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Detection of Isolated Mitochondria-Associated ER Membranes Using the Sigma-1 Receptor

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Abstract

The interface between the endoplasmic reticulum (ER) and mitochondria referred to as the MAM (*mitochondria-associated ER membrane*) plays important roles in many physiological functions. A specific marker for this important entity of cellular structure is urgently needed. Thus, we propose in this method chapter that the membrane-bound ER chaperone sigma-1 receptor serves as an ideal marker for the MAM. We describe in detail the preparation and purification of the MAM by using the sigma-1 receptor as the marker and demonstrate the uniqueness of this marker by using a variety of cells, peripheral and neuronal.

Keywords

Sigma-1 receptor; Chaperone; Endoplasmic reticulum; Mitochondria; MAM; SDS/PAGE electrophoresis; Fractionation

1 Introduction

Since its detection in the 1970s, the specialized area of the ER in physical contact with the outer mitochondrial membrane, termed *mitochondria-associated ER membrane* (MAM) [1–7], has emerged as a critical signaling junction within the cell. In fact, the transient contact the MAM provides [8] is used to facilitate critical cellular processes including phospholipid exchange, Ca^{2+} signaling, autophagosome formation, and cellular morphology [9], and has been implicated in several disease models. Just recently, point mutations in the presynaptic protein α -synuclein associated with Parkinson disease was determined to have reduced association with the MAM, in comparison to wild-type α -synuclein, and coincident with a decrease in MAM function and increased fragmentation of mitochondria [10]. Abrogation of MAM functioning has also been implicated in Alzheimer's disease [11–13], diabetes [14–16], and cancer [17].

Due to the increasingly critical implications of MAM localized proteins in the study of neurodegenerative disease, we provide a subcellular fractionation assay for the isolation of MAM by taking advantage of differential centrifugation. However, validation of isolated MAM can be problematic. Previous studies have identified several ER-resident proteins enriched at the MAM [9]; however, most of these proteins are also highly detectable in the bulk ER and other cellular compartments. Although phosphatidylethanolamine-N-methyltransferase-2 has emerged as a specific marker for the MAM, it is only reliable in

liver and primary hepatocytes [18]. Thus, after careful examination of several MAM-enriched proteins, we show that sigma-1 receptor (Sig-1R) is the most reliable protein marker for this subcellular local in various mammalian cell and tissue types.

2 Materials

2.1 Cell and Tissue Preparation

1. Cell scraper.
2. Phosphate buffered saline (PBS): 13.7 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄. Adjust pH to 7.4 and autoclave.
3. Surgeon's scalpel.
4. Hank's balanced salt solution (HBSS) without Ca²⁺ and Mg²⁺: 13.7 mM NaCl, 5.33 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄. Adjust pH to 7.4 and autoclave.

2.2 MAM Isolation Assay

1. Teflon glass homogenizer.
2. Pasteur pipettes, fire-polished.
3. Ultracentrifuge with swinging bucket and fixed angle rotors.
4. Large (16 × 76 mm) and small (13 × 51 mm) polycarbonate thick-walled tubes are preferred for use with the rotors.
5. Homogenization buffer (H-B): 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, and 0.25 M sucrose. Store at 4 °C.
6. Isolation medium (I-M): 5 mM HEPES, pH 7.4, 250 mM mannitol, 0.5 mM EGTA. Store at 4 °C.
7. Isolation medium 2 (I-M2): 25 mM HEPES, pH 7.4, 225 mM mannitol, 1 mM EGTA. Store at 4 °C.
8. Percoll medium: 25 mM HEPES, pH 7.4, 225 mM mannitol, 1 mM EGTA, 30 % Percoll (v/v). Make fresh before experiment.
9. SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and western blotting equipment.
10. *Optional*: 10,000 MWCO Centrifugation Filtration Unit.

3 Methods

3.1 Cell and Tissue Preparation

3.1.1 Cell Preparation

1. Culture adherent cells in 15-cm dishes until 90–100 % confluent (see Note 1).
2. Place cells on ice and wash once with ice-cold PBS.

3. Collect cells in ice-cold PBS by scraping and place into conical tube.
4. Centrifuge cells at $500 \times g$ to pellet and discard supernatant. Remove excess supernatant by inverting the tube on a Kimwipes for 30–60 s.

3.1.2 Tissue Preparation

1. Use standard procedures to dissect tissue and place on ice (see Note 2).
2. Using a surgeon's scalpel, cut the tissue into small pieces and place into conical tube with HBSS.
3. Centrifuge tissue at $500 \times g$ to pellet and discard supernatant. Remove excess supernatant by inverting the tube on a Kimwipes for 15–30 s.

3.2 MAM Isolation Assay

To avoid protein degradation during the process, the subcellular fractions should be prepared on ice and centrifuged at 4 °C. Store fractions at –80 °C, if not used immediately.

1. Suspend pelleted cells/tissue in H-B (approximately 9 v/w of wet pellet) and homogenate very slowly with a Teflon glass homogenizer for 20–30 strokes (see Note 3). Return homogenate to original conical tube.
2. Centrifuge homogenate at $600 \times g$ for 5 min. Remove supernatant and place into new conical or eppendorf tube(s). Suspend pellet in H-B and homogenate again (7–15 strokes).
3. Centrifuge homogenate at $600 \times g$ for 5 min. Combine resulting supernatant with previous one from **step 2**. Suspend pellet containing cell debris and nuclei in 0.5 ml I-M and store as *PI* fraction.
4. Centrifuge supernatant at $10,300 \times g$ for 20 min. Collect supernatant containing microsome and cytosol in large polycarbonate thick-walled tube(s) and place on ice until **step 9**. Thoroughly suspend pellet containing crude mitochondria (mitochondria with intact MAM) in 500 μ l of I-M.
5. Layer the crude mitochondria suspension on top of 3 ml of Percoll medium (Fig. 1) in a small polycarbonated thick-walled tube (see Note 4). Centrifuge at $95,000 \times g$ for 30 min using a swinging bucket rotor with deceleration set to zero as not to disturb the gradient.
6. Collect the MAM and mitochondria (Fig. 2) using fire-polished Pasteur pipettes (see Note 5). The MAM is observed as a diffuse white layer above the

¹Four to six 15-cm dishes are recommended to obtain an adequate protein concentration in each fraction; however, the researcher should optimize for the number of cells.

²Standard dissection procedures can be found in [19].

³Homogenization should be done very slowly and thoroughly as not to destroy the mitochondria and cause dissociation of the MAM. One stroke includes both the up and down movement.

⁴Tube size may vary depending on the rotor used. The researcher should optimize the volume of Percoll medium so that it fills approximately $\frac{2}{3}$ of the tube. It is also important that the two layers do not significantly mix. Gently layer crude mitochondria on top of Percoll medium so that the interface between the two layers is viewed as a sharp line.

⁵Take care when collecting MAM and mitochondria. Place the tip of the fire-polished Pasteur pipette on the wall of the tube and collect from the top of the layer to avoid contamination.

mitochondria. Dense bands containing mitochondria are recovered at approximately $\frac{3}{4}$ – $\frac{2}{3}$ of the way down the tube.

7. Dilute the MAM with five times volume I-M2 and centrifuge at $6300 \times g$ for 10 min. Place supernatant containing the MAM into a large polycarbonate thick-walled tube. *Optional:* Resuspend pellet in I-M2 and store as *Crude MAM* (MAM attached to mitochondria or aggregated MAM membranes), otherwise, discard.
8. Centrifuge the MAM and microsome/cytosol from **step 4** at $100,000 \times g$ for 1 h using a fixed angle rotor.
9. Dilute mitochondria with five times I-M. Wash three times by centrifugation at $10,500 \times g$ for 10 min (*see Note 6*). Discard all of the supernatant. Collect pelleted *Mitochondria* fraction by suspending in I-M2 (50–200 μ l to obtain 0.5–2 μ g/ μ l protein concentration).
10. Collect the MAM: The MAM appears as a loose floating white material (Fig. 3). Gently remove most of the supernatant. Using a fire-polished Pasteur pipette, carefully collect the *MAM* and suspend in H-B (50–200 μ l to obtain 0.5–2 μ g/ μ l protein concentration; *see Note 7*).
11. Collect microsome and cytosolic fractions: Collect supernatant containing *Cytosolic* fraction (*see Note 8*). Add 200 μ l of H-B to tube and suspend pelleted *Microsome* fraction by sonicating on ice for 10 s (repeat if necessary).
12. Subcellular fractions (*P1*, *Mito*, *MAM*, *P3*, *Cyto*) are analyzed by SDS-PAGE and western blot analysis (Fig. 4).

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References

1. Area-Gomez E, Del Carmen Lara Castillo M, Tambini MD, Guardia-Laguarta C, de Groof AJC, Madra M, Ikenouchi J, Umeda M, Bird TD, Sturley SL, Ea S. Upregulated function of mitochondria-associated ER membranes in Alzheimer disease. *EMBO J*. 2012; 31:4106–4123. [PubMed: 22892566]
2. Csordás G, Renken C, V'arnai P, Walter L, Weaver D, Buttle KF, Balla T, Mannella CA, Hajn'oczky G. Structural and functional features and significance of the physical linkage between ER and mitochondria. *J Cell Biol*. 2006; 174:915–921. [PubMed: 16982799]
3. Cui Z, Jean E, Chen MH, Voelker DR, Vance DE. Cloning and expression of a novel phosphatidylethanolamine N-methyltransferase. A specific biochemical and cytological marker for a unique membrane fraction in rat liver. *J Biol Chem*. 1993; 268:16655–16663. [PubMed: 8344945]

⁶This washing step is necessary to remove Percoll medium from the sample. In the presence of Percoll; however, the mitochondrial fraction will not pellet. Thus, after each centrifugation only remove the top $\frac{2}{5}$ – $\frac{2}{3}$ of the supernatant to ensure that mitochondria are retained. Washing is complete when a pellet is visible. All of the supernatant can be removed at this time.

⁷Take particular care when collecting the MAM so that only the pellet is recovered. Further dilution of the MAM in H-B may not be necessary. It is up to the research to optimize for desired concentration.

⁸*Optional:* The cytosolic fraction can be concentrated using a 10,000 MWCO centrifugal filtration unit.

4. Franke WW, Kartenbeck J. Outer mitochondrial membrane continuous with endoplasmic reticulum. *Protoplasma*. 1971; 73:35–41. [PubMed: 5000179]
5. Goetz JG, Genty H, St-Pierre P, Dang T, Joshi B, Sauvé R, Vogl W, Nabi IR. Reversible interactions between smooth domains of the endoplasmic reticulum and mitochondria are regulated by physiological cytosolic Ca^{2+} levels. *J Cell Sci*. 2007; 120:3553–3564. [PubMed: 17895372]
6. Guardia-Laguarta C, Area-Gomez E, Rüb C, Liu Y, Jordi M, Becker D, Voos W, Ea S, Przedborski S. α -Synuclein is localized to mitochondria-associated ER membranes. *J Neurosci*. 2014; 34:249–259. [PubMed: 24381286]
7. Hayashi T, Su T, Rizzuto R, Hajnoczky G. MAM: more than just a housekeeper. *Trends Cell Biol*. 2009; 19:81–88. [PubMed: 19144519]
8. Hedskog L, Moreira C, Filadi R, Rönnbäck A, Hertwig L, Wiehager B. Modulation of the endoplasmic reticulum-mitochondria interface in Alzheimer's disease and related models. *Proc Natl Acad Sci U S A*. 2013; 110:7916–7921. [PubMed: 23620518]
9. Ja L, Tata JR. A rapidly sedimenting fraction of rat liver endoplasmic reticulum. *J Cell Sci*. 1973; 13:447–459. [PubMed: 4357366]
10. Ca M, Marko M, Penczek P, Barnard D, Frank J. The internal compartmentation of rat-liver mitochondria: tomographic study using the high-voltage transmission electron microscope. *Microsc Res Tech*. 1994; 27:278–283. [PubMed: 8186446]
11. Morré DJ, Merritt WD, Ca L. Connections between mitochondria and endoplasmic reticulum in rat liver and onion stem. *Protoplasma*. 1971; 73:43–49. [PubMed: 5112775]
12. Pinton P, Giorgi C, Pandolfi PP. The role of PML in the control of apoptotic cell fate: a new key player at ER-mitochondria sites. *Cell Death Differ*. 2011; 18:1450–1456. [PubMed: 21475307]
13. Ea S, Area-Gomez E. Mitochondria-associated ER membranes in Alzheimer disease. *Mol Cell Neurosci*. 2013; 55:26–36. [PubMed: 22922446]
14. Sebastián D, Hernández-Alvarez MI, Segalés J, Sorianello E, Muñoz JP, Sala D, Waget A, Liesa M, Paz JC, Gopalacharyulu P, Matej O, Pich S, Burcelin R, Palacín M, Zorzano A. Mitofusin 2 (Mfn2) links mitochondrial and endoplasmic reticulum function with insulin signaling and is essential for normal glucose homeostasis. *Proc Natl Acad Sci U S A*. 2012; 109:5523–5528. [PubMed: 22427360]
15. Shore GC, Tata JR. Two fractions of rough endoplasmic reticulum from rat liver. II Cytoplasmic messenger RNA's which code for albumin and mitochondrial proteins are distributed differently between the two fractions. *J Cell Biol*. 1977; 72:726–743. [PubMed: 838773]
16. Spijker, S. Dissection of rodent brain regions. In: Li, KW., editor. *Neuroproteomics*. Humana Press; Totowa, NJ: 2011. p. 13-26.
17. Tubbs E, Theurey P, Vial G, Bendridi N, Bravard A, Chauvin M, Ji-Cao J, Zoulum F, Bartosch B, Ovize M, Vidal H, Rieusset J. Mitochondria-associated endoplasmic reticulum membrane (MAM) integrity is required for insulin signaling and is implicated in hepatic insulin resistance. *Diabetes*. 2014; 63:3279–3294. [PubMed: 24947355]
18. Wang HJ, Guay G, Pogan L, Sauvé R, Nabi IR. Calcium regulates the association between mitochondria and a smooth subdomain of the endoplasmic reticulum. *J Cell Biol*. 2000; 150:1489–1498. [PubMed: 10995452]
19. Zorzano A, Liesa M, Palacín M. Role of mitochondrial dynamics proteins in the pathophysiology of obesity and type 2 diabetes. *Int J Biochem Cell Biol*. 2009; 41:1846–1854. [PubMed: 19703653]

Fig. 1.
Crude mitochondrial fraction layered over Percoll medium

Fig. 2.

Ultracentrifugation was used to separate mitochondria and MAM using a Percoll gradient.

(a) The MAM can be found as a diffuse white band towards the *top* of the tube, while (b) mitochondria are found in several dense bands layered *below*. (c) Cellular debris collects on the bottom of the tube

Fig. 3. Ultracentrifugation was used to pellet the purified MAM and is found as a loose white floating material at the bottom of the tube

Fig. 4.

Western blotting analysis of subcellular fractions collected by the MAM isolation assay protocol in (a) HEK cells. Known nuclear (NP62), mitochondrial (ATP synthase, Cytochrome c), MAM-enriched (Sig-1R, BiP, IP₃ R3), and ER (ERp57) proteins were used to validate the fractions. Of the MAM-enriched proteins visualized, Sig-1R is the most reliable and is significantly less detectable in other cellular compartments. Cellular distributions of MAM-enriched proteins, Sig-1R and BiP, are also shown for (b) CHO cells, (c) Astrocytes, and (d) Neurons. Overall, Sig-1R was the most reliable protein marker for the MAM when compared to other MAM-enriched proteins