Nanodomains can persist at physiologic temperature in plasma membrane vesicles and be modulated by altering cell lipids

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Running title: Nanodomains in giant plasma membrane vesicles
Abstract

The formation and properties of liquid-ordered lipid domains (rafts) in the plasma membrane are still poorly understood. This limits our ability to manipulate ordered lipid domain-dependent biological functions. Giant plasma membrane vesicles (GPMVs) undergo large-scale phase separations into coexisting liquid-ordered and -disordered lipid domains. However, large-scale phase separation in GPMVs detected by light microscopy is observed only at low temperatures. Comparing Förster Resonance Energy Transfer (FRET)-detected versus light microscopy–detected domain formation, we found that nanodomains, domains of nanometer size, persist at temperatures up to 20°C higher than large-scale phases - up to physiologic temperature. The persistence of nanodomains at higher temperatures is consistent with previously reported theoretical calculations. To investigate the sensitivity of nanodomains to lipid composition, GPMVs were prepared from mammalian cells in which sterol, phospholipid, or sphingolipid composition in the plasma membrane outer leaflet had been altered by cyclodextrin-catalyzed lipid exchange. Lipid substitutions that stabilize or destabilize ordered domain formation in artificial lipid vesicles had a similar effect on the thermal stability of nanodomains and large-scale phase separation in GPMVs, with nanodomains persisting at higher temperatures than large-scale phases for a wide range of lipid compositions. This indicates it is likely that plasma membrane nanodomains can form under physiologic conditions more readily than large-scale phase separation. We also conclude that membrane lipid substitutions carried out in intact cells are able to modulate the propensity of plasma membranes to form ordered domains. This implies lipid substitutions can be used to alter biological processes dependent upon ordered domains.
Introduction

The conditions under which sphingolipid-and-cholesterol-rich ordered domains form in the plasma membrane of mammalian cells remain unclear. With a few notable exceptions (1, 2), ordered domains remain difficult to directly observe in intact cells. Under many conditions ordered domains may not even be a constitutive feature of the plasma membrane, but rather one induced in the presence of a stimulus (3, 4). Giant plasma membrane vesicles (GPMV) represent a natural membrane system in which the domain-forming properties of plasma membrane lipids and proteins can be investigated much more readily than in intact cells (5, 6). They have the advantage over artificial lipid vesicles that they contain a complex, natural mixture of lipids and proteins. At low temperatures, the lipids in GPMV undergo a phase separation, in which classical, large-scale co-existing Ld and Lo phases form and are easily visualized by light microscopy. These phases have properties very similar to those formed in phase-separating artificial vesicles with simple lipid mixtures in terms of their properties and association with specific proteins. Although large-scale phase separation is only seen in GPMV at lower temperatures, it has been predicted that nanodomains that decrease in size as temperature increases would persist to much higher temperatures in GPMV (7). Consistent with this, in some artificial lipid vesicles nanodomains exist under conditions that large-scale phase separation is not observed, and domain size can decrease as temperature increases (8-10). Using GPMV, studies have shown that modulating the conditions under which cells are grown, modulating cholesterol levels, or adding molecules that can act as anesthetics modulate the stability of ordered domains in GPMV (11-14). Combined, these studies show that GPMV are a highly useful system for examining the principles behind lipid domain formation in natural membranes.

In this report we investigate the relationship between nanodomain and large-scale phase separation in GPMV, and how the formation of both large scale phases and nanodomains is affected by alteration of plasma membrane lipid composition. We find that ordered nanodomains persist to much higher temperatures than large-scale phase separations. Using methods to modify plasma membrane composition we find that both large-scale phase separation and nanodomain thermal stability respond to lipid
composition in a fashion analogous to what has been previously reported for domain formation in artificial vesicles. However, in some cases nanodomains respond to a different extent than large-scale phase separation do. Thus, this study provides novel insights into both the inherent ability of plasma membrane lipids to form ordered nanodomains and how the properties of such domains be altered by lipid substitutions in intact cells.
Materials and Methods

**Materials:** Brain sphingomyelin (bSM), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), cholesterol and 3β-hydroxy-5,24-cholestadiene (desmosterol) were purchased from Avanti Polar Lipids (Alabaster, AL). 5-cholesten-3α-ol (epicholesterol), 5β-cholestan-3β-ol (coprostanol) and 4-cholesten-3-one (cholestenone) were from Steraloids (Newport, RI). 7-dehydrocholesterol (Fluka brand), lanosterol, 1,6-diphenyl-1,3,5-hexatriene (DPH), and methyl-β-cyclodextrin (MβCD) were from Sigma-Aldrich. Methyl-α-cyclodextrin (MαCD) was purchased from AraChem (Budel, the Netherlands). DMEM medium, MEMα medium, fetal bovine serum (FBS), calf bovine serum (CBS), Dulbecco's phosphate-buffered saline (DPBS) (200 mg/L KCl, 200 mg/L KH₂PO₄, 8 g/L NaCl, and 2.16 g/L Na₂HPO₄), penicillin and streptomycin, and trypsin with EDTA, were from Thermo Fisher Scientific (Waltham, MA). Bovine serum albumin (BSA) was from Millipore (Kankakee, IL). Octydecylrhodamine B (ODRB), dithiothreitol (DTT), 4 (w/v) % paraformaldehyde (PFA) and 3, 3’-dilinoleyloxacarbocyanine perchlorate (FAST DiO) was from Invitrogen. Agarose (AMRESCO 0710) was from Cole-Palmer Scientific (Vernon Hills, IL). Glass bottom four compartment Cell View cell culture dishes were from Greiner Bio-One (Germany). 10 x 10 mm microscope slide coverslips were from TED PELLA, INC. (Redding, CA). Lipids were stored at -20 °C. PFA and DTT were stored as small aliquots at -20 °C to avoid thaw-freeze cycles. Sterol purity was confirmed by TLC as previously(15).

**Cell Culture:** Rat basophilic leukemia (RBL-2H3) cells were a kind gift from Dr. Barbara Baird (Cornell University). Chinese hamster ovary (CCL-61 CHO K-1) cells were obtained from the American Type Culture Collection (Manassas, VA). RBL-2H3 cells were grown in DMEM medium supplemented with 10% FBS (v/v) and 100 U/ml penicillin and streptomycin. CHO cells were grown in MEMα medium with 10% CBS (v/v). All cells were maintained in a humidified incubator with 5% CO₂ at 37 °C.

**Lipid/Sterol Substitution in Cultured Cells:** Cells were seeded 1 day prior to GPMV formation and/or lipid/sterol substitution, and were 80-90% confluent the next day. Cells were seeded on 100 mm
dishes for FRET experiments and on 35 mm dishes for microscopy imaging. Lipid-loaded MαCD and sterol-loaded MβCD solutions were prepared and lipid/sterol substitution carried out similarly to as described previously (15, 16). Lipid-loaded MαCD and sterol-loaded MβCD solutions were prepared 1-day prior to use. Phospholipids or sterols were dried under nitrogen followed by high vacuum for at least 1 h. They were then hydrated with 40 mM (RBL-2H3) or 50 mM (CHO cells) MαCD for phospholipids, or 2.5 mM MβCD, for sterols, in serum-free DMEM in a 70°C water bath for 5 min. Concentrations after hydration were 1.5 mM bSM (RBL-2H3 cells) or 2 mM bSM (for CHO cells), 3 mM POPC, 0.1 mM cholesterol or 0.4 mM sterol (7-dehydrocholesterol, 4-cholesten-3-one, coprostanol, epicholesterol, desmosterol, lanosterol or cholesterol). Sterol-loaded MβCD solutions were then sonicated (Cole-parmer ultrasonic cleaner, IL) for 5 min until no visible particles remained. Next phospholipid-loaded MαCD or sterol-loaded MβCD solutions were incubated at 55°C for 30 min and then at room temperature (for phospholipid-loaded MαCD solutions) or at 37°C (for sterol-loaded MβCD solutions) overnight. 10 mM MβCD in serum-free media (cholesterol depletion) was also prepared using the protocol for sterol-loaded MβCD solutions. For lipid/sterol substitution, RBL-2H3 cells were washed twice with 10 ml (100 mm dishes) or 2 ml (35 mm dishes) DPBS, and then 2 ml (100 mm dishes) or 0.5 ml (35 mm dishes) of the prepared phospholipid or sterol-loaded CD solution was added.

The dishes were then incubated at room temperature for 1 h (for phospholipid-loaded MαCD solutions) or at 37°C for 2 h (for sterol-loaded MβCD solutions) while gently shaken. Next, cells were washed by DPBS once, and 10 ml (100 mm dishes) or 2 ml (35 mm dishes) complete culture media was added. Samples were then placed in a 37°C incubator for 2 h recovery. Phospholipid and sterol substituted-cells were used immediately for GPMV formation.

**GPMV Formation/Isolation:** Giant plasma membrane vesicles (GPMV) were prepared following previous protocols (17). Cells that had been treated as above, or untreated controls were used. PFA and DTT were freshly added to 10 mM HEPES, 150 mM NaCl, 2 mM CaCl₂, pH7.4 at a final concentration 25
mM PFA and (unless otherwise noted) 2 mM DTT. We define this as GPMV buffer. GPMV were
isolated from unsubstituted, lipid-substituted and sterol-substituted cells. Cells were first washed twice
with DPBS and then twice with GPMV buffer lacking PFA and DTT. Next 2.7 ml (in 100 mm dishes, for
FRET experiment) or 0.5 ml (in 35 mm dishes for microscopy) of GPMV buffer was added and incubated
at 37 °C for 2 h, sometimes with gentle shaking. Shaking was found to be necessary for CHO cells. The
GPMV-containing supernatant was gently harvested by pipetting to avoid removal of intact cells, and
then centrifuged at 100 x g for 5 min to remove contaminating cells, which pelleted. The supernatant from
this centrifugation was used for the FRET measurements. For microscopy, the supernatant was
centrifuged a second time at 500 x g for 10 min to concentrate the GPMV. For microscopy imaging, the
final GPMV pellet, which cannot be seen, plus a small amount of solution (10-20 μl) above the pellet,
was resuspended in GPMV buffer. GPMV were used for FRET or microscopy on the same day as they
were prepared.

In experiments to separate larger from smaller GPMV from RBL-2H3 cells, a second centrifugation
was performed at 500 x g for 10 min. The pellet and supernatant were collected separately. The pellet
contained GPMV that were large enough to see by microscopy. The supernatant contained
submicroscopic vesicles. Lipid concentration in the preparations and vesicle fraction was measured with
the DPH assay. About 60-70% of total GPMV lipid was in the submicroscopic fraction. FRET vs.
temperature was then measured for the total GPMV, large GPMV and small GPMV fractions as described
above.

**Estimation of GPMV Lipid Concentration by DPH:** Lipid concentration in GPMV for FRET
experiments was estimated by measurement of DPH fluorescence under conditions in which DPH was in
excess (18, 19). A 1 mM lipid stock solution of lipid vesicles containing 1:1 bSM:POPC were prepared to
construct a standard curve. To do this 0.5 μmol bSM and 0.5 μmol POPC were mixed and then dried
under nitrogen followed by high vacuum for 1 h. Then, the lipids were dissolved in 30 μl ethanol and then
970 μl phosphate-buffered saline (PBS) (1 mM KH₂PO₄, 10 mM Na₂HPO₄, 137 mM NaCl, and 2.7 mM KCl (pH 7.4)), and incubated at 70°C (in a water bath) for 5 min. 5 μl of 0.2 mM DPH/ethanol was added to either an aliquot from the GPMV stock solution (700μl - 1ml) or a range of vesicle aliquots from the 1 mM bSM/POPC vesicle stock solution after dilution to 700μl - 1 ml with GPMV buffer. After incubation for 5 min at room temperature in the dark the intensity of DPH fluorescence as a function of lipid concentration was measured at excitation 358 nm and emission 430 nm. Generally, GPMV from RBL-2H3 cells contained 15-25 μM lipid and had a volume about 2.7 ml when isolated from one 100 mm plate. GPMV lipid concentration prepared from CHO cells contained 4-6 μM and had a volume of 2.5 ml when isolated from one 100 mm plate.

**Artificial Lipid Vesicles for FRET Experiments:** Multilamellar vesicles (MLVs) were prepared containing 1 μmol of lipid (2:1 DOPC:CHOL, 2:1 POPC:CHOL, 1:1:1 bSM: DOPC:CHOL or 1:1:1 bSM: POPC:CHOL, all mol:mol). The lipids were mixed and then dried under nitrogen followed by high vacuum for 1 h. Then lipids were dispersed at 70°C (in a water bath) for 5 min in 1 ml PBS, mixing in a multitube vortexer (VWR International) while incubating at 55°C for 15 min. Then to prepare large unilamellar vesicles (LUVs) from the MLVs, the MLVs were subjected to seven cycles of freezing using a mixture of dry ice and acetone followed by thawing to about room temperature using warm water, and then passed through 100-nm polycarbonate filters (Avanti Polar Lipids) 11 times. Samples were diluted to 200 μM with PBS, and transferred to 12-well cell culture plates before addition of FRET probes, incubation and fluorescence measurements analogous to those using GPMV (see below).

**Measurement of the Temperature Dependence of FRET:** FRET measurements were made with DPH as the FRET donor and ODRB as the FRET acceptor. The “F sample” with FRET acceptor was prepared by adding 10 μl from a 500 μM ODRB dissolved in ethanol to 1 ml of GPMV or lipid vesicles that had been transferred to 12-well Greiner bio-one polystyrene cell culture plates, and after mixing...
incubating at 37°C for 1 h in the dark. The “Fo sample” lacking FRET acceptor was also incubated at 37°C for 1 h. (It did not seem to matter whether 10 μl ethanol was added or not (data not shown).) Before fluorescence measurements, DPH (1.8 μl from a 3.4 μM stock solution in ethanol) was added to both the “F sample” and “Fo sample” and incubated at room temperature for 5 min in the dark. Background fluorescence before adding DPH was negligible (<1% of samples containing DPH). To initiate measurements, samples were placed in a cuvette were transferred to a temperature controlled sample spectrofluorimeter (Horiba PTI Quantamaster) sample holder and cooled to 16°C, and DPH fluorescence (excitation 358 nm, emission 430 nm, and using 10 nm bandpass slits) was measured after increasing temperature at 4°C intervals up to 44°C (for GPMV) or 64°C (for lipid vesicles). Between readings temperature was increased at a rate of 4°C/5 min. The ratio of DPH fluorescence intensity in the presence of ODRB to that in its absence (F/Fo) was then calculated. T_{end}, the measure of the temperature above which segregation of lipids into ordered and disordered domains was fully lost (i.e. the “melting” of ordered domains is complete) was estimated by finding the minimum value of a polynomial fit applied to the normalized F/Fo using Excel software (Microsoft Corporation, Redmond, WA) and utilities available at www.wolframalpha.com. (The minimum F/Fo represents T_{end} because in the absence of domains there is a small decrease in FRET vs. temperature, i.e. F/Fo increases as temperature is increased (20, 21).

Control experiments showed that the amount of ODRB bound to GPMV (as judged by pelleting of GPMV and GPMV-bound ODRB) was almost identical at 16°C and 40°C, showing the changes in FRET at different temperatures did not reflect changes in binding to GPMV (data not shown).

**Labeling of GPMV for Microscopy.** RBL-2H3 GPMV samples for light microscopy were either labeled before GPMV were prepared from cells, or after GPMV were isolated from cells. CHO GPMV were only stained after isolation from cells (since CHO cells were too sensitive to incubation in DPBS). For labeling before GPMV preparation, cells (untreated, phospholipid-substituted, or sterol-substituted) were washed once with DPBS, and after removing the DPBS by aspiration 5 μg/ml dye FAST DiO
(diluted into DPBS from a 500 μg/ml stock solution in ethanol) in 1 ml DPBS was added to the cells and incubated at 4°C for 10 min. After removal of dye-containing solution by aspiration, the cells were washed 5 times with DPBS and once with GPMV buffer lacking PFA and DTT. Then GPMV were prepared as described above. For labeling after GPMV preparation, to 100-500 μl of untreated, phospholipid-substituted, or sterol-substituted GPMV was added 5 μg/ml FAST DiO or 5 μM ODRB (diluted 100-fold from their stock solutions in ethanol), and then incubated at room temperature for 30 min. The labeled GPMV were pelleted by centrifugation under 500 x g for 10 min and after removal of the supernatant re-suspended in GPMV buffer for microscopy imaging.

**GPMV Imaging:** 70 μl of GPMV were placed in each chamber of a glass bottom 4 compartment Cell View cell culture dish and each chamber was then covered with 10 mm microscope slide coverslip slightly trimmed to fit in the dish. Each chamber was sealed with agarose (AMRESCO). Chamber temperature was controlled with CL-100 cooling system (Warner Instrument). An AMG EVOS fluorescence microscope (Thermo Fisher Scientific) was used to examine the samples and take pictures of the GPMV. Pictures of GPMV were taken over a range of 2-30°C at intervals of 4°C, incubating 5 min after the sample reached the desired temperature prior to taking pictures. Microscopy was performed the same day GPMV were prepared, although no difference was seen when GPMV were stored for up to 2d at 4°C (data not shown). For each experiment, at the temperatures at which some but not all GPMV had domains, the domain status of 50 GPMV was measured for three separate fields. The results shown are for three experiments, unless otherwise noted. To define the transition from temperatures at which domains form to those at which they do not form, the fraction of GPMV with domains was measured as a function of temperature, fit to a sigmoidal curve (Slidewrite, Advanced Graphics Software, Encinitas, CA), and then T mid, the value at which 50% of the GPMV contained domains, and T end, which was defined as the point at which <1% of the GPMV contained domains calculated.
**TLC Analysis of GPMV Lipids:** Unsubstituted, lipid-substituted, or sterol-substituted GPMV were concentrated by centrifugation at 20,000 x g at 4°C for 30 min, and then the lipids in the pellet (about 80% yield of total lipid) were extracted by adding 3:2 (v:v) hexanes/isopropanol. The organic solvent was dried under N₂ gas and lipids were dissolved in 1:1 chloroform/methanol for loading onto TLC plates. Lipids were chromatographed in 54:18:18 (v:v) chloroform/methanol/acetic acid and detected as described previously(16).

**Results**

**Assays for Domain Formation**

Co-existing large liquid ordered (Lo) and liquid disordered (Ld) domains/phases in GPMV can be visualized by light microscopy. Large Ld domains can be detected as regions enriched in a fluorescence probe that partitions favorably into Ld domains, while Lo domains appear depleted in that type of fluorescent probe. GPMV are heterogeneous, and the parameter conventionally used to estimate domain levels is the fraction of GPMV containing co-existing ordered and disordered domains (22). When temperature is increased, the fraction of GPMV with domains large to visualize decreases from near 100% to 0%. A transition midpoint temperature (T mid or T misc) was defined as the temperature at which 50% of GPMV show co-existing ordered and disordered domains (22). The transition endpoint (T end) was defined as the temperature above which <1% of GPMV contained ordered domains.

To detect nanodomains in GPMV FRET was used. A FRET assay developed to detect ordered domains in bacteria (1), using DPH as donor and ODRB as acceptor, was adapted to use in GPMV. In membranes with co-existing ordered and disordered domains, there is partial segregation of DPH (which tends to equally partition between ordered and disordered domains (23)) from ODRB (which localizes preferentially in disordered domains (24)). As a result, the fraction of DPH fluorescence not quenched by the FRET acceptor, which equals the ratio of DPH fluorescence in the presence of ODRB to that in the absence of ODRB (F/Fo), is elevated relative to F/Fo in membranes fully in the Ld state. (The fraction of
FRET equals the fraction of fluorescence quenched, 1-F/Fo.) Samples with co-existing domains at lower temperatures generally show a marked decrease in F/Fo at elevated temperatures. The reason is that the average distance between DPH and ODRB decreases when Lo domains disappear as they melt/mix with Ld lipids at elevated temperatures. (Since Lo domains are ordered, we refer to the order to disorder transition associated with miscibility at high temperature as a melting transition.)

To test the ability of this assay to detect ordered domain formation, FRET was measured in artificial lipid vesicles containing mixtures of SM, PC, and cholesterol known to form co-existing Lo and Ld domains (25, 26)(Supplemental Figure S1, diamonds, squares). As expected in such samples F/Fo was elevated at low temperature and decreased at elevated temperatures. In control Ld state vesicles in which lipids are homogeneously mixed F/Fo was largely temperature independent (Supplemental Figure S1, triangles, circles).

**Phase Separation and Nanodomain Formation In GPMV from RBL-2H3 Cells**

GPMV were prepared from RBL-2H3 cells using the DTT/PFA protocol and domain formation examined by microscopy and FRET. In agreement with prior studies (22) Figure 1A and the examples of GPMV in Supplemental Figure S2 show that GPMV formed co-existing large Lo and Ld phases at low temperatures, and these phases disappeared as temperature was increased. Both T mid and T end increased as the concentration of DTT used to prepare the GPMV was increased, again in agreement with previous studies (22). The transition midpoint temperature was as high as 19°C and the transition endpoint was as high as 25°C when GPMV were prepared with 5 mM DTT (Table 1). At the widely used concentration of 2 mM DTT, the transition midpoint temperature was 15°C and the endpoint was 23°C. These values are close to those reported previously (22).

FRET showed behavior similar to that measured by microscopy in the sense that higher DTT levels resulted in increased formation and increased thermal stability of ordered domains as measured by T end (Figure 1B, Table 1). (FRET curves were too incomplete to estimate T mid.) T end measured by FRET
was much higher than that detected by microscopy, as high at 38°C with 2-5 mM DTT. This shows nanodomains persist to much higher temperatures than large-scale phase separation.

Control experiments showed that the difference between domain stability by microscopy and FRET did not reflect use of a different fluorescent probes in the two methods. When ODRB was used in microscopy experiments to stain Ld domains, large-scale phase separation appeared to be, if anything, slightly less thermally stable, than when detected by FAST DiO (Supplemental Figure S3A). It should be noted that under some conditions, illumination can induce large domain formation (27), but large-scale phase separation observed were present as soon as samples were visualized, indicating that illumination was not a factor in their formation.

We found that the GPMV preparations contained some sub-microscopic vesicles. FRET values for entire GPMV preparations and sub-microscopic vesicles in the GPMV preparations were very similar to that for the (more easily pelleted) large GPMV (Supplemental Figure S3B). This shows that the difference between T end by microscopy and FRET did not reflect a difference in physical properties of larger and smaller vesicles.

The formation of both large-scale phase separation and nanodomains in these samples involved an equilibrium process. For phase separation, this was shown by the observation that changes in domain formation were reversible upon cooling and upon reheating. However, the transition temperature decreased a few degrees during cooling and during a second reheating step (Supplemental Figure S3C). The transition temperature was also not affected significantly if samples were visualized up to at least two days after GPMV were prepared (data not shown). FRET changes vs. temperature were also reversible, being almost superimposable with the original temperature scans, although a small decrease in thermal stability might have occurred after one round of heating and cooling. (Supplemental Figure S3D).

**Effect of Altering Phospholipid/Sphingolipid Composition Upon Domain Stability**

In previous studies (16, 19) we used cyclodextrins (CDs) to manipulate plasma membrane phospholipid and sphingolipid composition in a fashion that should stabilize or destabilize domain
formation (as predicted by the effect of lipid composition upon domain formation in artificial vesicles). To investigate whether changing lipids in cells affects plasma membrane domain formation, GPMV were prepared from RBL-2H3 cells in which phospholipid composition had been altered by incubation with MαCD/lipid mixtures under conditions that result in nearly complete replacement of endogenous outer leaflet phospholipid and sphingomyelin with exogenous lipid (16, 19). (MαCD does not carry sterols, and so does not perturb cell sterol content (28).) Figure 2A and Table 2 shows that replacement of plasma membrane outer leaflet lipids with SM prior to GPMV preparation stabilized large-scale ordered domain formation in GPMV while substitution with the unsaturated lipid POPC destabilized it, as predicted from the effect of these lipids upon domain formation in artificial lipid vesicles. These results are consistent with previous studies showing that the stability of large scale domain formation in GPMV from cells grown at different temperatures is modulated in a fashion that likely reflects changes in lipid composition(13). Analogous effects of lipid substitution on nanodomain formation were observed by FRET (Figure 2B and Table 2). TLC of the lipids of GPMV prepared from cells before and after phospholipid/sphingolipid exchange confirmed PC/SM ratio was altered as expected (Supplemental Figure S4). Omitting the 2h recovery step in complete serum-containing medium after the lipid exchange using MαCD/lipid mixtures did not greatly affect large-scale domain formation (Supplemental Figure S5). This indicates that the changes in domain-forming properties after exchange are stable for hours after the exchange step. This is not a surprise, as these types of changes in lipid composition reverse only slowly after lipid exchange (16, 19).

**Effect of Altering Sterol Composition Upon Domain Stability**

Experiments were also carried out to determine if sterol (or steroid if the molecule lacks a OH group) substitution altered GPMV ordered domain formation in a fashion similar to which it affects ordered domain formation in artificial lipid vesicles. Previous studies by our group (20, 22, 29-31) and others (32, 33) have shown that sterols can stabilize or destabilize ordered domain formation in artificial vesicles depending on sterol structure. Sterol substitution in cells using sterols with a range of domain-
stabilizing or destabilizing properties has been used in many studies to determine if membrane domain formation influences a biological process (15, 34). Sterol substitution was carried out as previously (15), treating RBL-2H3 cells with sterol-loaded MβCD at cyclodextrin concentrations too low to bind phospholipid (35). We previously found this leads to efficient replacement of endogenous cholesterol with exogenous sterol (15). GPMV were prepared from the sterol-substituted RBL-2H3 cells. Figure 3A and 3C and Table 2 shows that sterol substitution altered the thermal stability of large-scale phase formation. Relative to untreated cells largest reductions in large-scale domain stability were observed with 4-cholesten-3-one and coprostanol, previously shown to strongly inhibit ordered domain formation (20, 29). Substitution with most other sterols tested (desmosterol, lanosterol, and epicholesterol) or simple depletion of cholesterol decreased the thermal stability of large-scale domain formation to a moderate degree. Exchange using cholesterol-loaded MβCD, which can increase cholesterol membrane concentration (15), or with 7-dehydrocholesterol stabilized large-scale domain formation. It should be noted that the effects of increasing or decreasing cholesterol levels in GPMV upon large scale phase separation are in agreement with previous studies (11, 12).

The effect of sterol substitutions upon nanodomain formation was also examined (Figure 3B and 3D and Table 2). The pattern observed for the various sterols was similar to that for large-scale domain formation, but also showed some important differences. Unlike what was observed for phase separation, epicholesterol destabilized nanodomain formation much more than lanosterol and desmosterol, and to an extent similar to the destabilization observed with cholestenone and coprostanol. Also, cholesterol replacement restored T end to values similar to that before cholesterol depletion, but did not stabilize nanodomain formation relative to GPMV from untreated cells, unlike its effect upon T end for large-scale phase separation. It should be noted that differences between the lipid-dependence of nanodomain and large-scale phase separation can also be seen in artificial lipid vesicles in which different sterols have been incorporated (29, 30, 33). Interestingly, the order of sterol substitution effects on nanodomain stability (but not phase separation) is very close to that observed for these sterols when FRET-detected
ordered domain stability is measured in asymmetric artificial lipid vesicles (20), and to the order in which these sterols support endocytosis (15).

**GPMV From CHO Cells Show Similar Stability Patterns as Those From RBL-2H3 Cells**

To see if GPMV properties and response to lipid changes were specific to RBL-2H3 cells, some experiments were repeated in CHO cells. Large phases (Figure 4A) and nanodomains (Figure 4B) were also observed in GPMV prepared from CHO cells. They were slightly more stable than those formed by RBL-2H3 cells (Figure 2A). The effect of lipid substitution upon the stability of nanodomain formation was also examined in CHO cells (Figure 4B), and again nanodomain stability was slightly greater than in RBL-2H3 cells. In addition, substitution affected both large-scale phase separation and ordered nanodomain formation as expected, with decreased stability upon substitution with POPC, and increased stability upon substitution with SM. Note that the apparent T end for CHO cells appears a bit higher that for RBL-2H3 cells (compare Figure 2 and 4). This is consistent with the higher fraction of SM relative to PC in CHO cells relative to RBL-2H3 cells (Supplemental Figure S4).

**Relationship Between Large-scale and Nanodomain Transition Temperatures**

Figure 5 illustrates the relationship between T end for large-scale phase formation and nanodomain formation in RBL-2H3 cells under the various experimental conditions studied here. There is a significant correlation between T end values for phase and nanodomain stability ($r^2=0.60$) with T end being between about 15-25°C higher for nanodomains than for large-scale domain formation. The lipid dependence of the difference between these parameters suggests that although the two parameters are related, they sometimes respond differently to changes in lipid structure.
Discussion

Nanodomain and Large Domain Formation and Properties in GPMV

The primary conclusions of this report are that: 1. ordered lipid nanodomains are present in GPMV at temperatures well above those in which there is large-scale phase separation; 2. alteration of plasma membrane lipid composition and properties in intact cells results in corresponding changes in the composition and properties of the GPMV prepared from the cells; and 3. altering GPMV lipid composition sometimes alters nanodomain thermal stability differently than that of large phase separation.

Effect of Lipid Substitution upon Nanodomain Formation

Changing plasma membrane lipid composition affected the thermal stability of ordered domains in GPMV prepared from cells subjected to lipid exchange. The changes in stability as a function of lipid composition were similar to the effect of the same changes in lipid composition upon the stability of ordered domains in asymmetric artificial lipid vesicles(21). In addition, sterols found to stabilize or destabilize ordered domain formation in asymmetric lipid vesicles had the analogous effect upon ordered nanodomain stability in GPMV (20).

The effect of sterol structure upon nanodomains matches the pattern for the effect of sterol structure upon endocytosis (15). One caveat is that there can be differences in the extent of exchange for different sterols (15) which complicates quantitative comparison of the extent of altered stability in GPMV to that of artificial vesicles with well-defined compositions. Nevertheless, the overall pattern, shows that the physical principles controlling ordered domain formation by lipids in simple bilayers (at least when they have asymmetry mimicking that in cells) are likely to apply to the more compositionally complex lipid bilayers in natural membranes.
Nanodomain Formation and Stability in GPMV

The observation that nanodomains, here meaning regions of distinct lipid composition and/or properties that are in the nanometer size range, can exist under conditions that large-scale domains are not stable is consistent with the behavior of lipids in artificial lipid vesicles. Conditions under which nanoscale domains form but there is no large-scale phase separation have been reported for a number of lipid mixtures (8-10). The reasons that nanodomains might not merge into larger domains has been much discussed in the literature, although no consensus has been reached (9, 36-38). It has been proposed in one study that whatever factor opposes the merger of nanodomains becomes predominant when line tension between Lo and Ld domains falls below 0.3 pN (9, 37).

It has also been found by FRET that some (but not all) lipid mixtures show a relatively gradual temperature dependent decrease in ordered domain size as temperature is increased (8). A decrease in domain size as temperature is increased has also been observed as a lipid mixture approaches a critical point, and has been proposed that transient sub-microscopic domains exist above the critical point, including in the case of GPMV (7, 36). The prediction for the temperature dependence of domain size above the critical point temperature is in good agreement with our experimental results. It has been proposed that domains of 22nm size would be present at 37°C in GPMV, 14°C higher than the critical temperature, which would be close to T mid. The slightly higher values observed for the persistence of nanodomains above the critical temperature (15-25°C) may reflect the ability of FRET to detect domains smaller than 22 nm in size. Roughly speaking FRET can detect domains larger than Ro for the FRET donor-acceptor pair being used, about 4 nm for the FRET probes used in this report (8).

It should be noted that if large scale phases collapse and are replaced by nanodomains whenever line tension at domain boundaries drops below a critical value, then nanodomains could exist for different reasons in membranes with different lipid compositions. In the case of membranes that are approaching the critical point temperature, ordered and disordered domain compositions and properties approach each other, and thus line tension at domain boundaries may fall below the value allowing large scale phases to form. Thus, nanodomains might form before the critical point temperature is reached. In other words,
whereas some lipid mixtures may have low line tensions at domain boundaries over a wide range of compositions, others may only have low line tensions at domain boundaries near critical points.

**Biological Implications**

The observation that as temperature is increased large-scale domain formation disappears in GPMV well before nanodomains suggests that in biological membranes nanodomains are more likely to form than large-scale domains. The observation that altering lipids in intact cells by exchange not only alters GPMV lipid composition, but also GPMV properties, shows that when plasma membrane lipid composition is modulated by exchange the capacity of the plasma membrane to form domains is also likely to be modulated. Even in the absence of detailed measurement of lipid composition changes, the extent to which nanodomains are altered in GPMV by lipid exchange should be a useful measure of the extent to which the capacity to form domains in intact cells is altered by lipid substitution. Extrapolating from the effect of lipid composition upon large-scale phase separation to lipid behavior in cells may be less accurate, since some of more biologically-relevant lipid compositions can form nanodomains but lack the capacity to form large-scale phase separations, even in artificial lipid vesicles (10). Presumably, some factors that influence the ability of nanodomains to form differ from the factors that allow nanodomains to merge into larger domains.

Furthermore, the results of this study also show that the change in both plasma membrane composition and behavior persists for hours after the lipid exchange step. This means that experiments on plasma membranes carried out hours after a lipid substitution step should still reflect the effects of the lipid substitution. This should enhance the utility of lipid exchange as a method to probe the functions of membrane domains *in vivo*.

On the other hand, in view of the highly perturbed structure of GPMV it must be kept in mind that the fact that nanodomains persisted to physiological temperature in these studies does not mean that they are present in intact cells. This perturbation includes changes in the lipid asymmetry found in plasma membranes. At present, it is known at least some of the asymmetry of phosphatidylserine is lost in
GPMV(39). In addition, it is not clear if GPMV lipids represent a biased subset of plasma membrane lipids. Experiments using milder methods to prepare GPMV, and experiments in intact cells, will be needed to further investigate these issues.

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Table 1: T mid and T end values for large-scale phase separation and nanodomain formation for RBL-2H3 GPMV prepared at different DTT concentrations. The data was fit a sigmoidal curve of % GPMV with domains vs. temperature, assuming the limit at extremely low temperature would equal 100% of vesicles containing domains. T mid was defined as temperature at which 50% of the vesicles would have domains, and T end the temperature above which less than 1% of vesicles contain domains. Mean values and standard deviations from results from three preparations are shown.

| DTT conc. (mM) | Phase Separation | Nanodomains |
|---------------|------------------|-------------|
|               | T mid            | T end       | Tend        |
| 0             | 0.5 ± 2.0        | 5.4 ± 2.4   | 26.7 ± 2.1  |
| 0.5           | 10.6 ± 2.5       | 15.1 ± 3.9  | 31.4 ± 2.5  |
| 1             | 12.5 ± 2.0       | 19.2 ± 3.6  | 34.6 ± 1.4  |
| 2             | 14.8 ± 0.7       | 22.7 ± 0.4  | 38.2 ± 1.1  |
| 5             | 19.1 ± 0.9       | 24.7 ± 5.0  | 37.4 ± 1.5  |
Table 2: T mid and T end values for large-scale phase separation and nanodomain formation for RBL-2H3 GPMV prepared using 2 mM DTT after different substitutions or additions. Mean values and standard deviations from results from three preparations are shown, except for untreated samples (samples without any lipid substitutions) for which n=9.

| Substitution        | Phase Separation | Nanodomain Formation |
|---------------------|------------------|-----------------------|
|                     | T mid            | T end                 | Tend                  |
| Untreated           | 12.2 ± 1.3       | 18.6 ± 2.1            | 38.5 ± 2.4            |
| POPC                | 4.4 ± 1.9        | 7.6 ± 1.6             | 33.3 ± 2.1            |
| SM                  | 20.1± 1.3        | 25.9± 2.4             | 42.5± 1.1             |
| Coprostanol         | 2.6 ± 1.1        | 7.6 ± 3.3             | 32.9 ± 2.1            |
| 4-Cholesten-3-one   | -1.3 ± 0.8       | 11.1 ± 1.1            | 32.9 ± 2.7            |
| Desmosterol         | 10.1± 1.5        | 14.9± 2.1             | 37.1± 3.5             |
| Epicholesterol      | 10.9± 1.5        | 18.0± 2.7             | 31.4± 0.2             |
| Lanosterol          | 11.5± 1.8        | 18.5± 3.9             | 34.5± 1.8             |
| 7-Dehydrocholesterol| 21.9± 1.5        | 25.7± 1.8             | 44.1± 2.9             |
| Cholesterol depleted| 2.9± 2.5         | 17.8± 1.8             | 30.3± 1.4             |
| Cholesterol loaded  | 16.8± 1.2        | 20.5± 3.6             | 37.1± 1.3             |
| (0.1 mM)            |                  |                       |                       |
| Cholesterol loaded  | 19.7± 2.8        | 24.1± 2.4             | 38.8± 2.2             |
| (0.4 mM)            |                  |                       |                       |
Figure 1: Temperature Dependence of Phase Separation and Nanodomain Formation in RBL-2H3

**GPMV: Effect of DTT concentration.** A. Phase separation assayed by fluorescence microscopy. B. Nanodomain formation assayed by FRET. Samples for microscopy contained FAST DiO. Samples for FRET contained DPH and ODRB. GPMV prepared with DTT/25 mM PFA. F/Fo values are normalized to 1 at the highest temperature at which measurements made (44°C). Unnormalized F/Fo values 44°C for this and later figures are shown in Supplemental Table S1. Symbols: (Diamonds) no DTT; (squares) 0.5 mM DTT; (triangles) 1 mM DTT; (circles) 2 mM DTT; (crosses) 5 mM DTT. In this and following figures mean values and standard deviations (up bars only) for n=3 are shown, unless otherwise noted.
Figure 2: Effect of Phospholipid Exchange in Cells Upon Phase Separation and Nanodomain Formation in RBL-2H3 GPMV: A. Phase separation assayed by fluorescence microscopy. B. Nanodomain formation assayed by FRET. F/Fo values are normalized to a value of 1 at the highest temperature at which measurements made. Symbols: (Diamonds) untreated; (squares) after exchange with POPC; (triangles) after exchange with bSM. Samples prepared using 2 mM DTT and 25 mM PFA.
Figure 3: Effect of Sterol Exchange in Cells Upon Phase Separation and Nanodomain Formation in RBL-2H3 GPMV: A. and C. Phase separation assayed by fluorescence microscopy. Symbols: (Diamonds) untreated; (filled squares) 7 dehydrocholesterol; (filled triangles) coprostanol; (circles) desmosterol; (crosses) epicholesterol; (open square) lanosterol; (open triangle) 4-cholsten-3-one. B. and D. Nanodomain formation assayed by FRET. Symbols: (Diamonds) untreated; (filled squares) cholesterol-depleted; (filled triangles) exchange with 0.1 mM cholesterol; (circles) exchange with 0.4 mM cholesterol.
cholesterol. Samples prepared using 2 mM DTT and 25 mM PFA. n=3; except n=9-10 for the untreated samples.
Figure 4. Temperature Dependence of, and Effect of Phospholipid Exchange Upon, Phase Separation and Nanodomain Formation in CHO Cells. A. Phase separation assayed by fluorescence microscopy. B. Nanodomain formation assayed by FRET. Symbols: (Diamonds) untreated; (squares) after exchange with POPC; (triangles) after exchange with bSM. Samples prepared using 2 mM DTT and 25 mM PFA.
Figure 5: Correlation between T end for large-scale phase separation and T end for nanodomain formation for RBL-2H3 GPMV. Data is from Tables 1 and 2. $r^2 = 0.60$