Biochimica et Biophysica Acta 1793 (2009) 1848–1859

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Mitochondrial m-calpain plays a role in the release of truncated apoptosis-inducing factor from the mitochondria ☆

Taku Ozaki [a], Tetsuro Yamashita [b], Sei-ichi Ishiguro [a,*]

[a] Department of Biochemistry and Biotechnology, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561, Japan
[b] Department of Food science and Biochemistry, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

ARTICLE INFO

Article history:
Received 25 May 2009
Received in revised form 19 September 2009
Accepted 6 October 2009
Available online 13 October 2009

Keywords:
Calpain
Mitochondria
Apoptosis
AIF
VDAC
Grp75

ABSTRACT

Calpains, calcium-dependent cysteine proteases, are involved in a variety of cellular processes. We have reported on the characteristics of mitochondrial μ-calpain and have shown that ERp57-associated mitochondrial μ-calpain cleaves the apoptosis-inducing factor (AIF) to a truncated form (tAIF). In addition, we found an unknown mitochondrial calpain. In this study, we identified and characterized this undescribed mitochondrial calpain in rat liver mitochondrial intermembrane space. The mitochondrial μ- and unknown calpains were separated by DEAE-Sepharose column chromatography. We immunoprecipitated the unknown calpain with anti-calpain small subunit and identified it as calpain 2 (m-calpain large subunit) by nanoflow-LC-MS/MS analysis and database searching. Because the identified mitochondrial calpain was stained with anti-m-calpain large subunit antibody, we named it mitochondrial m-calpain. The Ca^{2+} dependency of mitochondrial m-calpain was similar to that of cytosolic m-calpain. Immunoprecipitation analyses showed that mitochondrial m-calpain is associated with a 75-kDa glucose-regulated protein, a member of the heat shock protein 70 family. We also investigated the involvement of mitochondrial m-calpain in the release of tAIF from mitochondria. Calpain inhibitor, PD150606, an anti-voltage-dependent anion channel (VDAC), and anti-Bax antibodies prevented the release of tAIF from mitochondria. In addition, we found that mitochondrial m-calpain truncated VDAC in Ca^{2+}-dependent manner. This cleavage of VDAC promotes the mitochondrial accumulation of Bax and the release of tAIF from mitochondria. The accumulated Bax in mitochondrial outer membrane was co-immunoprecipitated with VDAC. Our results demonstrated that mitochondrial m-calpain plays a role in the release of tAIF from mitochondria by cleaving VDAC, and tAIF is released through VDAC-Bax pores.

1. Introduction

The calpains (E.C. 3.4.22.17) form a superfamily of intracellular cysteine proteases with numerous isoforms that are found in almost all eukaryotes and some bacteria [1–4]. At present, 14 human genes have been identified as members of the calpain catalytic subunit family and two genes identified for calpain regulatory subunits [4,5]. Although their physiological roles have not been fully determined, they function in Ca^{2+} signaling by modulating the biological activities of their substrates through limited proteolysis [5].

The calpain family can be divided into the ubiquitous and the tissue-specific calpains [4]. Two major ubiquitous calpains, viz. μ-calpain and m-calpain large catalytic subunits (molecular mass, ~80 kDa), are associated with a common regulatory small subunit (molecular mass, ~30 kDa) [2]. The μ- and m-calpains share about 62% identical residues of their catalytic subunits and differ in the Ca^{2+} concentrations required for their activation *in vitro*; μ- and m-calpains are activated by 5–50 μM and 0.2–1 mM Ca^{2+}, respectively. These two calpains have a specific endogenous inhibitor, calpastatin [6–9]. Knockout of the μ-calpain large subunit gene results in viable mice with reduced platelet aggregation and impaired tyrosine phosphorylation in the platelets [10]. Knockout of the m-calpain large subunit is embryonically lethal [11,12].

Although calpains are considered to be cytoplasmic enzymes, we have shown that calpains also exist in the mitochondria and are involved in the truncations of mitochondrial aspartate aminotransferase (AAT) and apoptosis-inducing factor (AIF) [13–15]. The biochemical characteristics of mitochondrial μ-calpain are similar to cytosolic μ-calpain [14], and ERp57 chaperone associates with

Abbreviations: AIF, apoptosis-inducing factor; tAIF, truncated AIF; VDAC, voltage-dependent anion channel; Grp75, glucose-regulated protein 75; AK2, adenylate kinase 2; PDH, pyruvate dehydrogenase; GAPDH, glyceraldehyde phosphate dehydrogenase; OM, outer membrane; IMS, intermembrane space; IM, inner membrane; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid

☆ The cost of publication of this article was defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* Corresponding author. Division of Cell Technology, Department of Biochemistry and Biotechnology, Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki 036-8561, Japan. Tel./fax: +81 172 39 3780.
E-mail address: is1019@cc.hirosaki-u.ac.jp (S. Ishiguro).

0167-4889/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2009.10.002

mitochondrial μ-calpain [15]. We have also shown that ERp57-associated mitochondrial μ-calpain cleaves AIF to a truncated form, tAIF, and releases it from the mitochondrial inner membrane (IM) into the intermembrane space (IMS). Recently, other investigators also reported that μ-calpain is present in the mitochondria [16–19]. It is becoming increasingly apparent that calpain 10 exists in the mitochondria and is involved in calcium-induced mitochondrial dysfunction by its cleavage of Complex I subunits of the electron transport chain [20].

However, we and other investigators expected that one or more unidentified calpains exist in the mitochondria. Beer et al. [21] suggested that two Ca^{2+}-activated proteases exist in rat liver mitochondria, one was half-maximally activated with 25 μM Ca^{2+} and the other by 750 μM Ca^{2+}. Furthermore, Tavares et al. [22] found two calpain activities in the IMS and three in the matrix. In our earlier study, we found two types of mitochondrial calpains by casein zymography; one is mitochondrial μ-calpain and the other is an unknown mitochondrial calpain [14,15]. We also demonstrated that the unknown mitochondrial calpain is associated with a regulatory small subunit. Calpastatin is not present in the mitochondria, so we expected that the unknown mitochondrial calpain is associated with an unidentified regulatory molecule such as ERp57.

AIF, one of the apoptosis-related proteins, is known to be a key factor in caspase-independent apoptosis [23]. It is anchored to the outer face of the mitochondrial IM and its proteolytic processing by mitochondrial μ-calpain is required to become a soluble and apoptogenic protein [14,15,24]. The released tAIF from mitochondria to the cytosol translocates into nucleus where it causes chromatin condensation and large scale (~50 kb) DNA fragmentation in a caspase-independent fashion [25,26]. This apoptogenic function of AIF seems essential in some types of cell death. In fact, AIF seems to be implicated in retinal degeneration in eyes with retinitis pigmentosa [27,28] and brain neuronal injury including hypoxia and ischemia [16,29].

However, the mechanisms that cause the release of tAIF from mitochondrial IMS into the cytoplasm have not been determined. We considered that the inhibition of the release of tAIF from mitochondria can prevent these tAIF-mediated insults.

The purpose of this study was to identify the unknown mitochondrial calpain and its regulatory molecules and to examine its functions in the mitochondria. We tested whether the unknown mitochondrial calpain plays a role in the release of tAIF from mitochondria.

2. Materials and methods

2.1. Materials

Antibodies purchased were rabbit polyclonal antibodies against μ-calpain large subunit domain IV (Sigma Aldrich Corp., St. Louis, MO); anti-m-calpain large subunit domain I, III, and IV (Abcam, Inc., Cambridge, MA); anti-calpain small subunit domain V (Abcam); anti-calpain 10 domain T (Sigma Aldrich); anti-Grp75 (Santa Cruz Biotechnology Inc., CA); anti-adenylate kinase 2 (Santa Cruz); anti-glyceraldehyde phosphate dehydrogenase (Santa Cruz); anti-VDAC (Calbiochem); anti-Bax (Abcam); anti-AIF (Abcam); anti-pyruvate dehydrogenase (Molecular Probes, Eugene, OR); goat polyclonal anti-VDAC C-terminal (Santa Cruz); mouse monoclonal anti-TGN-38 (trans-Golgi network-38) (Abcam); rat monoclonal anti-ZO-1 (Chemicon, Temecula, CA); and normal rabbit IgG (Santa Cruz). Anti-VDAC antibody recognizes amino acid residues 185–197 of human VDAC. Rabbit anti-ERp57 antiserum was prepared against the 17-amino acid C-terminal peptide (VIQEEKPKKKKKAQEDL) of human ERp57 as described [30]. Rabbit anti-calnexin antisera were obtained as described [31]. Calpain inhibitors, calpeptin and PD150606, were purchased from Calbiochem (Cambridge, MA), and VDAC inhibitor, DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid), was purchased from Sigma Aldrich.

2.2. Preparation of rat liver mitochondria and submitochondrial fractionations

Rat liver mitochondria and mitochondrial compartments were prepared as described in detail [14]. To obtain mitochondrial unknown calpain, the mitochondrial IMS fraction was prepared from 30 Sprague–Dawley rats (8 weeks old). The purity of the mitochondrial compartments was determined by immunoblot analysis with the following antibodies; anti-mitochondrial porin (VDAC) antibody for mitochondrial outer membrane (OM), anti-adenylate kinase 2 (AK2) for intermembrane space (IMS), anti-AIF antibody for inner membrane (IM), pyruvate dehydrogenase (PDH) antibody for matrix, anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody for cytosolic fraction, anti-ZO-1 (tight junction-associated protein) for plasma membrane, anti-calnexin for ER, and anti-TGN-38 for Golgi apparatus. High purities of the mitochondrial compartments were obtained.

2.3. Partial purification of mitochondrial unknown calpain

A partial purification of mitochondrial unknown calpain was accomplished by DEAE-Sepharose CL-6B (Amersham Pharmacia Biotech) column chromatography. All chromatographic procedures were performed at 4 °C. The prepared IMS fraction was dialyzed overnight against 2 L of buffer A (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol) containing 50 mM NaCl (buffer B) at 4 °C. The dialyzed proteins were applied to a DEAE-Sepharose CL-6B column (26.4×400 mm) pre-equilibrated with buffer B 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 [14]. Unbound proteins, containing mitochondrial unknown calpain, were eluted with 600 ml of buffer B. Mitochondrial μ-calpain was eluted with a linear gradient of 50–250 mM NaCl in buffer A in a total volume of 1.2 L. Four activity peaks, unbounded protein fraction at 120 mM NaCl, at 150 mM NaCl, and at 200 mM NaCl, were detected. The fractions containing calpain 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, m-calpain large subunits, anti-calpain small subunit, anti-ERp57, and anti-calpain 10 antibodies and for calpain assay using EGTA and calpain inhibitors. The protein concentration was measured by the method of Bradford et al. [32] with bovine serum albumin as the standard.

2.4. Western blotting

Western blotting was performed as described in detail [14,15]. After electrophoretic transfer of the proteins to a nitrocellulose membrane, the membranes were treated with the primary antibodies followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit or rabbit anti-goat IgG secondary antibodies (DAKO, Cambridge, UK). Immunoreactive signals were developed with an ECL western blotting detection kit (Amersham Biosciences) and quantified with a Luminescent Image Analyzer, LAS-3000 (Fujifilm Co., Tokyo, Japan).

2.5. Immunoprecipitation of mitochondrial unknown calpain

A rabbit polyclonal anti-calpain small subunit antibody was used to immunoprecipitate mitochondrial unknown calpain. We added the antibody (1 μg/tube) to calpain activity peak 1 pooled from DEAE-Sepharose CL-6B column chromatography (1 mg protein/tube), which contained mitochondrial unknown calpain, in a total volume of 500 μl of buffer B. After standing at 4 °C for 18 h, excess protein G-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) was added (12 μl/tube) and allowed to stand for another 2 h at 4 °C. The sample