Intracellular cholesterol transport

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Abstract The intracellular movement of cholesterol in mammalian cells may involve complex pathways by which the sterol moves to various cellular sites and mediates transcriptional regulation, enzyme activation, and protein degradation. Current evidence indicates that there are three distinct pathways modulating intracellular cholesterol trafficking. The movement of endogenously synthesized cholesterol from the endoplasmic reticulum appears to be distinct from movement of exogenous, low density lipoprotein (LDL)-derived cholesterol to the plasma membrane. In addition, steroidogenic cells possess a third mechanism by which cholesterol is transported to the mitochondria to initiate steroid hormone synthesis. In this review, we have outlined the current knowledge of cholesterol transport mechanisms and pathways and have described approaches that may help define cholesterol trafficking mechanisms in molecular detail. The use of genetic and molecular biologic techniques can potentially reveal gene products that are involved in intracellular cholesterol transport and regulation as well as those that may secondarily affect this process.— **Liscum, L., and N. K. Dahl.** Intracellular cholesterol transport. *J. Lipid Res.* 1992. **33:** 1239–1254.

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INTRODUCTION

In mammalian cells, cholesterol plays a dual role as an essential structural component of cellular membranes and a regulator of gene transcription (1), protein degradation (2), and enzyme activity (3). Cholesterol is obtained by two distinct but coordinately regulated processes. Mammalian cells synthesize cholesterol, and the rate-limiting enzyme in cholesterol biosynthesis is the endoplasmic reticulum enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (4). However, when supplied with serum lipoproteins, mammalian cells acquire cholesterol by the receptor-mediated endocytosis of low density lipoproteins (LDL). Upon internalization, LDL is delivered to lysosomes where LDL-derived cholesteryl esters are hydrolyzed to unesterified cholesterol (5).

Cellular levels of free, unesterified cholesterol are tightly controlled. The cellular proteins that control the acquisition of cholesterol are principally the LDL receptor and HMG-CoA reductase. The deposition of excess cholesterol in the form of cholesteryl esters is controlled by acyl CoA:cholesterol acyltransferase (ACAT). When cells require additional cholesterol, they express high levels of LDL receptors and HMG-CoA reductase and low levels of ACAT activity. As cellular levels of free cholesterol rise, LDL receptor levels and HMG-CoA reductase activity are suppressed and ACAT is activated (6). Although receptor-mediated internalization of LDL and the control of cellular cholesterol metabolism have been extensively studied (6, 7), many issues remain unresolved. These include what mechanisms mediate the transcriptional and post-translational regulatory responses elicited by cholesterol, how is cholesterol transported throughout the cells and by what pathway(s), and which gene products control this transport and regulation.

This review will examine what is known about intracellular cholesterol trafficking and discuss experimental approaches that may prove valuable. Recent reviews by Dawidowicz (8), Phillips, Johnson and Rothblat (9), van Meer (10), Voelker (11, 12), Reinhart (13), Pagano (14), and Johnson et al. (15) may also prove interesting to the reader.

Evidence for cholesterol sorting and transport mechanisms

The question of how cholesterol is transported throughout the cell and the pathway of cholesterol's movement have been difficult to address. Many of the reagents and techniques used successfully to study protein transport in intact cells (such as antibodies, analysis of glycosylation

and proteolytic trimming, crosslinking reagents) are not practical for the study of cholesterol transport (8). Valuable information about cholesterol movement has been gained by examining the in vitro transfer of cholesterol between isolated biological membranes and phospholipid vesicles. Cholesterol moves between lipid vesicles of various compositions, lipid vesicles and isolated cell membranes, and lipid vesicles and cultured cells (reviewed in refs. 8, 9). These studies have shown that the net transfer of cholesterol is spontaneous and occurs down a cholesterol concentration gradient. The process is temperature-dependent and is affected by the lipid composition of the donor and acceptor membranes. Desorption of cholesterol from donor membranes appears to be rate-limiting for transport and is followed by aqueous diffusion of cholesterol to acceptor membranes.

Given the ability of cholesterol to move spontaneously between membranes (9), is it necessary to invoke specific cellular cholesterol sorting and transport mechanisms? We can draw several arguments in favor of distinct cellular components that control cholesterol movement from our knowledge of the cellular distribution of cholesterol, the comparison of normal and genetically defective cholesterol transport, and inhibition by pharmacological agents.

Cholesterol is not uniformly distributed among subcellular membranes (16, 17). Cholesterol/phospholipid molar ratios vary dramatically in rat liver membranes, from 0.06 in the endoplasmic reticulum to 0.76 in the plasma membrane (16). This distribution would not be expected if intracellular cholesterol movement were spontaneous. In addition, spontaneous cholesterol transfer between membranes has a $T_{1/2}$ of 1–2 h (9), which is slow when compared to the sterol movement observed in vivo (18–20). Wattenberg and Silbert (21) have shown that the cholesterol distribution can be qualitatively accounted for by the partitioning properties of cholesterol in isolated membrane fractions. For example, cholesterol has a higher affinity for plasma membranes relative to other cellular membranes due to the former's higher sphingomyelin content and degree of fatty acyl saturation (21). However, the quantitative in vivo differences in cholesterol distribution are significantly larger than in vitro partitioning differences. Therefore, although spontaneous diffusion may play a role in the intracellular movement of cholesterol, other factors, such as active transport, are important as well.

Genetic evidence that cellular factors mediate intracellular cholesterol transport comes from the analysis of mammalian cells with specific impairments. These include fibroblasts from individuals with Niemann-Pick disease, type C (NPC) (22–25) and Niemann-Pick type D (NPD) (26), mutant BALB/c mice (27, 28), and Chinese hamster ovary (CHO) mutants selected for defective cholesterol trafficking (29, 30). The fact that genetic muta-tions exist that affect the transport of cholesterol indicates that cholesterol movement is not random or diffuse, but is precisely mediated.

Pharmacological evidence for regulated intracellular cholesterol transport comes from studies with energy poisons (18, 31) and hydrophobic amines, such as U18666A (3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one) (32, 33) and imipramine (34). These experiments indicate that drug-sensitive steps are involved in the movement of endogenously and exogenously derived cholesterol. Based upon these genetic and pharmacological experiments, we conclude that intracellular cholesterol movement is not random but is highly controlled and directed.

WHAT DO WE KNOW ABOUT INTRACELLULAR CHOLESTEROL TRAFFICKING?

Emerging evidence indicates that at least three distinct pathways modulate intracellular cholesterol trafficking. The movement of endogenously synthesized cholesterol from the endoplasmic reticulum to the plasma membrane appears to be distinct from movement of LDL-derived, exogenous cholesterol to the plasma membrane (**Table 1**). In addition, steroidogenic cells possess a third mechanism for cholesterol transport to the mitochondria which is functionally independent of the previous two pathways. These three pathways will be discussed in detail and possible mechanisms for cholesterol movement will be considered. These include aqueous diffusion, vesicle-mediated transport, and transport via a soluble carrier protein or lipid. As noted by Reinhart (13), all of these mechanisms may work, together or separately, to mobilize cholesterol within the cell.

TABLE 1. Characteristics of cholesterol transport from endoplasmic reticulum to plasma membrane (endogenously derived) and lysosomes to plasma membrane (exogenously derived)

	Endoplasmic Reticulum to Plasma Membrane	Lysosomes to Plasma Membrane
$T_{1/2}$	10 min to 1 h	2 to 40 min
Inhibited by:	<15°C Energy poisons	NPC mutation U18666A Imipramine Stearylamine RV-538 Sphinganine
Not inhibited by:	NPC mutation Colchicine Cytochalasin B NH₄Cl Cycloheximide Monensin Brefeldin A	Energy poisons Colchicine Cytochalasin B NH₄Cl Cycloheximide Monensin? Leupeptin

Methods

First, let us consider the methods that have been developed to study the transport of endogenously synthesized and exogenously derived cholesterol in cultured cells. In general, cells are cultured in medium containing lipoprotein-deficient serum to induce high levels of cholesterol synthesis and LDL receptor activity. For endogenously synthesized cholesterol, cells are pulse-labeled with a precursor of cholesterol, such as [³H]acetate, which labels newly synthesized cholesterol within minutes (18). Some studies take advantage of this rapid labeling by first incubating cells to steady state with a radiolabeled precursor (i.e., [¹⁴C]acetate) and then pulse-labeling for short times with the precursor containing another isotope (i.e., [³H]acetate) to measure rapid transport. In subcellular fractionation, the ³H/¹⁴C ratio reflects the enrichment of a membrane fraction in newly synthesized cholesterol (31, 35).

To measure the transport of exogenous, LDL-derived cholesterol, we use LDL that contains [³H]cholesteryl linoleate ([³H]CL-LDL) (25, 36). LDL can be readily labeled with [³H]sterol esters using dimethyl sulfoxide (37). Alternatively, the hydrophobic core of LDL can be extracted and the particle can be reconstituted with a wide variety of hydrophobic molecules (38). [³H]CL-LDL prepared by either method binds specifically to the LDL receptor and is internalized and delivered to lysosomes where hydrolysis releases [³H]cholesterol. During continuous exposure to [³H]CL-LDL, the lysosomal content of [³H]cholesterol plateaus coincident with the initial appearance of [³H]cholesterol in other cell membranes. The plateau is indicative of a steady state in which the production of unesterified [³H]cholesterol in lysosomes (by continual receptor-mediated endocytosis and hydrolysis) is balanced by the movement of [³H]cholesterol out of the lysosome.

The arrival of [³H]cholesterol at particular cell membranes can be measured by subcellular fractionation (20, 25, 31–33, 35, 39) or by rapid isolation of the plasma membranes using cationic beads (18). The amount of [³H]cholesterol at the cell surface can also be estimated by taking advantage of the fact that cholesterol will spontaneously desorb from cell membranes. If an excess of small unilamellar vesicles (25) or high density lipoproteins (15, 40, 41) is available in the medium to serve as an acceptor, a time-dependent increase in desorbed [³H]cholesterol is seen that is indicative of the amount of [³H]cholesterol in the plasma membrane. In addition, [³H]cholesterol can be detected at the surface of intact cells by cholesterol oxidase treatment and subsequent quantitation of [³H]cholestenone (19, 36, 42, 43).

None of these techniques is flawless. Widely varying differences in kinetics of cholesterol movement have been reported, and many differences may be attributed to the method used to detect [³H]cholesterol at the plasma membrane. Plasma membrane isolation using either subcellular fractionation or cationic beads results in a membrane fraction that may not be completely free from contamination by other intracellular membranes. Determining plasma membrane [³H]cholesterol content by measuring efflux of [³H]cholesterol is indirect and incapable of measuring rapid transport since the rate of efflux is a function of the rates of cholesterol transport to the plasma membrane and desorption of cholesterol from this membrane.

Cholesterol oxidase sensitivity is a rapid method of determining plasma membrane [³H]cholesterol content; however, concerns have arisen as to whether plasma membrane cholesterol is the only cholesterol pool subject to oxidation (for discussion see refs. 8, 43, 44). The cholesterol in cultured cells is normally refractory to cholesterol oxidase treatment. To make plasma membrane cholesterol accessible to oxidation, cells must be subjected to either glutaraldehyde fixation and incubation in a low salt buffer (45) or sphingomyelinase treatment (46). After either treatment, 80–90% of cellular cholesterol is susceptible to oxidation and postulated to be at the cell surface. However, theoretical calculations of plasma membrane cholesterol content (based on cholesterol/phospholipid ratios of purified plasma membranes and morphometric measurements) predict that only 40% of cellular cholesterol resides in the plasma membrane (44). This discrepancy can be explained if cholesterol oxidase is reaching an intracellular pool of cholesterol or if the theoretical calculations are skewed by faulty assumptions or contaminated membrane fractions. In addition, the amount of membrane comprising intracellular versus plasma membrane may vary according to cell type. Cholesterol oxidase incubation does increase plasma membrane permeability to ions (15, 19), although the cytosolic enzyme lactate dehydrogenase is not released from treated cells (19). It is also possible that intracellular cholesterol is transferred to the plasma membrane during the 37°C treatment with cholesterol oxidase; however, newly synthesized cholesterol residing in the endoplasmic reticulum remains cholesterol oxidase-resistant despite incubation at 37°C for 45 min (47). Lange and colleagues (48) have provided detailed subcellular fractionation data indicating that the plasma membrane of cultured human fibroblasts contains 50% of cellular phospholipid and 90% of cellular cholesterol. These data appear to validate the use of cholesterol oxidase to measure cell surface cholesterol. Morphometric analysis of the same cell type may confirm that the plasma membrane accounts for 50% of cellular membranes.

Transport of endogenously synthesized cholesterol

Cholesterol synthesis follows a complex metabolic pathway that is tightly regulated in response to cell require-

ments. Cholesterol is synthesized in the endoplasmic reticulum. Subcellular fractionation indicates that HMG-CoA reductase and the enzymes catalyzing the conversion of lanosterol and zymosterol to cholesterol co-fractionate with endoplasmic reticulum markers (20, 49, 50). The final destination of this endogenously synthesized cholesterol varies according to the cell type and cellular requirement for cholesterol. Cholesterol is required for proper functioning of all mammalian cell membranes, although the plasma membrane contains most of the cellular free cholesterol (17). Cholesterol can be targeted to ACAT in the endoplasmic reticulum, and when ACAT is activated a large portion of the cholesterol that is esterified is newly synthesized cholesterol (32). In hepatocytes, cholesterol is needed for lipoprotein and bile acid synthesis, both of which are initiated in the endoplasmic reticulum (51, 52). In addition, in steroid hormone-producing cells, cholesterol may be directly transported to the mitochondria, fueling steroid hormone synthesis (53). The mechanisms controlling these alternate transport pathways remain unknown.

Recent evidence suggests that cholesterol transport from its site of synthesis to the plasma membrane is a fast, energy-requiring process that proceeds independently of the secretory protein pathway. Cholesterol appears to be transported to the plasma membrane as rapidly as it is synthesized and does not linger in intracellular membranes (17). The half-time for this movement is from 10 min (18, 31) to 18 min (20). However, when cells are incubated at 15°C, cholesterol synthesis continues but the newly synthesized cholesterol doesn't reach the plasma membrane (31). Instead, the nascent cholesterol accumulates in the endoplasmic reticulum and in a lipid-rich vesicle fraction (31, 39). When isolated from vesicular stomatitis virus-infected cells, the lipid-rich vesicle fraction also contains viral G protein, but it is likely that G protein and nascent cholesterol reside in separate vesicles (39). Urbani and Simoni (39) have shown that the fungal metabolite Brefeldin A, which disassembles the Golgi apparatus, blocks G protein transit to the plasma membrane without affecting cholesterol transport. Additional evidence that cholesterol bypasses the Golgi apparatus en route from the endoplasmic reticulum to the plasma membrane is that monensin does not perturb this process (31). Drugs that affect the cytoskeletal network (colchicine, cytochalasin B), lysosomal function (NH_4Cl), and protein synthesis (cycloheximide) also have no effect on cholesterol transport (31).

Urbani and Simoni (39) speculate that newly synthesized cholesterol is released from the endoplasmic reticulum in vesicles spatially distinct from those carrying proteins destined for the Golgi apparatus. Some facet of this sorting, budding, and transport requires metabolic energy, although a small amount of transfer continues in the presence of energy poisons (31). Kaplan and Simoni

(31) suggest that energy may be required for exiting the endoplasmic reticulum but cholesterol in transit may be able to continue to the plasma membrane despite energy depletion. The possibility that energy poisons inhibit cholesterol transport by blocking the synthesis of other lipids needed for vesicle formation has not been eliminated (31).

The dogma that cholesterol is synthesized to completion in the endoplasmic reticulum before being transported to the plasma membrane may be an oversimplification. The lipid-rich vesicles have been found to carry cholesterol and its precursor, zymosterol (20). In addition, Lange and Muraski (54) and Echevarria et al. (55) reported that both newly synthesized lanosterol and zymosterol are present in the plasma membrane. One interpretation of these data is that cholesterol synthesis is not completed in the endoplasmic reticulum, but takes place in heterogeneous compartments within the cell. Instead, Lange, Echevarria, and Steck (20) found that plasma membrane zymosterol is efficiently returned to the endoplasmic reticulum, perhaps via lipid-rich vesicles, and is converted to cholesterol. The significance of this bidirectional movement of cholesterol precursors is unclear. The presence of zymosterol at the plasma membrane may result from indiscriminate sorting within the endoplasmic reticulum.

Transport of exogenously derived cholesterol

Receptor-mediated internalization of cholesteryl ester-laden LDL particles supplies the cell with exogenous cholesterol. Cholesterol that is produced by lysosomal hydrolysis of LDL-derived cholesteryl esters must insert into and traverse the lysosomal membrane. Then the sterol must be transported through the cytoplasm and become incorporated into the appropriate acceptor membrane(s). So far, there is little information on the regulation of any of these steps. Because of the high cholesterol concentration in the plasma membrane and the observed rapid kinetics, one possible scenario is that the bulk of exogenous cholesterol is transported from lysosomes directly to the plasma membrane. As plasma membrane cholesterol levels rise, the excess cholesterol is internalized and brought to the endoplasmic reticulum for cholesteryl ester formation via ACAT or desorbed into the extracellular mileau.

Evidence from Xu and Tabas (56) supports this idea. They found that ACAT is not directly stimulated by LDL-derived cholesterol, but by expansion of cellular cholesterol levels. They calculate that, in macrophages, the cellular cholesterol levels have to reach a critical threshold of ~25% above basal levels before ACAT is stimulated. Then a mixture of cellular and lipoprotein-derived cholesterol is esterified, and the mixture reflects the proportion of original cellular and lipoprotein-derived cholesterol in the cell. This finding implies that the cell

has the capability of expanding its pool of unesterified cholesterol by 25% (i.e., by incorporating more cholesterol into membranes) before reversing the expansion to return to basal levels.

The kinetics of LDL-cholesterol movement was determined in CHO cells by Brasaemle and Attie (19) using brief cholesterol oxidase treatment as a measure of [³H]cholesterol arrival at the plasma membrane. They found that [³H]cholesterol reached the plasma membrane (i.e., became cholesterol oxidase-susceptible) within 2 min of [³H]cholesteryl linoleate hydrolysis in the lysosome. Using high density lipoproteins as an acceptor of LDL-cholesterol desorbed from Fu5AH rat hepatoma cells, Johnson et al. (40) found that a maximum of 40–50 min is required for transport of LDL-cholesterol from lysosomes to plasma membranes. The time required for LDL-cholesterol transport is independent of the concentration of acceptors for cholesterol in the medium, sterol content of the cell, and the level of ACAT activity (40). Since the transport of LDL-cholesterol to the plasma membrane is not affected by activation or inhibition of ACAT, LDL-cholesterol may not mix with the ACAT-accessible pool of cholesterol (i.e., endoplasmic reticulum) during its journey from the lysosomes to the plasma membrane.

As suggested by Brasaemle and Attie (19), the rapid transport of cholesterol indicates a catalytic mechanism for cholesterol movement between lysosomes and plasma membranes, and is inconsistent with passive diffusion. While not providing direct evidence in favor of a particular cholesterol transport mechanism, Johnson and coworkers (40) note that the kinetics of exogenous cholesterol movement are consistent with vesicular trafficking. Thus, the underlying mechanism of trafficking of endogenously and exogenously derived cholesterol may be the same, although LDL-derived cholesterol is not likely to be targeted to the endoplasmic reticulum for vesicular packaging prior to transport to the plasma membrane.

Facilitated transport of LDL-cholesterol is underscored by studies in fibroblasts from individuals with the lysosomal storage disease, Niemann-Pick type C. NPC first came to light as a defect in cholesterol metabolism when Pentchev and colleagues (22) found that the LDL-mediated stimulation of cholesterol esterification is impaired in NPC cells. Subsequently, it was found that, although LDL receptor activity is normal and LDL-derived cholesteryl esters are hydrolyzed to free cholesterol, the LDL-derived cholesterol does not effectively suppress endogenous cholesterol synthesis or LDL receptor activity in NPC cells (23, 24). Since NPC fibroblasts exhibit normal regulatory responses in response to 25-hydroxycholesterol and excess endogenously synthesized cholesterol, the defect in cholesterol metabolism appears to be specific for LDL-derived cholesterol (24). Indeed, LDL-derived cholesterol accumulates in lysosomes

of NPC fibroblasts by filipin staining (57), subcellular fractionation of [³H]CL-LDL-labeled cells (23, 25), and mass measurement (25).

The gene defects in NPC and NPD remain unresolved. Movement of LDL-derived cholesterol from lysosomes to plasma membranes is affected, while the movement of endogenously synthesized cholesterol from the endoplasmic reticulum to the plasma membrane is normal in NPC cells (25). In addition, the reciprocal movement of cell surface cholesterol to ACAT is normal in NPC cells (58). This could signify that two distinct pathways shuttle cholesterol from the endoplasmic reticulum and lysosomes to the plasma membrane, and only one pathway is defective in NPC cells. Alternatively, the two flows of cholesterol may normally merge into one; however, the NPC defect does not allow LDL-cholesterol to exit the lysosome and join the normal flow.

One difference between the movement of endogenous and exogenous cholesterol was revealed by our finding that the transport of LDL-derived cholesterol from lysosomes to the plasma membrane is insensitive to energy poisons (59) at concentrations shown to block vesicular transport of nascent cholesterol (18, 31). If both pathways are vesicle-mediated, one possible explanation for this difference is that an energy-dependent sorting step is required in the endoplasmic reticulum before vesicle budding can occur; this sorting step may not be requisite in the pathway originating in the lysosome. The movement of LDL-cholesterol from lysosomes to the plasma membrane is not altered by agents that affect cytoskeletal organization (colchicine, cytochalasin B, nocodazole), lysosomal function (NH₄Cl, leupeptin) and does not require continuous ongoing protein synthesis (59).

The hydrophobic amines, such as U18666A (32, 33) and imipramine (34), are one type of pharmacological agent that continues to intrigue us (**Fig. 1**). Early studies in many laboratories focused on U18666A's effects on cho-
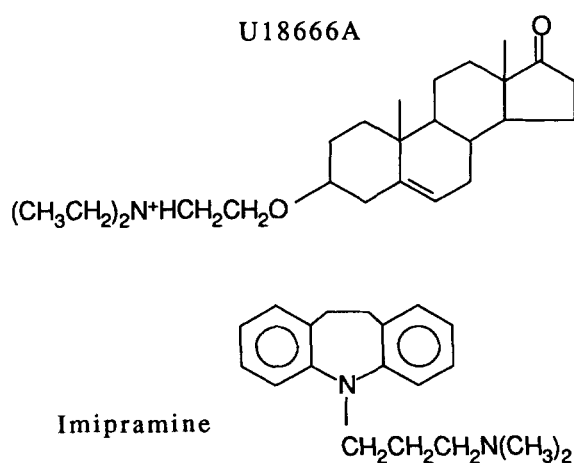

Fig. 1. Chemical structure of U18666A and imipramine.

lesterol synthesis (60–62). U18666A first came to our attention from the work of Panini, Sexton, and Rudney (61) who showed that U18666A abolishes LDL-mediated suppression of HMG-CoA reductase in rat intestinal epithelial cells whereas polar sterol-mediated suppression is unaffected by U18666A. Our studies indicate that U18666A also blocks LDL-mediated stimulation of cholesterol esterification and suppression of LDL receptor activity in CHO cells. The regulation of these activities by 25-hydroxycholesterol is not affected by U18666A (32). The characteristics of U18666A-treated CHO cells appear to be very similar to the NPC phenotype. When we looked more closely at the transport of LDL-derived cholesterol, we found that LDL-cholesterol accumulates in the lysosomes of U18666A-treated CHO cells resulting in impaired movement of LDL-cholesterol to other cell membranes.

The mechanism by which U18666A retards the movement of LDL-cholesterol remains unknown. U18666A might inhibit the activity, synthesis, or turnover of a protein or lipid that regulates LDL-cholesterol trafficking. U18666A exhibits the lysosomotrophic properties of a weak basic amine; however, this is unlikely to explain its action on cholesterol transport since other lysosomotrophic agents, such as NH_4Cl and chloroquine, have minimal effect on cholesterol movement (59).

Since our initial discovery that U18666A inhibits LDL-cholesterol movement, Rodriguez-Lafrasse and colleagues (34) reported that a second hydrophobic amine, imipramine, has inhibitory effects on LDL-cholesterol transport that are strikingly similar to those of U18666A. Most recently, Roff et al. (63) showed that stearylamine, RV-538, and sphinganine also induce NPC-like defects in normal human fibroblasts. The observation with sphinganine, a natural constituent in cells, has stimulated the search for an endogenous hydrophobic amine that could be responsible for sluggish cholesterol efflux from lysosomes in cultured NPC fibroblasts. Alternatively, U18666A and other hydrophobic amines may act by forming a complex with an acidic component that is essential for LDL-cholesterol transport. The activity or amount of this critical molecule may be genetically defective in NPC.

Sterol Carrier Protein 2 (SCP₂)

Cholesterol transport via a soluble carrier protein or lipid is an attractive hypothesis. A sterol carrier may be involved in the bulk transport of cholesterol, in the precise delivery of cholesterol to specific membrane microdomains, or cholesterol delivery to sterol-regulated nuclear transcription elements. For example, a sterol carrier could mediate the delivery of cholesterol desorbed from lysosomal membranes to the membrane microdomain surrounding HMG-CoA reductase, which would lead to enhanced HMG-CoA reductase degradation while leaving the membrane's cholesterol/phospholipid ratio essentially unaltered. A sterol carrier that functioned to maintain precise cholesterol/phospholipid ratios of the cellular membranes would be analogous to the phosphatidylinositol/phosphatidylcholine transfer protein, which has been postulated to play a role in maintaining the correct phosphatidylinositol to phosphatidylcholine ratio at a specific intracellular sites (64). The name "carrier" implies a binding and transfer function but a soluble protein or lipid may also facilitate cholesterol movement by influencing the rate of cholesterol desorption from specific membranes.

One highly touted candidate for a sterol carrier is the nonspecific lipid transfer protein, SCP₂. SCP₂ is a soluble protein of 13.2 kDa that has been purified from a variety of sources and is purported to play a role in the intracellular movement of sterols and phospholipids (65–67; also reviewed in 13, 68). Recent cloning of a rat liver cDNA encoding SCP₂ has revealed a single rat gene, approximately 20–30 kb in size (69). SCP₂ appears to be synthesized as a 15.3 kDa precursor that is processed to yield the mature 13.2 kDa protein (69–71). In addition, there is evidence for multiple larger gene transcripts that yield SCP₂-related proteins of 58.3 and 30 kDa (72, 73). The SCP₂-related proteins are of relatively low abundance and their functions are unknown. Only the 13.2 kDa moiety is thought to mediate sterol transfer.

Most of the evidence that SCP₂ is involved in intracellular cholesterol movement is not unequivocal, however, as it emanates from in vitro studies. The first in vivo evidence for a physiological role of SCP₂ was provided by Yamamoto et al. (71) using COS African green monkey kidney cells containing expression vectors for cytochrome P-450 cholesterol side chain cleavage (P-450scc) enzyme and adrenodoxin. Cells expressing cytochrome P-450scc and adrenodoxin synthesize and secrete low levels of progestin. When a cDNA encoding the human SCP₂ is co-expressed, progestin secretion increases threefold. This finding supports the notion that SCP₂ mediates cholesterol movement to mitochondria for the initial steps in steroidogenesis.

Strong evidence that SCP₂ plays a physiological role in other aspects of cholesterol movement is lacking. For example, Reuber H35 hepatoma cells express only 6% of the SCP₂ present in rat hepatocytes, yet both cell types show similar kinetics of esterification of endogenously synthesized cholesterol or exogenously derived cholesterol (74). This suggests that SCP₂ has little direct effect on cholesterol trafficking to ACAT. However, it may be argued that the low level of SCP₂ present in the hepatoma cells is sufficient for normal intracellular trafficking of cholesterol.

We therefore examined several aspects of cholesterol metabolism in cells that do not express the 13.2 kDa, or active form, of SCP₂. CHO cells deficient in peroxisomes, designated ZR-82, were isolated by Zoeller and Raetz (75) and found to lack the 13.2 kDa SCP₂ (76). We have found

that ZR-82 cells exhibit normal LDL and mevalonate stimulation of cholesterol esterification (N. K. Dahl and L. Liscum, unpublished observation). This indicates that the absence of SCP$_2$ does not impede ACAT activation or mobilization of endogenous or exogenous cholesterol to ACAT in the endoplasmic reticulum. Furthermore, we have found that endogenously synthesized cholesterol is transported to the plasma membrane and desorbed to the medium with similar kinetics in wild-type and ZR-82 cells (N. K. Dahl and L. Liscum, unpublished observation).

Our results are difficult to interpret, however, because the 58 kDa SCP$_2$-related protein is present in ZR-82 cells, albeit at reduced levels (76). The possibility that the 58 kDa SCP$_2$-related protein mediates some of SCP$_2$'s action has not been eliminated. To convince skeptics that SCP$_2$ plays a role in the bulk transfer of cholesterol or is crucial to cholesterol-mediated regulation of cellular cholesterol metabolism, it may be necessary to repeat these experiments in a cultured cell line that lacks higher molecular weight SCP$_2$-related proteins in addition to the 13.2 kDa SCP$_2$.

The role of the Golgi apparatus in cholesterol transport

The role of the Golgi apparatus in intracellular cholesterol transport remains ambiguous. Evidence described earlier indicates that vesicles laden with nascent cholesterol bypass the Golgi complex en route to the plasma membrane. However, the Golgi may play a role in the transport of exogenously derived cholesterol since the transport of LDL-cholesterol from lysosomes to the plasma membrane appears to be sensitive to monensin (59). This is not strong evidence for LDL-cholesterol trafficking through the Golgi complex because the ionophoretic effects of monensin are not Golgi-specific (77, 78).

Using filipin fluorescence and electron microscopy, Blanchette-Mackie et al. (79) examined normal and NPC fibroblasts that had been incubated in the absence or presence of LDL. They found that in normal cells there is cholesterol enrichment in Golgi membranes after prolonged incubation with LDL. In NPC cells, the Golgi cholesterol enrichment occurs after short LDL incubation, along with massive cholesterol accumulation in the lysosomes. Blanchette-Mackie and colleagues speculate that the Golgi complex may serve as a "postlysosomal depot for the cellular distribution of exogenously derived cholesterol." Perhaps a lesion at the Golgi complex could serve as a bottleneck resulting in LDL-cholesterol deposition in lysosomes.

Cholesterol trafficking in the liver

The liver plays a key role in regulating whole body cholesterol balance and cholesterol trafficking in hepatocytes is likely to be more complex than in other cell types. In addition to endogenous biosynthesis and receptor-mediated endocytosis of LDL, the liver acquires cholesterol by internalizing chylomicron remnants, which carry dietary and biliary cholesterol that has been absorbed and packaged by the intestine. Cholesterol within the hepatocytes has several possible fates. It can be: *i)* incorporated into cell membranes; *ii)* converted to cholesteryl esters that are packaged into very low density lipoprotein (VLDL) particles and secreted into the sinusoid; *iii)* metabolized to bile acids that are secreted into the bile canaliculus; or *iv)* directly secreted into the bile. Factors regulating the flux of cholesterol into these diverse pathways have yet to be delineated (discussed by Turley and Dietschy (80) and Cooper (81)). The difficulties of examining cholesterol trafficking are compounded when studying hepatic cholesterol balance in the intact animal. Methodological differences may explain disparate observations on the balance of newly synthesized and exogenously derived cholesterol incorporated into bile acids and lipoproteins.

In the normal liver, hepatic cholesterol synthesis changes to balance the arrival of dietary sterol and LDL. While the relative proportions of newly synthesized and dietary cholesterol secreted into the bile changes, the total amount of cholesterol recruited for secretion in bile or metabolized to bile acids remains constant (82–84). Regulation of the amount of cholesterol secreted in bile is important since lithogenic bile that is supersaturated with cholesterol can lead to cholesterol gallstone disease (80). Bilhartz, Spady, and Dietschy (84) have shown that when cholesterol synthesis is uncoupled from hepatic requirements, the excess newly synthesized cholesterol is preferentially targeted to secretion in the bile. This preferential targeting may result from vesicles containing bile acids forming in close proximity to cholesterol synthesis or from compartmentalization of various cellular cholesterol pools. As with other aspects of cholesterol movement, the mechanisms controlling cholesterol availability for different pathways remain enigmatic.

Cholesterol trafficking in steroidogenic cells

The mitochondria have gone unmentioned in the discussion of cholesterol trafficking so far for two reasons. In most cell types, the cholesterol content of mitochondrial membranes is extremely low, and the pathways for cholesterol movement do not intersect the mitochondria. However, steroidogenic tissues, such as the adrenal cortex, Leydig cells, and corpus luteum, have the unique capability to direct cholesterol flow to the mitochondria in response to the appropriate peptide hormones (reviewed in ref. 85). Within the mitochondrial inner membrane, cholesterol is converted to pregnenolone by a cytochrome P-450scc-catalyzed reaction. This is the first and rate-limiting step in steroid hormone production.

Steroidogenic tissues use both endogenous and exogenous sources of cholesterol as substrate for pregnenolone synthesis (36, 86, 87). Cholesterol is also recruited by

hydrolysis of cytoplasmic cholesteryl ester lipid droplets. In MA-10 Leydig tumor cells, cholesterol derived from lipid droplets appears to be transported to mitochondria via the plasma membrane (88). Freeman has shown that the cholesterol content of the Leydig cell outer mitochondrial membrane is high when compared to mitochondria in nonsteroidal tissues. Nevertheless, extramitochondrial cholesterol is translocated to mitochondria and converted to steroid hormones without equilibrating with mitochondrial cholesterol (87).

Cholesterol transport to the mitochondria can be divided into two processes: i) mobilization of exogenously and endogenously derived cholesterol to the outer mitochondrial membrane, and ii) translocation of cholesterol from the outer to an inner mitochondrial membrane pool accessible to cytochrome P-450scc. Mobilization of cholesterol to the outer mitochondrial membrane occurs in response to a peptide hormone-induced rise in cAMP levels (reviewed in ref. 89) and requires the action of a cAMP-dependent protein kinase (90). One action of the cAMP-dependent protein kinase is to stimulate cholesteryl ester hydrolase activity (91). The movement of cholesterol to the outer mitochondrial membrane is not inhibited by cycloheximide (92, 93), but is blocked by agents that disrupt microfilaments and microtubules (88, 92, 94). Cholesterol transfer to the outer mitochondrial membrane is also thought to be mediated by SCP_2 (discussed above). The potential role of SCP_2 in mediating cholesterol movement is reviewed by Reinhart (13). The mechanism of this controlled delivery has not been elucidated.

The rate-limiting step in steroidogenesis is not the enzymatic conversion of cholesterol to pregnenolone, but the translocation of cholesterol from the outer to an inner mitochondrial membrane pool accessible to cytochrome P-450scc (95, 96). When steroidogenic cells are stimulated with hormone in the presence of cycloheximide, cholesterol accumulates in the outer mitochondrial membrane and is not transferred to the inner mitochondrial membrane (93). Therefore, it has been proposed that a labile protein is required for cholesterol delivery to the membrane microdomain containing cytochrome P-450scc. Three cycloheximide-sensitive proteins have been described that affect the rate of steroidogenesis. Epstein and Orme-Johnson (97) have described a 30 kDa candidate (designated pp30) that is present only in steroidogenic tissues (reviewed in ref 85). Their evidence suggests that, in ACTH- or cAMP-stimulated cells, pp30 is synthesized on cytoplasmic ribosomes as a larger precursor of 37 kDa, denoted as p37. p37 is phosphorylated prior to translocation into mitochondria. Within mitochondria, pp37 is cleaved very rapidly by a matrix protease to yield pp32, which is clipped by a second protease generating the mature pp30 (98, 99). pp30 is an integral membrane protein, probably residing in the inner mitochondrial membrane (98). In the absence of ACTH or cAMP stimulation, p37

is not phosphorylated prior to translocation into mitochondria; thus p30 is found in the mitochondria of control cells (98). pp30 fits some of the requirements for a cholesterol delivery protein because it is rapidly synthesized upon cAMP stimulation, phosphorylated by cAMP-dependent protein kinase, and resides in the mitochondrion. Also consistent with this role, Stocco and Chen (100) have found that a Leydig tumor cell line that constitutively produces steroids has constitutively high levels of a protein structurally similar to pp30. Additionally, Stocco and Sodeman (99) have identified high molecular weight precursors of these mitochondrial phosphoproteins. But as both groups note, pp30 is not a labile protein and the possibility that the short-lived precursors of pp30 directly or indirectly facilitate cholesterol transfer must be considered (98).

Steroidogenesis activator proteins (SAP) of 2.2 and 3.2 kDa have been purified by Pedersen and Brownie (101, 102) from adrenal and Leydig cells, respectively. Consistent with a role in cholesterol delivery, SAP levels in steroidogenic tissues quickly increase in response to peptide hormones or cAMP, and this increase can be blocked by cycloheximide (103). Turnover studies indicate that SAP is labile (103). SAP is a cytosolic protein (102), which seems inconsistent with a role in inner mitochondrial cholesterol delivery; however, SAP could bind to the outer mitochondrial membrane and cause cholesterol desorption. SAP appears to correspond to the carboxy-terminus of the glucose-regulated heat shock protein GRP78. Mertz and Pedersen (103) speculate that physiologically active SAP is cleaved from GRP78 co-translationally in response to a rise in cAMP levels.

A third protein implicated in cholesterol delivery within mitochondria is an 8.2 kDa protein isolated by Yanagibashi and colleagues (104) from adrenal cortex. In in vitro assays, the 8.2 kDa protein increases the cholesterol content of mitochondria and stimulates pregnenolone synthesis. Sequence analysis of the 8.2 kDa protein revealed that it is des-(Gly-Ile)-endozepine, which binds to the adrenal benzodiazepine receptor of the outer mitochondrial membrane (105). Furthermore, Krueger and Papadopoulos (106) have shown that several pharmacological agents that bind the peripheral benzodiazepine receptor stimulate pregnenolone synthesis and promote translocation of cholesterol from the outer to the inner mitochondrial membrane. Perhaps ligand binding to the cytosolic domain of the benzodiazepine receptors causes an activation or conformation change of the inner mitochondrial domain.

Arguments can be made in favor of each of these candidates playing a role in modulating steroid biosynthesis, although evidence that any of them directly or indirectly participate in stimulus-induced physiological mitochondrial cholesterol translocation is still lacking. Additionally, the possibility that they function together cannot be excluded.

FUTURE DIRECTIONS FOR RESEARCH

The directed movement of cholesterol involves a complex pathway by which the sterol moves to various cellular sites and mediates transcriptional regulation, enzyme activation, and protein degradation. Alternatively, a regulatory intermediate may be produced in response to cholesterol insertion into one distinct cellular compartment. These processes may potentially involve multiple gene products, which must be identified to understand cholesterol transport and regulation at the molecular level. We would like to highlight selected approaches that may prove fruitful in the near future.

Niemann-Pick disease, type C

One or more gene products involved in cholesterol movement should be revealed through the study of NPC and NPD fibroblasts. It is well established that NPC is an autosomal recessive disease with a spectrum of clinical manifestations (107–109). It is conceivable that more than one single gene defect can give rise to the clinical entity of NPC. Through complementation analysis, it will be possible to determine whether one or more gene defects are responsible for the observed wide clinical heterogeneity (age of onset, presenting signs, and pattern of organ system involvement) seen in NPC. However, there is no evidence so far for multiple complementation groups (110). The finding that a given biochemical phenotype is constant within families with multiple affected siblings (111) is consistent with genotypic variation. Most significantly, it should be possible to identify the mutant gene(s) responsible for the NPC phenotype by transfecting a cDNA library from normal cells into NPC cells and isolating revertants. In this manner, both complementing and suppressing genes can be analyzed. However, the usefulness of primary human fibroblasts in delineating the precise NPC gene defect is limited. Primary cultures of human fibroblasts have a limited lifespan and are not easily grown at clonal density nor do they not lend themselves to simple genetic manipulation. For these reasons, we have transformed normal and NPC fibroblasts by infecting them with a helper-free recombinant retrovirus, ψCRIP, that carries the SV40 large T antigen (N. K. Dahl, D. Jefferson, and L. Liscum, unpublished results). Our preliminary results indicate that transformed NPC fibroblasts maintain the NPC phenotype of impaired LDL-stimulation of cholesterol esterification. Since these cells grow at clonal density, they may prove valuable in DNA-mediated complementation or suppression of the NPC defect.

Somatic cell mutants

To identify multiple cell components involved in intracellular cholesterol movement and regulation requires more comprehensive genetic analysis. An approach taken by Cadigan, Spillane, and Chang (29) and our laboratory (30) has been to isolate CHO cell mutants that express defective mobilization of LDL-derived cholesterol. The advantages of isolating and characterizing CHO mutants defective in cholesterol metabolism have been well described (112, 113). CHO cells are an immortalized cell line in which mutant analysis is simplified by virtue of their haploid state for the expression of many genes (114). Complementation analysis of a family of mutants has the potential of revealing multiple alleles that produce the desired phenotype. Finally, exogenous DNA can readily be introduced into mutant CHO cells, thus facilitating the cloning of complementing and suppressing genes.

Cadigan et al. (29) reported the isolation of CHO-derived cholesterol transport (CT) mutants from 25-RA cells. 25-RA cells were derived from mutagenized CHO cells previously selected for the ability to grow in 25-hydroxycholesterol. Isolation of CT cells was achieved by selecting mutagenized 25-RA cells under cholesterol starvation conditions. Cholesterol transport-defective cells survived cholesterol starvation presumably due to an increased reservoir of LDL-derived cholesterol accumulated in lysosomes which slowly leached out during the 5–6 days of cholesterol starvation. CT cells exhibited reduced LDL-stimulation of cholesterol esterification and an accumulation of unesterified cholesterol as detected by filipin immunofluorescence (29), both of which are indicative of an NPC phenotype.

Dahl et al. (30) isolated cholesterol transport-defective mutants from wild-type CHO cells using the polyene antibiotic amphotericin B. Amphotericin B complexes with sterol-rich membranes forming aqueous pores resulting in cell lysis (115–117). The two-step selection is depicted in **Fig. 2**. Selection I isolated mutants defective in several facets of LDL metabolism. Mutagenized CHO cells were cultured in medium containing lipoprotein-deficient serum, mevinolin (to inhibit endogenous cholesterol synthesis), and LDL. After 24 hr, cells were treated with amphotericin B. Cells capable of binding, internalizing, and hydrolyzing LDL-derived cholesteryl esters survive the initial selection if they are defective in the transport of LDL-derived cholesterol (phenotypically similar to NPC). However, two additional types of non-transport-defective mutants could also survive selection I: cells defective in binding or internalizing LDL (phenotypically similar to familial hypercholesterolemia), and cells with normal LDL binding and internalization that are unable to hydrolyze LDL-derived cholesteryl esters (phenotypically similar to Wolman's and cholesteryl ester storage diseases). Selection II was devised to isolate cells with normal LDL receptor activity and cholesteryl ester hydrolysis. Cells were cultured in medium containing lipoprotein-deficient serum and r[25-HC oleate]LDL (LDL from which the endogenous cholesteryl esters had been removed, and replaced with 25-hydroxycholesteryl oleate). After 24 hr, cells were
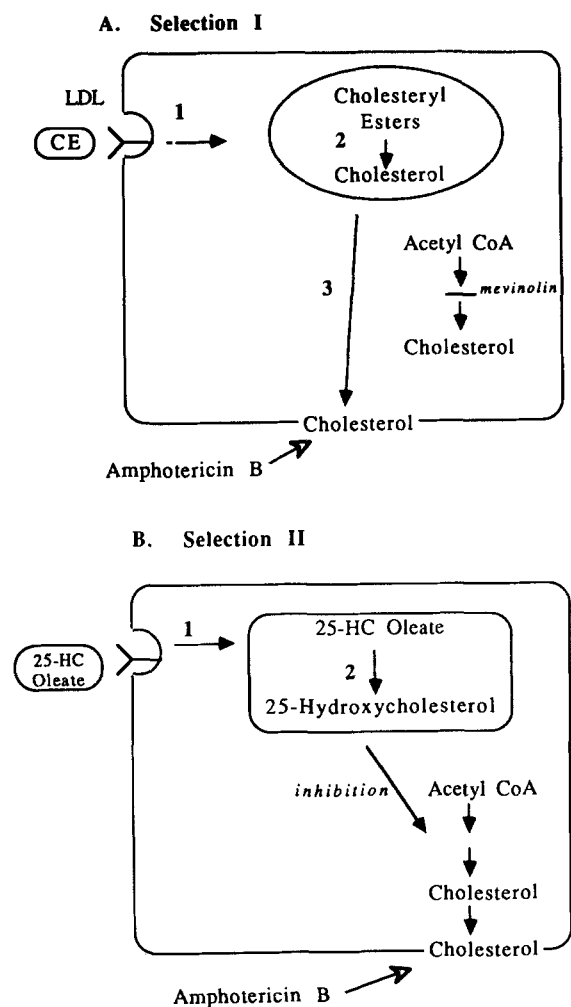
A. Selection I

B. Selection II

Fig. 2. Depiction of Selection I (panel A) and Selection II (panel B) for the isolation of cholesterol transport-defective CHO cells. For Selection I (panel A), mutagenized CHO cells were incubated in medium containing lipoprotein-deficient serum and mevinolin (to inhibit cholesterol synthesis). LDL was added, and 24 h later the cells were treated with amphotericin B. To survive amphotericin B treatment, cells must have a defect at one of the three steps that alter the delivery of LDL-derived cholesterol to the plasma membrane: *1*, LDL binding and internalization; *2*, lysosomal cholesteryl ester hydrolysis; or *3*, mobilization of LDL-derived cholesterol to the plasma membrane. For Selection II (panel B), survivors of Selection I were incubated in medium containing lipoprotein-deficient serum supplemented with r[25-HC oleate]LDL for 24 h followed by amphotericin B treatment. To survive, cells must be able to internalize the LDL (step 1) and hydrolyze the 25-hydroxycholesteryl oleate (step 2). The unesterified 25-hydroxycholesterol that is released rescues the cells from amphotericin B killing by suppressing endogenous cholesterol synthesis thus lowering cellular cholesterol levels. Presumably, cells surviving this dual selection procedure would exhibit normal binding, internalization, and hydrolysis of LDL (steps 1 and 2) but defective transport of LDL-derived cholesterol to the plasma membrane (step 3).

treated with amphotericin B. To survive, cells must be able to internalize r[25-HC oleate]LDL and hydrolyze 25-hydroxycholesteryl oleate. The 25-hydroxycholesterol that is released rescues the cells from amphotericin B killing by suppressing endogenous cholesterol synthesis and

lowering plasma membrane cholesterol levels. This combined selection resulted in mutants that are able to bind and internalize LDL normally, and to hydrolyze cholesteryl esters in the LDL core, but are defective in the movement of LDL-derived cholesterol to the plasma membrane. Biochemical analysis of our cholesterol transport mutants revealed a mutant phenotype that is identical to NPC, consisting of an accumulation of free cholesterol in lysosomes in response to LDL coincident with sluggish LDL-mediated regulation of cholesterol metabolism.

Complementation analysis of both sets of cholesterol transport mutants will indicate whether LDL-cholesterol transport is a multistep process and whether blockage of any one step gives rise to a similar phenotype. With a battery of mutant CHO cells, it should be possible to characterize many potential genes involved in cholesterol transport, as well as genes that may secondarily affect this process.

Mouse peritoneal macrophages

Another system that has generated interest is the mouse peritoneal macrophages. Tabas and colleagues (118) described the differential trafficking of cholesterol delivered via native human LDL or hypercholesterolemic rabbit β-VLDL. They determined that LDL and β-VLDL are internalized by the same receptor, but are targeted to different lysosomes. Native LDL is delivered to perinuclear vesicles where it is promptly degraded and the LDL-derived cholesteryl esters are hydrolyzed. However, internalized β-VLDL appears in a distinct set of widely distributed vesicles, where protein degradation and cholesteryl ester hydrolysis are relatively sluggish. To sort out the differential targeting, large, intestinally derived apoE-rich β-VLDL was compared with small, hepatically derived β-VLDL (119). The endocytic distribution was found to correlate with the content of apoE in the lipoprotein particle. The large apoE-rich β-VLDL is relatively resistant to acid-mediated release from macrophage LDL receptors, when compared to smaller β-VLDL. This may be due to multivalent interaction with the receptor.

β-VLDL and LDL not only target to separate endocytic compartments, but also produce different physiological consequences. β-VLDL-derived cholesterol stimulated ACAT to form cholesteryl ester lipid droplets, while LDL-derived cholesterol does not (118). Tabas and coworkers (118) first speculated that this could be due to the proximity of the β-VLDL-containing vesicles to ACAT in the endoplasmic reticulum, or to a protein or lipid that facilitates cholesterol release from the vesicles in which β-VLDL has been hydrolyzed. It is also possible that a protein or lipid causes the sequestration of cholesterol in the LDL-containing vesicles, mimicking cholesterol accumulation in NPC lysosomes. But recent results indicate that one factor influencing the ability of LDL to stimulate cholesterol esterification is efflux of cholesterol from LDL-

incubated cells. That is, the above experiments were performed by incubating macrophages with sixfold higher levels of LDL than β-VLDL (on a protein basis) in order to deliver equivalent amounts of cholesterol (118). The high concentration of LDL acts as an acceptor for cholesterol desorbed from plasma membranes. Therefore, LDL incubation does not increase cell cholesterol levels to a threshold necessary to stimulate ACAT (56). The critical threshold theory brings to the forefront our lack of knowledge of how cellular and membrane cholesterol levels are maintained. What "switch" turns on and off cholesterol acquisition and esterification? In macrophages, a mechanism must exist that allows flexible free cholesterol levels.

Hydrophobic amines

Identification of the target of U18666A inhibition of LDL-cholesterol transport might reveal one facet of the mechanism used to mobilize LDL-cholesterol from the lysosome. A somatic cell genetic approach to identify the target of U18666A inhibition is to isolate a U18666A-resistant CHO line. Due to impaired LDL-cholesterol transport, CHO cells acutely treated with U18666A are unable to grow with LDL as the sole cholesterol source. U18666A-resistant (U18R) cells were selected for the ability to grow in increasing concentrations of U18666A and a limited supply of LDL (33). In addition, U18R cells are resistant to U18666A inhibition of LDL-[³H]cholesterol transport to the plasma membrane and LDL-mediated regulatory events.

LDL stimulation of cholesterol esterification deserves special attention. In several studies, we noted a distinction between LDL stimulation of cholesterol esterification and the other LDL-mediated regulatory responses. In NPC fibroblasts, LDL stimulation of cholesterol esterification is more profoundly defective than are other LDL-regulatory responses (23, 24). Similarly, LDL stimulation of cholesterol esterification in CHO cells is the regulatory response most sensitive to inhibition by U18666A (32). It appears as though any perturbation in LDL-derived cholesterol metabolism influences LDL stimulation of ACAT most dramatically. Surprisingly, U18R cells were 100-fold resistant to U18666A inhibition of LDL stimulation of cholesterol esterification whereas other LDL-mediated regulatory responses exhibited 7- to 10-fold resistance (33). Once again, alteration in LDL-derived cholesterol metabolism dramatically affected LDL stimulation of cholesterol esterification. U18R cell express a dominant phenotype (33) and may be useful for identifying a gene conferring U18666A resistance by gene transfer experiments.

Cell-free systems

The reconstitution of membrane trafficking in cell-free and permeabilized-cell systems blends cell biology and biochemistry by allowing molecular characterization of vesicular transport along endocytic and secretory pathways (reviewed in 120–122). Thus, investigators have been able to study the energy, cofactor and cytosolic protein requirements, and kinetics of vesicular transport. First applied to the study of protein targeting, reconstitution systems have been successfully adapted to the study of membrane lipid trafficking. The first successes were in phospholipid transport. For example, Voelker (123) examined phosphatidylserine synthesis and translocation to mitochondria in saponin-permeabilized CHO cells. This analysis was possible because phosphatidylserine is synthesized in the endoplasmic reticulum and decarboxylated to phosphatidylethanolamine in the mitochondria. By following the conversion of [³H]phosphatidylserine into [³H]phosphatidylethanolamine, Voelker was able to detect phosphatidylserine translocation from the endoplasmic reticulum to the mitochondria. Translocation required ATP, but did not require cytosol or ongoing phosphatidylserine synthesis, and was unaffected by Ca^{2+}, GTP, or GTPγS. Since phosphatidylserine transfer was unaffected by dilution, Voelker postulated that it did not involve a freely diffusible complex.

The difficulties inherent in establishing a cell-free reconstitution system are compounded when studying sterol trafficking due to the following considerations. a) There are no simple methods to detect the transfer of cholesterol between cellular membranes. Cholesterol is not metabolized by multiple compartments within the cell, unlike newly synthesized proteins that undergo modification in a predictable manner as they pass through the secretory pathway. One metabolic step that may be exploitable in a cell-free system is ACAT-catalyzed esterification of cholesterol in the endoplasmic reticulum. Otherwise, it is necessary to detect cholesterol movement by tedious fractionation of intracellular membranes. b) There is a need to distinguish active, directed sterol transport from spontaneous transfer that is observed in broken-cell preparations. To demonstrate physiologically relevant cholesterol movement, it is necessary to show that the in vitro transfer is either energy-dependent, inhibitable by pharmacological agents (that do not merely alter membrane fluidity), or defective in extracts from cholesterol transport-defective cells.

Moreau et al. (124) achieved this goal by demonstrating ATP- and cytosol-dependent transfer of newly synthesized phospholipids and sterols in a cell-free system prepared from rat liver. Lipid transfer was observed from [³H]acetate-labeled transitional elements of the endoplasmic

reticulum to purified Golgi apparatus immobilized on nitrocellulose strips. Two kinetically different components of transfer were detected. *i)* An ATP-dependent component of transfer was observed to be linear with time before reaching steady-state levels at 60 min. All of the major lipid species present in the endoplasmic reticulum transferred in similar molar ratios with the exception of triacylglycerides, which were not transferred. The ATP-dependent transfer, postulated to be mediated by transition vesicles, was facilitated by cytosol and inhibited by GTPγS and N-ethylmaleimide. Most significantly, transfer appeared to be acceptor-specific, i.e., Golgi vesicles but not endoplasmic reticulum or plasma membranes served as acceptors. *ii)* An ATP-independent transfer was also linear with time but did not reach steady state. ATP-independent transfer did not require cytosol and was unaffected by the above-mentioned inhibitors. This transfer may be mediated by monomeric diffusion.

The lipid movement reconstituted by Moreau et al. (124) fulfills several important criteria: it is energy- and cytosol-dependent and acceptor-specific. The ATP-dependent lipid transfer from endoplasmic reticulum to Golgi closely parallels the transfer of protein between these compartments (120); the transition vesicles may contain both nascent lipid and protein. This lipid transport should be sensitive to Brefeldin A and therefore distinct from bulk sterol transport from the endoplasmic reticulum to the plasma membrane, which is insensitive to Brefeldin A (39). Thus, there may be three separate pathways for transporting nascent cholesterol: monomeric diffusion, protein-rich transition vesicles mediating movement to the Golgi, and lipid-rich vesicles transporting sterol to the plasma membrane. Reconstitution of additional aspects of sterol transport will allow for identification of cellular factors mediating cholesterol transport and more precise biochemical analysis of cell lines bearing defects in intracellular cholesterol movement. ▮▮

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