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Mitochondrial localization of μ-calpain ☆

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Abstract

Calcium-dependent cysteine proteases, calpains, have physiological roles in cell motility and differentiation but also play a pathological role following insult or disease. The ubiquitous calpains are widely considered to be cytosolic enzymes, although there has been speculation of a mitochondrial calpain. Within a highly enriched fraction of mitochondria obtained from rat cortex and SH-SY5Y human neuroblastoma cells, immunoblotting demonstrated enrichment of the 80 kDa μ-calpain large subunit and 28 kDa small subunit. In rat cortex, antibodies against domains II and III of the large μ-calpain subunit also detected a 40 kDa fragment, similar to the autolytic fragment generated following incubation of human erythrocyte μ-calpain with Ca^{2+}. Mitochondrial proteins including apoptosis inducing factor and mitochondrial Bax are calpain substrates, but the mechanism by which calpains gain access to these proteins is uncertain. Mitochondrial localization of μ-calpain places the enzyme in proximity to its mitochondrial substrates and to Ca^{2+} released from mitochondrial stores.

Keywords: Apoptosis; Apoptosis inducing factor; Bax; Calcium; Cell death; Cortex; Necrosis; Neuroblastoma; Permeability transition; Protease

Calpains are a family of non-lysosomal calcium-dependent cysteine proteases found in all eukaryotes (for review see [1]). The predominant calpain isoforms found in the mammalian central nervous system include the ubiquitous μ- and m-calpains (calpains 1 and 2, respectively), which consist of a unique 80 kDa subunit and a common small 28 kDa subunit. Physiological functions of the calpain system are not well understood, but calpain activation is implicated in cell adhesion and motility, exocytosis, signal transduction, cell cycle progression, and regulation of gene expression [1–5]. Calpain overactivation is associated with the pathogenesis of a wide range of disorders including cataract formation, diabetes mellitus, muscular dystrophies, neurodegenerative disorders including Alzheimer's disease and Huntington's disease, prion disorders, and secondary degeneration resulting from acute insults including myocardial infarction, stroke, traumatic brain injury, and spinal cord injury [6–8]. Implicated in both apoptotic and necrotic death [9–12], recent studies reveal that calpain activation is an essential component of necrosis and caspase-independent cell death [13–16].

In unstimulated cells, the m- and μ-calpains are widely distributed within the cytoplasm [17–20]. Following stimulation, the calpains translocate to the plasma membrane [19,21–23], where the resultant interaction with phosphoinositides reduces the $[Ca^{2+}]$ required for activation [24,25]. Calpains have also been localized to other subcellular organelles, including endoplasmic reticulum, Golgi apparatus, and the nucleus [20,26–28], but have not previously been localized to a highly enriched mitochondrial fraction.

Evidence for a possible association between calpain and mitochondria includes the presence of calpain activity and small subunit immunoreactivity in a heavy membrane/

☆ *Abbreviations:* AIF, apoptosis inducing factor; BCA, bicinchoninic acid; VDAC, voltage-dependent anion channel; AEBSF, 4-[2 aminoethyl]-bensenesulfonyl fluoride, HCl; BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,-*N*′,*N*′-tetraacetic acid tetraacetoxy-methyl ester; MIB, mitochondrial isolation buffer; TTBS, Tris-buffered saline containing 0.05% Tween 20.

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crude mitochondrial fraction [29,30]; the observation that mitochondrial proteins including apoptosis inducing factor (AIF) and mitochondrial Bax are calpain substrates [31,32]; and that cyclosporine A, which binds to cyclophilin D to block the mitochondrial permeability transition pore, can prevent calpain activation in some situations [33,34]. As a result there is speculation of a specific, but uncharacterized, mitochondrial calpain [11,35]. In the present study, we identified μ-calpain in mitochondria-enriched fractions obtained from rat cortex and human SH-SY5Y neuroblastoma cells. Both the large 80 kDa μ-calpain and small 28 kDa subunit were enriched in the mitochondrial fraction, along with smaller fragments consistent with calpain autolysis. The mitochondrial localization of μ-calpain is consistent with previous studies demonstrating that calpain can function as a death-related protease via the cleavage of mitochondrial proteins [11,31,36,37].

Materials and methods

Reagents. Bicinchoninic acid (BCA) protein assay kit and the Supersignal West Pico chemiluminescent substrate were purchased from Pierce (Rockford, IL). Percoll was purchased from Amersham Biosciences (Piscataway, NJ). Primary antibodies included rabbit anti-voltage-dependent anion channel (VDAC, Affinity Bioreagents, Golden, CO, USA), mouse anti-calnexin (BD Transduction Laboratories, San Jose, CA); mouse anti-actin, clone AC-40 (Sigma); mouse anti-β 2 subunit of Na^+/K^+ATPase (BD Transduction Laboratories); antibodies against domains II, III (mouse IgG_1) of the μ-calpain large subunit and against the common small subunit (mouse IgG_1) (EMD/Calbiochem, San Diego, CA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and IgM, and anti-rabbit IgG, secondary antibodies were purchased from Zymed (San Francisco, CA) and Jackson Immunoresearch (West Grove, PA). Reagents for Western blotting including acrylamide, bis, nitrocellulose, and mol. wt. standards were from Bio-Rad (Hercules, CA). Purified human erythrocyte μ-calpain and AEBSF (4-[2 aminoethyl]-bensenesulfonyl fluoride, HCl) were obtained from EMD/Calbiochem (San Diego, CA). EDTA-free protease inhibitor tablets were from Roche Applied Science (Indianapolis, IN). BAPTA-AM (1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,-*N*′,*N*′-tetraacetic acid tetraacetoxy-methyl ester) were obtained from Molecular Probes/Invitrogen (Carlsbad, CA). Cell culture media was obtained from GIBCO/Invitrogen (Carlsbad, CA). Animals were obtained from Harlan Sprague–Dawley (Indianapolis, IN). All other reagents were from Sigma Chemical Company (St. Louis, MO) unless otherwise indicated.

Mitochondrial isolation from rat cortex. All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee. All results were replicated in a minimum of three independent experiments. Male Sprague–Dawley rats (250–300 g, approximately 3 months of age) were used in all studies. The mitochondrial isolation procedure for rat cortex was modified from method A of Sims [38], a combination of differential centrifugation and Percoll density gradient centrifugation. Following sodium pentobarbital anesthesia, the rats were decapitated and the brains rapidly removed. The cortices were dissected out, minced, and homogenized (10% w/v) in mitochondrial isolation buffer (MIB) (215 mM mannitol, 75 mM sucrose, 20 mM Hepes, 1 mM EGTA, and 1× complete protease inhibitor, pH adjusted to 7.2 with KOH) in a glass dounce homogenizer. The homogenate was centrifuged at 1300*g*, 3 min, 4 °C. The supernatant was collected and the pellet was resuspended in MIB and centrifuged again. The pooled supernatants were centrifuged at 13,000*g*, 10 min, 4 °C, and the pellet was resuspended in MIB and then mixed with an equal volume of 30% Percoll in isolation buffer. The resultant homogenate was layered on a discontinuous Percoll gradient with the bottom layer containing 40% Percoll solution in isolation buffer, followed by a 26% Percoll solution, and finally the sample in a 15% Percoll solution. The density gradients were centrifuged at 44,500*g* for 25 min, 4 °C. Following centrifugation, fraction 3 was removed from the interface between the 23% and 40% Percoll, diluted fourfold in MIB, and centrifuged at 24,200*g* for 15 min. The resultant mitochondrial pellet was resuspended in MIB with 0.1% Triton-X and incubated on ice for 10 min prior to sonication for 12 s, and centrifuged at 14,000*g* for 10 min. The supernatant was used for subsequent analysis.

Mitochondrial isolation from cultured cells. SH-SY5Y cells were obtained from the American Type Culture Collection (Manasas, VA) and propagated in Eagle's minimum essential medium with non-essential acids, 10% fetal bovine serum, and 1% penicillin–streptomycin until 80% confluency was achieved. Method B of Sims [38], which involves discontinuous Percoll density gradient without differential centrifugation, was used to isolate the mitochondria from the cultured cells due to the improved yield of this method. The cells were trypsinized with Trypsin–EDTA, pelleted at 2000 rpm at 4 °C, 10 min, and following the addition of mitochondrial isolation buffer with protease inhibitors and incubation on ice for 10 min, the cells were dounce homogenized, mixed with an equal volume of 24% Percoll, and then layered on a Percoll density gradient containing layers of 40% and 26% Percoll. The density gradient was centrifuged at 30,700*g* for 10 min. The layer between the 40% and 26% Percoll was collected, diluted 1:5 in MIB and centrifuged at 16,700*g*. The pellets were diluted in MIB and spun at 14,000*g* for 10 min and the final pellets were resuspended in MIB.

For cultures treated with the Ca^{2+} chelator BAPTA-AM prior to mitochondrial isolation, 25 μM BAPTA-AM (Molecular Probes) was added to SH-SY5Y cells for 30 min prior to trypsinization. The remainder of the procedure was as indicated above for the Sims B protocol.

Western blotting. Sample buffer was added to the mitochondrial fractions based on relative protein concentrations determined from the BCA protein assay and all lanes were loaded with the same amount of protein (5–20 μg). Samples were separated by SDS–PAGE, 10% Tris–acrylamide, for proteins with a mol. wt. >30 kDa (μ-calpain large subunit, PSD-95, VDAC, calnexin, Na^+/K^+ATPase, actin, and PSD-95) or at 12.5% Tris–acrylamide for lower molecular weight proteins (small calpain subunit), along with molecular weight markers. Following SDS–PAGE, polypeptides were transferred electrophoretically onto 0.45 μm nitrocellulose membranes. Membranes were incubated at room temperature for 1 h in 5% non-fat milk in 50 mM Tris–saline containing 0.05% Tween 20 at pH 7.5 (TTBS). The blots were incubated overnight in the primary antibody in TTBS at 22 °C. After overnight incubation in primary antibody, the membranes were rinsed three times in TTBS and incubated in peroxidase-conjugated species-appropriate secondary antibodies. The blots were rinsed thoroughly in TTBS, incubated in the Pierce SuperSignal Pico chemiluminescent substrate, and visualized using a Kodak Image Station 2000R and 1D software (Kodak, Rochester NY).

Immunohistochemistry. The human neuroblastoma cell line SH-SY5Y was used for immunohistochemical analysis of the mitochondria and μ-calpain co-localization. MitoTracker Red 580, 1 mM, was added in fresh culture medium to SH-SY5Y cells grown to 80% confluency. Following 15 min incubation at 37 °C, the cells were rinsed 3× in PBS and then fixed for 5 min in acetone, 4 °C. The acetone-fixed cells were rinsed in PBS and then incubated with primary antibody overnight, followed by detection with the species appropriate FITC-tagged secondary antibody. The cells were coverslipped and photographed using an Olympus AX70 microscope equipped for epifluorescence and digital photography.

In vitro μ-calpain autolysis. To compare the lower mol. wt. bands detected with the μ-calpain antibodies with autolytic μ-calpain fragments, human erythrocyte μ-calpain was resuspended in 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 1 mM DTT at room temperature following the protocol of Gabrijelcic-Geiger et al. [39]. Following the addition of 0.5 mM Ca^{2+}, aliquots were taken at time intervals of 0, 1, 5, 15, 30, 60, and 120 min, and 25 mM EDTA was added to stop calpain activation. The aliquots were mixed with an equal volume of sample buffer, and then placed on a 10% SDS–PAGE gel for Western blotting, with 0.375 μg μ-calpain being added to each lane. The blot was probed with anti-calpain