MINIREVIEW

Recent Advances in Membrane Cholesterol Domain Dynamics and Intracellular Cholesterol Trafficking (44047)

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Abstract. **Cholesterol is distributed nonrandomly in and between biological membranes. Despite over two decades' investigation of these phenomena, the origin, regulation, and function of membrane cholesterol asymmetry are not known. Likewise, although pathways of cellular cholesterol absorption/utilization as well as *de novo* synthesis have been investigated in depth, parallel progress in elucidating pathways of intracellular cholesterol trafficking and final deposition of cholesterol within membranes remains undefined. Understanding the nature and regulation of these processes is essential to resolving molecular mechanisms of cholesterol uptake, reverse cholesterol transport, steroidogenesis, and modulation of membrane function. Based on the fundamental observation that cholesterol is not distributed uniformly in the cell, three key concepts have contributed to recent advances in this field: First, cholesterol is asymmetrically distributed across the cell surface plasma membrane, wherein it translocates rapidly. Second, cholesterol is distributed within the plane of biomembrane bilayers into dynamic and static domains, with the latter predominating. The exact nature and physiological functions of such cholesterol domains or pools remain an enigma. Third, regulation of the size and kinetics of biomembrane cholesterol domains may be determining factors in intracellular cholesterol trafficking, targeting, and efflux. Contributions of both cytosolic carrier proteins and vesicular processes are recognized.** [P.S.E.B.M. 1996, Vol 213]

In 1972, a breakthrough in membrane biology by Singer and Nicolson (1) led to the acceptance of the mosaic nature of protein distribution in fluid membrane lipid bilayers. Parallel and similar exciting advances have shown a growing appreciation that not only proteins but also lipids are nonrandomly distributed within membranes (2). Membrane proteins are now thought to reside in highly structured lipid domains (2–18), the nature of which is the subject of intense interest and debate (7, 19). A variety of physical techniques have been utilized to demonstrate the existence of protein and lipidic domains in biological and/or model membranes. These include fluorescence photobleaching recovery (6, 8–12, 20), fluorescence energy transfer (5), release from self-quenching (3, 4, 13, 16), fluorescence microscopy (21, 22), differential scanning calorimetry (23), NMR (24), x-ray and neutron diffraction (21, 25), electron microscopy (25, 26),

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elasticity (27), and other optical methods (24, 28), as well as enzymes (2, 14, 15) and sterol exchange proteins (17, 18). Interest in membrane domains has been so extensive that the Fogarty Foundation recently sponsored the First International Symposium on Membrane Domains, which resulted in publication of an entire volume of the journal *Molecular Membrane Biology* dedicated to this topic (16, 19–21, 24–26). Unfortunately, most of the previous work in the field of membrane domains has dealt only with protein domains or with phospholipid domains in phase separated model membrane systems. The subject of cholesterol domains within membranes is now of intense interest for a variety of physiological as well as biophysical reasons (4, 29–34).

Historical Perspective on the Asymmetric Distribution of Cholesterol in Biomembranes

Perhaps the most enigmatic of the lipid asymmetries concerns the nonuniform intramembrane and intracellular distribution of cholesterol. The suggestion that cholesterol is asymmetrically distributed across the plane of the membrane bilayer actually predated the 1972 Singer and Nicolson (1) hypothesis in which proteins were nonrandomly localized in a uniform lipid membrane bilayer. The first observation of an asymmetric lateral distribution of cholesterol also came prior to the Singer and Nicholson (1) model. In 1971, it was first reported by x-ray crystallography of myelin (35) and subsequently by freeze fracture electron microscopy of erythrocyte plasma membranes (36) that the transbilayer distribution of cholesterol is not uniform. Likewise, in 1965 it was first suggested by radioautography (37) and subsequently confirmed in 1973 by electron microscopy (38) that cholesterol is organized into lateral domains in erythrocyte plasma membranes. Ongoing observations indicate that plasma membranes are rich in cholesterol while intracellular membranes are relatively cholesterol poor (e.g., endoplasmic reticulum) or devoid of cholesterol (e.g., mitochondrial inner membrane). This finding suggests that intracellular pathways of cholesterol sorting and targeting must exist (4, 29–34). Research on the origin, regulation, and function of membrane transbilayer (3) and lateral (4) cholesterol distribution as well as intracellular cholesterol trafficking (4, 29) progressed steadily over the next two decades. The lateral (4) and transbilayer (3) asymmetry of cholesterol within membranes were first reviewed in depth only about 5 years ago. The object of this article is to highlight selectively some of the more recent advances in our understanding of the origin, regulation, and physiological function of these different membrane cholesterol domains, including potential role(s) in intracellular cholesterol trafficking.

Transbilayer Cholesterol Domains

Plasma Membrane Transbilayer Cholesterol Domains: Occurrence and Regulation. An asymmetric transbilayer distribution of cholesterol has been observed in plasma membranes from both normal and abnormal tissues. The cytofacial leaflet of plasma membranes from cultured tumorigenic L-cell fibroblasts (4, 39–41) is nearly 4-fold enriched in sterol (Table I). Likewise, the cytofacial leaflet of plasma membranes from normal tissues such as brain synaptosomes (15, 43, 44) and human erythrocytes (46, 47) also appears 3- to 7-fold enriched in sterol as compared to the exofacial leaflet (Table I) Transbilayer sterol domains might be regulated either by alterations in transbilayer migration rate (flip-flop) or in transbilayer

Table I. Plasticity of Plasma Membrane Transbilayer Cholesterol Distribution

Plasma membrane	Treatment	Cholesterol distribution (%)		Reference
		Exofacial leaflet	Cytofacial leaflet	
LM fibroblast	—	28 ± 6	72 ± 6	41
	Linoleic acid	72 ± 4[a]	28 ± 4[a]	41
	Linolenic acid	76 ± 8[a]	24 ± 8[a]	41
LM fibroblast	—	20 ± 3	80 ± 3	42
	Chronic ethanol	36 ± 2[a]	64 ± 2[a]	42
L fibroblasts	—	16 ± 2	84 ± 2	39, 40
	L-FABP expression	34 ± 4[a]	66 ± 4[a]	39, 40
Synaptosome	—	12 ± 2	88 ± 2	43
Synaptosome	—	11 ± 1	89 ± 1	44
	Ethanol	28 ± 1[a]	72 ± 1[a]	43
Synaptosome	Young mice	13 ± 1	87 ± 1	45
	Old mice	31 ± 2[a]	69 ± 2[a]	45
Erythrocyte	—	26 ± 3	75 ± 5	46
Erythrocyte	—	13	87	47

[a] $P < 0.05$ compared with no treatment.

equilibrium distribution of sterol. Almost nothing is known regarding factors modulating the cholesterol transbilayer migration rate. Only a single study has thus far examined this question and reported that chronic ethanol treatment of mice significantly increased the sterol transbilayer migration rate by 60%–70% (43). However, at least four potential factors affecting the plasma membrane equilibrium transbilayer sterol distribution have been examined in some detail.

First, culturing LM fibroblasts in the presence of polyunsaturated fatty acids such as linoleic acid (18:2) or linolenic acid (18:3) resulted in incorporation of these fatty acids in the LM cell plasma membrane phospholipids (41). Although LM cells can synthesize saturated and monounsaturated fatty acids (as well as chain elongate these fatty acids) *de novo,* they can neither synthesize polyunsaturated fatty acids *de novo* nor further desaturate medium-derived fatty acids. Incorporation of 18:2 or 18:3 fatty acids into LM cell phospholipids reversed the plasma membrane transbilayer sterol distribution. The exofacial leaflet became 3-fold enriched in cholesterol (Table I).

Second, the effect of chronic ethanol treatment on plasma membrane transbilayer sterol distribution was examined. Chronic ethanol treatment of LM fibroblasts or mice resulted in a transbilayer redistribution of cholesterol in plasma membranes and brain synaptic plasma membranes from the cytofacial to the exofacial leaflet (Table I), without any change in the total plasma membrane cholesterol/phospholipid ratio (43). The amount of cholesterol in the exofacial leaflet was increased 2- to 3-fold (Table I).

Third, aging dramatically altered mouse brain synaptic plasma membrane transbilayer sterol distribution (Table I). In young mice (3–4 months) 13% of synaptic plasma membrane sterol was located in the exofacial-leaflet while in aged mice (24–25 months) 2.4-fold more sterol was exposed in the exofacial leaflet (Table I).

Fourth, the molecular mechanism regulating transbilayer sterol distribution is not known. Because the spontaneous transbilayer migration rate of sterols is fast, on the order of minutes (3, 4, 16, 30, 43), this cannot account for an asymmetric transbilayer sterol distribution under equilibrium conditions. Therefore, other factor(s) must be operative and several potential mechanisms regulating transbilayer sterol distribution have been proposed:

Sphingomyelin. A potential role of sphingomyelin (e.g., sphingomyelin-cholesterol complexes) has been postulated but largely discounted. Preferential interaction of sphingomyelin with cholesterol cannot account for the observed enrichment of cholesterol in the cytofacial leaflet since the transbilayer distribution of cholesterol (enriched in cytofacial leaflet) is opposite to the transbilayer localization of sphingomyelin (enriched in exofacial leaflet) in the plasma membrane (3, 29, 48).

The Formation of Transbilayer Dimers. Transbilayer tail-to-tail interactions of cholesterol have been detected (2, 49, 50). Both cholesterol (49) and fluorescent NBD-cholesterol (50) form transbilayer coupled cholesterol dimers. The role of such transbilayer dimers in regulating transbilayer or lateral cholesterol distribution in either model or biological membranes is unknown and represents a new area of research in the field of cholesterol domains.

Plasma Membrane Proteins. Other potential candidates include proteins interacting with cholesterol within the plasma membrane (3, 4, 51). In a parallel development, proteins containing basic regions that interact with and cause domain formation of negatively charged phospholipids have been documented. These include protein kinase C, myristoylated alanine-rich C kinase substrate, pp50^{src}, influenza hemagglutinin (52–57), or peptides (e.g., Alzheimer's amyloid fragments) (58). Whether such protein- or peptide-induced formation of acidic phospholipid domains in membranes is also associated with formation of transbilayer cholesterol gradients is not known. However, at least one recent report indicates that vesicular stomatitis virus G or M proteins induced formation of membrane acidic phospholipid domains along with recruitment of cholesterol into these domains (51). Whether such induced cholesterol rich lateral domains in one leaflet of the membrane may also result in a different equilibrium transbilayer distribution of cholesterol remains to be determined.

Cytoplasmic Proteins. Finally, cytoplasmic proteins capable of interacting with cholesterol may also elicit transbilayer cholesterol domain alterations including transbilayer migration rate, rate of desorption, or transbilayer distribution. Two cytosolic proteins binding cholesterol with micromolar K_d and 1:1 stoichiometry for cholesterol have been reported: liver fatty acid binding protein (L-FABP, reviewed in Ref. 59) and sterol carrier protein-2 (SCP-2), a ubiquitous protein found in nearly all tissues examined (60). SCP-2 and, less universally, L-FABP affect the initial rate and half-time of sterol transfer between membranes. SCP-2 has a basic amphipathic helical region, a putative membrane interaction domain, shown to be essential for SCP-2 activity in sterol transfer (61). SCP-2 and L-FABP both appear to interact with membranes in order to function in intermembrane sterol transfer (see following sections) (62, 63). Although it is not known if SCP-2 or L-FABP directly alter plasma membrane transbilayer sterol distribution *in vitro,* evidence with transfected cells indicates that L-FABP expression can influence this phenomenon (32, 64). L cells (which do not express L-FABP) were transfected

with an L-FABP expression construct, and the effect on plasma membrane transbilayer sterol distribution and fluidity gradient was determined (39, 40). L cells cultured with a serum-containing medium showed a symmetric plasma membrane transbilayer sterol distribution, as expected (Table I). However, when these cells were transfected with the cDNA encoding L-FABP, L-FABP expression induced a redistribution of sterol across the bilayer (Table I). Since nothing is known of the plasma membrane transbilayer sterol distribution of normal tissues containing L-FABP (liver, intestine), it remains to be determined whether L-FABP expression normally tends to "deregulate" or decrease the plasma membrane transbilayer cholesterol distribution. Likewise, it is not known if SCP-2 may have the same or opposing effects as L-FABP on plasma membrane transbilayer sterol distribution in these tissues. L-FABP and SCP-2 are concomitantly present in liver and intestine. Similar considerations hold for concomitant expression of SCP-2 and B-FABP (65) in brain. How does each of these proteins affect synaptosomal plasma membrane transbilayer sterol distribution? Finally, it should be noted that both the expression of such proteins in brain and liver (66, 67; Myers-Payne SC *et al,* in preparation), respectively, and their interactions with ligand (68; Myers-Payne SC *et al.,* in preparation) are altered by chronic ethanol or acute ethanol treatment of mice or rats. Chronic ethanol administration also alters the transbilayer distribution of sterol in mouse brain synaptosomes (Table I). Unfortunately, it is difficult to resolve whether altered levels and/or function of L-FABP and SCP-2 represent primary or secondary contribution(s) to altered membrane transbilayer cholesterol domains in these tissues.

Transbilayer Structural Domains

Structural Consequences of Plasma Membrane Transbilayer Cholesterol Domains. Major determinants of membrane lipid fluidity include phospholipid composition, fatty acid unsaturation, and cholesterol/phospholipid ratio. All three lipid species are asymmetrically distributed across the plasma membrane bilayer. The more "fluid" phospholipids (phosphatidylcholine and sphingomyelin) are enriched in the exofacial leaflet, while the less fluid phospholipids (phosphatidylethanolamine and phosphatidylserine) are enriched in the cytofacial leaflet. In contrast, the more "fluid" unsaturated fatty acids appear enriched in the cytofacial leaflet. As shown in Table I, the "rigidifying" cholesterol appears enriched in the cytofacial leaflet. In the cytofacial leaflet, enrichment of unsaturated fatty acids with concomitant enrichment of less fluid phospholipids and cholesterol are expected to have have opposing effects on fluidity. It is therefore not simple to predict hemileaflet fluidity based on leaflet lipid composition alone. If the transbilayer distribution of cholesterol is the major determinant of plasma membrane individual leaflet fluidity, it would seem that the leaflet enriched with cholesterol would be the less fluid leaflet. This possibility was tested with selective fluorescence quenching techniques (40–43, 45, 69–71) and membrane impermeant probes (72, 73). The structural consequence of a cholesterol enrichment in the cytofacial leaflet of plasma membranes from cultured tumorigenic LM fibroblasts is that this leaflet is less fluid than the exofacial leaflet (Table II). For example, diphenylhexatriene limiting anisotropy is a static parameter (directly related to the order parameter) that reflects the 'wobbling in a cone angle' of diphenylhexatriene or the "restriction to motion" experienced by diphenylhexatriene in the membrane. Diphenylhexatriene limiting anisotropy in the cytofacial leaflet of LM fibroblasts cultured in chemically defined medium was significantly ($P < 0.05$) greater than in the exofacial leaflet. Likewise, plasma membranes of "normal" tissues also appear to have a higher diphenylhexatriene limiting anisotropy in the cytofacial leaflet, indicating a lower fluidity in that leaflet. These "normal" tissues include synaptosomes and erythrocytes which also have a enrichment of cholesterol in the cytofacial leaflet. Although the transbilayer sterol distribution in spermatozoa membranes is not known, the transbilayer structure of the spermatozoa plasma membrane, outer acrosomal membrane, and inner acrosomal membrane (71) all have a more rigid cytofacial leaflet than exofacial leaflet (Table II). On the basis of the latter findings, one might predict that the sperm membrane inner leaflet would also be enriched with cholesterol. Thus, it appears that cholesterol transbilayer distribution appears closely associated with transbilayer fluidity gradients in plasma membranes.

Regulation of Plasma Membrane Transbilayer Structural Domains. A key question in membrane biology is whether the plasma membrane transbilayer structure is "fixed" (static) or dynamic and under cellular regulation. Increasing data indicate that the plasma membrane transbilayer fluidity gradient is a dynamic, rather than a fixed, parameter that appears to follow the transbilayer cholesterol distribution (Table I).

First, the plasma membrane fluidity gradient is regulated by exogenously supplied lipids. For example, culturing LM cells in the presence of polyunsaturated fatty acids such as linoleic acid (18:2) or linolenic acid (18:3) resulted in incorporation of these fatty acids in the LM cell plasma membrane phospholipids (41) and reversal of the plasma membrane transbilayer

Table II. Plasma Membrane Transbilayer Structure as Determined by Diphenylhexatriene

Plasma membrane	Treatment	Limiting anisotropy		Reference
		Exofacial leaflet	Cytofacial leaflet	
LM fibroblasts	—	0.148 ± 0.008	0.194 ± 0.009[a]	41
	Linoleic acid	0.133 ± 0.006[b]	0.132 ± 0.005[b]	41
	Linolenic acid	0.144 ± 0.004	0.154 ± 0.004[b]	41
	Low metastatic[c]	0.95 ± 0.04		74
	High metastatic[c]	1.35 ± 0.03[a]		74
Synaptosome	Neonate mice	0.214 ± 0.006[b]	0.234 ± 0.005[a,b]	75
	Young mice	0.237 ± 0.001	0.27 ± 0.002[a]	75
	Young mice[d]	0.191 ± 0.007	0.236 ± 0.007[a]	45
	Old mice[d]	0.235 ± 0.010[b]	0.238 ± 0.008[a]	45
Synaptosome	Control mice	0.208 ± 0.005	0.268 ± 0.004[a]	75
	Lupus mice	0.255 ± 0.008[a]	0.278 ± 0.005[a]	75
Erythrocyte	Control	0.257	0.281	76
	Control[e]	0.091 ± 0.001	0.147 ± 0.003[a]	72
	Acanthocytosis	Decrease	No Change	77
	Cholesterol	Decrease	No Change	77
Spermatozoa	Plasma Membrane	0.196 ± 0.012	0.259 ± 0.001[a]	71
	Outer acrosomal membrane	0.117 ± 0.006	0.252 ± 0.001[a]	71
	Inner acrosomal membrane	0.216 ± 0.004	0.253 ± 0.001[a]	71

[a] $P < 0.05$ versus exofacial leaflet.
[b] $P < 0.05$ versus no treatment.
[c] Ratio of inner/outer leaflet limiting anisotropy.
[d] Steady-state anisotropy.
[e] Glutathione-pyrene fluorophore.

sterol distribution (Table I). Concomitantly, the plasma membrane cytofacial leaflet became more fluid as indicated by a decreased diphenylhexatriene limiting anisotropy (Table II). Loss of cholesterol from L-cell fibroblast plasma membranes also resulted in a fluidization of the plasma membrane cytofacial leaflet (39,40). In contrast, enrichment of erythrocyte plasma membranes with cholesterol resulted in the exofacial leaflet becoming more rigid without change in the limiting anisotropy of fluorophore in the cytofacial leaflet (Table II). Thus, it would appear that the plasma membrane cytofacial leaflet cholesterol level may be near maximal and can only be lowered, while the exofacial leaflet may have a minimal cholesterol content that is either maintained constant or may be increased by nutritional regulation.

Second, developmental age modulates synaptosomal plasma membrane transbilayer fluidity (Table II) as well as transbilayer sterol distribution (Table I). The transbilayer difference in synaptosomal limiting anisotropy of diphenylhexatriene is greater in young (6-month-old) versus neonatal mice (Table II). Furthermore, in synaptosomal plasma membranes from older animals (24 months) this difference is essentially obliterated (Table II). Thus, the magnitude of the synaptosomal plasma membrane transbilayer fluidity difference obeys the following order: neonate > young > aged.

Third, the plasma membrane transbilayer fluidity gradient may be dramatically altered by disease or aging. For example, the metastatic potential of cultured LM cells is highly correlated with the ratio of cytofacial/exofacial leaflet diphenylhexatriene limiting anisotropy (Table II). Synaptosomal plasma membranes from mice affected by central nervous system lupus erythematosus have a much less fluid exofacial leaflet than unaffected animals (Table II). This results in a substantial reduction in the synaptosomal plasma membrane transbilayer fluidity difference in the lupus mice. A similar effect is noted with chronic ethanol administration (Table III). In both aging and chronic ethanol treatment the reduction in synaptosomal plasma transbilayer fluidity gradient (Table I) correlated with reduction in synaptosomal plasma membrane transbilayer sterol distribution (Tables II and III). There have been no reports on transbilayer sterol distribution in synaptosomal plasma membranes from lupus mice. Interestingly, both lupus and chronic ethanol as well as aging share in common compromised central nervous system function. Finally, hypercholesterolemia results in acanthocytic erythrocytes. It is thought that this shape change is due to enrichment of cholesterol in the exofacial leaflet of the erythrocyte membrane. As might be expected, acanthocytic erythrocyte plasma membranes displayed an increased rigidity of the exofacial leaflet as compared to normal erythrocytes (Table II).

Fourth, acute administration of ethanol or plasma membrane hemileaflet selective drugs selectively fluidized the exofacial or cytofacial leaflet of cell plasma membranes (Tables III). Ethanol and/or barbiturates fluidized the exofacial leaflet of plasma membranes

Table III. Effects of Alcohols and Leaflet Selective Drugs on Plasma Membrane Leaflet Fluidity Determined by Diphenylhexatriene

Plasma membrane	Treatment	Limiting anisotropy		Reference
		Exofacial leaflet	Cytofacial leaflet	
L fibroblasts	—	0.202 ± 0.006	0.198 ± 0.003[a]	78
	Acute alcohol	0.148 ± 0.004[b]	0.194 ± 0.003[a]	78
Synaptosome	—	0.225 ± 0.001	0.248 ± 0.002[a]	69
	Chronic ethanol	0.230 ± 0.001[b]	0.240 ± 0.001[a,b]	69
	—	0.245 ± 0.002	0.271 ± 0.002[a]	73
	Acute ethanol	0.233 ± 0.002[b]	0.268 ± 0.003[a]	73
Intestinal microvillus	—	0.125 ± 0.005	0.255 ± 0.005[a]	79
	Benzyl alcohol	0.106 ± 0.005[b]	0.251 ± 0.004[a]	79
Erythrocyte	—	0.266 ± 0.001	0.242 ± 0.004[a]	80
	Benzyl alcohol	0.242 ± 0.002[b]	0.205 ± 0.011[b]	80
LM fibroblasts	—	0.119 ± 0.002	0.155 ± 0.002[a]	(81, 82)
	Phenobarbital	0.11 ± 0.001[b]	0.155 ± 0.002[a]	(81, 82)
	Phenobarbital	0.110 ± 0.002[b]	0.150 ± 0.002[a]	(81, 82)
	Prilocaine	0.115 ± 0.002	0.147 ± 0.002[a,b]	(81, 82)

[a] $P < 0.05$ versus exofacial leaflet.
[b] $P < 0.05$ versus no treatment.

from L-cell or LM-cell fibroblasts as well as mouse brain synaptosomes (Table III). In contrast, prilocaine and benzyl alcohol selectively fluidized the plasma membrane cytofacial leaflet of LM-cell fibroblasts and erythrocytes, respectively (Table III).

Function of Transbilayer Domains

Receptor Effector Coupling and Ion Transport. Since the plasma membrane transbilayer cholesterol distribution is an important determinant of transbilayer fluidity gradients, the functions of proteins residing in one or both leaflets may be modulated by redistribution of transbilayer cholesterol/fluidity gradients (2, 83, 84). For example, receptor proteins (e.g., glucagon receptor) and effector proteins (e.g., adenylate cyclase) are located in exofacial and cytofacial leaflets, respectively. There is considerable evidence that maximal receptor-activated adenylate cyclase activity may require an optimal transbilayer fluidity gradient (84). In addition, ion transporters comprised of subunits need to be in an associated/aggregated state to be maximally functional. This association may be modulated by the plasma membrane individual leaflet fluidity and/or transbilayer fluidity gradient. Hence, membrane leaflet cholesterol content may define specific ion transporter subunit association. Indeed, it has been suggested that spontaneous organization of cholesterol into domains may result in lateral segregation of proteins into specific cholesterol-rich or cholesterol-poor regions in the membrane (85). Alternatively, it has been proposed that proteins may organize cholesterol into lateral domains (51). Individual leaflet cholesterol content may affect the formation of such lateral domains. Recently, the Na^+,K^+-ATPase was examined in plasma membranes from L-cell fibroblasts transfected with the cDNA encoding L-FABP. As pointed out above, these cells have an altered plasma membrane transbilayer cholesterol distribution with 2.1-fold higher percentage sterol in the outer leaflet compared with control cells. This alteration was associated with a 42% decrease in plasma membrane Na^+,K^+-ATPase specific activity and 46% decrease in [^{3}H]-ouabain binding (39). Active Na^+,K^+-ATPase is comprised of several types of subunits that are organized into functional enzyme in the plasma membrane. Antisera against Na^+,K^+-ATPase–specific subunits showed that the L-FABP–expressing cells actually had more total Na^+,K^+-ATPase protein. However, the proportions of the different types of subunits were inadequate for maximal Na^+,K^+-ATPase activity. These data suggested that altered plasma membrane transbilayer sterol distribution may affect Na^+,K^+-ATPase activity by altering transbilayer fluidity gradients and/or by altering recruitment of Na^+,K^+-ATPase subunits to the plasma membrane from intracellular sites.

Cellular Cholesterol Influx. Although in most cells it is accepted that cholesterol uptake takes place primarily *via* the LDL receptor–mediated endocytic pathway, there is some evidence that LDL free cholesterol may enter the cell *via* an exchange pathway (86). The low cholesterol content of the plasma membrane exofacial leaflet has been suggested as a potential potent driving force for this pathway (86). The acceptor of this free cholesterol pathway of LDL-derived cholesterol localizes cholesterol to a compartment/pool in the plasma membrane that is readily accessible for efflux from the cell surface. It was further suggested than an *n*-ethylmaleimide sensitive factor appears to function in LDL-cholesterol influx by drawing the free cholesterol into specific plasma membrane microdomains (86).

Cellular Cholesterol Efflux or Reverse Cholesterol Transport. Cholesterol efflux from tissue cell membranes (both intracellular and plasma membranes) to vascular acceptors (blood cells and/or HDL) appears to occur by molecular sterol transfer, a process termed "reverse cholesterol transport" (87–89). The mechanism(s) of reverse cholesterol transport is not understood (89). Potential contributors to reverse cholesterol transport include: intracellular mobilization of cholesterol, transfer of intracellular cholesterol to the plasma membrane, transbilayer migration across the plasma membrane, lateral cholesterol domains in the plasma membrane, desorption from the exofacial leaflet, and transfer to acceptor membrane or lipoprotein. Some advances have been made in our understanding of these parameters.

With regard to intracellular mobilization of cholesterol, it is important to recognize that 80%–94% of cellular cholesterol is localized to the plasma membrane (89). Furthermore, greater than two-thirds of Fu5AH cellular cholesterol is in a rapidly exchangeable sterol pool (89). This observation is consistent with the plasma membrane cholesterol transbilayer migration rate being rapid relative to the rate of cholesterol desorption from the plasma membrane. The disparity wherein one-third of cholesterol in fibroblasts is not exchangeable while 94% of cholesterol is in the plasma membrane indicates that slowly equilibrating pools of cholesterol must exist in the plasma membranes of these intact cells (89).

Cholesterol efflux studies wherein lipoproteins or other particles function as acceptors now clearly indicate that the nature of the acceptor particle dramatically affects sterol transfer (86, 90–93). Thus, the rate of cholesterol desorption from the cellular plasma membrane is not necessarily the rate-limiting step in cholesterol transfer to acceptor lipoproteins, apoproteins, peptides, cyclodextrins, etc. Agents such as phorbol-12-myrisate-13-acetate and cyclodextrins influence cholesterol transfer either by inducing intracellular translocation of cholesterol to be plasma membrane (92, 93) or by altering plasma membrane cholesterol microdomains.

Despite the above observations regarding the importance of the acceptor in reverse cholesterol transport, present dogma suggests that the rate of cholesterol desorption from the membrane is the rate-limiting step in sterol transfer between membranes and that the nature of the acceptor membrane or lipoprotein has no effect (4). However, as discussed in the following sections this basic assumption does not hold and needs to be reevaluated.

In summary, three potentially important components of cellular cholesterol efflux relate to membrane cholesterol domains: the transbilayer domain equilibrium sterol distribution in the plasma membrane, the transbilayer migration rate of sterol, and the rate of cholesterol desorption from the plasma membrane surface. Since the above discussion indicates that the rate of cholesterol desorption from the plasma membrane exofacial leaflet is not necessarily the rate-limiting step in sterol transfer, altered transbilayer migration rate of the plasma membrane cholesterol could be an important mechanism in reverse cholesterol transport. However, at present data supporting this possibility are lacking. The other major transbilayer factor potentially affecting reverse cholesterol transport is the plasma membrane equilibrium transbilayer cholesterol distribution. It would seem reasonable to predict that greater exposure of cholesterol in the exofacial leaflet of the plasma membrane would lead to increased accessibility of cholesterol to desorb from the cell plasma membrane and transfer to acceptor lipoproteins in the serum. Again, direct proof of this interesting possibility remains to be obtained. Finally, all of the consideration for plasma membrane transbilayer cholesterol domains must be extended to transbilayer distribution of cholesterol in intracellular membranes. To our knowledge, there have been no reports of intracellular membrane transbilayer cholesterol domains. Knowledge of such domains would be critical to understanding intracellular mobilization of cholesterol prior to transfer of that cholesterol to the cell surface.

Lateral Cholesterol Domains in Biological Membranes

What Are Lateral Cholesterol Domains? Because the exact nature of the nonuniform lateral cholesterol distribution in biomembranes is not known, definition of lateral cholesterol structures depends on one's point of view (7, 19). Biophysicists and biologists use different criteria when defining domains. For example, a biologist might define cytoskeleton-membrane protein interactions that impede protein motion in the membrane as domains. In contrast, biophysicists studying lipid bilayers or monolayers think of domains as regions of the bilayer differing in lipid composition or lipid packing. Thus, the terms defining cholesterol domains, pools, packing array, etc., are all essentially describing the same thing but from different viewpoints. Although an actual microscopic morphological picture of cholesterol lateral distribution in biological membranes has, to our knowledge, not appeared, such pictures have been obtained for model membranes (21, 25, 26, 94). Furthermore, cholesterol rich and cholesterol poor plasma membrane subfractions have been isolated from liver, kidney, intestine, and spermatozoa (4). The following scenarios offer views for different types of laterally segregated cho-

lesterol domains that can be envisioned in biological membranes.

New Methods for Detecting Cholesterol Domains in Plasma Membranes. In addition to those methods already reviewed earlier (3, 4), several advances in the use of fluorescent sterols to examine sterol exchange dynamics in plasma membranes of erythrocytes (16, 95) and cultured fibroblasts (40, 96, 97) have allowed resolution of sterol kinetic domains based on mass of sterol rather than just polarization change. Concomitantly, radiolabeled cholesterol exchange kinetics have also been used to confirm and/or further elucidate lateral cholesterol domains in erythrocytes (95) and synaptosomal plasma membranes (98).

Another recent exciting development is the use of bacterial cytolysins (hemolysins) such as θ-toxins as probes for detection of lateral cholesterol domains in membranes. Such toxins specifically bind to cholesterol and have revealed multiple cholesterol domains in membranes not detectable with cholesterol oxidase or other techniques (99). These θ-toxins may provide unique probes for examining cholesterol organization in membranes. They have been used to reveal two classes of cholesterol in the erythrocyte, lymphoma B, and BALL-1 cell surface plasma membrane (18, 99, 100), as well as in model membranes (17, 99).

Lateral Phase Separation of Cholesterol from Phospholipids in Plasma Membranes: Pure Cholesterol Phases. Lateral phase separation of lipids is the lateral segregation of different types of lipids into structures/domains. These phases can represent fluid-fluid immiscible, fluid-solid immiscible, reversed micellar, etc., structures. Phase-separated lipids may either be enriched in a particular lipid species or may be comprised of only a single lipid species. In either case, the physical structure (e.g., the fluidity) of the laterally phase-separated lipids is expected to differ markedly in model membrane systems. Because biological membranes contain several hundred different types of lipid species in a fluid/gel phase, it is thought that lateral phase separation in biomembranes must represent areas of fluid-fluid immiscibility. Furthermore, the relationship of transbilayer cholesterol domains to lateral cholesterol domains is unclear. In some instances, model membrane data indicate that transbilayer coupling of cholesterol domains may occur (2, 49, 50).

Although lateral phase separations of phospholipids are easily achieved in model membrane systems, pure cholesterol phases laterally segregated from phospholipids in biomembranes have been difficult to demonstrate. Three sets of evidence consistent with the existence of phase-segregated cholesterol have been obtained in model and biomembranes. First, spectroscopic evidence with model membranes indicates a pure cholesterol phase does not form below 50 mol% cholesterol (101, 102). Such a pure cholesterol phase may represent crystalline cholesterol in the membrane. It might be expected that cholesterol in a crystalline phase might be much less exchangeable or dynamic than unsegregated cholesterol. Indeed, kinetic exchange studies with radiolabeled or fluorescent sterols in model membranes demonstrate a substantial poorly or nonexchangeable cholesterol domain (4). Second, although most biomembranes have less than 50 mol% cholesterol, several types of cell surface membranes including erythrocyte plasma membranes (16, 95, 102), brain myelin (102, 103), lymphocytes (102), and select areas of cell surface membranes such as intestinal microvillus (104), bile canaliculus (84, 102), kidney brush border (84), sperm peri-acrosomal plasma membrane regions (64, 105–108), and perhaps caveolae (109) have equal to or more than 50 mol% cholesterol. Third, as shown in Table I, even for membranes containing less than 50 mol% cholesterol, enrichment of that cholesterol in the cytofacial leaflet renders the cholesterol content in that leaflet as comprising 50 or more mol% of total lipid in that leaflet. Recent studies examining sterol exchange kinetics in human erythrocytes showed that the majority, 67% of cholesterol, resided in a nonexchangeable sterol domain. In most cases, the size of this cholesterol domain is not affected by SCP-2. However, SCP-2 does increase the rate of cholesterol transfer and concomitantly decreases the half-time for cholesterol transfer. In the human erythrocyte the nonexchangeable cholesterol domain was so kinetically stable that it was even refractile to SCP-2–mediated stimulation of sterol transport rate (16, 95). Thus it seems reasonable to predict that, in cholesterol-rich cell surface membranes or membrane leaflets, cholesterol may segregate into lateral phases. Despite these exciting observations clearly demonstrating the potential for the existence of pure cholesterol phases in biological membranes, direct physical evidence in biomembranes is lacking.

Plasma Membranes: Lateral Cholesterol Enriched Phases. The plasma membrane cholesterol/phospholipid molar ratio is quite high. This ratio is 0.6 in L cells (39), 0.73 in brain synaptosomes (43), 0.43–0.9 in liver bile fronts (102), 0.9–1.0 in lymphocytes, and 0.8–1.0 in erythrocytes (95, 102, 110), 1.0 in myelin (102). Several sets of evidence are consistent with laterally segregated cholesterol domains in plasma membranes.

As shown in Figure 1, exchange studies demonstrate that this cholesterol is distributed asymmetrically in the lateral plane of the plasma membrane bilayer. These domains have been described as representing lateral cholesterol domains because their kinetics of exchange are considerably slower than the

transbilayer migration rate of cholesterol in the plasma membranes (see above). Plasma membranes from L cells (40, 96, 97), brain synaptosomes (98), and erythrocytes (16, 95) appear remarkably similar in their lateral cholesterol domain sizes as resolved by exchange kinetics between labeled and unlabeled plasma membrane vesicles at 37°C. The methodology involved in exchange assays has been reviewed earlier (4, 111). In all cases, 51%–81% of the cholesterol resides in a single domain, F_3, that is nonexchangeable. This might be expected since these cell surface membranes have a relatively high cholesterol/phospholipid ratio, ranging between 0.6 and 1.0. What is more remarkable is the fact that the remaining cholesterol appears to be exchangeable, usually in one exchangeable domain, with a very wide range of exchange half-times of 3.6 ± 0.2, 8.8 ± 0.9, and 23 ± 7 hr for L cells, synaptosomes, and erythrocytes, respectively. In some cases, resolution of an additional small domain containing 4% ± 2% of total sterol with a very rapid exchange half-time, near 23 ± 2 and 32 ± 9 min, respectively, for L-cell and erythrocyte plasma membranes, was resolvable (Fig. 1). In summary, one generalization may be made from these limited observations of plasma membrane lateral sterol domain data. The half-time of exchange of the primary exchangeable sterol domain, but not domain size, apparently correlated directly with the cholesterol/phospholipid ratio in the plasma membrane. This conclusion is opposite to that observed with model membranes comprised of phospholipid and cholesterol where the half-time of exchange was independent of cholesterol/phospholipid ratio ranging from 0.5 to 1.0 (95).

As pointed out earlier (4), fluorescent sterols in organic solvents have one lifetime, while in plasma membranes as well as model membranes they have two lifetimes. The two lifetimes do not represent the transbilayer distribution of the fluorescent sterol. More importantly, the molar fractions of the two lifetimes correlated with the size to the kinetically resolved sterol domains above.

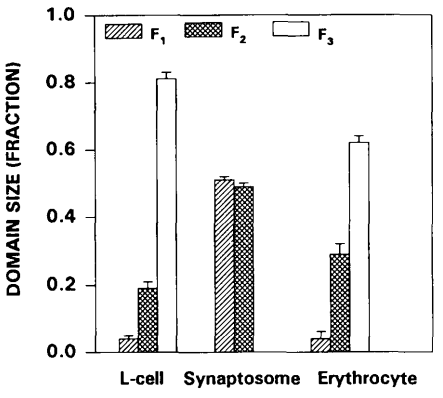

Figure 1. Plasma membrane cholesterol domains determined by dehydroergosterol and/or [^{3}H]-cholesterol exchange kinetics. F_1, F_2, and F_3, the fractional size of respective sterol domains.

A relatively new method utilizing θ-toxins has been employed to detect plasma membrane lateral sterol domains in intact cells. θ-toxins bind specifically to cholesterol and reveal two cholesterol domains in plasma membranes of intact cells. These domains did not reflect the transbilayer distribution of cholesterol in the membrane (17). Furthermore, such domains in intact cells were not detectable with cholesterol oxidase (99). θ-toxins revealed high- and low-affinity classes of cholesterol in the erythrocyte and lymphoma B cell surface plasma membrane (18, 99, 100), as well as in model membranes (17, 99). In the case of erythrocytes, the proportion of the two classes of plasma membrane cholesterol binding sites was 15%–18% and 82%–85% (100), while in lymphoma B cells it was 3% and 97% (100). The multiple classes of cholesterol domains in these cell surface membranes were confirmed by electron microscopic observations (99, 100). Of interest in this regard are additional observations with model membranes indicating that θ-toxins reveal high-affinity cholesterol binding sites only when the cholesterol/phospholipid ratio exceeded 0.4 (17). Furthermore, the specific phospholipid composition and fatty acid composition of the phospholipids dramatically affected the cholesterol domains revealed by θ-toxins (17, 99).

Cholesterol heterogeneity has been observed in epithelial cell plasma membranes. Exchange assays and erythrocyte oxidase assays have shown that 15%–30% of intestinal (112) and renal (113, 114) brush border membrane cholesterol, respectively, is rapidly exchangeable or oxidizable. This cholesterol pool appears to interact only weakly with other membrane lipids (114). The size of this cholesterol domain is in the range of the exchangeable sterol domain reported for L-cell, synaptosome, and erythrocyte plasma membranes (Fig. 1).

Recent data with deoxygenation-induced sickling in erythrocytes provided further insights into lateral cholesterol domains (115). Human erythrocyte membranes have a 1:1 molar ratio of cholesterol/phospholipid. Kinetic exchange assays revealed that only 33% of erythrocyte membrane cholesterol was exchangeable (96). Both normal and sickle cell erythrocyte membranes had similar sized exchangeable sterol domains (16). Deoxygenation of sickle cells, but not normal erythrocytes, resulted in increased uptake of the fluorescent sterol, dehydroergosterol. Membrane spicules from the deoxygenated sickle cell erythrocytes had 3-fold higher dehydroergosterol content than the remaining erythrocyte. More important, nearly all of this spicule membrane dehydroergosterol was rapidly exchangeable (115). Thus, erythrocyte spicule membranes may represent an area of the erythro-

cyte plasma membrane enriched in exchangeable sterol domain. Alternately, deoxygenation in sickle cells alters membrane structure to convert nonexchangeable sterol domain to exchangeable sterol domain capable of forming spicules (115).

Function of Plasma Membrane Lateral Cholesterol Domains

Sperm Capacitation. Sperm must be capacitated before they can complete the acrosomal reaction to fertilize an oocyte. Cholesterol is enriched in the sperm head. Unless cholesterol is shed from this periacrosomal region of the sperm plasma membrane head piece the sperm is not capacitated and cannot complete the acrosomal reaction (64, 105–107). Interestingly, a serum lipid transfer protein-I stimulated sperm capacitation by enhancing cholesterol loss (88).

Caveolae Formation. The function of cholesterol in caveolae is not known. However, caveolae are small plasma membrane invaginations that function in transcytosis of molecules across capillary endothelial cells and in utilization of GPI-linked proteins to concentrate small molecules in caveolae for transcytosis (109).

Cholesterol Absorption and Efflux. In the case of intestinal microvillus membranes, an intrinsic microvillar plasma membrane protein is required to potentiate cholesterol entry (104). This integral membrane protein must interact with membrane cholesterol domains. The size of the exchangeable cholesterol domains and/or the half-time of sterol transfer through the exchangeable domains may be important to cholesterol efflux. Cholesterol efflux from the cell is also known as reverse cholesterol transport (87, 88). This is an important process related to atherosclerosis and other serum lipoprotein disorders. In addition, the size of the exchangeable cholesterol domains and/or the half-time of sterol transfer through the exchangeable domains may be important to cholesterol uptake by the cell via the well known LDL receptor–mediated pathway. In this pathway cholesterol and cholesteryl esters enter the cell *via* the lysosomal compartment where they are subsequently hydrolyzed to free cholesterol (29). The latter molecular cholesterol apparently trafficks first to the plasma membrane and thereafter to the endoplasmic reticulum for re-esterification and subsequent storage. In both of the latter cases molecular cholesterol transfer must occur through the plasma membrane cholesterol compartment.

Membrane Protein Function. As reviewed earlier (4), the size of the exchangeable domain(s) may be correlated with size of cholesterol "poor" regions of the cell surface membrane. With rare exception (103) most proteins reside in relatively cholesterol poor lateral domains (4). Thus, the size of the exchangeable or sterol "poor" lateral domain may regulate the concentration of proteins in that domain which may in turn determine the association or aggregation state of subunit proteins (i.e., receptor-effector coupled systems, ion transporters, etc.).

Annular Phases around Proteins. Cholesterol enrichment in an annulus surrounding select proteins has been reviewed earlier (4). Basically, most proteins are localized in cholesterol-poor domains. However, the acetylcholine receptor appears associated with an annulus of cholesterol (103). A recent publication shows that vesicular stomatitis virus G and M proteins induce the partition of cholesterol into domains comprised of acidic phospholipids (51).

Cholesterol Domains in Subcellular Membranes

Subcellular organelle membranes contain much lower cholesterol/phospholipid ratios than observed with plasma membranes. This ratio is 0.10–0.25 in nuclear membranes (102), 0.16–0.23 in microsomes (4, 102, 110), 0.02–0.1 in mitochondria (4, 102, 110), and 0.6 in lysosomes (102). Major changes in membrane functions occur in the cholesterol/phospholipid molar range 0.2–0.5 or more encompassed by intracellular organellar membranes (4, 102). The cholesterol/phospholipid ratio of microsomes and mitochondria from normal tissues is remarkably stable, implying a mechanism discriminating against cholesterol (102). For example, exposure to plasma lipoprotein cholesterol enriched liver mitochondrial membranes by 95% to alter the ratio from <0.05 to <0.10, much lower than that of plasma membranes (102). This was in contrast to what is observed with microsomes and mitochondria from hepatoma cells where lipoproteins enriched 2.2- and 7.2-fold the cholesterol content versus organelles from liver (102). The specific activities of mitochondrial enzymes such as succinate-cytochrome c reductase increase with cholesterol enrichment (102). Finally, abnormal regulation of cholesterol metabolism in tumor cells appears to lie in cholesterol transport within the tumor cells (116, 117).

Almost nothing is known regarding subcellular organelle membrane sterol domains. With rare exception, almost all previous reports of cholesterol domains in biomembranes were with cell surface plasma membranes (97, 118). Therefore, the same techniques whereby the fluorescent sterol dehydroergosterol was used to elucidate plasma membrane sterol domains (16, 40, 95, 96), were adapted to examine sterol domains in other subcellular membranes (97). The cholesterol/phospholipid ratio of plasma membranes, microsomes, and mitochondria from L cells grown in chemically defined medium is 0.6 (39), 0.2 (110), and 0.02 (110), respectively. The corresponding ratios in membranes from L cells grown in 10% serum-containing medium were 0.54, 0.24, and 0.17 (119). As observed for plasma membranes from a variety of tis-

sues (Fig. 1), the L-cell fibroblast microsomal membranes had an exchangeable domain comprising 41% ± 3% of total sterol (Fig. 2). The half-time of exchange in the exchangeable domain, near 2 hr, is similar to that observed in plasma membranes (Fig. 3). In the case of mitochondria, cholesterol exchange data reflect only the outer membrane since the inner mitochondrial membrane contains little if any cholesterol. Mitochondria had an exchangeable lateral sterol domain comprising 16% ± 5% of total sterol, considerably smaller than that observed with plasma membranes and microsomes (Fig. 2). The exchange half-time of this domains, 51 ± 9 min, was more than 2-fold faster than that observed in plasma membranes or microsomes. Other data with cholesterol oxidase indicate the existence of at least two and possibly three cholesterol pools in adrenal mitochondria (118). Furthermore, increasing data indicate that the outer and inner mitochondrial membranes may not be completely separated. In fact, contact sites between the outer and the inner mitochondrial membranes have been demonstrated (120). Digitonin, a glycoside that interacts with cholesterol, treatment removed most of the outer mitochondrial membrane. However, it was not possible to completely remove all of the outer mitochondrial membrane. These observations suggested (i) the existence of attachment sites between outer and inner mitochondrial membranes; and (ii) the existence of cholesterol domains in the outer mitochondrial membrane. The latter possibility was confirmed by exchange data (see Fig. 5 below) showing that only 16% of mitochondrial membrane sterol was exchangeable and the remaining 84% was nonexchangeable.

Several interesting observations may be made from these data on spontaneous sterol exchange. First, the size of the lateral sterol domains in L-cell subcellular membranes did not correlate directly with their sterol/phospholipid ratio. Second, the half-times of cholesterol exchange of the exchangeable lateral sterol

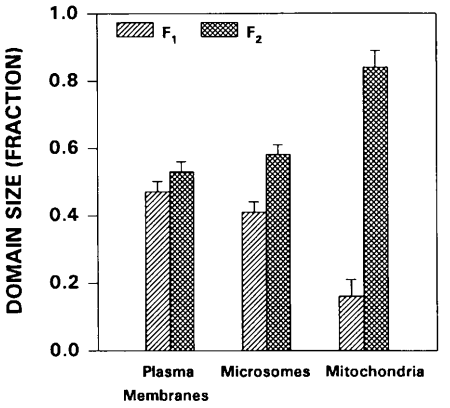

Figure 2. L-cell subcellular membrane cholesterol domains determined by dehydroergore exchange kinetics. F_1 and F_2, the fractional size of respective sterol domains (97).

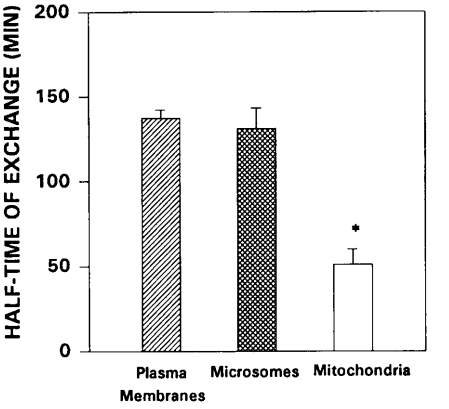

Figure 3. Half-times of dehydroergosterol exchange in L-cell subcellular membranes (119).

domains in L-cell subcellular membranes were independent of sterol/phospholipid ratio above 0.2. This conclusion is consistent with that observed with model membranes with cholesterol/phospholipid ratio ranging from 0.5 to 1.0 (95).

Intraorganellar Transfer of Cholesterol from the Outer to Inner Mitochondrial Membrane: the Rate-Limiting Step in Steroidogenesis. Mitochondrial steroidogenesis is one of the more fascinating processes in which cholesterol trafficking is the rate-limiting step (121, 122). Subsequent steps in steroidogenesis occur in the microsomes. Details of the latter transport processes remain to be clarified. With regard to the initial step in steroidogenesis, biosynthesis of all hormonal steroids occurs in the inner mitochondrial membrane *via* the cholesterol side chain cleavage enzyme. However, the inner mitochondrial membrane is devoid of cholesterol. There is strong association of progesterone production with induction of SCP-2 in steroidogenic cells wherein the rate-limiting step is the transfer of cholesterol substrate from the outer mitochondrial membrane to the cholesterol deficient inner mitochondrial membrane where oxidation occurs (123, 124). Thus, the rate-limiting step(s) of mitochondrial steroidogenesis appears to be the transfer of cholesterol from the outer to the inner mitochondrial membrane and/or the replenishment of outer mitochondrial membrane cholesterol from other intracellular sites. The mechanism(s) whereby this occurs is not known, but the possibilities fall into three major categories, all of which require a protein mediator:

First, a protein may alter the transbilayer migration rate and/or equilibrium transbilayer distribution of cholesterol in the outer mitochondrial membrane. This could then make more cholesterol accessible to desorption from the intermembrane face of the outer mitochondrial membrane. Although there are no reports of transbilayer distribution of cholesterol in the outer mitochondrial membrane, expression of L-FABP, a cholesterol-binding protein, in transfected L cells re-

sulted in an altered transbilayer distribution of sterol in the plasma membrane (39, 40).

Second, a protein such as SCP-2 may stimulate cholesterol transfer from extramitochondrial sources to mitochondria and/or from outer to inner mitochondrial membranes (121, 122). Since immunoelectron microscopy localized SCP-2 in peroxisomes, cytosol, and mitochondria (125), both possibilities must be considered. Indeed, expression of 15-kDa SCP-2 in transiently transfected COS cells co-expressing 15-kDa SCP-2 and P450scc enzyme showed both increased SCP-2 expression and 2.5-fold stimulation of steroid production (124). However, immunocytochemical data on the intracellular site of increased SCP-2 expression in these cells are lacking. Consistent with a role of SCP-2 in steroidogenesis are data from isolated membrane organelles. SCP-2 stimulates the initial rate of sterol transfer from plasma membranes and endoplasmic reticulum 12-fold and 3-fold, respectively (see Fig. 6 below). However, other recent data indicate that the role of SCP-2 in ovarian steroidogenesis is predominantly in transfer of cholesterol to mitochondria rather than between outer and inner membranes within mitochondria (122, 126). Other proteins potentially involved in transfer of cholesterol from the outer to the inner mitochondrial membrane include the steroidogenesis inducing protein, peripheral benzodiazepine receptor, and steroidogenic acute regulatory protein (122). A multitude of data have recently accumulated consistent with a close correlation of steroidogenesis with steroidogenic acute regulatory protein induction (127–129). In cells transfected with steroidogenic acute regulatory protein, the magnitude of increase in steroid production was much greater than observed in cells transfected with SCP-2. Very important in this regard, the gene defect in congenital lipoid adrenal hyperplasia, an autosomal recessive disease with undetectable synthesis of adrenal and gonadal steroids, is due to mutations in the steroidogenic acute regulatory protein (128). Although these data convincingly demonstrate a central role for steroidogenic acute regulatory protein in steroidogenesis, the mechanism whereby this membrane bound protein may stimulate steroidogenesis remains to be resolved. A potentially significant finding in this regard is a recent report indicating that steroidogenic acute regulatory protein binds a fluorescent sterol with K_d in the low nanomolar range (130). Thus, it is possible that this protein may act like the other sterol transfer proteins that require an intact sterol binding site for activity (i.e., SCP-2 and L-FABP).

Intracellular Cholesterol Trafficking

As detailed in several excellent preceeding reviews (29–33, 131–134), the intracellular distribution of cholesterol is markedly nonuniform. The cholesterol/phospholipid ratio of intracellular organelles can vary over a nearly 30-fold range: 0.03, mitochondrial membrane; 0.08, endoplasmic reticulum; 0.49, lysosomal membrane; 0.4–0.76, plasma membrane (4, 134). Clearly intracellular pathways must exist that direct cholesterol to specific organelles. Intracellular cholesterol trafficking appears to occur by two major routes. In the *de novo* route, cholesterol is synthesized in the endoplasmic reticulum and/or peroxisomes and subsequently trafficks rapidly (half-time near 10–20 min) to the plasma membrane and other cellular sites by at least three potential pathways: spontaneous transfer, energy requiring vesicular transfer, and/or cytosolic lipid transfer proteins (see following sections). The route of exogenous cholesterol entry into the cell is thought to be a slower (half-time near 40 min) and is also thought to occur by at least two pathways: First, the LDL receptor mediated endocytic pathway will transfer exogenously derived cholesterol out of the lysosomal compartment to the plasma membrane and subsequently to the endoplasmic reticulum (4, 32, 134). Second, the molecular exchange of free cholesterol may occur from LDL to the cell surface plasma membrane wherein in may localize to select pool(s) (86). Finally, it should be cautioned that the pathways of cholesterol trafficking between two membrane populations are not necessarily direct. For example, it is though that exogenous cholesterol trafficks from the lysosomal membrane to the endoplasmic reticulum *via* the plasma membrane with a possible intermediate step *via* the Golgi apparatus.

Although the above reviews (4, 29–33, 132–134) on intracellular cholesterol trafficking indicate that this is an important problem, the potential role of the donor and acceptor membrane properties remain unresolved. Factors intrinsic to the membrane include transbilayer and lateral domains, transbilayer migration rate, desorption rate from the donor membrane, and adsorption rate in the acceptor membrane. Factors extrinsic to the membrane include sterol binding proteins and any other protein, peptide, drug, hormone, etc., that may alter the intrinsic factors. Finally, regulation of the intrinsic membrane properties involved in cholesterol trafficking is far from clear. Part of the difficulty is conceptual. First, the basic assumption in previous studies is that the rate of cholesterol desorption from the donor membrane is the rate-limiting step in spontaneous sterol transfer between membranes. As pointed out below, this assumption is not correct. Second, only recently has the potential involvement of plasma membrane cholesterol domains in intracellular cholesterol trafficking been recognized (4, 86). Equally important, neither the existence of cholesterol domains in intracellular membranes nor their potential role in intracellular cholesterol trafficking has been taken into account. Third, the mechanism(s) whereby

the cholesterol transfers between membranes and specifically targets to certain membranes (e.g., plasma membrane) is unresolved. The respective roles of spontaneous transfer, protein mediated transfer, vesicular transfer, and membrane cholesterol domains (lateral as well as transbilayer) remain to be clarified. Several approaches have been used to advance the problem: (i) isolated membrane vesicles, (ii) pharmacological treatment of intact cells, (iii) genetic mutant cells, and (iv) transfected cells.

Is the Rate of Desorption from the Donor Membrane Really the Rate Limiting Step in Spontaneous Sterol Transfer between Isolated Membrane Vesicles? As reviewed previously (4), the basic assumption that the rate of cholesterol desorption from a donor membrane is the rate-limiting step is based on model membrane studies using assays requiring the physical separation of donor and acceptor membranes. By necessity in these assays the donor and acceptor membranes had to differ in some parameter (size, charge, glycolipid content, etc.). Consequently, it was not possible to determine the rate of sterol transfer in the appropriate control—that is, identical or very similar donor and acceptor membranes not requiring physical separation in the assay.

The first hint that this fundamental assumption may need to be revised came from initial model membrane sterol exchange studies with the fluorescent sterol, dehydroergosterol, in an assay not requiring separation of donor and acceptor membranes (48). Dehydroergosterol is a probe molecule used for cholesterol that has many advantages and similarities to cholesterol (3, 4). In the key experiment, the rate of sterol transfer was first determined between similar small unilamellar vesicles (48). Sterol transfer at 37°C between fluid dimyristoylphosphatidylcholine (DMPC) and fluid DMPC vesicles was rapid. In contrast, sterol transfer at 37°C between gel phase distearoylphosphatidylcholine (DSPC) and gel phase DSPC vesicles was too slow to be measurable. If the rate of desorption of sterol from the donor membrane were the rate-limiting step in sterol transfer then changing the acceptor vesicles should have no effect. On the contrary, sterol transfer at 37°C between fluid dimyristoylphosphatidylcholine (DMPC) and gel phase DSPC vesicles was not measurable. When the opposite experiment was performed, sterol transfer at 37°C between gel phase distearoylphosphatidylcholine (DSPC) and fluid phase DMPC vesicles did occur. Additional revelations that the above findings also extended to biological membranes come from isolated L-cell subcellular membrane vesicles and fluorescent sterol exchange assays (119). When plasma membranes were the cholesterol donors, the initial rate of spontaneous sterol transfer decreased up to 4-fold when plasma membrane acceptors were replaced with mitochondrial acceptors (Fig. 4, left). Dependency on acceptor membrane type was also noted with microsome donors (Fig. 4, center) and mitochondria (Fig. 4, right). In summary, properties of the acceptor as well as the donor membrane are important in spontaneous sterol exchange. The rate of desorption of sterol from the donor membrane is not necessarily the rate-limiting step in spontaneous sterol transfer. Both the "off-rate" from the donor and the "on-rate" to the acceptor membrane must be considered.

Spontaneous Sterol Transfer between Dissimilar Intracellular Membrane Fractions. The initial rate of spontaneous sterol transfer between isolated L-cell membrane fractions differed more than 3-fold, depending on the acceptor membrane used (Fig. 4) (119). For example, the rate of sterol transfer from plasma membranes to microsomes, 0.48 ± 0.06 pmol/min, was more than 3-fold faster than to mitochondria (0.15 ± 0.03 pmol/min). The latter rate was 9-fold slower than from mitochondria to microsomes. These observations are consistent with faster sterol trafficking from plasma membranes or mitochondria to endoplasmic reticulum for sterol esterification rather than to mitochondria for oxidation.

Equally important, the initial rate of spontaneous sterol transfer between isolated L-cell membrane fractions differed depending on the direction of sterol transfer examined (Fig. 4). While the spontaneous rate of sterol transfer from plasma membranes to microsomes was 0.48 ± 0.06, this rate did not differ from that in the reverse direction from microsomes to plasma membranes (0.42 ± 0.06). In contrast, the spontaneous rate of sterol transfer from plasma membranes to mitochondria, 0.15 ± 0.03, was 4-fold slower than that in the reverse direction from mitochondria to plasma membranes (0.62 ± 0.13). This finding is surprising in view of the several-fold lower cholesterol/phospholipid ratio in mitochondria compared with that

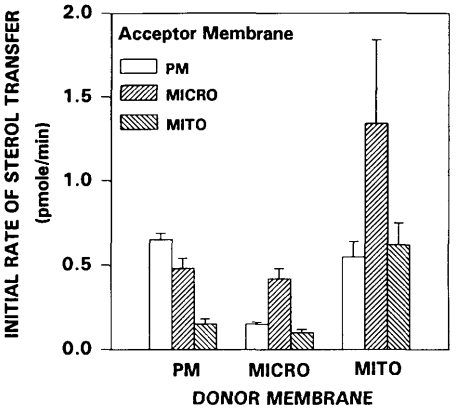

Figure 4. Initial rate of sterol transfer between L-cell subcellular membranes determined (119).

in plasma membranes. Essentially, sterol spontaneously moved up a concentration gradient from mitochondria to plasma membranes and less so from mitochondria to microsomes.

The spontaneous rate of sterol transfer from microsomes to mitochondria, 0.10 ± 0.02, was 13-fold slower than that in the reverse direction from mitochondria to microsomes (1.34 ± 0.50). Again, this finding is surprising in view of the nearly similar cholesterol/phospholipid ratio in mitochondria and microsomes isolated from L cells cultured in serum containing medium (97). Why would sterol movement be so different if the donor and acceptor have similar sterol/phospholipid ratios? The mechanism whereby this occurs is not known. Interestingly, an earlier study with isolated intracellular organelle membranes showed that the distribution of cholesterol between the organelle membranes can be qualitatively but not quantitatively accounted for by the relative affinities of the organellar membranes for cholesterol (135). For example, plasma membranes have a higher affinity for cholesterol but this affinity is not high enough to account for the magnitude of the cholesterol/phospholipid ratio observed in this membrane.

Effect of Pharmacological Treatment of Intact Cells on Cholesterol Trafficking in Intact Cells. The transport of cholesterol synthesized *de novo* is not inhibited by drugs that affect vesicular transport, lysosomal function, protein synthesis, or the Golgi apparatus (29). The trafficking of exogenously derived cholesterol is not altered by drugs that inhibit vesicular trafficking or by energy poisons (29). Although the mechanism(s) whereby cholesterol leaves the lysosomes and trafficks to the plasma membrane is not understood, this would suggest a potential role of cytosolic lipid transfer proteins. Several such candidate proteins are discussed below. A variety of pharmacological agents affecting lysosomes have been examined. Pharmacological agents that affect lysosomal pH (e.g., hydrophobic amines, progesterone) can interfere with this process (29). Hydrophobic amines such as U18666A and the steroid progesterone also appear to inhibit cholesterol movement from plasma membrane to endoplasmic reticulum by an unknown mechanism (29). There is as yet no evidence to suggest that these drugs may possibly affect donor membrane sterol domains and/or interact with lipid transfer proteins. However, it has been shown that another drug that inhibits microsomal esterification of plasma membrane–derived cholesterol may do so, in part, by blocking ligand binding to a sterol transfer protein (136).

Potential Role of Sterol Domains in Spontaneous Sterol Transfer between Isolated Intracellular Membrane Fractions. The effect of cholesterol transfer between dissimilar membranes on cholesterol domains in the donor membranes was examined (97). Different acceptors significantly affected the size of the exchangeable sterol domain in plasma membrane donor membranes (Fig. 5, left), microsomal donor membranes (Fig. 5, center), or mitochondrial donor membranes (Fig. 5, right). For example, substitution of microsomal acceptor membranes by plasma membrane acceptor membranes significantly reduced the size of the exchangeable sterol domain by 47% in donor microsomes (from 0.41 ± 0.04 to 0.22 ± 0.04, Fig. 5, center). Likewise, substituting plasma membrane acceptors with microsomal acceptors reduced the size of the exchangeable sterol domain in plasma membrane donors (Fig. 5, left). Simple aqueous diffusion of the cholesterol released from the membrane surface cannot account for this difference since the domain sizes and half-times of exchange for plasma membrane–plasma membrane and for microsomes-microsomes are very similar (97). Postulation of a direct interaction between the donor and acceptor membranes does account for these observations, since these interactions would be the same for plasma membrane-microsome and microsome-plasma membrane combinations. Perhaps a diffusible component (e.g., a protein) that interacts better with one organellar membrane than another may play a role (see below). In summary, not only does the nature of the acceptor membrane contribute to the kinetics of spontaneous sterol trafficking between isolated plasma membranes, but in addition the nature of the acceptor membrane may modulate the apparent size of the spontaneously exchanging sterol domain in the donor membrane.

Relative Role of Spontaneous Cholesterol Transfer in Intracellular Cholesterol Trafficking. Several points may be made from the preceeding sections' sterol exchange data in terms of potential sterol trafficking pathways. (i) Clearly, specific unknown factor(s) must be operative that would direct spontaneous movement of cholesterol up a concentration

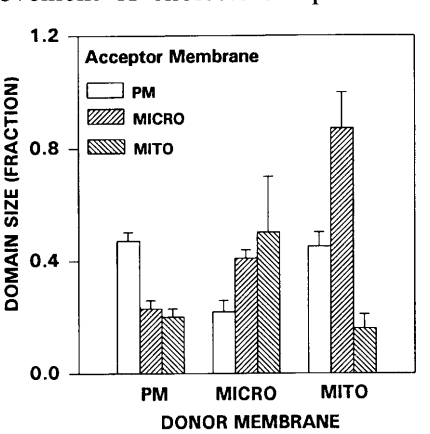

Figure 5. Effect of L-cell acceptor membrane on exchangeable sterol domain size in donor membranes (97).

gradient. (ii) Plasma membrane cholesterol spontaneously moves better to microsomes rather than mitochondria. (iii) The paucity of cholesterol in intracellular membranes and the enrichment of cholesterol in plasma membranes cannot be based on spontaneous cholesterol movement between these membrane fractions. The last conclusion is based on the following. First, the rate of aqueous diffusion of cholesterol is relatively slow (89). Second, the rate of spontaneous cholesterol transfer from microsomes to the plasma membrane (half-time 49 ± 3 min) (97) and mitochondria to the plasma membrane (half-time 188 ± 38 min) (97) is also relatively slow. These times are in the range of 1–2 hr reported for other membranes *in vitro* (89). Third, in intact cells the half-time for spontaneous cholesterol transfer is fast. In CHO fibroblasts the half-time of transfer for exogenously derived cholesterol from lysosomal membranes to the plasma membrane is near 2 min (137). The half-time of endogenously derived cholesterol transfer from endoplasmic reticulum to plasma membrane of intact cells is also fast, near 10–18 min (138–140). The latter half-times obtained in intact cells are about 3–5 times faster than observed with isolated plasma membranes and isolated microsomes *in vitro* above. In summary, it appears unlikely that spontaneous sterol transfer is a major contributor to rapid intracellular cholesterol trafficking (32). However, as described in the following sections the cytosolic sterol carrier proteins can stimulate intermembrane cholesterol trafficking nearly 30-fold *in vitro*.

Role of Sterol Binding Proteins in Intracellular Cholesterol Trafficking

Candidate Cytosolic Proteins Potentially Involved in Cholesterol Transfer between Membranes: Studies with Plasma Membranes *in Vitro*. As mentioned above, the relative roles of vesicular versus cytosolic protein mechanisms in intracellular sterol trafficking have not been resolved. The effects of several of the cytosolic lipid binding proteins are summarized in Table IV. The SCP-2 (also called nonspecific lipid transfer protein), the liver sterol carrier protein (also called fatty acid binding protein, L-FABP, or sterol and squalene carrier protein), the brain fatty acid binding protein (B-FABP), and the heart fatty acid binding protein (H-FABP) all stimulate sterol transfer, but by different mechanisms. SCP-2 (4, 62, 96, 97, 119) and less so L-FABP (4, 40, 63, 97, 119) stimulate the initial rate of sterol transfer but differently affect the exchangeable sterol domain fraction in plasma membranes. SCP-2, but not L-FABP, decreases the half-time of sterol exchange between plasma membranes (Table IV). In contrast, L-FABP but not SCP-2 increases the size of the exchangeable domain (Table IV). Furthermore, SCP-2, but not L-FABP, stimulated sterol transfer between model membranes (141). In contrast, neither brain fatty acid–binding protein (B-FABP) nor heart fatty acid–binding protein (H-FABP) enhance the initial rate of sterol transfer (Table IV). But, by increasing the size of the exchangeable sterol domain B-FABP and H-FABP concomitantly increase the half-time of sterol transfer for the exchangeable domain. Finally, these effects appear specific because the closely related intestinal fatty acid binding protein (I-FABP) was without effect.

Mechanism Whereby Cytosolic Proteins Enhance Intermembrane Sterol Transfer *in Vitro*: Binding of Cholesterol and Aqueous Carrier. Cytosolic lipid binding proteins may participate in intracellular sterol trafficking by either binding cholesterol and acting as carriers or by enhancing desorption of sterol from membranes with or without a sterol binding site. At least two classes of cytosolic lipid transfer proteins are considered to be potential candidates: SCP-2 and L-FABP. Because the cytosolic concentration of these proteins is quite high, near 40 and 400 μM, respectively, and may be even higher in select subcellular sites, these proteins could be important participants in sterol trafficking between membranes. However, because of the low solubility of sterols in aqueous buffers it has been difficult to determine if either cytosolic lipid transfer protein actually binds cholesterol, much less acts as a carrier of cholesterol

Table IV. Effect of Cytosolic Lipid Binding Proteins on Sterol Transfer between L-Cell Fibroblast Plasma Membranes

Protein	Effect on			Reference
	Initial rate of sterol transfer	Half-time of sterol transfer (min)	Exchangeable domain (fraction)	
SCP-2	Increase	Decrease	Increase/no	4, 62, 96, 97, 119
L-FABP	Increase	Decrease	No	4, 40, 63, 97, 119; Jolly C, *et al.*, Submitted
B-FABP	No	Increase	Yes	Pu L, Schroeder F, In preparation
H-FABP	No	Increase	Yes	Pu L, Schroeder F, In preparation
I-FABP	No	No	No	4, 40, 63

between membranes. The critical micellar concentration of cholesterol is 20–30 nM (4). Recent advances with fluorescent sterols as well as modifications of older lipid binding assays have led to the conclusion that both SCP-2 (60, 142, 143) and L-FABP (144–146) bind cholesterol with micromolar K_d and stoichiometry of 1 mole sterol per mole of protein (Table V). More important, when the sterol binding site was chemically blocked, SCP-2 (62) and L-FABP (63) were unable to stimulate sterol transfer between plasma membranes. Although these data are consistent with either of these proteins acting as aqueous sterol carriers, they do not provide definitive proof. SCP-2 and L-FABP could also act by a direct membrane effect wherein the proteins bind to the membrane surface, interact with cholesterol therein, and enhance sterol desorption from the membrane. This possibility is explored in the following section.

Mechanism Whereby Cytosolic Proteins Enhance Intermembrane Sterol Transfer *in Vitro:* Direct Membrane Effect. The other major mechanism whereby SCP-2 or L-FABP may stimulate intermembrane sterol transfer is by facilitating membrane fusion or by enhancing sterol desorption from membrane surface. Fusion as a viable mechanism was disproven (62, 63). However, other data show that the direct membrane effect mechanism appears operative (62): (i) Fluorescence lifetime analysis of CPM–SCP-2, a SCP-2 with covalently attached fluorophore, showed the appearance of a new lifetime component in the presence of plasma membranes. (ii) Differential polarized phase fluorometry showed that the CPM–SCP-2 became more immobilized upon interacting with plasma membranes. (iii) When donor and acceptor plasma membranes were separated by a dialysis membrane in a dialysis chamber, SCP-2 stimulated the transfer of [^{3}H]-cholesterol across the dialysis membrane equally whether the dialysis membrane was permeant or impermeant to the SCP-2 protein. Basically similar data were obtained with L-FABP (63). Thus, SCP-2 and L-FABP both required a sterol-binding site for activity and appeared to stimulate sterol transfer from plasma membranes by interacting directly with the membranes, binding sterol therein, and enhancing its desorption. Although about 30% of SCP-2 used in the assay bound to the plasma membranes at equilibrium, the fraction of SCP-2 or L-FABP bound will depend on the respective protein concentrations and the concentration of plasma membrane. However, the data made it unlikely that either protein acted as an aqueous sterol carrier of cholesterol.

Because SCP-2 appears to act through a direct membrane interaction mechanism to enhance sterol transfer, the direction of sterol transfer between two different populations of membranes will be dependent on the cholesterol "off" and "on" rates (Fig. 6. Several possible SCP-2 effects may be considered. If the on/off rates of the donor and acceptor membranes are equal, then SCP-2 stimulates sterol transfer only if it binds to the donor to accelerate the "off" rate 1 (Fig. 6). If the "off" rate from the donor (1 in Fig. 1) is greater than the "on" rate in the acceptor (3 in Fig. 6), then SCP-2 will stimulate sterol transfer only if it binds to the acceptor membrane. If the "on" rate of the acceptor (3 in Fig. 6) is faster than the "off" rate of the donor (1 in Fig. 1), then SCP-2 will stimulate sterol transfer only if it binds to the donor membrane. In summary, the presence of binding sites for the transfer protein in a particular membrane (donor or acceptor) as well as the spontaneous "on" and "off" rates of cholesterol from the membranes both will determine the degree and direction of sterol transfer enhancement.

Cytosolic Proteins Potentially Involved in Cholesterol Transfer between Intracellular Membranes: Studies with Membrane Domains *in Vitro?* Because of the complexities of examining intermembrane sterol transfer in intact cells (e.g., multiple potential intracellular acceptors), a reductionist approach was utilized. The possibility that SCP-2 or L-FABP could be involved in intracellular membrane sterol trafficking and/or targeting was examined in isolated plasma membrane (PM), and microsome (MICRO) and mito-

Table V. Cytosolic Binding Sterol Proteins: Sterol Affinities and Stoichiometries

Ligand	Assay	Sterol carrier protein-2			Liver fatty acid binding protein		
		K_d (μ*M*)	B_{max} (mole/mole)	Reference	K_d (μ*M*)	B_{max} (mole/mole)	Reference
[^{3}H]-cholesterol	Lipidex-1000	0.3	—	60	0.78 ± 0.18	0.47 ± 0.16	144
[^{3}H]-cholesterol	Liposomes	—	—	—	1.53 ± 0.28	0.83 ± 0.17	144
[^{3}H]-cholesterol	Lipid Droplets	—	1.0	147	—	—	—
Dehydrorgosterol	Fluorescence	1.7	0.9	60	0.37	0.83	145, 146
Dehydrorgosterol	Light Scatter	1.2	1.0	142	0.88	1.0	145, 146
Dehydrorgosterol	Lifetime	1.6	1.0	142	0.40	1.1	145, 146
Dehydrorgosterol	Energy Transfer	1.3	1.0	142	—	—	—
Choleslatrienol	Light Scatter	—	—	—	0.32	1.0	145, 148

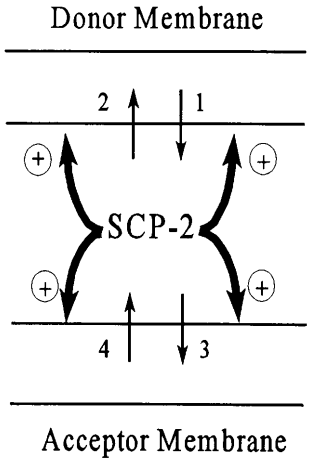

Figure 6. SCP-2–mediated intermembrane sterol transfer. 1 and 3, cholesterol "off" rate from the donor membrane and "on" rate into the acceptor membrane, respectively; 2 and 4, cholesterol "on" rate into the donor membrane and "off" rate from the acceptor membrane, respecitvely; ⊕, possible sites of stimulation by cholesterol.

chondria (MITO) membranes. The fluorescent sterol dehydroergosterol exchange assay again proved most effective (97, 119). The value of this technique in modeling studies in intact cells is best exemplified as follows: SCP-2 stimulated the initial rate of sterol transfer from microsomes to plasma membranes 5-fold *in vitro* (Fig. 7). This observation is entirely consistent with a recent report in which SCP-2 antisense oligonucleotides inhibited SCP-2 synthesis when inserted into intact human fibroblasts, resulting in a 5-fold decreased initial rate of cholesterol transfer from endoplasmic reticulum to plasma membrane (149). Both studies convincingly demonstrate for the first time that SCP-2 has a role in sterol trafficking between endoplasmic reticulum and plasma membrane (i.e., it may participate in reverse cholesterol transport).

The same assay was applied to a variety of donor/acceptor membrane combinations where SCP-2 and/or L-FABP stimulated the initial rate of intermembrane

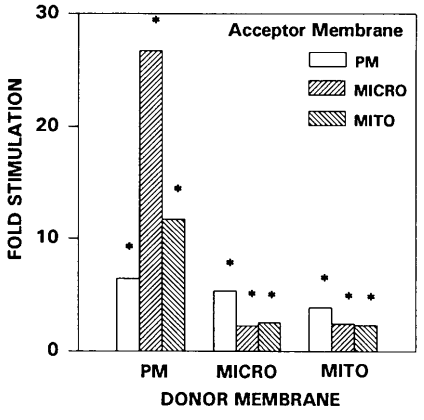

Figure 7. SCP-2 stimulation of the initial rate of sterol transfer between subcellular membranes isolated from L-cell fibroblasts. *$P < 0.05$ compared with no SCP-2 added (119).

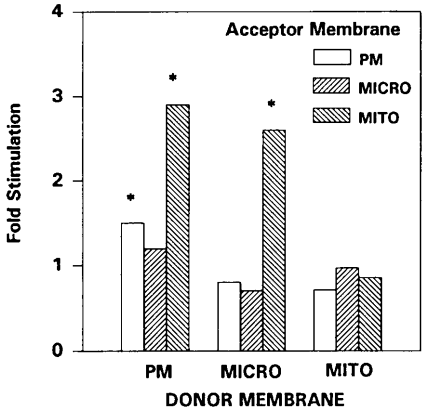

Figure 8. L-FABP stimulation of the initial rate of sterol transfer between subcellular membranes isolated from L-cell fibroblasts. *$P < 0.05$ compared with no L-FaBP added (119).

sterol transfer *in vitro*. SCP-2 (Fig. 7) and L-FABP (Fig. 8) both stimulated the initial rate of intermembrane sterol transfer to the highest extent when plasma membrane vesicles were the sterol donor. However, the magnitude and specificity of the effects of these proteins on stimulating the initial rate of sterol transfer between isolated L-cell intracellular membranes differed markedly. (i) The effect of SCP-2 (Fig. 7) was 3- to 9-fold larger than that of L-FABP (Fig. 8). (ii) SCP-2 (Fig. 7), but not L-FABP (Fig. 8), stimulated the initial rate of sterol transfer when mitochondria were the donor. (iii) SCP-2 participated in sterol targeting to specific membranes. For example, SCP-2 stimulated the initial rate of sterol transfer from plasma membrane to microsomes 26-fold, while in the reverse direction it stimulated the initial rate of sterol transfer from microsomes to plasma membranes only 5-fold. Likewise, SCP-2 stimulated the initial rate of sterol transfer 12-fold from plasma membranes to mitochondria, but only 4-fold in the reverse direction from mitochondria to plasma membranes. Thus, SCP-2 was five and four times more efficient in stimulating sterol transfer from plasma membrane to microsomes and plasma membranes to mitochondria, respectively, than in the reverse direction. (iv) L-FABP was effective in stimulating the initial rate of intermembrane sterol transfer for only three combinations: 1.5-fold between plasma membrane donor and mitochondria acceptors; 3-fold between plasma membrane donor and mitochondria acceptor; 2.6-fold between microsomes and mitochondria. Although this would make it appear to be a much less effective stimulator of intermembrane sterol transfer than SCP-2, the 10-fold higher cytosolic content of L-FABP (near 400 μM) (4, 150, 151) than SCP-2 (near 40 μM) (13, 152), as well as differential localization of L-FABP (153) and SCP-2 (125) within the cell, makes both L-FABP and SCP-2 candidates involved in intracellular sterol trafficking and targeting, perhaps between different organelles as indicated by the data presented here.

The possibility that SCP-2 and/or L-FABP stimulated intermembrane sterol transfer between different donor-acceptor membrane combinations through altered sterol domains was examined with the above assay (97). In simple phospholipid model membrane studies SCP-2 (but not L-FABP) effectively stimulated intermembrane sterol exchange both by increasing the rate of exchange and by increasing the size of the rapidly exchangeable sterol domain in the donor membrane (141). Likewise, in an earlier study (4) it was reported that SCP-2 increased the rate of sterol transfer as well as the size of the rapidly exchangeable sterol domain in isolated L-cell plasma membrane vesicles. However, the latter conclusion was based on dynamics of fluorescent sterol polarization change without conversion (*via* a standard curve) of polarization change to mass of sterol transfer. When the assay is properly calibrated to measure molecular sterol transfer, SCP-2 did not affect the plasma membrane molecular sterol domain size (Fig. 9, left) (97). L-FABP significantly increased molecular sterol domain size only in plasma membrane-plasma membrane exchanges (Fig. 9, left) (97). In heterogeneous exchanges, SCP-2 did not increase the size of the exchangeable molecular sterol domain in any heterogeneous combination of plasma membrane, microsomes, or mitochondria. In heterogeneous exchanges, L-FABP appeared to increase the size of the exchangeable molecular sterol domain only for the plasma membrane-microsome donor-acceptor, from 0.23 ± 0.03 to 0.31 ± 0.02 (97). However, this effect was not statistically significant ($P < 0.01$). Thus, in contrast to earlier studies on sterol domains based only on multiexponential curve fitting to polarization data (4), correction of polarization data using standard curves to calculate the mass of sterol transferred, revised our understanding of how SCP-2 and L-FABP transfer sterols. SCP-2 and L-FABP stimulated intermembrane sterol transfer primarily by stimulating the rate of sterol transfer, rather than by larger alterations in the size of the exchangeable sterol domains in donor biomembranes (97).

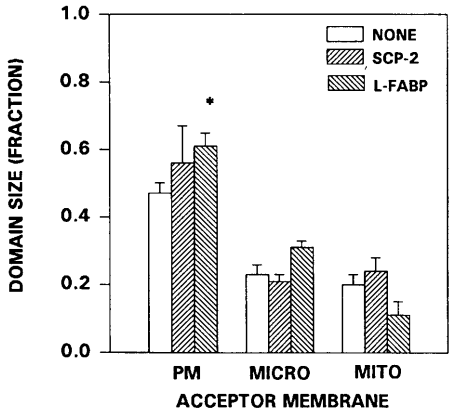

Figure 9. Effect of SCP-2 and L-FABP on intracellular membrane exchangeable sterol domain size in plasma membrane donor membranes (97).

Cytosolic Proteins Potentially Involved in Cholesterol Transfer between Intracellular Membranes: Studies with Transfected Intact Cells Expressing L-FABP. Several studies utilizing transfected cells have provided valuable information consistent with L-FABP involvement in intracellular cholesterol trafficking. The impact of transfected cell studies lies in their ability to resolve primary from secondary effects. Unlike the spontaneous genetic mutant cell lines described below, the transfected cells are based on a single genetic alteration in L-FABP resulting in primary alteration in expression of this protein.

Role of L-FABP Expression in Modulating Plasma Membrane Cholesterol Domains in Intact Cells. L-cell fibroblasts express very low levels of endogenous intracellular fatty acid binding protein and sterol carrier protein (136). These cells were transfected with cDNA encoding L-FABP and cholesterol uptake was measured in the intact cells (136, 154). Expression of L-FABP in the L cells dramatically altered spontaneous cholesterol domain dynamics in isolated plasma membranes (40). The pool size of the remaining plasma membrane spontaneous exchangeable sterol domains was decreased while at the same time the half-time of exchange of the spontaneous rapidly exchangeable sterol domain in the plasma membrane was shortened nearly 8-fold.

Role of L-FABP Expression in Modulating Cholesterol Uptake in Intact Cells. Uptake of extracellular radiolabeled cholesterol or the fluorescent sterol dehydroergosterol was enhanced in transfected L cells expressing L-FABP (136, 154). For example, the uptake of fluorescent sterol was enhanced up to 39% in transfected L cells expressing high L-FABP compared with low expression cells (155, 156).

Role of L-FABP Expression in Regulating the Intracellular Cholesterol Distribution in Intact Cells. The equilibrium distribution of cholesterol between the plasma membranes and intracellular membranes was shifted in the transfected cells such that the plasma membrane cholesterol/phospholipid ratio was decreased by 50% (136, 154).

Role of L-FABP Expression in Cholesteryl Ester Metabolism in Intact Cells. Since L-FABP can bind fatty acids (151, 157–159), fatty acyl CoAs (160; Frolov A, *et al.*, Biochemistry, in press; Jolly C *et al.*, submitted), and sterols (57, 145, 148) it seems likely that L-FABP may modulate the esterification of cholesterol to cholesteryl esters. *In vitro* studies show that L-FABP expression stimulates microsomal acyl CoA cholesteryl acyl transferase (ACAT) (136). In transfected L cells expressing L-FABP, the cellular mass of intracellular cholesteryl ester was increased by 54% (154). Likewise, the cellular mass of cholesteryl ester

derived from exogenous radiolabeled cholesterol or oleic acid increased significantly, by 2.0-fold (136). Finally, the transfer of cholesterol from the plasma membrane to the endoplasmic reticulum for esterification by ACAT was enhanced significantly, by 20% (136).

A New Method for Resolving Cholesteryl Esters Derived from Exogenous Versus Endogenous Sterols: Role of L-FABP Expression in Intact Cells. As pointed out earlier (161), endogenously synthesized cholesterol is preferentially targeted to cholesteryl esters compared with exogenously cholesterol. L cells cultured in the presence of dehydroergosterol readily take up dehydroergosterol and replace up to 80% or more of endogenous sterol with dehydroergosterol without ill effect (4, 162). In control L-cells cultured without radiolabeled cholesterol or dehydroergosterol, cholesteryl ester mass represented from 14% of total cholesterol (154) to 44% of total cholesterol (Murphy EJ, Schroeder F, submitted), depending on experimental conditions. Consistent with the presence of two pools of cholesteryl esters (161), in L cells cultured without dehydroergosterol but with 10% fetal bovine serum containing radiolabeled cholesterol, only 2% of radiolabeled cholesterol was esterified to cholesteryl ester (136). In contrast, in L cells cultured for 3 days with dehydroergosterol over 28% of dehydroergosterol was esterified to dehydroergosteryl ester, well within the range of cholesteryl ester mass of control cells. This results is consistent with at least three possibilities: (i) dehydroergosterol may enter the pathway for steryl esters of endogenous origin, (ii) dehydroergosterol may be esterified more readily by ACAT, or (iii) dehydroergosterol may be hydrolyzed more slowly by cholesteryl ester hydrolase. Which of these possibilities is operative remains to be determined.

Since exogenous cholesterol does not equilibrate with endogenously synthesized cholesterol in terms of the cholesteryl ester pools (161), it is not known which of these pools is affected by L-FABP expression. Through use of radiolabeled cholesterol and acetate one can quantitate the contribution of these pools to total cholesteryl ester mass (161). However, with radiolabel technology it is difficult to resolve which fatty acids are esterified to sterol of exogenous versus endogenous origin. Therefore, a new method was derived to resolve the sterol esters derived from exogenously supplied sterol (155, 156). To this end, the fluorescent sterol dehydroergosterol proved particularly useful to discriminate esterification of exogenous versus endogenously synthesized sterol ester (155, 156). L cells cultured in the presence of dehydroergosterol readily esterify dehydroergosterol (Figs. 10–12, Table VI) (155, 156). The L cell–derived dehydroergosteryl esters may be resolved by HPLC and detected by

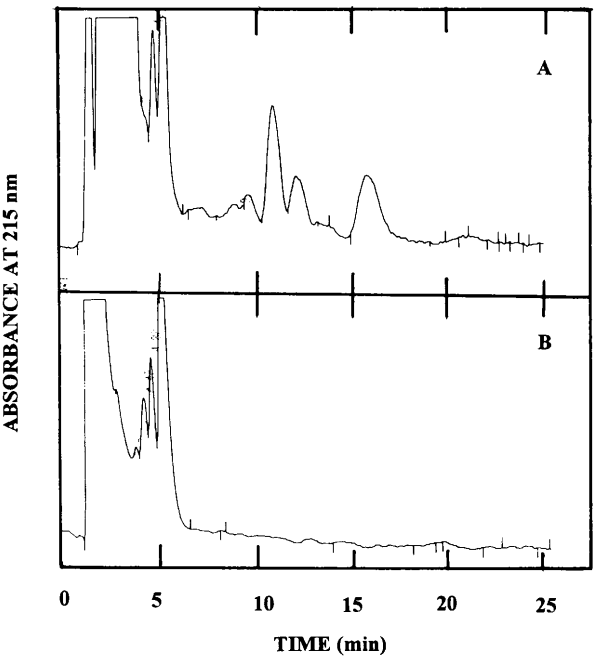

Figure 10. HPLC separation of dehydroergosteryl esters and detection by absorbance at 215 nm. (A) A C-18 reversed phase HPLC tracing of dehydroergosteryl esters isolated from L-cells cultured in the presence of dehydroergosterol for 3 days. (B) A C-18 reversed phase HPLC tracing of cholesteryl esters isolated from L-cells cultured in the absence of dehydroergosterol but in the presence of 10% fetal bovine serum for 3 days (155, 156).

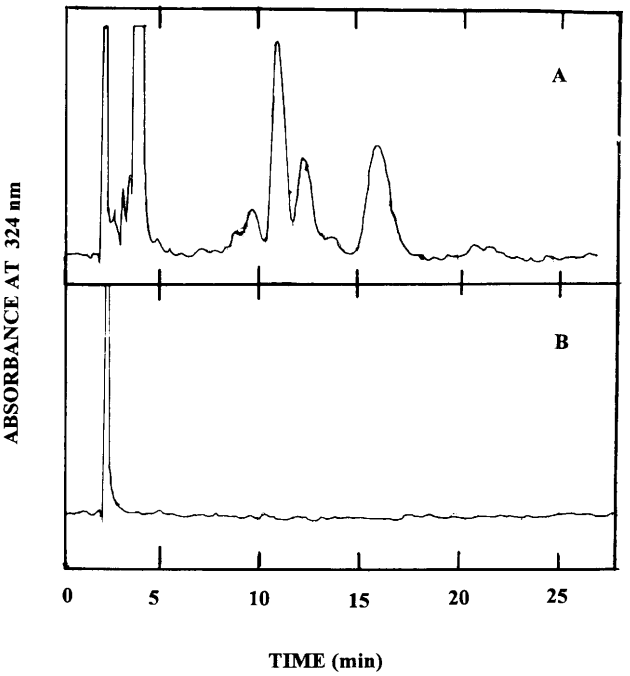

Figure 11. HPLC separation of dehydroergosteryl esters and detection by absorbance at 324 nm. All conditions were as described in Figure 10 (155, 156).

three different methods which are insensitive to the concomitant presence of cholesteryl esters. First, dehydroergosteryl esters may be detected by measuring

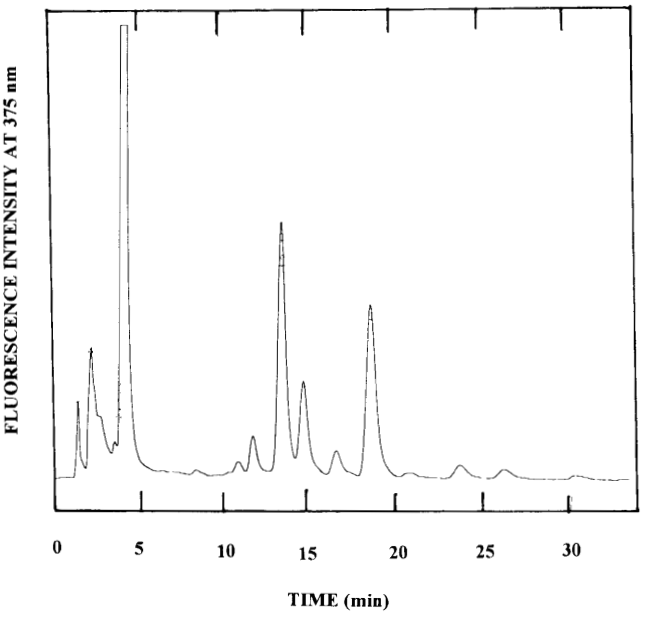

Figure 12. HPLC separation of dehydroergosteryl esters and detection by fluorescence emission at 375 nm. All conditions were as described in Panel A of Fig. 10. Baseline intensity (not shown) was obtained for cholesteryl esters from L cells (155, 156).

absorbance at 215 nm (Fig. 10A). At this wavelength, the corresponding cholesteryl esters have over 20-fold lower response factors as noted by the nearly flat baseline in cholesteryl esters extracted from L cells cultured without dehydroergosterol (Fig. 10B). Alternatively, dehydroergosteryl esters can be resolved by HPLC and detected with absorbance at 324 nm (the absorbance maximum of dehydroergosterol) can be used to detect dehydroergosteryl esters with greater sensitivity (Fig. 11A) and even lower background (Fig. 11B). Finally, dehydroergosteryl esters can be resolved by HPLC and detected by measuring fluorescence emission at 375 nm (Fig. 12) with baseline resolution and maximal sensitivity. Basically, the percent dehydroergosteryl ester composition obtained by detecting absorbance at 215 nm (Fig. 10A), absorbance at 324 nm (Fig. 11A), or fluorescence emission at 375 nm (Fig. 12) did not differ significantly. However, the fluorescence detection method resolved additional minor dehydroergosteryl esters not clearly resolved by the other methods. The total mass of dehydroergosterol esterified was significantly higher by 46% in high versus low expression L cells transfected with the cDNA encoding L-FABP. This compares favorably with more than 90% increase in radiolabeled cholesteryl ester mass in high-expression cells cultured with medium containing radiolabeled cholesterol (136).

The fatty acid composition of dehydroergosteryl esters represents the steryl ester pool of exogenously derived sterol, namely dehydroergosterol. The effect of L-FABP expression on this pool was determined (Table VI). The fatty acids esterified to dehydroergosterol in the low-expression L cells did not differ significantly from those esterified to cholesterol (data not shown). However, in the transfected L cells expressing high L-FABP the dehydroergosteryl fatty acid ester composition was basically similar with one exception (Table VI). In high expression cells the level of linoleic acid (18:2) esterified to dehydroergosterol was significantly decreased. Thus, L-FABP expression modulated the type of fatty acid esterified to exogenously derived dehydroergosterol.

In summary, L-FABP expression in transfected L-cell fibroblasts clearly demonstrates a role for this protein in regulation of membrane cholesterol domains and in cholesterol trafficking/metabolism in intact cells.

Cytosolic Proteins Potentially Involved in Cholesterol Transfer between Intracellular Membranes: Studies with Transfected Intact Cells Expressing SCP-2. With regard to effects of SCP-2 expression on cholesterol trafficking in intact transfected cells, four studies have been reported.

Transiently Transfected COS Cells Co-Expressing 15-kDa SCP-2 and P450scc Enzyme. Transiently transfected COS cells co-expressing 15-kDa SCP-2 and P450scc enzyme showed both increased SCP-2 expression and 2-fold stimulation of steroid production (124). There is strong association of progesterone production with induction of SCP-2 in steroidogenic cells wherein the rate-limiting step is the transfer of cholesterol substrate from the outer mitochondrial membrane to the cholesterol deficient inner mitochondrial membrane where oxidation occurs (123, 124).

Transfection of Human Fibroblasts with SCP-2 Antisense Oligonucleotides. Transfection of human fibroblasts with SCP-2 antisense oligonucleotides resulted in a 5-fold decrease in cholesterol transport from the endoplasmic reticulum to the plasma membrane (149).

Transfection of L-Cell Fibroblasts with cDNA Encoding SCP-2 Alters Cholesterol Uptake. Transfection of L-cell fibroblasts with cDNA encoding SCP-2 resulted in the production of stably transfected cells suitable for examining the effects on cholesterol uptake (163, 164). As detailed in preceeding sections, cellular cholesterol is derived from three sources: First, the primary uptake pathway is mediated *via* the LDL-receptor. Second, molecular exchange from serum lipoproteins accounts in most cells for only a small portion of cholesterol uptake. However, in the reverse direction this may be an important pathway of cholesterol efflux. Third, cholesterol may be synthesized *de novo*. As pointed out above, cholesterol from these three different sources may not necessarily equilibrate in all cellular cholesterol pools

Table VI. Fatty Acid Composition of Dehydroergosteryl Esters from Transfected L Cells Expressing L-FABP[a]

L-FABP expression	Fatty acid						
	14:0	16:0	18:1	18:2	20:4	22:6	x
Low (4)[b]	4.25 ± 1.10	31.94 ± 0.72	18.21 ± 0.84	37.81 ± 1.18	4.81 ± 1.62	1.18 ± 0.72	0.69 ± 0.69
High (5)	2.49 ± 1.14	33.85 ± 0.80	19.61 ± 1.16	33.86 ± 0.88[c]	6.65 ± 0.37	2.90 ± 0.58	0.16 ± 0.16

[a] Determined from absorbance at 215 nm or at 324 nm.
[b] Values in parenthesis refer to *n*.
[c] $P < 0.05$.

(161). How SCP-2 may affect these three different pathways in intact cells is not known but transfected L cells expressing SCP-2 have provided some useful information.

Uptake of exogenous cholesterol by the LDL receptor pathway is thought to proceed by hydrolysis of cholesterol ester in the lysosomes, release of cholesterol from the lysosomes, transfer to the plasma membrane, transfer to endoplasmic reticulum, and reesterification by ACAT in the endoplasmic reticulum. At which point in this pathway SCP-2 may work is not known. Neither is the role of the 15-kDa versus 13.2-kDa SCP-2. The gene for SCP-2 encodes for two gene products a 15- and 58-kDa SCP-2. Since the latter is exclusively localized in the peroxisomes, expression of the 15-kDa SCP-2 is potentially more relevant to the cholesterol uptake pathway. The 15-kDa SCP-2 contains a 20–amino acid amino terminal leader (signaling) presequence, which is rapidly cleaved to form the 13.2-kDa SCP-2. Since the function of the presequence is not known, stable transfectants expressing either the 15- or 13.2-kDa SCP-2 forms were prepared. In both cases, the expression product detected was the 13.2-kDa SCP-2, indicating rapid post-translational cleavage of the 15- to the 13.2-kDa SCP-2. Two types of experiments were performed. First, the uptake of exogenous [^{3}H]-cholesterol was examined. Expression of the 15-kDa, but not the 13.2-kDa, form of SCP-2 stimulated the extent of [^{3}H]-cholesterol uptake from the medium by 35% compared with control or mock-transfected cells. Thus, expression of the 15-kDa SCP-2 (even though it is rapidly converted to the 13-kDa SCP-2) not only facilitates cholesterol uptake but also intracellular esterification. Since expression of the 13-kDa SCP-2 did not affect exogenous cholesterol uptake, it would appear that the 20–amino acid presequence in the 15-kDa SCP-2 was essential to proper targeting of the protein in the cell. Second, the mass of cholesterol (nmol/mg protein) in control L cells, 73.3 ± 2.5, also did not differ significantly from L cells transfected with the 13.2-kDa SCP-2 (Murphy EJ, Schroeder F, submitted). However, the mass of cholesterol was significantly increased to 90.9 ± 4.3 nmol/mg in 15-kDa SCP-2–expressing cells (Murphy EJ, Schroeder F, submitted). Clearly, expression of the normal gene product, the 15-kDa SCP-2, stimulated cholesterol uptake and altered the equilibrium cholesterol content of the transfected L cells by 24%.

Transfection of L-Cell Fibroblasts with cDNA Encoding SCP-2 Alters Cholesteryl Ester Metabolism. Transfection of L-cell fibroblasts with cDNA encoding SCP-2 resulted in the production of stably transfected cells suitable for determining cholesteryl ester metabolism (164; Murphy EJ, Schroeder F, submitted). Three types of experiments were performed. First, expression of the 15-kDa, but not the 13.2-kDa, form of SCP-2 increased [^{3}H]-cholesteryl-ester levels (from exogenously derived [^{3}H]-cholesterol) by 30%, compared with control or mock-transfected cells (164). Second, both 13.2-kDa and 15-kDa SCP-2 expression increased cholesteryl ester mass, from 57 ± 3.4 nmol/mg protein in control cells to 129 ± 2.2 and 113 ± 6.7 nmol/mg protein, respectively (Murphy EJ, Schroeder F, submitted). Third, the transfer of exogenously derived [^{3}H]-cholesterol from the plasma membrane to the endoplasmic reticulum for esterification was determined. Cells were preloaded with [^{3}H]-cholesterol and then treated with sphingomyelinase containing buffer (without serum) to release plasma membrane [^{3}H]-cholesterol, which then trafficked to the endoplasmic reticulum for reesterification by ACAT. Expression of both the 15- and the 13-kDa SCP-2 stimulated transfer of plasma membrane cholesterol (released by sphingomyelinase treatment) for esterification by ACAT. These data are consistent with the interpretation that expression of the 15-kDa SCP-2 results in localizing a 13.2-kDa SCP-2 within the cell such that enhanced cholesterol trafficking from the lysosomes to the plasma membrane ensues. Expression of 13.2 kDa from either the 15- or 13.2-kDa cDNA constructs enhances transfer of cholesterol from the plasma membrane to the endoplasmic reticulum. Although these experiments show that SCP-2's may function in intracellular trafficking of exogenously derived cholesterol, there are still questions as to the mechanism. For example, immunoelectron microscopic localization of SCP-2 showed reactivity with peroxisomes, mitochondria, endoplasmic reticulum, and cytosol but not in

Golgi, lysosomes, or nuclei (125). The observation that SCP-2 needs to interact with membranes (e.g., plasma membranes; see preceeding sections) to enhance sterol transfer is difficult to reconcile with the immunoelectron microscopic data showing no SCP-2 bound to plasma membranes, Golgi, or lysosomes. How then could expression of the 15-kDa SCP-2 enhance cholesterol transfer from lysosomes to plasma membranes? How then could expression of 15- or 13.2-kDa SCP-2 enhance cholesterol transfer from plasma membranes to endoplasmic reticulum?

In summary, data obtained with cultured cell lines transiently transfected with cDNA encoding 15-kDa SCP-2, transiently transfected with antisense oligonucleotides to SCP-2, and stably transfected with cDNA encoding 15- or 13-kDa SCP-2 all indicate that SCP-2 may function in intracellular cholesterol trafficking between plasma membranes and endoplasmic reticulum, endoplasmic reticulum and plasma membrane, and, in the case of the 15-kDa SCP-2, the lysosomal compartment and the plasma membrane. These results obtained with intact cells containing SCP-2 expression constructs are consistent with studies in preceeding sections using isolated intracellular membrane fractions that showed that 13-kDa SCP-2 induced up to 27-fold enhancement of sterol transfer from plasma membranes to microsomes and 5-fold enhancement of sterol transfer in the reverse direction, primarily by altering the rate of transfer rather than by changing membrane cholesterol domain size.

Studies of Cholesterol Trafficking in Intact Cells: The Use of Genetic Mutants with Lipid Abnormalities. Clearly, spontaneous genetic mutant cell lines provide excellent models for examining intracellular sterol trafficking pathways in human diseases. Which parts of the complex intracellular cholesterol trafficking pathways are altered in these cells is the subject of intense investigation. However, results with spontaneous genetic mutant cell lines indicate that abnormalities in cholesterol and/or other lipid metabolism may not necessarily be associated with SCP-2 expression. Instead, defects in other aspects of cholesterol trafficking must account for at least some of the observations with genetic mutant cells. Specifically, these mutant or transformed cell lines include Zellweger's fibroblasts (32, 149, 165), infantile Refsum's syndrome (165), Leber congenital amaurosis (165), Niemann-Pick C fibroblasts (165–167), and hepatoma cells (102). Published reports indicate that these mutant cell lines display abnormalities in cholesterol metabolism and, in some cases, express low or undetectable levels of the 13-kDa SCP-2 as follows.

Human Zellweger syndrome fibroblasts as well as CHO cell mutants with Zellweger syndrome phenotype are deficient in peroxisomes (29, 32, 33, 149, 165, 168) and lack SCP-2 (32, 165). In human Zellweger syndrome fibroblasts, the initial rate of cholesterol transfer from the endoplasmic reticulum to the plasma membrane was 2-fold slower than in normal fibroblasts (149). However, maximal values of cholesterol transfer were the same for both cell lines. These data nevertheless indicate a role of SCP-2 in sterol trafficking from endoplasmic reticulum to plasma membranes. In contrast, opposite results were obtained with CHO cell mutants with Zellweger phenotype. The CHO mutants had normal LDL and mevalonate stimulated cholesterol esterification (32), normal transport of endogenously synthesized cholesterol to the plasma membrane (32), and normal cellular efflux of lysosomally derived cholesterol (165, 169, 170). The latter observations may be due to the fact that, although 13.2-kDa SCP-2 was not detectable in CHO mutants with Zellweger phenotype, these mutants did express the 15-kDa SCP-2, in amounts higher than in wild type cells (165). As pointed out, the 15-kDa SCP-2 is the precursor of the 13.2-kDa SCP-2. In normal tissues the 15-kDa SCP-2 is usually present in such low amounts as to be undetectable. This exciting result suggests that the CHO mutant Zellweger phenotype cells may have a defect either in the SCP-2 gene (resulting in inappropriate post-translational processing), or a defect in post-translational processing of the 15-kDa SCP-2 to form the mature 13.2-kDa SCP-2. The observation that the 15-kDa SCP-2 is capable of eliciting cholesterol transfer between membranes (171, 172), suggests that the apparent higher expression of 15-kDa SCP-2 in CHO mutants with Zellweger phenotype may possibly compensate for the lack of 13-kDa SCP-2 such that lysosomal trafficking of cholesterol was unaltered.

It has been suggested that Niemann-Pick cells have abnormal lysosomal cholesterol trafficking and this may be due, in part, to decreased levels of SCP-2 (142). In Niemann-Pick C fibroblasts intracellular movement of LDL-derived cholesterol from lysosomes to plasma membranes appears affected (166). In addition, this study concluded that SCP-2 was reduced in Niemann-Pick C fibroblasts but did not have a role in sterol trafficking from lysosomes to plasma membranes (166). However, these observations were in contrast to those reported in another study wherein the Niemann-Pick C fibroblasts did not differ in trafficking of lysosomally derived cholesterol compared to control human fibroblasts (165). More important, the level of SCP-2 reported in these cells appeared only slightly different from that observed in "normal" human fibroblast control cells (165). The basis for these differences between the two studies is not known. Finally, it should be noted that other pathways of intracellular movement of cholesterol are normal in Niemann-Pick C fibroblasts: from endoplasmic reticulum to plasma membranes (166); from plasma membranes to endoplasmic reticulum (167).

Infantile Refsum's syndrome and Leber congenital amaurosis fibroblasts are inherited peroxisome deficiency disorders (165, 173). The level of SCP-2 reported in these cells was the same as/or comparable to that observed in "normal" human fibroblast control cells (165). Furthermore, the lysosomally derived cholesterol trafficking in these cells was unaltered compared with normal human fibroblasts. Whether SCP-2 plays a role in intracellular trafficking of lysosomally derived cholesterol in these cells cannot be resolved from existing data (165).

Hepatoma cells have abnormal intracellular cholesterol distribution, cholesterol synthesis, and low SCP-2 levels compared with normal liver tissue (102, 123). There was no change in either the rate of esterification of exogenously derived cholesterol or in cholesterol accumulation in hepatoma cells compared with normal hepatocytes (123). These observations do not favor a role for SCP-2–mediated cholesterol trafficking in hepatoma cells.

In summary, the studies of intracellular cholesterol trafficking in mutant cell lines offer exciting potential models for examining intracellular cholesterol trafficking pathways. However, with regard to SCP-2 these investigations also illustrate the complexities and the difficulties in resolving the contributions of SCP-2 to intracellular sterol trafficking in mutant cells. A consistent pattern clearly identifying the absence of a role for SCP-2 in intracellular cholesterol trafficking in these mutant cells is not apparent. Consequently, whether SCP-2 functions in intracellular sterol trafficking in these mutant cells requires further clarification by taking advantage of the opportunities presented by these fascinating genetic mutants.

Studies of Cholesterol Trafficking in Intact Cells: Reconciliation of Observations in Intact Genetic Mutant Cells with Data from Intact Cells Transfected with cDNA Encoding SCP-2. Reconciliation of the data obtained in intact mutant cell lines with both the *in vitro* experiments with isolated intracellular organelle membranes and with intact transfected cell studies above requires recognition of the complexities involved in intact cell studies. Several considerations must be taken into account:

Both protein-mediated and vesicular pathways of intracellular cholesterol trafficking occur. As pointed out in preceeding sections, cholesterol may transfer between intracellular organelles by both vesicular and/or protein mediated pathways. Thus, blocking one pathway may result in compensation by another pathway. An excellent recent example of this was reported with normal versus Zellweger fibroblasts (149). In normal fibroblasts with a normal complement of SCP-2, both vesicular and SCP-2–mediated transfer of cholesterol from endoplasmic reticulum to plasma membrane were operative. Injection of normal fibroblasts with SCP-2 antisense oligonucleotides reduced the SCP-2–mediated, rapid (10 min maximal values of cholesterol transport) pathway of cholesterol transport by 81%. Concomitantly, a simultaneous increase in the vesicular mediated slower (20 min maximal values of cholesterol transport) pathway of cholesterol transport was observed.

Multiple sterol carrier proteins exist, sometimes in the same cell type (see above). In the latter case, a decrease in one sterol carrier protein could also be compensated by another sterol carrier protein. The specific pathways mediated by these multiple sterol carrier proteins remain to be resolved.

Multiple forms of SCP-2 exist. SCP-2 occurs in at least three different molecular weight forms differing dramatically in intracellular localization (125). In most of the above studies measurement of SCP-2 levels reported only the level of the 13.2-kDa SCP-2. To date, only one study with mutant cell lines clearly considered the possibility of multiple roles for the 13.2- and 58-kDa SCP-2 forms in mutant cells (32). Based on existing evidence at the time, it was suggested that only the 13.2-kDa SCP-2 functioned in sterol transfer (32). More recent evidence indicates that this is not the case. The 13.2- (123, 174), 15- (123, 174), and 58- (175) kDa forms of SCP-2 all enhance sterol transfer between model membranes *in vitro*. The 13-kDa protein stimulates sterol transfer between biological membranes (4, 33, 96), cholesterol synthesis from lanosterol, cholesterol esterification by ACAT, cholesterol oxidation by side-chain cleavage enzyme or cholesterol-7α-hydroxylase by transfer of liposomal substrate sterol to organelles containing the enzyme (33) *in vitro*. The peroxisomal 58-kDa SCP-2 transfers sterol, stimulates certain enzyme activities in the final steps of cholesterol biosynthesis, and has intrinsic thiolase activity *in vitro* (125, 165, 175, 176). In addition to these *in vitro* based studies, there are strong direct correlations among the expression of 13.2-kDa SCP-2, cholesterol synthesis, and pulmonary surfactant formation in developing lung and alveolar type II cells (177). Likewise, there is a close association between SCP-2 gene expression and cholesterol accumulation in rat peritoneal macrophages during foam cell formation (178).

The nature of the genetic defect in many of the mutant cells remains to be determined and may not be the SCP-2 gene. The genetic defect in Zellwegers syndrome, Niemann-Pick C disease, hepatoma, and mutant cell models is not known. In fact, the Niemann-Pick C (NPC) gene defect is not even located on the same chromosome as SCP-2 (121). It must be considered that altered levels of SCP-2 detected in these cells may represent a secondary effect in response to whatever is the "real" or "primary" gene defect.

Because the mutant cells are deficient in organelle

synthesis or organelle function, multiple lipid metabolism pathways are affected. The above mutant cell lines appear to have an intracellular organelle abnormality (lysosomes, peroxisomes, or mitochondria) or are deficient in specific intracellular organelles (peroxisomes). These organelles have many functions in lipid metabolism. Observed abnormalities in lipid metabolism therefore represent the net result of loss of multiple lipid synthetic functions. For example, peroxisomal deficiency correlates with complete or partial loss of the ability of the cell to make ether lipids, bile acids, isoprenoids (especially dolichols), cholesterol, and to oxidize very long chain fatty acids (29, 32, 33, 165, 168). Although studies from available mutant cell lines are complex, they provide opportunities for additional investigation to resolve the defects in intracellular trafficking pathways in human diseases, whether or not they are related to primary or secondary effects on SCP-2 function in intracellular sterol trafficking.

In conclusion, studies on intracellular cholesterol trafficking using mutant cell lines deficient in specific organelles or with abnormal organelle function will provide many valuable insights on the functions of those organelles and/or specific proteins in those organelles. The contribution(s) of SCP-2 to intracellular sterol trafficking in these cells remain(s) to be clearly resolved in many cases. Furthermore, pathways of cholesterol trafficking other than SCP-2 mediated must be explored.

Summary

Much progress has been made in elucidating pathways of intracellular cholesterol trafficking and targeting. Understanding the nature and regulation of these processes is essential to resolving molecular mechanisms of cholesterol uptake, reverse cholesterol transport, steroidogenesis, and regulation of membrane function. Cholesterol is not distributed uniformly in the cell, but instead is distributed asymmetrically within biomembranes both in the transbilayer as well as lateral plane of the plasma membrane. The latter domains are characterized by being either dynamic (exchangeable) or static (nonexchangeable), with the latter predominating. Future exciting areas of investigation are legion. The exact nature of cholesterol domains or pools remains to be resolved not only in model membranes but, more importantly, in biological membranes. We have only begun to investigate the existence of cholesterol domains in subcellular organelles. Similar techniques that have demonstrated the existence of lateral sterol domains in the cell surface plasma membrane and more recently to endoplasmic reticulum and mitochondria now need to be extended in more depth to these and other intracellular membranes. Although we are beginning to understand some of the factors (vesicular transport, sterol carrier proteins, etc.) involved in intracellular sterol trafficking, we have almost no insights regarding the mechanism(s) of selection or targeting of cholesterol to specific membranes at this time. Proteins and factors regulating the size and kinetics of biomembrane cholesterol domains may be determining factors in intracellular cholesterol trafficking, targeting, and efflux. Do specific receptors for these proteins exist? How are cholesterol and other lipids sorted from vesicular protein? How is the vesicular cholesterol selectively targeted in endocytosed membrane recycling as well as *de novo* synthesized cholesterol transported by vesicular pathways? Are the vesicular and protein mediated pathways of cholesterol trafficking truly separate, do they compensate for one another, do the proteins play a role in the vesicular pathway and vice versa? Which steps in which pathways are altered in human genetic diseases of intracellular lipid trafficking? Membrane cholesterol domains exist in interesting times.

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