated proteins in the supernatant were collected. The protein concentration was measured, and the samples were subjected to immunoblotting as described above.

2.7 Bioinformatics: Sequence alignments

C2 domain sequences were gathered from UniProtKb (www.uniprot.org). Multiple sequence alignments were performed using Clustal Omega (EMBL-EBI) and/or PSI-BLAST (NCBI) and percent identity and similarity matrices corresponding to the alignments were evaluated. Alignments were downloaded to Jalview alignment editor [45] for visualization.

2.8 Experimental rigor

All experiments were replicated a minimum of three times with representative results presented. For immunoblots, any adjustments in brightness/contrast were applied to the entire image. Blot lanes were not spliced from different images of a gel. All blots included a loading control, typically β-actin.

3. Results

3.1 Autoproteolytic processing of CAPN5 demonstrates Ca^{2+} dependence of CAPN5 activity.

As substrates of CAPN5 have not yet been identified and purified proteolytically active recombinant CAPN5 is not available, we sought to determine if CAPN5 autolysis occurred, and if it could be used to monitor CAPN5 enzyme activity. Previously, proteolysis of CAPN5 was observed following treatment of SH-SY5Y cells with maitotoxin which activates calcium channels [14, 46]. However, it was uncertain if the observed proteolysis was the result of CAPN5 autolysis or degradation of CAPN5 by other proteases, including other calpains known to be activated by the influx of calcium caused by maitotoxin [47].

Several variants of full-length human CAPN5 constructs were successfully overexpressed in stably transfected human neuroblastoma SH-SY5Y cells. This approach was necessitated by the relatively low levels of CAPN5 in SH-SY5Y cells in the absence of transfection, as shown by immunoblotting for CAPN5 with the antibody N1C1 (GeneTex) against an epitope in the central region of CAPN5 (Fig. 2A). CAPN5 was only faintly detected in non-transfected SH-SY5Y cells but was present at higher levels in cells stably expressing CAPN5 or CAPN5-3xFLAG (Fig. 2B).

Endogenous CAPN5 was also detected in mouse platelets where calpains are implicated in cell signaling, aggregation, clot retraction, and α-granule secretion [48, 49]. However, the specific contribution of CAPN5 to these functions has not been determined. The molecular weight of human and mouse CAPN5 is calculated as 73.2 kDa, with CAPN5-3xFLAG having an estimated mol. wt. of 76.5 kDa. On SDS-PAGE, CAPN5 in mouse platelets and SH-SY5Y cells migrated at or slightly above a 75 kDa mol. wt (Fig. 2A). Stably expressed CAPN5-3xFLAG in SH-SY5Y cells migrated slightly above stably expressed CAPN5 without the tag (Fig 2B), consistent with the 3 kDa molecular weight of the 3xFLAG C-terminal tag. CAPN1 was examined as an internal control and was also present in platelets and SH-SY5Y cells.

Following incubation of cell extracts with 1 mM $CaCl_2$, 37°C, 30 minutes, several bands of lower molecular weight were detected with anti-CAPN5, with a prominent band at ~61

kDa and a faint band at ~44 kDa (Fig. 2B), with additional faint bands observed. The lower molecular weight bands are thought to result from sequential autolytic processing of the N-terminal fragment. Autolysis was not a consequence of the FLAG tag itself because autolysis of CAPN5 was also observed with a C-terminally 3xHA-tagged construct (results not shown) and in cells stably expressing CAPN5 without the tag (Fig. 2B). Together, these results demonstrate that the presence of the 3xFLAG tag on the C-terminus of CAPN5 did not alter the proteolytic processing in response to elevated calcium. Incubation of cell extracts with 1 mM Ca^{2+} also resulted in a loss of CAPN1 immunoreactivity, detected using an antibody against the N-terminus of CAPN1.

Treatment of the cells with maitotoxin (0.8nM, 1h, 37°C) resulted in decreased levels of the full-length CAPN5 bands, as detected with anti-CAPN5 or anti-FLAG, but only when the CAPN5 catalytic Cys (C81) was unaltered (Fig. 3). The presence of autolytic CAPN5 fragments in untreated cells (lane 1) is the result of calcium influx associated with mechanical lifting/scraping of the cells from the cell culture dishes [50]. The MEM culture media contained 1.8 mM $CaCl_2$. Treatment with EGTA to sequester Ca^{2+} in the culture media abolished the appearance of the fragments of CAPN5 following maitotoxin treatment (Fig. 3, lane 3) and mechanical lifting (Fig. 3, lane 4), further supporting an essential role for calcium in the autoproteolysis of CAPN5. Although the anti-FLAG antibody confirmed changes to the full-length CAPN5 immunoreactivity, CAPN5 fragments were not observed with the anti-FLAG antibody suggesting that a C terminal part of the protein is removed. These data clearly demonstrate that the immunoreactive CAPN5 fragments result from calcium-induced autoproteolytic processing when the enzyme is active and thus can serve as a measure of CAPN5 catalytic activity or activation.

As a positive control for the effectiveness of the maitotoxin in the presence or absence of EGTA, the autoproteolysis of the large subunit of calpain-1 (CAPN1) was also monitored using an antibody against the N-terminus of CAPN1, which is known to be removed by autolysis [44]. CAPN1 immunoreactivity also decreased following treatment of the cells with maitotoxin, and as expected EGTA protected against autolytic processing of CAPN1 (Fig. 3).

To further evaluate the influence of Ca^{2+} on CAPN5 activity, SH-SY5Y cells stably expressing CAPN5-3xFLAG were suspended in HEPES buffer, lysed, and incubated with various concentrations of $CaCl_2$ (Fig. 4). The calcium concentration required for half-maximal activity for autoproteolysis of CAPN5 was estimated to be 80 μM when assessed by the decrease in the intensity of the full-length CAPN5 immunoreactivity, with a Hill coefficient estimate of 0.94 which is similar to that pf PKCα and γ [51]. Measuring the increased appearance of the autolytic ~61 kDa fragment of CAPN5 yielded an estimate of approximately 200 μM calcium. This higher estimate may result from multiple intermediate cleavage events, further processing of fragments, differences in transfer efficiency, or the lower sensitivity of detection of CAPN5 breakdown products.

Loss of CAPN1 immunoreactivity was also evaluated as a positive control. We observed half-maximal activity at approximately 50 μM calcium, based on loss of immunoreactivity of the N-terminus of the large subunit. This is comparable to estimates observed using the

purified enzyme [36, 52]. Together, the above results demonstrated that CAPN5 is activated by Ca^{2+} resulting in its autoproteolytic processing. Without the option to use purified enzyme, we acknowledge that only a relative, apparent calcium sensitivity is derived with this approach due to contributions of cellular calcium and the assortment of other calcium binding proteins present in a cell lysate.

To determine if small molecule proteinase inhibitors attenuate the Ca^{2+}-induced autolysis of CAPN5, a series of known inhibitors for other calpains and the proteasome were screened (Fig. 5). CAPN5 autolysis was reduced by the tri-leucine aldehyde inhibitors ALLN (N-acetyl-Leu-Leu-NorLeu-al or MG101, 20 μM and 100 μM), MG132 (Z-Leu-Leu-Leu-al, 10 μM) known to inhibit both the proteasome and calpain [53, 54], and by Bortezomib (0.05 and 1 μM) thought to be a more specific proteasome inhibitor [55, 56]. In contrast, other known calpain inhibitors MDL28170 (Z-Val-Phe-al, 20 μM), Z-Leu-Leu-Tyr-fluoromethylketone (Z-LLY-FMK, 20 μM), calpeptin (Z-Leu-NorLeu-al, 10 μM), and acetyl-calpastatin B peptide (1 μM, 10 μM and 50 μM), failed to inhibit CAPN5 autolysis, although each was effective in preventing autoproteolysis of CAPN1 that was assessed as a control. MG132 also inhibited CAPN1 proteolysis as expected, while Bortezomib did not. Leupeptin (Ac-Leu-Leu-Arg-al) provided modest inhibition at 100 μM. As PD150606 was ineffective for the control CAPN1, this suggests a problem with the inhibitor stock and no conclusion can yet be made for CAPN5.

3.2 Influence of the C2 domain on CAPN5 activation

Although the C-terminal domain of CAPN5 is annotated as a C2 domain, its structure and function have not previously been examined. The Protein Data Bank contains a 3D structure of the protease core domain of CAPN5, but not of the C2 domain [13]. To identify potential Ca^{2+} binding residues in the C2 domain, this region was modeled *in silico*. The RaptorX [57] and Phyre2 [58] modeling servers, which utilize template-based approaches, returned highly similar models for the CAPN5 C-terminal domain (residues 518–640). Both models (Fig. 6A) adopt a type II topology C2 membrane interaction domain fold characterized by two antiparallel four-stranded beta sheets arranged in a beta-sandwich [26, 31], and their structures closely match other known C2 domains, including the extended synaptotagmin-2 C2A domain (Fig. 6B). Three loop regions at one end of the beta sandwich frequently contain a constellation of acidic residues (Asp/Glu) to mediate binding of several calcium ions and are designated Ca^{2+} binding regions (CBR) 1–3.

In this region of the CAPN5 C2 models (Fig. 6C), two Asp residues (D531 and D589), along with a Glu residue (E590), are present suggesting that the domain might coordinate binding of Ca^{2+} ions to mediate membrane interaction. The potential significance of these acidic residues is also supported by a bioinformatics approach. Examination of CAPN5 orthologs across a range of species showed conservation of several acidic and basic residues within the C2 domain (Supplemental figure 1). To further assess the conservation of these acidic residues (Asp or Glu) the human CAPN5 genomic coding sequence variants (both synonymous and non-synonymous) reported in dbSNP [59], ExAC [60] and the Cancer Genome Atlas [61] were analyzed.

Considering all sequence variants, allele frequencies, and whether the variants were validated, as of the 31 December 2020 versions of the cited databases only D531, D589, and D600 had no sequence variants [62]. Thus, the structural model developed here, conservation of residues during evolution, and their apparent stability in the human genome argue for the potential importance of D531 and D589 for CAPN5 function and suggest they might coordinate binding of Ca^{2+} ions to contribute to membrane interaction via the C2 domain.

To test the possible significance of these residues in the calcium dependent membrane association of CAPN5, Asp531 and Asp589 were each mutated to Asn, and Glu590 was changed to Gln. This approach has been used successfully in previous characterizations of calcium binding sites in C2 domains [63–66]. If the mutation of a putative calcium binding residue did abrogate calcium coordination, it could also impair CAPN5 association with the membrane and result in the shift of CAPN5 localization from the membrane to the cytosol.

Immunofluorescence, using an antibody against the C-terminal 3xFLAG tag was used to selectively detect full-length CAPN5 and not the autoproteolytic fragments. CAPN5-3xFLAG, whether proteolytically active WT or inactive C81A, was associated with the plasma membrane. In addition, diffuse punctate immunoreactivity was observed in the cytosol and nucleus (Fig. 7). Of the mutations to selected amino acid residues, the altered cellular localization of $CAPN5_{D589N}$-3xFLAG was the most striking. It was localized to the cytoplasm without any clear enrichment at the plasma membrane. CAPN5-3XFLAG with either the D531N or E590Q mutations exhibited some enrichment at the plasma membrane, albeit to a lesser degree by comparison with WT and C81A CAPN5.

Permeabilized cells, with and without incubation with 2 mM $CaCl_2$, were also separated into cytosolic and membrane enriched fractions for evaluation of CAPN5 localization by subsequent immunoblotting (Fig. 8). Detection of the Na^+/K^+ ATPase, a plasma membrane marker; ERK (extracellular signal regulated kinase), a cytosolic marker; and TOMM20, a mitochondrial marker, confirmed successful fractionation of the cells.

The exogenously expressed, FLAG tagged CAPN5 with an unmutated (WT) C2 domain was present in both membrane-enriched and cytosolic fractions, with a slightly greater abundance in the membrane fraction in the absence of added calcium. In contrast, CAPN5-3xFLAG with mutations of the putative acidic Ca^{2+} binding residues (D531N, D589N, and E590Q) were most abundant in the membrane fraction as compared to the cytosol. This was most dramatic for $CAPN5_{D589N}$, which was almost exclusively cytosolic.

In the presence of 2 mM added Ca2+, a similar distribution of WT and mutant CAPN5 variants was observed, except that autolytic CAPN5 fragments were readily detected in the membrane fraction, with only faint bands detected in the cytosolic fraction (Fig. 8A). The autolytic fragments were most abundant for WT CAPN5, followed by $CAPN5_{E590Q}$, $CAPN5_{D531N}$, and $CAPN5_{D589N}$, although this also reflects their relative abundance in the membrane fraction.

To evaluate the possible influence of CAPN5 activity on localization, we examined the localization of WT and catalytically dead (C81A) CAPN5, with or without the D589N

mutation. (Fig. 8B). In the absence of added Ca^{2+}, the membrane/cytosol distribution of $CAPN5_{C81A}$ was similar to that of WT CAPN5, being present in both the cytosolic and membrane fractions. $CAPN5_{D589N}$ and $CAPN5_{C81A/D859N}$ also exhibited similar distributions, being enriched in the cytosolic fraction and barely detectable in the membrane fraction. This distribution persisted in the presence of added Ca^{2+}, with autolytic fragments detected for only WT CAPN5. The similar localization of CAPN5 and $CAPN5_{C81A}$, and of $CAPN5_{D589N}$ and $CAPN5_{C81A/D859N}$, suggests that the CAPN5 activation does not alter localization. Together, these results demonstrate that D589, and to a lesser extent D531, have important roles for CAPN5 association with membranes which may facilitate CAPN5 activation and autoproteolysis.

4. Discussion

Although identified over twenty years ago [4], relatively little is known regarding the properties of CAPN5. CAPN5 and catalytically inactive CAPN6 contain putative C2 domains as their C-terminal domain, in contrast to the penta-EF hand domain in classical calpains such as calpains 1 and 2. C2 domains represent the second most abundant Ca^{2+}-regulatory domain, after the EF hand domain [33]. Most C2 domains bind Ca^{2+}, although many do not [32, 33]. The purpose of this study was to characterize the autolytic processing of CAPN5 and to examine the contribution of the CAPN5 C2 domain to enzyme function and localization.

SH-SY5Y human neuroblastoma cells were chosen as a model system based on their neuronal-like phenotype [67], relatively high CAPN5 mRNA expression as compared to other cell lines (https://www.proteinatlas.org/ENSG00000149260-CAPN5/cell), and use in previous studies examining CAPN5 [8, 14, 68]. Although CAPN5 mRNA is expressed at high levels in SH-SY5Y cells, detectable CAPN5 protein levels are low consistent with the prediction of its rapid turnover (T1/2 = 2 min UniprotkB) relating to the destabilizing N terminal amino acid, Phe at position 2 [69, 70]. To facilitate analysis, SH-SY5Y cells were stably transfected to express several variants of CAPN5-3xFLAG. The data presented here characterized the calcium dependent, autoproteolytic processing of CAPN5 first suggested by Wang and colleagues [14]. Use of a catalytically dead variant (C81A) provided unequivocal evidence that the appearance of truncated CAPN5 immunoreactive products resulted from autoproteolysis. Notably these products were detected with antibodies directed at the central portion of CAPN5 but not with the anti-FLAG antibody directed at the 3xFLAG peptide positioned C terminal to the natural CAPN5 sequence. Autolysis of CAPN5 appears to proceed sequentially, with the initial appearance of a ~74 kDa fragment and later appearance of the 61 kDa band. This is similar to the sequential autolysis of the large subunits of calpains 1 and 2 [71, 72]. In contrast to calpains 1 & 2, where autolytic processing removes N terminal peptides, the sequential removal of peptides from CAPN5 appears to begin at the C terminus. The predominant 61 kDa CAPN5 product is13-15 kDa smaller than the intact protein, suggesting that most of its C2 domain could be removed by this process. Interestingly, N-terminal CAPN5 fragments remain associated with the membrane through mechanisms which remain to be determined.

Although the physiological relevance of the observed autoproteolysis is not yet known, it provides a key tool for assessing the characteristics of the catalytic activity of intact CAPN5, as to date only the proteolytic core has been studied [12, 13, 16]. Isolated catalytic domains have proven useful for understanding mechanisms of enzyme activation and screening of inhibitors of the catalytic domain [73–75]. However, the activity of the catalytic core domain is a typically a fraction of that of the complete enzyme [20]. Adding various amounts of calcium to cellular lysates resulted in estimates of 80 and 200 μM Ca^{2+} required for half-maximal loss of the full-length CAPN5 or appearance of its 63kDa N terminal product, respectively. In contrast, the Ca^{2+} required for half-maximal activity of the CAPN5 protease core was 12 mM [13], suggesting that either the additional domains on full-length CAPN5 or the presence of modifying factors in the cell reduced the Ca^{2+} threshold required for enzyme activation.

By comparison, monitoring loss of the N-terminus of CAPN1 provided an estimate of 50 μM Ca^{2+} for half-maximal CAPN1 autoproteolysis. This is within published estimates of the calcium concentration (0.8–150 μM) required for half-maximal calpain 1 activity, based on either autolysis or substrate proteolysis [43, 76]. However, these values may be influenced by the presence of the endogenous inhibitor calpastatin in SH-SY5Y cells [77]. Also, other cellular proteins and phospholipids may alter the Ca^{2+} threshold for calpains 1 and 2 [21, 34, 78, 79]. The availability of stable, active recombinant CAPN5 and identification of other substrates would be expected to allow more detailed analysis of the Ca^{2+}-sensitivity of CAPN5 that may reflect both the binding sites in PC1/PC2 and binding to other domains including the C2 domain.

Selective small molecule inhibitors are often essential for dissecting physiological roles of proteolytic enzymes [80–82]. Although the PC1/PC2 domains of CAPN5 include the catalytic Cys-His-Asn triad, they are just 46% identical/60% similar to the PC1/PC2 of CAPN1 (residues 55–354). A screen of small molecule inhibitors aimed to better characterize the catalytic activity of CAPN5 relative to known calpains. Tri-leucine peptidyl aldehydes are effective against both classical calpains and the proteasome, while di-leucine peptidyl aldehydes are more selective towards calpains [53]. Interestingly a similar pattern was observed for CAPN5 with the tri-Leu aldehydes MG132 and ALLN [83] inhibiting both CAPN5 and calpain 1. In contrast, the di-Leu aldehydes Z-LLY-FMK, Calpeptin, and Leupeptin preferentially inhibited CAPN1 and were less effective against CAPN5. MDL28170 and a single inhibitory domain of calpastatin (acetyl-calpastatin 184–210) [84, 85] were selective for calpain 1 and less or ineffective against CAPN5. Bortezomib, thought to be a more specific inhibitor of the 26S proteasome [55], also inhibited CAPN5. The results obtained here, through monitoring autolytic cleavage of the full-length CAPN5 in response to small molecule inhibitors, are consistent with the catalytic core adopting a more open conformation as reported by Mahajan and colleagues from their detailed structural analysis of the isolated core domains [16].

Previously we reported that the CAPN5 protein, in striking contrast to other calpains, was predominantly non-cytosolic and instead is enriched in crude mitochondrial and synaptic fractions and in the nucleus [8, 68]. Although nuclear localization sequences were identified, a mechanism for membrane association had not been experimentally investigated.

The C terminal, putative C2 domain provided a likely candidate. Models produced with RaptorX/Phyre2 aligned well with a structurally characterized C2 domain of an extended synaptotagmin, ESyt2-C2A, and it was hypothesized that it could provide calcium dependent membrane binding as a member of the PFAM C2 clan/PKC-C2 family (PFAM: PF00168) [29]. Substitution of each of three residues potentially important for calcium binding demonstrated that loss of D589 had the most significant impact on loss of CAPN5 from the membrane both by immunofluorescence and biochemical fractionation. Notably the appearance of the autoproteolytic product of CAPN5 was significantly decreased when D589 was mutated suggesting that membrane association enhances CAPN5 autoproteolytic activity. Mutation of D531 also resulted in both decreased membrane association and reduced autoproteolytic activity, consistent with predictions from the invariance of these residues in currently known human genomes and their alignment with key residues of other C2 domains.

Based on the finding that mutations to putative Ca^{2+} binding acidic residues in the CAPN5 C2 domain result in both decreased membrane association and reduced autolytic activity, and that autolytic fragments of CAPN5 were most prevalent in the membrane-enriched fraction, we propose that Ca^{2+}-regulated membrane localization of CAPN5 facilitates enzymatic activity. However, alternative explanations are possible, as discussed in greater detail below.

The results obtained with Asp to Asn mutations in the C2 domain of CAPN5 are similar to PKCα, where Asp residues in the C2 domain are critical for Ca^{2+} binding, translocation to the membrane, and resultant enzyme activation [26, 63, 66, 86, 87]. PKCα is normally present in the cytosol and translocates to the plasma membrane following elevations in intracellular Ca^{2+} [88]. In contrast, Ca^{2+} CAPN5 is present in both the cytosol and membrane fractions in resting cells, and elevations in Ca^{2+} do not markedly shift this distribution although they result in CAPN5 autolysis. Also, the enzyme/cytosol distribution of WT CAPN5 is similar to that of catalytically inactive CAPN5 (C81A). It could therefore be argued that the association of CAPN5 with the membrane is independent of Ca^{2+} binding and enzyme activation. As an example, C2 domains of PKCε and PTEN associate with membranes via Ca^{2+}-independent electrostatic mechanisms [89][90]. However, these proteins are not Ca^{2+}-regulated, and mutations of C2 domain Asp residues in PKCε did not alter lipid binding [91].

The C2 domain of PKCα, when expressed alone, is predominantly located to the plasma membrane [63]. Mutation of critical Asp residues to Asn abolishes the membrane association. The presence of other domains in the full-length protein is thought to restrict exposure of the C2 domain prior to activation, resulting in cytosolic localization in the absence of PKCα activation. In contrast, CAPN5 is able to associate with membranes in the absence of activation, similar to the PKCα C2 domain alone. This presents the question of how membrane associated CAPN5 is activated. For PKCα, translocation to the membrane and interaction with phosphatidylserine is essential for full enzyme activity [88]. A sequential model has been proposed in which the initial binding of Ca^{2+} to a subset of Asp residues in the C2 domain induces the initial association with membrane surfaces, while subsequent Ca^{2+} binding to additional Asp residues triggers membrane penetration

and activation [92][87]. CAPN5 appears to associate with the membrane while inactive and becomes activated by elevations in Ca^{2+}. This is consistent with a sequential model, in which resting levels of intracellular Ca^{2+} are sufficient to promote membrane association, while elevated Ca^{2+} levels are required for CAPN5 activation and autolysis.

While the results of the present study support the above sequential model, they do not allow us to determine with certainty whether the association of CAPN5 with membranes is the result of Ca^{2+}-dependent or -independent mechanisms. Although mutation of putative Ca^{2+} binding acid residues resulted in decreased membrane association and reduced CAPN5 autolysis in the presence of added Ca^{2+}, calcium binding was not demonstrated and it is possible that the results are due to structural alterations or changes in electrostatic interactions induced by the mutations, independent of Ca^{2+} binding. The precise mechanisms involved in CAPN5 association with membranes, and the mechanism by which Ca^{2+} promotes CAPN5 autolysis remains to be determined.

There are reports of activation of classical calpains by phospholipids [93], with the strongest evidence supporting an interaction of CAPN2 with phosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PIP2) [94]. Calpain activity at membranes has long been recognized with many membrane proteins identified as calpain substrates [79, 95–97]. More surprising perhaps for CAPN5 is that despite loss of a C terminal peptide and possibly most of the C2 domain, autolyzed CAPN5 remained predominantly associated with the membrane fraction. This suggests it may have an additional mechanism to bind to membranes, such as S-acylation (Gal et al., in preparation).

Membrane association of CAPN5 appears to be one of its inherent properties and is not unique to SH-SY5Y cells based on data found in two published quantitative proteomic studies [98, 99]. Using highly purified red blood cells (RBCs), Bryk & Wiśniewski documented protein concentrations from both whole cells and white ghosts, a crude membrane fraction from RBCs. Data for CAPN1 and CAPN5 are found within their results ([98], supplemental table 3). Enrichment of CAPN5 in the membrane is evident by comparing its reported protein concentration (pmol/mg) in the white ghosts relative to the whole cell and shows about a 10-fold enrichment in the membrane. They also reported protein concentrations for subcellular fractions of hepatocytes ([99]- Supplemental table 2 - sheet 4-subcellular fractionation) that includes data for CAPN1, CAPN2 & CAPN5. CAPN5 concentration was highest in membranes (by approximately 9.5 or 32 fold relative to the lysate). In contrast CAPN2 concentration was highest in the cytosol as would be expected. For the DEK1 transmembrane calpain from plants, autolysis is reported to release the catalytic domain with functional consequences on the transmembrane fragment remaining in the membrane [100]. For CAPN5 it appears that the catalytic domain remains membrane associated, at least in part.

In summary, the results of the present study demonstrate an apparent calcium concentration requirement and inhibitor sensitivity for CAPN5 to undergo Ca^{2+}-activated autoproteolysis with loss of C terminal residues. Our results also suggest that CAPN5 association with the membrane occurs, at least in part, through calcium binding to the C2 domain, although alternative explanations are possible. CAPN5 activation, as monitored via autolysis, may

occur exclusively at the membrane with the truncated CAPN5 remaining associated with the membrane. Determining the physiological substrates of CAPN5 and the functional consequences of their cleavage remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. S.W. Whiteheart and Dr. Smita Joshi, Department of Molecular and Cellular Biochemistry, University of Kentucky, for the mouse platelets in Fig 2. We also thank Dr. Ben L. King for his ideas, guidance and expertise for the bioinformatics analysis. DEC thanks AJ Bolduc and other former undergraduates for their efforts with the CAPN5 project at UMaine. This research was supported by the National Institutes of Health [Grant number R01NS095229 (JWG, DEC)]. KEL received partial support through an Honors College INBRE thesis fellowship provided by an Institutional Development Award (IDeA) from NIGMS [Grant number P20GM103423]. The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations Used:

3xFLAG	Peptide with 3 tandem FLAG repeats (Asp-Tyr-Lys-Asp-His-Asp-Gly- Asp-Tyr-Lys-Asp-His-Asp-Ile-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys)
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
ALLN	N-acetyl-Leu-Leu-NorLeu-al
CAPN1	Calpain 1 large subunit
CAPN2	Calpain 2 large subunit
CAPN5	Calpain 5
CAPNS1	Calpain small subunit
CBSW	Calpain beta sandwich domain
CNS	central nervous system
DEK1	Defective kernel 1
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EMBL-EBI	European Molecular Biology Laboratory- European Bioinformatics Institute
ERK	extracellular signal regulated kinase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
NP-40	nonyl phenoxypolyethoxylethanol-40

PC1	Protease core domain 1
PC2	Protease core domain 2
PIP2	phosphatidylinositol 4,5-bisphosphate
PSI-BLAST	Position-Specific Iterative-Basic Local Alignment Search Tool
RBCs	Red blood cells
SDS	Sodium Dodecyl Sulfate
TBS	Tris-buffered saline
TOMM20	Translocase of outer mitochondrial membrane 20
TTBS	Tris-buffered saline with 0.05% Tween 20 detergent
WT	Wild-type
Z-LLY-FMK	Carboxybenzyl-Leu-Leu-Tyr-fluoromethylketone

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Highlights

- Calpain 5 undergoes autoproteolysis upon activation by calcium
- Membrane localization appears essential for Calpain 5 activation
- Half-maximal CAPN5 autolysis required 80 μM Ca^{2+}
- C2 domain acidic residues contribute to membrane localization of CAPN5

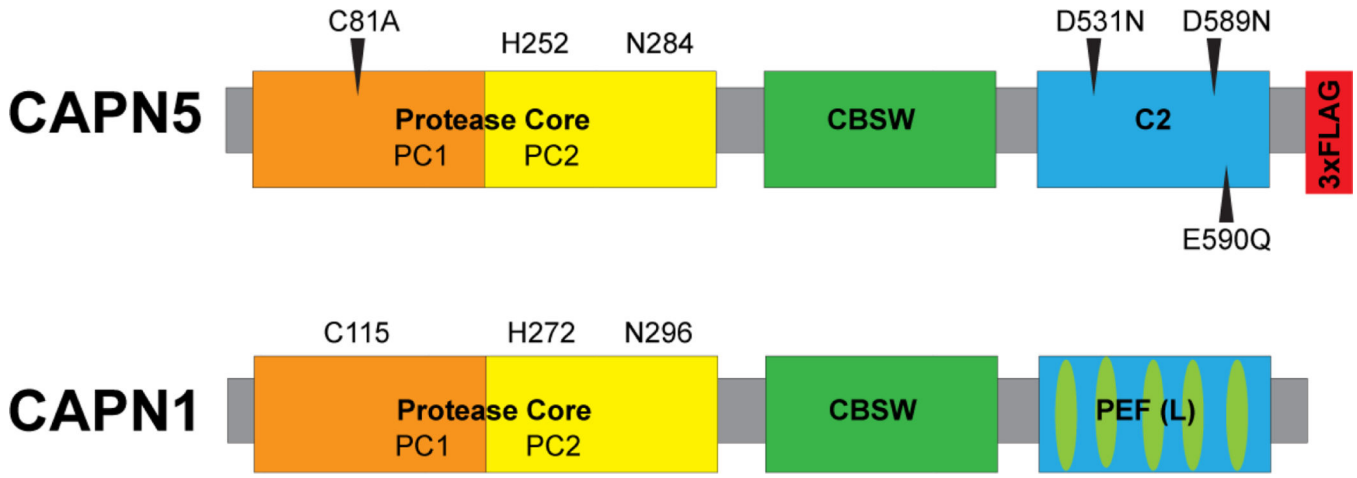

Figure 1. Domain structure and nomenclature of human CAPN5 and CAPN1.

PC1 and PC2 refer to protease core domains and contain the catalytic Cys-His-Asn triad, previously domains I and II, respectively. CBSW refers to calpain-type β-sandwich domain, previously the C2-like domain or domain III. PEF (L) is the penta-EF hand domain on the large subunit of calpain 1, characteristic of classical calpains (previously domain IV). There is a second PEF hand domain on the small subunit (CAPNS1) which forms a heterodimer with CAPN1 to form the active enzyme, calpain 1. CAPN5 lacks a PEF domain, but contains a unique C2 domain (previously domain T). CAPN5 is not known to require a subunit or dimerization for activity, in contrast to classical calpains. For most studies, we utilized a CAPN5 construct containing a 3xFLAG tag on the C-terminus. The arrowheads illustrate mutations introduced into CAPN5. Mutation of the Cys in the protease core (C81A) results in a catalytically dead protein. Mutations of the putative calcium binding acidic residues in the C2 domain (D531N, D589N, E590Q) were introduced to determine their influence on CAPN5 activity and localization.

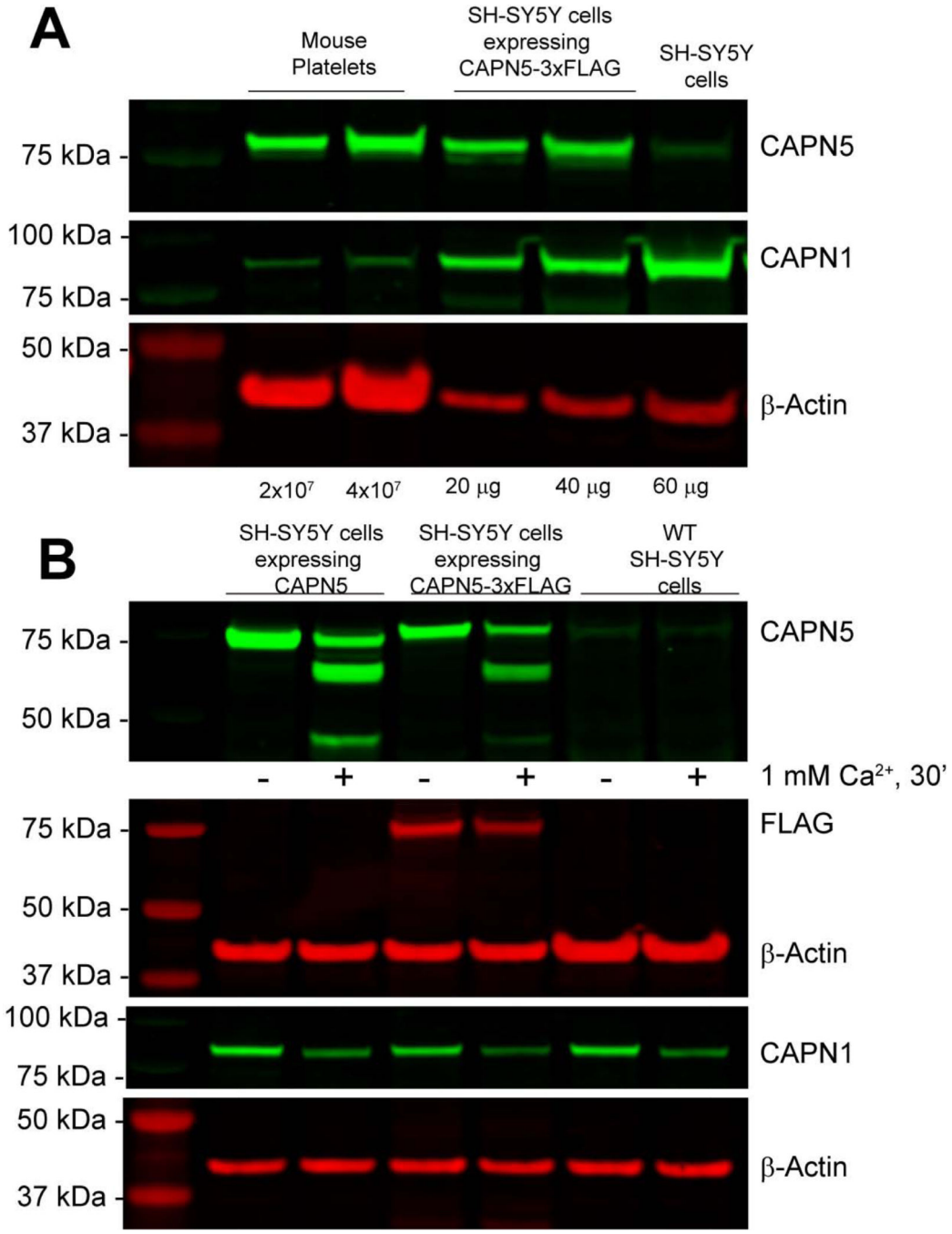

Figure 2. CAPN5 expression levels and autolysis.

CAPN5 and CAPN1 expression was examined by immunoblotting in untreated SH-SY5Y cells, SH-SY5Y cells stably expressing CAPN5 or CAPN5–3xFLAG, and in isolated mouse platelets. **(A)** CAPN5 was faintly detected in untreated SH-SY5Y cells, was more abundant in cells expressing the CAPN5–3xFLAG construct, and was also present in mouse platelets (β-actin levels are known to be high in these cells [101]). CAPN1 was faintly detected in mouse platelets but was more strongly detected in SH-SY5Y where its levels reflected protein loading and did not appear to be influenced by the presence of the CAPN5–3xFLAG

construct. **(B)** Cells were permeabilized and incubated as indicated with 1 mM $CaCl_2$ for 30 min. The resultant blot was immunostained with antibodies against CAPN5, FLAG, and β-actin. Molecular weight standards are also shown. In cells expressing CAPN5, a band of CAPN5 immunoreactivity was evident at ~75 kDa corresponding to full-length CAPN5, close to the calculated molecular weight of 73.2 kDa. This band was also evident, although faint, in WT SH-SY5Y cells. In cells expressing CAPN5–3xFLAG, the immunoreactive band migrated at ~76 kDa, consistent with its calculated molecular weight of 76.5 kDa, and was also detected with anti-FLAG. Incubation of SH-SY5Y cells with 1 mM $CaCl_2$ for 30 minutes resulted in the appearance of CAPN5 immunoreactive bands of ~61 and ~45 kDa. These bands were of similar molecular weight in extracts from cells expressing CAPN5 and CAPN5–3xFLAG and were not detected with anti-FLAG. This suggests that the lower molecular weight bands resulted from cleavage events which occur between the epitope recognized by anti-CAPN5 in the central region of the protein and the C-terminus. For the CAPN5 immunoblot and accompanying FLAG and β-actin immunoreactivity, 20 μg protein was loaded into each lane for SH-SY5Y cells expressing CAPN5 or CAPN5–3xFLAG, and 60 μg protein was utilized for WT SH-SY5Y cells. For the CAPN1 immunoblot, 20 μg protein was loaded for each lane of cell extracts.

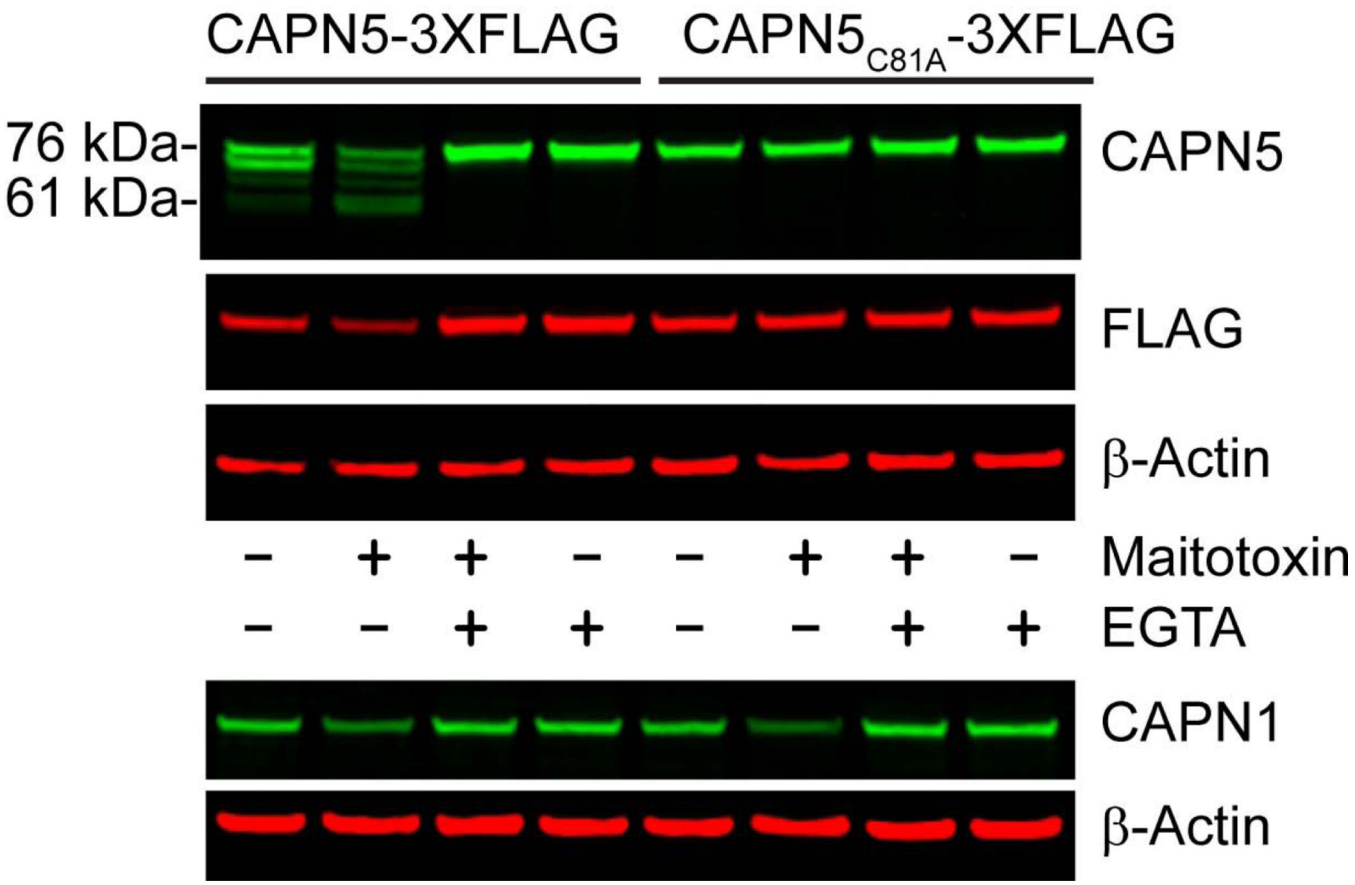

Figure 3. CAPN5 autolysis following treatment with maitotoxin.

SH-SY5Y cells, stably transfected with CAPN5–3xFLAG or with $CAPN5_{C81A}$-3xFLAG, were treated with maitotoxin (0.8nM) in the media, in the presence or absence of 5mM EGTA for 60 minutes. The culture media was removed, and the cells were processed for SDS-PAGE and immunoblotting. CAPN5 breakdown products were evident in untreated cells, with breakdown exacerbated by maitotoxin treatment. EGTA diminished the levels of CAPN5 breakdown products with or without maitotoxin. Breakdown products were not detected in cells expressing the catalytically inactive $CAPN5_{C81A}$-3xFLAG mutant with or without maitotoxin.

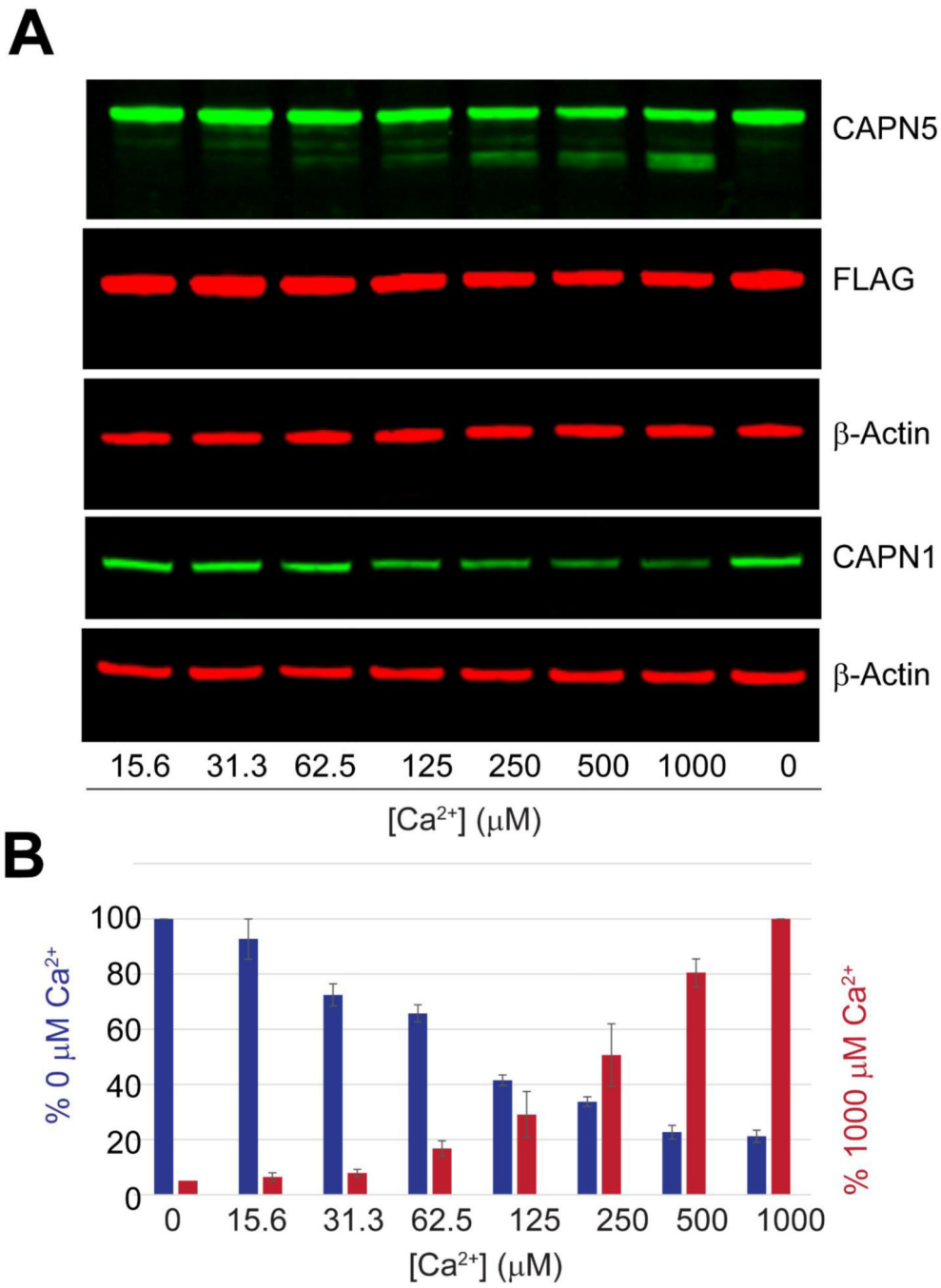

Figure 4. Calcium activation of CAPN5.

(A) Extracts from SH-SY5Y cells expressing WT CAPN5–3xFLAG were treated with 16 μM to 1 mM $CaCl_2$ at 37°C for 30 minutes. This resulted in the loss of full-length CAPN5 (~76 kDa) and the appearance of lower mol. wt bands, indicative of CAPN5 activation and autoproteolysis. Endogenous CAPN1 was also evaluated as a control, using an antibody against the N-terminus known to be removed by autolysis. The decrease in CAPN1 immunoreactivity is indicative of activation and autoproteolysis. Quantitative CAPN5 results are shown in (B). The blue bars represent full-length CAPN5 as % of the

level in the absence of added Ca^{2+}. The red bars indicate levels of the 61 kDa breakdown product as % of the 1 mM Ca^{2+} level. The results are mean ± SEM, n=4, or n = 3 for 31 μM when measuring loss of substrate and 500 μM when measuring gain of product because one value for each was >3x the standard deviations from the mean value.

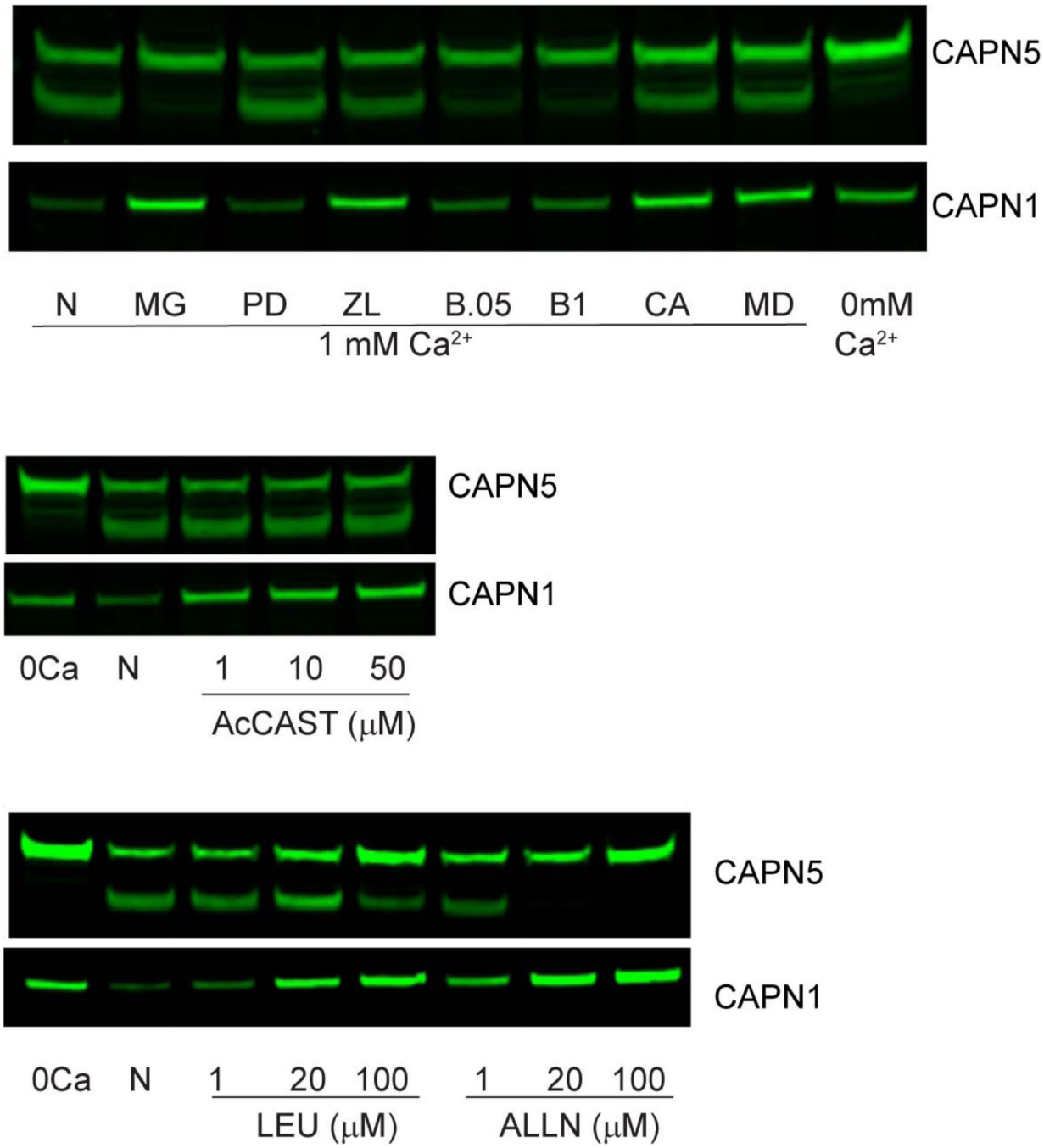

Figure 5. Small molecule inhibitors of CAPN5.

SH-SY5Y cells expressing CAPN5–3xFLAG were incubated with small molecule inhibitors in the media overnight. Following treatment, cells were washed with HEPES buffer and resuspended in HEPES buffer containing 1% NP40 and 1 mM $CaCl_2$ and the respective small molecule inhibitors. Following incubation, cells were lysed and processed for immunoblotting. Loss of full-length CAPN5 and increased intensity of the CAPN5 breakdown products is indicative of CAPN5 autoproteolysis in the presence of 1 mM Ca^{2+} without added inhibitors (N, no treatment). Reduced intensity of the breakdown product

and increased intensity of full-length CAPN5 following treatment with small molecule inhibitors is indicative of CAPN5 inhibition. Endogenous CAPN1 was also included as a positive control for the small molecule inhibitors. Preservation of CAPN1 immunoreactivity following incubation with 1 mM $CaCl_2$ is indicative of CAPN1 inhibition. Abbreviations: N, no added inhibitor; MG, MG132 10 μM; PD, PD150606 100 μM; ZL, ZLLY-FMK, 20 μM; B.05, Bortezomib .05 μM; B1, Bortezomib 1 μM; CA, Calpeptin 10 μM; MD, MDL28170 20 μM; AcCAST, Acetyl Calpastatin 184–210; LEU, Leupeptin. CAPN5 inhibition was evident with MG132, Bortezomib, and higher doses of ALLN. CAPN1 inhibition was observed with MG132, Z-LLY-FMK, Calpeptin, MDL28170, Acetyl Calpastatin, Leupeptin, and ALLN.

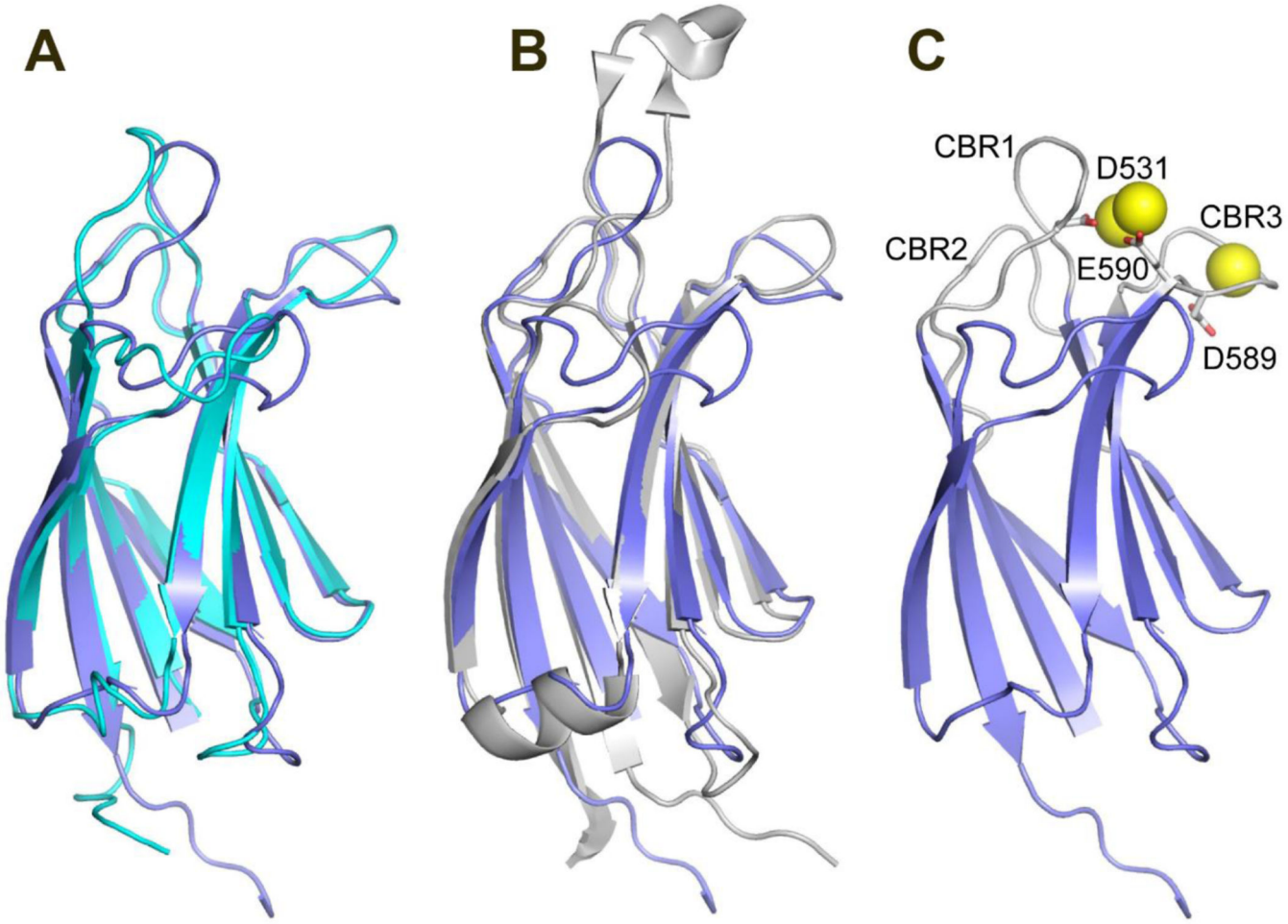

Figure 6. Homology models of the CAPN5 C-terminal domain.

A) Superposition of the final models from the RaptorX (purple) and Phyre2 (cyan) homology modeling servers based on the C-terminal 123 residues of CAPN5. The models are shown in a ribbons representation and both adopt the eight-stranded beta-sandwich characteristic of a C2 membrane interaction domain. **B**) Superposition of the CAPN5 C2 model from RaptorX (purple) with the crystal structure of the extended synaptotagmin-2 C2A domain (gray), which binds three calcium ions (not shown). **C**) CAPN5 C2 model with calcium ions (yellow spheres) placed based on superposition with the extended synaptotagmin-2 C2A domain. The side chains for residues mutated to potentially disrupt calcium ion binding (D531, D589, E590) are shown in a stick representation. The three calcium binding region (CBR) loops characteristic of C2 domains are colored gray and labeled.

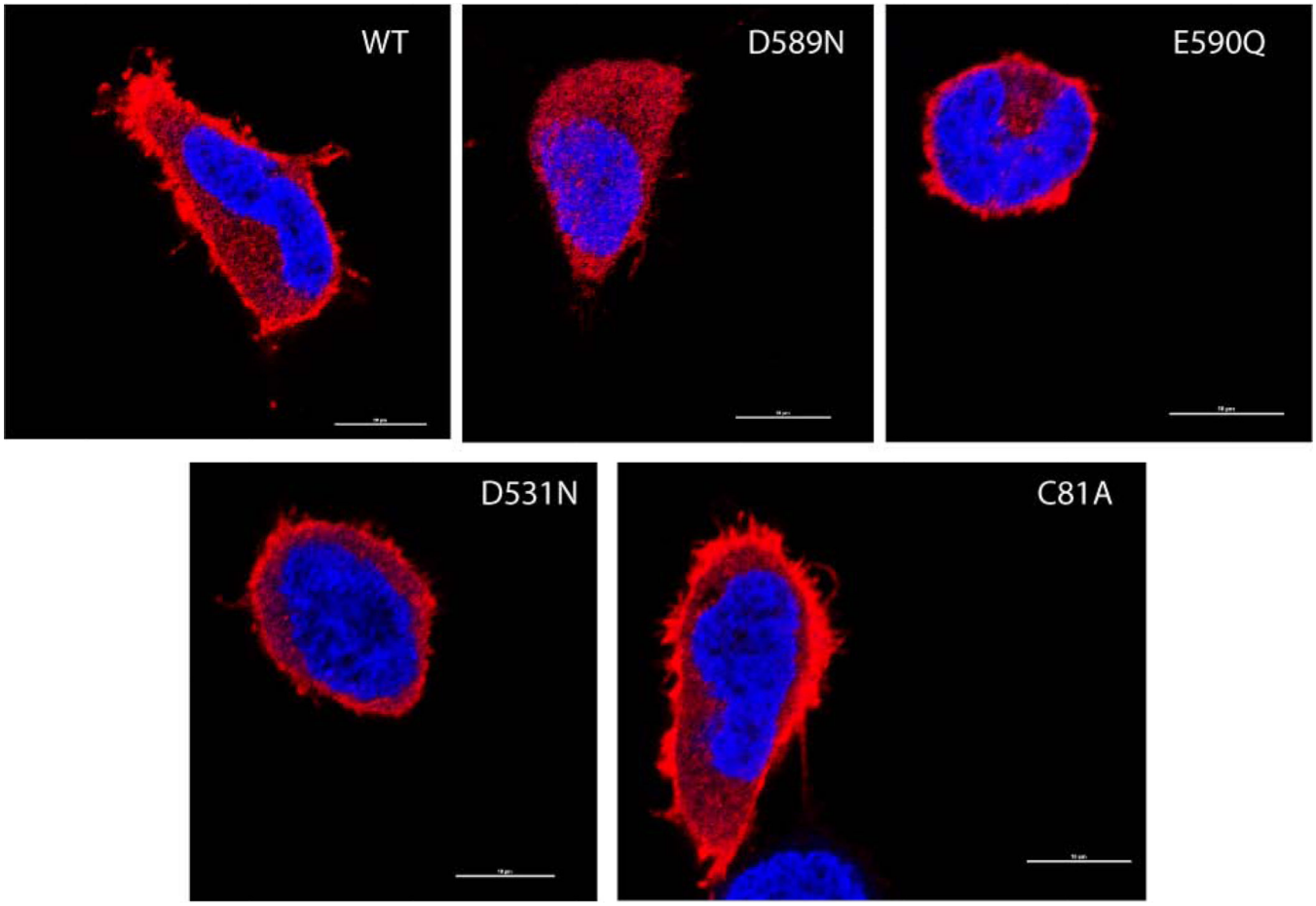

Figure 7. Localization of CAPN5 is altered when putative calcium binding residues are mutated in its C2 domain.

CAPN5 localization was visualized using an anti-FLAG antibody to ensure detection of only full-length CAPN5–3XFLAG. SH-SY5Y cells stably expressing catalytically active CAPN5–3xFLAG with WT C2 domain or mutants of residues predicted to bind Ca^{2+} within the C2 domain (D531N, D589N, or E590Q) were examined along with catalytically dead $CAPN5_{C81A}$-with a WT C2 domain. CAPN5–3xFLAG, whether proteolytically active WT or inactive C81A, was most abundantly associated with the plasma membrane, with diffuse punctate immunoreactivity located in the cytosol and nucleus. $CAPN5_{D589N}$ exhibited the most dramatic alteration in localization, being present in the cytosol but without enrichment at the plasma membrane. CAPN5 with either D531N or E590Q exhibited some enrichment at the plasma membrane, albeit to a lesser degree by comparison with WT and C81A CAPN5, although this may also reflect the observed lower expression level of each C2 domain mutant/variant. Nuclei were stained (blue) with Hoechst 33258. Scale bars=10 μm.

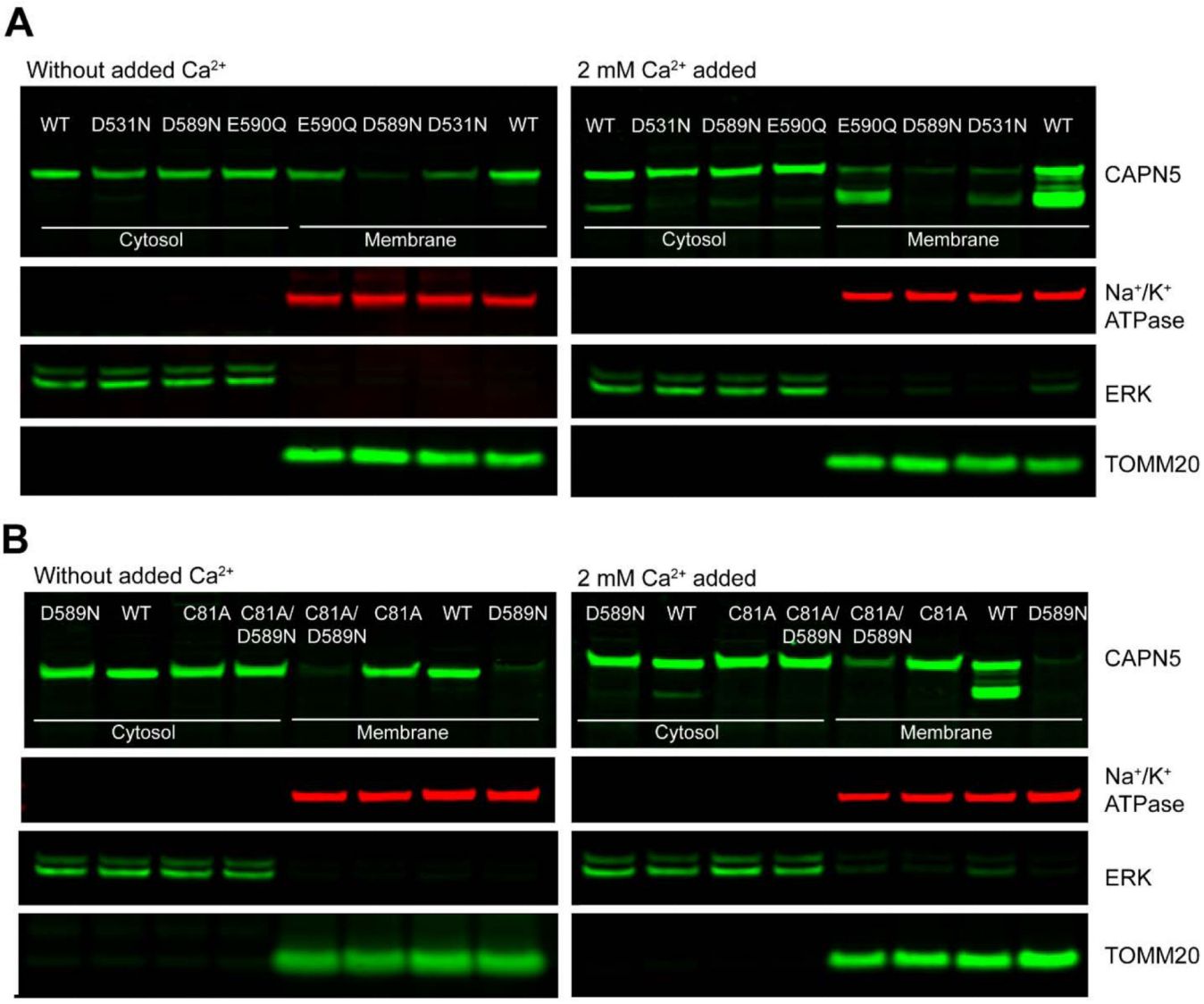

Figure 8. Cellular fractionation reveals the presence of both full-length and autoproteolyzed CAPN5 associated with the membrane.

(A) SH-SY5Y cells, stably expressing WT CAPN5–3xFLAG with the WT sequence or mutants of acidic C2 domain residues predicted to bind Ca^{2+} (D589N, D531N, and E590Q), were permeabilized with or without 2 mM $CaCl_2$ followed by separation of cytosolic and membrane-enriched fractions. B) SH-SY5Y cells, stably expressing CAPN5–3xFLAG with the WT sequence, catalytically inactive C81A, the C2 domain mutant D589N, or both C81A/ D589N double mutant. For WT and C81A CAPN5–3xFLAG, 10 μg was loaded per lane. It was necessary to load more lysate protein (30 μg/lane) for immunodetection of D589N, D531N, E590Q and C81A/D589N to compensate for their lower expression level. In parallel the fractions were also probed for the Na^+/K^+ ATPase as a marker of the plasma membrane, ERK as a marker of the cytosol (20 μg protein/ lane), and TOMM20 as a mitochondrial marker. In the absence of added Ca^{2+}, WT CAPN5–3xFLAG and $CAPN5_{C81A}$-3xFLAG were present at similar levels in cytosolic and membrane fractions. $CAPN5_{D531N}$ and $CAPN5_{E590Q}$ were prevalent in the cytosolic fraction. $CAPN5_{D589N}$ and

$CAPN5_{C81A/D589N}$ were largely restricted to the cytosol, with only a faint band being detected at the membrane. This distribution was similar for cells permeabilized in the presence of 2 mM Ca^{2+}. However, in the presence of added Ca^{2+}, lower molecular weight CAPN5-immunoreactive bands were observed, predominantly in the membrane fractions. These proteolytic fragments of were most pronounced for WT CAPN5 followed by E590Q. The autolytic fragments were not detected for D589N, catalytically dead C81A, or for C81A/D589N mutants of CAPN5.

Key Resources Table

Reagent or Resource	Source/Identifier
Antibodies	
CAPN5	GeneTex Cat# GTX103264, RRID:AB_1949829
CAPN1	Abcam Cat# ab28257, RRID:AB_725818
FLAG	Sigma-Aldrich Cat# F3165, RRID:AB_259529
β-actin	Sigma-Aldrich Cat# A2228, RRID:AB_476697
Na^+/K^+ ATPase	Abcam Cat# ab7671, RRID:AB_306023
ERK1	Santa Cruz Biotech. Cat# sc-94, RRID:AB_2140110
IrDye 800CW Goat anti-rabbit IgG	LI-COR Biosciences Cat# 926-32211, RRID:AB_621843
AlexaFluor 680 Goat anti-mouse IgG	Thermo Fisher Cat# A-21058, RRID:AB_2535724
Chemicals	
MG132	Sigma-Aldrich, Cat#M7449
PD150606	Sigma-Aldrich, Cat#D5946
Calpain Inhibitor IV, Z-LLY-FMK	Millipore, Cat#208724
Bortezomib	Selleckchem, Cat#S1013
Calpeptin	Sigma-Aldrich, Cat#03-04-0051
Calpain Inhibitor III-MDL28170	Calbiochem, Cat#208722
Acetyl Calpastatin 184-210	Sigma-Aldrich, Cat#CH6H11E4F558
Leupeptin	APExBIO, Cat#A2570
Calpain Inhibitor I-ALLN	APExBIO, Cat#A2602
Experimental Models: Cell Lines	
SH-SY5Y human neuroblastoma	ATCC Cat# CRL-2266, RRID:CVCL_0019