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Comparative proteomics of flotillin-rich Triton X-100-insoluble lipid raft fractions of mitochondria and synaptosome from mouse brain

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SUMMARY

There is increasing evidence that lipid rafts may play important roles in brain neuronal cell functions including signal transduction. Meanwhile, the results suggesting possible presence of rafts in intracellular organelles such as mitochondria have been also reported. In this study, we compared proteins in raft-like structure of mitochondria to those of synaptosomal rafts and analyzed age-related alterations in protein in both mitochondrial and synaptosomal lipid rafts. A low density Triton X-100-insoluble fraction was prepared from cerebral cortical synaptosome and mitochondria of mouse and analyzed by two-dimensional (2-D) gel electrophoresis in combination with mass spectrometry. Co-localization of flotillin and cholesterol in Triton X-100-insoluble fraction of mitochondria was shown by Western blot analysis. Differential display of proteins using computer-aided image analysis revealed that the composition of protein in flotillin-rich Triton X-100-insoluble fraction of mitochondria was similar to that found in synaptosome. Several protein spots on 2-D gels varied in quantity depending on the age of the mouse, including the guanine nucleotide-binding protein G(O) alpha subunit, as identified by peptide mass fingerprinting.

Key words: synaptosome, mitochondria, rafts, flotillin, proteomics.

INTRODUCTION

Lipid rafts are membrane compartments that are rich in glycosphingolipid, cholesterol, and acylated proteins, and are resistant to solubilization with non-ionic detergent^{1,2}. Rafts have been proposed to be important in various cellular functions including lipid transport, signal transduction, ion channel activation, and cell growth. There is increasing evidence that lipid rafts contain amyloid β -protein (A β)^{3,4}, amyloid β protein precursor³, β -secretase⁵, and presenilins^{3,6}, suggesting that analysis of lipid rafts may provide valuable insights into neuronal function and the pathophysiology of some neurodegenerative diseases. Recent evidence suggests that lipid rafts are also present in synaptosome^{7,9}.

Other evidence indicates that lipid rafts are present not only at the plasma membrane, but also on the membranes of

internal organelle, where they could assist in trafficking between membrane compartments¹⁰⁻¹². Bini et al.¹³ reported that mitochondrial membranes might contain rafts. Recently, Szabo et al.¹⁴ hypothesizes a role for lipid rafts in ion channel regulation given the apparent presence of rafts in intracellular organelles such as the endoplasmic reticulum, mitochondria or vesicles. However, quantitative proteomics of lipid rafts using stable isotope-labeling with amino acids in cell culture appeared to contradict this possibility¹⁵. Thus, the question as to whether lipid raft-like structure exists in mitochondria remains open.

The purpose of the present study was to isolate detergent-resistant membrane structures from mitochondria and synaptosome of mouse cortices and to compare their protein compositions using proteomic analysis. To date, two methods have commonly been employed to isolate lipid rafts, using either high pH or non-ionic detergents. Eckert

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Abbreviations : CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CHCA, α -cyano-4-hydroxy-trans-cinnamic acid; DTT, dithiothreitol ; G α O, guanine nucleotide-binding protein G(O) alpha subunit; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); PVDF, polyvinylidene difluoride; TFA, trifluoroacetic acid.

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et al.⁹) reported that fractions likely to be lipid rafts of brain synaptosomes showed greater enrichment in protein and lipid markers of rafts when isolated with detergent than other methods. In addition, the pH/carbonate-resistant preparation was shown to be less specific in isolating raft proteins than the detergent-resistant methods¹⁵).

Flotillin-1 was initially identified as a caveolae-associated integral membrane protein in murine lung and brain tissue¹⁶). Although flotillin-1 is abundant in brain, neurons do not express caveolin and do not possess caveolae. Therefore, flotillin-1 has been used as a biochemical marker for rafts in neural tissue. In support of this, flotillin-1 was recently localized in rat brain preparations ultrastructurally to cholesterol-rich microdomains- presumably rafts¹²).

Accordingly, in our current study, we isolated the low density Triton X-100-insoluble fraction from both mitochondria and synaptosome from mouse cortices using flotillin as a raft marker. We then analyzed and contrasted the protein composition of these fractions primarily using 2-D gel electrophoresis in combination with mass spectrometry.

MATERIALS AND METHODS

1. Materials

IPG Buffer (pH 4.0–7.0), Immobiline DryStrips (pH 4.0–7.0, 18 cm long), horseradish peroxidase-conjugated anti-mouse IgG, and a detection kit for enhanced chemiluminescence (ECL-plus) were purchased from Amersham Biosciences. TFA, α -cyano-4-hydroxy-trans-cinnamic acid (CHCA) and iodoacetamide were obtained from Sigma. Trypsin (sequencing grade) was procured from ProMega. Protease inhibitor cocktail tablets and antibody for G α O were obtained from Roche Molecular Biochemicals and Santa Cruz Biotechnology, respectively. SYPRO Ruby Gel Stain was purchased from Bio-Rad Laboratories, Inc. Pro-Q Diamond phosphoprotein gel stain and Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) were obtained from Molecular Probes. Horseradish peroxidase-conjugated anti-rabbit IgG were purchased from MBL. Antibody directed towards flotillin-1 was from Transduction Laboratories. A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce.

2. Preparation of synaptosomal and mitochondrial proteins

The cerebral cortices were obtained from 6-month-old and 30-month-old C57BL/6 mice. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology. All efforts were made to minimize the number of animals used and their suffering. Based on the method described by Ueda et al.¹⁷, subcellular fractionation of mouse cerebral cortex was performed as follows. Cerebral cortical tissue was homogenized in 10 volumes of

the isolation medium A (0.32 M sucrose, 3 mM HEPES, pH 7.3, 2 mM pervanadate, protease inhibitor cocktail) and centrifuged for 10 min at 1,500 $\times$ g to remove nuclei and debris. The supernatant was then centrifuged for 10 min at 14,500 $\times$ g to obtain crude fractions containing mitochondria and synaptosome. The pellet was suspended with the isolation medium A and followed by sedimentation on a discontinuous sucrose gradient of 0.32 M and 1.2 M (2 h at 85,000 $\times$ g) to separate the myelin fraction at the upper floating layer, the endoplasmic reticulum/Golgi membranes as an upper band, the synaptosomal fraction at a lower band, and the mitochondrial fraction in the pellet. The isolated synaptosomes and mitochondria were washed twice for 10 min at 14,500 $\times$ g with isolation medium A. These final pellets were the use for raft preparation for both mitochondrial and synaptosome fractions.

3. Preparation of raft-enriched membrane fractions

Raft proteins were obtained by buoyant-density fractionation over a discontinuous sucrose-density gradient as follows. Synaptosome and mitochondria, isolated as described above, were lysed in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% Triton X-100, protease inhibitor cocktail, and 2 mM pervanadate. The lysate was subjected to Dounce homogenization using a loose-fitting pestle, rotated for 30 min at 4°C, adjusted 40% sucrose, and then overlaid with 36% and 5% sucrose. Buoyant-density centrifugation was performed at 250,000 $\times$ g for 18 h at 4°C in a Beckman SW 55 Ti rotor. Fractions of 400 μ l were collected from the top down. Protein concentration was determined by BCA assay (Pierce). Lipids were extracted in 2:1 chloroform/methanol from each fraction in sucrose density gradient and total cholesterol levels were measured using the Amplex Red cholesterol assay method¹⁸). Aliquots of fractions subjected to SDS-PAGE followed by immunoblotting. Triton X-100-insoluble flotillin-rich fractions, detected as a light-scattering band around the 5–36% interface were collected, diluted with Tris-buffered saline and concentrated by centrifugation.

4. SDS-PAGE and Western blot analysis

Equal volumes of the harvested fractions in sucrose density gradient were applied to SDS-PAGE on 10% polyacrylamide gel. For immunoblotting, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane with a semidry blotting apparatus at 100 mA for 2 h at room temperature using buffer containing 25 mM Tris, 192 mM Glycine, 0.1% SDS and 20% methanol. In some experiments, the protein spots on the 2-DE gels were also electroblotted onto a PVDF membrane as described above. Immunoreactive spots were detected using an enhanced chemiluminescence detection kit (ECL plus) and peroxidase-conjugated secondary antibodies.

5. 2-Dimensional (2-D) gel electrophoresis

Pelleted lipid raft fractions were solubilized in 2% SDS and subjected to acetone precipitation to remove lipids. After centrifugation, the pellets were rinsed with cold acetone twice, dried, resuspended in 8 M urea, thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), sulphobetaine 10, IPG buffer, Orange G and 65 mM dithiothreitol (DTT) and sonicated. 2-D electrophoresis was performed according to our previously reported method with slight modifications as described in detail on our web site (http://www.proteome.jp/2D/2DE_method.shtml)¹⁹. The isoelectric focusing (IEF; first dimension) was carried out on nonlinear immobilized pH gradients (IPGs; pH 4–7; 18-cm IPG strips; Amersham Pharmacia Biotechnology, Uppsala, Sweden). Passive sample application during rehydration was performed by placing the Immobiline DryStrip gel side down in a rehydration tray that contains the sample in an appropriate rehydration solution overnight. As the strips hydrated, proteins in the sample were absorbed and distributed over the entire length of the strip. The IEF was performed at 20°C using PowerPhoreStar Pro 3800 (Anatech, Tokyo, Japan) with the following voltage program: 500 V for 2 h, 700 V for 1 h, 1000 V for 1 h, 1500 V for 1 h, 2000 V for 1 h, 2500 V for 1 h, 3000 V for 1 h, 3500 V for 18 h. After completion of electrofocusing, IPG strips were equilibrated for 45 min in 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.05 M Tris-HCl, pH 6.8, 2% (w/v) DTT. In the second-dimensional SDS-PAGE, equilibrated IPG strip was placed on top of the 7.5% polyacrylamide gel (18 cm×20 cm×1 mm), and proteins on the strip were separated electrophoretically with a Tris-Tricine buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS) at a constant current of 30 mA/gel until the dye front reached the bottom of the gel. Gels were stained with SYPRO Ruby. The fluorescent stained gel images were acquired using the Molecular Imager FX laser scanning fluorometer (Bio-Rad, Richmond, USA) and analyzed using PDQuest software (Bio-Rad, Richmond, USA). pI/Mr calibration of all protein spots was carried out using 2D-protein pI marker and MW marker as the internal standard. In some experiments, 2-D gels were stained with Pro-Q Diamond phosphoprotein gel stain followed by SYPRO Ruby gel stain to detect the relative phosphorylation level of the protein in each spot. After Pro-Q Diamond staining, the fluorescent gel images were acquired using the Molecular Imager FX laser scanning fluorometer in the 555 nm long-pass emission filter setting with the 532 nm excitation laser. For SYPRO Ruby-stained gels, 640 nm band-pass emission filter was used with the 488 nm excitation laser.

6. Peptide mass fingerprinting

Protein spots on the SYPRO Ruby-stained 2-D gel were excised by ProteomeWorks Spot Cutter (Bio-Rad, Richmond, USA) and FluoroPhoreStar 3000 (Anatech Co.,

Tokyo, Japan). The excess dye was removed from the gel pieces in 50% acetonitrile and 50% ammonium bicarbonate, and finally dehydrated in absolute acetonitrile, and dried up. The proteins were digested with 5 µg/ml trypsin, 50% ammonium bicarbonate, 30% acetonitrile overnight at 30°C according to the method described in our web site. After digestion, peptide fragments in the supernatants were subjected to MALDI-TOF mass spectrometry for peptide mass fingerprinting (PMF). The MALDI-TOF-MS analysis was performed using the Shimadzu AXIMA-CFR mass spectrometer operated in reflectron positive ion mode. The background noise was removed by subtraction of mass signals generated from a digest of the control gel. Protein identification was carried out using the MS-fit search engine in Protein Prospector (UCSF) by sending a query of the PMF data.

RESULTS

1. Sucrose density gradient ultracentrifugation

Triton X-100-insoluble lipid raft preparations isolated from either the mitochondria or synaptosome fractions could be identified as insoluble white light-scattering band at the interphase between the 5% and 36% sucrose layer. Fig. 1 summarizes the protein and cholesterol contents from both fractions. As can be seen, the lipid raft marker protein, flotillin, and the lipid marker, cholesterol were highly enriched in the low-density Triton X-100-insoluble fractions of both mitochondria and synaptosomes.

2. 2-Dimensional (2-D) gel electrophoresis

The flotillin-rich Triton X-100-insoluble fractions of synaptosome and mitochondria were collected and processed for analysis by 2-D gel electrophoresis. To conquer difficulties with solubilization of membrane proteins, the pelleted Triton X-100-insoluble proteins were solubilized in a small volume of SDS. Because high amounts of lipids in rafts resulted in arcing or dissolution of the gel strip, the removal of lipids by acetone precipitation was required to improve the spot resolution. The acetone-precipitated proteins were sufficiently solubilized in IEF sample buffer containing thiourea and Triton X-100 which are used for membrane protein mixtures. For the initial analysis, acetone-precipitated proteins were separated by first-dimensional IEF on pH 3–10 linear IPG strips in combination with 7.5% polyacrylamide gels for second-dimensional SDS-PAGE. Since the well-focused protein spots were mainly located in acidic range, we performed subsequent detailed analysis using IPG strips with a narrower range (pH 4–7). Typical SYPRO Ruby-stained 2-D gel images of synaptosomal and mitochondrial proteins in 6 month-old mice are presented in Fig. 2. A number of major raft protein components appeared in both preparations. Unique spots present only in mitochondria or in synaptosome are indicated by arrow heads in Fig. 2. A quantitative differential analysis using the

Fig. 1. Fractionation of Triton X-100 insoluble complexes of mitochondria and synaptosome by sucrose density gradient centrifugation.

Mitochondrial (A) or synaptosomal (B) proteins were treated with Triton X-100, sonicated, subjected to sucrose density gradient centrifugation, and fractionated from the top down. Fractions were analyzed for cholesterol (bar graph; μg/ml) and protein (line graph; mg/ml) as described under "MATERIALS AND METHODS". Equal volumes of each fraction of the gradient were subjected to SDS-PAGE and immunoblotted using specific antibodies directed towards flotillin. The raft marker flotillin was detected in the low-density region of the gradient. The results are representative of three independent experiments.

PDQuest software demonstrated that several protein spots showed age-related changes in relative abundance between the 6 month and 30 month-old rats (Fig. 2, No. 1–8).

3. Protein identification

Protein spots were excised from the SYPRO Ruby-stained 2-D gel, and subjected to in-gel digestion with trypsin. The resulting peptide mixtures were analyzed by MALDI-TOF mass spectrometry using a database search.

Fig. 2. Typical 2-D electrophoretograms of mitochondrial and synaptosomal proteins in flotillin-rich Triton X-100-insoluble fractions.

A low density Triton X-100-insoluble fraction was prepared from cerebral cortical synaptosome and mitochondria as described under "MATERIALS AND METHODS". After removal of lipids from the flotillin-rich fractions by acetone precipitation, solubilized proteins were separated using 4–7 linear IPG strips and 7.5% SDS-PAGE. Gels were stained with SYPRO Ruby, scanned using the Molecular Imager FX laser scanning fluorometer, and analyzed using PDQuest software. Normalization in ppm was done on total quantity of all valid spots on the 2DE protein profile. A: mitochondria. B: synaptosome. Arrow heads represent unique protein spots present only in mitochondria or in synaptosome. Histograms of age-related abundance of protein spots indicated by the arrows (No. 1–8) are shown in the left side insets.

The proteins isolated in Triton X-100-insoluble fractions of synaptosome and mitochondria were highly reproducible across triplicated experiments. These are summarized in Fig. 3 and Table 1. Contactin precursor, V-ATPase A and B subunits, heat-shock cognate 71 kDa protein, and guanine nucleotide-binding protein G(O) α subunit were present in both mitochondrial and synaptosomal flotillin-rich fractions. By contrast, α -internexin was observed in synaptosomal rafts fractions, whereas mitochondrial flotillin-rich fraction contained mitofilin and NADH-ubiquinone oxidoreductase 30 kDa subunit (a mitochondrial precursor).

Fig. 3. Mitochondrial and synaptosomal proteins identified by MALDI-MS.

Low density Triton X-100-insoluble fractions from synaptosome and mitochondria were analyzed on 2-D gels. Selected spots were excised, digested with trypsin and analyzed by MALDI-MS and identified using proteomic data bases. Panel A summarizes the mitochondrial fractions; panel B summarizes the synaptosome fractions.

4. Western blotting

We confirmed the identity of guanine nucleotide-binding protein G(O) α subunit has been confirmed by 2-D immunoblotting with a specific antiserum (Fig. 4). The distribution of guanine nucleotide-binding protein G(O) α subunit in sucrose density gradient was distinct from that of flotillin (Fig. 1 and 4), suggesting that its localization in membrane rafts is probably due to post-translational modification. As shown in Fig. 2 insets, concentration of this protein differed both mitochondria and synaptosome as a function of the age of the mouse.

DISCUSSION

In these experiments, we demonstrated that flotillin,

which is well known as a protein marker of lipid raft in neural cells, co-localized in the low-density Triton X-100-insoluble fractions prepared from both synaptosome and mitochondria. The significantly high abundance of flotillin in the mitochondrial membrane fraction argues strongly against it being present merely due to contamination of mitochondrial preparation by synaptosome. The results of our proteomic analysis performed on the cholesterol-rich/flotillin-rich membrane preparations of synaptosome and mitochondria revealed the similarity in protein composition, suggesting the existence of raft-like structures not only on synaptosomal but also on mitochondrial membrane in neural cells.

Mitofilin, which has been assigned as a mitochondria-associated membrane protein, was detected only in the flotillin-rich membrane preparation of mitochondria. In contrast, α -internexin was detected only in the synaptosomal membrane preparation. This also argues against the high abundance of flotillin in the mitochondrial membrane preparation being the result of simple contamination.

Selective recruitment of protein components from the cytoplasm to a membrane micro-domain is thought to be important in the regulation of signal transduction and ion channeling. Cholesterol-rich lipid rafts have been proposed as platforms for interaction of signal-transducing enzymes with substrates, and recent studies have suggested a novel role of the lipid rafts in regulation of ion channels on the membrane¹⁴. Mitochondrial membrane has been thought to contain calcium-induced, cardiolipin-rich domains that may serve as platforms for the recruitment and/or exclusion of ion channels and ion signaling molecules²⁰. Van Blitterswijk et al.²¹ noted the involvement of glycosphingolipid-containing vesicles in mitochondrial signal transduction. Our proteomic analysis in this paper also supports the existence of lipid raft-like micro-domain structures on mitochondrial membranes.

Neural cell surface protein F3/contactin, guanine nucleotide-binding protein G(O) α -11 subunit, vacuolar ATP synthase catalytic subunit A and B, and heat-shock cognate 71 kDa protein were observed in both mitochondrial and synaptosomal flotillin-rich membrane fractions. Mitochondrial inner membrane protein/mitofilin, NADH-ubiquinone oxidoreductase 30 kDa subunit (mitochondrial precursor) were detected only in mitochondrial preparations. Guanine nucleotide-binding protein G(O) α -1 and α -2 subunits, and 66-kDa neurofilament protein/ α -internexin were localized only in synaptosomal rafts. We also observed age-related changes in localization of isoelectric isoforms of guanine nucleotide-binding protein G(O) α subunits on both synaptosomal and mitochondrial raft-like structure, suggesting G-protein-coupled signal transduction may be altered at both synaptosomal and mitochondrial membrane in neural cells of aged mouse brain. To assess the possible involvement of phosphorylation, we used a phosphoprotein-

Table 1. Summary of protein identification. Protein spots indicated with arrows in Fig. 3 were excised from the gels and subjected to peptide mass fingerprinting as described in MATERIALS AND METHODS. The identification was performed using MS-Fit of Protein Prospector.

Protein name	SWISS-PROT accession No.	pI	Mr	MOWSE Score	Location
contactin precursor (neural cell surface protein F3)	P12960	5.8	113389	2.129E+07	S, M
guanine nucleotide-binding protein G(O) alpha subunit					
alpha-1 subunit	P18872	5.3	40085	7343	S
alpha-2 subunit	P18873	5.7	40037	7351	S
alpha-11 subunit	P21278	5.7	42025	1.237E+04	S, M
vacuolar ATP synthase catalytic subunit A (V-ATPase A subunit 1) ubiquitous isoform	P50516	5.6	68269	5.943E+04	S, M
vacuolar ATP synthase catalytic subunit B (V-ATPase B2 subunit) brain isoform	P50517	5.6	56551	5.259E+05	S, M
alpha-internexin (66 kDa neurofilament protein)	P46660	5.2	55870	2.670E+06	S
heat-shock cognate 71 kDa protein	P08109	5.4	70872	3.096E+04	S, M
mitochondrial inner membrane protein (mitofilin)	Q8CAQ8	6.2	83901	2.348E+05	M
NADH-ubiquinone oxidoreductase 30 kDa subunit (mitochondrial precursor)	Q9DCT2	6.4	30208	5.160E+05	M

S and M represent synaptosome and mitochondria, respectively.

A

B

Fig. 4. Anti-GαO Western blot analyses.

Panel A: Proteins in Triton X-100-insoluble fraction from mitochondria (a) or synaptosome (b) were separated by 2-DE, transferred to PVDF membrane, and immunoblotted with anti-GαO antibody. Immunoreactive spots indicated by arrows (No. 1–8) correspond with protein spots in Fig. 2. Panel B: Sucrose-density gradient fractions obtained in Fig. 1 were subjected to SDS-PAGE followed by Western blot analysis using anti-GαO antibody.

specific fluorescent stain, Pro-Q Diamond, but the protein spots of guanine nucleotide-binding protein G(O) alpha subunits on the 2-D gels were negative in the staining, suggesting that the isoelectric isoforms of guanine nucleotide-binding protein G(O) alpha subunits might be the results of post-translational modifications other than phosphoryla-

tion. We are currently exploring this possibility through mass spectrometric analyses on the post-translational modification of the isoelectric isoforms of the protein.

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