N Terminus of Calpain 1 Is a Mitochondrial Targeting Sequence*

The ubiquitous m- and μ-calpains are thought to be localized in the cytosolic compartment, as is their endogenous inhibitor calpastatin. Previously, μ-calpain was found to be enriched in mitochondrial fractions isolated from rat cerebral cortex and SH-SY5Y neuroblastoma cells, but the sub mitochondrial localization of μ-calpain was not determined. In the present study, submitochondrial fractionation and digitonin permeabilization studies indicated that both calpain 1 and calpain small subunit 1, which together form μ-calpain, are present in the mitochondrial intermembrane space. The N terminus of calpain 1 contains an amphipathic α-helical domain, and is distinct from the N terminus of calpain 2. Calpain 1, but not calpain 2, was imported into mitochondria. Removal of the N-terminal 22 amino acids of calpain 1 blocked the mitochondrial calpain import, while addition of this N-terminal region to calpain 2 or amino acids of calpain 1 blocked the mitochondrial import of μ-calpain, while addition of this N-terminal region to calpain 2 or μ-calpain was not imported following mitochon-
drial import, but was removed by autolysis following calpain activation. Calpain small subunit 1 was not directly imported into mitochondria, but was imported in the presence of calpain 1. The presence of a mitochondrial targeting sequence in the N-terminal region of calpain 1 is consistent with the localization of μ-calpain to the mitochondrial intermembrane space and provides new insight into the possible functions of this cysteine protease.

Calpains (EC 3.4.22.17) are a family of Ca^{2+}-activated cysteine proteases, including both ubiquitous and tissue-specific isoforms, that cleave their substrate proteins at discrete sites to modulate activity (1–3). The best characterized, and the pre-
dominant calpains in the central nervous system, are the clas-
sical m- and μ-calpains. Their physiological roles have not been fully elucidated but include cell motility, cell differentiation, membrane fusion, platelet activation, and signal transduction (3). Also extensively investigated have been the pathological roles of calpains in cell death, where calpains can cleave key structural proteins and contribute to the release of death-relat-
ed proteins such as apoptosis-inducing factor (AIF)^3 (4–9).

At present, it is unclear whether the μ- and m-calpains have distinct or overlapping functions. They are each heterodimers consisting of a unique 80-kDa large catalytic subunit (calpain 1 or 2) and a common 28-kDa small regulatory subunit (calpain small subunit 1 or 2) (2). In vitro, the substrates of m- and μ-calpains are similar, if not identical (10). Knock-out of the μ-calpain large subunit, calpain 1, results in viable mice with reduced platelet aggregation and impaired tyrosine phospho-
rylation in platelets, but not overt phenotype (11). Knock-out of the m-calpain large subunit, calpain 2, or of calpain small sub-
unit 1 (CSS1) is embryonically lethal (12–14).

Both m- and μ-calpains are considered to be cytosolic enzymes (2, 3, 15, 16). An association of m- and μ-calpains with subcellular organelles including endoplasmic reticulum and Golgi apparatus has been observed, but this association is hydrophobic, and the calpains are largely localized to the cyto-
plasmic surface of the organelle membranes (17–19). Previ-
ously, calpain-like activity was observed in both the mitochon-
drial matrix and intermembrane space of rat liver mitochondria (20). We recently observed an association between μ-calpain and mitochondria (21), but whether this represented a hydro-
phobic association on the external mitochondrial surface or a sub mitochondrial localization of μ-calpain was not deter-
mined. An atypical calpain, calpain 10, was recently localized to the mitochondrial matrix (22). The results of the present study demonstrate that μ-calpain, including both the large and small subunits, is present in the mitochondrial intermembrane space. The results further demonstrate that the μ-calpain large sub-
unit, calpain 1, contains a mitochondrial targeting sequence in its N terminus, and that CSS1 is imported into mitochondria by atting to the calpain 1 large subunit. The identification of a mitochondrial targeting sequence in calpain 1 and localization of μ-calpain to the mitochondrial intermembrane space provides new insight into its possible functions.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Bicinchoninic acid protein assay kit and the Supersignal West Pico chemiluminescent substrate were

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3 The abbreviations used are: AIF, apoptosis-inducing factor; AEBSF, 4-[2 ami-
nocyanoethyl]bensensulfonyl fluoride; BCA, bicinchoninic acid; β-gal, β-galac-
tosidase; CSS1, calpain small subunit 1; CSS2, calpain small subunit 2; GFP, green fluorescent protein; IMS, mitochondrial intermembrane space; IVT, in vitro transcription and translation; MOPS, 3-(N-morpholino)propanesul-
fonic acid; mHSP70, mitochondrial 70-kDa heat shock protein; VDAC, voltage-dependent anion channel.
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purchased from Pierce. Percoll was purchased from Amersham Biosciences (Piscataway, NJ). Reagents for Western blotting including acrylamide, bis, nitrocellulose, and molecular weight standards were from Bio-Rad. AEBSF (4-[2 aminoethyl]benzenesulfonyl fluoride, HCl) was obtained from EMD/Calbiochem. EDTA-free protease inhibitor tablets were from Roche Applied Science (Indianapolis, IN). Cell culture media was obtained from Gibco/Invitrogen (Carlsbad, CA). Animals were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Primary antibodies included mouse anti-AIF (E-1 clone, Santa Cruz Biotechnology), anti-voltage-dependent anion channel (VDAC, Affinity Bioreagents, Golden, CO); antibodies against domain III (mouse IgG1) of calpain 1, domain III of calpain 2 (rabbit polyclonal), and against CSS1 (mouse IgG1) (EMD/Calbiochem, San Diego, CA), and against CSS2 (rabbit polyclonal, Triple Point Biologics, Forest Grove, OR). Species-appropriate IRDye secondary antibodies were obtained from Rockland Immunochemicals (Gilbertsville, PA). DsRed2-mito was from Clontech (Mountain View, CA). All other reagents were from Sigma-Aldrich unless otherwise indicated.

Mitochondrial Isolation and Subfractionation—All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee. Mitochondrial isolation from SH-SY5Y human neuroblastoma cells and from rat cerebral cortex was as described previously (21) and from rat brain total RNA, with the 5’ primer harboring T7 promoter sequence upstream to AAT GCC ATT TAT TCT GAT GAG GAA TTT ATT TCC TCT TGG GC-3’ and reverse: 5’-GAA GCA GCG AAT GGA GAG TTT ATT TAC ATT CCT TCT TGG GC-3’) purified PCR product was used for in vitro transcription and translation reaction. Human calpain small subunit 2 (CSS2) cDNA clone was purchased from OpenBiosystems MHS1011-62749, accession no. BC006000. Rat Calpain 10 DNA was PCR-amplified using mammalian expression rat calpain 10 (OpenBiosystems cat. MRN1768-98078701) plasmid as a template, with primers harboring Kpn1 and Xho1 at the 5’ end and N40-GFP, N60-GFP reverse primer: 5’-TGC AAC CAC-3’ and reverse: 5’-GGCG CGG CCC GGG T CAT GCA AAC ATG GTC AGC TGC AAC CAC-3’).

N-terminal 40 and 60 amino acid constructs were prepared by PCR amplification of 120 and 180 bp, respectively and cloned into a modified plent6/V5-D-TOPO (Invitrogen) containing green fluorescent protein (GFP) coding sequence, a kind gift from Dr. George Smith, University of Kentucky, with BamH1 and Spe1 at the 5’ and 3’ regions to result in a GFP fusion protein (N40 and N60 forward: 5’-GGC GCG GAA TTC ATG GCC AGG GAG CTT GGC GGC CTC CAT GAG AAT GCC AAC A3-3’ and reverse: 5’-GGC CCG GCC CCC GGG T CAT GCA AAC ATG TGC AGC TGC AAC CAC-3’).

For proteinase K treatment, the mitochondrial fraction or mitoplasts (2–4 mg/ml) were suspended in the extraction buffer, pH 7.4, and incubated with proteinase K (50\(\mu\)g/ml) for 30 min at 37 °C. Phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM to terminate the proteinase K activity, followed by 10 min of incubation. Mitochondria or mitoplasts were pelleted by centrifugation at 13,000 \(\times\) g for 10 min, and analyzed by SDS-PAGE and Western blotting. To confirm the activity of the proteinase K, the mitochondrial or mitoplast fraction was solubilized with 0.2% Triton X-100, incubated with proteinase K, centrifuged at 13,000 \(\times\) g, and the supernatant analyzed by Western blotting as described previously (21, 24).

For digitonin permeabilization, isolated mitochondria (1 mg/ml) were incubated with 0.05–0.4 mg/ml digitonin for 30 min. at 4 °C. Low digitonin concentrations (0.05–0.2 mg/ml) cause selective permeabilization of the mitochondrial outer membrane while higher concentrations (0.2–0.4 mg/ml) result in permeabilization of the inner and outer mitochondrial membrane and of the mitochondrial matrix. Following digitonin treatment, the samples were centrifuged at 22,800 \(\times\) g for 20 min. The supernatant was subjected to SDS-PAGE and Western blotting.

Construction of Plasmids and in Vitro Translation and Transcription—Human calpain 1 cDNA was kindly provided by Dr. Rodney P. Guttmann (University of Kentucky, Lexington, KY). Rat calpain 2 and \(\Delta 86\) CSS1 cDNAs were a generous gift from Dr. Peter L. Davies, Queens University. CSS1 was generated by PCR amplification from library grade cDNA (SMART cDNA Library construction kit, cat. 634901, Clontech) which was generated from P90 rat brain total RNA, with the 5’ primer harboring T7 promoter sequence apart from CSS1 5’ sequence (forward: 5’-GAA TTG TAA TAC GAC TCA GTA TAG GGC GGC AAA CAT GTT CTT GTT GGT GAA TTC TTC TGC A A3-3’ and reverse: 5’-GAA GCA GCG AAT GGA GAG TTT ATT TAC ATT CCT TCT TGG GC-3’) purified PCR product was used for in vitro transcription and translation reaction. Human calpain small subunit 2 (CSS2) cDNA clone was purchased from OpenBiosystems MHS1011-62749, accession no. BC006000. Rat Calpain 10 DNA was PCR-amplified using mammalian expression rat calpain 10 (OpenBiosystems cat. MRN1768-98078701) plasmid as a template, with primers harboring Kpn1 and Xho1 at the 5’ (forward: 5’-GGG CGG GCC CTC CAT GCA AAC ATG GTC AGC TGC AAC CAC-3’) and reverse: 5’-GGC GCG GAA TTC ATG GCC AGG GAG CTT GGC GGC CTC CAT GAG AAT GCC AAC A3-3’)

For digitonin permeabilization, isolated mitochondria (1 mg/ml) were incubated with 0.05–0.4 mg/ml digitonin for 30 min. at 4 °C. Low digitonin concentrations (0.05–0.2 mg/ml) cause selective permeabilization of the mitochondrial outer membrane while higher concentrations (0.2–0.4 mg/ml) result in permeabilization of the inner and outer mitochondrial membrane and of the mitochondrial matrix. Following digitonin treatment, the samples were centrifuged at 22,800 \(\times\) g for 20 min. The supernatant was subjected to SDS-PAGE and Western blotting.
TCAGCCACGAGA-3’) and cloned into Blue script SK(+) at EcoRV and EcoRI. Substitution of 40 amino acids of rat calpain 2 with rat calpain 1 was achieved by introducing a restriction site NaeI by site directed mutagenesis (Stratagene) at amino acid position 2 (forward site 1: 5’-CGAATTCTGACAGA-TGGC CGGCATCGAGATGAAACTG-3’ and reverse site 1: 5’-CAGTTTCGAGATGAAACTG-3’ and reverse site 2: 5’-CTTGAAGGCGCCGCGCCGCGCTTCGAGT-3’ and reverse site 2: 5’-CTTGAAGGCGCCGCGCCGCGCTTCGAGT-3’). All constructs were sequenced prior to use. In vitro transcription and translation was performed as per the instructions of the TNT T7/T3-coupled reticulocyte lysate system (L5010, Promega). IVT proteins were labeled by [35S]methionine (specific activity of 10 mCi/ml, cat. 51006 MP Biomedicals) per IVT.

**Mitochondrial Import Assay**—Mitochondria were isolated from rat cortex as described above. In the presence and absence of ATP, 25 μg of mitochondria were resuspended in 25 μl of 2× import buffer (0.25 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.22 mM mannitol, 5 mM NADH, 20 mM sodium succinate, 0.25 mM ATP, 10 mM creatine phosphate, 0.1 mg of creatine kinase (Roche Applied Science) per ml, and 20 mM MOPS-KOH (pH 7.2) and incubated with 25 μl of reticulocyte lysate-synthesized protein. ATP, creatine phosphate, and creatine kinase were added as an ATP regeneration system, except where indicated (−ATP). Samples were incubated at 30°C for 50 min. Assays were terminated by centrifugation at 14,000 × g for 5 min at room temperature (25–27). The pellet was washed and resuspended in 25 μl of 1× sample buffer, and the supernatant fractions were resuspended in 25 μl of 3× sample buffer, for electrophoretic analysis 25% of the sample volume was run on 10% SDS-PAGE. The presence and absence of imported protein was deciphered by fluorescence detection on Typhoon 9600 (Molecular Dynamics) at 532 nm excitation at normal sensitivity.

**Subcellular Localization of Calpain 1 N-terminal GFP Expression Constructs**—B35 rat neuroblastoma cells were grown on 35-mm cover glass-bottom cultures dishes precoated with fibronectin (Millipore). N40-GFP, N60-GFP, or GFP (Invitrogen). At 48-h post-transfection, mammalian expression constructs were transfected with a Lipofectamine 2000 (Invitrogen). At 48-h post-transfection, live cell imaging was performed with a Leica SP5 inverted confocal microscope with an environmental chamber. Images were captured sequentially using an argon gas laser for N40-GFP and a HeNe gas laser for Mito-DSRed2.

**In Silico Analysis**—The protein sequences of human calpain 1 (Swiss-Prot/TrEMBL Accession Number P07384) and human calpain 2 (P17655) were compared using the SIM local similarity program based on the algorithm of Huang and Miller (28), using the web interface, with the resultant comparison visualized using LALNVIEW (29). For analysis of the amphipathic nature of the N terminus of calpain 1 and additional proteins, the Emboss pepwheel helical wheel plot was utilized.

**RESULTS**

**Mitochondrial Subfractionation**—To determine the mitochondrial localization of calpain 1, we examined the resistance of calpain 1 and CSS1 to proteinase K digestion in mitochondria and mitoplasts. In mitochondria isolated from SH-SY5Y cells, Calpain 1 and CSS1 were protected following incubation of the mitochondrial fraction with proteinase K (50 μg/ml, 37°C, 30 min.), as were the mitochondrial proteins AlF and mHSP70. All proteins were digested by proteinase K in Triton X-100 (1% w/v) solubilized mitochondria. This indicates that calpain 1 and CSS1 are localized internal to the outer mitochondrial membrane. In mitoplasts obtained following incubation of the mitochondria in hypo-osmotic media, calpain 1 and CSS1 are largely lost, and the small amount of calpain 1 remaining is digested by proteinase K, indicating that these proteins are not localized to the mitochondrial matrix and that the calpain 1 remaining with the mitoplasts may be associated with the outer face of the inner mitochondrial membrane. Mitoplast localized AlF was also digested by proteinase K whereas mHSP70 was protected. This is consistent with the localization of mHSP70 to the mitochondrial matrix and AlF to the inner mitochondrial membrane, exposed to the intermembrane space. During the preparation of the mitoplasts, we also examined the presence of calpain 1 in the supernatant (S) or pellet fractions (P) following the initial incubation of mitochondria (M) in hypo-osmotic media (B). Following incubation of mitochondria isolated from SH-SY5Y cells in 10 mM Hepes/KOH and centrifugation at 1900 × g, calpain 1 was found predominantly in the supernatant, while AlF was localized to the pellet. This finding suggests that calpain 1 is unanchored in the intermembrane space, and is consistent with the anchoring of AlF to the inner mitochondrial membrane.

**FIGURE 1. Mitochondrial subfractionation and proteinase K digestion.** In the mitochondrial enriched fraction obtained from SH-SY5Y cells (A), calpain 1 and CSS1 were protected from proteinase K (50 μg/ml, 37°C, 30 min.), as were the mitochondrial proteins AlF and mHSP70. All proteins were digested by proteinase K in Triton X-100 (1% w/v) solubilized mitochondria. This indicates that calpain 1 and CSS1 are localized internal to the outer mitochondrial membrane. In mitoplasts obtained following incubation of the mitochondria in hypo-osmotic media, calpain 1 and CSS1 are largely lost, and the small amount of calpain 1 remaining is digested by proteinase K, indicating that these proteins are not localized to the mitochondrial matrix and that the calpain 1 remaining with the mitoplasts may be associated with the outer face of the inner mitochondrial membrane. Mitoplast localized AlF was also digested by proteinase K whereas mHSP70 was protected. This is consistent with the localization of mHSP70 to the mitochondrial matrix and AlF to the inner mitochondrial membrane, exposed to the intermembrane space. During the preparation of the mitoplasts, we also examined the presence of calpain 1 in the supernatant (S) or pellet fractions (P) following the initial incubation of mitochondria (M) in hypo-osmotic media (B). Following incubation of mitochondria isolated from SH-SY5Y cells in 10 mM Hepes/KOH and centrifugation at 1900 × g, calpain 1 was found predominantly in the supernatant, while AlF was localized to the pellet. This finding suggests that calpain 1 is unanchored in the intermembrane space, and is consistent with the anchoring of AlF to the inner mitochondrial membrane.

**TABLE 1. Mitochondrial subfractionation and proteinase K digestion.**

| Protein | M | S | P |
|---------|---|---|---|
| Calpain 1 | + | + | - |
| CSS1 | + | + | - |
| AlF | + | + | - |
| mHSP70 | + | + | - |

**Mitochondrial Localization of μ-Calpain**
Mitochondrial Localization of μ-Calpain

AIF in the inner mitochondrial membrane and projection into the IMS (30), and the mitochondrial matrix localization of mHSP70 (31).

**Digitonin Permeabilization**—An additional method to examine sub-mitochondrial localization is digitonin permeabilization, in which low concentrations of digitonin selectively permeabilize the outer mitochondrial membrane with higher concentrations resulting in inner membrane permeabilization (32). Incubation of mitochondria isolated from SH-SY5Y neuroblastoma cells with digitonin concentrations as low as 0.05 mg/ml resulted in the release of calpain 1 (Fig. 2A). With higher digitonin concentrations, membrane associated proteins AIF and VDAC were released into the soluble fraction. For CSS1, there appeared to be two phases to the release. Much of the protein was released with the 0.05 mg/ml digitonin, similar to calpain 1, while additional protein was released with 0.4 mg/ml digitonin. This suggests that within the intermembrane space, CSS1 may bind to proteins or lipids additional to calpain 1. Similar results were obtained following digitonin permeabilization of mitochondria isolated from rat cerebral cortex (Fig. 2B), except that CSS1 could not be examined in the rat because of the species specificity of the primary antibody.

**Mitochondrial Import of Calpain**—Although calpains 1 and 2 share a high degree of homology, the amino terminus of mammalian calpain 1 contains an additional 11 amino acids that are not present in calpain 2 (Fig. 3). Based on our previous localization of calpain 1 to mitochondria, we hypothesized that this N terminus of calpain 1 represents a mitochondrial targeting sequence. Five programs that predict subcellular or mitochondrial location of protein sequences (MitoProt (33), MitPred (34), Target P (35), WoLFPSORT (36), and Predotar (37)) indicated a low probability that calpain 1 is localized to mitochondria. However, further analysis indicated that human calpain 1 contains an amphipathic helix or coiled-coil in its N-terminal domain (Fig. 3). This is a characteristic of pro-
Mitochondrial Localization of μ-Calpain

The various calpain 1 constructs utilized are illustrated in panel A. Fluorograms obtained following import of in vitro translated calpain 1 and related constructs, calpain 2, CSS1, CSS2, and β-Gal into mitochondria isolated from rat cerebral cortex are shown in panels B–J. Panel J represents an autoradiogram obtained following import of in vitro translated [35S]methionine-labeled proteins. Following import, mitochondria were pelleted by centrifugation and the supernatant (S) or pellet (P) fractions. The mitochondrial content of each fraction was confirmed by detection of VDAC by Western blotting and is shown below the fluorograms/autoradiogram in panels B–J. In panel A, calpain 1 refers to full-length human calpain 1. Δ22-Calpain 1 represents calpain 1 lacking the N-terminal 22 amino acids. N40- and N60-Calpain 1-GFP refer to chimeric proteins consisting of the N-terminal 40 or 60 amino acids of calpain 1 fused to GFP (panel B). Mitochondrial import of calpain 1 was observed in the presence, but not in the absence of ATP. β-Gal was not imported into mitochondria in the presence or absence of ATP (panel E). In contrast to calpain 1, calpain 2 was not imported into mitochondria, in the presence or absence of ATP. In the presence of ATP, Δ22-Calpain 2 was not imported (panel D), while chimeric proteins N40-Calpain 1-GFP and N60-Calpain 1-GFP were imported (panel E). Neither CSS1 or CSS2 were imported into mitochondria when translated in the absence of calpain 1 (panel F). CSS1, but not CSS2, was imported when co-translated with calpain 1 (panel G). The co-import of CSS1 was not observed with the chimeric proteins N40- or N60-Calpain 1-GFP (panel H). In panel I, calpain 10 import is included as a positive control. Addition of the N-terminal 22 amino acids from calpain 1, or replacement of the N-terminal 40 amino acids of calpain 1 with those of calpain 1 results in mitochondrial import of the chimeric protein. When co-incubated with rat calpain 1, a truncated calpain small subunit (ΔΔ86CSS1) was imported by mitochondria. The molecular weight of imported calpain 1 (shown in the same lane as the ΔΔ86CSS1) appears identical to that of in vitro translated calpain 1 prior to import, indicating that the calpain 1 N terminus is not processed following import. Together, these results demonstrate that calpain 1 contains a mitochondrial targeting domain in its N terminus, and that mitochondrial import of CSS1 requires the co-import of calpain 1. All constructs were derived from rat cDNAs, with the exception of the human calpain 1 constructs in panels B, D, G, and H; and the human CSS2 construct in panels F and G. Rat calpain 1 constructs were utilized in panels I and J.

FIGURE 4. Mitochondrial import of calpain 1 and CSS1. The various calpain 1 constructs utilized are illustrated in panel A. Fluorograms obtained following import of in vitro translated calpain 1 and related constructs, calpain 2, CSS1, CSS2, and β-Gal into mitochondria isolated from rat cerebral cortex are shown in panels B–J. Panel J represents an autoradiogram obtained following import of in vitro translated [35S]methionine-labeled proteins. Following import, mitochondria were pelleted by centrifugation and the in vitro translated proteins detected in the supernatant (S) or pellet (P) fractions. The mitochondrial content of each fraction was confirmed by detection of VDAC by Western blotting and is shown below the fluorograms/autoradiogram in panels B–J. In panel A, calpain 1 refers to full-length human calpain 1. Δ22-Calpain 1 represents calpain 1 lacking the N-terminal 22 amino acids. N40- and N60-Calpain 1-GFP refer to chimeric proteins consisting of the N-terminal 40 or 60 amino acids of calpain 1 fused to GFP (panel B). Mitochondrial import of calpain 1 was observed in the presence, but not in the absence of ATP. β-Gal was not imported into mitochondria in the presence or absence of ATP (panel E). In contrast to calpain 1, calpain 2 was not imported into mitochondria, in the presence or absence of ATP. In the presence of ATP, Δ22-Calpain 2 was not imported (panel D), while chimeric proteins N40-Calpain 1-GFP and N60-Calpain 1-GFP were imported (panel E). Neither CSS1 or CSS2 were imported into mitochondria when translated in the absence of calpain 1 (panel F). CSS1, but not CSS2, was imported when co-translated with calpain 1 (panel G). The co-import of CSS1 was not observed with the chimeric proteins N40- or N60-Calpain 1-GFP (panel H). In panel I, calpain 10 import is included as a positive control. Addition of the N-terminal 22 amino acids from calpain 1, or replacement of the N-terminal 40 amino acids of calpain 1 with those of calpain 1 results in mitochondrial import of the chimeric protein. When co-incubated with rat calpain 1, a truncated calpain small subunit (ΔΔ86CSS1) was imported by mitochondria. The molecular weight of imported calpain 1 (shown in the same lane as the ΔΔ86CSS1) appears identical to that of in vitro translated calpain 1 prior to import, indicating that the calpain 1 N terminus is not processed following import. Together, these results demonstrate that calpain 1 contains a mitochondrial targeting domain in its N terminus, and that mitochondrial import of CSS1 requires the co-import of calpain 1. All constructs were derived from rat cDNAs, with the exception of the human calpain 1 constructs in panels B, D, G, and H; and the human CSS2 construct in panels F and G. Rat calpain 1 constructs were utilized in panels I and J.

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The other component of μ-calpain, CSS1, does not contain an amphipathic domain at the N terminus (Fig. 3). CSS2, an alternate small subunit present in rat brain (40), also lacks an amphipathic N termin-
nus. Other mechanisms exist for importing proteins into the IMS include a cysteine-dependent folding trap mechanism (41, 42), and proteins that bind to lipids or proteins on the inner or outer mitochondrial membrane (43). CSS1 does not contain cysteine residues, ruling out the folding trap mechanism for import. The 28-kDa CSS1 is similar in size to Class 3 IMS proteins, such as 30-kDa cytochrome c heme lyase in which the mitochondrial targeting domain is an internal hydrophilic domain (43). CSS1 also contains regions of polar hydrophilic amino acids which have been hypothesized to serve as a tether to other molecules (2). However, CSS1 was not directly imported into mitochondria following in vitro translation (Fig. 4). When calpain 1 and CSS1 were translated simultaneously, both were imported into mitochondria (Fig. 4). CSS1 was not co-imported with N40- or N60-calpain1-GFP indicating that the association of calpain 1 and CSS1 during import involves domains other than the N terminus of calpain 1. A truncated form of CSS1 lacking the N-terminal 86 amino acids, Δ86 CSS1 (45, 46), was co-imported with calpain 1. When incubated with the large subunit, Δ86 CSS1 results in full calpain activity and is similar in size to the small subunit following autolysis, in which the N-terminal 91–93 amino acids are removed (45, 46). CSS2, an alternate small subunit present in rat brain (40), was not imported into mitochondria in the presence or absence of calpain 1 (Fig. 4). The import of [35S]methionine-labeled calpain 1, an alternate small subunit present in rat brain (40), was not imported into mitochondria in the presence or absence of calpain 1 (Fig. 4). The import of [35S]methionine-labeled calpain 1, and lack of import of [35S]-calpain 2, indicates that the inability of mitochondria to import calpain 2 following labeling with the FluoroTect Green-Lys system was not the result of spatial interference (Fig. 4).

Lack of N-terminal Processing of Calpain 1—To determine if the N terminus of calpain 1 is processed following mitochondrial import, mitochondria from SH-SY5Y cells were incubated in 20 mM Tris, 1 mM EDTA, 100 mM KCl, and 0.1% 2-mercaptoethanol for 2 h at room temperature, in the presence or absence of 0, 0.5, or 5 μmol of Ca2+ /mg protein. Following incubation, the mitochondrial extracts were probed using a mouse monoclonal antibody against domain III of calpain 1 (Calbiochem) and a rabbit polyclonal antibody whose epitope is located in the first 30 amino acids of the N-terminal end of calpain 1, and which does not recognize amino-processed forms (ab28257, Abcam, rabbit polyclonal). Mitochondrial calpain 1 reacted with both antibodies prior to incubation with Ca2+. Following incubation with 5 μmol of Ca2+/mg protein, immunoreactivity with the antibody against the N-terminal epitope was lost, while a slight decrease in molecular weight was observed with the antibody against domain III (Fig. 5). Together, these results indicate that the N terminus of calpain 1 is not processed following mitochondrial import, but is lost following calpain activation.

Subcellular Localization of Calpain 1 N-terminal GFP Expression Constructs—The N40- and N60-Calpain 1-GFP and GFP expression constructs were co-transfected with DsRed2-mito into B35 rat neuroblastoma cells to visualize the subcellular localization of the chimeric proteins (Fig. 6). DsRed2-mito (Clontech) is a fluorescent protein containing a mitochondrial targeting sequence from subunit VIII of human cytochrome c oxidase. The ratio of the expression constructs was 3:1 for the various GFP constructs versus DsRed2-mito. Most cells were transfected with the GFP vector and ~30% of the cells expressed both GFP and DsRed2-mito. The absorption spectra of DsRed2 includes a shoulder between 480 and 500 nm, while the excitation maximum of eGFP is 488 nm. As a result, a faint DsRed2 signal is visible in the green channel (not shown). To address this, we imaged cells transfected with N40-GFP, N60-GFP, or GFP mammalian expression constructs (ratio of 1:3 for the DsRed2-mito and GFP constructs). At 48-h post-transfection, live cells were visualized using a Leica SP5 inverted confocal microscope equipped with an environmental chamber. Images were captured sequentially using an argon gas laser for N40-GFP and a HeNe gas laser for Mito-DsRed2. Images represent individual Z sections captured using a ×100 objective with oil. In cells not transfected with DsRed2-mito (upper panel), punctate fluorescence is evident in cells transfected with N40-GFP and N60-GFP, in contrast to the diffuse localization of GFP. In cells co-transfected with N40-GFP and DsRed2-mito, the co-localization of the two signals demonstrates the mitochondrial localization of N40-GFP. Scale bar, 10 μm.

**FIGURE 5. Calpain 1 N terminus immunoreactivity.** Mitochondria isolated from SH-SY5Y cells were incubated for 2 h at room temperature in the presence or absence of 0.5 or 5 μmol of Ca2+/mg protein. Western blots of the mitochondrial extracts were probed using a mouse monoclonal antibody against domain III of calpain, then stripped and reprobed using a rabbit polyclonal antibody whose epitope is located in the first 30 amino acids of the N-terminal end of calpain 1. Mitochondrial calpain 1 reacted with both antibodies prior to incubation with Ca2+ (left lane). Incubation with 5 μmol Ca2+/mg protein, but not lower Ca2+ concentrations, resulted in a downward shift in electrophoretic mobility observed with the antibody against domain III, and loss of immunoreactivity with the antibody specific for the N-terminal epitope. These results indicate that mitochondrial calpain 1 contains an intact N terminus, and that calpain activation results in the loss of the N-terminal domain via autolysis.

**FIGURE 6. Mitochondrial localization of calpain 1 N-terminal GFP chimeric proteins.** B35 rat neuroblastoma cells were co-transfected with DsRed2-mito and N40-GFP, N60-GFP, or GFP mammalian expression constructs (ratio of 1:3 for the DsRed2-mito and GFP constructs). At 48-h post-transfection, live cells were visualized using a Leica SP5 inverted confocal microscope equipped with an environmental chamber. Images were captured sequentially using an argon gas laser for N40-GFP and a HeNe gas laser for Mito-DsRed2. Images represent individual Z sections captured using a ×100 objective with oil. In cells not transfected with DsRed2-mito (upper panel), punctate fluorescence is evident in cells transfected with N40-GFP and N60-GFP, in contrast to the diffuse localization of GFP. In cells co-transfected with N40-GFP and DsRed2-mito, the co-localization of the two signals demonstrates the mitochondrial localization of N40-GFP. Scale bar, 10 μm.
DISCUSSION

There has been considerable speculation regarding the possible mitochondrial localization of one or more calpains, but the identity of the mitochondrial calpains has been unknown. Ca\(^{2+}\)-induced calpain activity in liver mitochondria was previously observed, but the calpains were not identified (20, 47). In subcellular fractionation studies of rat brain, m-calpain activity was greatest in the soluble cytoplasmic fraction, while \(\mu\)-calpain activity was greatest in the crude mitochondrial P2 fraction (48, 49). We previously localized \(\mu\)-calpain to mitochondria from rat brain and SH-SY5Y neuroblastoma cells, but the sub mitochondrial localization was not determined (21). Arrington and colleagues identified calpain 10, an atypical calpain, in the mitochondrial outer membrane, IMS, inner membrane, and matrix fractions of rat and rabbit kidney mitochondria (22). They detected calpain 1 in the cytosol, but not in the mitochondrial fractions, and noted that calpain 1 does not contain an identifiable mitochondrial targeting signal. Ozaki et al. (50) recently identified a mitochondrial calpain in both the IMS and matrix of swine liver mitochondria, but concluded that this calpain was distinct from the m- and \(\mu\)-calpains based on size, enzymatic activity, and pH optimum. In contrast, Cao et al. (69) found calpain 1 in the IMS of rat brain mitochondria, but did not evaluate the localization of the small calpain subunit.

In the present study, mitochondrial subfractionation and digitonin permeabilization studies indicate that both components of \(\mu\)-calpain, calpain 1 and CSS1, are localized to the mitochondrial IMS. Calpain 1 contains a mitochondrial targeting sequence in its N terminus, while CSS1 is imported into mitochondria via a “piggyback” with calpain 1.

Most mitochondrial proteins, including all proteins in the intermembrane space, are nuclear-encoded and are imported into mitochondria following translation. Three import mechanisms have been identified for protein translocation into the IMS (39). One IMS import mechanism is similar to that of mitochondrial matrix proteins and involves an N terminus that has the potential to form an amphipathic helical structure. This amphipathic N terminus interacts with the translocase of the outer membrane (TOM) complex (38, 51–53). In addition to the amphipathic helix, these N-terminal domains also have patches of negatively charged amino acids and are rich in positive amino acids, the latter to direct the imported protein to negatively charged matrix face of the inner membrane via the translocase of the inner membrane (TIM) complex. Alternatively, some small proteins are able to diffuse through the translocase without direct interaction, but then become trapped in the IMS following protein folding and the formation of disulfide bonds as a result of protein oxidation in the IMS (for review see Ref. 41). An additional import mechanism is interaction with TOM via an internal sequence, and following import the proteins stably bind to proteins or lipids within the IMS (43).

The N terminus of calpain 1 includes an amphipathic helical domain in the first 24 amino acids, contains two adjacent glutamates to form a negative patch, and contain a positively charged amino acids at positions 20 (lysine) and 22 (arginine). In most proteins directed to the IMS, the N-terminal amphipathic helical domain is processed following protein import via a matrix protease such as mitochondrial processing peptidase, and a hydrophobic sorting domain following the amphipathic helical region anchors the cleaved protein in the inner membrane. Examples include AIF, endonuclease G, HtrA2, and Smac. Our results indicate that the N terminus of calpain 1 is not processed following import, but is lost following calpain activation.

The piggyback import mechanism for CSS1 appears unique for mitochondrial proteins, although it has been observed for nuclear protein import (55, 56). This mechanism enables CSS1 to be localized to both the mitochondria and cytosol. When co-expressed with calpain 1, CSS1 is imported into mitochondria. If CSS1 translation is spatially or temporally distinct from that of calpain 1, it remains in the cytosol where it can associate with calpain 2.

Whether all or only a fraction of calpain 1 is mitochondrial is not certain. The mitochondrial import studies indicate that most, if not all, of the newly translated protein is imported into mitochondria. Similarly, live cell imaging of cells transfected with a construct encoding the N terminus of calpain 1 fused to GFP illustrate a mitochondrial localization. However, in cells fixed with 4% paraformaldehyde, immunocytochemistry reveals both mitochondrial and non-mitochondrial localization of calpain 1 (21). This suggests approximately half of the \(\mu\)-calpain is mitochondrial, although aldehyde and other mild fixation conditions can result in an artifactual redistribution of some proteins (57, 58). Other proteins imported into the IMS via an N-terminal targeting sequence, such as AIF, endonuclease G, and Smac, are exclusively mitochondrial (39).

Both m- and \(\mu\)-calpains were previously thought to be located in the cytosol, the same cellular compartment as their endogenous inhibitor, calpastatin (2, 3). The cytosolic localization of calpastatin has been demonstrated in several previous studies (50, 59–62), although one recent study found calpastatin in smooth muscle mitochondria (63). In most tissues, calpastatin activity exceeds the combined activities of m- and \(\mu\)-calpains (64). Thus, it was unclear how m- and \(\mu\)-calpains became activated. The mitochondrial IMS localization of \(\mu\)-calpain may provide protection from inhibition by cytosolic calpastatin. Purified \(\mu\)-calpain requires 3–50 \(\mu\)M Ca\(^{2+}\) for half-maximal activity (10). Acyl-CoA binding protein, a calpain activator protein, is also present in the mitochondrial IMS and may lower the Ca\(^{2+}\) activation requirements of \(\mu\)-calpain (65, 66). This suggests the possibility that mitochondrial \(\mu\)-calpain may be activated by transient increases in Ca\(^{2+}\) in the IMS (for a recent review, see Ref. 67).

IMS \(\mu\)-calpain is also likely to be activated during mitochondrial permeability transition (mPT), when Ca\(^{2+}\) stored in the mitochondrial matrix is released into the intermembrane space following the opening of a nonspecific channel in the inner membrane permeable to solutes up to 1500 Da (68). Previous studies demonstrated that AIF is released from mitochondrial during mPT, and that AIF is a \(\mu\)-calpain substrate (9, 69). However, it was unclear whether the \(\mu\)-calpain was endogenous to mitochondria or exogenous, gaining access following an.
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increase in outer mitochondrial membrane permeability. The IMS localization of μ-calpain provides additional support for its role in AIF processing following mPT (9, 70, 71).

Following activation of μ-calpain, the N-terminal 26 amino acids of calpain 1, and the N-terminal 91 amino acids, of CSS1, are removed via autolysis (72, 44). It has been speculated that autolysis serves an important role in calpain function (2). The results of the present study demonstrate that by removing the N-terminal 26 amino acids of calpain 1, autolysis removes the mitochondrial targeting domain. Following activation, the large and small subunits of μ-calpain dissociate. Because μ-calpain is unanchored in the IMS, activated μ-calpain may be released into the cytosol following outer mitochondrial membrane permeabilization associated with mPT and Bax/Bak/Bid-mediated permeabilization (54). Autolytic removal of the N terminus of calpain 1 would prevent mitochondrial reuptake of the activated protease.

In summary, by identifying a mitochondrial targeting sequence on the N-terminal of calpain 1 and localizing calpain 1 and CSS1 to the mitochondrial IMS, the present study removes much of the previous uncertainty and confusion regarding the identity of mitochondrial calpains. μ-Calpain appears responsible for the calpain activity previously observed in the IMS, while calpain 10 accounts for the calpain activity in the mitochondrial matrix (22). The mechanisms involved in the regulation of mitochondrial μ-calpain activity, along with its substrates and functions, remain to be determined.

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