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Detergent-resistant membrane subfractions containing proteins of plasma membrane, mitochondrial, and internal membrane origins

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Abstract

HEK293 cell detergent-resistant membranes (DRMs) isolated by the standard homogenization protocol employing a Teflon pestle homogenizer yielded a prominent opaque band at approximately 16% sucrose upon density gradient ultracentrifugation. In contrast, cell disruption using a ground glass tissue homogenizer generated three distinct DRM populations migrating at approximately 10%, 14%, and 20% sucrose, named DRM subfractions A, B, and C, respectively. Separation of the DRM subfractions by mechanical disruption suggested that they are physically associated within the cellular environment, but can be dissociated by shear forces generated during vigorous homogenization. All three DRM subfractions possessed cholesterol and ganglioside G_{M1}, but differed in protein composition. Subfraction A was enriched in flotillin-1 and contained little caveolin-1. In contrast, subfractions B and C were enriched in caveolin-1. Subfraction C contained several mitochondrial membrane proteins, including mitofilin and porins. Only subfraction B appeared to contain significant amounts of plasma membrane-associated proteins, as revealed by cell surface labeling studies. A similar distribution of DRM subfractions, as assessed by separation of flotillin-1 and caveolin-1 immunoreactivities, was observed in CHO cells, in 3T3-L1 adipocytes, and in HEK293 cells lysed in detergent-free carbonate. Teflon pestle homogenization of HEK293 cells in the presence of the actin-disrupting agent latrunculin B generated DRM subfractions A–C. The microtubule-disrupting agent vinblastine did not facilitate DRM subfraction separation, and DRMs prepared from fibroblasts of vimentin-null mice were present as a single major band on sucrose gradients, unless pre-treated with latrunculin B. These results suggest that the DRM subfractions are interconnected by the actin cytoskeleton, and not by microtubes or vimentin intermediate filaments. The subfractions described may prove useful in studying discrete protein populations associated with detergent-resistant membranes, and their potential interactions in cell signaling.

Keywords: Detergent-resistant membrane; Actin; Flotillin; Caveolin; Mitofilin; Porin

1. Introduction

Many different intracellular signaling systems are now thought to be localized to stable membrane elements called lipid rafts, which are enriched in cholesterol, various sphingolipids, and lipid-modified proteins [1–3]. A key biochemical tool used to investigate lipid raft composition has been the generation of detergent-resistant membrane (DRM) preparations, based on the property of the cholesterol-enriched membranes to survive extraction with cold Triton X-100 [4]. While DRMs do not appear to represent isolated lipid rafts, their study has provided insight into the population of proteins in various cells that are selectively associated with detergent-resistant, ordered lipid structures [5]. Isolation of DRMs from cultured cells or tissues is typically achieved by underlaying a dense suspension of detergent-containing homogenate beneath continuous or discontinuous sucrose gradients. Ultracentrifugation then allows flotation of DRMs. To circumvent the possible generation of artifact ordered lipid structures by surfactants, and to remove loosely associated proteins, investigators have used alkaline carbonate buffer without detergent to generate floating membrane preparations [6,7].

In preparing DRM fractions from HEK293 cells by the established Triton X-100 extraction procedure [4,8], the use of a ground glass homogenizer instead of Teflon® generated three discrete floating bands as compared with the usual single band at approximately 16% sucrose. These DRM subfractions had unique

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protein compositions, and could be generated by pre-treatment of cells with latrunculin B followed by gentle homogenization. The results suggest that the DRM subfractions represent unique membrane populations derived from different intracellular sites that are linked together by the actin cytoskeleton.

2. Materials and methods

2.1. Materials

Latrunculin B, vinblastine, cytochalasin D, and ganglioside G_{M1} were obtained from Sigma. SurfactAmp® Triton X-100 was purchased from Pierce. TRex293 cells, derived from HEK293 human kidney cell line, were obtained from Invitrogen. Fibroblast cell lines derived from vimentin knockout mice and wild-type littermates were kindly provided by Dr. Robert Evans, University of Colorado Health Sciences Center. CHO-K1 cells and 3T3-L1 preadipocytes were obtained from American Type Culture Collection. All cell lines were cultured in DMEM medium containing 10% FBS.

2.2. DRM preparation

Preparation of DRM fractions was carried out as previously described [8], with a minor modification in the homogenization step. All steps were carried out on ice. Cells from 2 approximately 70% confluent 150 mm culture dishes were washed twice with PBS, thoroughly drained, and scraped in 1 ml of homogenization buffer: 25 mM PIPES, 150 mM NaCl, 40 μM leupeptin, 8 μM pepstatin A, 250 μM PMSF, 0.5 mM benzamidine–HCl, 100 μM sodium vanadate, 0.2 μM microcystin, pH 6.6, containing 1% Triton X-100. The volume was adjusted to 2.0 ml with PBS, and the scraped cells were homogenized 20 strokes using a 15 ml ground glass homogenizer (Norman D. Erway Glass Blowing, Oregon, WI). The volume was adjusted to 4.0 ml with 80% sucrose in homogenization buffer minus Triton X-100, and the sample was layered under an 8.5 ml 5% to 30% sucrose gradient in homogenization buffer minus Triton X-100. Centrifugation was carried out for 15–18 h at 5 °C in an SW-41 rotor at 188,000 ×*g*. DRM subfractions were collected by aspiration and diluted 3-fold with homogenization buffer minus Triton X-100. The diluted DRM fractions were centrifuged at 4 °C and 20,000 ×*g* for 30 min to sediment the membranes. The membrane pellets were taken up to 5% of the original volume in homogenization buffer minus Triton X-100, frozen at −70 °C for later assays, or processed directly for gel electrophoresis. To prepare detergent-free lipid rafts, cells were lysed in sodium carbonate, pH 11, without Triton X-100 as previously described [6], then adjusted to 40% sucrose and centrifuged under a sucrose gradient as described above.

2.3. Biotinylation of cell surface proteins

Subconfluent HEK293 cells in a 150 mm dish were washed twice with ice cold PBS, and labeled on ice with 2 mg of Sulfo-NHS-LC-Biotin (Pierce) exactly as described in the manufacturer's protocol for cell surface labeling. After 30 min in the cold, the unreacted reagent was removed by four rapid washes with cold PBS, and the labeled cells were combined with 2 non-labeled dishes for preparation of DRM subfractions by the standard ground glass homogenization protocol. Biotin-labeled proteins were detected in protein blots using alkaline phosphatase-conjugated ExtrAvidin (Sigma).

2.4. Gel electrophoresis and protein immunoblotting

Samples were electrophoresed in pre-cast 10% polyacrylamide slab gels in MOPS buffer (Invitrogen), using the sample and running buffers provided. Gels were stained with Coomassie blue R250, or transferred to nitrocellulose membranes for immunoblot analysis. For the latter, alkaline phosphatase second antibodies were employed, and bands were visualized using BCIP/NBT substrate. Flotillin-1 mouse monoclonal antibody, caveolin-1 polyclonal antibody, and anti-LYN mouse monoclonal antibody, clone 42, were obtained from BD Transduction Laboratories. Anti-talin 8d4 mouse monoclonal antibody and anti-porin rabbit polyclonal antibody (V2139) were purchased from Sigma. Anti-vinculin mouse monoclonal clone VIN-11-5 was from ICN. Mouse monoclonal antibody α6-F against the α-subunit of Na,K-ATPase was obtained from the Developmental Studies Hybridoma Bank, University of Iowa.

2.5. Assays

Protein was determined with the bicinchoninic acid procedure [9] using bovine serum albumin as the standard. Total cholesterol was measured using the cholesterol esterase/cholesterol oxidase procedure [10]. Ganglioside G_{M1} content of DRM fractions was assayed using a dot-blot method previously described [11]. A series of dots containing known amounts of commercial G_{M1} were employed to construct a standard density curve. Sucrose concentration in density gradients was determined colorimetrically following reaction with sulfuric acid and phenol [12].

2.6. Mass spectrophotometry

Mass spectrophotometric analyses were performed in the Proteomics Core Laboratory at The University of Toledo Health Science Campus. DRM subfractions isolated from HEK293 fibroblasts were subjected to SDS-PAGE and colloidal Coomassie blue staining. Bands were cut from the gels and digested with sequencing-grade, modified trypsin (Promega) overnight at 37 °C. Peptides were extracted with 60% acetonitrile:0.1% TFA, and concentrated to approximately 15 μl using a vacufuge. A 2 μl aliquot of the sample was separated on a reverse phase column (75 μm id × 5 cm × 15 m Aquasil C18 Picofrit column, New Objectives). The eluent was directly introduced into an ion-trap mass spectrometer (LCQ-Deca XP Plus, Finnigan) equipped with a nano-spray source. The mass spectrometer was operated on a double play mode where the instrument was set to acquire a full MS scan (400–2000 *m*/*z*) and a collision induced dissociation (CID) spectrum on the most abundant ion from the full MS scan.

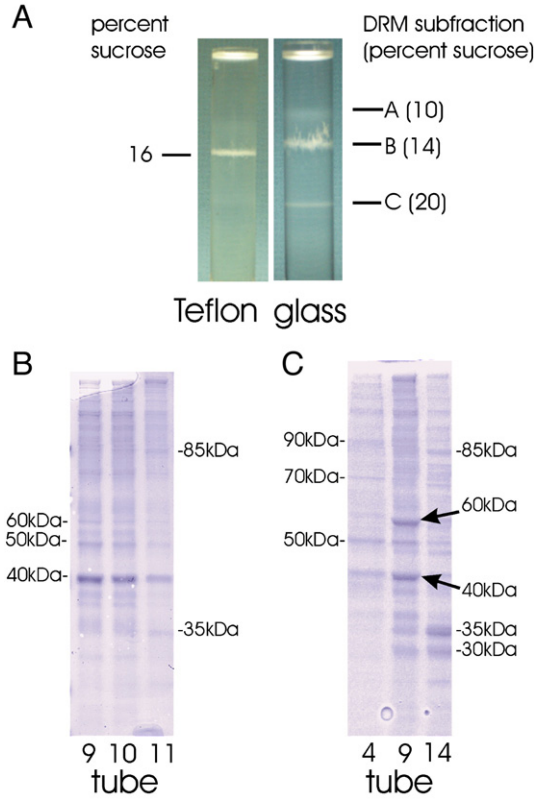

Fig. 1. Preparation and protein profiles of HEK293 cell DRM subfractions. A) Homogenization with a ground glass homogenizer (glass) resulted in isolation of three translucent to opaque zones at different buoyant densities upon ultracentrifugation in a 5% to 30% sucrose density gradient. Use of a Teflon homogenizer (Teflon) generated a single major zone at approximately 16% sucrose, and a weak, broad band at 20% sucrose. Panels B and C: 10 μl aliquots of the indicated sucrose gradient fractions, concentrated as described in the Materials and methods section, were electrophoresed on a 10% SDS-PAGE gel and stained with Coomassie blue. B) After fractionation, the Teflon-homogenized sample displayed enrichment of an approximately 50 kDa protein in the low buoyant density fraction of the single apparent opaque zone (tube 9), and enrichment of 85 kDa and 35 kDa proteins in the high buoyant density fraction (tube 11). C) Different major protein components were clearly evident in tubes 4, 9, and 14 from sucrose gradients of ground glass-homogenized HEK293 cells, representing the main protein-containing tubes of DRM subfractions A–C, respectively.

CID spectra were either manually interpreted or searched against an appropriate non-redundant database using the TurboSEQUEST program.

3. Results

Except where otherwise stated, results are representative of at least three independent experiments.

3.1. Generation of DRM subfractions

HEK293 cells were homogenized using a ground glass homogenizer (vigorous homogenization) and subjected to sucrose gradient flotation as described in the Materials and methods section. Under these conditions, two clearly defined opaque floating DRM bands were observed at 14% and 20% sucrose (Fig. 1, panel A, right). In addition, a faint, translucent band was observed at 10% sucrose. These results are representative of at least six independent experiments using HEK293 cells. In contrast, homogenization with a Teflon pestle homogenizer (gentle homogenization) generated a more tightly focused opaque region at about 16% sucrose, consistent with previous reports of an apparent single species of DRM under mild homogenization conditions [8]. There was also often a very faint, diffuse band at approximately 20% sucrose. Hereafter, the three bands generated by vigorous homogenization conditions are referred to as DRM subfractions A, B, and C in order of increasing buoyant density.

3.2. Cholesterol, ganglioside G_{M1}, and protein contents of DRM subfractions A, B, and C

Because cholesterol and G_{M1} are major lipid raft components found in DRM preparations, the subfractions were analyzed for potential differences in these two raft markers. Subfraction B contained the majority of total DRM cholesterol, G_{M1}, and protein (Table 1). However, a significant amount was also present in subfractions A and C, comprising together approximately 20 to 30% of total DRM cholesterol, G_{M1}, and protein. Similar cholesterol, G_{M1} and protein contents were obtained in a second, independent experiment.

3.3. Protein composition of DRM subfractions A, B, and C

Sucrose gradients containing DRM isolated by gentle or vigorous homogenization were fractionated into 0.5 ml fractions as described in the Materials and Methods section, and analyzed by SDS-PAGE followed by Coomassie staining. Three fractions (9–11) contained the bulk of the protein in the single DRM band observed in the gently homogenized sample (Fig. 1, panel B). There were subtle differences in the protein profiles of the three fractions: fraction 9 was relatively enriched in a 50 kDa protein, while fraction 11 contained prominent 35 kDa and 85 kDa proteins.

The DRM subfractions derived from vigorously homogenized HEK293 cells possessed clearly different complements of associated proteins (Fig. 1, panel C). DRM subfraction A was characterized by its relative enrichment in a protein band at approximately 50 kDa. Subfraction B had strongly stained bands at approximately 40 kDa and 60 kDa, while subfraction C was characterized by bands at approximately 30, 35, and 85 kDa. The DRM subfractions were analyzed for known lipid raft proteins by immunoblotting. Subfraction A from HEK293 cells was enriched in flotillin-1, and had little caveolin-1 compared with the other

Table 1
Cholesterol, G_{M1}, and protein content of DRM subfractions A, B, and C

DRM subfraction	nMol cholesterol	μg G_{M1}	μg protein
A	74 (7.76%)	0.75 (11.8%)	13 (2.48%)
B	745 (78.1%)	4.62 (72.6%)	420 (80.3%)
C	135 (14.1%)	0.99 (15.6%)	90 (17.2%)

DRM subfractions were obtained from 10 confluent flasks of HEK293 cells (55 mg total protein). Numbers in parentheses are the percentage of total DRM constituent found in the subfraction.

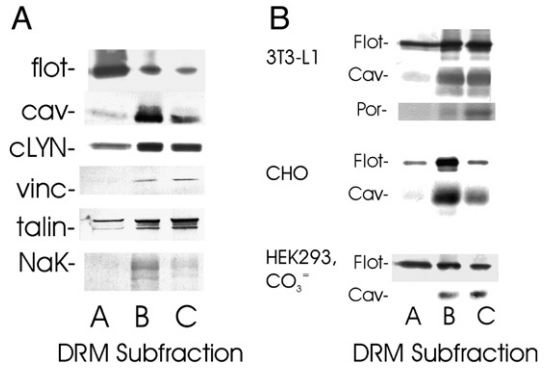

Fig. 2. Distribution of lipid raft proteins among DRM subfractions A–C. A) Aliquots of DRM subfraction A (15 µl/lane), B (3.5 µl/lane), and C (7 µl/lane), prepared from HEK293 cells were subjected to SDS-PAGE and protein immunoblot analysis as described in the Materials and methods section. B) DRM subfractions from 3T3-L1 adipocytes and CHO cells were analyzed as in panel A. HEK293 cells were homogenized in alkaline carbonate buffer as described in the Materials and methods section. flot = flotillin-1; cav = caveolin-1; vinc = vinculin; NaK = Na,K-ATPase; por = porin-2.

subfractions (Fig. 2, panel A). DRM subfractions B and C seemed to have similar levels of the proteins tested, with the exception of the plasma membrane sodium pump (NaK in Fig. 2, panel A), which was mainly found in subfraction B. To determine whether the flotillin-1 enrichment in subfraction A was general, and not uniquely associated with Triton-prepared DRM from HEK293 cells, the experiments shown in Fig. 2, panel B were carried out. Subfractions A from 3T3-L1 adipocytes, CHO cells, and detergent-free, carbonate-extracted HEK293 cells displayed greater flotillin-1 staining relative to caveolin-1, compared with the other subfractions.

Initial mass spectrophotometric analysis of the DRM subfractions from HEK293 cells (Table 2) demonstrated that the 60 kDa band in subfraction B was vimentin (Mr=57 kDa), and the 40 kDa band was actin (Mr=42 kDa). Consistent with the immunoblot experiments reported in Fig. 2, the 50 kDa Coomassie-stained band observed on SDS-PAGE was found to be a mixture of flotillins -1 and -2, demonstrating that the flotillins are major protein components of DRM subfraction A. The bands enriched in DRM subfraction C were found to be

Table 2
Mass spectrophotometric analysis of HEK293 cell DRM subfractions

DRM subfraction	Apparent mass (SDS-PAGE), kDa	Peptides/ total identified	Protein	Accession #	Calculated mass, Da
A	50	5/12	Flotillin-1	O75955	47,355
		5/12	Flotillin-2	Q14254	41,685
B	60	10/16	Vimentin	P08670	53,520
	40	4/8	Actin A	P03996	41,775
		3/8	Actin B	P02570	41,737
C	85	10/10	Mitofilin	Q16891	83,678
	35	4/7	Porin-2	P45880	38,093
		2/7	BAP37	Q99623	33,296
	30	8/16	Porin-1	P21796	30,641
		3/16	Prohibitin	P35232	29,804

several proteins of the mitochondrial outer membrane (prohibitin, porin-1, and porin-2) as well as the mitochondrial inner membrane protein, mitofilin. The DRM subfractions isolated from 3T3-L1 adipocytes were subsequently immunoblotted for porin. Consistent with the proteomic analysis of the HEK293 cells, DRM subfraction C from adipocytes was also enriched in porin (Fig. 2, panel B, top).

The association of Na,K-ATPase with DRM subfraction B (Fig. 2, panel A, bottom) suggested that this DRM subfraction was derived from plasma membrane. To explore this further, HEK293 cells were labeled with a cell impermeant biotin probe as described in the Materials and methods section. Upon isolation of DRM subfractions from the labeled cells, biotinylated proteins were almost exclusively found in DRM subfraction B (Fig. 3), suggesting that this subfraction was derived from plasma membrane.

Because DRM subfraction A appeared to be novel in its abundance of flotillin relative to caveolin, its composition was investigated in further detail by mass spectrophotometric analysis of pooled, concentrated samples isolated from a total of ten 150 mm culture dishes of HEK293 cells (Fig. 4). In

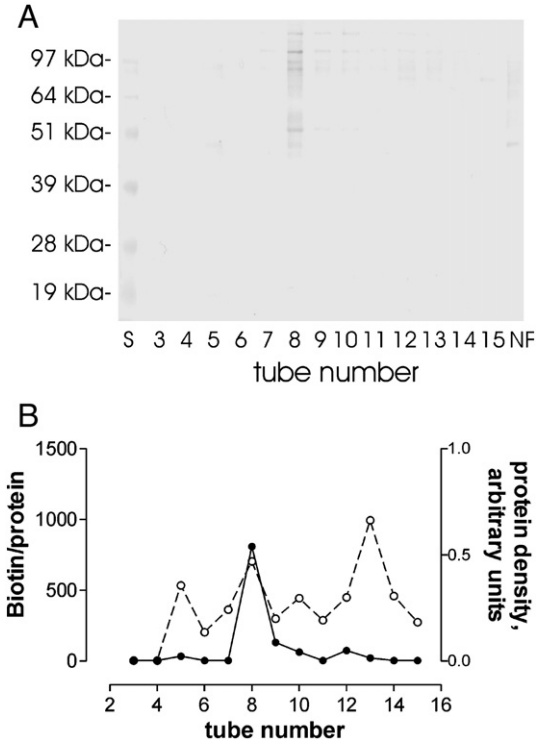

Fig. 3. Distribution of surface-biotinylated proteins to DRM subfraction B. Subconfluent HEK293 cells were surface labeled with Sulfo-NHS-LC-Biotin as described in the Materials and methods section. DRM subfractions were prepared, subjected to SDS-PAGE/electroblotting, and probed with alkaline phosphatase-conjugated ExtrAvidin. Protein loads were adjusted so that the lanes representing the peak tube of each DRM subfraction (5, 8, and 13 for DRM subfractions A–C, respectively) contained approximately 2 µg of total protein. Lanes representing tubes 3–6 were loaded with 10 µl; tubes 7 and 8 with 1 µl, and tubes 9–15 with 5 µl. A 5 µl sample of non-floating fraction was also loaded (NF). A) The ExtrAvidin-stained electroblot. B) ExtrAvidin-stained lanes were scanned employing NIH ImageJ densitometric software and normalized for protein content per lane (filled circles), the latter determined by densitometric scanning of a replicate Coomassie-stained gel (hollow circles).

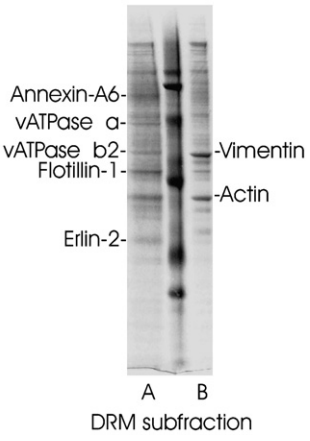

Fig. 4. Analysis of protein components selectively associated with DRM subfraction A. DRM subfractions A and B were isolated from ten 150 mm dishes of HEK293 cells. Ten micrograms of protein derived from each fraction was applied to SDS-PAGE followed by proteomic analysis as described in the Materials and methods section. Five proteins were found to be enriched in DRM subfraction A, and were identified by mass spectrophotometric analysis, as indicated on the left side of the figure.

addition to flotillin, four other bands were specifically enriched in this preparation relative to the DRM subfraction B (compare lanes A and B of Fig. 4). These were identified as annexin A6 (accession number: P08133), vacuolar ATP synthase catalytic subunit a (P38606), vacuolar ATP synthase subunit b2 (P21281), and c8orf2 protein (O94905). The latter was recently named erlin-2, because of its association with endoplasmic reticulum-derived lipid raft microdomains [13].

One explanation for three discrete DRM subfractions would be the presence of multiple weakly associated proteins that impart different buoyant densities to various DRMs. Because this association could occur artifactually during cell lysis and DRM preparation, more vigorous methods were applied to the preparation of the floating membrane fractions. Increasing Triton X-100 concentration to 3% did not substantively alter the distribution of flotillin-1 to the location of DRM subfraction A, nor porin-2 to subfraction C (Fig. 5). Upon homogenization of cells with carbonate in the absence of detergent [6], loosely

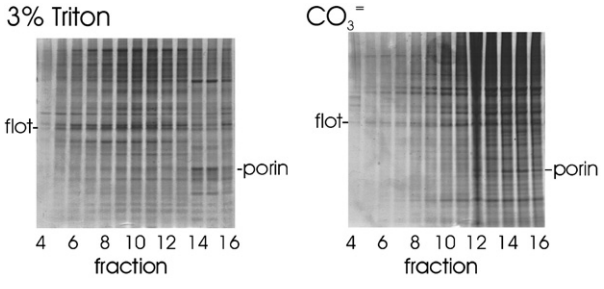

Fig. 5. Differential distribution of flotillin-1 and porin-2 within subfractions is maintained in HEK293 DRMs prepared by 3% Triton X-100 or alkaline carbonate extraction. DRMs were prepared from 3% Triton X-100 or carbonate lysates as indicated. The sucrose gradients were fractionated into 0.5 ml samples and 10 µl samples were analyzed by SDS-PAGE and Coomassie blue staining. The flotillin-1 and porin-2 bands remained enriched in DRM subfractions A (tubes 4 and 5) and C (tubes 14 and 15), respectively.

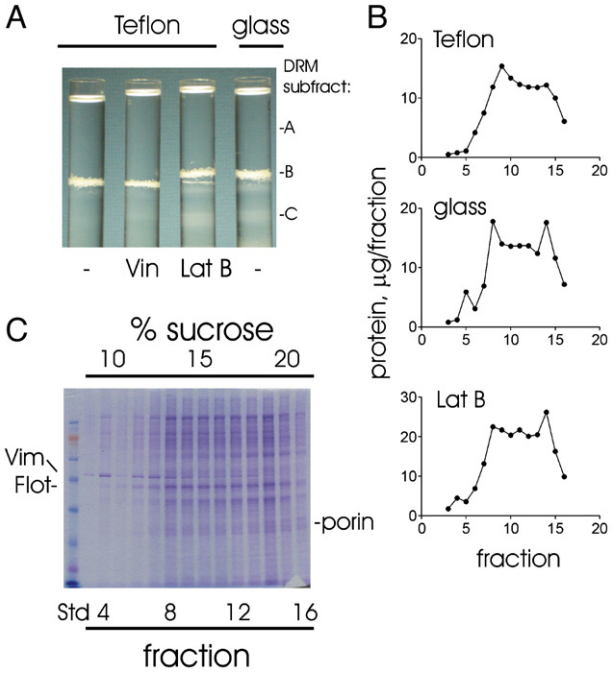

Fig. 6. Latrunculin B pre-treatment generates DRM subfractions. HEK293 cells were pre-treated with 2 µM latrunculin B or 10 µM vinblastine for 1 h. DRM fractions were prepared after homogenization of cell lysates with a Teflon pestle homogenizer. A) Pre-treatment with latrunculin B, but not with vinblastine, generated DRM subfraction bands in sucrose gradients. B) Pre-treatment with latrunculin B resulted in accumulation of protein in the sucrose gradient region corresponding to DRM subfraction A (tubes 4 and 5). C) Ten microliter aliquots of sucrose gradient fractions were analyzed by SDS-PAGE and Coomassie blue staining. Vim = vimentin, Flot = flotillin-1, and porin = porin-2.

associated proteins should be solubilized from the floating membrane fraction, arguing against an effect of these proteins in DRM subfraction generation. However, even with alkaline extraction, flotillin was a major protein present in the lower density fractions, while porin-2 remained in fractions 14–16 (Fig. 5). These results are consistent with the presence of DRM

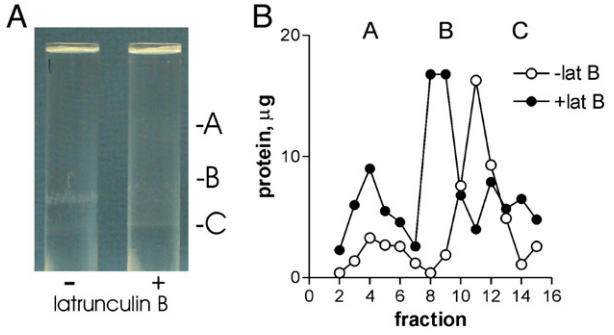

Fig. 7. DRM subfractions are not separated in fibroblasts from vimentin-null mice. DRM fractions were prepared from vimentin-null fibroblasts using a Teflon pestle homogenizer. One sample of fibroblasts was pre-treated with 2 µM latrunculin B for 1 h before homogenization. A) Sucrose gradients containing DRM fractions from fibroblasts with or without latrunculin pre-treatment. Note the single major band in the minus latrunculin sample, and the diffuse dispersal of the band in the latrunculin B-treated sample. A band corresponding in location to DRM subfraction C is evident in the latter sample. B) Protein determination of the fractionated sucrose gradients depicted in panel A.

subfractions, even under conditions that should remove weakly associated proteins.

3.4. DRM subfractions are generated by disruption of the actin cytoskeleton

Generation of the DRM subfractions by vigorous homogenization suggested that they might be released from larger cytoskeletal fragments by mechanical shearing. To explore this possibility, HEK293 cells were cultured for 1 h in the presence of 10 μM vinblastine, a microtubule-disrupting agent, or 2 μM latrunculin B, which destabilizes f-actin. The cells were then homogenized using a Teflon pestle, and DRMs were prepared by the standard procedure. Exposure to latrunculin B resulted in a pattern of DRM bands that was nearly identical to those generated by a ground glass homogenizer (Fig. 6, panel A). In this experiment, DRM subfraction A was extremely faint and not readily visualized. However, protein determination of the fractionated sucrose gradients clearly demonstrated a small protein peak between fractions 4 and 5, for both ground glass homogenized and latrunculin-generated DRMs (Fig. 6, panel B). The minor quantity of protein in these peaks is consistent with the observation that DRM subfraction A is relatively protein-poor (Table 1). SDS-PAGE confirmed the accumulation of flotillin in DRM subfraction A, as well as porin in subfraction B (Fig. 6, panel C). Vinblastine pre-treatment did not alter the single major DRM band observed in Teflon-homogenized samples (Fig. 6, panel A). This was consistently observed in several independent experiments.

In several experiments, DRM subfraction A was enriched in vimentin as well as flotillin (see, for example, Fig. 6, panel C). In other experiments (*e.g.*, Fig. 4), little vimentin was present in this subfraction. Vimentin-based intermediate filaments could provide a structural framework to interconnect the DRM subfractions. To determine whether loss of vimentin facilitates separation of the DRM subfractions, fibroblasts derived from vimentin-null mice were subjected to the classic DRM isolation protocol using Teflon pestle, gentle homogenization. Under these conditions, a single predominant DRM band was observed, as well as a faint band migrating at the correct location for DRM subfraction C (Fig. 7, panel A). In DRMs obtained from vimentin-null fibroblasts pre-treated with latrunculin B, the subfraction C band was more intense, and the major band was dispersed into poorly resolved lower density fractions that approximated the location of DRM subfractions B and A derived from HEK293 cells. Protein determination of the fractionated sucrose gradients demonstrated that latrunculin pre-treatment resulted in increased protein content at the gradient positions for DRM subfractions A and C (Fig. 7, panel B).

4. Discussion

Recent studies indicate that DRMs comprise a mixture of lipid microdomains containing different protein constituents [14–16]. However, until now it has not been possible to effect physical separation of discrete DRM subfractions by a simple biochemical approach. Importantly, flotillins, annexin-A6, vimentin, and caveolin-1, proteins that define the various DRM subfractions described in the present work, are previously identified lipid raft-associated proteins [17]. Consistent with their integral association with pre-existing ordered lipid structures, flotillin and porin were enriched in subfractions A and C, respectively, of DRMs prepared by alkaline carbonate extraction without detergent (Figs. 2 and 5).

The present study provides evidence for separate DRM fractions of mitochondrial, plasmalemmal, and endoplasmic reticulum/endosomal origins. Several proteins were enriched in DRM subfraction A compared with the main DRM component, subfraction B. Among these were the SPFH proteins flotillin-1 and erlin-2. Flotillin-1 has previously been shown to be important in organizing the internal vesicle protein complex responsible for insulin-stimulated GLUT-4 transport to the cell surface of adipocytes [18]. Importantly, flotillin-1 was also enriched in DRM subfraction A prepared from 8-day differentiated 3T3-L1 adipocytes (Fig. 2, panel B). Erlin-2 is a lipid raft constituent that has recently been demonstrated to be predominantly associated with endoplasmic reticulum [13]. Also present in DRM subfraction A derived from the HEK293 kidney cell line were annexin-A6 and the vacuolar ATPase subunits a and b2. Annexin-A6 has been identified as a resident lipid raft protein [17], and appears to be predominantly localized to endosomal compartments [19]. Vacuolar ATPases are resident membrane proteins of intracellular vesicles or plasma membrane. Importantly, the kidney vATPase b2 subunit is localized to intracellular vesicles, except under certain conditions, including chronic carbonic anhydrase inhibition, which results in its association with the plasma membrane [20]. These results, together with the relative absence of Na,K-ATPase (Fig. 2, panel A), and cell surface-labeled proteins (Fig. 3), suggest that DRM subfraction A is derived from intracellular membranes.

DRM subfraction C was notable for its abundance of the mitochondrial membrane proteins mitofilin and porins. The presence of mitochondrial proteins in this subfraction requires further comment, as mitochondrial protein association with DRMs or lipid rafts has been controversial. Foster et al. [17] found that much of the mitofilin and porin content of lipid rafts was not removable by treatment with the cholesterol depleting drug, methyl-β-cyclodextrin. They therefore proposed that the mitochondrial proteins observed in DRM preparations were likely to be associated by non-specific absorption. It is noteworthy that the DRM subfraction C is specifically enriched in mitofilin and porins. It does not appear to contain major amounts of other inner or outer mitochondrial membrane proteins [21] that might also be expected to non-specifically associate. Several recent studies have argued for the existence of lipid rafts in mitochondria. DRMs prepared from Jurkat T-cells were enriched in mitofilin and porins, but not in abundant mitochondrial matrix proteins [22]. DRMs have recently been prepared from isolated mouse brain mitochondria. Mitofilin and porins were found to be major components of the mitochondrial DRMs [23]. These observations and others have led to the idea that mitochondria possess lipid raft-like structures [24]. Interestingly, mitofilin is a mitochondrial inner membrane protein, and so would not be likely to participate in cytoplasmic signaling or transport functions. However, it has recently been shown to localize to regions of the inner membrane that abut the

outer membrane and regulate mitochondrial cristae morphology [25]. It is conceivable that mitofilin localization in an ordered lipid domain could facilitate the communication between the inner and outer mitochondrial membranes that is required for proper cristae formation.

The present study suggests that membranes containing the DRM subfractions are linked by the actin cytoskeleton. Inspection of data from a number of previously published studies suggested that the single DRM band isolated by conventional procedures might not represent a single homogeneous population. This was evident by heterogeneity of distribution of specific proteins within the higher and lower density fractions when the apparent single DRM band was divided upon fractionation of the sucrose density gradient. Indeed, differences were observed in the protein compositions of the low and high buoyant density fractions of the single apparent DRM band isolated from HEK293 cells utilizing the gentle homogenization procedure (Fig. 1, panel B). Vigorous mechanical disruption resulted in physical separation of three floating opaque zones, suggesting that a structural component of some sort had been broken, allowing separation of a precursor or precursors into the separate DRM subfractions. Importantly, the cholesterol/protein and G_{M1}/protein ratios were very similar for DRM subfractions B and C; and several-fold higher for subfraction A, consistent with its low buoyant density (Table 1). These results indicate that all three DRM subfractions are enriched in two well-accepted lipid components of DRMs [3], and argue against contamination of the DRM fractions with a non-DRM membrane component. The presence of either flotillin-1 or caveolin-1 in each of the three floating bands also supports their designation as DRM subfractions.

The requirement for physical disruption suggested that one or more of the classic cytoskeletal components might be responsible for linking the DRM subfractions together. To test this hypothesis, major cytoskeletal elements were disrupted. Heterogeneity of DRMs isolated following gentle homogenization was then assessed. The microtubule-disrupting agent vinblastine was without effect, and fibroblasts isolated from vimentin-null mice contained a single major DRM fraction. These studies suggested that disruption of microtubules or intermediate filaments had at best a minor effect in separating the DRM subfractions. However, pre-treatment of HEK293 cells (Fig. 6) or vimentin-null fibroblasts (Fig. 7) with the f-actin-disrupting drug, latrunculin B, resulted in the generation of DRM subfractions. In separate experiments, preincubation of HEK293 cells with 10 μM cytochalasin D, another f-actin-disrupting drug, was also capable of separating DRM into subfractions (data not shown). It therefore seems likely that actin microfilaments interconnect the various DRM subfractions, and by extension the intracellular ordered lipid structures from which they are putatively derived.

5. Description of the method and future applications

Here it is shown that including a ground glass homogenization step in the standard technique for preparation of DRMs results in separation of discrete floating membrane fractions containing proteins of internal membrane, plasma membrane, and mitochondrial origin. The experiments also present evidence for a microfilament-based interconnection of lipid ordered structures associated with plasma membrane, endoplasmic reticulum/endosomes, and mitochondrial membranes. While much work remains to establish the nature of these biochemical preparations, recently published observations suggest that an intracellular association of ordered lipid structures in different intracellular sites could be important for signaling. Direct interaction between the endoplasmic reticulum and mitochondria has recently been proposed to be important in calcium-dependent, mitochondria-mediated apoptosis [26,27], and direct interactions between the junctional endoplasmic reticulum and the plasma membrane may be important in calcium signaling [28]. Recently, caveolin-1 in subconfluent BeWo choriocarcinoma cells has been shown to be associated with mitochondria, both by immunofluorescence, and by western blotting of mitochondria preparations. Caveolin-1 relocated to the plasma membrane when cells reached confluence [29]. The presence of mitochondria-associated caveolin-1 is consistent with its detection in DRM subfraction C (Fig. 2). It is hoped that the preparation described in the present work will prove useful to explore such putative interactions at the biochemical level.

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