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Characteristics of Mitochondrial Calpains

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Calpains are considered to be cytoplasmic enzymes, although several studies have shown that calpain-like protease activities also exist in mitochondria. We partially purified mitochondrial calpain from swine liver mitochondria and characterized. Only one type of mitochondrial calpain was detected by the column chromatographies. The mitochondrial calpain was stained with anti-μ-calpain and calpain small subunit antibodies. The susceptibility of mitochondrial calpain to calpain inhibitors and the optimum pH differ from those of cytosolic μ- and m-calpains. The Ca^{2+}-dependency of mitochondrial calpain was similar to that of cytosolic μ-calpain. Therefore, we named the protease mitochondrial μ-like calpain. In zymogram analysis, two types of caseinolytic enzymes existed in mitochondria and showed different mobilities from cytosolic μ- and m-calpains. The upper major band was stained with anti-μ-calpain and calpain small subunit antibodies (mitochondrial calpain I, mitochondrial μ-like calpain). The lower band was stained only with anti-calpain small subunit antibody (mitochondrial calpain II, unknown mitochondrial calpain). Calpastatin was not detected in mitochondrial compartments. The mitochondrial calpain processed apoptosis-inducing factor (AIF) to truncated AIF (tAIF), releasing tAIF into the intermembrane space. These results indicate that mitochondrial calpain, which differs from μ- and m-calpains, seems to be a ubiquitous calpain and may play a role in mitochondrial apoptotic signalling.

Key words: apoptosis-inducing factor (AIF), calpastatin, mitochondrial calpain, mitochondrial intermembrane space, μ-calpain-like protease.

Calpains (EC 3.4.22.17) are calcium-dependent neutral cysteine proteases that are widely distributed in animal tissues and are involved in a variety of cellular processes involving calcium ions (*1*, *2*). The calpain family is made up of the ubiquitous calpains, viz., μ-calpain (calpain 1), m-calpain (calpain 2) and calpain 10 (*3*), and the tissue-specific calpains, viz., calpain 3 (muscle-specific p94) (*4*), calpain 8 (stomach nCl-2) (*5*), calpain 9 (digestive tubule nCl-4) (*6*), lens Lp82 and Lp85 (*7*, *8*), retinal Rt88 (*9*) and corneal Cn94 (*10*). The μ- and m-calpains are known to co-exist in most cells; a low micro-molar order-Ca^{2+}-requiring form (μ-calpain) and a high milli-molar order-Ca^{2+}-requiring form (m-calpain) (*11*).

Mitochondria are involved in life phenomena such as apoptosis, metabolism of many different substances, disease, aging, storage of Ca^{2+} and what not. An overload of Ca^{2+} in the mitochondria under pathologic conditions initiates a series of destructive cycles that can lead to irreversible cell damage. During ischaemia, the intracellular Ca^{2+} homeostasis is altered, but mitochondria are able to buffer cytosolic Ca^{2+}, suggesting that the mitochondria have Ca^{2+}-transporting capability (*12*, *13*).

We have shown that 90 min of ischaemia or hypoxia of the rat retina led to a 20% decrease in the mitochondrial aspartate aminotransferase (mAAT) activity but the cytosolic AAT (cAAT) activity remained unchanged (*14*). AAT has the highest activity among the enzymes for glutamate metabolism (*15–19*). The decrease of mAAT activity under ischaemic conditions was prevented by calpain specific inhibitors, e.g. by calpeptin and calpain inhibitor peptide, but not by other protease inhibitors (*14*). The abnormal increase of intracellular calcium ions in ischaemic retinal cells may cause an influx of Ca^{2+} into the mitochondria, thus affecting the different mitochondrial enzymes through the action of mitochondrial calpain. We have suggested that the decreased mAAT activity in ischaemic and hypoxic rat retinas might be induced by calpain-catalysed proteolysis in mitochondria, and a specific mitochondrial calpain may exist in mitochondria.

Both μ- and m-calpain have been purified from the cytosolic fraction of retinal cells (*11*), and it is reasonable to consider these as cytoplasmic proteases. However, calpain-like activity has also been found in the mitochondrial fraction (*14*, *20–23*). Some reports suggested that cytosolic μ-calpain translocates into mitochondria (*24–26*) and calpain 10 localizes in mitochondrial matrix (*27*). However, a specific mitochondrial calpain have not been identified, and obviously the substrate in mitochondria remains unknown.

Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein involved in caspase-independent cell death (*28*), while it functions as an anti-apoptotic factor via its oxidoreductase activity (*29*). Previous reports have demonstrated that AIF is anchored to the outer face of the mitochondrial inner membrane (IM) that needs to be

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cleaved for becoming a soluble and apoptogenic protein (*30*, *31*), and that incubation of isolated mitochondria with different amounts of Ca^{2+} induces mitochondrial AIF release by cytosolic μ-calpain (*24*). However, it is difficult to consider that cytosolic μ-calpain, a ~110 kDa heterodimer with regulatory subunit, enters the mitochondrial intermembrane space (IMS) across the mitochondrial outer membrane (OM) without a requirement for cytosolic factors. We supposed that AIF is directly cleaved by another endogenous mitochondrial IMS protease. Thus, we tested the hypothesis that the activation of specific mitochondrial calpain induces proteolysis of AIF and release from mitochondrial IM.

The purpose of this study was to determine the distribution of calpain activity in the mitochondria, and to compare the properties of partially purified mitochondrial calpain to that of the cytosolic μ- and m-calpains by examining its Ca^{2+}-dependency, its inhibition by calpain inhibitors, its optimum pHs, its distribution by immunoblotting techniques and to determine its enzymatic property by casein zymography and the target molecule of mitochondrial calpain in addition to mAAT (*14*).

MATERIALS AND METHODS

Subcellular Fractionation—All of the procedures were carried out at 4°C on freshly prepared swine liver. Swine livers were washed with homogenizing buffer to remove blood and homogenized in 5 volumes of homogenizing buffer (20 mM Tris–HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose and 5 mM 2-mercaptoethanol) using a glass-Teflon homogenizer. The nuclear, mitochondrial, lysosomal and microsomal fractions were separated by differential centrifugation at $600 \times g$ for 5 min, $4{,}500 \times g$ for 10 min, $20{,}000 \times g$ for 20 min and $100{,}000 \times g$ for 60 min, respectively. Each fraction was washed three times with homogenizing buffer or with homogenizing buffer containing 0.14 M NaCl or with homogenizing buffer containing 0.01% Triton ×-100, and centrifuged under the same conditions except for the supernatants. The supernatants were used as the soluble fractions. Each sample was resuspended in 500 μl of buffer A (20 mM Tris–HCl, pH 7.5 containing 1 mM EDTA, 1 mM EGTA and 5 mM 2-mercaptoethanol).

Subfractionation of mitochondria—All of the procedures were carried out at 4°C, following the method of Parson *et al*. (*32*). The mitochondrial fraction obtained by differential centrifugation was resuspended in 2 volumes of 20 mM potassium phosphate buffer containing 0.2 mg/ml bovine serum albumin (Wako Pure Chemical Industries, Osaka, Japan) at pH 7.4 and allowed to stand at 4° C for 1 h. The resuspended sample was centrifuged at $3{,}000 \times g$ for 10 min. The supernatant was centrifuged at $105{,}000 \times g$ for 30 min, and the pellet was used as the OM fraction and the supernatant was used as the IMS fraction. The remaining pellet was sonicated (15 sec × 4) and centrifuged at $77{,}000 \times g$ for 60 min, and this pellet was used as the IM fraction and the supernatant was used as the matrix fraction. The purity of the mitochondrial compartments was determined by immunoblot analysis [anti-gliceraldehyde phosphate dehydrogenase (GAPDH) for cytosolic fraction, anti-mitochondrial porin (VDAC) for OM, anti-AIF for IM, anti-pyruvate dehydrogenase (PDH) for matrix]. High purities of the mitochondrial compartments were observed (results not shown).

Assay of Calpain Activity—Calpain activity was assayed using Succinyl-Leu-Tyr-7-amino-4-methylcoumarin (Succ-Leu-Tyr-AMC; BACHEM, Bubendorf) as a substrate (*33*). Succ-Leu-Tyr-AMC is cleaved by calpain to form a fluorescent product. Samples were dialysed against 100 volumes of buffer A before use. The assay was performed as follows: 50 μl of each sample (100 μg of protein) was added to 450 μl of substrate solution (100 mM Tris–HCl, pH 7.5, 10 mM 2-mercaptoethanol, 100 mM KCl, 5 mM $CaCl_2$ contaning 20 μM Succ-Leu-Tyr-AMC) and incubated at 25°C. After incubating for 20 min, 500 μl of ice-cold 100 mM EDTA was added, and the mixture was rapidly boiled at 80°C for 3 min to stop the reaction. The samples were kept on ice for 20 min. Then the intensity of the fluorescence of each sample was measured with a fluorophotometer (excitation: 380 nm; emission: 460 nm). The calpain activity was defined as the increased intensity of fluorescence/min. We also assayed each fraction for calpain by column chromatography.

The protein concentration was measured by the Lowry *et al*. method (*34*).

Chromatographic Procedures—Partial purification of μ-calpain (calpain 1), m-calpain (calpain 2) and mitochondrial calpain was accomplished by three different column chromatographic procedures in the following order; DEAE-Sepharose CL-6B, Sephacryl S-300 and Sepharose 6B (Amersham Pharmacia Biotech) column chromatography. All chromatographic procedures were performed at 4°C. The μ- and m-calpains were purified from the cytosolic fractions of swine liver. The fractions were treated with 60% saturated $(NH_4)_2SO_4$ for 1 h at 4°C. After centrifugation at 18,000 rpm for 30 min, the pellet was resuspended in 6 ml of buffer A and the solution was applied to a DEAE-Sepharose CL-6B (26.4 × 400 mm) column pre-equilibrated with buffer B (20 mM Tris–HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA and 5mM 2-mercaptoethanol, 50mM NaCl). The adsorbed protein was eluted with a linear gradient of NaCl (50–400mM) using a total volume of 2 l.

Two Ca^{2+} protease peaks, one at 120 mM NaCl and the other at 250 mM NaCl, were detected. The first peak was assigned to μ-calpain, and the latter to m-calpain based on their Ca^{2+} requirements. From these chromatographic procedures, μ- and m-calpain were completely separated, and the following purification steps were carried out on each fraction.

The peak fractions were concentrated by Amicon PM-10 membrane (Millipore Co., Bedford, MA, USA). The samples were applied to a Sephacryl S-300 (19 × 1000 mm) column equilibrated with buffer C (20 mM Tris–HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA and 5 mM 2-mercaptoethanol, 0.14 M NaCl). The fractions containing calpain activity were collected and concentrated by Amicon PM-10 membrane. The concentrate was applied to a Sepharose 6B (19 × 1000 mm) column equilibrated with buffer C.

The fractions containing calpain activity were collected and concentrated to approximately 4 ml.

Mitochondrial calpain was purified by the same procedures. The mitochondrial IMS fraction from swine liver was treated with 60% saturated $(NH_4)_2SO_4$ for 1h at 4°C. After centrifugation at 18,000 rpm for 30 min, the pellets were resuspended in 6 ml of buffer A. The solution was applied to a DEAE-Sepharose CL-6B (26.4 × 400 mm) column pre-equilibrated with buffer B. The adsorbed protein was eluted with a linear gradient of NaCl (50–400 mM) with a total volume of 2 l.

The calpain activity appeared as one peak at 150 mM NaCl. The peak fraction was collected and concentrated by Amicon PM-10 membrane. The concentrate was applied to a Sephacryl S-300 (19 × 1000 mm) column equilibrated with buffer C. The fractions containing calpain activity were collected and concentrated by Amicon PM-10 membrane. The concentrate was applied to a Sepharose 6B (19 × 1000 mm) column and equilibrated with buffer C. The fractions containing calpain activity were collected and concentrated to approximately 4 ml.

Final purity of the mitochondrial calpain was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli (*35*), with 12% acrylamide gel. After electrophoresis, proteins were detected with a silver staining kit (Wako).

Calcium Activation, Optimum pH, and Inhibitor Experiments—All of the assays for μ-, m- and mitochondrial-calpain activities were carried out using Succ-Leu-Tyr-AMC as a substrate. The samples were μ-, m- and mitochondrial-calpains that were partially purified using ammonium sulfate, DEAE-Sepharose, Sephacryl S-300 and Sepharose 6B column chromatography as described.

To compare the susceptibilities of μ-, m- and mitochondrial-calpains to different calpain inhibitors and other protease inhibitors, the assay buffer (100 mM Tris–HCl, pH 7.5, 10 mM 2-mercaptoethanol, 100 mM KCl, 5 mM $CaCl_2$ and 20 μM Succ-Leu-Tyr-AMC) containing 1 μM calpain inhibitor I, calpeptin (Calbiochem, Cambridge, MA, USA), leupeptin, chymostatin or 10 μM pepstatin and *O*-phenanthrolin (Wako) was used.

The Ca^{2+} requirement of the calpains was determined by a Ca^{2+}-EGTA buffer system (*21*). Each sample (25 μg protein) was added to 450 μl of assay buffer (100 mM Tris–HCl, pH 7.5, 10 mM 2-mercaptoethanol, 100 mM KCl, 1mM EGTA, 20 μM Succ-Leu-Tyr-AMC) in the presence of different concentrations of $CaCl_2$ (0–1000 μM).

For the optimum pH assay, the pH of the assay buffer (100 mM Tris–HCl, 10 mM 2-mercaptoethanol, 100 mM KCl, 5 mM $CaCl_2$, 20 μM Succ-Leu-Tyr-AMC) was varied from 6.0 and 8.8.

Western Blotting—Western blotting was performed according to Towbin *et al*. (*36*). Each sample was electrophoresed on 10% acrylamide gels for anti-μ-, m-calpains, calpain 10 and AIF, 12% gels for anti-calpastatin, and 15% gels for anti-calpain small subunit antibodies. After electrophoretic transfer of proteins to nitrocellulose membrane, the membrane was treated with blocking buffer, phosphate buffered saline (0.14 M NaCl and 10 mM phosphate buffer, pH 7.4) containing 0.5% bovine serum albumin, 1% rabbit or goat serum and 0.05% Tween 20 for 2 h at room temperature. The membrane was then incubated with the appropriate primary antibody and horseradish peroxidase (HRP)-conjugated secondary antibodies at 4°C overnight, respectively. Antibody sources and dilutions were: mouse monoclonal antibodies against μ-calpain (domain III) (1: 5000; Alexis Biochemicals, San Diego, CA, USA), calpain small subunit (1: 4000; Chemicon International Inc., Temecula, CA, USA), goat polyclonal antibodies against calpastatin (1: 4000; Santa Cruz Biotechnology Inc., CA, USA), rabbit polyclonal antibodies against μ-calpain domain IV (1:5000; Sigma Aldrich Corp., St. Louis, MO, USA), m-calpain (domain III) (1: 4000; Chemicon), calpain 10 (domain T) (1: 5000; Sigma Aldrich), AIF (1: 5000; Abcam Inc., Cambridge, MA, USA), HRP-conjugated rabbit anti-mouse IgG, rabbit anti-goat IgG and goat anti-rabbit IgG antibodies (1: 5000; DAKO). The antibodies were diluted with blocking buffer. Washing was performed after each step with phosphate-buffered saline containing 0.05% Tween 20. Immunoreactive signals were developed with the ECL Western blotting detection kit (Amersham Biosciences) and the immunoreactive bands were quantified with a Luminescent Image analyzer, LAS-3000 (Fujifilm).

Zymography—Zymogram analysis was carried out according to Raser *et al*. (*37*) and Arthur *et al*. (*38*). Briefly, 60 μl of each sample was diluted in 0.1 M Tris–HCl (pH 6.8) containing 20% glycerol, 10 mM 2-mercaptoethanol and 10 mM EDTA and loaded onto 10% polyacrylamide gel containing 1 mg/ml casein (Sigma Aldrich). The electrophoretic run was carried out for 3 h at 4°C and 125 V using 25 mM Tris–HCl (pH 8.0) containing 125 mM glycine, 1 mM EDTA and 10 mM 2-mercaptoethanol. After electrophoretic run, the gels were incubated overnight at room temperature in calcium proteolysis buffer (20 mM Tris–HCl, pH 7.4 containing 10 mM 2-mercaptoethanol and 20 mM $CaCl_2$) or non-calcium proteolysis buffer (20 mM Tris–HCl, pH 7.4 containing 10 mM 2-mercaptoethanol, 5 mM EDTA and 1 mM EGTA) under gentle shaking. The gels were stained with Coomassie Brilliant Blue for 30 min and destained overnight. The bands of caseinolytic activity appear white on a stained background.

Determination of AIF Cleavage by Endogenous Mitochondrial Calpain—Isolated swine liver mitochondria were incubated at 37°C for 5–120 min in suspending buffer (20 mM Tris–HCl, pH 7.5, 0.25 M sucrose and 5 mM 2-mercaptoethanol) supplemented with 1 mM $CaCl_2$. After incubation, 5 μM calpeptin was added to each sample. Then, we performed the subfractionation of mitochondria as described earlier. Each sample was separated by SDS-PAGE, and then proteolytic processing of AIF and activation of mitochondrial calpain were detected by immunoblotting.

Trypsin Treatment of the Mitochondrial Surface—To eliminate contamination of cytosolic proteins by mitochondrial fraction, trypsin treatment of the mitochondrial surface was performed following the method of Matocha *et al*. (*39*). Isolated swine liver mitochondria were suspended in homogenizing buffer and 50 μg/ml of

TPCK-trypsin (Worthington, Freehold, NJ, USA) were added to the suspensions. After 30 and 60 min on the ice, trypsin activity was arrested by the addition of 200 μg/ml of soybean trypsin inhibitor (Sigma Aldrich). Mitochondria were reisolated and washed by centrifugation at 10,000 × *g* for 10 min at 4°C. Then, we performed the subfractionation of mitochondria as described earlier.

Assay of Glutamate Dehydrogenase Activity—The purity of the mitochondrial compartments was determined by assaying for glutamate dehydrogenase (GDH) (*40*), a specific mitochondrial matrix enzyme. For the assay, 1 μl of each sample (8 μg of protein) was added to 193 μl of substrate solution (50 mM Tris–HCl, pH 8.0, 2.5 mM EDTA, 0.2 mM NADH, 1 mM ADP, 100 mM ammonium acetate containing 7 mM 2-oxoglutarate) and incubated at 25°C. The equilibrium of the reaction lies far in favour of glutamate formation. The measurements were, therefore, made with oxoglutarate and NH_4^+ as substrate that served as a measure of the GDH activity. The GDH activity was defined as the decrease of intensity of absorbance at 340 nm/min. GDH activity was present in mitochondrial matrix (47.5%), IM (45.4%), IMS (4.5%) and OM (2.6%). GDH activity existed mainly in the matrix and IM. Therefore, the possibility of a contamination of the mitochondrial IMS by the matrix was eliminated.

RESULTS

The subcellular distribution of calpain activity in swine liver was determined (Fig. 1A). The main calpain activity was detected in the soluble fraction, but enzyme activity was also present in the mitochondrial fraction. This distribution was similar to that for retinal calpain activity as suggested by our previous report (*14*). The distribution of calpain activity in subfractions of swine liver mitochondria was then examined in more detail (Fig. 1B). A significant amount of the specific activity was also detected in the mitochondrial IMS and matrix subfractions.

To compare the properties of mitochondrial calpain with cytosolic μ- and m-calpains, the enzymes were partially purified. Cytosolic μ- and m-calpains were separated by DEAE-Sepharose CL-6B column; one at 120 mM NaCl (μ-calpain) and another at 250 mM NaCl (m-calpain) in the cytosolic fraction of swine liver (Fig. 2A). The purification steps were carried out separately for each calpain with Sephacryl S-300 column chromatography (Fig. 2B and C). Mitochondrial calpain was purified by the same methods used for the cytosolic calpains. The calpain activity appeared as one peak at 150 mM NaCl (Fig. 2D). The eluted activity peak slightly differed from cytosolic μ-calpain. No peak was observed at 250 mM NaCl. The peak fractions were used for further purification by Sephacryl S-300 column chromatography (Fig. 2E). By this column chromatography, single peak of calpain activity appeared. We obtained 27-fold purification of mitochondrial calpain. We carried out SDS-PAGE to determine the purity of mitochondrial calpain (Fig. 3A). Immunoblot analysis showed that μ- and mitochondrial-calpains were positive to monoclonal anti-μ-calpain antibody (domain III) (Fig. 3B).

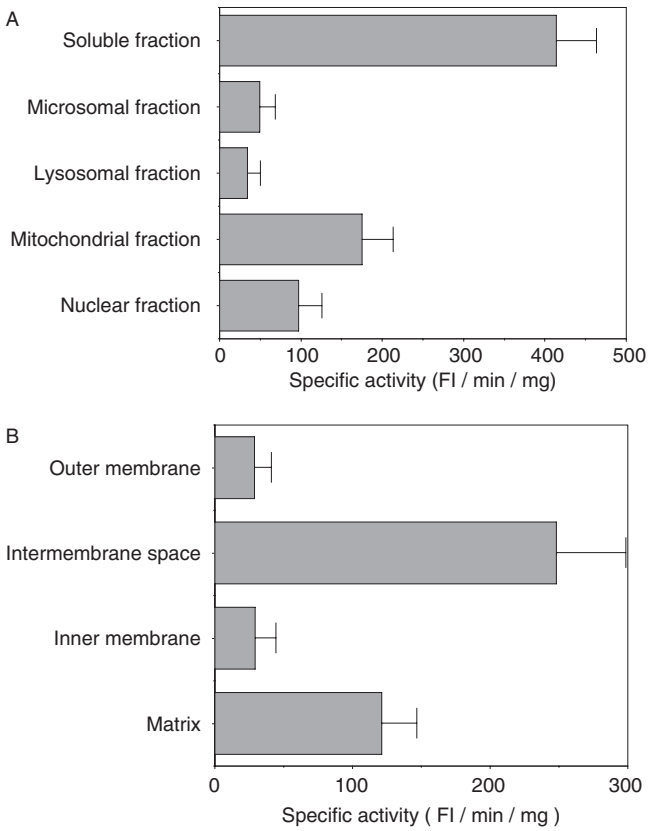

Fig. 1. **Subcellular distribution of calpain activity in swine liver.** (A) Major calpain activity is found in the soluble fraction, however, relatively high specific activity of calpain is also seen in the mitochondrial fraction. Total calpain activity was 790 ± 40 (mean ± SD, $n = 5$) FI/min/mg crude liver protein. (B) Distribution of calpain activity in mitochondrial subfractions; the outer membrane, the intermembrane space, the inner membrane and matrix fractions as described in the Materials and Methods section. Prominent specific activity of calpain is found in the intermembrane space subfraction.

Approximate molecular mass of cytosolic μ-calpain and mitochondrial calpain was 78 kDa and 75 kDa, respectively. On the other hand, mitochondrial calpain was not stained with monoclonal anti-m-calpain antibody (domain III) (Fig. 3C). The samples tested were Sepharose 6B-purified μ-, m- and mitochondrial-calpains. Cytosolic μ-, m-calpains associate with 30 kDa regulatory small subunits, however, mitochondrial calpain associates with 26 kDa subunit (Fig. 3D).

It seems unlikely that cytosolic μ-calpain binds to the cytosolic surface of the mitochondrial OM because mitochondrial fractions were washed three times with homogenizing buffer or with homogenizing buffer containing 0.14 M NaCl or homogenizing buffer containing 0.01% Triton ×-100. Nevertheless, mitochondrial calpain was detected by Western blot analysis (data not shown). After trypsin treatment of the mitochondrial surface, mitochondrial calpain and its regulatory small subunits were still localized in mitochondrial compartments (data not shown). It is also difficult to consider that only the cytosolic μ-calpain is contaminated in the mitochondrial fraction (Fig. 3). Furthermore, the molecular forms of

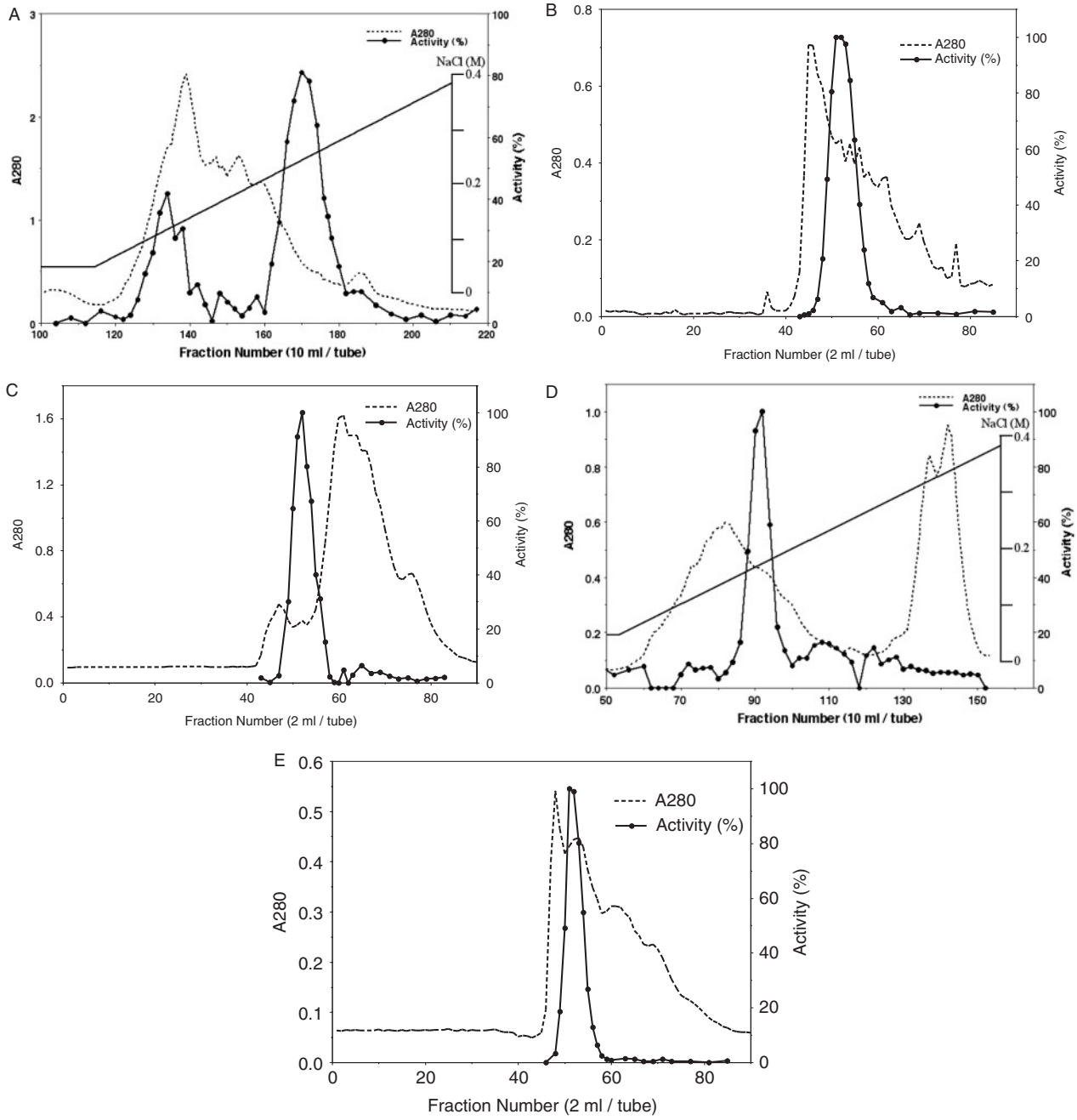

Fig. 2. **Purification of cytosolic μ-, m-calpains and mitochondrial calpain from swine liver.** Details are described in the Materials and Methods section. (A) DEAE-Sepharose CL-6B column chromatography of cytosolic μ- and m-calpains. Two peaks of calpain activity, one at 120 mM NaCl and the other at 250 mM NaCl are present. The former peak was assigned to μ-calpain and the latter to m-calpain. (B) Sephacryl S-300 column chromatography of cytosolic μ-calpain. The sample purified with DEAE-Sepharose CL-6B column chromatography was loaded onto Sephacryl S-300 column and eluted with buffer A containing 0.14 M NaCl. A single peak of calpain activity is obtained. (C) Sephacryl S-300 column chromatography of cytosolic μ-calpain. The sample purified with DEAE-Sepharose CL-6B column chromatography was loaded onto Sephacryl S-300 column and eluted with buffer A containing 0.14 M NaCl. (D) DEAE-Sepharose CL-6B column chromatography of mitochondrial calpain. One peak of calpain activity is present at 150 mM NaCl. (E) Sephacryl S-300 column chromatography of mitochondrial calpain. The sample purified with DEAE-Sepharose CL-6B column chromatography was loaded onto Sephacryl S-300 column and eluted with buffer A containing 0.14 M NaCl. A single peak of calpain activity is obtained.

mitochondrial calpain and its regulatory small subunits differ from those of cytosolic μ-calpain, so the possibility of cytosolic contamination in the mitochondrial fractions was very low.

The enzymatic properties of partially purified mitochondrial calpain were determined by the effects of various inhibitors on the proteolytic activity (Fig. 4A). The susceptibilities of μ-, m-, and mitochondrial-calpains

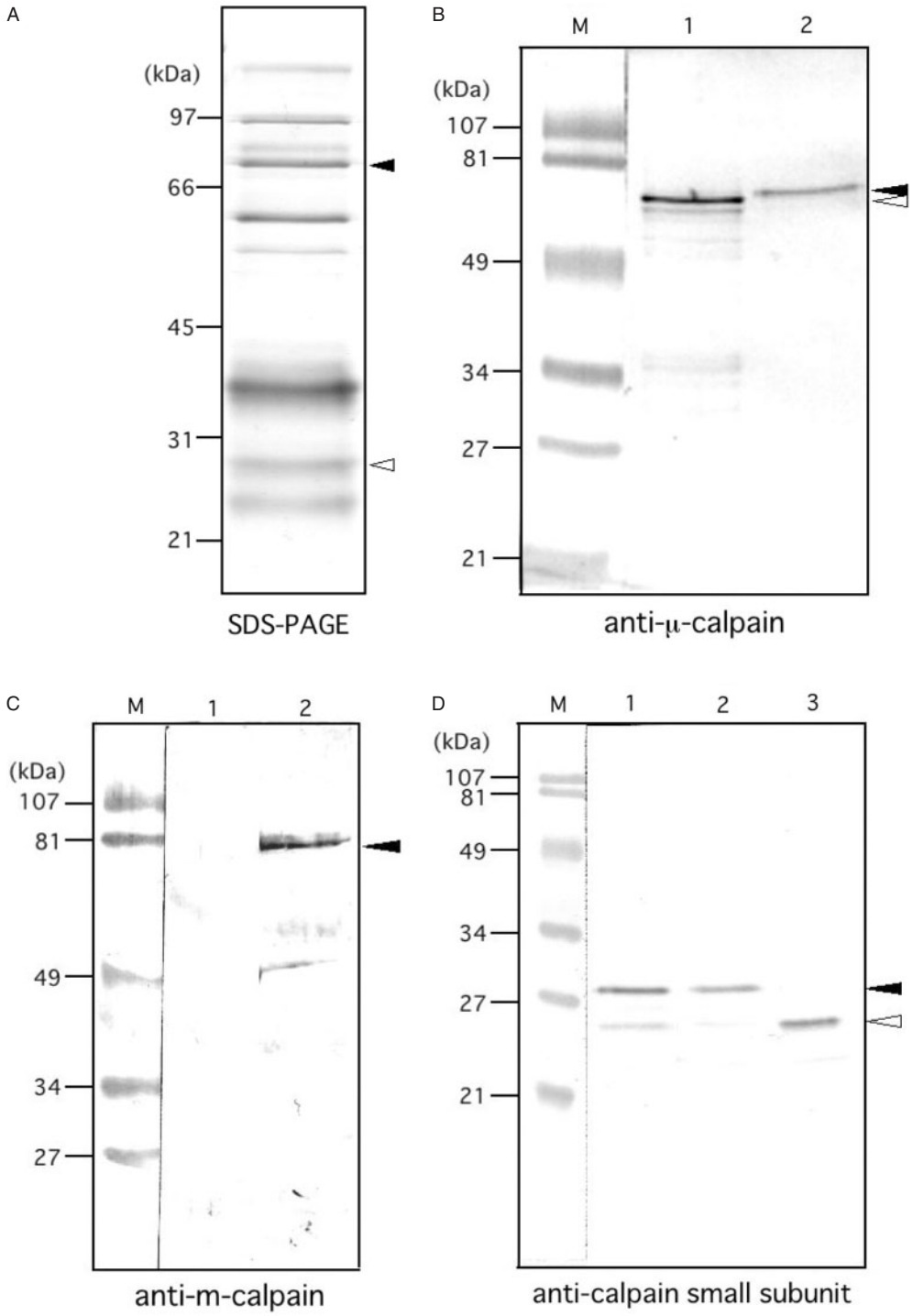

Fig. 3. **SDS-PAGE and Western blot analysis of partially purified cytosolic μ-, m-calpains and mitochondrial calpain.** (A) SDS-PAGE of partially purified mitochondrial calpain. Partially purified mitochondrial calpain (5 μg) was electrophoresed and silver-stained as described under Materials and Methods section. Solid and open arrowheads indicate mitochondrial calpain (75 kDa subunit) and its regulatory subunit (26 kDa subunit), respectively. (B) Immunoblot for anti-μ-calpain (domain III) antibody. Lane 1, partially purified mitochondrial calpain (10 μg); 2, partially purified cytosolic μ-calpain (10 μg). Mitochondrial calpain has a molecular weight of ~75,000 (open arrowhead) and cytosolic μ-calpain (solid arrowhead) has a molecular weight of ~78,000. (C) Immunoblot for anti-m-calpain (domain III) antibody. Lane 1, partially purified mitochondrial calpain (10 μg); 2, partially purified cytosolic m-calpain (10 μg). No band is present in partially purified mitochondrial calpain preparation. Solid arrowhead indicates 80 kDa. (D) Immunoblot for anti-calpain small subunit antibody. Lane 1, partially purified μ-calpain (10 μg); 2, partially purified m-calpain (10 μg); 3, partially purified mitochondrial calpain (10 μg). Partially purified cytosolic μ- and m-calpains associate with 30 kDa regulatory small subunit, however, mitochondrial calpain associates with 26 kDa small subunit. Solid and open arrowheads indicate 30 and 26 kDa, respectively. Lane M, molecular weight standards.

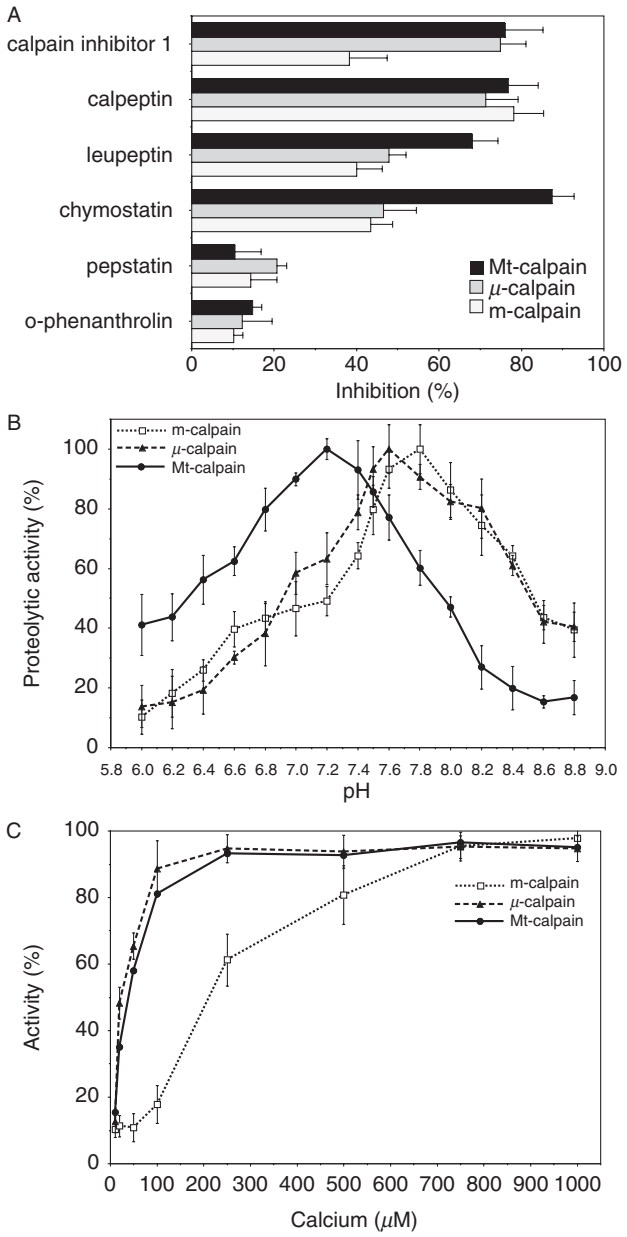

Fig. 4. **Comparison of cytosolic μ-, m-calpains and mitochondrial calpain enzymatic properties.** The samples used were partially purified μ-, m-, and mitochondrial calpains (25 μg/reaction mixture). Data are the means ± SD ($n = 4$). (A) Effects of calpain inhibitors on μ-, m- and mitochondrial-calpain activities. Treatment with calpain inhibitors (1 μM calpain inhibitor I, calpeptin, leupeptin, chymostatin) specifically inhibits the three enzymes, whereas acid protease inhibitor (10 μM pepstatin) and metalloprotease inhibitor (10 μM *O*-phenanthroline) do not. (B) Effects of pH on μ-, m- and mitochondrial-calpain activities. The optimum pHs for μ-, m-, and mitochondrial calpains were 7.6, 7.8 and 7.2, respectively. The optimum pH of mitochondrial calpain is different from that of μ- and m-calpains. (C) Calcium requirement of μ-, m- and mitochondrial-calpains for hydrolysis. Assays of calpain hydrolytic activity were performed at different Ca^{2+} concentrations. Calcium dependency of mitochondrial calpain is similar to that of cytosolic μ-calpain.

to calpain inhibitor I, calpeptin, and leupeptin were similar. Mitochondrial calpain was more susceptible to chymostatin than μ- and m-calpains. These three enzymes were not inhibited by acid protease inhibitor (pepstatin) and metalloprotease inhibitor (*O*-phenanthrolin). The optimum pH of μ-, m-, and mitochondrial-calpains was about 7.6, 7.8 and 7.2, respectively (Fig. 4B). The dependency of μ- and mitochondrial-calpains to calcium was similar (Fig. 4C). Therefore, we named the partially purified mitochondrial protease as mitochondrial μ-like calpain.

Casein zymography is a technique commonly used to evaluate calpain activity. Using this technique, we evaluated calpain activity in the cytosolic fraction and mitochondrial compartments from swine liver. As shown in Fig. 5A, zymogram of cytosolic fraction revealed one upper band for μ-calpain and another lower band for m-calpain. Erythrocytes were used as controls for μ-calpain. Two bands in the mitochondrial IMS and one band in matrix were detected, and the upper major band in IMS had same mobility as one band in matrix. The active bands observed in IMS and matrix had different mobilities from cytosolic μ- and m-calpains. No clear bands were detected in mitochondrial OM and IM (data not shown). Pretreatment with calpeptin inhibited the proteolytic activity in IMS and matrix (Fig. 5B). In a non-calcium proteolysis buffer, no distinct band appeared in cytosolic fraction, IMS and matrix (data not shown). These results demonstrate that these two mitochondrial proteases in the each fraction are calpain isoforms.

The caseinolytic bands of each sample following zymography were subjected to SDS-PAGE and immunoblotting with anti-μ-calpain, m-calpain, calpain small subunit and calpain 10 antibodies (Fig. 5C). Cytosolic μ-calpain (band No. 1) and upper mitochondrial calpains (bands No. 3 and 5) were detected with anti-μ-calpain domain III, and domain IV antibodies, however, upper and lower mitochondrial calpains were not with anti-m-calpain (domain III) and anti-calpain 10 (domain T) antibodies. Cytosolic μ-, m-calpains and two mitochondrial calpains remain associated with calpain small subunits. These results revealed that two types of mitochondrial calpains reside in mitochondria, activated in Ca^{2+}-dependent manner, inhibited by calpeptin and have different mobilities from cytosolic μ- and m-calpains. We tentatively call mitochondrial calpain stained with anti-μ-calpain antibody (bands No. 3 and 5) mitochondrial μ-like calpain (mitochondrial calpain I). In the similar way, we call mitochondrial calpain not stained with anti-μ-, m-calpains and calpain 10 antibodies (bands No. 4) unknown mitochondrial calpain (mitochondrial calpain II).

Western blot analysis using anti-μ-calpain (domain III), calpain small subunit and calpastatin antibodies were performed in swine liver cytosolic fraction and mitochondrial compartments (Fig. 6). Two bands at about 80 and 78 kDa were detected in the cytosolic fraction, mitochondrial IMS and matrix, but only one band at about 80 kDa exists in mitochondrial OM and IM (Fig. 6A). About 30- and 28-kDa small subunits were detected in cytosolic fraction, however, 30 kDa small subunit was in OM and IM, and 26 kDa in IMS and matrix (Fig. 6A). Interestingly, calpastatin, the specific

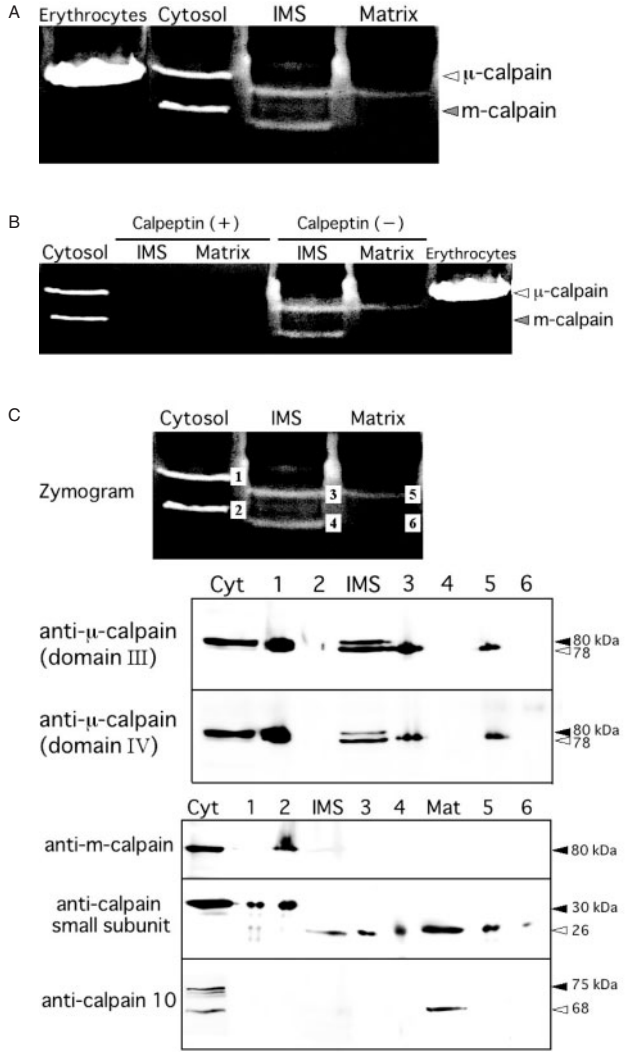

Fig. 5. **Casein zymogram and immunoblots for the cytosolic and mitochondrial fractions.** (A) Casein zymogram for μ-, m- and mitochondrial-calpains. Samples used were swine erythrocytes (300 μg), liver cytosol (250 μg), mitochondrial IMS (400 μg) and matrix (400 μg). Erythrocytes were used as controls for μ-calpain. The cytosolic fraction revealed upper band for μ-calpain (open arrowhead) and lower band for m-calpain (solid arrowhead). In IMS, upper clear and lower faint band were detected. One upper band was also in matrix. The activity bands in IMS and matrix had different mobilities from cytosolic μ- and m-calpains. (B) Casein zymogram of calpeptin-treated mitochondrial calpain. Calpeptin inhibited the active enzymatic bands in IMS and matrix. (C) Following zymography, protein from the active enzymatic bands were extracted and subjected to SDS-PAGE, Western blotting and immunostaining techniques. In order, from top panel to bottom, antibodies used were anti-μ-calpain domain III, domain IV, m-calpain (domain III), calpain small subunit and calpain 10 antibodies. As a positive control, cytosolic fraction, mitochondrial IMS and matrix were used (20 μg). Cytosolic μ-calpain (band No. 1) and mitochondrial calpain bands No. 3 and 5 were stained with anti-μ-calpain domain III and IV antibodies (solid arrowhead, 80 kDa; open arrowhead, 78 kDa). Only cytosolic m-calpain (band No. 2) was stained with anti-m-calpain antibody (solid arrowhead, 80 kDa). Every band contains calpain small subunits (solid arrowhead, 30 kDa; open arrowhead, 26 kDa). Every band was not stained with anti-calpain 10 (domain T) antibody (solid arrowhead, 75 kDa; open arrowhead, 68 kDa).

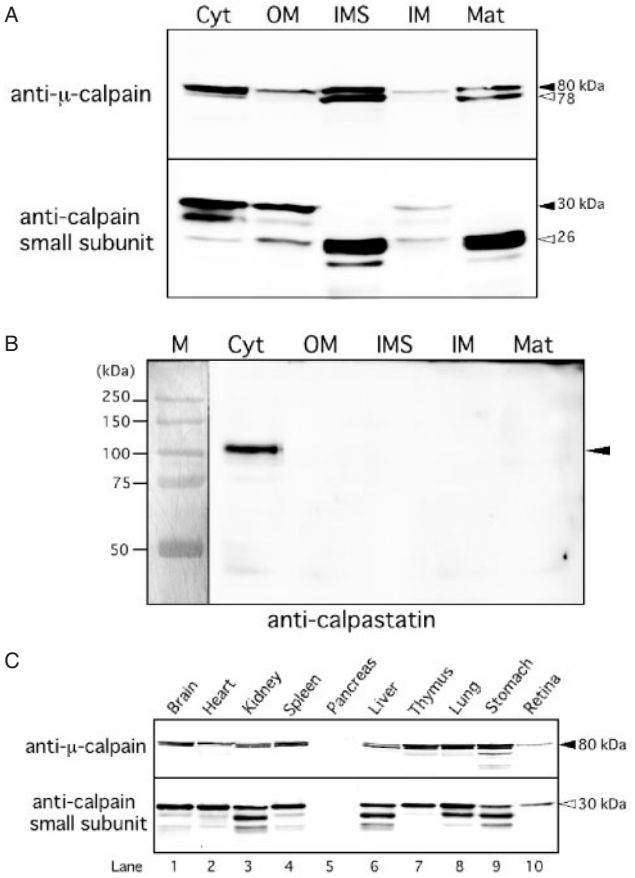

Fig. 6. **Mitochondrial distribution and tissue distribution of mitochondrial calpain.** Each containing 40 μg of protein/lane was applied (A–C). (A) Immunoblot for anti-μ-calpain (domain III) and calpain small subunit antibodies in swine liver mitochondrial compartments. Two bands at about 80 (solid arrowhead) and 78 kDa (open arrowhead) were detected in IMS and matrix, but only 80 kDa bands exist in OM and IM. Cytosolic calpain associated 30- and 28-kDa small subunit, however, mitochondrial calpain associated 30 kDa small subunit in OM and IM, and 26-kDa in IMS and matrix (solid arrowhead, 30 kDa; open arrowhead, 26 kDa). (B) Immunoblot for anti-calpastatin antibody. Calpastatin was not in the mitochondrial compartments. Solid arrowhead indicates ~110 kDa. Lane M, molecular weight standards. (C) Tissue distribution of mitochondrial calpains. Mitochondrial fractions from various swine tissues were subjected to Western blot analysis with anti-μ-calpain (domain III) and calpain small subunit antibodies. Lane 1, brain; 2, heart; 3, kidney; 4, spleen; 5, pancreas; 6, liver; 7, thymus; 8, lung; 9, stomach; 10, retinas. Almost all tissues contain mitochondrial calpain catalytic large and its regulatory small subunits except pancreas. Solid and open arrowheads indicate 80 kDa catalytic large subunit and 30 kDa regulatory small subunit, respectively.

endogenous inhibitor of calpains, was not detected in the mitochondrial compartments (Fig. 6B). Tissue distribution of mitochondrial calpain was examined by Western blot analysis in various swine tissues using anti-μ-calpain (domain III) and calpain small subunit antibodies (Fig. 6C). Mitochondrial calpain and its regulatory small subunit were detected in brain, heart, kidney,

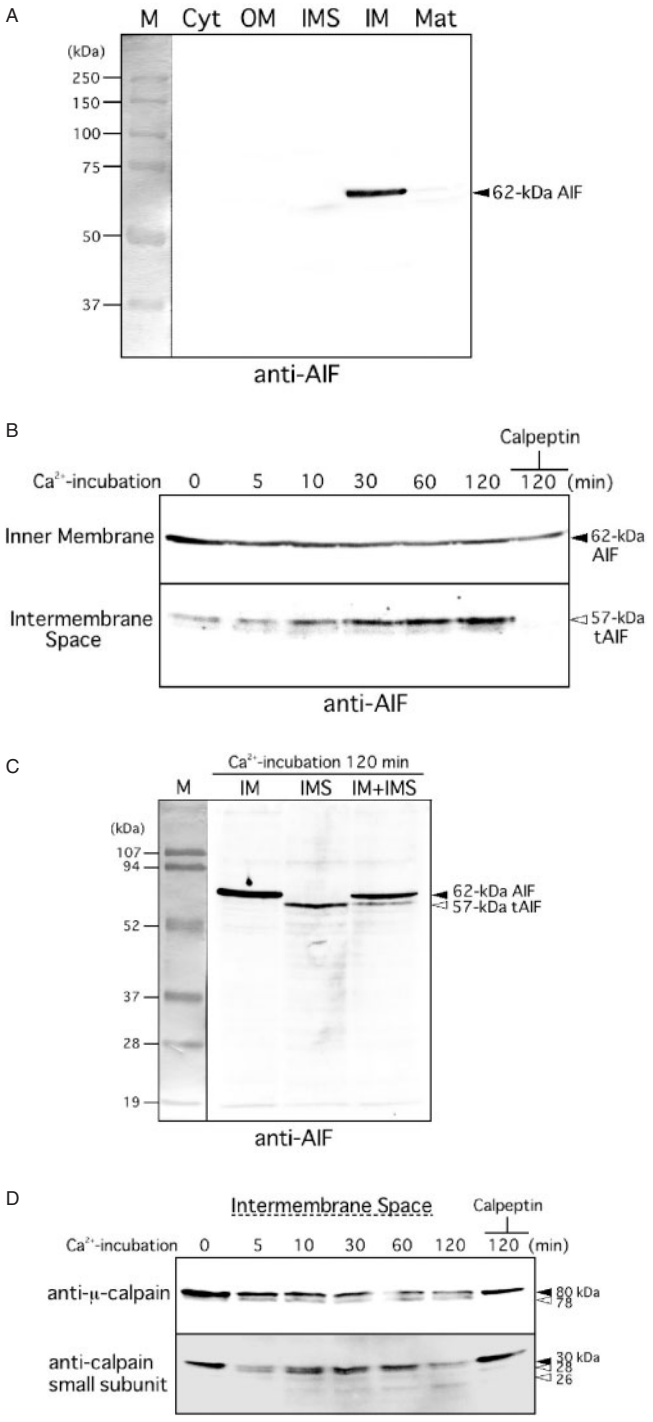

Fig. 7. **Endogenous mitochondrial calpain directly cleaves AIF and releases it from inner membrane.** (A) Immunoblot for AIF in mitochondrial compartments from fresh swine liver (20 μg/lane). The 62 kDa intact AIF exists in IM (solid arrowhead). (B–D) We added 1 mM calcium to isolated mitochondria from fresh swine liver and incubated at 37°C for 5–120 min with or without 5 μM calpeptin, and then performed the subfractionation of mitochondria. The autolysis of mitochondrial calpain and proteolytic processing of AIF were determined by immunoblot techniques. (B) Immunoblot for AIF in IM and IMS incubated with calcium for each time (20 μg/lane). No distinct decrease in 62 kDa intact AIF was detected, however, the amount of 57 kDa released tAIF increased in time-dependent manner in IMS. Calpeptin completely prevented the cleavage of AIF and release into IMS. (C) Immunoblot for AIF in IM and IMS incubated with calcium for 120 min (20 μg/lane). We can separate clearly between 62 kDa intact AIF (solid arrowhead) in IM and 57 kDa tAIF (open arrowhead) in IMS. (D) Upper panels showing immunoblot for the intact mitochondrial calpain catalytic subunit (stained with anti-μ-calpain antibody) at 80 kDa (solid arrowhead) and its autolysis fragments at 78 kDa (open arrowhead). Lower panels showing immunoblot for the intact calpain regulatory subunit at 30 kDa (solid arrowhead), and its autolysis fragments at 28 and 26 kDa (open arrowheads). Each containing 40 μg of protein/lane was applied. In IMS, mitochondrial calpain autolysed from 80 to 78 kDa and its regulatory small subunit was also autolysed from 30 to 28 kDa after 5 min. Calpeptin completely inhibited the autolysis of mitochondrial calpain and its regulatory small subunits. Lane M, molecular weight standards.

spleen, liver, thymus, lung, stomach and retina but not in the pancreas.

To investigate whether the proteolytic processing of intact AIF is caused by activated mitochondrial calpain in isolated mitochondria, we performed Western blot analysis (Fig. 7). About 62 kDa intact AIF was detected in IM (Fig. 7A). In IMS, the amount of 57 kDa truncated AIF (tAIF) increased in time-dependent manner (Fig. 7B). In IM, however, no distinct decrease in 62 kDa intact AIF was detected. Thus, only little amount of tAIF was released in respect to the amount of intact AIF in a Ca^{2+}-dependent manner. Calpeptin completely prevented the cleavage of AIF and release into IMS. Figure 7C showed the mixture of IM and IMS incubated with Ca^{2+} for 120 min. We can separate clearly 62 kDa intact form in IM and 57 kDa truncated form in IMS. We found that mitochondrial calpain in IMS was autolysed from 80 kDa intact form to 78 kDa autolysed form after 5 min incubation with Ca^{2+} (Fig. 7D). The regulatory small subunit in IMS was also autolysed from 30 kDa intact form to 28- and 26-kDa autolysed forms after 5 min. Calpeptin inhibited the autolysis of mitochondrial calpain and its regulatory small subunit. This indicates that mitochondrial calpain in IMS was activated in response to mitochondrial Ca^{2+} influx, as a result, auto-catalytic hydrolysis of mitochondrial calpain and its regulatory small subunits occurs during activation. On the other hand, calpain activity in IM fraction was very low as shown in Fig. 1B. These results revealed that endogenous mitochondrial calpain I in IMS mainly plays a role in direct cleavage of intact AIF to a slightly smaller form (tAIF), releasing the molecules from IM to IMS in response to mitochondrial Ca^{2+} influx.

DISCUSSION

In the present study, we examined various differences in properties of three calpains isolated from swine liver: cytosolic μ-, m-calpains and mitochondrial calpain. Reportedly, investigators have demonstrated calpain-like activities in isolated mitochondria (*14*, *20–23*), however, mitochondrial calpain has not been identified, and the characteristics have been unknown. This is the first report describing enzymatic characteristics of mitochondrial calpain. The calpains are generally considered to be cytoplasmic enzyme, however, this study showed

that mitochondrial calpain exists in the mitochondrial compartments. We found that the majority of calpain activity in mitochondria is localized to IMS and matrix (Fig. 1C). Mitochondrial calpain was stained with anti-μ-calpain domain III and IV (Figs 3B and 5C), and inhibited by calpain specific inhibitor calpeptin (Fig. 4A). The calcium dependency of μ-calpain and mitochondrial calpain was similar (Fig. 4C). These results suggest that some characteristics of μ-calpain and mitochondrial calpain are similar. However, one of the important findings in this study was that μ-calpain and mitochondrial calpain have various differences in the properties. The lower optimum pH (Fig. 4B), higher sensitivity to chymostatin (Fig. 4A) and higher mobility in zymography (Fig. 5A) suggested that the properties of mitochondrial calpain differ from μ-calpain, and that mitochondrial calpain is different calpain isoform from μ-calpain.

Zymogram analysis demonstrates that two types of caseinolytic bands reside in swine liver mitochondria. Two enzymes were Ca^{2+}-dependent and inhibited by calpeptin, and have different mobility from μ- and m-calpains (Fig. 5A and B). The upper bands (mitochondrial calpain I) observed in IMS and matrix were stained with anti-μ-calpain antibody (Fig. 5C). The mitochondrial calpain I had a higher mobility than μ-calpain and a lower mobility than m-calpain. This was consistent with observation on DEAE-Sepharose column chromatography that mitochondrial calpain (at 150 mM NaCl) elutes later than μ-calpain (at 120 mM NaCl) and faster than m-calpain (at 250 mM NaCl) (Fig. 2A and D), suggesting that mitochondrial calpain I has a greater negative charge than μ-calpain and a positive charge than m-calpain or unknown factors may bind to the mitochondrial calpain. For this reason, it is considered that the partially purified mitochondrial calpain is the identical enzyme with the mitochondrial calpain I observed in zymography. The lower caseinolytic enzyme (mitochondrial calpain II) was not stained with anti-μ-, m-calpains and calpain 10 antibodies. It would be additional calpain isoform or alternative splicing variant of known calpains. One possibility is that mitochondrial calpain I and II have been derived from μ-calpain by novel post-transcriptional modification and/or processing. Further molecular analysis of mitochondrial calpain I and II may provide an important clue in understanding its functions in mitochondria.

In present experiments, calpastatin, the specific endogenous inhibitor of calpains, was not detected in mitochondrial compartments (Fig. 6B). This suggests that the activating mechanism of mitochondrial calpain differs from that of cytosolic calpains. We observed calpain molecules in mitochondrial OM, IMS, IM and matrix, however, mitochondrial calpain activity was present only in IMS and matrix in calpain assay (Fig. 1B) and zymography (Fig. 5). Furthermore, our data identified differences in susceptibility to Ca^{2+} overload between soluble mitochondrial calpain in IMS and membrane-bound mitochondrial calpain in IM (Fig. 7D), *viz*., soluble mitochondrial calpain is prominently activated by Ca^{2+} overload but membrane-bound mitochondrial calpain is not. These results suggest that membrane-associated forms of mitochondrial calpain remain pre-activated state or endogenous inhibitor of mitochondrial calpain exists in membranes in place of calpastatin. It is an important task to investigate the activation mechanism of mitochondrial calpain in more detail, particularly as to interactions with mitochondrial membranes or regulatory molecules.

Our earlier study showed that 90 min of ischaemia or hypoxia caused a 20% decrease in mAAT activity and resulted in a 2.5-fold increase in mitochondrial calpain activity (*14*). We suggested that under ischaemic or hypoxic conditions, the activation of mitochondrial calpain take place because of the elevated mitochondrial Ca^{2+} that leads to the decrease of mAAT activity. It has been reported that Ca^{2+} accumulates in the mitochondria during ischemia (*12*, *41*, *42*). As shown in this study, calpain activity is relatively low in the mitochondrial matrix (Fig. 1B). This may explain why 90 min of ischaemia or hypoxia caused only a mild decrease in mAAT activity.

In this study, we found that endogenous mitochondrial calpain was able to directly cleave AIF to a slightly smaller form, releasing the molecules from IM to IMS (Fig. 7). The endogenous mitochondrial protease responsible for apoptosis-dependent AIF processing has not yet been identified. It is considered that unknown proteases localized in the mitochondrial IMS or IM is involved in this process. Our findings support this consideration. It is most likely that AIF is directly cleaved by endogenous mitochondrial calpain in mitochondrial IMS rather than cytosolic μ-calpain Polster *et al*. (*24*) suggests that Bid induces an outer membrane pore large enough for AIF (57 kDa theoretical molecular mass) to release it to cytosol from mitochondria, and they detected calpain activity and multiple calpain isoforms in preparations of isolated liver and brain mitochondria. This report is supportive of our findings which indicate a role for endogenous mitochondrial calpain in AIF cleavage. Our study demonstrates that AIF is a specific substrate of mitochondrial calpain, and the activation of mitochondrial calpain may play a role in mitochondrial apoptotic signal via proteolytic processing of AIF.

In summary, this study indicates that mitochondrial calpain, which differs from cytosolic calpains, seem to be ubiquitous, exist in physiological state mitochondria and mediators of mitochondrial apoptotic signaling via direct cleavage of AIF.

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