Effects of cholesterol depletion and increased lipid unsaturation
on the properties of endocytic membranes

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ABBREVIATIONS

DAF, decay accelerating factor; DHE, dehydroergosterol; ERC, endocytic recycling compartment; GPI, glycosylphosphatidylinositol; LDL, low density lipoprotein; LE/LY, late endosomes/lysosomes; MβCD, methyl-β-cyclodextrin; SCD1, stearoyl-CoA desaturase1; SE, sorting endosomes; Tf, transferrin; TX-100, Triton X-100.
ABSTRACT

Lipid analogs with dialkylindocarbocyanine (DiI) headgroups and short or unsaturated hydrocarbon chains (e.g., DiIC_{12} and FAST DiI) enter the endocytic recycling compartment efficiently, while lipid analogs with long, saturated tails (e.g., DiIC_{16} and DiIC_{18}) are sorted out of this pathway and targeted to the late endosomes/lysosomes (Mukherjee, S, T.T. Soe, and F.R. Maxfield. 1999. J. Cell Biol. 144: 1271-84). This differential trafficking of lipid analogs with the same polar headgroup was interpreted to result from differential partitioning to different types of domains with varying membrane order and/or curvature. Here, we investigate the system further by monitoring the trafficking behavior of these lipid analogs under conditions that alter domain properties. There was a marked effect of cholesterol depletion on the cell-surface distribution and degree of internalization of the lipid probes. Furthermore, instead of going to the late endosomes/lysosomes as in control cells, long chain DiI analogs, such as DiIC_{16}, were sorted to the recycling pathway in cholesterol-depleted cells. We confirmed that this difference was due to a change in overall membrane properties, and not cholesterol levels per se, by utilizing a CHO cell line that overexpressed transfected stearoyl-CoA desaturase 1, a rate-limiting enzyme in the production of monounsaturated fatty acids. These cells have a decrease in membrane order since they contain a much larger fraction of unsaturated fatty acids. These cells showed alteration of DiI trafficking very similar to cholesterol-depleted cells. Using cold Triton X-100 extractability of different lipids as a criterion to determine the membrane properties of
intracellular organelles, we found that the endocytic recycling compartment has abundant detergent-resistant membranes, in contrast to the late endosomes and lysosomes.
INTRODUCTION

Lipids and proteins associated with the cell surface vary in their lateral and transbilayer distribution, as well as the rate at which they are internalized from the plasma membrane. Once inside the cell, they can potentially be delivered to a large variety of organelles by selective partitioning in a series of sorting steps associated with vesicle or tubule formation (1). Although many specific peptide motifs and protein-protein interactions that determine the distribution and trafficking of transmembrane proteins have been characterized (1,2), the principles underlying lipid sorting and trafficking remain relatively unclear. While the intracellular destinations and sorting decisions for a variety of lipids and lipid analogs have been investigated in the recent years (1,3), a coherent general set of sorting rules for lipids are yet to emerge.

Part of the difficulty in understanding lipid sorting and distribution in the cell arises from the fact that this is the result of a complex interplay between the specific chemistries of individual lipid molecules (e.g., their size, hydrophobicity, headgroup to acyl chain cross-sectional ratio, charge on the headgroup, acyl chain unsaturation, etc.) as well as the biophysical properties of the membrane bilayer as a whole (e.g., its composition, thickness, tension, fluidity and curvature) [reviewed in (4,5)]. Adding to the complexity is the fact that a typical biological membrane is composed of hundreds of different lipid classes, with varying permutations of head groups and acyl chains, in addition to varying amounts of rigid, relatively planar structures like cholesterol. In addition, membranes contain a variety of proteins, both transmembrane and
peripheral, which vary in shapes, sizes, charge distribution, and propensity for aggregation among themselves or with other proteins and/or lipids. As a final measure of the complexity, many of these components are distributed non-randomly in the bilayer, varying both in the lateral and the transbilayer dimensions.

Biophysical studies in model membrane systems of precise composition have clarified to a great extent, the ways in which classes of lipid molecules interact among themselves and with other classes of lipids or cholesterol (6). However, these studies are carried out in relatively simple, well-defined, two or three component systems. Thus, although there is no doubt that similar principles are at play in various biological membranes, these membranes are too complex to allow a simple extrapolation of the insights obtained from model systems.

In contrast to the plasma membrane, very little is known about the biophysical properties of most intracellular membranes. Although lateral membrane domains or “rafts” have been shown in several studies to exist on the plasma membranes of mammalian cells, whether such domains exist in endocytic organelles as well, and if they do, how their properties compare with the domains on the cell surface remains an open question. Glycolipid and cholesterol enriched ‘rafts’ have been proposed to play a role in biosynthetic protein and lipid sorting (7). Also, experiments have utilized the ability of BODIPY-labeled lipid analogs to form excimers in a concentration-dependent manner to suggest a redistribution of lipids within seconds after the initiation of endocytosis (8). Lipid rafts have been shown to exist as early in the biosynthetic pathway as the endoplasmic reticulum (7,9).
In the current study, we have utilized several of fluorescent lipid analogs of the dialkyldiindocarbocyanine (DiI) series, whose trafficking behavior in normal fibroblasts was investigated previously (1). In the previous study, we found that, in general, lipids that have a propensity to partition into the more disordered lipid domains, or ones with a propensity to enter membranes of concave curvature, trafficked preferentially to the endocytic recycling compartment (ERC), while those with opposite propensities trafficked predominantly to the late endosomes/lysosomes (LE/LY). In order to explain the results of the above studies, we had proposed a working hypothesis based on differential partitioning of endocytosed membrane-associated molecules into coexisting membrane domains, as defined by varying fluidities and/or curvatures.

It is well known that both membrane fluidity (or ‘membrane order’), as well as curvature, are strongly modulated by the amount of cholesterol present in the bilayer (10-13). Thus, in this paper, we test our working hypothesis by following the trafficking of the DiI analogs in cells whose membrane properties have been altered by depleting the amount of cholesterol in the bilayer. In order to ensure that the effect of cholesterol depletion was an overall alteration of membrane structure and dynamics, rather than a specific cholesterol interaction effect, we utilized an alternate method to alter membrane fluidity, without changing its cholesterol content. This was achieved by utilizing a cell line (CHO-SCD1 cells) overexpressing an enzyme, stearoyl-CoA desaturase 1 (SCD1), which alters the amount of monounsaturated fatty acid chains in the membrane lipids (35).
In addition to following the trafficking itineraries of the lipid analogs in this study, we also investigate the membrane properties of intracellular endocytic organelles, namely the ERC and the LE/LY, by monitoring their insolubility in cold Triton X-100 (TX-100). Resistance to cold triton extraction is a criterion often used in cell biology research as phenomenological evidence for molecules residing in membrane domains, termed ‘rafts’, that are believed to exist in the so-called liquid ordered or \( L_\text{O} \) phase (14,15).

Our interest in this study is to look for any general sorting rules for lipids that may emerge from these comparative studies, and also, to investigate the types of lateral lipid distributions (domains) that may occur in various endocytic organelles. In these studies, we are able to follow the fate of cholesterol directly, by using the fluorescent cholesterol analog, dehydroergosterol (DHE) (16,17).
MATERIALS AND METHODS

Materials

All fluorescent probes and anti-Alexa 488 were obtained from Molecular Probes Inc. (Eugene, OR). Human Tf was obtained from Sigma Chemical Co. (St. Louis, MO). It was iron loaded and passed through a Sephacryl S-300 gel filtration system as described previously (18). Alexa 488 was then conjugated to the iron-loaded Tf following manufacturers’ instructions. Labeled transferrin was dialyzed thoroughly to remove the unbound dye. Monoclonal antibody against DAF was provided by Dr. S. Tomlinson (Medical University of South Carolina, SC) (19). DHE-loaded MβCD was prepared as described previously (17). DiI-LDL was a gift from Dr. Ira Tabas (Columbia University, NY). Lipid analogs and free fatty acids were transferred as monomers from fatty acid-free BSA carriers (1). All tissue culture supplies were from GIBCO-BRL (Gaithersburg, MD). All other chemicals were from Sigma Chemical Co.

Cells and cell culture

TRVb-1 is a modified CHO cell line that lacks endogenous Tf receptor and expresses the human Tf receptor (20). DAFTb-1 cells are a derivative of TRVb-1 cells. In addition to the human Tf receptors, they also express the GPI-linked DAF (21). They were grown in bicarbonate buffered Hams F-12 medium supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 200 μg/ml geneticin as a selection for the
transfected Tf receptors. The CHO-SCD cell line was established by transfecting CHO cells with pcDNA3.1/Hygro-mSCD1 using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to manufacturer’s instruction. Hygromycin resistant clones were pooled for experiments. CHO and CHO-SCD cells were grown in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin. 200 U/ml hygromycin was used as a selection for the transfected SCD1. All cells were kept in a 5% CO2 environment in humidified incubators at 37°C. Cells for microscopy were grown for 2 days on 35 mm plastic tissue culture dishes whose bottoms were replaced with poly-D-lysine coated coverslips (22). All experimental manipulations as well as microscopy were carried out in these dishes.

**Cholesterol depletion**

*Metabolic depletion* – TRVb-1 cells were grown for 2 days in metabolic depletion medium [Hams F-12 medium similar to the growth medium but with 5% lipoprotein-deficient serum in place of FBS, supplemented with 200 µM mevalonate, and 10 µM mevastatin (23)] to block cholesterol synthesis and deplete cholesterol stores (24).

*Depletion by MβCD* – Cells were incubated with 10 mM MβCD in Medium 1 (150 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 2 g/l glucose, and 20 mM Heps, pH 7.4) for 1 hr prior to labeling.
Endocytosis assays

Control TRVb-1 cells were labeled with 10 nM DiI labeling solution for 2 min at 37°C, rinsed several times with Medium 1 and then incubated with 5 µg/ml Alexa 488-Tf for 30 min at 37°C. For cholesterol depletion experiments, cells were prelabeled with 5 µg/ml Alexa 488-Tf for 1 hr at 37°C, extracted with 10 mM MβCD for 1 hr at 37°C, labeled with 50 nM DiI for 2 min, and chased for 30 min at 37°C. Alexa 488-Tf was present in all the steps except for the 2 min labeling with DiI. Cells that were cholesterol depleted metabolically were single labeled with DiI. At the end of the chase period, the cells were rinsed with Medium 1 and fixed with 2% paraformaldehyde.

The effects of free saturated long chain fatty acids {heptadecanoic (C17:0) and nonadecanoic acid (C19:0)} on DiIC$_{16}$ trafficking were monitored in both TRVb-1 and CHO-SCD cells, under conditions of growth in normal media as well as after acute cholesterol depletion using MβCD. For these experiments, free fatty acids were first loaded on to 1% w/v fatty acid free BSA, at a final stock concentration of 0.3 mM fatty acid, using established protocols (25). The cells were then cholesterol depleted (or not for control) with MβCD for 30 min, rinsed, labeled for 1 min with DiIC$_{16}$, rinsed, and incubated with or without the fatty acid for 45 min at 37°C, followed by imaging of the live cells.

Wide-field Fluorescence microscopy
Fluorescence microscopy and digital image acquisition were carried out using a Leica DMIRB microscope (Leica Mikroskopie und Systeme GmbH, Germany) equipped with a Princeton Instruments (Princeton, NJ) cooled CCD camera driven by Image 1/MetaMorph Imaging System software (Universal Imaging Corporation, PA). All images were acquired using a high-magnification oil immersion objective (63×, 1.4 NA). Alexa 488-conjugated proteins were imaged using a standard fluorescein filter cube [470-nm (20-nm bandpass) excitation filter, 510-nm longpass dichromatic filter, and 537-nm (23-nm bandpass) emission filter], DiI probes using a standard rhodamine filter cube [535-nm (50-nm bandpass) excitation filter, 565-nm longpass dichromatic filter, and 610-nm (75-nm bandpass) emission filter], and DHE using a filter cube obtained from Chroma Technology Corp. (Brattleboro, VT) [335-nm (20-nm bandpass) excitation filter, 365-nm longpass dichromatic filter, and 405-nm (40-nm bandpass) emission filter] (17).

Fluorescence cross-over was measured using single-labeled samples of each probe, and images were corrected for background (26) and cross-over (1).

Confocal Microscopy

Confocal microscopy was performed using an Axiovert 100M inverted microscope equipped with an LSM 510 laser scanning unit and a 63× 1.4 NA plan Apochromat objective (Carl Zeiss, Inc.). Cells labeled with DiI were excited with a 1.0 mW helium/neon laser emitting at 543 nm, and a 560 nm long pass filter was used for collecting emissions. Alexa 488-
conjugated proteins were excited with a 25-mW argon laser emitting at 488 nm and a 505-530
band pass filter was used for emissions. The two channels were scanned alternately in a line-
by-line fashion, having only one laser line and one detector channel on at each time. Summation
projection of all background corrected confocal slices were produced using the MetaMorph
software.

**Image analysis**

*Correlation measurements* – Six images were selected in which each of the cells was double
labeled with Alexa 488-Tf and FAST DiI, DiIC$_{12}$, DiIC$_{16}$ or DiIC$_{18}$ in normal media, and
DiIC$_{16}$ or DiIC$_{18}$ in cholesterol depletion media. They were first background corrected by
subtracting the average fluorescence in regions within the image that contained no cells from the
overall fluorescence (26). A threshold was then applied to each image such that only cell-
associated pixels were used in calculating the correlation coefficient. A correlation plot and a
correlation coefficient for all selected pixels above the threshold for each image were generated
by MetaMorph.
RESULTS

Cholesterol depletion interferes with the normal trafficking of the DiI analogs

Endocytic fates of the DiI derivatives were determined after their initial incorporation in the plasma membrane. Fluorescent transferrin (Tf), bound to its receptor (TfR), was used as a marker for the ERC (27,28). Exit from the ERC is the slowest step in the endocytic recycling itinerary of the TfR, resulting in the ERC being the most brightly labeled structure at steady state (18). It has been shown previously (29) that cholesterol depletion severely inhibits the internalization of TfR from the cell surface. Thus, under these conditions, most of the Tf remains at the cell surface, and the bright cell surface fluorescence impedes the detection of the central ERC fluorescence. In order to obtain a clear definition of the ERC in cholesterol depleted cells, we labeled the cells first with Alexa488-Tf, allowing enough time to load the ERC, followed by cholesterol extraction and labeling with the DiI analog.

Figure 1 shows control (normal growth medium) and cholesterol depleted cells that were labeled for 2 min with DiIC\textsubscript{16} (Fig. 1A-D) and chased for 30 min. Our previous work showed that DiIC\textsubscript{16}, which has some preference for ordered membrane domains due to its long and saturated tails, was sorted away from the endocytic recycling pathway and delivered to the LE/LY after 30 min internalization (1) (Fig. 1A-B). Several changes in lipid traffic were seen upon cholesterol depletion. First, a very large fraction of DiIC\textsubscript{16} in cholesterol depleted cells
was found in the plasma membrane (the ring stain in Fig. 1D). Second, most of the internalized DiIC\textsubscript{16} localized to the ERC, indicated by extensive colocalization with internalized Tf (Fig. 1C-D). This was clearly different from the route taken by DiIC\textsubscript{16} in control cells that resulted in its accumulation in the LE/LY (Fig. 1A-B). Even after 30 min chase, very few punctate structures representing the LE/LY were seen labeled with DiIC\textsubscript{16} in cholesterol depleted cells. To be sure that the change in intracellular trafficking upon cholesterol depletion was related to the order preferences of the hydrocarbon tails, we confirmed these results with DiIC\textsubscript{18}, which has a higher affinity for the ordered domains than DiIC\textsubscript{16} (30). Cells labeled with DiIC\textsubscript{16} and DiIC\textsubscript{18} behaved identically, both under normal growth conditions, and upon cholesterol depletion (data not shown).

In order to avoid possible artifacts, cholesterol depletion was carried out in two different ways. The approach used for the cells shown in Figure 1 was to treat cells grown in normal medium with methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD), an efficient cholesterol chelator known to form soluble inclusion complexes with cholesterol (31). In CHO cells, incubation with 10 mM M\(\beta\)CD for 30 min or longer reduced total cellular cholesterol by 40-50% (29,32). The other method to reduce cholesterol was metabolic depletion, in which the cells were grown for two days in medium containing lipid-depleted serum, an inhibitor of cholesterol biosynthesis (mevastatin) and supplemented with low levels of mevalonate in order to maintain basic metabolism of other essential isoprenoids (23,24). Both methods produced similar degrees of cholesterol depletion.
and resulted in similar trafficking alterations (not shown). Cholesterol depletion of the cells shown in all figures here was performed using MβCD.

In CHO cells, the ERC is a collection of narrow tubular elements that appears as an unresolved central spot by epifluorescence microscopy (Fig. 1). We used confocal scanning microscopy to examine in more detail the extent of colocalization between DiIC$_{16}$ and Tf in cholesterol depleted cells. Shown in Figure 2 are single plane images (A-C) and summation projections of all focal planes (D-F). In cholesterol depleted cells, DiIC$_{16}$ was seen to colocalize extensively with Tf in ERC tubules in the juxta-nuclear region of the cell. To make sure that the morphology of LE/LY was not disrupted by cholesterol depletion, we double labeled the cholesterol depleted cells to steady state with DiI-LDL and Tf. The LE/LY, appearing as small punctate dots labeled by LDL (Fig. 2G), distributed throughout the cell and separated from Tf (Fig. 2H) in a fashion identical to that in control cells. Unlike the control cells, however, these LDL-containing endosomes did not contain significant amounts of DiIC$_{16}$.

Figure 3 shows control and cholesterol depleted cells that were labeled for 2 min with either FAST DiI (Fig. 3A-D) or DiIC$_{12}$ (Fig. 3E-H) and chased for 30 min. As reported previously, both FAST DiI and DiIC$_{12}$ are delivered to the ERC in control cells, and they show significant co-distribution with Tf (Fig. 3A-B, E-F) (1). However, when cholesterol was depleted, labeling of the ERC by FAST DiI and DiIC$_{12}$ was reduced, and most of the label was retained at the plasma membrane (Fig 3C-D, G-H). Many patches of fluorescence were seen,
especially in the FAST DiI (Fig. 3D), and these patches on the plasma membrane were relatively stable over time (at least up to 20 min). We have previously reported patching of DiIC\textsubscript{12} and C\textsubscript{6}-NBD-SM into the more disordered regions of the plasma membrane upon cholesterol depletion (32). To verify that the patches of FAST DiI were indeed on the plasma membrane, we utilized a confocal microscope to optically section through a cholesterol-depleted cell labeled with FAST DiI (Fig. 4A-D). At each section, the fluorescent patches were seen at or near the cell border, consistent with patching on the cell surface. Very little FAST DiI was seen inside the cells.

The effect of cholesterol depletion on the DiI trafficking is reversible (Fig. 4 E,F). Cells were first incubated with M\textsubscript{β}CD for 1 hr. They were then labeled with FAST DiI for 2 min, chased for 30 min in either Medium 1 (Fig. 4E) or Medium 1 supplemented with cholesterol-loaded M\textsubscript{β}CD (Fig. 4F). Repletion of cholesterol with an exogenous source resulted in a uniform plasma membrane distribution and intracellular accumulation for FAST DiI, demonstrating that the change in FAST DiI distribution upon cholesterol depletion treatment was truly an effect of reduction in cholesterol levels.

Quantification of the overlap of the distribution of different DiI analogs relative to Tf

Although Fig. 1 shows the redirection of DiIC\textsubscript{16} trafficking from the LE/LY to the ERC visually, we quantified this redirection to give statistical validity to our results. Fig 5A shows the correlation plots of the distribution of different DiI derivatives relative to Tf, for control and
cholesterol depleted cells. Fig. 5B shows a plot of the correlation coefficients obtained from an average of several such correlation measurements, for each experimental condition. Correlation data (relative to Tf) for the disordered domain preferring DiI analogs (DiIC$_{12}$ and FAST DiI) were not calculated since these analogs were primarily retained on the plasma membrane upon cholesterol depletion. The correlation coefficients for the DiIC$_{16}$ and DiIC$_{18}$ compared to Tf increase significantly upon cholesterol depletion.

Retention of disorder-preferring lipids on the plasma membrane of cholesterol depleted cells is due to impaired internalization

In the pulse-chase protocols used to examine lipid trafficking, the higher surface labeling with FAST DiI and DiIC$_{12}$ in cholesterol depleted cells could have been a consequence of either reduced internalization or increased recycling. In order to distinguish between these possibilities, we required a lipid analog that could be easily stripped from the plasma membrane. For this, we chose C$_6$-NBD-SM, which has very similar membrane partitioning behavior and trafficking pattern as DiIC$_{12}$ and FAST DiI. We labeled the cells with C$_6$-NBD-SM for 2 min at 37°C and then immediately back-exchanged on ice with defatted BSA to remove cell-surface probes (33,34). Fig. 6 shows intracellular accumulation of C$_6$-NBD-SM after 2 minutes of internalization in normal and cholesterol depleted cells. It can be seen that C$_6$-NBD-SM uptake
was severely impeded in cholesterol depleted cells. Fig 6G shows that this decrease in
internalization upon cholesterol depletion is not accounted for by a reduction in incorporation of
C₆-NBD-SM into the plasma membrane. Upon cholesterol depletion, whereas the total amount
of cell-associated C₆-NBD-SM is reduced only marginally (~74% of normal), the amount
internalized is reduced to ~17% of normal.

_DiIC₁₆ trafficking is altered in CHO-SCD cells_

SCD1 is a membrane-bound, rate-limiting enzyme in the biosynthesis of
monounsaturated fatty acids. Since membrane fluidity is controlled in part by the ratio of
saturated to unsaturated fatty acid chains in lipids, SCD1 plays an important role in regulating
membrane biophysical properties (35). We wanted to confirm that the effects we were seeing on
the trafficking of lipid analogs upon cholesterol depletion were truly due to a change in
membrane biophysical (fluidity) properties and not due to a specific interaction with cholesterol
itself. For this, we utilized a CHO cell line that overexpressed the SCD1 gene. CHO-SCD cell
membranes have a dramatically different lipid acyl chain composition compared to their parental
CHO cells. In particular, the SCD1 expressing cells showed a 71% increase in 18:1 (unsaturated)
to 18:0 (saturated) fatty acid ratio in plasma membrane compared to the parental cells (36).
Interestingly, there were no significant differences between the parental CHO cells and the
CHO-SCD cells, in terms of the amounts of total cholesterol, free cholesterol, cholesterol esters,
and phosphatidylcholine, the major phospholipid class in these cells (35). In this way, we were able to manipulate the membrane properties of the early endocytic pathway without directly altering cholesterol levels.

We examined DiIC$_{16}$ trafficking in CHO-SCD cells. Instead of being delivered to the LE/LY as in parental CHO cells (Fig. 7B), the majority of DiIC$_{16}$ was found in the ERC of SCD1 overexpressing cells (panels D-F). This alteration in DiIC$_{16}$ trafficking was very similar to that observed in cholesterol depleted cells (Fig. 1C-D). Other endocytic routes were not affected in CHO-SCD cells, as shown by normal delivery of DiIC$_{12}$ and Tf to the ERC (panels G-I) and LDL to the LE/LY (panels J-L). Interestingly, although the trafficking of DiIC$_{16}$ was changed in CHO-SCD cells, Tf internalization was relatively normal (Fig. 7F, I, L), suggesting that SCD1 overexpression did not affect clathrin-mediated endocytosis to the same extent as was seen in cholesterol depleted cells.

Altered DiIC$_{16}$ trafficking in cholesterol depleted or CHO-SCD cells are not reversed by addition of exogenous free long chain saturated fatty acids

We attempted to determine whether the DiI trafficking changes upon cholesterol depletion or in CHO-SCD cells could be reversed by increasing the membrane order, using an approach that did not involve adding back cholesterol to cells. For this, we chose long chain
saturated free fatty acids, specifically heptadecanoic acid (C17:0) or nonadecanoic acid (C19:0), which were expected to rigidify the membrane bilayer. These odd-carbon fatty acids were chosen since they are known to be poor substrates for the endogenous stearoyl-CoA desaturase. Cells were loaded with a 0.3 mM stock solution of free fatty acid bound to 1% w/v fatty acid free BSA [as described in (25)]. The cells were first cholesterol depleted (or not) with methyl-β-cyclodextrin for 30 min, rinsed, labeled for 1 min with DiIC16, rinsed and then incubated with the BSA-loaded fatty acid (or not) for 45 min at 37°C, followed by imaging of the live cells. We found that free long chain saturated fatty acids do not significantly rescue the altered trafficking of DiIC16 caused by cholesterol depletion or overexpression of SCD1 (Supplementary Figures 1 and 2). It appears that these fatty acids, though long and saturated, would only have their rigidifying effect on the membrane when incorporated into lipids. By themselves, they probably behave more like detergents, and if anything, have further fluidizing effects on the bilayer. This indeed seems to be the case, since these fatty acids, by themselves, appear to alter the trafficking of DiIC16 in a manner very similar to that induced by cholesterol depletion or increased lipid fatty acyl chain unsaturation, as seen in CHO-SCD cells (see panels E and F in Supplementary Figure 1).

Cold TX-100 solubility shows different profiles in the intracellular compartments

The above results show that modulation of membrane biophysical properties affects the
endocytic trafficking of various lipid analogs. Since the trafficking of these lipid analogs is possibly dependent on their partitioning into separate membrane domains (1,5), and since the amount of cholesterol in the bilayer plays a major role in the formation of these domains (37,38), we addressed the membrane properties of various organelles through which these lipid analogs traffic. For this, we examined the cold Triton X-100 (TX-100) insolubility of various membrane markers. TX-100 insolubility has often been used as diagnostic of the presence of specialized lipid domains, enriched in cholesterol and order-preferring lipids, in the membrane bilayer.

Fig. 8 shows the cold triton extractability of the two main destinations of endocytosed material – namely the ERC and the LE/LY. To examine the cold TX-100 solubility of the ERC (panels A-H), we double labeled DAFTb-1 cells, which express the GPI-anchored protein Decay Accelerating Factor – DAF, with either anti-DAF and DHE or Tf and DHE. DHE is a naturally fluorescent sterol that has been shown to closely mimic the behavior of cholesterol (16,17,39). On the plasma membranes of CHO cells expressing DAF and transmembrane human TfR (DAFTb-1 cells), both DAF and DHE have been shown to be resistant to cold TX-100 solubilization, where TfR is almost completely solubilized (21,32). All three molecules, Tf (bound to the TfR), DAF, and DHE, accumulate in the ERC at steady state (Fig. 8A, B and E, F) (17,23). Fig. 8G shows that after extraction with cold TX-100, Tf was completely extracted from the ERC. In contrast, a large fraction of DAF and DHE molecules in the ERC were retained after treatment with the detergent (panels C, D, H), similar to the detergent resistance in the plasma membrane.
In panels I and J, we determined whether DiIC$_{16}$, which has been shown to be retained on the plasma membrane after cold TX-100 extraction (32), is also retained in its final endocytic destination, the LE/LY. For this, DiIC$_{16}$ was chased for 30 min after a brief pulse and accumulated in the LE/LY. An optical section (with confocal microscopy) near the middle of the cells showed DiIC$_{16}$ labeling of the LE/LY (panel I). When cells were treated with cold TX-100, complete removal of DiIC$_{16}$ from the LE/LY was observed (panel J), although it was retained on the plasma membranes of the same cells.

Cold TX-100 extraction of DiIC$_{18}$-labeled plasma membranes showed that CHO-SCD cells were much more susceptible to cold detergent extraction than the parental CHO cells (36), consistent with the notion that there are more liquid disordered domains in the plasma membrane of CHO-SCD cells. To see whether the ERC membrane also became more fluid upon SCD1 overexpression, we let DHE accumulate in the ERC and extracted CHO-SCD cells with cold TX-100. In contrast to the images of the control cells shown in Fig 8D, H and K, DHE in the ERC of CHO-SCD cells was completely extracted, leaving only the label in the plasma membrane (Fig 8L).
DISCUSSION

Overall, we observed a distinct change in the endocytic trafficking destinations of lipid analogs (as exemplified by the DiI analogs) upon cholesterol depletion. It is interesting to note that almost all endocytic trafficking destinations are affected. Specifically, the internalization of disordered domain preferring lipids was most severely affected upon cholesterol depletion. Using CHO-SCD cells, in which the membrane fluidity is altered without a change in cholesterol content, we were able to show that the alteration in trafficking of the lipid analogs was because of changes in membrane order and not specifically due to the chemical entity, cholesterol.

Our results with cold Triton X-100 extraction of labeled cells showed that the ERC not only contains high amounts of cholesterol (16,17) but also forms “raft-like” domains, as defined by resistance to cold TX-100 extraction. The LE/LY, by contrast, do not contain these rafts, which is not surprising, since they do not contain much cholesterol (16,17,40).

Hypotheses for differential sorting at multiple endocytic destinations

Sorting at the plasma membrane:

The plasma membranes of cells growing in normal media were always found to be uniformly labeled, no matter what lipid analog they were labeled with – including ordered domain preferring lipids such as DiIC\textsubscript{16}, disordered domain preferring lipids such as FAST DiI, or the fluorescent cholesterol analog, DHE (1,17). In contrast, cholesterol depletion creates the separation of membrane domains on the plasma membrane, with regions more or less enriched in
fluid- or rigid domain preferring lipids (32).

In this paper, when we examine the internalization profiles of these phase-separated lipid analogs, we find that the ordered domain preferring lipid, DiIC$_{16}$, appears to enter the cells quite efficiently, whereas the disordered domain preferring lipids, such as DiIC$_{12}$ or FAST DiI, are almost completely prevented from entering the cell when cellular cholesterol is reduced. Such prevention of internalization has previously been reported for some transmembrane proteins such as the TfR (29,41), whereas the internalization of ricin (41) appears to continue relatively unhindered. We do not understand the basis for this difference in internalization rates.

Sorting in the sorting endosome:

We have shown previously that lipid analogs, varying solely in the length and degree of unsaturation of their hydrophobic tails, are delivered to different intracellular destinations following internalization by endocytosis (1). More precisely, lipids containing either short or unsaturated hydrocarbon chains (DiIC$_{12}$ and FAST DiI), which partition into the more disordered parts of the membrane bilayer, efficiently entered the ERC. In contrast, lipid analogs with long and saturated tails (DiIC$_{16}$ and DiIC$_{18}$), which preferentially enter the more ordered domains, were sorted out of this pathway and were instead targeted to LE/LY. Similar behavior is observed for short chain lipid analogs such as C$_6$-NBD-SM, which recycle efficiently upon internalization (33,34), whereas some lipid analogs containing long, saturated acyl chains (e.g.,
Rh-PE and some glycosphingolipids (42,43) are targeted to late endocytic compartments.

This differential trafficking can be rationalized by the propensities of the lipid analogs to partition into membrane domains of varying fluidity and/or curvature (1). Briefly, this hypothesis states that a membrane-bound molecule can be effectively excluded from the recycling pathway (and targeted to the LE/LY) by constraining it in the part of the membrane that surrounds the spherical part of a sorting endosome. This can happen by (a) the tubules of the sorting endosomes could be enriched in more disordered domains, so that lipid analogs that preferentially partition into these domains would be efficiently recycled; (b) the bilayer fluidity in both the vesicular and the tubular parts could be roughly similar, except that the necks joining the tubules to the vesicle, that are under high curvature stress, would be specifically enriched in lipids that can be accommodated in regions of high curvature (disordered domain preferring); (c) various endosomal structures containing inward invaginations, with curvatures opposite to the emanating tubules, may represent a specialized domain that enhances the segregation of some membrane components from the recycling pathway.

In this study, we show that whereas cholesterol depletion redirects the ordered domain preferring lipids, such as DiIC<sub>16</sub>, from the LE/LY to the ERC, the trafficking destination of whatever disordered domain preferring lipid that does enter the cells (the majority is retained at the plasma membrane) is not affected. This observation is very similar to that of Puri et al (44), who demonstrated that cholesterol could modulate membrane traffic along the endocytic pathway in sphingolipid-storage diseases. An elevated cholesterol level in normal fibroblasts
was responsible for the redirected trafficking of the sphingolipid analogs from a recycling route (via the trans-Golgi network) into the LE/LY pathway (44). On the other hand, depletion of cellular cholesterol eliminated the labeling of the LE/LY by the sphingolipid analogs in sphingolipid-storage disease cells and concomitantly enhanced the labeling of the Golgi complex. It thus appears that cholesterol depletion somehow alters the properties of the recycling tubules of the SE, or the mechanism by which LE/LY-destined lipids are specifically retained in the central spherical part of the SE, such that molecules like DiIC$_{16}$, which were previously restricted from entering the recycling tubules, can now do so, resulting in an alteration in their overall endocytic destination.

Interestingly, the GPI-anchored proteins, human folate receptor $\alpha$ (FR$\alpha$) and decay accelerating factor (DAF), are trafficked almost exclusively to the ERC (negligible delivery to the LE/LY), upon internalization from the plasma membrane. This is in spite of the fact that the GPI anchor resembles DiIC$_{16}$ or DiIC$_{18}$ (i.e., containing long saturated chains). Thus, the sorting signal for the GPI-anchored proteins could be more related to their curvature preference, than their preference to partition into the more rigid domains.

Sorting at the ERC:

Cholesterol depletion increases the rate at which GPI-anchored proteins exit from the
ERC by almost 3-fold, without affecting the rate of exit of C\textsubscript{6}-NBD-SM (23). One possible interpretation of this result is that the ordered-domain preferring GPI-anchored proteins and the disordered domain preferring molecules such as C\textsubscript{6}-NBD-SM and Tf, reside in different regions of the ERC, distinguished by their cholesterol content. Thus, a treatment such as cholesterol depletion would specifically affect the ordered domains, thereby releasing the GPI-anchored proteins from the slowed recycling, while having no significant effect on the recycling rates for membrane constituents for which the recycling rate was not initially dependent on cholesterol content.

In this paper, we present a direct demonstration that the ERC does indeed contain raft-like domains, by using cold TX-100 extraction of cells, and show that, both the GPI-anchored protein, DAF, as well as the fluorescent cholesterol analog, DHE, are significantly retained upon extraction.
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FIGURE LEGENDS

Figure 1. Trafficking of DiIC<sub>16</sub> in control and cholesterol depleted cells. For control cells (panels A, B), they were labeled with DiIC<sub>16</sub> for 2 min, and chased in the presence of Alexa 488-Tf for 30 min. For cholesterol depletion experiments (panels C, D), cells were labeled with Alexa 488-Tf for 1 hr, incubated with 10 mM MβCD for 1 hr in the presence of Alexa 488-Tf, labeled with DiIC<sub>16</sub> for 2 min, and chased in the presence of Alexa 488-Tf for 30 min. All steps were done at 37°C. The cells were then fixed and imaged using a wide-field microscope. Panels A, C: Alexa 488-Tf; panels B, D: DiIC<sub>16</sub>. Bar, 10 μm.

Figure 2. Co-localization of DiIC<sub>16</sub> with Alexa 488-Tf in the ERC of cholesterol depleted cells. Cells were labeled with Alexa 488-Tf for 1 hr, incubated with 10 mM MβCD for 1 hr in the presence of Alexa 488-Tf, labeled with either DiIC<sub>16</sub> for 2 min (panels A-F) or DiI-LDL for 5 min (panels G-I), and chased in the presence of Alexa 488-Tf for either 30 min (panels A-F) or 20 min (panels G-I). All steps were done at 37°C. The cells were then fixed and imaged using a scanning confocal microscope. Panels A-C are single plane images and D-I are summation projections of all the focal planes. Panels A, D: DiIC<sub>16</sub>; panel G: DiI-LDL; panels B, E, H: Alexa 488-Tf; panels C, F, I: Merge. Bar, 10 μm.
Figure 3. Trafficking of FAST DiI and DiIC\textsubscript{12} in control and cholesterol depleted cells. Cells were labeled as described in Figure 1. Control and cholesterol depleted cells are shown in panels A, B, E, F, and panels C, D, G, H, respectively. Panels A, C, E, G: Alexa 488-Tf; panels B, D: FAST DiI; panels F, H: DiIC\textsubscript{12}. Bar, 10 µm.

Figure 4. FAST DiI distribution in cholesterol depleted and repleted cells. Cells were incubated with 10 mM M\textbeta CD for 1 hr, labeled with FAST DiI for 2 min, and chased for 30 min. Optical sections were then taken through the cells by scanning confocal microscopy. Panel A shows a summation projection of all the focal planes. Planes B-D show three representative planes through the cell (B: bottom focal plane; D: top). Panels E-F shows the reversibility of cholesterol depletion on the trafficking of FAST DiI. Cells were incubated with 10 mM M\textbeta CD for 1 hr, labeled with FAST DiI for 2 min, and chased for 30 min in Medium 1 (panel E) or Medium 1 supplemented with cholesterol-loaded M\textbeta CD (panel F). They were then imaged using a wide-field microscope. Bar, 10 µm.

Figure 5. Correlation of the distribution of various DiI analogs relative to Tf, in normal and cholesterol depleted cells. Panel A shows one representative correlation plot for each labeling condition discussed in the paper [cells double-labeled with Tf and either (i) FAST DiI, DiIC\textsubscript{12}, DiIC\textsubscript{16} or DiIC\textsubscript{18} in normal media, or (ii) DiIC\textsubscript{16} or DiIC\textsubscript{18} in cholesterol depletion media].
Panel B shows the correlation coefficients obtained from six replicates of the above measurements.

**Figure 6.** *C<sub>6</sub>*-NBD-SM internalization is blocked in cholesterol depleted cells. Control cells (panels A-C) or cholesterol depleted cells (incubated with 10 mM MβCD for 1 hr at 37°C; panels D-F) were labeled with 30 μM *C<sub>6</sub>*-NBD-SM for 2 min at 37°C, immediately washed with ice-cold Medium 1 and incubated with back-exchange medium (5% fatty acid free BSA in Medium1) on ice for 1 hr, during which six washes of ice cold back-exchange medium were applied. The cells were then fixed and taken to a scanning confocal microscope. Panels A, D: single-plane DIC; panels B, E: single plane images; panels C, F: summation projections of all the focal planes. Bar, 10 μm. For the quantification shown in panel G, imaging was carried out using the wide field microscope and 25X oil immersion objective (0.75 NA), in order to include a large number of cells in one field and to acquire fluorescence from entire cell thickness. Both the total and internal fluorescence power of the control cells were defined as 100%. The fluorescence power of the cholesterol depleted cells was normalized to that of the control cells in each case.

**Figure 7.** Trafficking of Tf, DiIC<sub>16</sub>, DiIC<sub>12</sub>, and LDL in CHO and CHO-SCD cells. Cells were labeled with DiIC<sub>16</sub> (panels A-F), or DiIC<sub>12</sub> (panels G-I) for 2 min, or DiI-LDL for 5
min (panels J-L), and chased in the presence of Alexa 488-Tf for 30 min (panels A-I) or 20 min (panels J-L). All steps were done at 37°C. The cells were then fixed and imaged using a scanning confocal microscope. Images shown are summation projections of all the focal planes.

Panels A-C, CHO cells; panels D-L, CHO-SCD cells. Panels A, D, G, J, single-plane DIC; panels B, E: DiIC_16; panel H: DiIC_12; panel K: DiI-LDL; panels C, F, I, L: Tf. Bar, 10 µm.

**Figure 8. Cold TX-100 extraction of DAF, DHE, and Tf in the ERC and DiIC_16 in the LE/LY.**

Panels A-D, cells were labeled for 1 min with DHE-loaded MβCD, incubated for 1 hr with Alexa 488 Anti-DAF and chased for additional 1 hr in Medium 1. They were subsequently incubated for 15 min with anti-Alexa 488 to quench Alexa 488 fluorescence on the cell surface.

Panels E-H, cells were labeled for 1 min with DHE-loaded MβCD, followed by 1 hr incubation with Alexa 488-Tf. All incubations were done at 37°C. The cells were then washed with ice-cold Medium 1, incubated with Medium 1 (panels A, B, E, F) or 1% TX-100 (panels C, D, G, H) on ice for 30 min, and fixed. Images shown are wide field epifluorescence images. Panels A, C: DAF; panels E, G: Tf; panels B, D, F, H: DHE. For panels I, J, cells were labeled with DiIC_16 for 2 min and chased in Medium 1 for 30 min at 37°C. They were then incubated with either Medium 1 (panel I) or 1% TX-100 for 30 min on ice (panel J) and fixed and single confocal sections cutting through the middle of the cell were obtained. For panels K and L, control CHO (panel K) and CHO-SCD (panel L) cells were first labeled for 5 min with DHE at 37°C as
described above and chased at 37°C for one hour. They were then chilled on ice, and the TX-
100 extraction was carried out as described above. Bar, 10 μm.
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: Effect of saturated long chain fatty acids on TRVb-1 cells. Panel A: Untreated cells labeled with DiIC₁₆; panel B: cholesterol depleted cells labeled with DiIC₁₆; panel C: cholesterol depleted cells labeled with DiIC₁₆, and further incubated with fatty acid 17:0 for 45 min; panel D: cholesterol depleted cells labeled with DiIC₁₆, and further incubated with fatty acid 19:0 for 45 min; panel E: untreated (non-cholesterol depleted) cells labeled with DiIC₁₆, and further incubated with fatty acid 17:0 for 45 min; panel F: untreated (non-cholesterol depleted) cells labeled with DiIC₁₆, and further incubated with fatty acid 19:0 for 45 min.

Supplementary Figure 2: Effect of saturated long chain fatty acids on CHO-SCD cells. Panel A: Untreated cells labeled with DiIC₁₆; panel B: cholesterol depleted cells labeled with DiIC₁₆; panel C: cholesterol depleted cells labeled with DiIC₁₆, and further incubated with fatty acid 17:0 for 45 min; panel D: cholesterol depleted cells labeled with DiIC₁₆, and further incubated with fatty acid 19:0 for 45 min; panel E: untreated (non-cholesterol depleted) cells labeled with DiIC₁₆, and further incubated with fatty acid 17:0 for 45 min; panel F: untreated (non-cholesterol depleted) cells labeled with DiIC₁₆, and further incubated with fatty acid 19:0 for 45 min.
min.
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**Figure 1:**

(A) Control DAF

(B) Tx-100 extract DHE

(C) DAF

(D) Tx-100 extract DHE

(E) Control Tf

(F) Tx-100 extract DHE

(G) Tf

(H) DHE

(I) Control DilC16

(J) Tx-100 extract DilC16

(K) Cho

(L) Cho-SCD

Control

Tx-100 extract

Tf

DilC16

Cho

Cho-SCD
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