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83. The authors would like to thank J. Kim, T. Sato, A. Wurmser, and S. Scott for helpful comments. D.J.K. is supported by Public Health Service grant GM53396 from NIH and S.D.E. is supported by NIH grant CA58689 and is an Investigator with the Howard Hughes Medical Institute.

REVIEW: CELL BIOLOGY

How Cells Handle Cholesterol

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Cholesterol plays an indispensable role in regulating the properties of cell membranes in mammalian cells. Recent advances suggest that cholesterol exerts many of its actions mainly by maintaining sphingolipid rafts in a functional state. How rafts contribute to cholesterol metabolism and transport in the cell is still an open issue. It has long been known that cellular cholesterol levels are precisely controlled by biosynthesis, efflux from cells, and influx of lipoprotein cholesterol into cells. The regulation of cholesterol homeostasis is now receiving a new focus, and this changed perspective may throw light on diseases caused by cholesterol excess, the prime example being atherosclerosis.

Sterols are ubiquitous components of cell membranes in eukaryotes. Mammalian cells produce their own cholesterol and receive cholesterol by uptake from lipopro-

teins. Cells also continuously lose cholesterol to the outside circulation. Regulation of synthesis, influx and efflux keeps cellular cholesterol levels precisely controlled. But why cholesterol homeostasis is under such stringent control has not been obvious from our understanding of cholesterol function. Cholesterol was simply thought to rigidify the fluid membrane, thus reducing passive permeability and increasing the mechanical durability of the lipid bilayer. Cholesterol was also postulated to facilitate post-Golgi protein

sorting by regulating the thickness of the lipid bilayer (1). These functions have now been expanded by the key role that cholesterol plays in lipid raft assembly and function. Lipid rafts function to segregate and concentrate membrane proteins (2). These dynamic assemblies are involved in sorting and distributing lipids and proteins to the cell surface (3), where they play an important role in signal transduction and in generating cell surface polarity (4). For these functions, it is crucial that not only the total cellular level of cholesterol but also its distribution between membranes and within a given membrane are tightly regulated. Here, we will review the movements of cholesterol within the cell and the regulation of cellular cholesterol from this new perspective.

What Are Lipid Rafts?

Lipid rafts are constituted of cholesterol and sphingolipids (sphingomyelin and glycosphingolipids) in the exoplasmic leaflet of the bilayer [for reviews on rafts, see (2, 3, 5)].

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Cholesterol condenses the packing of sphingolipid molecules by occupying the spaces between the saturated hydrocarbon chains of the sphingolipids. The association of cholesterol with sphingolipids is most likely strengthened by hydrogen bonding between the 3'-OH group of the sterol and the amide function of the sphingolipid ceramide backbone. The exoplasmic assemblage of sphingolipids and cholesterol is thought to be linked to the underlying cytoplasmic leaflet (6) and forms a separate phase, a liquid-ordered phase, which is dispersed in the liquid-disordered phase constituting the more loosely packed fluid matrix of the membrane (see Fig. 1 for a schematic presentation of the transbilayer assembly). Cholesterol partitions preferentially into the liquid-ordered phase, as compared to the liquid-disordered matrix of the membrane, and is essential for the maintenance of the two lipid phases.

Lipid rafts incorporate distinct classes of proteins (2). They are glycosylphosphatidylinositol (GPI)-anchored proteins, doubly acylated peripheral membrane proteins, cholesterol-linked proteins, and transmembrane (often palmitoylated) proteins (Fig. 1). The transmembrane domains of proteins have to be intercalated with raft lipids in such a way that the tight packing of the liquid-ordered phase is maintained. Removal of cholesterol from rafts (e.g., by cyclodextrin treatment) leads to dissociation of the raft proteins from the lipids (4). Raft size has been difficult to determine, but recent studies using photonic force microscopy in live fibroblasts have demonstrated that plasma membrane rafts have a distinct size with a diameter of about 50 nm, corresponding to about 3500 sphingomyelin molecules (7). Moreover, the diffusion of raft proteins is confined to the size of the raft. The small size implies that each raft carries only a limited set of proteins, probably not more than 10 to 30. Raft size, however, will depend on the concentration of sphingolipids and cholesterol in the membrane. Considering two co-existing lipid phases in bilayers, the major lipid phase forms a continuum in which its components diffuse freely (8, 9). If the minor phase were the liquid-ordered phase, its components would be confined to the size of the individual rafts. However, if the concentration of the raft lipids is increased to exceed a critical value, the rafts would coalesce to form the connected phase while the liquid-disordered phase would become dispersed like "islands in an archipelago" (8). Thus, the lipid phases determine the boundaries for lateral diffusion of proteins in each phase. By modulating lipid concentrations and external conditions, the threshold for raft coalescence can be influenced (5). Such changes may play a role in regulating membrane properties both under physiological conditions and in the pathogen-

esis of diseases such as lipid storage disorders and atherosclerosis.

A subclass of plasma membrane rafts is contained within membrane invaginations called caveolae (10). They are formed in cell types that express caveolins. These proteins bind cholesterol and form hairpin structures embedded in the bilayer. Polymerization of caveolins is thought to bend the membrane to form caveolae (11). The structure of caveolae is dependent on cholesterol; the caveolae disappear after cholesterol removal. One complication in analyzing caveolar constituents is the difficulty of separating caveolae from plasma membrane rafts biochemically. For instance, after detergent extraction both caveolae and raft components of the plasma membrane are aggregated together in detergent-resistant membranes (DRMs) [see (4)

for the distinction between individual and clustered rafts, DRMs, and caveolae]. Because of this, many studies of caveolar function remain difficult to interpret.

Biosynthetic Cholesterol Transport

Cholesterol is synthesized in the endoplasmic reticulum (ER), while most sphingolipids receive their headgroups in the Golgi complex. Accordingly, nascent cholesterol partitions first preferentially into nonraft membrane but seems to become incorporated into rafts in the Golgi (12). From the Golgi lipid, rafts are distributed to the cell surface (13), where both cholesterol and sphingolipids are enriched (14, 15) (Fig. 2). In polarized cells, such as epithelial cells and neurons, there are raft pathways to the apical and axolemmal plasma membrane domains, respectively

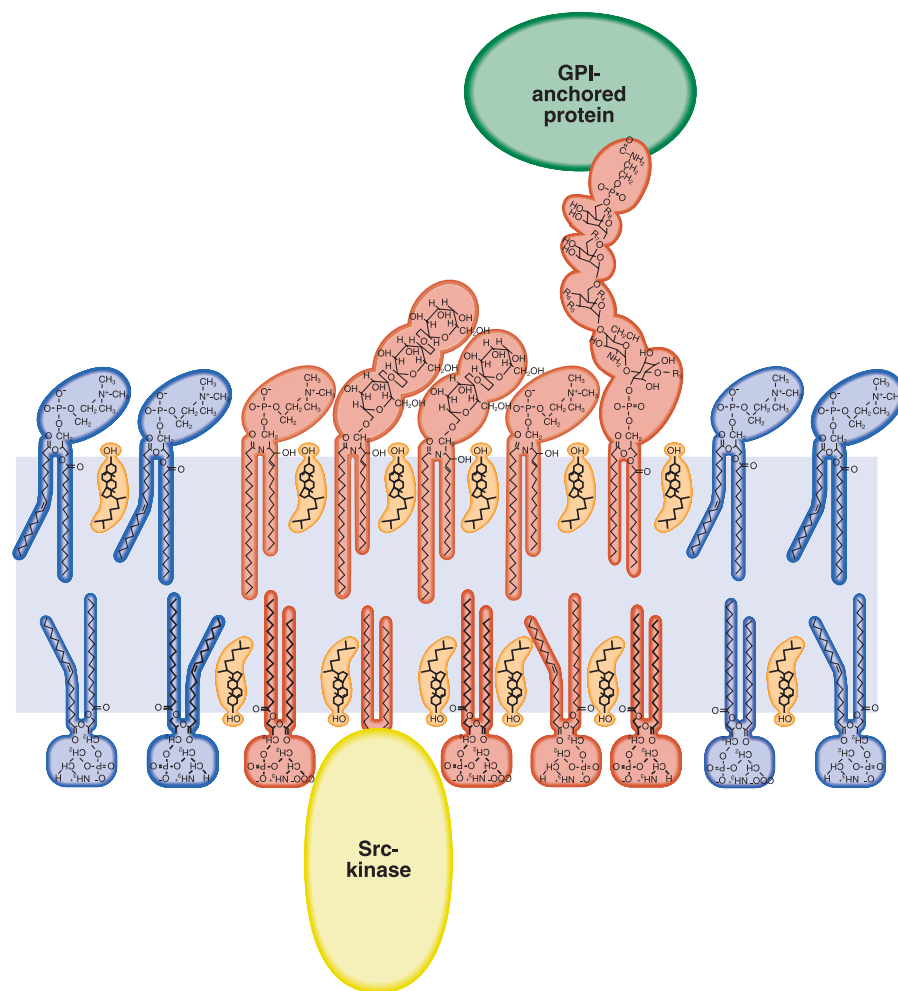


Fig. 1. Model of a raft with two intercalated proteins. A GPI-anchored protein is attached to the exoplasmic leaflet, and a doubly acylated Src-kinase to the cytoplasmic leaflet. Lipids within the liquid-ordered phase are shown as red and in the liquid-disordered phase as blue. Cholesterol, indicated by orange, partitions preferentially into the liquid-ordered phase. The outer leaflet of the raft is enriched in glycosphingolipids and sphingomyelin, and the corresponding inner leaflet is illustrated as containing glycerolipids with predominantly saturated fatty acyls. The degree of acyl chain saturation in the putative inner raft leaflet remains open. The coupling between the exo- and cytoplasmic leaflets is hypothetical; the connecting mechanisms remain to be established, but antibody patching of a GPI-anchored protein leads to copatching of the associated Src-family kinase (85).

(13). However, the basolateral and the somato-dendritic membrane domains are also supplied with rafts. Caveolae are present only basolaterally in epithelial cells (16). On the basis of the high mole fraction of raft lipids in the apical membrane of epithelial cells (14), one would predict that in these membranes rafts would constitute the connected phase and would allow free diffusion of raft proteins over the entire membrane.

If rafts were the main transport vehicle for cholesterol to the plasma membrane, one would assume that the secretory pathway would be employed for surface transport. However, under conditions in which secretory protein transport out of the ER is blocked, the majority of biosynthetic cholesterol uses a pathway that bypasses the Golgi complex to reach the plasma membrane (12). This route could either involve an unknown membrane transport pathway from the ER to the cell surface or a carrier protein-mediated transport pathway (17). Candidates for the latter function have been identified, including the sterol-binding protein 2 [(18), but see (19)] and a cytosolic caveolin-chaperone complex [(20, 21), but see (12)]. Considering possible short-cuts for the transport of newly synthesized cholesterol from the ER to the plasma membrane, the juxtaposition of the smooth ER and the cell surface could facilitate carrier-mediated transfer.

An interesting mechanism for cholesterol transport exists in adrenal and gonadal cells utilizing cholesterol as a precursor for steroid hormone synthesis (22). Cholesterol is transported to the mitochondria presumably via the cytosol because it is unlikely that there is a vesicular pathway connected to the mitochondria. An alternative mechanism has been suggested to involve membrane-membrane contacts between the ER and mitochondria for interorganelle transport of phospholipids (23). Obviously, such a mechanism may also apply for cholesterol transfer. The key protein shuttling cholesterol across the outer and inner mitochondrial membranes is the steroidogenic acute regulatory protein (StAR). Its structure has recently been determined (24) and it has a binding pocket for cholesterol.

Influx of Exogenous Cholesterol

Cholesterol can be taken up from lipoproteins in the circulation by mechanisms involving desorption—transfer of cholesterol from the lipoprotein to the exoplasmic leaflet of the plasma membrane bilayer—or by receptor-mediated uptake (25). The best understood and quantitatively the most important process is the one involving serum low-density lipoprotein (LDL), and its LDL-receptor. LDL is released from its receptor in the sorting endosomes (Fig. 2) and the LDL-receptor recycles to the cell surface. Cholesterol esters are hydrolyzed from LDL, and free chole-

sterol is continuously cycled to the plasma membrane (26, 27). The main mechanism responsible for exit of LDL-cholesterol from late endosomes or lysosomes involves the NPC1 protein defective in Niemann-Pick type C disease, in which cholesterol and other lipids accumulate in lamellar bodies derived from lysosomes (28, 29). The NPC1 protein has been localized to late endocytic structures (30); however, its cycling itinerary and its function have not yet been pinpointed. The protein appears to change its localization with increased cholesterol loading of late endosomes or lysosomes and accumulates in cholesterol-laden lysosomes

and the Golgi (30–32). The NPC1 protein is a multispanning membrane protein that contains a putative sterol-sensing domain in the membrane spans 4–8 (33). Mutations in this domain can inactivate the protein (34). Similar domains are found in membrane proteins involved in the regulation of cholesterol synthesis (see below). One potential function for the sterol-sensing domain is direct cholesterol binding. This should be possible to test using a photoactivatable cholesterol reagent that can be introduced into cells and used to label potential cholesterol binding proteins (35). The NPC1 protein may function to remove cholesterol from the degra-

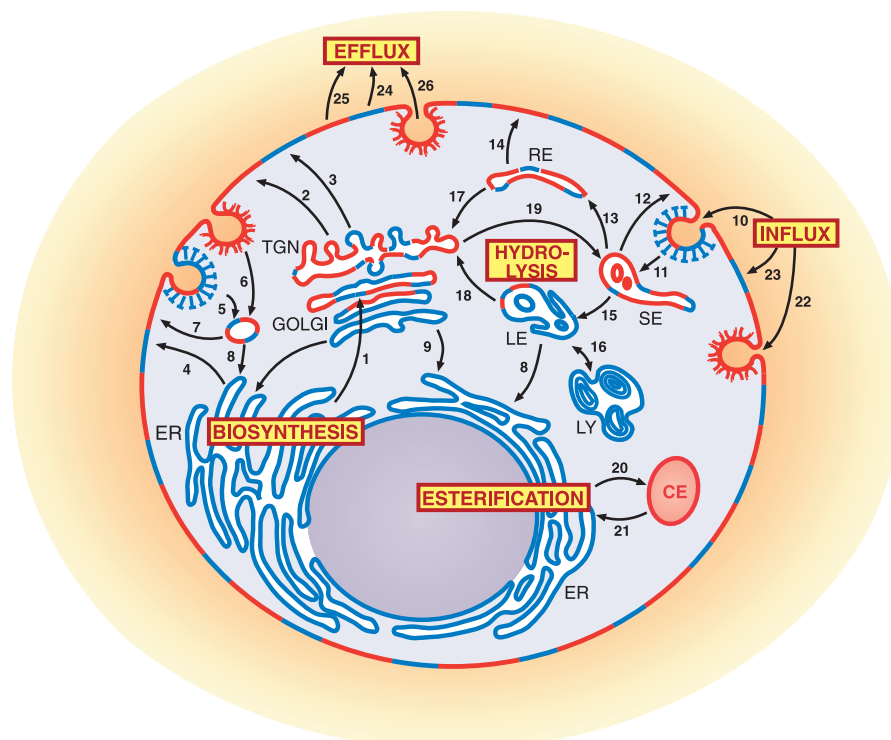


Fig. 2. Schematic presentation of cellular cholesterol distribution, processing, and trafficking circuits. Cholesterol is synthesized in the endoplasmic reticulum (ER). Part of it is transported via the Golgi complex (1) and the trans-Golgi network (TGN) to the plasma membrane, where it is distributed either to raft (2, red) or nonraft (3, blue) microdomains. The majority of cholesterol, however, takes a Golgi-bypass route (4) to the cell surface. Cholesterol can be internalized from the plasma membrane by endocytosis via clathrin-coated vesicles (5) or other pathways, including caveolae (6). Endocytosed rafts are found in sorting and recycling endosomes. From the endocytic circuits, cholesterol may be recycled to the surface (7) or transported back to the ER (8). Also, retrograde routes from the Golgi complex (9) recycle cholesterol to the ER. There may also be a route involving transport via caveolae to the ER. Caveolae have been shown to internalize the simian virus 40 (86). This is delivered to a peripheral compartment from where caveolin returns, and the virus is packaged into tubules that move to the ER (87). Cholesterol is endocytosed in LDL via clathrin-coated pits (10) and transported to sorting endosomes (SE; 11). From there, it can be recycled to the surface either via a rapid route (12) or through slower circuits involving recycling endosomes (RE; 13, 14). Cholesterol is also transported to late endocytic structures [15, late endosomes (LE) and lysosomes (LY)] that can fuse with each other (16). Sorting, recycling, and late endosomes communicate with the exocytic pathway at the level of the TGN (17 through 19), thus exchanging cholesterol between the endocytic and exocytic routes. Cholesterol esters in LDL are hydrolyzed prior to release from the endocytic organelles, but cholesterol returning to the ER may become re-esterified. Cholesterol esters (CE) are deposited in cytosolic lipid droplets (20) from where cholesterol can be mobilized upon ester hydrolysis (21). Cholesterol and cholesterol esters can also be exchanged directly between circulating lipoproteins and the plasma membrane. Caveolae have been implicated in the uptake of cholesterol esters from HDL (22), and free cholesterol can be taken up from LDL (23). Cholesterol can be released from cells, both from nonraft (24) and raft domains (25), the latter potentially involving caveolae (26). In some cases, this may involve endocytic uptake and resecretion of lipoproteins.

dativ endosomal compartments by facilitating sterol transport to the Golgi or other destinations in vesicular carriers (29, 36). Homologs of the NPC1 protein are found in many eukaryotes, for instance, *Caenorhabditis elegans*, *Drosophila*, and *Saccharomyces cerevisiae* (37–39). Because these organisms also employ sterols to assemble rafts from sphingolipids (40–42), these eukaryotes provide useful alternative strategies for finding out how cells handle sterols.

LDL-cholesterol is not only cycled to the cell surface but is also transported to the ER, where cholesterol may become esterified by the enzyme acyl-coenzyme A:cholesterol acyl-transferase (ACAT) (17, 43). This esterification is activated as a means to detoxify excess free cholesterol, and the esters are deposited in cytosolic lipid droplets. Two pathways have been implicated in the delivery of endocytosed LDL cholesterol to the ER. One involves a retrograde route through the Golgi complex. The other pathway is brefeldin A insensitive but cytochalasin D-inhibitable (44, 45). This retrograde connection from the endosomes to the ER thus bypasses the Golgi and could be carrier protein-mediated (Fig. 2). Recent studies have identified a protein similar to the mitochondrial StAR protein that is localized to late endosomes (46) and may thus be involved in shuttling cholesterol out of the endosomes and feeding it into the putative carrier pathway. Transport along actin filaments would require motors not yet identified.

Cholesterol Efflux from Cells

Cellular cholesterol is continuously lost by release of cholesterol to circulating lipoproteins. Such a loss can be quite rapid, up to 0.1% of total cholesterol per minute (47). The release from the plasma membrane can take place by desorption of cell surface cholesterol into lipoproteins or be induced after high-density lipoprotein binding to membrane receptors (48). Some tissues, mainly the liver and the intestine, release cholesterol to the circulation mostly as esters by synthesizing and secreting lipoproteins (49). Yet an additional mechanism of cholesterol removal is by membrane shedding, a process releasing plasma membrane vesicles that may be enriched in raft lipids (50).

Obviously, cholesterol behaves differently with respect to efflux whether it is in rafts or in the liquid-disordered matrix. Several studies have shown that raft cholesterol is more slowly extracted by cyclodextrin and by HDL (51, 52). Thus, nonraft cholesterol is the most likely source for desorptive efflux. One candidate protein involved in cholesterol efflux has been identified by studies of Tangier's disease. This genetic disease is caused by the loss of function of an ATP-bind-

ing cassette transporter ABCA1 (formerly ABC1) (53–55). This defect manifests itself by increased catabolism of HDL caused by decreased efflux of cellular cholesterol. The increase in cellular cholesterol levels leads to increased deposits of cholesterol esters in cytoplasmic lipid droplets. One possible function of ABCA1 could be to promote translocation of cholesterol from the cytoplasmic to the exoplasmic bilayer leaflet from where efflux would take place. In erythrocyte membranes, cholesterol has been found to exhibit transbilayer translocation with a half-time of about 50 min (56), so that efficient efflux may indeed require a specific flippase. ABCA1 has also been found to promote Ca^{2+} -induced translocation of phosphatidylserine to the exoplasmic leaflet (57), which may favor the release of phospholipids and cholesterol to HDL. The ABCA1 protein has been localized both to the plasma membrane and to the Golgi complex (57) and could also be involved in the transport of cholesterol from the Golgi to the cell surface, possibly involving rafts (58). The transporter may facilitate the formation of transport carriers by regulating lipid asymmetry.

Regulation of Cellular Cholesterol Levels

The best understood mechanism involved in cholesterol homeostasis is the control of cholesterol biosynthesis. The control center for this process is in the ER. Brown and Goldstein have unraveled the exquisite regulatory circuits that control the levels of the enzymes involved in cholesterol and fatty acid synthesis, as well as the uptake of plasma LDL (59, 60). This control system involves membrane-bound transcription factors called sterol regulatory element-binding proteins (SREBPs) that activate genes upregulating cholesterol uptake and synthesis. The SREBPs cycle between the ER and the Golgi, and can be released from the membrane by two proteolytic cleavages. The movement of SREBP is dependent on a co-factor, the SREBP cleavage-activating protein (SCAP) that contains a sterol-sensing domain regulating the transport. When cholesterol levels fall, the SREBP-SCAP complex moves from the ER to the Golgi, where the first cleavage takes place, and this is then followed by the second cleavage to liberate the active SREBP for transport to the nucleus. Recent studies have shown that sterol deprivation renders the ER membrane competent to discharge the SREBP-SCAP complex into budding vesicles for transport to the Golgi (61). Brown and Goldstein postulate that sterols cause the SREBP-SCAP complex to bind to an ER-retention protein. Sterols could bind directly to SCAP and thereby cause a conformational change that activates binding to the retention protein, but whether this is the mechanism is not yet known. This is the first example of an ER exit process that is under metabolic control.

Another open issue is how the small cholesterol pool (possibly not more than 0.5% of total cholesterol) in the ER can sense the bulk pool peripherally (62). One potential mechanism is provided by the sequestration of cholesterol into rafts in the peripheral compartments. The rafts in the post-Golgi compartments, including the plasma membrane, seem to be kept away from the ER by restricting retrograde raft traffic (63). Increasing cholesterol levels beyond saturation of rafts would lead to an increased cholesterol concentration in the liquid-disordered phase peripherally. This latter pool of cholesterol would be free to move back to the ER, thus blocking transport of SREBP-SCAP to the Golgi. This would slow down cholesterol synthesis and lead to removal of excess cholesterol by storage as ester droplets. This hypothesis assumes that it is the cholesterol in the liquid-disordered matrix of the membrane that is connected to the ER and therefore can be sensed. Support for this model comes from studies demonstrating that treatment of living cells with sphingomyelinase degrading the major raft sphingolipid in the plasma membrane, leads to a rapid increase in ER cholesterol, as evidenced by cholesterol ester production and inhibition of SREBP cleavage (64, 65). Raft destruction moves cholesterol into the nonraft pool, which flows back to the ER. In other words, although the total cholesterol level remains the same in the cell, the ER sensors react by suppressing SREBP cleavage.

The late endosome and lysosome system is low in raft lipids, and like the ER, it also plays a role in raft lipid homeostasis (66). Late endosomes and lysosomes degrade endocytosed sphingolipids and remove cholesterol released from endocytosed LDL for transport to other destinations (17). The accumulation of one raft lipid (e.g., cholesterol in Niemann-Pick type C disease due to defective egress or glucosylceramide in Gaucher's disease caused by defective degradation) leads to the accumulation of other raft lipids in the lysosomes (29, 66). This causes the formation of abnormal lysosomes containing lipid lamellae (67). The accumulation of rafts in the degradative compartments may disturb membrane transport through late endosomes and lysosomes and eventually give rise to the disease symptoms seen in different lipidoses involving raft lipids. Pagano and co-workers (32) have shown that a glycosphingolipid carrying the fluorescent BODIPY moiety is endocytosed and normally distributed to the Golgi. However, in sphingolipid storage diseases, the lipid probe accumulates in late endosomes and lysosomes instead. This trapping in the degradative compartments could be due to the preferential association of sphingolipids with cholesterol. Normal breakdown of sphingo-

lipids seems to require removal of endocytosed cholesterol from the degradative compartments. The activation of sphingolipid hydrolases requires acidic phospholipids (68), of which the major one is lysobisphosphatidic acid. This lipid is localized to the internal membranes of late endosomes and seems to play a role not only in sphingolipid hydrolysis but also in cholesterol exit from late endosomes and lysosomes (69). The formation of the internal membrane invaginations in endosomes must involve rearrangements of the lipid bilayer to bend the membrane (70). The geometric shapes of the lipids will play a crucial role in such processes; cone-shaped lipids will bend the membrane toward the lumen if accumulating in the luminal leaflet of the bilayer. Because lipid rafts tend to be flat, the enrichment of rafts in late endosomes and lysosomes may flatten the membranes and cause the production of lamellar bodies. If internal vesicles are crucial for sphingolipid degradation (71), then raft accumulation may impair the function of the degradative endosomes and lysosomes.

Because lipid rafts are composed of cholesterol and sphingolipids one would expect cellular cholesterol and sphingolipid concentrations to be coordinately regulated to control raft content of membranes. There are indeed reports indicating that sphingomyelin and cholesterol levels are controlled together with oxysterols acting as potential sensors (72) although the regulation of phosphatidylcholine synthesis is also linked to cholesterol loading. Oxysterols are produced by sterol hydroxylases from cholesterol. The molecule 25-OH-cholesterol is known to bind to the oxysterol-binding protein (OSBP) that belongs to a large family of proteins with unknown function (73). OSBP localizes to the Golgi complex or to the cytosol, and the distribution of the protein is regulated by the cholesterol content; depletion leads to translocation of OSBP to the Golgi complex and to activation of both cholesterol and sphingomyelin synthesis (74, 75).

In contrast to other lipid constituents of cell membranes, cholesterol is not metabolized in most tissues of the body. Rather, it is turned over by efflux from the cells. The liver plays a key role in eliminating the cholesterol that has been removed from the periphery by incorporation into circulating high-density lipoproteins. These are taken up by scavenger receptor B1 in the liver (76), and then part of the released cholesterol is metabolized to bile salts and eliminated by secretion, together with phospholipids into the bile (77, 78). This process is thought to involve the formation of phosphatidylcholine-containing lipid vesicles that incorporate cholesterol and are produced by the action of the secreted bile salts. Bile salts are detergents, which can release the phospholipid vesicles into the bile but presumably have to leave the raft-

covered bile canalicular (apical) membrane untouched. Otherwise, the hepatocytes would be permeabilized.

Perspectives

Here, we have given a brief overview of how cholesterol is distributed in cells. The transport network for cholesterol employs both the secretory and endocytic pathways for protein transport and encompasses cytosolic connections, which may be independent of membrane traffic. With the exception of the SREBP-SCAP system, the complex control mechanisms for regulating cholesterol levels in different cellular compartments remain largely unknown. There is thus an exciting future for the field of cholesterol research. We now start to get an insight into why sterols are so important for cell membrane function. Eukaryotic life seems to depend on sterols and this is probably due to their key function in lipid rafts. The list of raft-dependent functions in cells is continuously expanding. Cholesterol and lipid rafts not only participate in distributing proteins to the cell surface and to other organelles, they also play a significant role in many signaling cascades (4) and in the activation of immune responses (79).

However, cholesterol is not solely beneficial to the cell. Excess cholesterol is toxic and therefore cells have to employ a number of safety devices to limit the concentration of free cholesterol. Breakdown of cholesterol homeostasis causes disease states, the most common being atherosclerosis. Another common disease that potentially involves cholesterol metabolism is Alzheimer's disease. The lipoprotein allele apoE4 is associated with an increased incidence of Alzheimer's disease (80). Depletion of plasma membrane cholesterol in hippocampal neurons inhibits the formation of A β (81), the cleavage product of the amyloid precursor protein that is a key factor in the pathogenesis of the disease. Recent studies also demonstrate that a number of infectious diseases involving viruses, bacteria, and parasites make use of rafts to populate the host cells (82). HIV, for instance, uses lipid rafts both to enter and to leave its host cell (83, 84). It is thus hoped that future cholesterol research will not only lead to an increased understanding of normal cell functions but also to novel insights that will help us to combat a variety of disease states.

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88. We thank L. Liscum, M. Zerial, V. Olkkonen, and G. van Meer for helpful discussions and for critical reading of the manuscript. Supported by the Deutsche Forschungsgemeinschaft and the Academy of Finland.

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