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RESEARCH ARTICLE

Oxidation–reduction respiratory chains and ATP synthase complex are localized in detergent-resistant lipid rafts

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In order to detect and identify ubiquitous lipid raft marker proteins, we isolated lipid rafts from different mouse organs, including the liver, lung, large brain, and kidney, and analyzed their proteins *via* 2-DE. Many protein spots were determined to be ubiquitous in all of the lipid rafts, and were annotated *via* LC and MS/MS. Twelve proteins were identified as ubiquitous raft proteins, and most of these were determined to be mitochondrial proteins, including mortalin, prohibitin, voltage-dependent anion channel, ATP synthase, NADH dehydrogenase, and ubiquinol-cytochrome c reductase. *Via* immunoblotting, these proteins were shown to exist in detergent-resistant lipid rafts prepared using different organ tissues. Since these oxidation–reduction respiratory chains and ATP synthase complex were detected in detergent-resistant lipid raft fractions which had been isolated from the plasma membrane but not from the mitochondria, and found in the cell surface when determined by immunofluoresence and immunohistochemistry, we conclude that plasma membrane lipid rafts might contain oxidation–reduction respiratory chains and ATP synthase complex.

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1 Introduction

If signaling molecules are randomly located within the plasma membrane, it could be quite difficult for signals to be efficiently and rapidly transduced into the cells. Therefore, specific membrane compartments are required in order to concentrate and organize these signal molecules. These specific membrane compartments, also commonly referred to as the lipid rafts, are composed of cholesterol and glycolipids [1, 2], and predominantly harbor a host of receptors and signaling molecules, allowing them to mediate a variety of cellular events, including cellular signaling, pathogenic invasion, immune responses, and cholesterol homeostasis [3–6]. Because the lipid rafts are loaded with many receptors for epidermal growth factor (EGF) [7], platelet-derived growth factor [8], nerve growth factor [9], insulin [10], insulin-like growth factor [11], transforming growth factor [12], and tumor necrosis factor [13], these rafts might act as platforms which initiate various cellular signal pathways.

Due to cholesterol, the lipid rafts are known to be insoluble in the presence of nonionic detergents [14]. Therefore, lipid rafts have previously been isolated from other cellular

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Abbreviations: VDAC, voltage-dependent anion channel; **OXPHOS**, oxidative phosphorylation system

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organelles, on the basis of their detergent insolubility and their low density. However, the raft hypothesis has been challenged on the grounds that the rafts might simply be artifacts formed in the presence of detergent [15, 16]. For example, the mitochondrial protein, prohibitin, and the endoplasmic reticular protein, NADH cytochrome b_5 reductase, have been extracted from detergent-resistant lipid rafts [15], thereby indicating that the lipid rafts harbor large amounts of contaminants originating from the mitochondria, endoplasmic reticulum, Golgi complex, and endosomes.

Lipid rafts are known to harbor many integral and peripheral proteins, including a variety of receptors and their downstream signaling molecules, as well as many lipid-modified proteins. A long-standing question as to how these membrane proteins are targeted to lipid rafts has yet to be satisfactorily answered. It is tempting to speculate that there might be a scaffolding protein for the recruitment of different proteins into the lipid rafts. Caveolin-1 could be considered to be a strong lipid raft scaffolding protein [17], as it is not only acylated for targeting to the lipid rafts, but also interacts with different signaling molecules, including endothelial nitric oxide synthase [18], EGF receptor [19], H-Ras [20], *etc*. Caveolin-1 is a structural protein which assists in the formation of flask-shaped plasma membrane invaginations, called caveolae, in the lipid rafts [21]. Indeed, *via* the transient expression of caveolin-1, caveolae have been shown to appear from immune cells which do not naturally express caveolin-1 [22]. However, caveolin-1 is also clearly not a requisite protein for the targeting of different signaling molecules to lipid rafts, because these signaling molecules are known to remain in the lipid rafts in caveolin-1-deficient cells [23].

Lipid raft proteins might be expressed in all organs, and might also be required for the architecture of lipid rafts. In order to address this issue, raft-associated proteins from different organs must be compared and identified. Recently, a host of raft proteins have been identified *via* proteomic analysis in a variety of organs or cell types [24–26]. However, relatively few differential proteomic analyses have focused specifically on the lipid rafts. Despite recent technical developments in differential quantitative proteomics *via* stable isotope-labeling with amino acids in cell cultures [27] and isotope-coded affinity tag techniques [28], 2-DE remains the method of choice for differential proteomics, because protein spots can be visualized relatively easily by silver staining, and these spots can be annotated *via* MALDI and TOF-MS [29], or by ESI-MS/MS [30]. Here, we have conducted comparisons among the 2-D gel profiles of the lipid rafts isolated from a variety of mouse organs, in order to determine which lipid raft proteins are ubiquitously expressed in all tissues. Twelve proteins were detected in all of the assessed rafts, and were subsequently annotated *via* ESI-MS/MS. Among these, ATP synthase α and β, and oxidative phosphorylation system (OXPHOS) complexes I, II, and III were further shown *via* immunoblotting to exist in the rafts.

2 Materials and methods

2.1 Cell line, animal, and antibodies

Human embryonic kidney 293 cells were grown in DMEM containing 10% fetal bovine serum. Female C57BL/Ksj mice were maintained on a controlled lighting schedule, with a 12-h dark period. Twenty-wk-old mice were used in all of the experiments in this study. The antiflotillin-1, clathrin heavy chain, insulin receptor β subunit, and Ras antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Antibodies against the NADH-ubiquinone oxidoreductase 17 kDa subunit (for OXPHOS I), the succinate-ubiquinol oxidoreductase 70 kDa subunit (for OXPHOS II), and the ubiquinol-cytochrome c reductase core I subunit (for OXPHOS III) were purchased from Molecular Probes (Eugene, OR, USA), and the anticytochrome c, LAMP-1, Bcl-2 and β'-COP antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 The isolation of lipid rafts

Lipid rafts were extracted from 20-wk-old mouse liver, lung, kidney, and cerebral tissue. After extraction, each of the tissues was rinsed in ice-cold PBS buffer, minced with scissors, and then briefly ground with a mechanical grinder. Five hundred microliters of each of the tissue samples were then lysed with an equal volume of lysis buffer (25 mM HEPES, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF) containing various detergents (1% Triton X-100, Brij 35, or Brij 98), and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), homogenized using a Dounce homogenizer (Kontes, Vineland, NJ), and incubated for 30 min at 4°C. The raft isolation was then conducted, as previously described [31]. High-pH/carbonate-resistant lipid rafts isolation was performed in a similar way with the exception that the cells were lysed in 100 mM sodium carbonate, pH 11, and sequentially homogenized by using a Dounce homogenizer (10 strokes) and a probe sonicator (three 20 s bursts; Benson Sonifier), as previously described [20].

2.3 Immunoblotting

The proteins in the lysates were resolved by SDS-PAGE and transferred onto PVDF membranes (Millipore) and blocked for 1 h at 4°C with 5% w/v dry milk in TBS buffer solution. Incubation with primary and secondary antibodies was then conducted for 1 h at room temperature. The signals were visualized with ECL reagents (Intron, Seoul, Korea).

2.4 IEF and 2-DE

The preparation of the samples for 2-DE proceeded as was previously described. After quantification, we conducted the IEF of each sample containing equal protein contents (150 μg) on a linear wide-range IPG (pH 3–10; 24-cm long

IPG strips) with a total focusing time of 81 780 Vh, at 20°C, using the IPGphor system in accordance with the manufacturer instructions (Amersham Biosciences, Piscataway, NJ). The second dimension was then conducted on lab-made SDS-PAGE gels (11% polyacrylamide, 0.26% 1,4-bis(acryloyl)piperazine (PDA)/25.5 cm × 19.6 cm × 1 mm), under constant current, in three steps (step 1. 5 w/gel; step 2. 10 w/gel; step 3. 15 w/gel) at 20°C, using an Ettandalt 6 system (Amersham Biosciences). The analytical gels were stained using the PlusOne™ silver staining kit (Amersham Biosciences) in accordance with the manufacturer instructions, with the exception of the glutaraldehyde treatment.

2.5 In-gel protein digestion and ESI-MS/MS

Proteins were subjected to in-gel trypsin digestion [32]. The excised gel spots were then destained using 100 μL of destaining solution (1:1 = 30 mM potassium ferricyanide:100 mM sodium thiosulfate, v/v) with agitation for 5 min. After the solution had been removed, the gel spots were incubated using 200 mM ammonium bicarbonate for 20 min. These gel pieces were then dehydrated using 100 μL of ACN, and dried in a vacuum centrifuge. The dried gel pieces were then rehydrated with 20 μL of 50 mM ammonium bicarbonate containing 0.2 μg modified trypsin (Promega) for 45 min on ice. After the solution had been removed, we added 30 μL of 50 mM ammonium bicarbonate. Digestion was then conducted overnight at 37°C. The peptide solution was desalted with a C_{18} nano column (IN2GEN).

The MS/MS of peptides generated *via* in-gel digestion was conducted by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The source temperature used was 80°C. A 1 kV potential was then applied to precoated borosilicate nanoelectrospray needles (EconoTip™, New Objective, USA) in an ion source, combined with a 0–5 psi nitrogen back pressure, in order to ensure a stable flow rate (10–30 nL/min). The cone voltage used was 40 V. We also used a quadrupole analyzer in order to select precursor ions for fragmentation within the hexapole collision cell. Ar was used as a collision gas at a pressure of $(6–7) \times 10^{-5}$ mbar, with a collision energy of 20–30 V. The product ions were analyzed with an orthogonal TOF analyzer, and were fitted with a reflector, a microchannel plate detector, and a time-to-digital converter. These data were processed with a Mass Lynx Windows NT PC system.

In order to identify the proteins, all MS/MS spectra recorded on tryptic peptides derived from the spots were searched against protein sequences from the NCBInr databases, using the MASCOT search program (www.matrixscience.com).

2.6 Isolation of mitochondria and plasma membrane

Plasma membrane isolation was conducted on the basis of the methods of Cushman and Wardzala [33], with slight modifications. Mouse kidneys were excised, homogenized in the presence of four volumes of 0.25 M STM (0.25 M sucrose, 5 mM Tris–HCl, pH 7.4, 1.0 mM $MgCl_2$), and filtered through four gauze layers. The filtrates were then centrifuged at 12 000 rpm with a JA20 rotor for 30 min, resuspended in TES buffer, loaded onto a sucrose cushion (38.5%), and centrifuged at 100 000*g* in a SW55Ti rotor for 60 min, at 4°C. Plasma membranes are collected from the top of the sucrose cushion, resuspended in TES buffer, and repelleted *via* 60 min of centrifugation at 31 000*g* (JA20, 4°C). The pellet, under a 38.5% cushion containing mitochondria, was then washed and resuspended in ice-cold mitochondrial isolation buffer (MIB; 250 mM mannitol, 0.5 mM EGTA, and 5 mM HEPES with 0.1% BSA w/v, pH 7.2). The resuspended mitochondrial pellets were then loaded onto a continuous Percoll gradient (30% v/v Percoll, 225 mM mannitol, 25 mM HEPES, 0.5 mM EGTA, and 0.1% w/v BSA (pH 7.2)). After 60 min of ultracentrifugation at 40 000*g*, the brownish mitochondrial bands were collected and washed with MIB *via* 10 min of centrifugation at 6300*g*. The resulting pellets were then used as mitochondrial fractions. Further lipid raft isolation procedures for prepared mitochondria and plasma membranes were similar to those described above, except that 300 μL of mitochondria and plasma membrane samples were lysed using 600 μL of sample lysis buffer harboring 1% Triton X-100.

2.7 Immunofluorescence and immunohistochemistry

For immunofluorescence, cells were fixed with 10% formaldehyde in PBS for 20 min. The cells were incubated with blocking buffer (5% BSA in PBS), and primary antibodies. The primary antibodies were detected with fluorescein-conjugated secondary antibodies. For immunohistochemistry, mouse kidney cortex specimens were washed with saline and embedded in optimal cutting temperature to make frozen sections. Standard 6-μm sections were stained using the Labeled Streptavidin Biotin kit (Dako) according to the manual provided by the manufacturer.

3 Results

3.1 Isolation of detergent lipid rafts from different mouse organs

In order to determine which lipid raft proteins were ubiquitously expressed in all organs, we isolated lipid rafts from different mouse organs, on the basis of detergent insolubility and low density. Liver, lung, kidney, and cerebral tissues were lysed for 30 min with 1% Triton X-100 at 0°C. The lysates were then subjected to discontinuous sucrose gradient ultracentrifugation, in order to isolate an opaque buoyant band which harbored detergent-resistant lipid rafts. The sucrose gradient was then fractionated into 12 fractions, from bottom to top, and each of the fractions was analyzed

via immunoblotting with antiflotillin-1 and clathrin heavy-chain antibodies. As shown in Fig. 1, flotillin-1 was concentrated predominantly in the lipid raft fractions of all organs, whereas the clathrin heavy chain was not found to be present, thereby suggesting that all lipid rafts from different mouse organs were well prepared.

3.2 2-D gel profile of raft proteins from different organs

Next, lipid raft proteins, prepared as described above, were resolved *via* 2-DE and visualized using silver staining. The protein composition of the lipid rafts was quite complex, evidencing approximately 400 spots. However, in most cases, major protein profiles are concentrated within a p*I* range between 4.5 and 8 (Fig. 2). The raft proteins that ubiquitously appeared in all of the organs were indicated by arrows in the 2-D gel profiles. Twelve protein spots that were found in all of the raft samples were excised from the 2-D gels for identification *via* ESI-MS/MS analysis. Figure 3 shows a representative MS/MS spectrum for the NADH-ubiquinone oxidoreductase 24 kDa subunit. The resultant MS/MS spectra

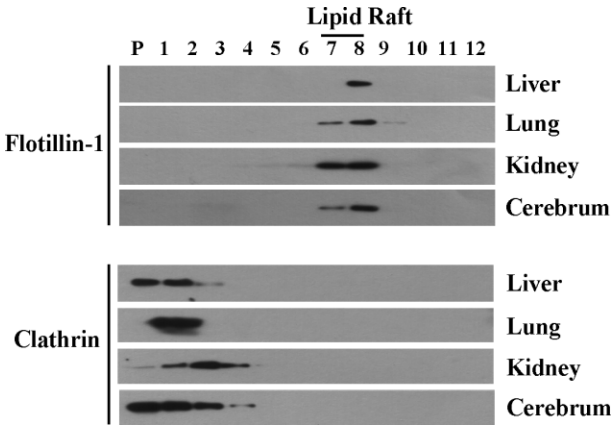

Figure 1. Isolation of detergent-resistant lipid rafts from various mouse organs. Lipid rafts from mouse liver, lung, kidney, and cerebral tissues were isolated on the basis of detergent insolubility and low density. After density gradient ultracentrifugation, each of the sucrose gradients was fractionated from the bottom to the top with 13 fractions, including the pellet (P), and then analyzed *via* immunoblotting with antiflotillin-1. Anticlathrin heavy chain was used as a nonraft marker.

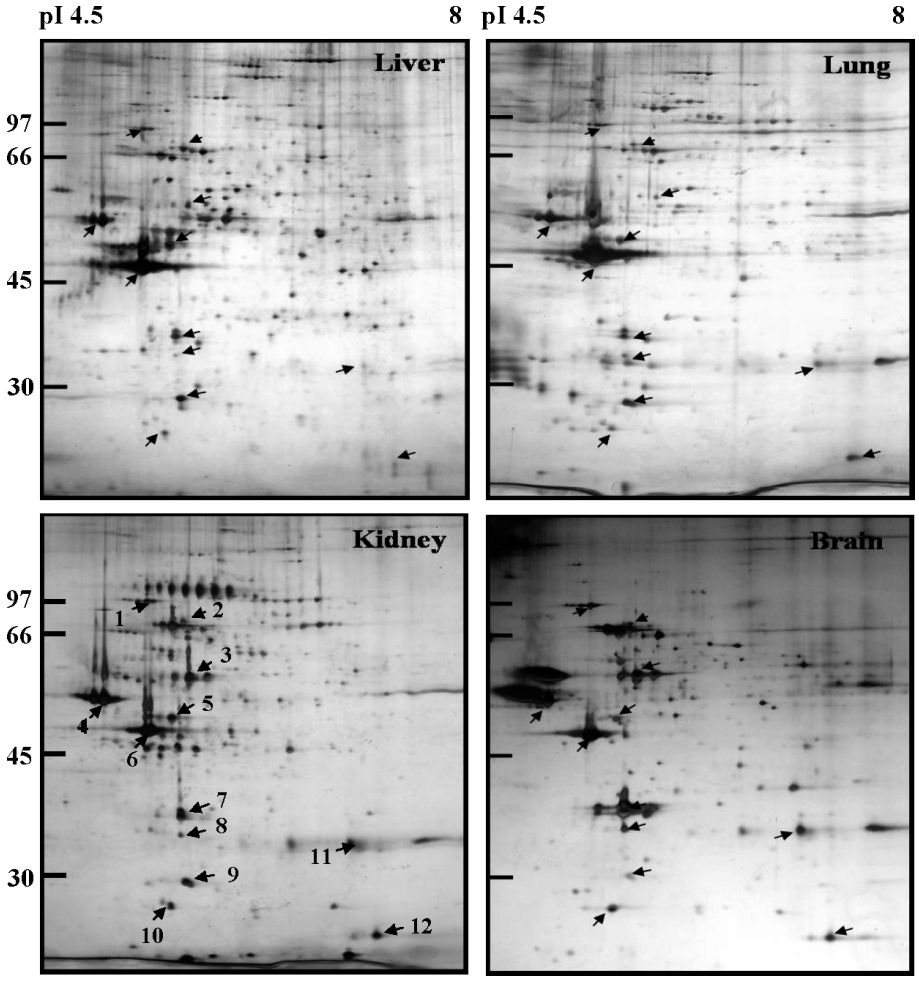

Figure 2. Lipid raft protein profile on 2-DE. Isolated lipid rafts from mouse liver, lung, kidney, and cerebral tissue were washed in washing buffer, and pelleted down by ultracentrifugation at 25 000 rpm, 4°C, for 30 min. Lipid raft samples prepared from each organ were then solubilized with 300 μL of IEF buffer containing 9 M urea and 2% CHAPS. An equal concentration (150 μg) of raft proteins was subjected to 2-DE, and the protein spots were visualized *via* silver staining. Among the protein profiles covering the entire p*I* range (pH 3–10), each of the figures above represent protein profiles within the p*I* range of 4.5–8, whereas most of the protein spots are concentrated. Black arrows and numbers indicate protein spots which were found ubiquitously in all lipid rafts prepared from the liver, lung, kidney, and cerebrum. Twelve protein spots were positively identified *via* ESI-MS (Table 1).

Table 1. Identification of ubiquitously expressed lipid raft proteins in various mouse organs

	Description	MW, (kDa)/p*I*	Gene symbol	Swiss-Prot accession no.	Sequence coverage (%)	Location
1	NADH dehydrogenase (ubiquinone)Fe-S protein 1	80.8/5.51	Ndufs1	Q5SUH2	14	MT
2	75 kDa glucose regulated protein; GRP 75; mortalin	73.7/5.81	Hspa9	Q9CQ05	18	MT
3	ATPase, H+ transporting, V1 subunit β, isoform 2	56/5.66	Atp6v1b2	P62814	27	LY
4	ATP synthase β-subunit	56.3/5.14	Atp5b	P56480	8	MT/LR
5	Ubiquinol-cytochrome c reductase complex core protein I	53.4/5.75	Uqcrc1	Q9CZ13	27	MT
6	β-Actin	39.4/5.78	Actb	P60710	34	LR
7	Guanine nucleotide binding protein β polypeptide 1	38.1/5.6	Gnb1	P62874	45	LR
8	Pyruvate dehydrogenase (lipoamide) β	39.2/6.41	Pdhb	Q9D051	26	MT
9	Prohibitin	29.8/5.57	Phb	P67778	41	MT/LR
10	NADH-ubiquinone oxidoreductase 24 kDa subunit	27/7.0	Ndufv2	Q9D6J6	40	MT
11	VDAC2	32.3/7.44	Vdac2	Q60930	36	MT/LR
12	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	29.6/8.91	Uqcrfs1	Q9CR68	16	MT

MT: mitochondria; LR: lipid raft; LY: lysosome.

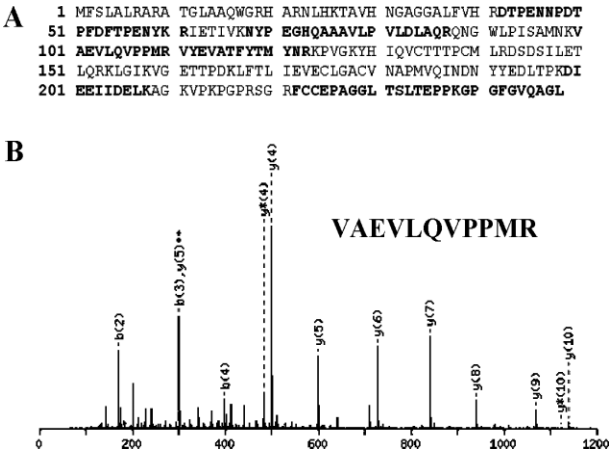

Figure 3. (A) Peptide sequence and MS spectrum of the tryptic digest of the protein with spot number 10, the mitochondrial precursor of the NADH-ubiquinone oxidoreductase 24 kDa subunit. Sequence coverage was 40% and the peptide sequences observed as the result of the experiments are noted by bolding. (B) A representative mass spectrum representing the peptide, VAEVLQVPPMR.

were then used for subsequent database search. The identified peptides were then employed in the calculation of sequence coverages of the original proteins. For example, this technique was able to obtain a 40% sequence coverage for the NADH-ubiquinone oxidoreductase 24 kDa subunit. Altogether, in a database search using the MS/MS data, 12 spots were found.

Table 1 lists the ubiquitous lipid raft proteins found by ESI-MS/MS. The proteins identified by MS/MS were found to correspond with the mass and p*I* values of selected protein spots in the gel images. Among these ubiquitous raft proteins, ATP synthase β, β-actin, $G_{\beta 1}$, prohibitin, and voltage-dependent anion channel (VDAC) were reportedly localized in the plasma membrane rafts.

3.3 Oxidation–reduction respiratory chain and ATP synthase complex in detergent-resistant lipid rafts

Unexpectedly, we were able to identify a variety of mitochondrial respiratory proteins from different lipid rafts originating from different organs. NADH dehydrogenase Fe-S protein 1, the NADH-ubiquinone oxidoreductase 24 kDa subunit, ubiquinol-cytochrome c reductase complex core protein 1, and ubiquinol-cytochrome c reductase Rieske iron-sulfur polypeptide 1 were identified from the detergent-insoluble lipid rafts, thereby implying that these mitochondrial respiratory proteins might be localized in the lipid rafts. In order to further confirm the localization of these mitochondrial respiratory proteins into the detergent-insoluble lipid rafts, we isolated the lipid rafts in the presence of 1% Triton X-100 from different organs and analyzed *via* immunoblotting with anti-ATP synthase α, ATP synthase β, the NADH-ubiquinone oxidoreductase 17 kDa subunit (for OXPHOS I), the succinate-ubiquinol oxidoreductase 70 kDa subunit (for OXPHOS II), and the ubiquinol-cytochrome c reductase core I subunit (for OXPHOS III) antibodies. As shown in Fig. 4A, these respiratory OXPHOS complexes and ATP synthase complex were located in the lipid raft fractions, as well as in the bottom fractions, when the detergent-resistant lipid rafts were isolated from different organs. This implies that all organs harbor respiratory chains and ATP synthase complex in their detergent-insoluble lipid rafts.

Since different proteins are found in lipid rafts depending on the isolation method [31], we tested the localization of oxidation–reduction chains and ATP synthase complex into lipid rafts after isolating lipid rafts from mouse kidney by

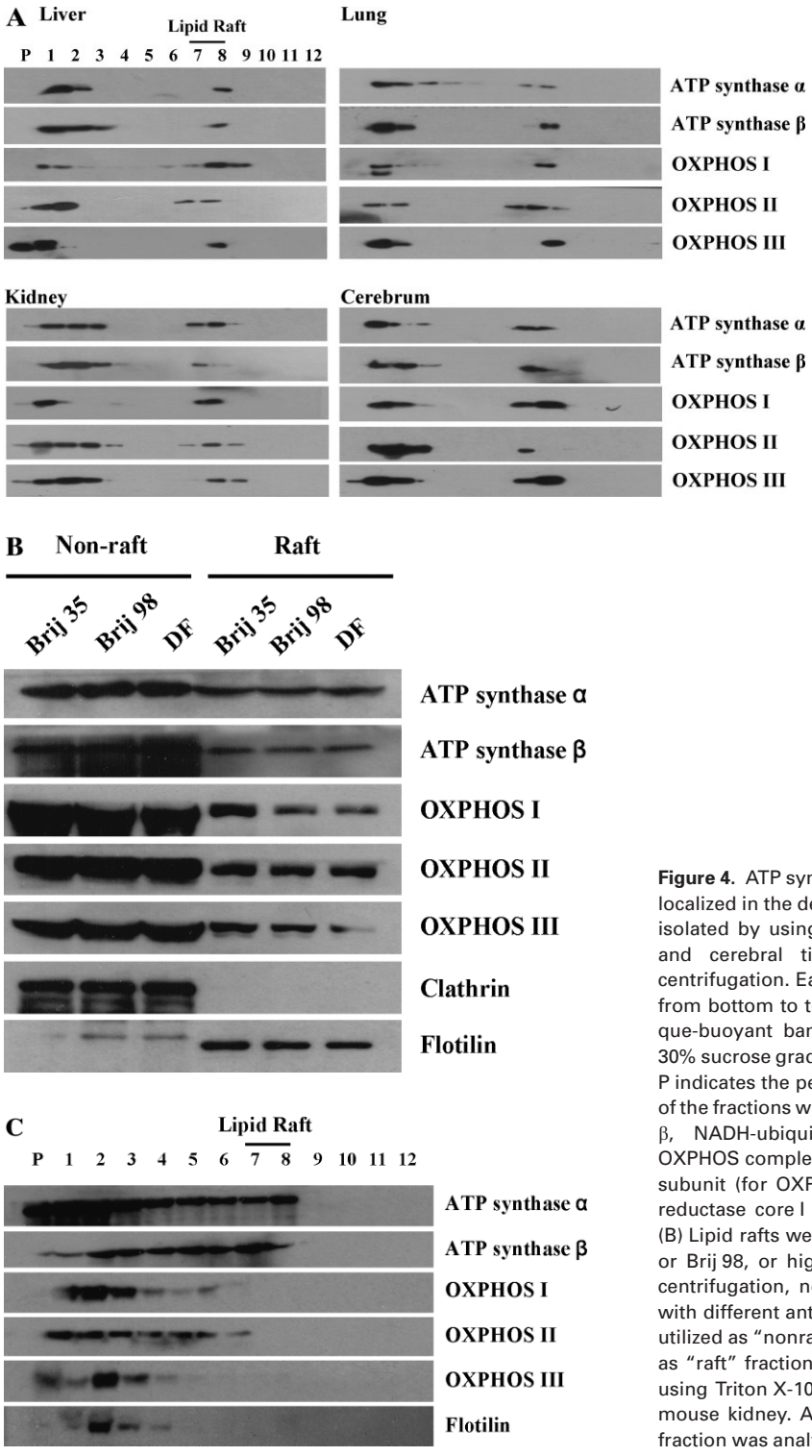

Figure 4. ATP synthase α, β and OXPHOS complex I, II, and III are localized in the detergent-resistant lipid rafts. (A) Lipid rafts were isolated by using Triton X-100 from mouse liver, lung, kidney, and cerebral tissues *via* sucrose density gradient ultracentrifugation. Each of the organ samples was then fractionated from bottom to top. Fractions 7 and 8, which contained an opaque-buoyant band found in the interface between the 5 and 30% sucrose gradient corresponding to the lipid raft fraction, and P indicates the pellet. After the preparation of the samples, each of the fractions was immunoblotted with anti-ATP synthase α and β, NADH-ubiquinone oxidoreductase 17 kDa subunit (for OXPHOS complex I), succinate-ubiquinol oxidoreductase 70 kDa subunit (for OXPHOS complex II), and ubiquinol-cytochrome c reductase core I subunit (for OXPHOS complex III) antibodies. (B) Lipid rafts were isolated from mouse kidney by using Brij 35 or Brij 98, or high pH/carbonate. After sucrose gradient ultracentrifugation, nonraft and raft fractions were immunoblotted with different antibodies. Combination of fractions 1 and 2 were utilized as "nonraft" fractions, and fractions 7 and 8 were utilized as "raft" fractions, respectively. (C) Lipid rafts were isolated by using Triton X-100 in the presence of 10 mM methyl-β-CD from mouse kidney. After sucrose gradient ultracentrifugation, each fraction was analyzed by immunoblots.

using different detergents such as Brij 35 or Brij 98, or by using detergent-free (high pH/carbonate) method. After sucrose gradient ultracentrifugation, nonraft (fraction numbers 1 and 2), and raft (fraction numbers 7 and 8) fractions were collected, and analyzed by immunoblots. As shown in Fig. 4B, oxidation–reduction chains and ATP synthase complex were found in lipid raft fractions irrespective of lipid raft isolation methods.

In order to determine whether the localization of oxidation–reduction chains and ATP synthase complex into lipid rafts is dependent on cholesterol, mouse kidney was treated with 1% Triton X-100 containing 10 mM methyl-β-CD, a cholesterol-sequestering agent. After sucrose gradient ultracentrifugation, each fraction was analyzed by immunoblots with anti-ATP synthase α, and β, and OXPHOS I, II, and III antibodies. As shown in Fig. 4C, all oxidation–reduction respiratory chains and ATP synthase complex disappear in the lipid raft fractions, indicating that the lipid raft localization of these proteins is dependent on cholesterol.

3.4 Oxidation–reduction chains and ATP synthase complex are found in the lipid rafts originating from plasma membrane not from mitochondria

Because many mitochondrial proteins have previously been observed in other raft proteomes from HeLa [25], THP-1 [34], and Jurkat cells [35], as well as in the liver raft proteome [24], it is quite tempting to speculate that the mitochondrial membrane does, indeed, harbor rafts. However, we noted no buoyant band when the mouse kidney mitochondria were extracted in the presence of Triton X-100 and subjected to sucrose gradient ultracentrifugation in order to obtain mitochondrial rafts. This indicates that mitochondrial lipid rafts are rarely, if ever, present. In order to confirm that respiratory chains and ATP synthase complex in the lipid rafts were not the result of contaminants originating from the mitochondria, we isolated mitochondria and plasma membranes from kidney tissues (Fig. 5A). Since the mitochondrial fractions did not contain insulin receptor (a plasma membrane marker), and plasma membrane did not have mitochondrial markers such as cytochrome c and Bcl-2, there was no cross-contamination between plasma membrane and mitochondria. Moreover, the mitochondrial and plasma membrane fractions did not show any β'-COP (a Golgi marker), and LAMP-1 (a lysosomal marker), indicating that these fractions have little contaminations originating from other intracellular organelles. The mitochondria and plasma membranes were subjected to sucrose gradient ultracentrifugation after being extracted with Triton X-100. Fraction numbers 7 and 8, both of which represent lipid rafts, were collected and precipitated *via* ultracentrifugation (25 000 rpm, 30 min, 4°C) after dilution with HEPES buffer. The precipitates (lipid rafts) were then analyzed *via* immunoblotting with anti-ATP synthase α and β, and OXPHOS I, II, and III antibodies. As shown in Fig. 5B, oxidative respiratory chains and ATP synthase complex were detected in lipid raft fractions originating from the plasma membrane and not from the mitochondria, thereby indicating that the proteins detected in the lipid raft fractions were not contaminants originating from mitochondria.

3.5 Finding of oxidation–reduction chains and ATP synthase complex in the cell surface

In order to make sure the surface localization of oxidation–reduction chains and ATP synthase complex, 293 cells were observed in immunofluorescence. It should be noted that the cells were not permeabilized, and only the cell surface is exposed to antibodies. As shown in Fig. 6A, ATP synthase α and β, and OXPHOS III appeared in the cell surface, and were exactly colocalized with 5'-nucleotidase. Since 5'-nucleotidase contain GPI lipid moiety that is required to anchor the proteins into the outer leaflet of plasma mem-

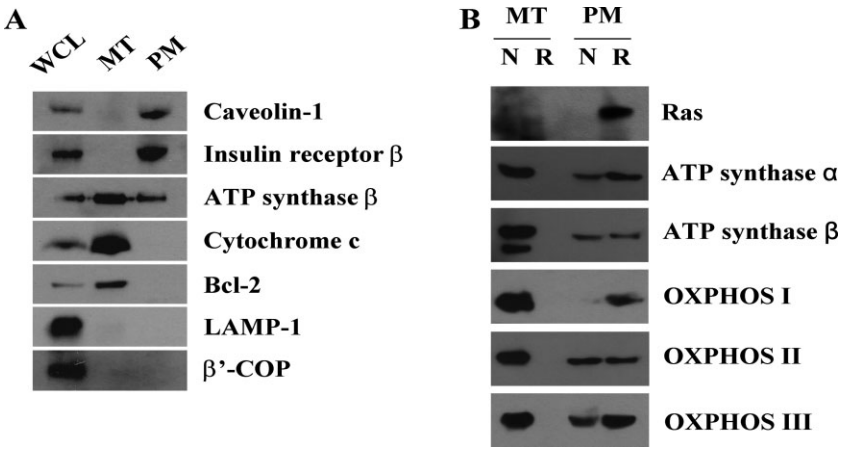

Figure 5. Confirmation of the association of oxidative respiratory chain complex in the plasma membrane-originated lipid rafts. (A) The plasma membrane and mitochondria were prepared from mouse kidneys, as described in Section 2. Proteins (15 μg) from each of the samples were analyzed *via* immunoblotting with the antibodies against the insulin receptor β subunit, cytochrome c, Bcl-2, β'-COP, LAMP-1, and ATP synthase α. (B) Lipid rafts isolated from the plasma membranes and mitochondria were analyzed *via* immunoblotting with antibodies which detect the signals of ATP synthase α, β and OXPHOS I, II, and III. Anti-Ras antibody was utilized as a marker for detergent-resistant lipid rafts. Combination of fractions 1 and 2 were utilized as "nonraft" fractions, and fractions 7 and 8 were utilized as "raft" fractions, respectively. N indicates nonraft, and R shows lipid raft.

brane lipid rafts, we conclude that ATP synthase α and β and OXPHOS III are indeed found in the plasma membrane rafts.

In order to further confirm the localization of oxidation–reduction respiratory chains and ATP synthase complex in the plasma membrane, mouse kidney cortex was analyzed by immunohistochemistry with anti-ATP synthase α and β, OXPHOS I, and III antibodies. As shown in Fig. 6B, ATP synthase α, and β, and OXPHOS I, and III appeared in the cell surface as well as in the intracellular organelles, mitochondria. It should be noted that these mitochondrial respiratory chains and ATP synthase complex appeared in the basal face but not in apical and lateral face of nephron tubules. With immunofluorescence and immunohistochemistry data, we can conclude that the oxidation–reduction chains and ATP synthase complex is localized in the plasma membrane lipid rafts.

4 Discussion

There has been a strong controversy regarding the reality of detergent-resistant lipid rafts because the lipid rafts harbor proteins originating from the mitochondria, the endo-

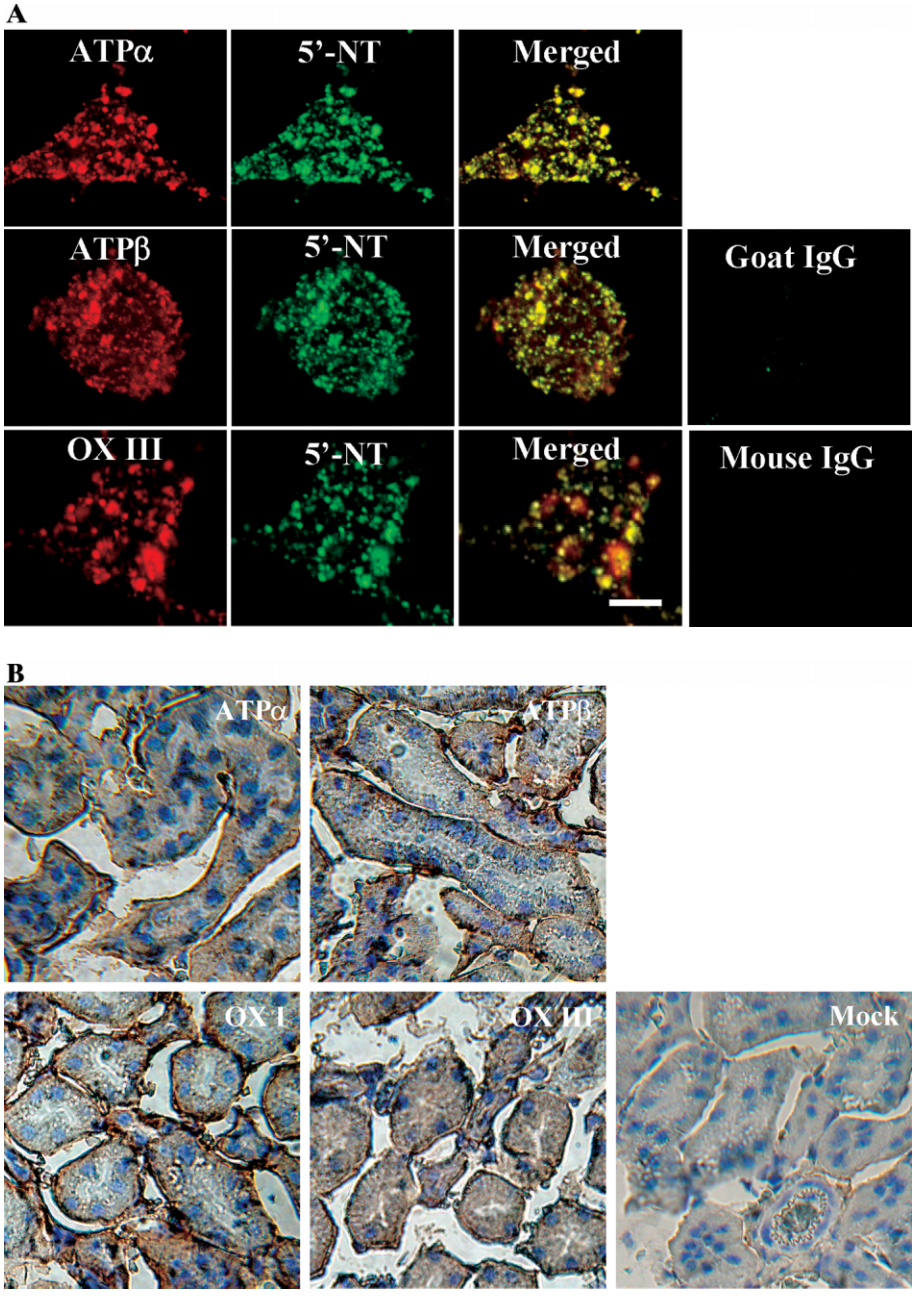

Figure 6. Localization of oxidation–reduction respiratory chains and ATP synthase complex on the cell surface. (A) Two hundred ninety-three cells were immunostained with anti-ATP synthase α, and β, and OXPHOS III antibodies. Cells were not permeabilized and only the cell surface is exposed to antibodies. Anti-5′-nucleotidase antibody was coincubated with anti-ATP synthase α, and β, and OXPHOS III antibodies in 293 cells to see the colocalization of 5′-nucleotidase and these mitochondrial proteins. Scale bar = 10 μm (B) Mouse kidney cortex was analyzed by immunohistochemistry with anti-ATP synthase α, β, and OXPHOS I, and III antibodies.

plasmic reticulum, and the Golgi complex. For example, the ATP synthase complex, an essential ATP-generating complex in mitochondria, has been present in lipid rafts originating from rat liver, HeLa, Jurkat, THP-1 [24, 25, 34, 35], and human umbilical vein endothelial cells [36]. Due to contaminants from the mitochondria, the endoplasmic reticulum, and the Golgi complexes, the presence of detergent-resistant lipid rafts has been challenged and they have been suggested to be artifacts resulting from extraction with nonionic detergents [15, 16]. Our differential proteomic analysis also indicated that all detergent-resistant lipid rafts from different organs contained primarily mitochondrial proteins, including mortalin, VDAC, prohibitin, ATP synthase β, ubiquinol-cytochrome c reductase complex core protein I, the NADH-ubiquinone oxidoreductase 24 kDa subunit, and ubiquinol-cytochrome c reductase (Table 1), raising a significant question as to whether the detergent-resistant lipid rafts are truly artifacts which harbor mitochondrial contaminants.

A recent proteomic analysis of integral plasma membrane proteins has demonstrated the existence of many mitochondrial proteins, including ATP synthase, NADH dehydrogenase, ubiquinol-cytochrome c reductase, and cytochrome c oxidase [37]. Because the plasma membrane was affinity-purified with streptavidin-conjugated magnetic beads, along with harsh washing with high-salt and high-pH substances after surface biotinylation, there is little chance of intracellular contamination. In another experiment, the contamination of other intracellular organelles from the detergent-resistant lipid rafts can be precluded by coating the surface of the cell with silica, obtaining the silica-coated plasma membranes, and then treating the silica-coated plasma membranes with Triton X-100. Proteomic analyses of the caveolin-enriched silica-coated lipid rafts also revealed mitochondrial proteins including ATP synthase and prohibitin [36]. Therefore, these repeated findings of mitochondrial proteins in detergent-resistant lipid rafts cannot be explained simply by mitochondrial contamination during the preparation of the lipid rafts.

Here, we have demonstrated that purified mitochondria contain no detergent-resistant lipid rafts, as we observed no buoyant bands after our sucrose-gradient ultracentrifugation. When the plasma membrane lipid rafts were prepared in the presence of Triton X-100, the lipid raft fractions were found to contain ATP synthase and electron transfer chains, including OXPHOS I, II, and III, whereas the mitochondrial raft fractions, if any, did not (Fig. 5A). This demonstrates that the mitochondrial proteins from the detergent-resistant lipid rafts originated from the plasma membrane, but not from the mitochondria.

Some mitochondrial proteins, including the VDAC, prohibitin, and ATP synthase, have been described in association with either the plasma membrane or the surface receptors [38–40]. For example, the ATP synthase complex has been located on the surface of a variety of mammalian cells and shown to function as a receptor for angiostatin [41], ApoAI [38], endothelial monocyte-activating polypeptide II [42], and the Vγ9Vδ2 T-cell receptor [43]. Indeed, ATP synthase α and β are expressed unambiguously in the plasma membrane of hepatocytes and adipocytes, determined by immunofluorescence, cell surface biotinylation, and cellular fractionation. Furthermore, plasma membrane-associated ATP synthase complex has previously been described as a detergent-resistant raft protein [24].

The oxidation–reduction chain and ATP synthase in the plasma membrane lipid rafts might be necessary for the synthesis of extracellular ATP [44]. Extracellular ATP is generated rapidly immediately after the addition of ADP and Pi into the cell media. Because the generation of extracellular ATP was prevented by the treatment of the cells with oligomycin (ATP synthase inhibitor), or *via* the neutralization of cells with anti-ATP synthase β antibody, the surface ATP synthase complex might constitute a prerequisite for the generation of extracellular ATP. Extracellular ATP generation can also be prevented by treating the cells with uncouplers (carbonyl cyanide *m*-chlorophenylhydrazone and KCN) or subjecting them to low pH conditions, thereby implying that a proton gradient through the plasma membrane lipid rafts is another prerequisite for the generation of extracellular ATP. More interestingly, some oxidative respiratory proteins have also been detected in the plasma membrane, and determined *via* cellular fractionation. Therefore, the detection of the oxidative respiratory chain, as well as the detection of ATP synthase in the plasma membrane lipid rafts, might be considered to be a novel concept with regard to the generation of ATP *via* the plasma membrane.

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