Biochemical and Biophysical Research Communications 504 (2018) 454–459

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Presence of calpain-5 in mitochondria

Takeshi Iwamoto [a], Eri Ishiyama [b], Kinji Ishida [b], Tetsuro Yamashita [c], Hiroshi Tomita [a], Taku Ozaki [a, *]

[a] Department of Chemistry and Biological Sciences, Faculty of Science and Engineering, Iwate University, Morioka, Japan
[b] Technical Support Center for Life Science Research, Iwate Medical University, Morioka, Japan
[c] Department of Biological Chemistry, Faculty of Agriculture, Iwate University, Morioka, Japan

ARTICLE INFO

Article history:
Received 9 August 2018
Accepted 24 August 2018
Available online 6 September 2018

Keywords:
Calpain
Mitochondria
Retina
Photoreceptor cell
Inner segment

ABSTRACT

Calpains are Ca^{2+}-dependent cysteine proteases that are widely distributed in animal tissues and modulate a variety of cellular processes. There are 15 members of the calpain family in mammals. In animal cells, there are three types of calpains, viz., calpain-1, calpain-2, and calpain-10 in the mitochondria. The three types of calpains have been shown to play significant roles in pathophysiological conditions, including in apoptosis- and necrosis-like cell death. One of the severe retinal diseases, autosomal dominant neovascular inflammatory vitreoretinopathy, is known to be induced by mutations of the calpain-5 gene. However, the distribution of calpain-5 in the retina has not been elucidated. Therefore, in the present study, we determined the localization of calpain-5 in the porcine retina. We detected calpain-5 in the inner segment of photoreceptor cells using immunohistochemistry. With immunoelectron microscopy, calpain-5 was localized in the mitochondria of photoreceptor cells. Western blot analyses showed that calpain-5 was present in each mitochondrial subfraction. Furthermore, we showed that the molecular weight of mitochondrial calpain-5 was slightly smaller than cytosolic one. Our results demonstrated that a novel mitochondrial calpian, calpain-5, was localized in the mitochondria of retinal photoreceptor cells.

1. Introduction

Calpains are Ca^{2+}-dependent cysteine proteases that specifically cleave substrates and regulate various intracellular functions such as cell movement, signal transduction, and gene expression [1]. In mammals, there are 15 members of the calpain family that can be classified into tissue-specific calpains (calpains-3, -6, -8, -9, -11, -12) and ubiquitous calpains (calpains-1, -2, -5, -7, -10, -13, -14, -15, -16) [2, 3]. Although calpains are important intracellular regulators, their overactivation or inactivation are involved in calpainopathies such as muscular dystrophy (CAPN3 mutations), type 2 diabetes (CAPN10 mutations), and eosinophilic esophagitis (CAPN14 mutations) [4–6].

Autosomal dominant neovascular inflammatory vitreoretinopathy (ADNIV) is a severe blinding disease caused by mutations of the calpain-5 gene. ADNIV is a hereditary autoimmune disease that mimics various blinding disease such as retinal degeneration, retinal neovascularization, intraocular fibrosis, and eventually leads to blindness [7]. The pathogenic mechanism has not been fully understood. However, it was suggested that overactivity of calpain-5 induced by CAPN5 mutations may be involved in ADNIV [8, 9]. Although calpain-5 is one of the ubiquitous calpains, the symptoms of ADNIV are restricted to the eye. Several studies indicate that calpain-5 localizes in photoreceptor synapses, the outer nuclear layer, inner segment, outer segment, and ganglion cells of the retina [7, 9, 10]. Although there are numerous mitochondria in photoreceptor synapses and the inner segment, the mitochondrial localization of calpain-5 has not been examined.

Most calpains are only present in the cytosol. However, in recent years, it was reported that calpains were localized in other organelles such as the mitochondria, endoplasmic reticulum, and Golgi apparatus [11–13]. We previously showed that mitochondrial calpains-1 and -2 play important roles for modulating mitochondria-mediated apoptosis [13–15]. Thus, investigating the subcellular localization of calpain-5 is necessary to elucidate its functions in physiological and pathological states.

Based on all of the previous findings, we hypothesize that

* Corresponding author. Department of Chemistry and Biological Sciences, Faculty of Science and Engineering, Iwate University, 4-3-5 Ueda, Morioka, Iwate, 020-8551, Japan.
E-mail address: tozaki@iwate-u.ac.jp (T. Ozaki).

https://doi.org/10.1016/j.bbrc.2018.08.144
0006-291X/

calpain-5 localizes in the mitochondria and plays significant roles in the physiological functions of photoreceptor cells. Therefore, the purpose of the present study was to determine the mitochondrial localization of calpain-5 in retinal photoreceptor cells.

2. Materials and methods

2.1. Immunohistochemistry

To show the expression of calpain-5 in photoreceptor cells of porcine retina, we performed immunohistochemistry using an anti-calpain-5 antibody (GTX103264; GeneTex, Irvine, CA, USA). Retinal sections (5-μm thick) were fixed with 4% paraformaldehyde, deparaffinized with xylene, rinsed with ethanol to remove xylene, and finally rinsed with water and rehydrated. Sections were treated with permeabilization buffer [0.5% Triton X-100 in phosphate-buffered saline (PBS)] for 15 min and rinsed with PBS. Sections were blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T) for 2 h at room temperature. Sections were then incubated with a rabbit polyclonal anti-calpain-5 antibody (1:500) diluted in PBS-T containing 5% skim milk overnight at 4 °C. Subsequently, sections were rinsed with PBS-T and incubated with AlexaFluor 488 goat anti-rabbit IgG (1:500, ab150077; Abcam, Cambridge, UK) diluted in PBS-T containing 5% skim milk overnight at 4 °C. Finally, sections were rinsed with PBS-T and nuclei were stained with 4′,6-diamidino-2-phenylindole. Fluorescence images were captured with a confocal laser microscope (C1si, Nikon, Tokyo, Japan).

2.2. Back-scattered electrons (BSE)

In order to observe the wide field morphology of porcine retina, we used BSE with a scanning electron microscope. All procedures were performed according to Ohta et al. [16]. Briefly, the anterior segment of porcine eyeballs was excised and fixed overnight with 2.5% glutaraldehyde - 2% paraformaldehyde - 0.1 M Na^+-phosphate buffer, pH 7.4. The fixed retinas were rinsed two times with 0.1 M phosphate buffer for 10 min, then post-fixed with 1% osmium tetraoxide for 2 h. The retinas were dehydrated by serial dilution in ethanol for 15 min each and embedded in Epon 812 (TAAB Laboratories). Ultrathin sections (100 nm) were prepared with an ultramicrotome (Leica, EM-UC6) and stained with 1% uranyl acetate and lead citrate. Images were captured with a scanning electron microscope (SU 8010, Hitachi, Tokyo, Japan).

2.3. Immunoelectron microscopy

All procedures were performed according to Akagi et al. [17]. Briefly, the anterior segment of porcine eyeballs was cut, the retinas removed, and fixed with 4% paraformaldehyde for 1 h. The fixed retinas were rinsed with 0.2 M Na^+-phosphate buffer, pH 7.4. After washing, the retinas were cut into 5 mm^3 cubes and permeated in 30% sucrose. The specimens were infused with a mixture of 20% PVP and 1.8 M sucrose in 0.1 M phosphate buffer, pH7.4, for 2 h at room temperature then overnight at 4 °C. After the retinas were completely permeated, they were frozen at −190 °C using liquefied propane gas. Ultrathin cryosections were prepared with an ultramicrotome (Leica, EM-FC6). Sections were washed three times with distilled water for 10 min, then three more times with 0.1 M TBS for 10 min. After washing, sections were blocked with 10% normal goat serum in 0.1 M TBS for 2 h at room temperature. After blocking, sections were incubated with the rabbit polyclonal anti-calpain-5 antibody (1:100) diluted in 0.1 M TBS for 48 h at 4 °C. Then, the sections were incubated with colloidal gold 10 nm anti-rabbit IgG (1:100, EMGAR10, BBI Solutions, Cardiff, CF14 5DX, UK) for 2 h at room temperature and floated on a mixture of 1% polyvinyl alcohol-0.1% uranyl acetate solution for embedding and electron staining, and dried overnight at room temperature. Images were captured with a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan).

2.4. Subcellular fractionation

Several organelles, including photoreceptor cell mitochondria, were obtained by differential centrifugation. The anterior segments of porcine eyeballs were cut and the retinas removed. Retinas were gently vortexed in two volumes of mitochondrial isolation buffer (KC010100, BioChain Institute, Newark, CA). The suspended solution was homogenized 40 strokes and then centrifuged at 600×*g* for 10 min. After centrifugation, the fraction containing nuclei was obtained from the pellet and the supernatant was centrifuged at 12,000×*g* for 15 min. After centrifugation, the pellet was resuspended in mitochondrial isolation buffer and centrifuged at 12,000×*g* for 15 min to obtain the pellet containing mitochondria. A pellet containing lysosomes was obtained from centrifuging the post-mitochondrial supernatant at 20,000×*g* for 20 min. This supernatant was then centrifuged at 100,000×*g* for 60 min to obtain the microsomal fraction, and the cytosol was obtained from the supernatant. The mitochondrial fraction was resuspended in mitochondrial storage buffer (KC010100, BioChain Institute). All steps were performed on ice.

2.5. Mitochondrial subfractionation

Fractions including the outer membrane (OM), intermembrane space (IMS), inner membrane (IM), and matrix of mitochondria were prepared according to Ozaki et al. [15]. Briefly, purified mitochondria were centrifuged at 10,000×*g* for 10 min. After centrifugation, the pellet was resuspended in two volumes of buffer A (20 mM K^+-phosphate buffer, pH 7.4, containing 0.2 mg/mL

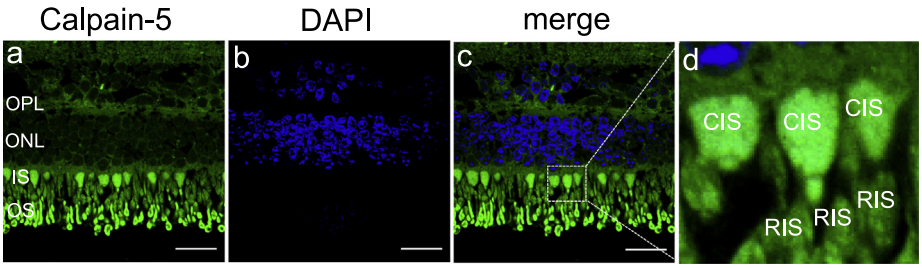

Fig. 1. Immunohistochemistry in the porcine retina. (a) Image of staining with the calpain-5 antibody (green). (b) Image of staining with 4′,6-diamidino-2-phenylindole (DAPI) (blue). (c) Merged image of calpain-5 antibody and DAPI staining. (d) Magnified view of the cone and rod ellipsoids. OPL: outer plexiform layer, ONL: outer nuclear layer, IS: inner segment, OS: outer segment, CIS: cone inner segment, RIS: rod inner segment. Scale bars: 20 μm.