Calpain-1 Cleaves and Activates Caspase-7*

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Caspase-7 is an executioner caspase that plays a key role in apoptosis, cancer, and a number of neurodegenerative diseases. The mechanism of caspase-7 activation by granzyme B and caspase-3 has been well characterized. However, whether other proteases such as calpains activate or inactivate caspase-7 is not known. Here, we present that recombinant caspase-7 is directly cleaved by calpain-1 within the large subunit of caspase-7 to produce two novel products, large subunit p18 and p17. This new form of caspase-7 has a 6-fold increase in \( V_{\text{max}} \) when compared with the previously characterized p20/p12 form. Zymography revealed that the smaller caspase-7 product (p17) is 18-fold more active than either the caspase-3-cleaved product (p20) or the larger calpain-1 product of caspase-7 (p18). Mass spectrometry and site-directed mutagenesis identified the calpain cleavage sites within the caspase-7 large subunit at amino acid 36 and 45/47. These proteolysis events occur \textit{in vivo} as indicated by the accumulation of caspase-7 p18 and p17 subunits in cortical neurons undergoing \( \text{Ca}^{2+} \) dysregulation. Further, cleavage at amino acid 45/47 of caspase-7 by calpain results in a reduction in nuclear localization when compared with the caspase-3 cleavage product of caspase-7 (p20). Our studies suggest the calpain-activated form of caspase-7 has unique enzymatic activity, localization, and binding affinity when compared with the caspase-activated form.

Apoptosis is a well-defined cellular destruction pathway that primarily utilizes a family of cysteine proteases, the caspases (1, 2). This cell death program can be initiated by cell death receptor activation (extrinsic pathway) or a variety of drugs or cellular stresses (intrinsic pathway) leading to activation of apical caspase-8, -9, and/or -10 (1, 3, 4). These initiator caspases in turn directly activate the executioner caspases, caspase-3 and -7, which through proteolysis of defined substrates are responsible for the dismantling of the cell and subsequent death (3, 4). Granzyme B, released by cytotoxic T lymphocytes to protect the host from pathogens and tumor cells, can also initiate this apoptotic cascade and therefore is considered an apical caspase mimic (5–7). All caspases, as well as granzyme B, preferentially cleave after aspartic acid residues, with many having well-defined consensus sequences, making substrate cleavage sites easy to predict and establish (3, 4, 7, 8).

Caspases exist in a latent form prior to activation. Both the initiator and executioner caspases are synthesized as a single chain protein, which require proteolytic cleavage to become active. Procaspase-7 is expressed as a 303-amino acid residue polypeptide chain. The activation and regulation of executioner caspase-7 by caspases and granzyme B has been extensively studied. Caspase-7 requires cleavage by caspase-3 and caspase-8/-10 or granzyme B, for activation (6, 9). Current evidence suggests that caspase-3 initially cleaves off the first 23 amino acids (propeptide, 2 kDa), followed by caspase-8/-10 or granzyme B cleaving between the large (20 kDa) and small (12 kDa) subunit after amino acid 198 to activate the enzyme. The large subunit containing the catalytic His-237 and Cys-285 (caspase-1 numbering convention), and the small subunit are involved in the formation of the substrate-binding region. \textit{In vitro}, granzyme B can also activate caspase-7 independently of caspase-3, but this does not appear to occur \textit{in vivo} (5, 6). Currently, there is no evidence that other classes of proteases play a role in activating or modulating caspase-7 activity.

Changes in intracellular \( \text{Ca}^{2+} \) levels influence apoptosis in a number of cell types (10–13). Because in many of these apoptotic cell models the \( \text{Ca}^{2+} \)-dependent cysteine proteases, calpains, are activated upstream of caspases (14–16), it is possible that calpains may activate and/or modulate caspase activity via direct cleavage. Studies directed at understanding calpains with respect to caspase activation are limited. Calpain-2 was shown to cleave procaspase-9, decreasing its activity (17). In the same study, calpain-2 treatment cleaved procaspase-7 to produce a single, novel fragment, but in this case the effect on enzymatic activity was not investigated (17). To improve our understanding of calpains and the role of calcium in cell death, we carried out studies directed at understanding how calpains activate or modulate caspase activity. We found that calpain treatment produced a large increase in caspase-7 activity. Calpain cleaves procaspase-7 to produce two large subunits of 18.5 and 17.2 kDa, the smaller of which has a robust increase in activity relative to the 20-kDa large subunit produced by caspase-3 cleavage of caspase-7. Both calpain cleavage sites in caspase-7 are identified using mass spectrometry. \( N \)-methyl-\( \text{d} \)-aspartate-induced \( \text{Ca}^{2+} \)-dependent cell death in primary cortical neurons produced calpain-derived caspase-7 cleavage products \textit{in vivo}. Lastly, the strictly cytosolic localization of the smaller calpain fragment confirms that a previously identified nuclear localization signal (18) is involved in caspase-7 cytosolic/nuclear distribution. Our data suggest that increases in \( \text{Ca}^{2+} \) leading to activation of calpains may significantly modulate caspase-7 activity and thus, apoptosis.

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MATERIALS AND METHODS

Plasmids and Enzymes—Caspase-1 through caspase-3 and caspase-5 through-10 plasmids and active caspase-7, -8, -10, and -11 were gifts from Guy Salvesen. Caspase-4 plasmid was a gift from Jean-Louis Lalanne. Mouse caspase-12 and human caspase-13 plasmids were cloned into pcDNA3 by PCR amplification and sequenced. Procaspase-7, truncated caspase-7, active caspase-1 through caspase-6 and granzyme B were purchased from Biomol. Calpain-1 was obtained from Calbiochem, and calpain-2 was obtained from Sigma.

Mutagenesis of Caspase-7 Construct—Site-directed mutagenesis was used to identify and evaluate calpain cleavage of caspase-7. Amino acids 35–37, 44–46, and 46–48 were deleted individually in the pcDNA3-caspase-7-FLAG construct (9). Amino acids 43–46 were subsequently deleted from the pcDNA3-caspase-7 (ΔΔ46–48)-FLAG construct to produce a ΔΔ43–48 mutant. The aspartate at amino acid 23 was mutated to an asparagine in pcDNA3-caspase-7-FLAG construct to use as control. Sites were deleted or mutated by polymerase chain reaction mutagenesis using the following primers: caspase-7

| Primer ID | Sequence |
|----------|----------|
| F9004 | 5′-CTCGGTCCCGG-GTGGCTTAGCATTCACTGA-3′ |
| R9013 | 5′-AACCCGGTTGTCTGGCTTAGCATTCACTGACCTTTTCCTTCTTC-3′ |

Site-directed mutagenesis was performed using 100 ng of pcDNA3-caspase-7-FLAG, 5.0 μM dNTPs (Promega), 0.2 mM MgCl₂, 0.2 mM dNTPs (Promega), 125 ng each of forward and reverse primers (Integrated DNA Technologies), 5.0% Me₂SO, and 1.0 mM Pfu buffer (Stratagene) for 16 cycles at 96 °C for 1 min, 55 °C for 1 min, and 65 °C for 20 min, then 65 °C for 7 min. Plasmids were DpnI (Promega)-treated, transformed into XL1-Blue supercompetent cells (Stratagene), and purified using the QIAprep Spin Miniprep Kit (Qiagen), and 0.2 mM dNTPs (Promega), 125 ng each of forward and reverse primers (Integrated DNA Technologies), 5.0% Me₂SO, and 1.0 μM Pfu polymerase (Stratagene) for 16 cycles at 96 °C for 1 min, 55 °C for 1 min, and 65 °C for 20 min, then 65 °C for 7 min. Plasmids were DpnI (Promega)-treated, transformed into XL1-Blue supercompetent cells (Stratagene), and purified using the QIAprep Spin Miniprep Kit (Qiagen). Mutations were confirmed by DNA sequencing.

Caspase-7 C-terminal Constructs—Caspase-7 constructs representing the C terminus of caspase-7 after cleavage with calpain (amino acids 37–303 and 48–303) or caspase-7 (amino acids 24–303) were made utilizing the pcDNA3-caspase-7-FLAG construct (9). The caspase-7 C-terminal products were amplified using polymerase chain reaction with the following primers:

| Primer ID | Sequence |
|----------|----------|
| F9004 | 5′-CTCGGTCCCGG-GTGGCTTAGCATTCACTGA-3′ |
| R9013 | 5′-AACCCGGTTGTCTGGCTTAGCATTCACTGACCTTTTCCTTCTTC-3′ |

Plasmids of caspase-7 constructs were made utilizing the pcDNA3-caspase-7-FLAG construct (9). The caspase-7 C-terminal products were amplified using polymerase chain reaction with the following primers:

| Primer ID | Sequence |
|----------|----------|
| F9004 | 5′-CTCGGTCCCGG-GTGGCTTAGCATTCACTGA-3′ |
| R9013 | 5′-AACCCGGTTGTCTGGCTTAGCATTCACTGACCTTTTCCTTCTTC-3′ |

Polymerase chain reactions were performed using 100 ng of pcDNA3-caspase-7-FLAG, 5.0 μM of 10X Pfu buffer (Stratagene), 0.2 mM dNTPs (Promega), 100 ng each of forward and reverse primers (Integrated DNA Technologies), 5.0% Me₂SO, and 1.0 μM Pfu polymerase (Stratagene) for 30 cycles at 95 °C for 1 min, 56 °C-62 °C for 1 min, and 72 °C for 3 min, then 72 °C for 10 min. Samples were purified using the Qiaquick PCR Purification Kit and Qiaquick Gel Extraction Kit (Qiagen). DNA inserts were then cleaved with XhoI/KPN1 (Promega), re-purified, and ligated to an XhoI/KPN1-treated pcDNA3 plasmid using T4 DNA ligase (Promega) overnight at 16 °C. Final constructs were verified using DNA sequencing.

In Vitro Protein Synthesis and Cleavage—Caspase constructs were translated with ³⁵S-labeled methionine (Promega coupled kit) and incubated with 9.0 units of calpain-1 in Nonidet P40 buffer (19) with 5 μM dithiothreitol (DTT)² and 20 mM CaCl₂ at 30 °C for 30 min. Reactions were terminated by addition of EDTA, SDS sample buffer, and boiling. One unit of calpain is defined as the amount of enzyme that will hydrolyze 1 pmol of Suc-LLVY-AMC in 1 min at 25 °C using the Fluorogenic Calpain Activity Assay kit (Calbiochem).

Caspase Activity Assays—Purified procaspase-7, ΔN caspase-7 (no prodomain) or active caspases were treated with calpain-1 (9.0 units) or calpain-2 (100 units) for 30 min at 30 °C in Nonidet P40 buffer with 5 μM DTT and 20 mM CaCl₂. In some cases, samples were co-incubated with granzyme B (200 units). One unit of granzyme B is defined as the amount of enzyme that will hydrolyze 1 pmol of Ac-IETD-pNA in 1 min at 30 °C. Following incubation, caspase activity was measured using appropriate fluorescently tagged substrates. Substrates used were: Ac-YVAD-AFC (Biomol) for caspase-1, -4, -5, and -11, Ac-VDVAD-AMC (Biomol) for caspase-2, (α-DEVD)-R110 (Cell Technology, Inc) and Ac-DEVD-AMC (Biomol) for caspase-3 and -7, Ac-IETD-AMC (Biomol) for caspase-6 and -8 and Ac-LEHD-AFC (Biomol) for caspase-10. Change in fluorescence was measured in a caspase assay buffer (20 mM Pipes, pH 7.2, 100 mM NaCl, 1% CHAPS, 10% sucrose, 10 mM DTT) at 37 °C for 1 h in a SpectraMAX Gemini fluorimeter (Molecular Devices Corporation). A Student’s t test or one-way analysis of variance with Tukey’s multiple comparison test was used to determine statistical significance.

Western Analysis—Superfect reagent (Qiagen) was used for transient transfections in human embryonic kidney 293T cells with the pcDNA3-caspase-7-FLAG constructs described above. Primary cortical cultures were made from ED16 mice and treated with AraC for 24 h at 3 DIV. Cultures were treated with 0, 100, or 250 μM N-methyl-d-aspartate for 3 h at 12 DIV. Cells were lysed with mammalian protein extraction reagent (MPER, Pierce) or fractionated into cytoplasmic and nuclear fractions with the NE-PER nuclear and cytoplasmic reagents (Pierce) in the presence of protease inhibitors (Complete mini, Roche Applied Science). Protein was determined using a BCA assay kit (Pierce). In some cases, samples were treated with 0.9 to 12.0 units of calpain-1 at 30 °C for 10 to 30 min with or without co-incubation with 200 units of gran-

² The abbreviations used are: DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; vMALDI, vacuum matrix-assisted laser desorption/ionization; LTQ, linear trap quadrupole; PARP, poly(ADP-ribose) polymerase.
zyme B. Samples were boiled in NuPAGE sample buffer (Invitrogen) with 50 mM DTT, resolved on a 12% polyacrylamide gel and transferred to nitrocellulose. Blots were probed with the following antibodies: polyclonal caspase-7 9492 (1:200, Cell Signaling), polyclonal-cleaved caspase-7 94915 (1:200, Cell Signaling), polyclonal DYKDDDD tag 2368 (1:200, Cell Signaling), polyclonal DYKDDDD tag T503 (4.0 µg/ml, Signalway Antibody), monoclonal α-tubulin T-6119 (1.0 µg/ml, Sigma), polyclonal calreticulin (1:20,000, Stressgene), and polyclonal poly(ADP-ribose) polymerase SA-253 (PARP; 1:3000, Biomol). Proteins were subsequently visualized using an enhanced chemiluminescence kit (Thermo Scientific).

**Mass Spectrometry**—Purified active caspase-7 was incubated with calpain-1 (9.0 units) at 30 °C for 30 min in 50 mM Hepes, pH 7.4, 250 mM NaCl, 5 mM EDTA, 20 mM CaCl2, and 5 µM DTT. The samples were concentrated and purified using ZipTipC18® pipette tips (Millipore) prior to mass spectrometric analysis following manufacturer’s instruction. Mass spectra of the samples were obtained by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Voyager DESTR plus instrument (Applied Biosystems) using 3.5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) as the matrix. Cytochrome c (Sigma) was used as external standard for calibration.

For the vMALDI experiment, ΔN caspase-7 was incubated with calpain-1 (9.0 units) at 30 °C for 30 min in 50 mM Hepes, pH 7.4, 250 mM NaCl, 5 mM EDTA, 20 mM CaCl2, and 5 µM DTT. The samples were then concentrated and purified using ZipTipC18® pipette tips (Millipore) prior to mass spectrometric analysis following manufacturer’s instruction. Mass spectra of the samples were obtained on a vMALDI-linear trap quadrupole (LTQ) mass spectrometer (Thermo Fisher) using manual data acquisition. Threshold values of 1800 counts for MS and 80 counts for MS2 were applied to collect spectra. Parent ion isolation width is 3 m/z units. Fragmentation setting of activation Q = 0.25, activation time = 30 ms, and relative collision energy of 30% were used. vMALDI-LTQ data were searched against a custom data base containing full-length human energy of 30% were used. vMALDI-LTQ data were searched against a custom data base containing full-length human

**RESULTS**

**Activity of Caspase-7 Is Increased by Calpain-1 Cleavage**—Previous studies have demonstrated that calpains are activated upstream of caspases in a variety of cell models (14–16). Therefore, we tested whether calpains can directly cleave caspases and the impact of cleavage on caspase activity. In *in vitro*-translated 35S-labeled procaspases were treated with active calpain-1 to determine which caspases are calpain substrates. Each caspase, with and without calpain treatment, was run on a single gel and developed by autoradiography. All of the caspases were run on four separate gels.

**Calpain-1 Directly Activates Caspase-7**

![FIGURE 1. Calpain-1 cleaves procaspases.](image_url)

*Calpain-1 cleaves procaspases. In vitro-translated 35S-labeled procaspases were treated with active calpain-1 to determine which caspases are calpain substrates. Each caspase, with and without calpain treatment, was run on a single gel and developed by autoradiography. All of the caspases were run on four separate gels.*

**Active caspases were also preincubated with calpain-1 to see the effect on activity. The activities of the majority of caspases were unchanged by calpain-1 preincubation (Fig. 2A; n = 3). Interestingly, the activity of caspase-1, one of the caspases that had numerous calpain cleavage products, was reduced by 69% following calpain-1 treatment (Fig. 2B; n = 3; *, p < 0.001).**

Surprisingly, the activity of active caspase-7 and active caspase-10 increased following calpain-1 pretreatment (Fig. 2C). Caspase-7 activity increased 4-fold (n = 3, ***, p < 0.001) and caspase-10 activity increased 27% (n = 3, **, p < 0.01) (Fig. 2C). A similar increase in caspase-7 activity was also observed following incubation with calpain-2 (data not shown). Additional experiments with active caspase-7 showed that variations in calpain-1 preincubation times (5 min to 1 h) and increases in the amount of calpain added did not further increase caspase-7 activity (data not shown).

Calpain-1 pretreatment produced two defined cleavage products and a substantial increase in caspase-7 activity. Therefore, we tested whether calpain-1 could activate procaspase-7 alone, because granzyme B can independently activate pro-
Calpain Directly Activates Caspase-7

Caspase-7 in vitro (5). When pro-caspase-7 was preincubated with calpain-1, caspase-7 was not activated as measured by a DEVD-rhodamine fluorescent substrate (Fig. 3A). As expected, granzyme B incubation alone activated procaspase-7 (Fig. 3A; n = 3, ###, p < 0.001) and calpain-1 and granzyme B co-incubation increased the activity by 2.4-fold (Fig. 3A; n = 3, ***, p < 0.001). When purified caspase-7 without the prodomain (∆N caspase-7, first 23 amino acid removed) was preincubated with calpain-1 there was also no evidence of calpain-1 independently activating ∆N caspase-7 (Fig. 3B). As with procaspase-7, when ∆N caspase-7 was incubated with granzyme B there was a significant increase in activity (Fig. 3B; n = 3, #, p < 0.05) and co-incubation with granzyme B and calpain-1 led to a 3.3-fold increase in caspase-7 activity relative to preincubation with granzyme B alone (Fig. 3B; n = 3, ***, p < 0.001). From these results it is clear that calpain-1 can only increase the activity of caspase-7 following granzyme B cleavage and activation between the linker of the small and large subunit.

Calpain-1 Produces Two Caspase-7 Cleavage Products with the Smaller Fragment Displaying Increased Activity—To identify the number and size of calpain cleavage products, purified procaspase-7 and ∆N caspase-7 was cleaved with increasing amounts of calpain-1, and protein was visualized with a Coomassie Blue stain (Fig. 4A). Two calpain cleavage products were produced, both smaller than ∆N caspase-7 and caspase-3-cleaved caspase-7 protein. Cleavage appeared to occur sequentially with lower amounts of calpain-1 producing the larger calpain fragment and higher amounts of calpain-1 producing more of the smaller calpain product. To determine which of the two calpain fragments produced are active, zymography of active caspase-7 with and without calpain-1 pretreatment was performed. After running samples on a denaturing gel, caspases were renatured and exposed to a DEVD fluorescently tagged substrate (Ac-DEVD-MCA) to visualize caspase-7 activity. Prior to calpain treatment, the caspase-3-cleaved active product of caspase-7 appeared as a blurry, faint activity band at ~19 kDa (Fig. 4B). Following calpain-1 treatment, the faint 19-kDa activity band disappeared and a distinct, robust activity band appeared at ~15 kDa (Fig. 4B). Subsequent SYPRO Ruby staining demonstrated that calpain treatment resulted in the disappearance of caspase-3-cleaved large subunit, p19 and the appearance of p16 and p15 (Fig. 4B, apparent migration on gel). While the larger calpain fragment, p16, appeared to have low activity, relatively equivalent to the activity observed for the caspase-3 fragment, the smaller calpain fragment had robust activity.
activity. Quantification of the change in caspase-7 activity intensity normalized to protein levels using densitometry demonstrates that the smaller calpain fragment is 18.4-fold more active than the caspase-3 fragment. Because the smaller, highly active calpain band was also present in the untreated active caspase-7 sample, this suggests that a calpain-like proteolysis may occur in the bacterial system used to make purified active caspase-7. Alternatively, production of a secondary translation product initiated at Met45 may be responsible for this product (9). Calpain-1 run alone does not have activity (data not shown).

In addition, the caspase-3-cleaved fragment and calpain-1-cleaved fragment that was not processed between the large and small subunit was observed around 34 kDa (Fig. 4B). Both proteins have low activity levels, suggesting that cleavage between the large and small subunit is necessary for the increase in activity observed with the calpain-1-cleaved large subunit.

Next we evaluated if calpain-cleaved caspase-7 cleaves endogenous protein substrates. Cellular lysates were treated with active caspase-7 (0–250 nM) incubated with and without calpain-1. The amount of PARP cleavage, a well-characterized substrate of caspase-7, was quantified using Western blot analysis and densitometry. Calpain-activated caspase-7 resulted in increased levels of cleaved PARP (54.3%) when compared with caspase-7 untreated (29.8%) (Fig. 4C). Because calpain cleavage of caspase-7 increased PARP protein processing, this suggests that calpain-cleaved caspase-7 is active against known caspase-7 substrates.

Identification of Three Calpain Cleavage Sites in Caspase-7 by Mass Spectrometry—To identify the calpain cleavage sites in caspase-7, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed using active caspase-7 treated with and without calpain-1. Untreated active caspase-7 has two distinct peaks at 12,558 and 19,918 Da, representing the small and large subunit, respectively (Fig. 5A, top graph). When caspase-7 was cleaved with calpain-1, two new peaks at 18,534 and 17,245 Da were observed (Fig. 5A, bottom graph). Expansion of the smaller peak exposed an additional peak very close in size (17,245 and 17,459 Da, Fig. 5A, inset). When calpain-1 was run alone, no peaks below 30 kDa were observed (data not shown). Based on the assumption that cleavage occurs near the N terminus of the large subunit, because the active cysteine is close to the C terminus (Fig. 5B), mass spectrometry masses were used to accurately predict calpain cleavage sites in the large subunit near the N terminus. Protein calculation software predicted that cleavage after Phe-36 would produce an 18,535-Da protein, cleavage after Met-45 would produce a
17,490-Da protein and cleavage after Ser-47 would produce a 17,246-Da protein. The calculated mass of two of these fragments is within 1 Da of the measured mass and the third within 31 Da. Also, two of these cleavage sites are composed of three amino acid sequences, which are predicted calpain cleavage sites (Fig. 5B, LFS and TMR in blue) (20).

**Sequencing the Calpain Cleavage Sites by vMALDI-LTQ™ Linear Ion Trap**—The MALDI-TOF data on calpain-treated caspase-7 samples suggest that cleavage occurs at Phe-36, Met-45, and Ser-47. However, this does not give sequence information. Therefore, analysis was performed on a vMALDI-LTQ™ quadrupole linear ion trap of caspase-7 treated with and without calpain. Fig. 6A shows the MS spectra of untreated caspase-7. Upon treatment with calpain is the appearance of a peak at m/z 1450.83 (Fig. 6B). This peak was not present in the untreated caspase-7 sample (Fig. 6A). This peptide was selected for MS/MS analysis to determine the sequence of the cleavage product. As shown in Fig. 6C, the tandem mass spectra of peptide AKPDRSSFVPSLF (m/z 1450.83) corresponds to cleavage at amino acid Phe-36.

**Confirmation of the Three Calpain Cleavage Sites in Caspase-7 by Site-directed Mutagenesis**—Calpain cleavage sites are not well conserved and recognition of the substrate depends on the three-dimensional structure of the substrate protein (20). We and others (17, 19) have previously shown that mutating a single amino acid is not adequate to prevent recognition of the substrate. Therefore we used deletion analysis in which we removed three amino acids in the region of cleavage. Controls for this approach are deletions in adjacent protein sequence, which are not recognized by calpains. We made deletions in caspase-7 at amino acids 35–37 (LFS site) and amino acids 46–48 (RSI site). Caspase-7, caspase-7 Δ35–37, and caspase-7 Δ46–48 C-terminal FLAG-tagged constructs were expressed in 293T cells. 293T lysates expressing wild-type or mutant caspase-7 proteins were treated with exogenous calpain-1. Calpain-1 treatment indicates that deletion of amino acids 35–37 eliminates production of the larger calpain fragment (supplemental Fig. S1A, top arrow) consistent with the mass spectrometry data mapping cleavage at amino acid Phe-36. Deletion of amino acids 46–48 significantly reduces production of the smaller calpain fragment suggesting cleavage occurs at Met-45 (supplemental Fig. S1A, bottom arrow). To confirm the calpain cleavage sites, C-terminal constructs of the predicted calpain fragments were made containing the C-terminal FLAG-tag (amino acids 37–303 and 48–303). The caspase-3-derived fragment was also made as a control (amino acids 24–303). When 293T lysates expressing these C-terminal calpain constructs were run on a gel with calpain-cleaved caspase-7, the calpain fragments appeared to be the same size as the C-terminal calpain constructs (supplemental Fig. S1B). We made deletion mutants at amino acids 44–46 (TMR site) as well as amino acids 43–48 (RSI and TMR site). Caspase-7, caspase-7 Δ35–37, caspase-7 Δ43–48, caspase-7 Δ44–46, and caspase-7 Δ46–48 C-terminal FLAG-tagged constructs were expressed in 293T cells and lysates and the deletion spanning both sites (Δ43–48) showed the greatest overall reduction in product (data not shown). These results suggest the smaller calpain product may be produced by calpain cleavage at both sites: Met-45 and Ser-47. These results could be influenced by altered conformation of the protein because of the deletion.

**Calpain-1 Products of Caspase-7 Are Produced in Vivo**—Because all of our experiments utilized *in vitro* systems, we investigated if calpain-derived caspase-7 products are produced in a cellular environment. Primary cortical neuronal cultures were treated with N-methyl-D-aspartate (NMDA; 0, 100, or 250 μM) for 3 h to increase Ca2+ levels and activate calpains (21–24). Treatment with NMDA resulted in an increase in both calpain-derived caspase-7 products (Fig. 7, arrows). Untreated lysates were also exogenously treated with calpain-1 as a positive con-
control (Fig. 7). The caspase-3 product of caspase-7 was also increased (Fig. 7, arrowhead). These data support that calpain products of caspase-7 are produced in vivo.

**Smaller Calpain Fragment of Caspase-7 Has Decreased Nuclear Localization**—Caspase-7 has been reported to have cytoplasmic, endoplasmic reticulum, and nuclear localization depending upon the cell type and mode of apoptotic induction. Therefore, we evaluated the localization of caspase-7 cleavage products. Particularly relevant to this experiment is the putative nuclear localization signal (Fig. 5B, KKKK amino acids 38–41) that is removed upon calpain cleavage (18). Caspase-7zymogen, caspase-3 fragment (amino acids 24–303) and calpain fragments (amino acids 37–303 and 48–303) were expressed in 293T cells, followed by cytosolic/nuclear fractionation. The caspase-7 24–303 fragment and caspase-7 37–303 fragment had an increased level of nuclear localization relative to procaspase-7. Consistent with deletion of a nuclear localization signal at amino acid 38–41, the caspase-7 48–303 fragment, the smaller calpain-derived fragment, was found in the cytoplasm (Fig. 8). This suggests that the putative nuclear localization signal may be necessary for caspase-7 to enter the nucleus and plays an important role in caspase-7 localization as the active protein is formed.

**The p17/p12 Caspase-7 Has Unique Substrate Specificity Compared with the p20/p12 Form**—Our results suggest the calpain-activated form of caspase-7 may have unique catalytic activity. Therefore, we examined the substrate specificity of the caspase-3-activated form of caspase-7 relative to the calpain form of caspase-7. We hypothesized that the p17 large subunit (see Fig. 9) may influence the turnover distinctly from the p20 large subunit. We examined substrates for all three classes of caspases and found that VDVAD-AMC and VDVAR-AMC where good substrates for caspase-7 p17 or p20 (Table 1). Strikingly, the caspase-7 p17/p12 increased 6-fold in $V_{\text{max}}$ when compared with p20/p12 form for the DEVD-AMC. The affinity $K_m$ decreased 2.4-fold but the $k_{\text{cat}}$ increased 6.1-fold (Table 1). Interestingly when we examined a caspase-2 substrate, VDVAR-AMC, we found the calpain form of caspase-7 (p17/p12) has an increased $V_{\text{max}}$ (3-fold) and higher affinity for the substrate (lower $K_m$) and increased $k_{\text{cat}}$ (3-fold). Our results suggest that the calpain-cleaved form of caspase-7 has a unique specificity.

**DISCUSSION**

The major findings of our study are that (i) caspase-7 is cleaved at Phe-36, Met-45, and Ser-47 by calpains to form a p18.5 and p17 large subunit (see Fig. 9A); (ii) deletion analysis of caspase-7 at these three sites prevents processing by calpain; (iii) the p17/p12 form of caspase-7 is more active than the p20/p12; (iv) the catalytic parameters for the calpain activated form of caspase-7 are distinct; and (v) localization of caspase-7 is modulated by calpain cleavage. Therefore, our results indicate that the activation and regulation of executioner caspase-7 by calpains is distinct from the activation of this enzyme by caspases. The activation of caspase-7 by calpain is summarized in Fig. 9B.

Caspase-7 and caspase-3 are both executioner caspases that were originally thought to have redundant properties based on their close phylogenetic relationship, structure, and sequence. While they have a number of similarities, such as identical small synthetic substrate specificity (25) and sensitivity to the inhibitor, XIAP (26, 27), they also have a number of distinct properties. Caspase-7 is catalytically less active than caspase-3 (28).
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![Diagram of the calpain cleavage sites of caspase-7](image)

A, caspase propeptide is 34 kDa in length. Cleavage by caspase-3 after D23 and caspase-8/-10 or granzyme B after D198 releases the active large subunit (p20). Cleavage by calpains after Phe-36 produces an 18.5 kDa active large subunit. Further cleavage by calpains after Met-45 or Ser-47 produces a 17.4 or 17.2 kDa large subunit, respectively, which has increased activity and decreased nuclear localization. B, model of the activation process of caspase-7 by calpain and caspase-8/-10 or granzyme B.

and has recently been shown to have a unique set of protein substrates (29). While caspase-3 and caspase-7 have some common substrates such as PARP and ROCK I, caspase-3 preferentially cleaves Bid and caspase-9 and caspase-7 more efficiently cleaves cochaperone p23 (29). Caspase-3 knockouts further support their unique cellular roles, because a caspase-3-deficient mouse (30) and MCF-7 caspase-3-deficient cells (31) undergo delayed apoptosis. An important structural difference, which may explain these functional differences, lies in the amino acid sequence of their N-peptide region. While they both have an aspartate cleavage site close to the N terminus necessary for enzyme activation, caspase-7 has an extra 30 amino acid stretch that follows containing a putative nuclear localization signal (KKKK) (18). This KKKK sequence is a highly conserved, seen in both human and *Xenopus* caspase-7, suggesting it may play an important functional role, although its identification as a nuclear localization signal is controversial (9, 18, 32, 33). A further distinction for N terminus of caspase-7 is the calpain cleavage sites.

The caspase-7 N-terminal peptide is believed to play an important role in the regulation and localization of caspase-7. The peptide is highly conserved and removed during apoptosis by caspase-3. While removal of the N-terminal peptide does not appear to affect catalytic activity *in vitro*, it does appear to play an important role in initiating cell death *in vivo* showing an early increase in DEVDase activity and cell death relative to the caspase-7zymogen (9). One hypothesis is that the caspase-7zymogen may be physically sequestered from its activators *in vivo* (caspase-8, -9, and -10) and is only released and activated after N-peptide removal by caspase-3 (9). An alternative explanation is that the caspase-7zymogen is primarily localized to the cytosol and caspase-3 cleavage results in increased nuclear compartmentalization (18), although this change in localization has not been observed in some studies (9).

We found that calpains cleave off a larger portion of the propeptide (45–47 amino acids) resulting in a caspase-7 large subunit that is much more active than its caspase-3-processed form. Cleavage by calpains may occur before caspase-3 cleavage, following caspase-3 cleavage or after caspase-8/-10 or granzyme B cleavage (Fig. 9B). While these three possibilities have not been fully explored *in vivo*, 293T cells expressing caspase-3-resistant caspase-7 (D23N) and calpain-1 do not show increases in caspase-3/7 activity relative to vector control, suggesting that caspase-3 removal of the propeptide precedes calpain cleavage (data not shown). Procaspase-7 is localized to both the cytosolic and nuclear compartment suggesting activation can occur in either compartment. This is consistent with the localization of caspases and calpains to both subcellular locations (19). It follows that calpain cleavage of caspase-7 can occur in either compartment. Caspase-7 can inactivate calpastatin, an endogenous inhibitor of calpains (34). Thus calpain activation of caspase-7 may initiate a vicious cycle of perpetually increasing calpain and caspase-7 activity.

Some studies indicate the KKKK motif of caspase-7 influences nuclear localization (18), while other studies suggest that the caspase-7zymogen and its caspase-3 cleavage product are strictly localized to the cytosol (9, 32, 33). One recent study found that sumoylation of a lysine in the N-terminal region of caspase-7, possibly within the KKKK motif, promotes nuclear localization (35). Our data support its function as a nuclear localization signal. We found some of the procaspase-7 in the nucleus. Initial cleavage of caspase-7 by caspase-3 or calpain produces the same amount caspase-7 in the nuclear fraction and further cleavage by calpains eliminates nuclear localization altogether. One hypothesis to explain this redistribution is that the caspase-3 N-terminal peptide (amino acids 1–23) physically blocks the KKKK site and that removal of this peptide by caspase-3 or caspase-1 increases exposure of this site, enhancing its ability to localize the protein to the nucleus. Subsequent removal of the KKKK motif by additional calpain cleavage eliminates the ability of the caspase-7 fragment to actively enter the nucleus, resulting in accumulation of this product in the cytosol. While our studies corroborate work done with *Xenopus* caspase-7 protein in a tadpole-derived myoblast cell line (XT-15–11), with the only clear difference being the amount of caspase-7zymogen localized to the nucleus, they conflict with previous studies using the human caspase-7 protein in a monkey kidney cell line (COS-7). One potential explanation for differences in localization may be the cellular model used for these studies. One concern is that the caspase-7 constructs we used may be activated and induce apoptosis when expressed at high levels, potentially affecting their cellular distribution. Because the endogenous caspase-7zymogen localized to the nucleus at
the same levels as the overexpressed FLAG-tagged caspase-7 zymogen and the highly active smaller calpain fragment did not have any nuclear localization, this suggests that overexpression of caspase-7 did not affect localization.

Interestingly the catalytic parameters are distinct for the p17/p12 form of caspase-7 when compared with p20/p12 form. This suggests that the conformation of caspase-7 p20/p12 is altered when additional cleavage by calpains occurs after amino acid 45. The atomic structure has been solved for both pro-caspase-7 and the active form cleaved at amino acid 23 (36, 37). However, the structures do not reveal density for residues before Ser-47, and therefore we cannot make predictions on the nature of the structural changes that may occur and alter the caspase-7 catalytic specificity.

The ability of calpains to cleave and modulate caspase-7 activity is clearly unique. While calpain-1 cleaved a number of caspases, their activity did not increase. Because caspase-3 has a catalytic specificity.

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