Proteolipid domains form in biomimetic and cardiac mitochondrial vesicles and are regulated by cardiolipin concentration but not monolyso-cardiolipin

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Edited by George M. Carman

Cardiolipin (CL) is an anionic phospholipid mainly located in the inner mitochondrial membrane, where it helps regulate bioenergetics, membrane structure, and apoptosis. Localized, phase-segregated domains of CL are hypothesized to control mitochondrial inner membrane organization. However, the existence and underlying mechanisms regulating these mitochondrial domains are unclear. Here, we first isolated detergent-resistant cardiac mitochondrial membranes that have been reported to be CL-enriched domains. Experiments with different detergents yielded only nonspecific solubilization of mitochondrial phospholipids, suggesting that CL domains are not recoverable with detergents. Next, domain formation was investigated in biomimetic giant unilamellar vesicles (GUVs) and newly synthesized giant mitochondrial vesicles (GMVs) from mouse hearts. Confocal fluorescent imaging revealed that introduction of cytochrome $c$ into membranes promotes macroscopic proteolipid domain formation associated with membrane morphological changes in both GUVs and GMVs. Domain organization was also investigated after lowering tetralinoleoyl-CL concentration and substitution with monolyso-CL, two common modifications observed in cardiac pathologies. Loss of tetralinoleoyl-CL decreased proteolipid domain formation in GUVs, because of a favorable Gibbs-free energy of lipid mixing, whereas addition of monolyso-CL had no effect on lipid mixing. Moreover, murine GMVs generated from cardiac acyl-CoA synthetase-1 knockouts, which have remodeled CL acyl chains, did not perturb proteolipid domains. Finally, lowering the tetralinoleoyl-CL content had a stronger influence on the oxidation status of cytochrome $c$ than did incorporation of monolyso-CL. These results indicate that proteolipid domain formation in the cardiac mitochondrial inner membrane depends on tetralinoleoyl-CL concentration, driven by underlying lipid-mixing properties, but not the presence of monolyso-CL.

Cardiolipin (CL)$^2$ is a unique anionic phospholipid that is localized to the inner mitochondrial membrane, where it plays a role in regulating bioenergetics, membrane structure, and apoptosis (1–6). Several mechanisms are proposed to explain how CL regulates inner mitochondrial membrane structure-function. These mechanisms include CL directly binding select enzymes, anchoring cytochrome $c$, promoting correct cristae morphology, and enhancing supercomplex formation (7–13). One poorly studied hypothesis is that CL facilitates the formation of distinct phase-segregated domains (14, 15). The biological advantage of CL-enriched domains would be to concentrate respiratory protein complexes for enhanced electron channeling, to augment membrane curvature, and to serve as docking platforms for apoptotic signals (16–22).

The limited understanding on the existence of CL-enriched domains comes largely from two- or three-component model membrane studies. Calorimetric studies with binary phospholipid mixtures in the presence of cytochrome $c$, truncated Bid, or creatine kinase suggested segregation of CL into distinct clusters (18). Studies with Brewster angle microscopy of phosphatidylcholine/phosphatidylethanolamine monolayers containing creatine kinase have also provided direct support for CL domains (23). Furthermore, microscopy studies with biomimetic CL-containing mixtures have demonstrated the unique

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$^2$The abbreviations used are: CL, cardiolipin; GUV, giant unilamellar vesicle; GMV, giant mitochondrial vesicle; MCL, monolysocardiolipin; NAQ, nonyl acridine orange; cyt c, cytochrome $c$; $\alpha$-syn, $\alpha$-synuclein; mtCK, mitochondrial creatine kinase; (18:1)$_2$CL, 1,1'-dioleoyl(9Z,9Z,12Z,15Z-tetra(9Z-octadecenoyl) cardiolipin; DOPC, 1,2-di(9Z-octadecenoyl)-sn-glycero-3-phosphocholine; 18:0–22:6 PC, 1-stearooyl-2-docosahexaenooyl-sn-glycero-3-phosphocholine; 16:0–20:4 PE, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine; DOPA, 1,2-dioleoyl-sn-glycero-3-phospho(1'-myo-inositol); DOPS, 1,2-dioleoyl-sn-glycero-3-phospho-l-serine; (18:2)$_2$CL, bovine heart cardiolipin; MIM, mitochondrial isolation medium; DRM, detergent-resistant membrane; DSM, detergent-soluble membrane; Chol, cholesterol; mN, millinewton.
ability of cytochrome c to regulate membrane morphology and induce CL domain formation (24, 25). CL domains have also been identified biochemically with detergent-extraction methods; similar to approaches used for the study of detergent-resistant “lipid raft” membranes (17).

There are several major gaps in knowledge regarding CL-enriched domain organization. The first major limitation is that many studies have not effectively modeled the inner mitochondrial composition and even relied on lipid raft models (24, 26). Approximately 70% of the inner mitochondrial membrane is accounted for by phosphatidylcholine (40%) and phosphatidylethanolamine (30%), whereas CL makes up about 15–20% of total phospholipids (7). Phosphatidylinositol, phosphatidic acid, and phosphatidylserine account for the remaining phospholipids, with very low levels of cholesterol. Thus, CL domain formation should be studied in the context of inner mitochondrial-mimicking membranes. Second, the underlying mechanistic factors that regulate CL domain formation are not defined. These are essential to establish because in a range of diseases, CL is subject to considerable physiochemical modifications that impair mitochondrial inner membrane structure-function (3, 27–29). For instance, with heart failure and type II diabetes, cardiac CL acyl chains undergo extensive remodeling that could disrupt CL domain formation (30–37). In Barth syndrome, there is a significant loss of cardiac tetraceneoyl-CL and increased levels of monolyso-CL (MLCL) (27, 38–42).

The first objective of this study was to determine the utility of biochemical detergent extraction to isolate CL domains. Subsequently, we investigated if CL domains can form in the presence of select proteins. These proteins included cytochrome c, creatine kinase, and α-synuclein, which are all associated with mitochondrial membranes. The approach relied on quantitative imaging of biomimetic giant unilamellar vesicles (GUVs) modeling the cardiac inner mitochondrial membrane, and the use of giant mitochondrial vesicles (GMVs) constructed for the first time with phospholipids extracted from rodent cardiac mitochondria. Next, we determined if decreased CL levels, or the accumulation of MLCL, as observed in various diseases, disrupted the organization of proteolipid domains. These studies relied on biomimetic mitochondrial GUVs as well as lipid monolayers mimicking the inner mitochondrial membrane lipid composition to quantify lipid-lipid mixing. In parallel, we constructed GMVs using cardiac tissue harvested from control and acyl-CoA synthetase 1 knockout mice to determine how CL remodeling impacted the organization of native phospholipids and thereby proteolipid domains.

Results

Mitochondrial CL microdomains cannot be isolated with detergent-extraction methods

We first determined if different detergents were equally efficacious at extracting CL-enriched microdomains from rat cardiac mitochondria. Rat myocardial tissue was utilized to ensure a high abundance of material for microdomain isolation. We relied on Triton X-100, Nonidet P-40, and digitonin at various concentrations (0.01, 0.05, and 0.5% w/v). Increasing the concentration of all three detergents led to an overall decrease of phospholipid levels, as measured by TLC, in the insoluble detergent-resistant membrane (DRM) (I) fractions (Fig. 1, A–C). This decrease was paralleled by an increase in the amount of lipids in the detergent-soluble membrane (DSM (S)) fractions (Fig. 1, A–C). The increase in the concentration of Triton X-100 from 0.01 to 0.05% caused a larger reduction in the amount of phospholipids present in the DRM fraction than Nonidet P-40 or digitonin (Fig. 1, A–C). At a concentration of 0.5%, phospholipids in the DRM fraction were appreciably reduced with all three detergents (Fig. 1, A–C).

Both DSM (S) and DRM (I) fractions were assayed for protein content. Triton X-100 and Nonidet P-40 had a higher concentration of protein in their DSM fractions than their DRM fractions, at all detergent concentrations (Fig. 1, D and E). Digitonin had more protein in the DRM fraction than the DSM fraction at lower detergent concentrations, but at 0.5% digitonin, very little protein was found in the DRM fraction (Fig. 1F). Overall, these findings challenge the notion that CL microdomains can be effectively isolated with biochemical detergent extraction. Therefore, we used a more controlled and less invasive model system to study CL microdomains.

The presence of cytochrome c induces proteolipid microdomain formation and morphological changes in GUVs and GMVs

We constructed GUVs, modeling the inner mitochondrial membrane, and GMVs to study CL microdomain organization. GMVs were constructed from native phospholipids extracted from isolated cardiac mitochondria. To visualize GUVs and GMVs, we employed two fluorescent probes, Texas Red DHPE (Texas Red), which is known to report on liquid-disordered domains of lipid membranes (43, 44), and nonyl acridine orange (NAO), which is suggested to report on CL (45, 46). Structures of the fluorescent probes are presented in Fig. S1, A and B.

We first visualized GUVs and GMVs in the absence of cytochrome c (− cyt c) using Texas Red (Fig. 2, A and B, left panels), which revealed a homogenous distribution of the fluorophore throughout the membrane. The addition of 17 μM cyt c to GUVs and GMVs containing Texas Red (Fig. 2, A and B, right panels) revealed fluorophore sequestration and the formation of proteolipid microdomains. The average fluorophore area was calculated using the equatorial cross-sectional view of GUVs and GMVs and was determined to decrease by 1.3–1.4-fold upon the addition of cyt c (Fig. 2, C and D). The formation of phase-segregated domains was accompanied by clear membrane morphological changes with the GUVs and GMVs. To exemplify, the addition of cyt c decreased the average diameter of GUVs and GMVs with Texas Red (Fig. 2, E and F, respectively) by 1.8-fold.

We next visualized vesicles using the fluorescent probe NAO. Similar observations to Texas Red were made when GUVs and GMVs were visualized by NAO. In the absence of cyt c, the NAO fluorophore was homogenously distributed throughout the membrane of GUVs and GMVs, and no microdomains were observed (Fig. 3, A and B, left panels). Upon the addition of 17 μM cyt c, strong phase segregation of NAO (Fig. 3, A and B, middle panels) was observed. Quantification revealed that 17 μM cyt c decreased the average fluorophore
area by up to 2.2–3.5–fold for GUVs and GMVs visualized by NAO (Fig. 3, C and D, respectively). Similarly, the diameter of the GUVs and GMVs also decreased upon the addition of cyt c by 1.5–2.5 (Fig. 3, E and F, respectively). We conducted co-localization analyses to confirm that the phase-separated domains were forming in regions where cytochrome c was associating. These studies revealed overlap (average Pearson's correlation coefficient of 45.1%) between NAO and an Alexa 647 antibody against cytochrome c (Fig. S2).

We also tested if cytochrome c-induced lipid microdomain formation depended on protein concentration. The addition of 3.8 μM cyt c promoted lipid microdomain formation as observed by phase segregation of NAO in GUVs and GMVs (Fig. 3, A and B, right panels). The addition of 3.8 μM cyt c to GUVs and GMVs also decreased the average area occupied by NAO (Fig. 3, C and D) as well as the average vesicle diameter (Fig. 3, E and F). Overall, Texas Red and NAO showed similar results (Figs. 2 and 3); therefore, for the subsequent imaging studies we used NAO given that is suggested to be CL specific (45, 46).

**Figure 1. Detergent extraction is not an effective tool for isolating cardiac mitochondrial CL-enriched domains.** Isolated rat mitochondria were solubilized with (A) Triton X-100, (B) Nonidet P-40, or (C) digitonin at various concentrations (0.01, 0.05, or 0.5% w/v). Images to the right of each plot are representative TLC plates from one experiment. Quantification of protein levels in the DRMs in response to solubilization with (D) Triton X-100, (E) Nonidet P-40, or (F) digitonin at various concentrations (0.01, 0.05, or 0.5% w/v) is shown. DRMs are indicated as the insoluble (I) fraction and DSMs are indicated as the soluble (S) fraction. Data are average ± S.D. from two to three independent experiments.

**Cytochrome c interactions with biomimetic and native mitochondrial lipid monolayers decreases the elasticity modulus**

Given that cyt c decreased the diameters of GUVs and GMVs, we hypothesized that cyt c could be promoting changes in the elastic properties of the membrane. Therefore, we constructed biomimetic and native mitochondrial lipid monolayers, in the absence and presence of cyt c, to quantify the elasticity modulus (C<sub>e</sub>), which is an inverse measure of membrane elasticity. We first present raw surface pressure-area isotherms for both biomimetic (Fig. 4A) and native (Fig. 4B) mitochondrial monolayers in the absence (black lines) and presence (red lines) of 17 μM cyt c. We only tested one concentration of protein because we observed similar effects on microdomain organization with 3.8 and 17 μM cyt c.

The isotherms were used to calculate the elasticity modulus at surface pressures of 30 mN/m. The addition of cyt c to biomimetic (Fig. 4C) and native (Fig. 4D) mitochondrial monolayers decreased the elasticity modulus by nearly...
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2-fold. Therefore, these results demonstrate that cyt c regulates the elastic properties of biomimetic and native mitochondrial monolayers.

Lowering of (18:2)_4CL levels, rather than substitution of (18:2)_4CL with MLCL, disrupts proteolipid domain formation in GUVs

In several pathologies, such as cardiac ischemia-reperfusion injury, type 2 diabetes, and Barth syndrome, there is loss of (18:2)_4CL content and remodeling of CL acyl chains (27, 35, 47, 48).

There is a debate in the literature whether which of these is more consequential (49). Therefore, we studied how a reduction of (18:2)_4CL concentration and CL acyl chain composition influenced lipid microdomain organization in GUVs.

In the absence of cyt c, GUVs containing 20 mol % (18:2)_4CL (Fig. 5A, left panel), a decrease in (18:2)_4CL concentration by 50 mol % (Fig. 5B, left panel), or 15/5 mol % MLCL/(18:2)_4CL (Fig. 5C, left panel) displayed no lipid microdomain formation. This was consistent with our previous imaging experiments in which no lipid microdomains were observed in the absence of cyt c.
The addition of 3.8 μM cyt c to GUVs containing (18:2)4CL (Fig. 5A, right panel) promoted proteolipid microdomain formation. Upon decreasing (18:2)4CL concentration by 50%, we observed two distinct populations. One GUV population displayed proteolipid domains (Fig. 5B(I), right panel) and another group of GUVs that did not display formation of proteolipid microdomains (Fig. 5B(II), right panel) in the presence of cyt c. The distribution of these populations was determined from a total of 155 individual GUVs, where ~56% of GUVs displayed domain formation. Conversely, the other 44% of GUVs showed no clear microdomain formation because NAO was homogeneously distributed throughout the membrane of these vesicles. Strikingly, inclusion of MLCL promoted strong domain formation (Fig. 5C, right panel). Quantification of the GUVs showed that cyt c lowered the fluorophore area in nearly all cases (Fig. 5, D–F). Similarly, the addition of cyt c decreased the diameter of the GUVs (Fig. 5, G–I).

A reduction in (18:2)4CL concentration promotes favorable Gibbs-free energy of lipid mixing

Mechanistically, lipid domains are driven by the Gibbs-free energy of lipid mixing. Therefore, we used pressure-area isotherms to calculate the change in Gibbs-free energy between the absence and presence of cyt c. We first present the raw surface pressure-area isotherms for biomimetic mitochondrial monolayers containing (18:2)4CL (Fig. 6A), 50% depletion of (18:2)4CL concentration (Fig. 6B), or MLCL/(18:2)4CL (Fig. 6C) in the absence (black traces) and presence (red traces) of 3.8 μM cyt c. Secondary analyses revealed that a 50% loss of (18:2)4CL significantly shifted the change in Gibbs-free energy of mixing upon cyt c addition toward a more favorable value by 2.5-fold (Fig. 6D). In contrast, inclusion of MLCL had no effect on the thermodynamics of lipid mixing (Fig. 6D).

Proteolipid microdomain formation is not disrupted in GMVs synthesized using native mitochondrial phospholipids extracted from cardiac tissue of AcsllT−/− mice

To further investigate how CL acyl chain composition regulates proteolipid domain organization, we relied on GMVs constructed from cardiac-specific acyl-CoA synthetase knockout mice (AcsllT−/−). The rationale for this model was that CL acyl chains are notably remodeled, although total CL levels do not change (50). Specifically, previously published LC/MS analyses show that AcsllT−/− mice have increased incorporation of oleic acid (18:1) into CL at the expense of the loss of linoleic acid (18:2) (50).

Upon the addition of 3.8 μM cyt c, proteolipid microdomain formation was observed in GMVs constructed from control (Fig. 7A) and AcsllT−/− (Fig. 7B) mice. Proteolipid domains were similar in size between control and AcsllT−/− GMVs, as measured by the average area occupied by NAO (Fig. 7C). Furthermore, the diameter of the vesicles was also similar between the control and AcsllT−/− GMVs (Fig. 7D). Taken together, these results suggested that the remodeling of tetrailnoeoyl-CL to other acyl chains does not impair the formation of cytochrome c-induced microdomains visualized by NAO fluorescence.

We further investigated how the accumulation of oleic acid in CL regulated proteolipid domain formation. The rationale for using the tetraoleoyl-CL species was that oleic acid is heavily accumulated in CL at the expense of linoleic acid in the murine knockout of AcsllT−/− (50). We specifically constructed biomi-
metic GUVs containing tetraoleoyl-CL ((18:1)₄CL) in the absence and presence of cyt c. In the absence of protein, NAO was homogenously distributed throughout the entire membrane of biomimetic GUVs containing (18:1)₄CL (Fig. S3A). Upon addition of cyt c, NAO was sequestered into localized regions of the membrane (Fig. 3A). Similarly, cyt c-induced proteolipid domains were generally smaller in size, as indicated by a decreased average microdomain area and decreased vesicle diameter (Fig. 3, B and C).

**Lipid microdomains form in the presence of mitochondrial creatine kinase in GUVs**

We subsequently determined if other membrane-associated proteins could promote lipid microdomain formation in biomimetic mitochondrial GUVs. We tested the ability of purified recombinant α-synuclein (Fig. 8A) and mitochondrial creatine kinase (Fig. 8B) to induce proteolipid microdomains in GUVs. We selected α-synuclein (α-syn) because its size is similar to cytochrome c, and mitochondrial creatine kinase (mtCK) because it is previously reported, in simpler model membranes, to bind to CL and promote domain formation (18, 51, 52).

The addition of 3.8 μM α-syn to biomimetic mitochondrial GUVs containing (18:2)₄CL did not promote observable microdomains (Fig. 8A) because NAO was homogenously distributed throughout the entire GUV membrane. In contrast, mtCK promoted strong phase segregation of the NAO probe (Fig. 8B). Quantitatively, there was no change in the average area occupied by NAO for α-syn (Fig. 8C), whereas mtCK lowered the fluorophore area of GUVs (Fig. 8D). The diameter of the GUVs was maintained with α-syn (Fig. 8E) and lowered with mtCK (Fig. 8F).

(18:2)₄CL oxidizes the heme cofactor iron-metal center in cytochrome c more effectively than MLCL

Finally, we investigated if the loss of CL concentration had a stronger effect than MLCL on the oxidation of cytochrome c. The rationale for this study was that MLCL may lack the ability to bind in the proper orientation near redox-sensitive cofactors, resulting in dramatic changes in midpoint potentials and impediments to electron transfer (53, 54). By using UV-visual spectroscopy, we monitored the interaction of (18:2)₄CL (Fig. 9A) and MLCL (Fig. 9B) with the heme cofactors of cytochrome c.
Interactions between CL and the low spin, Fe(II)-heme center of the reduced protein, resulted in oxidation of the metal center to a high spin, Fe(III) center. These changes in cytochrome c’s redox properties were dose-dependent as demonstrated by a blue-shift in the Soret bands (400 nm, data not shown) and decrease in α-heme bands (540–560 nm, Fig. 9).

Plots of the extent of cytochrome c oxidation versus lipid concentration (Fig. 9C) show that a higher concentration of MLCL (closed circles) was needed to fully oxidize cytochrome c relative to (18:2)₄CL (open circles), and a dose-dependent effect of (18:2)₄CL concentration on the redox properties of cyt c.

**Discussion**

**Biochemical detergent extraction is ineffective for isolating CL-enriched domains**

One major advancement from this study was data demonstrating that detergent extraction was ineffective for isolating and studying CL-enriched domains. We investigated the efficacy of three different detergents for extracting CL-enriched domains because CL domains are reported to be isolatable using nonionic detergents at cold temperatures (17). Triton X-100 was used because it is the preferred detergent for lipid domain extraction (55, 56). Nonidet P-40 was selected due to its nonionic nature and digitonin was relied on given its use for isolation of mitochondrial supercomplexes (57). The analyses revealed that detergent extraction displayed no specificity for CL-enriched domains because all phospholipids and proteins were generally solubilized with increasing detergent concentrations. Thus, similar to conclusions made in the study of plasma membrane lipid rafts, detergent was not an effective tool for studying CL domains (58).

**CL-cytochrome c interactions promote domain formation and membrane morphological changes**

Given the limitations of detergent extraction, an imaging approach was used to study mitochondrial inner membrane domains. The novelty of our approach was in the combined analysis of native mitochondrial and biomimetic vesicles that represent or model the phospholipid composition of the inner mitochondrial membrane. The observation that proteolipid
domains form with cyt c, as measured with the fluorescent probe NAO, was in agreement with the literature. For instance, (14:0)4CL in raft/nonraft mixtures promoted phase separation in the presence of dynamin-related protein 1 (22). The observed morphological changes with CL were also consistent with a previous study that suggested interactions between CL and cytochrome c in raft/nonraft mixtures promoted membrane budding and ultimately formation of a folded structure (24). The positively charged NAO was likely interacting with negatively charged regions and thereby promoting the formation of cyt c multimers associated with structural modifications. Alternatively, the binding of NAO to the membranes may be simply a reflection of ion-ion interactions.

Supporting monolayer studies showed that the addition of cytochrome c was disrupting membrane packing accompanied by changes in the elasticity modulus. The morphological changes were of biological significance because CL domains are hypothesized to form in select regions of the membrane, specifically in areas of high membrane curvature (59). For instance, Renner and Weibel (16) reported that CL domains localized in

Figure 8. Lipid microdomain formation depends upon specific lipid-protein interactions. A and B, representative images of biomimetic GUVs in the presence of 3.8 μM (A) α-syn or (B) mtCK. Vesicles were composed of (18:0–22:6)PC/(16:0–20:4)PE/(18:2)4CL/DOPI/DOPS/Chol and were visualized with 0.1 mol % NAO. The average fluorophore area occupied by NAO (C and D) and the average GUV diameter (E and F) were determined for vesicles in the absence and presence of protein. Each dot represents a single GUV. Data are average ± S.D. from a total of 25–90 vesicles analyzed from 3 independent experiments. Asterisks indicate significance from vesicles in the absence of protein: ****, p < 0.0001. Scale bars are 10 μm.

Figure 9. The reduction of (18:2)4CL concentration has a greater effect on cytochrome c oxidation than MLCL. A and B, representative normalized UV-visible absorption spectra displaying the oxidation of the heme cofactor iron-metal center in cytochrome c upon increasing concentrations of (18:2)4CL (A) or MLCL (B). Cytochrome c was fully reduced with the addition of ascorbate (90 mM, solid red line). Full spectra (200–700 nm) were obtained at a pH of 7.4 in the presence of glucose oxidase (15 units), glucose (5 mM), and catalase (5 units) to ensure O2 and H2O2 free conditions. Blue shift of the Soret band at 400 nm (data not shown) and decreases in the α-heme bands at 518 and 550 nm (A and B) are characteristic of oxidation of the Fe(II) center to Fe(III) with increasing lipid concentrations. For comparison, cyt c was fully oxidized after the final addition of lipid using 3 mM K3[Fe(CN)6] (dashed red line). C, the relative change in absorbance at 550 nm, defined as the absorbance at a specific lipid concentration/absorbance of the fully reduced complex, was determined and plotted as a function of lipid concentration (μM). Data are average ± S.D. from three independent experiments.
regions of high curvature induced with microchambers using an *Escherichia coli* model. Thus, proteolipid domains in the inner mitochondrial membrane may be localized to regions of high curvature induced by strong interactions between cytochrome *c* and CL.

We did not observe proteolipid domain formation with the intrinsically disordered protein α-synuclein. The rationale for using α-synuclein was based on evidence that it can interact with mitochondria to diminish complex I activity (60). Furthermore, α-syn is known to bind acidic phospholipids although these interactions have been shown to be significantly augmented by the presence of phosphatidylethanolamine, a neutral phospholipid (61). The results with mitochondrial creatine kinase were consistent with previous literature demonstrating the ability of this protein to promote the formation of proteolipid domains (18, 52). Notably, creatine kinase is also hypothesized to localize to cristae sites where CL is enriched (62, 63).

**A reduction in tetralinoleoyl-CL concentration but not substitution with MLCL prevents proteolipid domain formation through changes in lipid miscibility**

In conditions such as Barth syndrome, cardiovascular diseases, obesity, and diabetes, CL levels are lowered or undergo changes in acyl chain composition. It is hypothesized that such changes may be detrimental to mitochondrial function. To exemplify, in cardiac ischemia-reperfusion injury, total (18:2)₄CL content is selectively depleted (32, 48, 64). Therefore, our data provide a potential mechanism by which mitochondrial function may become impaired. That is, perhaps a reduction in total (18:2)₄CL content disrupts formation of mitochondrial proteolipid domains. Furthermore, the loss of CL in ischemia is also associated with the loss of cyt *c*, which triggers apoptotic signaling cascades (65, 66). Thus, formation of CL-cyt *c* domains may be a critical mechanistic link and perhaps a therapeutic target for preventing the robust apoptosis observed with cardiac ischemia-reperfusion injury (65, 66).

The result that MLCL did not perturb proteolipid domain organization does not preclude other mechanisms by which MLCL may exert its effects on mitochondrial bioenergetics. For instance, MLCL is not as tightly bound as CL to respiratory proteins, which occurs in response to CL degradation in Barth syndrome (39). Furthermore, increasing levels of MLCL are likely to influence membrane properties outside of microdomain formation such as viscosity and permeability. Thus, it is plausible that MLCL could impair respiratory enzyme activities and electron channeling. Future studies will be needed to investigate how MLCL disrupts differing aspects of mitochondrial structural and thereby protein-lipid and protein-protein interactions.

There are several emerging studies suggesting that CL acyl chain composition is not a major driver of mitochondrial membrane structure-function. Our results with MLCL on microdomain formation and cytochrome *c* oxidation status were consistent with these previous studies. For example, in a yeast model, remodeled and unremodeled CL were indistinguishable when analyzing oxidative phosphorylation activity (49). Similarly, CL content varies between differing tissues, which further argues against the notion that CL acyl chain composition is a key regulator of bioenergetics function. In addition, restoration of WT CL levels in differing disease models does not rescue mitochondrial functional responses. For instance, in aging or in a previous study with the heart-specific acyl-CoA 1 synthetase knockout, restoration of (18:2)₄CL did not improve key mitochondrial end points (50, 67).

It is important to point out that in some cases of extreme CL remodeling, the acyl chains may be major drivers of changes in mitochondrial bioenergetics. We previously demonstrated that dietary docosahexaenoic acid promoted robust CL remodeling, which lowered cardiac respiratory enzyme activities through changes in protein-CL binding and formation of membrane microdomains (68).

Although our data suggest that acyl chain composition may not be a major driver of mitochondrial microdomains, additional studies are needed to understand how CL oxidation products influence mitochondrial inner membrane physical properties. The use of the 18:1-containing CL would be a limitation given that it is not prone to generating downstream second messengers. Future studies will need to specifically investigate how oxidation products, which are known to influence bioenergetics, could regulate mitochondrial structure and thereby function (69).

**Conclusion**

The data demonstrate that CL-specific microdomains cannot be effectively isolated via classical biochemical detergent extraction techniques. More importantly, membrane domains were formed in GUVs and newly constructed GMVs with cytochrome *c*. These domains were sensitive to a reduction in CL concentration but not substitution with MLCL. At a molecular level, the impairment in domain formation with the loss of tetralinoleoyl-CL was due to changes in the Gibbs-free energy of lipid mixing. Collectively, the results have strong implications for the ongoing debate about CL concentration and acyl chain composition and their role in regulating membrane biophysical organization.

**Experimental procedures**

**Materials**

(18:1)₄CL, 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC), 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (18:0–22:6 PC), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine (16:0–20:4 PE), 1,2-dioleoyl-sn-glycero-3-phospho-(1’-myo-inositol) (DOPI), 1,2-dioleoyl-sn-glycerol-3-phospho-1'-serine (DOPS), bovine heart cardiolipin ((18:2)₄CL)), bovine heart MLCL, and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). The fluorescent probes Texas Red DHPE and NAO were purchased from Life Technologies. More importantly, mitochondrial functional responses. For instance, in aging or in a previous study with the heart-specific acyl-CoA 1 synthetase knockout, restoration of (18:2)₄CL did not improve key mitochondrial end points (50, 67).

All other reagents and solvents (HPLC grade) were purchased from Sigma or Fisher Scientific. Lipid mixtures were handled under low light conditions and fresh stocks of lipid mixtures were used for each experiment to minimize oxidation.
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Isolation of mitochondria from rodent cardiac tissue

Mitochondrial isolations were performed on ice and all buffers and instruments were pre-chilled to 4°C. All animal experiments were conducted in accordance with guidelines established by the Guide for Care and Use of Laboratory Animals and with prior approval by the University’s Animal Care and Use Committee. Adult male C57BL/6 mice purchased from Jackson Laboratories were housed were housed on a 12:12-h light-dark cycle and had unrestricted access to standard chow and water. Adult male Sprague-Dawley rats (150–250 g) were obtained from Charles River and were housed on a 12:12-h light-dark cycle and had unrestricted access to standard chow and water. Acs1T1–/– mice were maintained as previously described (50). One animal was sacrificed per isolation and cardiac tissue was collected immediately following euthanasia. Mice were anesthetized by isoflurane. Rats were anesthetized with ketamine/xylazine (90 mg/kg of ketamine, 10 mg/kg of xylazine, i.p.) and after the absence of animal eye-blink, toe pinch, and righting reflexes, rats were sacrificed. Cardiac tissue was excised using an extraction buffer containing 25 mM HEPES (pH 7.2), sucrose (300 mM), and EDTA (1 mM). Rodent cardiac tissue was manually minced for 5 min and subjected to homogenization (7 strokes) with a Teflon Potter glass homogenizer. The homogenate was centrifuged at 500 × g for 5 min and the decanted supernatant was centrifuged again at 500 × g for 5 min. The supernatant was centrifuged and centrifuged at 10,000 × g for 15 min, and the resulting pellet was washed with MIM prior to centrifugation at 12,000 × g for an additional 15 min. The resulting mitochondrial pellets were resuspended in either 300 μl of MIM (for rat cardiac mitochondria) or in 150 μl of MIM (for murine cardiac mitochondria) and frozen at −80°C. Total mitochondrial protein concentrations were determined prior to freezing using a BCA protein assay (ThermoScientific, La Jolla, CA).

Extraction of rodent cardiac mitochondrial detergent-resistant (DRM) and detergent-soluble (DSM) membranes and analyses

Experimental procedures were modified from an established protocol for isolating cardiolipin-enriched DRMs (17, 70). Briefly, 1.0 mg of isolated mitochondria was incubated in a Beckman Coulter Centrifuge Tube (35563S) on ice for 10 min using an extraction buffer containing 25 mM HEPES (pH 7.5), 150 mM sodium chloride, 10 μg/ml of aprotinin and various concentrations of Triton X-100, digitonin, or Nonidet P-40 detergents. The solution was centrifuged in an Optima Max-XP Ultracentrifuge with a TLA 100.4 rotor at 20,000 × g for 3 min at 4°C. The detergent-soluble fraction was extracted from the centrifuge tube and the detergent-resistant pellet was resuspended in a solubilization buffer containing 50 mM Tris-HCl (pH 8.8), 1% SDS, and 5 mM EDTA. DNA was sheared from the detergent-resistant fraction by passage through a 1.00-ml syringe and 20½-gauge needle. The fractions were then transferred to a fresh glass tube for lipid extraction. TLC was conducted on DRM and DSM fractions as previously described (53). Protein levels in the fractions were also quantified using a standard BCA assay kit.

Extraction of native phospholipids from isolated rodent cardiac mitochondria

Experimental procedures were modified from the Bligh and Dyer method (71). Frozen aliquots of rodent cardiac mitochondria were thawed and transferred into a glass test tube. For every 10 mg/ml of mitochondria, samples were resuspended in 10 ml of 70/30 (v/v) CHCl3/MeOH (0.05% BHT) solution and vortexed for 45 s. Upon vortex mixing, 2.5 ml of 0.88% KCl was added to each sample and vortexed for an additional 45 s. The resulting mixture was centrifuged at 2000 rpm for 10 min at room temperature (23°C). Upon separation of phases, the bottom layer (organic) was aspirated and dried via a gentle stream of N2 gas. The extract was resuspended in 2.0 ml of chloroform and a second extraction was performed. Upon recovering the chloroform-containing organic layer, the sample was aspirated and dried via a gentle stream of N2 gas prior to being resuspended in HPLC grade chloroform (final concentration 0.1–0.25 μmol/25 μl). Total phosphorous analysis of extracted native mitochondrial phospholipids was conducted as previously described (31).

Construction of GUVs and GMVs

GUVs were constructed by co-dissolving lipids (39.9 mol % 18:0–22:6 PC, 30.0 mol % 16:0–20:4 PE, 20 mol % (18:2), CL, 5 mol % DOPI, 3 mol % DOPS, 2 mol % Chol), and either the fluorescent probe Texas Red DHPE or NAO (0.1 mol %) in chloroform (0.5 mg/ml). The concentration of PC, PE, CL, PI, PS, and Chol approximated ratios from shotgun lipidomic analyses (data not shown) and were consistent with the literature (7). For select GUV experiments in which CL’s content and acyl chain composition was altered, (18:2)2CL concentration was decreased 50% by mass or replaced with MLCL. GMVs were constructed by co-dissolving extracted native mitochondrial phospholipids in HPLC-grade chloroform. Roughly 10 μg of total lipid was spread onto the conductive side of a indium tin oxide-coated glass slide. The lipid-coated slide was subjected to dark vacuum for 1–2 h to remove excess solvent. Once the lipid film was dried, an electroformation chamber was assembled as described (72). GUVs and GMVs were constructed by electroformation at room temperature as previously described (26, 72, 73). Once electroformation was complete, vesicles were extracted from the chamber using a 20½-gauge needle/syringe and allowed to equilibrate briefly prior to microscopy sample preparation. For select experiments, the addition of cyt c (3.8–17 μM final concentration), as well as other proteins, to GUVs and GMVs occurred immediately prior to imaging. Samples were drawn into a rectangular capillary tube (VitroTubes) and mounted onto a glass microscope slide as previously shown (68, 74).

Confocal microscopy and image analysis

Imaging was conducted with an Olympus FV1000 Confocal Microscope using a ×60/1.35NA oil immersion objective (Olympus, Waltham, MA) and a Zeiss LSM 880 with Airyscan using a ×63/1.40NA oil immersion objective (Carl Zeiss
Microscopy, Thornwood, NY). The Texas Red DHPE probe and Alexa 594 secondary antibody were excited by a diode laser at 559 nm, whereas the NAO probe was excited with an argon laser at 488 nm. A diode laser at 633–635 nm excited the Alexa 647 primary antibody used for cyt c immunofluorescence. All acquired images were of GUV equatorial cross-sections. Lipid microdomain and colocalization analysis was conducted using NIH ImageJ software (74). After background subtraction, the average area occupied by the fluorophore was determined by adjusting the image threshold and analyzing particles that were $>0.1 \mu m^2$. We selected this threshold, because an area $<0.1 \mu m^2$ resulted in particles/domains that could not be accurately measured. The average vesicle diameter was determined by measuring the horizontal distance between the membrane edges. For colocalization measurements, we determined the fraction of cytochrome c colocalized with NAO using the Coloc 2 and JACoP plugins. Colocalization was quantified after background subtraction using Pearson’s correlation coefficient.

Proteolipid domains are regulated by CL concentration

Biomimetic mitochondrial monolayers were generated by co-dissolving lipids (40 mol % 18:0–22:6 PC, 30.0 mol % 16:0–20:4 PE, 20 mol % (18:2)4CL, 5 mol % DOPI, 3 mol % DOPS, 2 mol % Chol) in chloroform (10 µg/µl). For select lipid monolayer experiments in which CL’s content and acyl chain composition was altered, (18:2)4CL concentration was decreased 50% by mass or replaced with MLCL. Native mitochondrial monolayers were initially generated by aliquoting native mitochondrial phospholipids (0.25 µmol/25 µl) extracted from murine (C57BL6/J) cardiac tissue. Lipid monolayers were constructed by spotting either 9.0 nmol (biomimetic mitochondrial monolayers) or 6.0 nmol of total lipid (native mitochondrial monolayers) on a subphase of 10 mM sodium phosphate buffer (pH 7.4). Biomimetic and native mitochondrial lipid monolayers were analyzed in the presence and absence of cytochrome c (cyt c; 3.8–17 µM final concentration) by gently injecting protein underneath the subphase immediately after spotting lipid. Excess chloroform was allowed to evaporate for 10 min prior to monolayer compression. Pressure-area isotherms were analyzed at physiologically relevant surface pressures (i.e. 30 mN/m) as previously described (26, 68). Raw isotherms were used to calculate the surface elasticity modulus ($C_s^{-1}$),

$$C_s^{-1} = \frac{(-A)(d\pi/dA)}{A} \quad \text{(Eq. 1)}$$

$A$ represents the mean molecular area of the lipid mixture of interest at the indicated surface pressure ($\pi$) (75). The Gibbs-free energy of mixing ($\Delta G_{mix}$) was calculated by the following relationship to quantify lipid-lipid mixing,

$$\Delta G_{mix} = \Delta G_{ex} + \Delta G_{ideal} \quad \text{(Eq. 2)}$$

$\Delta G_{ex}$ represents the excess Gibbs-free energy of mixing and $\Delta G_{ideal}$ represents the ideal Gibbs-free energy of mixing, which were calculated by the following expressions,

$$\Delta G_{ex} = \int_{A_{12,n}}^a \left[ A_{12,n} - (X_1A_1 + X_2A_2 + X_nA_n) \right] d\pi \quad \text{(Eq. 3)}$$

$A_{12,n}$ and $X_n$ are the mean molecular area and mol fraction of each component, respectively, at a given surface pressure ($\pi$) (76). $\Delta G_{ideal}$ was determined by,

$$\Delta G_{ideal} = RT(X_1\ln X_1 + X_2\ln X_2 + X_n\ln X_n) \quad \text{(Eq. 4)}$$

$R$ represents the ideal gas constant and $T$ is temperature in Kelvin (76). All lipid mixtures were acquired multiple times on differing days to ensure reproducibility.

Overexpression and purification of recombinant α-synuclein

Electrocompetent E. coli BL21 STAR (de3) cells were transformed with expression plasmid pReceiver-B31 (GeneCopeia, Rockville, MD) encoding for the N-terminally His-tagged α-synuclein. Protein was expressed in 20-liter batch cultures of LB media, supplemented with 200 µg/ml of ampicillin for plasmid maintenance, and inoculated with a 1-liter overnight culture of transformed E. coli. Large-scale fermentations were carried out in a 30-liter carboy equipped with an air inlet hose connected to, and fitted with, a gas diffusion stone for proper aeration and agitation of the media during growth and induction. Batch cultures were grown at 37 °C to an $A_{600} = 0.9–1.2$. The culture was then chilled on ice for 20 min prior the addition of isopropyl 1-thio-β-D-galactopyranoside (final concentration 1 mm). Protein expression was induced at 16 °C for ~20–24 h. Cells were harvested via centrifugation, yielding 100–200 × g of cell paste. Cell paste was flash frozen in liquid N2 and stored at −80 °C until needed.

All purification steps were performed at 4 °C. 5–10 ml of nickel-nitrilotriacetic acid resin (ThermoFisher Scientific, Waltham, MA) was equilibrated with wash buffer (20 mM NaPO4, pH 7.5, 300 mM NaCl). Cell paste (20 g) was thawed, while stirring, in 300 ml of lysis buffer (20 mM NaPO4, pH 8.0, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 100 µg/ml of lysozyme) for 30 min. Cells were disrupted via sonication and the lysate was clarified by centrifugation (20 min). Clarified lysate was incubated with equilibrated resin (batch binding) for 2 h while rotating. After supernatant decantation, batch-bound resin was loaded into a glass column and washed with 100 ml of 1.5 M NaPO4 for 20 min. Cell paste was separated from higher molecular weight aggregates using centrifugal spin concentrators (30 kDa MWCO). The resulting flow-through contained the purified protein that was further concentrated (1.5–3 mg/ml) with a 3-kDa MWCO membrane. Protein concentration was determined using a BCA assay (ThermoFisher Scientific). Purified protein was aliquoted, flash frozen using liquid N2, and stored at −80 °C.

Overexpression and purification of human mtCK

The human sarcomeric mtCK localized in skeletal and heart muscle was expressed in bacteria as a tag-free protein and was
purified in a two-dimensional HPLC protocol as previously described (54). Purified protein was aliquoted and stored at −20 °C.

UV-visible spectroscopy and oxidation of cytochrome c

UV-visible spectra of cytochrome c were recorded with a Shimadzu 1800 spectrophotometer fitted with a thermostated cell holder. Stock solutions of proteins, lipids, and buffers/substrates were made fresh prior to use. The 3-ml reactions contained HEPES (30 mM, pH 7.4), catalase (10 μg/ml, glucose (50 μM), glucose oxidase (50 μg/ml), and cytochrome c (5 μM). Initial UV-visible spectra were recorded (200–800 nm) and ascorbate (final concentration 10 μM) was added to completely reduce the cytochrome c. Spectra were recorded every minute for 5 min after the addition of ascorbate to ensure all cytochrome c was reduced. 2–3 μl of 1 mg/ml of solubilized lipid (DOPC, (18:2)₄ CL, and MLCL) was subsequently titrated into the reaction mixture, and after a 3-min equilibration period, spectra were recorded. After all titrations were complete, K₅[FeCN₆] (55 μM, final concentration) was added, and after a 3-min equilibration period, the final spectra of fully oxidized cytochrome c were recorded.

Statistics

Data were analyzed with GraphPad Prism version 7.0. Data were ensured to display normalized distributions. Statistical analyses were conducted using unpaired two-tailed t-tests or one-way analysis of variance followed by a post hoc Bonferroni test. p values less than 0.05 were considered significant. Results are plotted as mean ± S.D. from multiple independent experiments.

Author contributions—E. R. P., U. S., R. A. C., A. J. C., D. A. B., and S. R. S. conceptualization; E. R. P., E. M. S., A. F., S. D., T. N. Z., and A. D. data curation; E. R. P., T. N. Z., and S. R. S. formal analysis; E. R. P. validation; E. R. P., E. M. S., A. F., S. D., T. N. Z., A. D., and S. R. S. investigation; E. R. P., E. M. S., A. F., S. D., T. N. Z., and A. D. methodology; E. R. P. writing—original draft; E. R. P., T. N. Z., U. S., R. A. C., A. J. C., D. A. B., and S. R. S. writing—review and editing; T. N. Z., U. S., R. A. C., and D. A. B. resources; D. A. B. and S. R. S. funding acquisition; S. R. S. project administration.

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15946 J. Biol. Chem. (2018) 293(41) 15933–15946