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Ca^{2+}-induced release of mitochondrial m-calpain from outer membrane with binding of calpain small subunit and Grp75

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ABSTRACT

Although mitochondrial μ- and m-calpains play significant roles in apoptotic cell death, their activating mechanisms have not been determined. The purpose of this study was to determine the core factors that are involved in activating mitochondrial outer membrane (OM)-bound calpains. To accomplish this, we solubilized OM-bound calpains and separated them by DEAE-Sepharose column chromatography, and identified them by immunoblots. We also determined the core factors that activated the OM-bound calpains and release them from the OM by calpain assays, immunoprecipitations, and immunoblots. The OM-bound m-calpain large subunit was not associated with the small subunit or with Grp75 chaperone. Free calpain small subunit was located in the IMS and caused the release of the OM-bound m-calpain large subunit from the OM together with Grp75, ATP, and Ca^{2+}. Our results showed that the activating mechanism of mitochondrial OM-bound m-calpain and the release of mitochondrial m-calpain from the OM have important implications in facilitating apoptotic cell death.

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Introduction

Calpains are Ca^{2+}-activated neutral cysteine proteases that modulate the functions of many specific substrates by limited proteolysis [1]. In humans, 14 calpain isoforms have been identified as the catalytic large subunit and two isoforms for regulatory small subunits [1,2]. Among them, μ-calpain and m-calpain large subunits, calpain small subunit, and calpain 10 exist in mitochondria [3].

The mitochondrial calpains play significant roles in pathophysiological conditions including apoptotic and necrotic cell deaths [3]. Endo et al. showed that mitochondrial calpains inactivate mitochondrial aspartate aminotransferase in the rat retina under ischemic and hypoxic conditions [4]. We found that mitochondrial μ-calpain modulates apoptotic cell death by limiting the cleavage of the apoptosis-inducing factor (AIF)[1] [5,6]. We also demonstrated that mitochondrial m-calpain plays a significant role in the release of truncated AIF from mitochondria through VDAC–Bax complex [7]. Very recently, we proved that the mitochondrial calpains induce photoreceptor apoptosis in the Royal College of Surgeon's (RCS) rats, which are widely employed as an animal model of human retinitis pigmentosa [8].

Although mitochondrial calpains are involved in a variety of pathological conditions [8–11], how they are activated has not been determined. Earlier, we found that the mitochondrial m-calpain large subunit was located mainly in the OM [7], but the calpain activity in the OM was very weak [5]. These observations suggested that mitochondrial m-calpain in the OM is regulated by not only Ca^{2+} but also other factors such as calpain activators, inhibitors, and molecular chaperones.

The purpose of this study was to determine the core factors that are involved in activating OM-bound calpains. We have shown that mitochondrial μ- and m-calpains are associated with the molecular chaperones such as ERp57 and Grp75, respectively, in the mitochondrial intermembrane space (IMS) [6,7]. In addition, we detected three types of calpain small subunits in the IMS using non-denaturing gels: the first was a mitochondrial μ-calpain large subunit-associated calpain small subunit, the second was a mitochondrial m-calpain-associated calpain small subunit, and the third was an unknown calpain large subunit-associated or free calpain small subunit [6]. To identify the activating factors of OM-bound calpains, we determined what kinds of calpain isoforms were present in the OM and investigated the effects of Ca^{2+}, ERp57, Grp75, and calpain small subunit.

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[1] *Abbreviations used:* AIF, apoptosis-inducing factor; tAIF, truncated AIF; VDAC, voltage-dependent anion channel; Grp75, glucose-regulated protein 75; GAPDH, glyceraldehyde phosphate dehydrogenase; OM, outer membrane; IMS, intermembrane space; IM, inner membrane; ATP, adenosine 5′-triphosphate; TTP, thymidine 5′-triphosphate; GTP, guanosine 5′-triphosphate; CTP, cytidine 5′-triphosphate.

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Materials and methods

Materials

The detergents used to solubilize the mitochondrial outer membrane (OM) were: sodium deoxycholate (Fluka, Milwaukee, WI), Nonidet P-40 and Octyl-glucoside (Sigma Aldrich Corp., St. Louis, MO), digitonin, CHAPS, *n*-dodecyl-*β*-D-maltoside, Triton X-100, and Tween 20 (Wako). Grp75 protein was purchased from Abcam (Cambridge, MA). Adenosine 5′-triphosphate (ATP), thymidine 5′-triphosphate (TTP), guanosine 5′-triphosphate (GTP) and cytidine 5′-triphosphate (CTP) were purchased from Sigma.

The antibodies purchased were: rabbit polyclonal antibodies against μ-calpain large subunit domain IV (Sigma); anti-m-calpain large subunit domain III (Abcam); anti-calpain small subunit domain V (Abcam); anti-Grp75 (Santa Cruz Biotechnology Inc., CA); anti-adenylate kinase 2 (Santa Cruz); anti-GAPDH (Santa Cruz); anti-VDAC (Calbiochem); anti-pyruvate dehydrogenase (Molecular Probes, Eugene, OR); mouse monoclonal anti-trans-Golgi network-38 (Abcam); rat monoclonal anti-ZO-1 (Chemicon, Temecula, CA); and normal rabbit IgG (Santa cruz). Rabbit anti-ERp57 antiserum was prepared against the 17-amino acid C-terminal peptide (VIQEEKPKKKKKAQEDL) of human ERp57 as described [12].

Subfractionation of rat liver mitochondrial outer membrane and intermembrane space

All of the procedures were carried out at 4 °C, following the method of Parson et al. [13]. The mitochondrial fraction obtained as described [5] was resuspended in two volumes of 20 mM potassium phosphate buffer, pH 7.4, and allowed to stand at 4 °C for 1 h. The resuspended sample was centrifuged at 3000×*g* for 10 min. The supernatant was centrifuged at 105,000×*g* for 30 min, and the pellet was used as the OM fraction, and the supernatant was used as the IMS fraction. The purity of the mitochondrial OM and IMS was determined by immunoblot analysis with the following antibodies; anti-VDAC antibody for mitochondrial OM, anti-adenylate kinase 2 (AK2) for intermembrane space (IMS), anti-AIF antibody for inner membrane (IM), pyruvate dehydrogenase (PDH) antibody for matrix, anti-GAPDH antibody for cytosolic fraction, anti-ZO-1 for plasma membrane, anti-calnexin for ER, anti-trans-Golgi network-38 for Golgi apparatus as shown (see Ref. [7]). High purities of the mitochondrial OM and IMS were obtained.

Solubilization and column chromatography of mitochondrial OM-bound calpains

Mitochondrial OM was prepared from 10 Sprague–Dawley (SD) rats (8 weeks-old) as described above. To prevent the contamination of cytosolic proteins, the mitochondrial surface was treated with proteinase K as described in detail [7]. DEAE-Sepharose CL-6B (Amersham Pharmacia Biotech) column chromatography was used to separate the calpain isoforms in the OM (Fig. 2A). The prepared OMs were suspended in solubilizing buffer (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, 1% octyl-glucoside, and 50 mM NaCl). The samples solubilized for 30 min at 4 °C and were then centrifuged at 20,000×*g* for 30 min. The supernatants (360 mg protein) were applied to a DEAE-Sepharose CL-6B column (26.4 × 400 mm) pre-equilibrated with buffer A (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.1% octyl-glucoside) containing 50 mM NaCl (buffer B) at a flow rate of 1.0 ml/min. All chromatographic procedures were performed at 4 °C. The amount of each fraction size was 10 ml/tube. Aliquots (50 μl) of the separated fractions were used to assay for calpain activity as described [5]. Unbound proteins were eluted with 770 ml of buffer B. The bound proteins were eluted with a linear gradient of 50–300 mM NaCl in buffer A in a total volume of 1.0 L. Four calpain-like activity peaks were detected. The fractions containing calpain-like activity were collected and concentrated by Amicon PM-10 membrane (Millipore Co., Bedford, MA). The concentrates were used for Western blot analyses with anti-μ-calpain and anti-m-calpain large subunits, anti-calpain small subunit, anti-ERp57 and anti-Grp75 (Fig. 2B). They were also used for immunoprecipitation with anti-μ-calpain large subunit, anti-calpain small subunit, and anti-ERp57 (Fig. 2C). The protein concentration was measured by the Bradford et al. method with bovine serum albumin (BSA) as the standard [14].

We also determined whether that the proteins in the mitochondrial intermembrane space (IMS) were involved in the activation of mitochondrial OM proteins. Fifty-five micrograms of IMS proteins were added to 50 μl of each fraction and incubated at 4 °C for 2 h. Then the calpain activity of each sample was measured (Fig. 3D).

Column chromatography of mitochondrial IMS-localized μ- and m-calpains

Rat liver mitochondrial IMS were prepared from 30 SD rats (8 weeks-old) as described above. Partial purification of mitochondrial μ- and m-calpains was accomplished by DEAE-Sepharose CL-6B column chromatography as we described [5–7] (Fig. 2A). The prepared IMS fraction was dialyzed overnight against 2 L of buffer C (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol) containing 50 mM NaCl (buffer D) at 4° C. The dialyzed proteins (200 mg protein) were applied to a DEAE-Sepharose CL-6B column (26.4 × 400 mm) pre-equilibrated with buffer D at a flow rate of 1.0 ml/min. The fraction size was 10 ml/tube. Aliquots (50 μl) of the fractions were used to assay for calpain activity as described [5]. Unbound proteins, containing mitochondrial m-calpain, were eluted with 700 ml of buffer D. Mitochondrial μ-calpain was eluted with a linear gradient of 50–300 mM NaCl in buffer C in a total volume of 1.1 L. Each fraction was used for the calpain assay in the presence of the OM proteins (Fig. 2B). Fifty micrograms of OM proteins were added to 50 μl of each fraction and incubated at 4 °C for 2 h. In contrast, 1% octyl-glucoside-solubilized OM proteins were added to each fraction and incubated. Then the calpain activity of each sample was measured as described [5].

Partial purification of calpain small subunit in mitochondrial IMS

Sepharose 6B column chromatography (Amersham Pharmacia Biotech) was used to partially purify the calpain small subunit from the mitochondrial IMS (Fig. 4A). Rat liver mitochondrial IMS were prepared from 30 SD rats (8 weeks-old) as described above. The prepared IMS fraction was dialyzed overnight against 2 L of buffer C (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol) containing 140 mM NaCl (buffer E) at 4 °C. The dialyzed proteins (20 mg protein) were applied to a Sepharose column (9 × 600 mm) pre-equilibrated with buffer E at a flow rate of 0.5 ml/min. The proteins were eluted with buffer E depending on their molecular weight. The molecular weight markers were normal rabbit IgG (140 kDa), BSA (66 kDa), and chymotrypsinogen A (24 kDa). The fraction size was 500 μl/tube. Each fraction was concentrated by Amicon PM-10 membrane (Millipore Co., Bedford, MA). The concentrates were used for Western blot analysis with anti-μ- and anti-m-calpain large subunits, anti-calpain small subunit, anti-ERp57, and anti-Grp75 antibodies (Fig. 4B).