MINIREVIEW

Recent Advances in Membrane Microdomains: Rafts, Caveolae, and Intracellular Cholesterol Trafficking

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Cellular cholesterol homeostasis is a balance of influx, catabolism and synthesis, and efflux. Unlike vascular lipoprotein cholesterol transport, intracellular cholesterol trafficking is only beginning to be resolved. Exogenous cholesterol and cholesterol ester enter cells via the low-density lipoprotein (LDL) receptor/lysosomal and less so by nonvesicular, high-density lipoprotein (HDL) receptor/caveolar pathways. However, the mechanism(s) whereby cholesterol enters the lysosomal membrane, translocates, and transfers out of the lysosome to the cell interior are unknown. Likewise, the steps whereby cholesterol enters the cytofacial leaflet of the plasma membrane caveolae, rapidly translocates, leaves the exofacial leaflet, and transfers to extracellular HDL are unclear. Increasing evidence obtained with model and isolated cell membranes, transfected cells, genetic mutants, and gene-ablated mice suggests that proteins such as caveolin, sterol carrier protein-2 (SCP-2), Niemann-Pick C1 protein, steroidogenic acute regulatory protein (StAR), and other intracellular proteins mediate intracellular cholesterol transfer. While these proteins bind cholesterol and/or interact with cholesterol-rich membrane microdomains (e.g., caveolae, rafts, and annuli), their relative contributions to direct molecular versus vesicular cholesterol transfer remain to be resolved. The formation, regulation, and role of membrane microdomains in regulating cholesterol uptake/efflux and trafficking are unclear. Some cholesterol-binding proteins exert opposing effects on cellular cholesterol uptake/efflux, transfer of cholesterol out of the lysosomal membrane, and/or intracellular cholesterol trafficking to select membranous organelles. Resolving these cholesterol pathways and the role of membrane cholesterol microdomains is essential to our understanding not only of processes that affect cholesterol metabolism, but also of the abnormal regulation that may lead to disease (diabetes, obesity, atherosclerosis, neutral lipid storage, Niemann-Pick C, congenital lipoid adrenal hyperplasia, etc.).

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Although great advances have been made in understanding vascular lipoprotein cholesterol transport, only recently has attention focused on cholesterol-rich, plasma membrane microdomains and their interaction with pathways of intracellular cholesterol transport and metabolism. The past 5 years have seen an exponential growth in our understanding of these domains and pathways (1–21). For summaries of the older literature, the reader is referred to previous reviews (22–25). Although much information available on the still-emerging pathways of intracellular cholesterol trafficking (Fig. 1) was obtained with radiolabeled cholesterol, recent dramatic advances in fluorescence imaging technology (multiphoton laser scanning microscopy) (4, 20) and confocal microscopy (4, 20, 26) now allow acquisition of real-time images, dynamics, and localization of naturally occurring, fluorescent sterols (dehydroergosterol) within living cells. Like cholesterol, dehydroergosterol is taken up primarily via the low-density lipoprotein (LDL)-receptor endocytic pathway and codistributes with cholesterol within subcellular membranes. Multiphoton laser scanning microscopy is especially promising in

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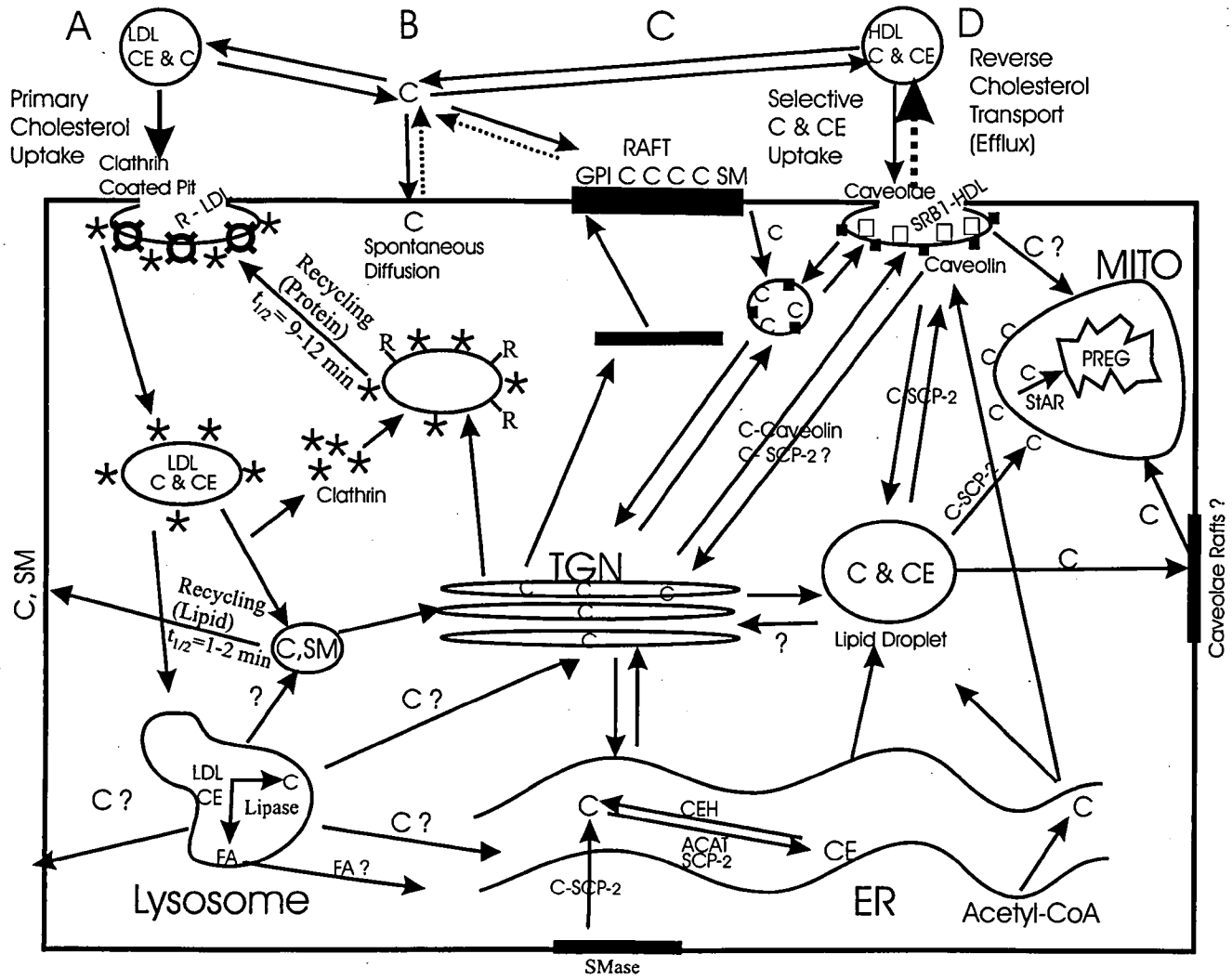

Figure 1. Plasma membrane domains, endocytic vesicles, and potential intracellular cholesterol trafficking pathways. (A) LDL receptor-mediated, clathrin-coated pit, endocytic, lysosomal pathway. (B) Spontaneous diffusion. (C) Rafts. (D) HDL receptor (SRB1)-mediated, caveolar, nonvesicular pathway and putative caveolar vesicle pathway. Symbols are as follows: LDL, low-density lipoprotein; C, cholesterol; CE, cholesteryl ester; R-LDL, LDL receptor; R, receptor; FA, fatty acid; TGN, trans-Golgi network; ER, endoplasmic reticulum; MITO, mitochondria; ACAT, acyl CoA:cholesterol acyltransferase; CEH, cholesteryl ester hydrolase; GPI, glycosylphosphatidylinositol; SM, sphingomyelin; SMase, sphingomyelinase; SCP-2, sterol carrier protein-2; StAR, steroidogenic acute regulatory protein; PREG, pregnenolone; HDL, high-density lipoprotein; SRB1, scavenger receptor B1.

that it provides direct visualization, real-time images of dehydroergosterol in living cells (Fig. 2). Confocal laser scanning microscopy has also proven useful in revealing that, at least in some cell types, the synthetic fluorescent sterols (NBD-cholesterol) (20, 21, 27) and/or fluorescent sterol esters (BODIPY-cholesteryl ester) (28) may be effective markers for imaging the dynamics of the "selective" cholesterol and cholesteryl ester uptake, respectively, mediated by high-density lipoproteins (HDL) along with the scavenger receptor B1 (SRB1) localized in plasma membrane caveolae in living cells. These imaging technologies provide data in intact cells that is not possible to see with radiolabels: colocalization to specific organelles, intracellular distribution, dynamics of sterol movement from and between organelles, and diffusion of sterols through cytosol and within membranes.

Historical Perspective

Earlier reviews focused on membrane cholesterol organization in terms of structural domains (transbilayer and lateral) (23, 25, 29, 30). With the exception of the acetylcholinesterase receptor and the budding site for the AIDS virus, it was generally accepted that the majority of plasma membrane proteins was localized in cholesterol-poor domains. Consequently, there was little incentive to determine the functional significance for the cholesterol-rich domains. Nevertheless, an early hypothesis envisioned that the membrane organization of cholesterol may be the key in forming the basis for membrane protein organization (31). Surprisingly, research in the past 5 years has yielded growing evidence that contrary to expectation, attests that cholesterol-rich plasma membrane microdomains are the sites wherein

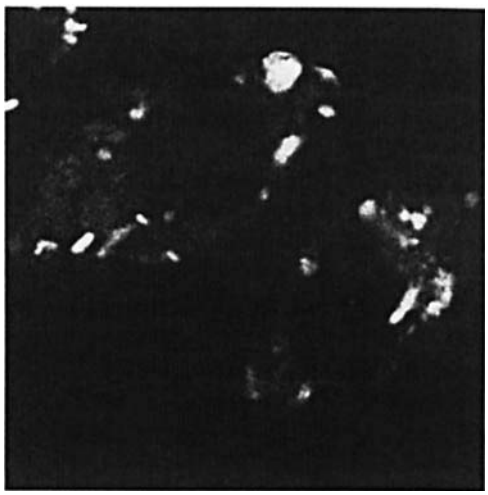

Figure 2. Multiphoton laser scanning microscopy of dehydroergosterol, a naturally occurring fluorescent sterol, in living L cell fibroblasts. The point made in this figure is that real-time, noninvasive imaging of sterols is now possible in living cells. The image was obtained by culturing L cells with 10% fetal bovine serum (FBS) medium containing 15 μg of dehydroergosterol/ml. Three-photon excitation was performed with a MIRA 900 Ti/Sa laser pumped at 12 W with a Sabre Ar ion laser (Coherent Inc., Sunnyvale, CA). Images were acquired at 63× (Axiovert 135 Inverted Microscope, Zeiss, NY) and a MRC1024MP Multiphoton Laser Scanning Imaging System (Bio-Rad, Hercules, CA). Dehydroergosterol fluorescence emission was detected near 375 nm through a dichroic filter.

proteins that function in diverse cellular processes (cholesterol uptake/efflux, signaling, T cell activation, B cell antigen signaling, antigen targeting, potocytosis [viruses and parasites], and sorting and trafficking through the secretory and endocytic pathways) are localized (9, 32–43, 43–56). The focus of the present review article is the role of lateral and transbilayer cholesterol domains and the proteins that mediate cholesterol uptake/efflux and intracellular trafficking.

Cellular Cholesterol Uptake via Plasma Membrane Lateral Microdomains. Although early work demonstrated the existence of lateral cholesterol-rich and cholesterol-poor domains in model membranes (23, 25), it was not until such domains were detected in the plasma membrane of the cell that their potential biological relevance became more apparent. Although cholesterol spontaneously transfers between vascular lipoproteins as well as from lipoproteins to the plasma membrane, this simple diffusive process is insufficient to account for the cellular uptake of lipoprotein lipids. Instead, the majority of cholesterol uptake and efflux occurs via a variety of specialized plasma membrane microdomains (Fig. 1).

Plasma membrane lateral microdomains for LDL uptake: clathrin-coated pits and the vesicular, lysosomal pathway. Most exogenous cholesterol and cholesterol ester comes from LDL particles taken up by cells via a specialized plasma membrane microdomain, the clathrin-coated pit (Fig. 1). LDL bind to the LDL receptors localized within the clathrin-coated pit, followed by formation of clathrin-coated vesicles, which subsequently become uncoated. Thereafter, a complex vesicular pathway (uncoated vesicles, sorting vesicles, recycling vesicles, etc.) selectively sorts both proteins and lipids (e.g., cholesterol and sphingolipid) for recycling back to the plasma membrane from the proteins and lipids (e.g., cholesteryl esters) that enter the lysosome for subsequent metabolism and trafficking to other intracellular sites (Fig. 1) (3).

Structure of the clathrin-coated pits/vesicles. The distribution of cholesterol to clathrin-coated pits has been investigated by use of histochemical stains such as filipin, a polyene macrolide antibiotic that binds cholesterol (57, 58). Based on the lack of filipin staining, it was originally concluded that clathrin-coated pit membranes were cholesterol free (59). However, the use of filipin to histochemically localize cholesterol may seriously underestimate the cholesterol content in certain types of membranes (59, 60). Indeed, removal of the clathrin coat enables filipin staining of cholesterol (61). Chemical analysis of isolated clathrin-coated vesicles and uncoated vesicles confirmed that these membrane microdomains/vesicles had approximately 56 and 50 μg cholesterol/mg protein, respectively, essentially the same as in the isolated plasma membrane (59 μg cholesterol/mg protein) (61). Thus, clathrin-coated pit and vesicle membranes derived therefrom are definitely not cholesterol-deficient membrane microdomains. However, the molar ratio of cholesterol/phospholipid of coated (0.3) and uncoated (0.36) vesicles membranes (61) was in the low range of that typically reported for the bulk (62) plasma membranes, 0.4 to 1.0 (25). Taken together, these results suggested that clathrin-coated pit membrane microdomains and vesicle membranes derived therefrom are not cholesterol deficient, but may be significantly enriched in phospholipid to a higher degree than for cholesterol. Other than the filipin staining and lipid analysis (cholesterol and phospholipid), nothing is known regarding the structural (transbilayer or lateral) organization of cholesterol and phospholipid species within the membranes of clathrin-coated pits, vesicles, or uncoated vesicles.

Differential sorting of cholesterol, cholesteryl esters, and proteins from clathrin-coated pits/vesicles. Early endocytosed vesicles containing LDL-unesterified cholesterol and cholesteryl ester enter a complex series of endocytic compartments, and certain types of lipids and ligand/receptor molecules return back to the plasma membrane via two distinct recycling pathways (63). These recycling vesicles are formed by budding off the sorting endosomal compartment or from the recycling endosomal compartment (63). One of these pathways (indicated by "recycling lipid" in Fig. 1) very rapidly (half-time of 1 to 2 min) recycles endocytosed lipids back to the plasma membrane, while the other pathway more slowly (half-time of 9 to 12 min) recycles receptor proteins (indicated by "recycling protein" in Fig. 1) such as the LDL receptor or transferrin back to the plasma membrane (63). Thus, endocytosed LDL-unesterified cholesterol and sphingomyelin (derived from the endocytosed plasma membrane) are selectively recycled

back to the plasma membrane without entering the late endosomes/lysosomal compartment. It has been suggested that some LDL-unesterified cholesterol is recycled from clathrin-coated pits without concomitant LDL-protein internalization (3). Vesicular recycling of LDL-unesterified cholesterol and sphingolipid back to the plasma membrane is very rapid with a halftime of 1 to 2 min (63, 64). Any remaining LDL-unesterified cholesterol, as well as the LDL-cholesteryl esters, are transferred via the vesicular pathway to the lysosome (3, 19, 65). The time required for transit of LDL-cholesteryl esters from the cell surface to the lysosome takes about 10 to 30 min (Fig. 1) (66).

Plasma membrane lateral microdomains: rafts. Plasma membrane microdomains termed "rafts" have now been identified as being distinct from clathrin-coated pits (Fig. 1). These rafts appear to represent a heterogeneous and/or transient group of plasma membrane microdomains (Fig. 3) that may be stabilized by certain proteins (39, 46, 48, 52–54, 56). Rafts are present not only in mammalian cells, but also in membranes of other phyla (67). While a variety of physiological functions are now becoming associated with rafts, cholesterol uptake has been clearly associated only with one subclass of plasma membrane rafts, i.e., caveolae (see below). For the purpose of the present review, rafts are defined as plasma membrane micropatches (domains) that do not contain clathrin or caveolin, but are rich in cholesterol, glycosphingolipids, GPI-anchored proteins, and signaling molecules (36, 68–70). The high concentration of cholesterol in rafts makes them susceptible to disruption by drugs that bind cholesterol, e.g., filipin. Caveolar rafts are a subclass of rafts defined as containing caveolin (see below). The remainder of this section describes only the noncaveolar rafts.

Structure of noncaveolar rafts. Rafts are flat, cholesterol-rich, sphingolipid-rich, GPI-linked protein-rich domains in cell plasma membranes. Rafts, visualized in cells by a variety of chemical and spectroscopic techniques, range in size from 70 to 370 nm (56, 71). They are insoluble in detergents such as Triton X-100 at 4°C (53, 56, 71). Clustering of glycosphingolipids and GPI-anchored proteins into rafts occurs in the presence (Fig. 1) or absence of proteins (e.g., in model membranes, Fig. 3). Raft formation

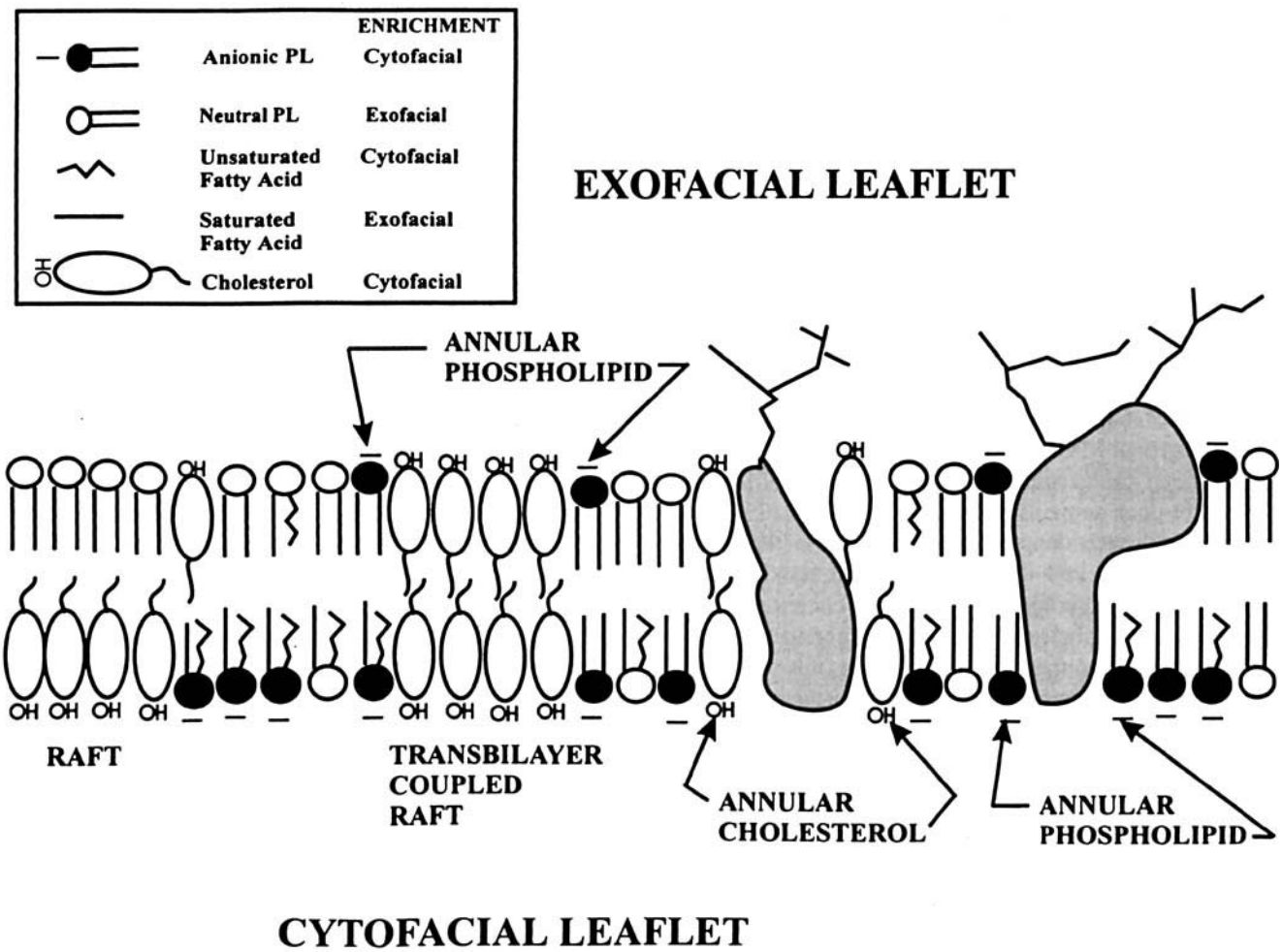

Figure 3. Plasma membrane lipid domains. •, Neutral zwitterionic phospholipids (PC, PE, and SP); ○, anionic phospholipids (PI, PIP, PIP_2, PS, etc.); straight lines and squiggly lines refer to saturated and unsaturated fatty acyl chains; ellipsoids refer to cholesterol. Annular phospholipid refers to the first molecular layer of phospholipid surrounding a cholesterol-rich domain or a protein. Annual cholesterol refers to the first molecular layer of cholesterol surrounding a protein.

is enhanced by cholesterol and detergent-resistant fractions enriched in cholesterol, and glycosphingolipid can be isolated from model membranes. These and other observations with model membrane systems led to the hypothesis that differential miscibility of lipids is the basis for formation of functional membrane rafts (53, 71). Lipid packing and transbilayer, as well as lateral asymmetry, are thought to be the driving force for raft formation. The detergent-resistant membrane rafts have a bilayer appearance and raft Src kinase is found only on the cytofacial leaflet, suggesting that rafts are present in both leaflets of the plasma membrane bilayer (Fig. 3) (46). Rafts are stabilized by the presence of specific proteins. It should be noted that while detergent-resistant fractions with similar lipid and protein composition have been isolated from plasma membranes, the nature of the *in vivo* correlate of such detergent-resistant membranes remains enigmatic (56).

Function of noncaveolar rafts. Certain proteins (e.g., reggie-1, reggie-2,GM3, sSrc, RhoA, and B cell antigen receptor) selectively associate with rafts, while others (clathrin, caveolin, and Ras) are excluded (50, 69, 70). Growing evidence links rafts to intracellular signal transduction, cell adhesion, and regulation of polarized intracellular sorting (39, 46, 70). While noncaveolar rafts and caveolae, as well as their associated functions, are often disrupted by cholesterol binding agents (filipin and nystatin), these drugs did not abolish or reduce GM3-dependent adhesion (a protein in rafts but not caveolae) of B16 melanoma cells (70). It is not yet clear whether the noncaveolar rafts have a role cellular cholesterol uptake/efflux.

Plasma membrane lateral microdomains for HDL cholesterol uptake via caveolar rafts: the nonvesicular, nonlysosomal pathway. Caveolae are a subclass of plasma membrane microdomains rafts (see above) that contain caveolin. Although caveolae are highly enriched in proteins that function in signal transduction (32–34, 37, 38, 41, 42, 49, 51, 72), the focus of this review is on their role in cellular cholesterol uptake/efflux.

Structure of plasma membrane caveolar rafts, caveolae. Caveolae were recognized as a morphologically distinct "small pockets, caves, or recesses" (also called "flask-shaped") at the cell surface plasma membrane as early as 1955 (41). Caveolin is localized at the cytoplasmic face of the caveolae and does not cross the caveolar membrane (9). Although caveolae have been isolated, their exact lipid composition is not well understood. Certain lipid classes appear to be enriched in caveolae as compared with the plasma membrane, including cholesterol, sphingomyelin, phosphatididylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylinositol P_2, ceramide, and diacylglycerol (9, 41). (However, this should not be taken to mean that other lipid fractions such as phosphatidylcholine are absent from caveolae, an issue that needs to be addressed by more in-depth analyses of caveolar lipids.) Quantitatively, caveolae contain disproportionate amounts of several lipid classes as compared with the bulk plasma membrane. While caveolae comprise 1% of the plasma membrane surface area, they contain 10% of plasma membrane cholesterol, i.e., they appear 10-fold enriched in cholesterol as compared with the bulk plasma membrane. This cholesterol is essential for maintaining caveolae morphology. Depletion of cholesterol by cholesterol-binding drugs (filipin) or by cholesterol oxidase disrupts caveolar structure and functions. Caveolae are also highly enriched in sphingomyelin (50%–96%), and in signaling lipids such as phosphatidylinositol P_2 (50%), ceramide (50%), and diacylglycerol (50%) (9). Almost nothing is known regarding the structural (lateral or transbilayer) organization of lipids in the caveolar membrane.

Caveolins: proteins that stabilize caveolae rafts. The caveolin gene family contains at least three members (caveolins 1, 2, and 3) and two isoforms (caveolin-1α and caveolin-1α) (9, 41, 47). Caveolin-1 and -2 are broadly distributed among many tissues, while caveolin-3 is primarily found in muscle. The majority of caveolar proteins are associated with caveolae, with lesser amounts in endoplasmic reticulum, Golgi, and cytosol (9).

Caveolae rafts are the plasma membrane microdomain through which the selective uptake of HDL cholesterol occurs. The cell surface scavenger receptor (SRB1) functions as an HDL receptor that mediates nonendocytic, selective uptake of cholesterol (Fig. 1) (20, 73). In the absence of the LDL receptor, overexpression of SRB1 mediates significant transport of sterols between HDL (and LDL) and the cell (74). SRB1 is concentrated primarily in caveolae (75). SRB1 interacts with HDL to mediate rapid (min) cholesterol cellular uptake by an as yet unresolved mechanism (9, 76). In the absence of serum HDL, cholesterol entering the cholesterol-rich caveolar domain slowly diffuses laterally to other microdomains within the plasma membrane (77).

It is important to note that caveolin/caveolae are essential for HDL-mediated cholesterol uptake. Cholesterol binding by filipin or oxidation by cholesterol oxidase disrupts caveolae shifts caveolin from the plasma membrane to the endoplasmic reticulum/Golgi, and inhibits HDL-mediated cholesterol uptake (9). Overexpression of caveolin-1 enhances HDL-mediated cholesterol uptake (9).

Mechanism whereby caveolin mediates selective uptake of HDL-derived cholesterol within the cell. Whether the transfer of HDL-derived cholesterol and cholesterol ester to Golgi and possibly other sites occurs by vesicular and/or protein-mediated, nonvesicular transfer is currently debated (9). Since direct evidence for a caveolin-containing cholesterol transport vesicle is lacking, an alternate nonvesicular mechanism has been proposed (9). Caveolin binds cholesterol (78), and a cytoplasmic complex of caveolin/cholesterol/chaperonins has been isolated (79). This may explain how caveolin, a relatively hydrophobic protein acylated with three palmitoyl groups, may transport cholesterol through the plasma membrane caveolae and therefrom through the cytoplasm to the Golgi/endoplasmic reticulum.

Caveolae rafts also function in the selective uptake pathway for HDL-cholesteryl ester. The scavenger receptor (SRB1) functions as an HDL receptor that mediates nonendocytic, selective uptake of cholesteryl esters (Fig. 1) (28, 80–82). This process is especially active in steroidogenic cells wherein the selective uptake of cholesteryl esters is an important source of cholesterol for steroidogenesis (28, 82). SRB1 interacts with HDL to mediate rapid (<5 min) cholesteryl ester uptake by a nonvesicular mechanism that does not go through the lysosome. Interestingly, the HDL-derived cholesteryl esters remain intact (i.e., are not hydrolyzed) and are directly stored in lipid droplets (82). The HDL-mediated uptake of fluorescent BODIPY-cholesteryl esters has been imaged in living cells (28, 82).

Mechanism whereby caveolin mediates selective uptake of HDL-cholesteryl ester. The mechanism whereby the hydrophobic cholesteryl esters leave the HDL, enter the plasma membrane caveolae, and translocate across the caveolar membrane is not known. Evidence suggests that the cholesteryl esters traffic to intracellular sites (lipid droplets and endoplasmic reticulum/Golgi) by a vesicular mechanism that does not involve the lysosome (82).

Caveolae rafts function in cellular fatty acid uptake. Cholesterol and fatty acid metabolism are coregulated (83–89). In fact, fatty acids elicit redistribution of intracellular cholesterol pools (90) and plasma membrane cholesterol transbilayer distribution (91). Thus, it is interesting to note that fatty acid uptake, as well as the "selective" uptake of cholesterol and cholesteryl ester, is mediated through plasma membrane caveolae. At least two putative fatty acid transport proteins, caveolin, and CD36 (also called fatty acid translocase [FAT]) are localized in caveolae (9, 92). Caveolin (93–95) and CD36/FAT (96) both bind fatty acids. Caveolin (95) and CD36/FAT (97, 98) transport fatty acids into the cell. Thus, caveolae represent a plasma membrane microdomain wherein transport proteins for fatty acids as well as cholesterol and cholesteryl esters are localized.

Plasma membrane lateral microdomains: desmosomes. Desmosomes are large plasma membrane domains localized at intercellular junctions. While desmosomes are insensitive to filipin (59), chemical analysis of isolated desmosomes reveals the presence of cholesterol. Although isolated desmosomes are relatively lipid poor, containing only 10% lipid vs 76% protein mass (99), cholesterol and phospholipid comprise 40% and 60% of lipid mass, respectively, of the lipids. This equates to a cholesterol/phospholipid molar ratio near 1.1 (99). Thus, desmosomes have low cholesterol and phospholipid content, but high cholesterol/phospholipid ratios in the range of those reported for plasma membranes (25)

Plasma membrane lateral microdomains: crystalline, pure cholesterol domains. Crystalline cholesterol domains have been reported to form in model membranes at very high cholesterol/phospholipid ratios (100). Plasma membranes from arterial smooth muscle cells enriched in cholesterol have been reported to contain crystalline cholesterol (101). Finally, crystalline cholesterol has also been detected in atherosclerotic plaques (102, 103). These data have been taken to suggest that crystalline cholesterol domains may play a role in atherosclerosis.

Plasma membrane cholesterol content regulates membrane fluidity and function. The ratio of cholesterol to phospholipid is a primary regulator of membrane structure, fluidity, and function (29, 104–110). However, nothing is known regarding the fluidity of membrane microdomain rafts. Since high cholesterol/phospholipid ratios make membranes very rigid and transport proteins function optimally in very narrow ranges of fluidity (104–107), it is an enigma as to how cholesterol-rich rafts, caveolae, etc. are the site wherein many signaling and transport proteins are localized and still maintain function within the cell membrane. The fluidity of lipids within the plasma membrane microdomains such as rafts and caveolae has not been determined.

Intracellular Cholesterol Trafficking of LDL-Derived Cholesterol from the Lysosome. Significant advances have been made in our understanding of the intracellular mechanism(s)/pathway(s) of cholesterol trafficking from the lysosome to intracellular sites for utilization or for efflux from the cell (1–21). The overall transit time of LDL-cholesterol ester from the plasma membrane clathrin-coated pit to the lysosome, intralysosomal hydrolysis, transfer of released cholesterol to trans-Golgi and/or endoplasmic reticulum, followed by transfer to the plasma membrane caveolae for efflux has been reported to be 37 to 50 min (66, 111, 112). However, some steps within this pathway(s) are very fast. For example, intralysosomal hydrolysis of cholesteryl ester by an acidic lipase occurs very rapidly and, once hydrolyzed, the released unesterified cholesterol appears at the plasma membrane within <2 min (Fig. 1) (66). From the available evidence 5 years ago, the mechanism of cholesterol transport out of the lysosome was still unclear (24, 25).

Mechanism(s) of cholesterol efflux from the lysosomal compartment: spontaneous diffusion. Cholesterol transits rapidly ($t_{1/2}$ <2 min) from the lysosome to the plasma membrane (66) and does not require intact cytoskeletal organization (113). This (24) suggested to some investigators that release of cholesterol from cholesteryl ester in the lysosomal matrix, entry of released cholesterol into the lysosomal membrane for translocation, and cholesterol desorption/vesiculation from the lysosomal membrane for trafficking to intracellular sites was rapid, diffusional, and did not require a protein (114). However, several lines of evidence indicate that this is not the case. First, Niemann-Pick C (NPC) disease is a genetic defect in a late endosomal/lysosomal membrane protein that results in cholesterol accumulation in the lysosome (see below). Second, spontaneous desorption of cholesterol from lysosomes and lysosomal membranes, purified mouse L cell fibroblasts (4, 115), and human fibroblasts (see below) was extremely

slow, with halftimes of >4 days and several days, respectively. This was 2 to 3 orders of magnitude slower than that observed with intact cells and 40- to 60-fold slower than from any other previously reported membrane. Therefore, these and other studies ruled out rapid, spontaneous diffusion as accounting for the rapid transit of cholesterol out of the lysosomal membrane. Further, these data suggested that protein(s) and/or vesicular mediated process(es) may be involved.

Protein-mediated cholesterol transfer from the lysosomal membrane: role of the Niemann-Pick C1 protein. The NPC1 protein is a membrane protein, contains a sterol sensing domain, and is associated, at least in part, with the lysosomal membrane (116, 117). The possibility that the defective protein may also alter spontaneous cholesterol transfer from the lysosomal membrane was examined. Lysosomal membranes were isolated from cultured normal (CWN) and NPC1 human fibroblasts exactly as described earlier for isolation of lysosomal membranes from L cell fibroblasts (115). The spontaneous transfer of sterol from these lysosomal membranes was then measured using a fluorescent sterol (dehydroergosterol) transfer assay as described earlier (115).

The basic characteristics of spontaneous sterol exchange between normal (CWN) lysosomal membrane donors and acceptors are depicted in Figure 4. In the absence of lysosomal membrane acceptors, dehydroergosterol polarization in lysosomal membrane donors was unaltered over the time period examined (not shown). However, addition of 10-fold excess acceptor lysosomal membranes elicited a slow spontaneous sterol exchange from lysosomal donor membranes to acceptor membranes (Fig. 4, bottom curve). The initial rate of molecular sterol transfer from normal CWN lysosomal membranes, 0.008 ± 0.002 pmol/min, was very slow and similar to that observed with L cell fibroblast lysosomal membranes (Table I). The spontaneous transfer of sterol from the NPC1 lysosomal membrane was also slow (Fig. 5, bottom curve), but exhibited a 2-fold faster initial rate of molecular sterol transfer, 0.015 ± 0.003 pmol/min (Table I). The slow molecular transfer of cholesterol from both CWN and NPC1 fibroblast lysosomal membranes was reflected in long half-times, i.e., days (Table II).

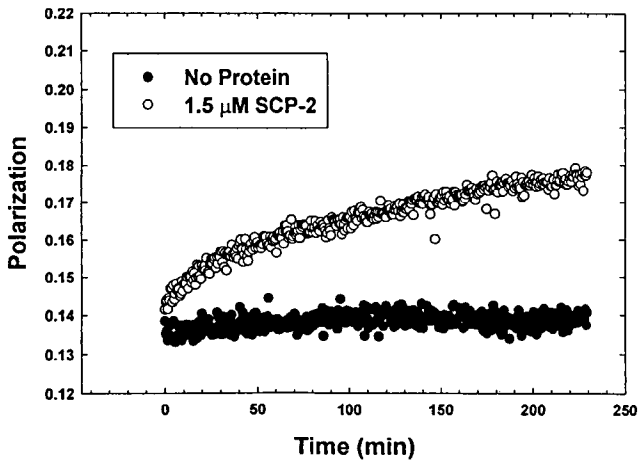

Figure 4. Effect of SCP-2 and on sterol exchange between lysosomal donor and lysosomal acceptor membranes isolated from normal human CWN fibroblasts. The purpose of this figure is to show that for normal human fibroblasts, spontaneous cholesterol transfer out of the lysosomal membrane is extremely slow, and cholesterol-binding proteins such as SCP-2 dramatically enhance cholesterol transfer out of the lysosomal membrane. The curves show the change in dehydroergosterol fluorescence polarization as a function of time after addition of a 10-fold excess of acceptor (dehydroergosterol-deficient) membranes to donor (dehydroergosterol-rich) membranes.

In summary, the NPC1 defect did not significantly slow the initial rate of spontaneous sterol transfer from the lysosomal membrane. Instead, spontaneous sterol transfer out of the NPC1 lysosomal membrane was enhanced. Therefore, it is unlikely that spontaneous sterol transfer contributed to the accumulation of cholesterol in the NPC1 lysosome. Finally, the observation of slow (half-times of days) spontaneous transfer of cholesterol from the lysosomal membranes of human fibroblasts was consistent with additional processes (vesicular and/or protein mediated) being required to account for the rapid movement of cholesterol out of the lysosomal compartment observed in intact cells.

Protein-mediated cholesterol transfer from the lysosomal membrane: involvement of caveolin. As indicated in the preceding sections, caveolin participates in the transfer of multiple lipids between plasma membrane and Golgi/endoplasmic reticulum. Caveolin-1 is associated with caveolar vesicles and with soluble, cytosolic caveolin/cholesterol/chaperonin complexes. The expression of caveolin-1 in liver of mice containing mutant (inactive) NPC protein was examined (118). Caveolin-1 expression was elevated 4.9- and 1.6-fold in livers taken from heterozygous (one normal and one mutant NPC allele) mice and mice homozygous (two mutant NPC alleles) for the NPC mutation, respectively (118). Caveolin-1 protein expression in heterozygous human NPC fibroblasts was increased 10-fold, while that of homozygous NPC fibroblasts was unchanged or slightly elevated (119). These data suggest that caveolin-1-containing vesicles and/or caveolin/cholesterol/

Table I. Initial rates of cholesterol transfer from lysosomal membranes isolated from mouse L-cell fibroblasts, normal CWN human fibroblasts, and NPC1 human fibroblasts

	Protein	Initial rate (pmol/min)[a]
L-Cell[b]	None	0.010 ± 0.002
	SCP-2	0.50 ± 0.08
CWN	None	0.008 ± 0.002
	SCP-2	0.185 ± 0.010
NPC	None	0.015 ± 0.003
	SCP-2	0.382 ± 0.020

[a] Units are per milliliter of medium, n = 3–9.
[b] Initial rates of molecular sterol transfer were taken from Ref. 115.

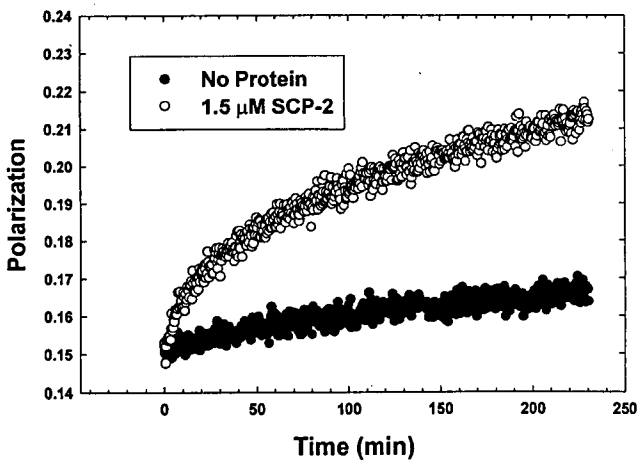

Figure 5. Effect of SCP-2 on sterol exchange between lysosomal donor and lysosomal acceptor membranes isolated from human NPC1 fibroblasts. The main point to be made in this figure is that the Niemann-Pick C defect does not result inhibit either spontaneous or SCP-2-mediated sterol transfer out of the lysosomal membrane. The curves show the change in dehydroergosterol fluorescence polarization as function of time after addition of a 10-fold excess of acceptor (dehydroergosterol-deficient) membranes to donor (dehydroergosterol-rich) membranes.

chaperonin complexes may be involved in the pathophysiology of NPC disease. Possibly the increased caveolin-1 represents a compensatory attempt by the cell to remove the cholesterol accumulated in the Golgi/endoplasmic reticulum and/or lysosomes. It remains to be determined if caveolin/caveolae participate in cholesterol trafficking out of the lysosomal compartment.

Protein-mediated cholesterol transfer from the lysosomal membrane: role of sterol carrier protein-2. Adult NPC mice are deficient in SCP-2 (120). Liver SCP-2 and SCP-x levels were reduced 70% to 90% and 83%, respectively, in adult NPC mice (120). Thus, the decreased removal of cholesterol from the lysosomal compartment in NPC disease may be due in part to decreased intracellular levels of SCP-2. SCP-2 mediates sterol transfer *in vitro*, in intact cells, and in animals (4). For example, SCP-2 is important in transfer of cholesterol from the endoplasmic reticulum for secretion into bile (121–123). In SCP-2 gene-ablated mice, overcompensatory upregulation (124) of liver fatty acid binding protein (L-FABP, another cholesterol binding protein) (125, 126) accounts for the hypersecretion of cholesterol in the bile (127). Despite the above data, there has been considerable controversy regarding a function of SCP-2 in intracellular cholesterol trafficking (4, 17). This controversy is based at least in part on interpretation of data regarding the intracellular localization of sterol carrier protein-2 (SCP-2).

Intracellular localization of SCP-2. Because SCP-2 contains a C-terminal peroxisomal targeting sequence and is present in high concentrations in peroxisomes, it has been suggested that this protein is exclusively peroxisomal (17, 128). The observation that cellular subfractionation of liver, testis, brain, kidney, lung, and intestine detected SCP-2 in cytosol and other membrane fractions in addition to peroxisomes (129, 130) is discounted and is attributed to the fragility of peroxisomes upon tissue homogenization. However, other data are not as easily dismissed.

Immunocytochemistry (immunogold electron microscopy and immunofluorescence microscopy), not subject to this potential artifact, detected highest concentration of SCP-2 in peroxisomes and, in addition, found significant quantities of extraperoxisomal SCP-2 in tissues expressing high SCP-2 levels (liver [130–134], steroidogenic tissues [135–137], tissues expressing lower levels of SCP-2 [intestine [132], and transfected cultured cells expressing SCP-2 [L cells and hepatoma cells [138–141]). Based on the above immunocytochemical data and the fact that peroxisomes represent <1% of cell volume, it has been estimated that as much as 50% of SCP-2 is extraperoxisomal (4, 140, 141).

Molecular basis for the intracellular localization of sterol carrier protein-2 (SCP-2). Recent data shed light on the apparent contradiction of predicted intracellular SCP-2 localization based on primary amino acid sequence (i.e., C-terminal SKL peroxisomal targeting sequence) versus the immunocytochemical data showing that a major portion of SCP-2 is extraperoxisomal (140, 141). The SCP-2 gene codes for a 15-kDa pro-SCP-2 precursor of the 13-kDa SCP-2. While both proteins share the same C-terminal SKL, pro-SCP-2 contains a 20-amino acid presequence. However, in all tissues and almost all cells examined, the N-terminal 20-amino acid extension in pro-SCP-2 is rapidly cleaved post-translationally to the mature SCP-2. The process is so rapid that 15-kDa pro-SCP-2 is not detectable. Overexpression of the 13-kDa SCP-2 (lacking the 20-amino acid N-terminal presequence) in transfected L cells followed by immunofluorescence confocal imaging showed that very little SCP-2 colocalized with peroxisomes. In contrast, when L cells and hepatoma cells were transfected with the 15-kDa pro-SCP-2 (completely post-translationally processed to the mature 13-kDa SCP-2) immunofluorescence confocal imaging revealed that about one-half (42% and 58%, respectively) of SCP-2 immunoreactivity was localized in peroxisomes. These data provided the first clue that the N-terminal presequence of the 15-kDa pro-SCP-2 was essential for the C-terminal SKL peroxisomal targeting sequence to interact with the PTS1 receptor of the peroxisome for import of the SCP-2.

Comparison of the tertiary and secondary structures of purified 13-kDa SCP-2 and 15-kDa pro-SCP-2 with anti-SCP-2 antibodies and circular dichroism showed that these proteins differed significantly. Furthermore, anti-SKL antisera and carboxypeptidase A (cleaves C-terminal Leu) showed that the C-terminal SKL and L of pro-SCP-2 were much more exposed at the surface of the protein than those of SCP-2.

In summary, predictions of exclusive SCP-2 peroxisomal targeting cannot be based solely on primary amino acid sequence alone. Secondary or tertiary folding also play

Table II. Cholesterol exchange dynamics from lysosomal membranes isolated from mouse L-cell fibroblasts, normal CWN human fibroblasts, and NPC1 human fibroblasts

Cells	Protein	$^1t_{1/2}$	$^2t_{1/2}$	f_1	f_2	f_3
L-CELL[a]	None	—	Days	—	—	1.000
	SCP-2	5.5 ± 0.8	154 ± 12	0.059 ± 0.008	0.363 ± 0.012	0.578 ± 0.022
CWN	None	—	Days	—	—	1.000
	SCP-2	10.1 ± 2.4	50.4 ± 6.5	0.022 ± 0.010	0.270 ± 0.010	0.708 ± 0.009
NPC	None	—	Days	—	—	1.000
	SCP-2	4.0 ± 2.1	195 ± 15	0.018 ± 0.009	0.30 ± 0.041	0.682 ± 0.02

Note. Here the half times $^1t_{1/2}$ and $^2t_{1/2}$ of the runs were in minutes. F_1 and f_2 refer to the respective fractional contributions due to each half-time, and f_3 refers to the nonexchangeable fraction. Values represent the mean ± SEM (n = 3).
[a] Taken from Ref. 115.

a role in determining the exposure of targeting sequences, especially for SCP-2.

Protein-mediated cholesterol transfer out of the human fibroblast lysosomal membrane: a potential role for SCP-2. Although immunocytochemical data showed that SCP-2 was not tightly associated with lysosomes (115), the SCP-2 N terminus contains an amphipathic helix that interacts with anionic phospholipid-containing membranes (142, 143) to allow SCP-2-mediated sterol transfer (4, 25). Based on the significant extraperoxisomal localization, the possibility that SCP-2-mediated cholesterol transfer out of the lysosomal membrane was considered. SCP-2 dramatically (as much as 50-fold) enhances the transfer of cholesterol out of the lysosomal membrane isolated from L cell fibroblasts (4, 115, 144). Therefore, the ability of SCP-2 to mediate sterol transfer out of lysosomal membranes isolated from normal CWN and NPC1 human fibroblasts was measured exactly as described earlier for lysosomes from L cell fibroblasts (115).

The basic characteristics of SCP-2-mediated exchange between normal (CWN) lysosomal membrane donors and acceptors are depicted in Figure 4. In the absence of lysosomal membrane acceptors, dehydroergosterol polarization in lysosomal membrane donors was unaltered over the time period examined (not shown). However, addition of 10-fold excess acceptor lysosomal membranes elicited a rapid, SCP-2-mediated sterol transfer from normal CWN lysosomal donor membranes to acceptor membranes (Fig. 4, top). The initial rate of SCP-2-mediated, molecular sterol transfer from normal CWN lysosomal membranes (0.185 ± 0.010 pmol/min) was enhanced 25-fold as compared with the absence of SCP-2 (i.e., spontaneous transfer; Table I). The SCP-2-mediated transfer of sterol from the NPC1 lysosomal membrane was accelerated (Fig. 5, top curve) such that the initial rate of molecular sterol transfer from NPC1 lysosomal membranes, 0.382 ± 0.020 pmol/min, was stimulated 25-fold as compared with the absence of SCP-2 (i.e., spontaneous transfer; Table I). However, the data clearly show that under the same conditions, the SCP-2-mediated initial rate of molecular sterol transfer from NPC1 fibroblast lysosomal membranes was 2.1-fold faster than from normal CWN fibroblast lysosomal membranes (Table I).

Multiexponential analysis of SCP-2-mediated sterol transfer from normal CWN fibroblast lysosomal membranes showed that SCP-2 dramatically altered the lysosomal sterol domains. SCP-2 reduced the fraction of nonexchangeable domain to 0.708 (Table II) by inducing formation of two exchangeable sterol domains: a small (2.2% of total), rapidly exchangeable ($t_{1/2}$ = 10.1 ± 2.4 min) sterol domain; and a larger (27% of total), slowly exchangeable ($t_{1/2}$ = 50.4 ± 6.5 min) sterol domain (Table II). While SCP-2 elicited similar qualitative effects in NPC1 fibroblast lysosomal membranes, these effects differed quantitatively from those in normal CWN fibroblast lysosomal membranes (Table II). The $t_{1/2}$ of the SCP-2 induced rapidly exchangeable sterol domain in NPC fibroblast lysosomal membranes (4.0 ± 2.1 min) was 2.5-fold faster than that of CWN fibroblast lysosomal membranes (Table II). Therefore, the rapidly exchangeable sterol domain revealed similar effects of NPC1 as observed with the initial rates (Table I). In contrast, the $t_{1/2}$ of the SCP-2-induced slowly exchangeable sterol domain in NPC fibroblast lysosomal membranes (195 ± 15 min) was nearly 4-fold slower than that of CWN fibroblast lysosomal membranes (Table II). Average $t_{1/2}$'s calculated from these data were about 14 min for lysosomes from normal CWN fibroblasts and 59 min (owing to a larger half-time in this fraction) for lysosomal membranes from NPC1 fibroblasts. Although the initial rate of SCP-2-mediated sterol transfer of cholesterol out of the lysosomal membrane is increased 2-fold more in lysosomes from NPC1 cells than normal CWN cells, the half-time to complete the SCP-2-mediated sterol transfer was 4.2-fold slower in NPC cells. Thus, while spontaneous sterol transfer from NPC lysosomal membranes was faster than from the normal CWN lysosomal membranes, the half-times revealed that it took 4-fold longer for SCP-2 to achieve half-maximum sterol transfer from the NPC lysosomal membranes. This was probably due to greater accumulation of cholesterol in the NPC lysosome/lysosomal membrane than that of the normal CWN lysosome/lysosomal membrane.

The following points may be made from these data: First, SCP-2 dramatically enhanced the molecular transfer of sterol from human fibroblast lysosomal membranes. Second, SCP-2 induced the formation of rapidly exchangeable sterol domains in human and mouse fibroblast lysosomal membranes. Third, the time frame wherein SCP-2 elicits

detectable sterol transfer from the lysosomal membranes is seconds to minutes. This time frame is similar to that reported in intact cells, <2 min (66), and is consistent with the possibility of rapid protein mediated sterol transfer out of the lysosomal compartment, in intact cells the transfer of cholesterol from lysosomes to the plasma membrane is microtubule and Golgi independent (24, 113, 145). Fourth, cholesterol accumulation in lysosomes of NPC1 cells does not result from a defect in spontaneous sterol transfer from the NPC1 fibroblast lysosomal membrane. Fifth, cholesterol accumulation in lysosomes of NPC1 cells does not result from a defect in SCP-2-mediated sterol transfer from the NPC1 fibroblast lysosomal membrane. However, the dramatic decrease in SCP-2 in NPC1 tissues may contribute to the slow removal of cholesterol from the lysosomal and/or Golgi/endoplasmic reticulum compartment. Concomitant upregulation of caveolin-1 may, however, in part compensate for the NPC1 defect and loss of SCP-2.

Vesicular-mediated efflux of cholesterol from the lysosomal membrane: role of the NPC protein. Important new insights into the role of vesicular transfer of cholesterol and other lipids from the lysosomal compartment have been made through study of NPC disease. NPC is characterized by abnormal accumulation of LDL-derived cholesterol in lysosomes and the Golgi complex (10, 146, 147). The NPC protein, recently cloned and shown to be a membrane protein (116), associates predominantly with late endosomes (Rab9 GTPase-positive vesicles), and to a lesser extent, with lysosomes and the trans-Golgi network (117, 148). The vesicular compartment wherein NPC1 protein resides contains LAMP2, a marker for both late endosomes and lysosomes (117). A mathematical model to determine the turnover times of endocytic cargo ([^{14}C]-sucrose) through the late endosomal compartment and the lysosome of normal human fibroblasts yielded turnover times of 1 hr and 24 hr, respectively (117). In NPC1 fibroblasts, the turnover time of the late endosomal vesicles was 47% slower, while that of the lysosomal compartment did not differ. These and the immunolocalization data were interpreted to indicate that the late-endosomal vesicles are involved in retrograde late endosomal vesicle transfer of cholesterol as well as other lipids from the lysosome to the Golgi (117). The accumulation of cholesterol in lysosomes and Golgi/endoplasmic reticulum of NPC1 cells was therefore interpreted to result from the reduced, late-endosomal vesicle-mediated transfer of cholesterol out of the lysosomal compartment. How the sucrose turnover times of the late endosomal compartment (1 hr) (117) relate to the reported rapid (<2 min) transit time (66) for cholesterol out of the lysosome and to the plasma membrane is unclear.

Intracellular Cholesterol Trafficking of Lysosomal Cholesterol to Other Intracellular Organelles/Membranes. Significant advances have been made in our understanding of the intracellular mechanism(s)/pathway(s) of cholesterol trafficking from the lysosome to intracellular sites for utilization or for efflux from the cell (1–21).

Cholesterol trafficking out of the lysosome and to other intracellular sites: plasma membranes, Golgi, endoplasmic reticulum, and mitochondria. As indicated by the many "unknowns" in Figure 1, there is considerable uncertainty on the route(s) and mechanism(s) of cholesterol movement once it leaves the lysosome (6, 10, 12, 13). Evidence has accumulated for several pathways whereby exogenously derived cholesterol traffics from the lysosome to intracellular sites:

Lysosomal cholesterol transiting to the plasma membrane by protein-mediated, nonvesicular transfer. Several studies are consistent with the possibility that protein-mediated, nonvesicular cholesterol transfer occurs between the lysosome and the plasma membrane. The rapid transit of lysosomally derived cholesterol to the plasma membrane in intact cells occurs with a half-time <2 min (66). Transfer of cholesterol from the lysosome to the plasma membrane of intact cells lacks a requirement for the intact cytoskeleton (113). While the specific protein(s) that mediate transfer of lysosomal cholesterol to the plasma membrane in intact living cells have not yet been identified, recent data on sterol transfer between isolated lysosomal and plasma membranes *in vitro* suggest that SCP-2 may at least in part serve this function[1]. The evidence supporting this possibility is as follows: Spontaneous sterol transfer from lysosomal membranes to the plasma membrane was extremely slow, with a $t_{1/2}$ of days. SCP-2-mediated sterol transfer from lysosomal to plasma membranes was detectable within 30 sec. SCP-2 decreased the $t_{1/2}$ of this transfer 364-fold from 4 days to 7 to 15 min. SCP-2 induced the formation of rapidly transferable sterol domains. Taken together, these data suggest that protein-mediated, nonvesicular cholesterol transfer may at least in part contribute to the transfer of lysosomal cholesterol to the plasma membrane.

Lysosomal cholesterol transiting from lysosomes: vesicular transfer via the Golgi to the plasma membrane and subsequently to the endoplasmic reticulum. The majority (two-thirds) of lysosomal cholesterol has been postulated to traffic by vesicular transfer to the Golgi followed by subsequent transfer to the plasma membrane and thence to the endoplasmic reticulum (10). The vesicular transit time for transfer of cholesterol from the trans-Golgi or endoplasmic reticulum to the plasma membrane is about 10 to 20 min (3, 4, 7). How these slower vesicular-mediated sterol transfer pathway(s) relate to the observed rapid ($t_{1/2}$ <2 min) (66) transfer of cholesterol from the lysosome to the plasma membrane in living cells is unclear. In contrast, SCP-2-mediated cholesterol transfer times are much faster (1, 2, 4), and in the case of the *in vitro* transfer of sterol from isolated lysosomal membranes to plasma membranes, it occurs in <30 sec[1]. This suggests that both rapid protein-mediated and

[1] A.M. Gallegos, B.P. Atshaves, S. Storey, A.L. McIntosh, A. Petrescu, and F. Schroeder, manuscript submitted for publication.

slower vesicle-mediated cholesterol occur. However, it is not yet known how the relative proportions are determined in a particular cell/tissue type.

Lysosomal cholesterol transiting from lysosomes: vesicular transfer via the Golgi to the endoplasmic reticulum without the plasma membrane intermediate. Another potential mechanism whereby cholesterol traffics to endoplasmic reticulum is via the Golgi without the plasma membrane as an intermediate (112, 149, 150). Pharmacological inhibitors and studies with the NPC defect revealed that in the presence of intact Golgi, cholesterol transports from the lysosome to the Golgi and thence to the endoplasmic reticulum (10). This transfer, thought to be a vesicle-mediated event (6), was estimated to account for one-third of cholesterol movement out of the lysosome (10). The remaining two-thirds of lysosomal cholesterol is thought to traffic by vesicular transfer first to the Golgi, thence to the plasma membrane, and finally to the endoplasmic reticulum (10). Finally, when the Golgi are pharmacologically disrupted, all the lysosomal cholesterol can traffic directly to the endoplasmic reticulum (10). Pharmacological disruption is thought to result in merging of the disrupted Golgi with the endoplasmic reticulum.

Lysosomal cholesterol transiting from lysosomes directly to the endoplasmic reticulum: protein-mediated transfer. *In vitro* studies of spontaneous sterol transfer from isolated lysosomal membranes and endoplasmic reticulum, performed using a fluorescent sterol transfer assay similar to that for other membranes (1, 2, 144), demonstrated that this process is very slow, 0.024 ± 0.003 pmol/min, with a $t_{1/2}$ of days (not shown). Thus, either vesicular- and/or protein-mediated transfer must account for sterol trafficking from the lysosome to the endoplasmic reticulum. The preceding sections suggest that complex vesicular-mediated pathways transport cholesterol (via Golgi and/or plasma membrane) to the endoplasmic reticulum of intact cells. However, when the Golgi are pharmacologically disrupted, then all the lysosomal cholesterol can traffic directly from the lysosome to the endoplasmic reticulum (10). While pharmacological disruption was interpreted to result in merging of the disrupted Golgi with the endoplasmic reticulum, an alternate possibility is that protein-mediated cholesterol transfer may be upregulated to compensate for the inhibition of vesicular transfer.

Rapid, SCP-2-mediated cholesterol transfer and vesicular cholesterol transfer exhibit such a compensatory relationship in intact cells. When fibroblasts are treated with antisense cDNA to SCP-2, vesicular cholesterol transfer is upregulated in intact cells (151). When SCP-2 is overexpressed in transfected L cell fibroblasts, the rapid protein-mediated transfer of cholesterol within the cell is enhanced and vesicular cholesterol transfer is inhibited in intact cells (21). To determine if SCP-2 expression actually increased cholesterol distribution to the plasma membrane of intact cells, the endoplasmic reticulum fraction was isolated from control and SCP-2-expressing L cells by methods basically a reported previously (1, 2). Extraction and analysis of the endoplasmic reticulum lipids showed that SCP-2 expression dramatically increased the cholesterol mass and percent composition by 2.2-fold (Table III). Consequently, SCP-2 expression increased the molar ratios of cholesterol/phospholipid and cholesterol/cholesteryl ester by 1.5- and 1.6-fold, respectively, in the endoplasmic reticulum (Table III).

Specificity of protein-mediated cholesterol accumulation in the endoplasmic reticulum. The preceding section demonstrated that SCP-2 expression elicited cholesterol redistribution to the endoplasmic reticulum, consistent with its observed effects on mediating cholesterol transfer between membranes *in* vitro. However, SCP-2 does not only bind and transfer cholesterol. Indeed, SCP-2 is rather promiscuous in terms of also binding phospholipids (152–154), as well as fatty acids (155–157), selectively transferring specific phospholipid classes (158, 159) and selectively enhancing the esterification (140, 160, 161) of specific types of fatty acids. Therefore, it was important to determine if the redistribution of cholesterol toward the endoplasmic reticulum was a specific effect and/or if it could be secondary to

Table III. Effects of SCP-2 expression on endoplasmic reticulum lipid mass and composition[a]

Lipid class	Mass (nmol/mg protein)		Composition (mol %)	
	Control	SCP-2	Control	SCP-2
Cholesterol	104.6 ± 14.4	235.0 ± 11.3[b]	27.9 ± 3.7	44.9 ± 2.2[b]
Triglyceride	12.7 ± 1.9	10.6 ± 1.9	3.4 ± 0.5	2.0 ± 0.4[c]
Cholesterol ester	74.7 ± 12.3	70.5 ± 7.9	19.9 ± 3.3	13.5 ± 1.5
Phospholipid	183.3 ± 45.1	207.2 ± 33.6	48.8 ± 11.0	39.6 ± 4.6
	Ratio (nmol/nmol)			
Neutral lipid/phospholipid	1.05 ± 0.18	1.53 ± 0.07[c]		
Cholesterol/phospholipid	0.69 ± 0.18	0.97 ± 0.16[c]		
Cholesterol/cholesterol ester	1.65 ± 0.38	2.70 ± 0.42[c]		
Triglyceride/cholesterol ester	0.17 ± 0.01	0.18 ± 0.02		

[a] Neutral lipid refers to cholesterol + triglyceride + cholesteryl ester. Values represent the mean ± SEM, n = 3–6.
[b] Indicates significant from control $P < 0.01$.
[c] Indicates significant from control, $P < 0.05$.

a major redistribution of other lipid classes that might interact with higher affinity for cholesterol.

Analysis of the other lipid components of the endoplasmic reticulum (Table III) showed that SCP-2's redistribution of cholesterol to the endoplasmic reticulum of transfected cells was relatively specific. The endoplasmic reticulum mass (nanomoles per milligram of protein) of triglyceride (Table III), cholesteryl ester (Table III), and phospholipid (Tables III and IV) was not significantly altered by SCP-2 expression (Table III). Nevertheless, SCP-2 expression significantly altered phospholipid mass distribution within the endoplasmic reticulum (Table IV). SCP-2 expression increased the mass of anionic phospholipids, phosphatidylserine and phosphatidylinositol, 3.2- and 1.4-fold, respectively, while it concomitantly decreased the proportion of phosphatidylcholine and phosphatidic acid (Table IV). These findings were consistent with SCP-2 also being a phospholipid transfer protein that transfers a wide variety of phospholipid classes (153, 158, 159). Two important points can be made from these observations: First, the increased anionic phospholipid content of the endoplasmic reticulum may at least in part potentiate the SCP-2-mediated increase in endoplasmic reticulum cholesterol content. Anionic phospholipids are essential for binding SCP-2 (142, 143) and thereby enabling SCP-2 to dramatically enhance cholesterol transfer (153, 162–164). Second, cholesterol preferentially partitions into membrane phospholipids rich in anionic phospholipids. The cytofacial leaflet of the plasma membrane contains almost all the anionic phospholipid as well as 80% of the membrane cholesterol (165–169). Thus, SCP-2's ability to transfer anionic phospholipids may indirectly contribute to the greater distribution of cholesterol to the endoplasmic reticulum in SCP-2-expressing cells.

Finally, it is known that phospholipid fatty acid composition also dramatically affects cholesterol distribution into membranes (175). Furthermore, SCP-2 enhances microsomal incorporation of fatty acids into phosphatidic acid, the precursor of phospholipids, with the following acyl chain selectivity: oleic acid > arachidonic acid > palmitic acid (140). Thus, SCP-2 selectively enhanced incorporation of unsaturated and polyunsaturated acyl chains into phospholipids. Based on these *in vitro* observations, one might expect increased cholesterol partitioning into the more unsaturated, more fluid phospholipid bilayer. However, SCP-2 expression elicited only small changes in the types of fatty acids esterified to endoplasmic reticulum phospholipids and did not significantly alter the ratios of unsaturated/saturated or polyunsaturated/monounsaturated fatty acids (Table V). Thus, the increased cholesterol mass of the endoplasmic reticulum in SCP-2-expressing cells was not due to an in direct effect mediated by SCP-2's ability to selectively alter phospholipid acyl chain composition in the endoplasmic reticulum.

Lysosomal cholesterol transiting to the mitochondria: SCP-2-mediated transport. The major source of cholesterol for mitochondrial steroidogenesis is the plasma membrane (14) and lipid droplets (11). SCP-2 enhances transfer of cholesterol from isolated lipid droplets to mitochondria in intact cells (11) and stimulates steroidogenesis in transfected cells overexpressing SCP-2 (176, 177). *In vitro* assays of spontaneous sterol transfer from a variety of membrane donors (lysosomal membrane donors [144], plasma membrane donors [1, 2], and endoplasmic reticulum donors [1, 2]) to mitochondria is very slow. However, SCP-2 enhances the transfer of cholesterol from each of these donors to the mitochondria (1, 2, 144). SCP-2 also enhances transfer of cholesterol from isolated lipid droplets to mitochondria *in vitro* (178). Since another protein, steroidogenic acute regulatory protein, has been shown to be responsible for the rapid transfer of cholesterol from the outer to inner mitochondrial membrane (where steroidogenesis occurs; see following section) (179), it is thought that the primary role of SCP-2 in steroidogenesis is to resupply the outer mitochondrial membrane with cholesterol (144, 176)[2].

Lysosomal cholesterol transiting within the mitochondria: steroidogenic acute regulatory protein (StAR)-mediated transport from outer to inner mitochondrial membrane. Although the inner mitochondrial membrane

[2] A.D. Petrescu, A.M. Gallegos, J.F. Strauss III, and F. Schroeder, manuscript submitted for publication.

Table IV. Effect of SCP-2 expression on L-cell endoplasmic reticulum phospholipid class distribution[a]

Phospholipid class	Mass (nmol/mg protein)		Composition (mol %)	
	Control	SCP-2	Control	SCP-2
PtdAci	23.5 ± 6.7	8.3 ± 2.7[b]	12.3 ± 3.5	4.0 ± 1.3[b]
PtdEtn	39.0 ± 5.8	42.7 ± 7.7	20.4 ± 3.1	20.6 ± 3.7
PtdSer	22.8 ± 4.9	73.2 ± 12.5[b]	11.9 ± 2.6	35.4 ± 6.0[b]
PtdIns	29.5 ± 7.4	41.3 ± 12.70	15.4 ± 3.9	20.0 ± 6.1
PtdCho	53.2 ± 9.6	25.3 ± 3.9[b]	27.8 ± 5.0	12.2 ± 1.9[b]
CerPCho	23.0 ± 7.8	16.2 ± 2.4	12.1 ± 4.1	7.8 ± 1.2
Total phospholipid	191.0 ± 17.7	206.9 ± 20.1		

[a] PtdAci, phosphatidic acid; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine; CerPCho, sphingomyelin. Values represent means ± SEM, n = 3–6.
[b] Indicates significant from control, $P < 0.01$.

Table V. Effect of SCP-2 expression on L-cell endoplasmic reticulum phospholipid fatty acid composition

Fatty acid	Composition (mol %)	
	Control	SCP-2
12:0	1.89 ± 0.19	2.36 ± 0.23
14:1 n-5	2.43 ± 0.21	2.54 ± 0.24
16:0	12.41 ± 1.04	13.12 ± 0.56
16:1 n-7	2.95 ± 0.23	1.64 ± 0.16[a]
18:0	23.17 ± 2.30	18.17 ± 0.81
18:1 n-9	26.87 ± 2.63	27.13 ± 2.10
18:2 n-6	5.28 ± 0.44	6.40 ± 0.61
18:3 n-6	1.31 ± 0.16	0.91 ± 0.16
20:0	2.70 ± 0.47	2.95 ± 0.54
20:1 n-9	0.69 ± 0.11	1.76 ± 0.10[a]
20:2 n-6	3.02 ± 0.53	5.39 ± 0.52[b]
20:3 n-6	2.76 ± 0.51	4.26 ± 0.72
20:4 n-6	7.60 ± 1.20	6.22 ± 0.51
22:3 n-3	0.48 ± 0.05	2.24 ± 0.52[b]
22:4 n-6	4.44 ± 0.28	2.56 ± 0.17[a]
22:5 n-6	0.96 ± 0.15	0.61 ± 0.27
22:6 n-3	0.72 ± 0.08	1.28 ± 0.14[a]
24:1 n-9	0.42 ± 0.07	0.46 ± 0.03
Unsaturated/saturated	1.49 ± 0.222	1.73 ± 0.198
PUFA/MUFA	0.79 ± 0.128	0.89 ± 0.128

[a] Indicates significant from control, $P < 0.05$.
[b] Indicates significant from control, $P < 0.01$.

contains the cellular machinery (enzymes) for steroidogenesis, ironically, the inner mitochondrial membrane is essentially devoid of cholesterol. Thus, transfer of cholesterol from the outer to the inner mitochondrial membrane is the rate-limiting step in mitochondrial steroidogenesis (180). StAR is responsible for the rapid transfer of cholesterol from the outer to inner mitochondrial membrane (where steroidogenesis occurs) (179, 181). Mutations that inactivate StAR in humans and StAR gene-ablated mice result in markedly reduced gonadal and adrenal steroidogenesis, a condition that leads to accumulation of cholesterol in lipid droplets and a disease termed congenital lipoid adrenal hyperplasia in humans (179) and (182) similar adrenal lipid accumulation in mice (183).

The mechanism whereby StAR elicits acute steroidogenesis is not yet completely clear. Removal of the N-terminal mitochondrial inner membrane targeting sequence does not inhibit cholesterol transfer from the outer to the inner mitochondrial membrane, thereby suggesting that StAR interaction with the outer mitochondrial membrane alone is sufficient to mediate sterol transfer (181, 184). StAR binds cholesterol, induces formation of a rapidly transferable sterol domain in mitochondrial membranes, and preferentially elicits sterol transfer from mitochondrial membranes isolated from steroidogenic MA-10 leydig cells as compared with those isolated from normal human fibroblasts[3].

Intracellular transfer of cholesterol between nonlysosomal membranes. This subject has been reviewed extensively in the past few years and will not be covered further here (4, 25).

Cholesterol Efflux through Plasma Membrane Lateral Domains: SRB1 and Caveolae. The majority of cellular cholesterol efflux occurs through the plasma membrane to SRB1 and HDL, a pathway termed "reverse cholesterol transport" (185). Recent evidence suggests that the HDL need not be tethered to the SRB1 to function as a cholesterol acceptor (76). Tangier disease cells are unable to efflux cholesterol to HDL (186) due to a genetic disorder affecting the ATP-binding cassette transporter (ABC1) (146). The intracellular localization of the ABC1 protein and how it relates to caveolae and SRB1 are the subject of active investigation. Cholesterol destined for efflux derives from several pathways.

Vesicular transfer of cholesterol to caveolae for efflux: exogenous cholesterol. Exogenous-derived cholesterol (from LDL cholesterol or LDL-cholesterol esters) effluxes by vesicular transfer of unesterified cholesterol, via the Golgi, with the entire process occurring in about 30 to 60 min (Fig. 1) (3). It should be noted that transfer of exogenous HDL-derived cholesterol to the trans-Golgi is bidirectional (9). The mechanism whereby caveolin mediates bidirectional cholesterol transfer between caveolae and Golgi is currently debated. Since caveolar vesicles can be isolated from cells, caveolin is thought to mediate vesicular cholesterol transfer, but such caveolin-coated vesicles separate from the plasma membrane have yet to be identified in intact cells (3, 9). Recent data from this laboratory showed that L cell fibroblasts have caveolin-1, SRB1, and exhibit HDL-mediated cholesterol efflux as determined by radiola-

[3] A.D. Petrescu, A.M. Gallegos, J.F. Strauss III, and F. Schroeder, manuscript submitted for publication.

bel as well as fluorescent sterol and confocal imaging of living cells (21). Confocal imaging of HDL receptor-mediated efflux of fluorescent sterols from the cytoplasm of L cell fibroblasts showed that this pathway is bidirectional (20, 21) and is comprised of two components, the major one accounting for 80% of the total, having a slow $t_{1/2}$ (near 10–20 min), consistent with a vesicular process (21). It should be noted, however, that caveolin is an essential component of the HDL-mediated cholesterol efflux process. L1210-JF lymphocytes, which do not express caveolin, transport cholesterol very slowly to the plasma membrane caveolae (9). In contrast, overexpression of caveolin in these cells results in rapid cholesterol transfer to plasma membrane caveolae. These studies, taken together with the presence of SRB1 in caveolae, suggest that caveolin mediates cellular cholesterol efflux through caveolae microdomains to HDL.

Nonvesicular transfer of cholesterol to the caveolae for efflux: exogenous cholesterol. It has been suggested that caveolin-dependent cholesterol transfer is bidirectional, nonvesicular, and rapid (1–2 min) (9). Consistent with this hypothesis, confocal imaging of HDL receptor-mediated efflux of fluorescent sterols from the cytoplasm of L cell fibroblasts deficient in SCP-2 showed that this pathway is bidirectional (21). Furthermore, mathematical analysis revealed the presence of two components: a rapid ($t_{1/2}$ near 1 min) process as well as a slower ($t_{1/2}$ near 10–20 min) vesicular process (21). While these data show that an underlying rapid sterol transfer (possibly caveolin mediated), rapid transfer is enhanced in SCP-2-expressing cells without alteration in the level or intracellular localization of caveolin (21).

Nonvesicular transfer of cholesterol to the caveolae for efflux: de novo synthesized cholesterol. Additional support for a protein-mediated mechanism comes from measurements showing that efflux of cholesterol synthesized *de novo* in endoplasmic reticulum is fast ($t_{1/2}$ of 1–2 min) (9, 16) due to rapid, nonvesicular transfer of cholesterol from the endoplasmic reticulum to plasma membrane caveolae (3, 187). A rapid, SCP-2-mediated cholesterol transfer pathway (for newly synthesized cholesterol) to the plasma membrane for efflux has been shown in intact fibroblasts (21) and fibroblasts treated with antisense cDNA to SCP-2 (151). Interestingly, transfer of cholesterol from the endoplasmic to the plasma membrane for secretion into bile is inhibited in liver of rats treated with antisense cDNA to SCP-2 (188).

Annular Cholesterol Domains. Annular cholesterol domains are the immediate layer or annulus of cholesterol surrounding proteins or lipid domains (Fig. 3) (189, 190). Such cholesterol-rich annuli have been demonstrated for the acetylcholine receptor (191, 192) and the oxytocin receptor (193). Vesicular stomatitis virus G and M proteins induce partition of cholesterol into acidic phospholipid-rich domains (194).

Conclusions and Future Perspectives. Building on the earlier findings reviewed 5 and 10 years ago for this journal (23, 25), the past 5 years have seen tremendous progress (1–21) in our understanding of membrane domains and intracellular cholesterol trafficking. Clearly, the majority of data support contributions from both protein-mediated and vesicular pathways for intracellular cholesterol trafficking. Nevertheless, important major challenges remain in this field: First, it will be important to resolve the lateral and transbilayer distribution and structure of lipids and proteins in the plasma membrane microdomains. Second, it will be essential to determine the existence and nature of microdomains in intracellular membranes. Third, by accepting the fact that both protein-mediated and vesicular pathways contribute to cholesterol trafficking within the cell, the question then becomes how to resolve the relative contributions of vesicular- and protein-mediated cholesterol transfer in the multiple pathways of intracellular cholesterol trafficking. By resolving these contributions, we will then have the tools necessary to identify factors regulating these pathways in normal cholesterol metabolism and disease.

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