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Intracellular sterol transport and distribution

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Sterols are important components of many biological membranes, and changes in sterol levels can have dramatic effects on membrane properties. Sterols are transported rapidly between cellular organelles by vesicular and nonvesicular processes. Recent studies have identified transmembrane proteins that facilitate the removal of sterols from membranes as well as soluble cytoplasmic proteins that play a role in their movement through the cytoplasm. The mechanisms by which these proteins work are generally not well understood. Cells maintain large differences in the sterol:phospholipid ratio in different organelles. Recent theoretical and experimental studies indicate ways in which the lipid environment can alter the chemical potential of sterols, which may help to explain aspects of their transport kinetics and distribution.

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Introduction

Cholesterol plays many important roles in mammalian cells. It is a major component of the plasma membrane and some organelle membranes. Changes in cholesterol levels in membranes can have profound effects on signal transduction processes and on the trafficking of membrane proteins and lipids. Excess cholesterol in macrophages in the wall of blood vessels is associated with the early stages of atherosclerosis, and high levels of cholesterol in these cells can lead to cell death in late stages of atherosclerosis [1]. Oxysterols produced by the oxidation of cholesterol are important signaling molecules that bind to nuclear receptors and affect gene expression [2].

The levels of cholesterol vary greatly among cellular organelles. It is estimated that cholesterol comprises ~30% of the lipid molecules in the plasma membrane (PM) [3]. In the endoplasmic reticulum (ER), which is the site of cholesterol synthesis, only ~1–10% of the lipid molecules are cholesterol [4,5•,6•]. In tissue culture fibroblasts, the endocytic recycling compartment (ERC) contains ~30–35% of the total cellular cholesterol, representing the major intracellular sterol pool [7].

Cholesterol moves rapidly among cellular organelles by vesicular transport as well as non-vesicular mechanisms. For non-vesicular transport, cholesterol must be associated with carriers because of its insolubility in aqueous solutions. Various proteins have been proposed as carriers of cholesterol. Additionally, there are a variety of integral membrane proteins that play important, but poorly defined, roles in the release of sterol from membranes, either for intracellular redistribution or for efflux from cells.

In this review we summarize recent information on these proteins. We also discuss how new ideas about the influence of lipid environment on the chemical potential of sterols can explain how the sterol:phospholipid ratio in different cellular organelles can vary so widely even though cells traffic sterols mainly by a non-vesicular, equilibration mechanism.

Regulation of cellular cholesterol levels

Mammalian cells acquire cholesterol by *de novo* synthesis in the ER and by endocytosis of lipoproteins. Cholesterol esters in the core of lipoproteins are hydrolyzed in late endosomes and lysosomes, and free cholesterol is released into the cell. Both the uptake and synthesis of cholesterol are tightly regulated by feedback mechanisms, chiefly involving the INSIG–SCAP–SREBP complex in the ER [8]. Excess free cholesterol can be esterified and stored in the cytoplasm in lipid droplets; the cholesterol esters in these droplets are hydrolyzed by neutral esterases, releasing cholesterol and fatty acids [9].

In most mammalian cells a small fraction of cholesterol is converted to oxysterols, and this is the only general pathway for biochemical metabolism of cholesterol (aside from esterification) [2]. In steroidogenic tissues cholesterol is the precursor for steroid hormones, and in hepatocytes cholesterol is converted to bile acids and secreted into the biliary circulation, which is the major pathway for cholesterol removal from the body. Cholesterol can be removed from cells by efflux to extracellular acceptors, especially apoA-I and high-density lipoprotein (HDL), which transports cholesterol to the liver for excretion [10].

Like mammalian cells, cells of the budding yeast, *Saccharomyces cerevisiae*, acquire sterols by biosynthesis or uptake. The major sterol in yeast is ergosterol rather

than cholesterol. Under aerobic growth conditions, yeast do not take up sterol and rely entirely on ergosterol biosynthesis in the ER to maintain their sterol complement. While yeast and mammalian cells differ in some aspects of sterol metabolism and function, yeast genetics has proven to be an excellent tool for investigating basic aspects of sterol metabolism [11–14].

Sterol transport

In yeast, sterols traffic between the ER and PM via an ATP-dependent mechanism that does not require *SEC* gene products, not even Sec18p/NSF, a protein needed at all stages of the classical secretory pathway [15••,16••]. Approximately 10^5 ergosterol molecules traffic in and out of the PM per second [17], a rate that is 10 times greater than needed to generate a new PM during yeast cell doubling. These recent results for the yeast system are consistent with older studies of cholesterol transport in mammalian cells, which showed that delivery of newly synthesized cholesterol to the PM was largely unaffected by ER-to-Golgi vesicular transport blocker, Brefeldin A [18]. It is worth noting that a Sec18p-independent vesicular transport pathway has been implicated in the transport of a membrane protein from the ER to the PM [19], and it is possible that an unusual (Sec18p-independent) vesicular pathway may also be used to some extent by sterols. Non-vesicular transport of lipids within cells requires that the lipids are extracted from the cytoplasmic leaflet of intracellular membranes and transported by lipid transfer proteins (LTPs). Sterols, like other lipid molecules with small polar headgroups, flip-flop rapidly and spontaneously across membranes [20], so they should have ready access to both leaflets of membranes. The basis of the ATP requirement for ER–PM sterol transport is not understood.

In mammalian cells, sterol that is introduced into the PM can be transported to various intracellular compartments, including the ER, where it is esterified [5•]. When dehydroergosterol, a naturally fluorescent sterol, is delivered to the PM of mammalian cells via a cyclodextrin carrier, it equilibrates with the sterol pool in the ERC by an ATP-independent (non-vesicular) mechanism with a half-time of 2–3 min [7]. In contrast, rates of cholesterol efflux from cells are typically much slower. Studies of cholesterol efflux to HDL and apoA-I generally show only a few percent of the cellular cholesterol pool released per hour [21].

Proteins involved in sterol transport

A number of transmembrane proteins have been shown to play an important role in facilitating sterol transport, but the precise mechanisms by which these proteins facilitate transport remain uncertain. The best studied of these are members of the ABC (ATP-binding cassette) superfamily of transporters, which facilitate the transfer of sterol to acceptors, mainly HDLs and their major associated apolipoprotein, ApoA-I [10,22]. Defects in one of these proteins, ABCA1, are responsible for Tangier disease, a rare autosomal recessive disorder that is characterized by a severe deficiency in HDL and reduced efflux of cholesterol, especially from macrophages [23]. In cell culture studies it has been shown that ABCA1 promotes the transfer of cholesterol and phospholipids to lipid-poor forms of ApoA-I, which bind to ABCA1 [22]. There is evidence that ABCA1 may function both at the PM and in endosomes [24]. ABCG1 also promotes cholesterol efflux to HDL particles [21]. ABCG5 and G8 are expressed in the liver and intestines, and the heterodimer of these proteins along with ABCB4 (MDR2) is involved in the biliary excretion of cholesterol and of dietary plant sterols [25,26]. These ABC proteins are involved in the export of cholesterol from mammalian cells. In yeast cells, the ABC proteins, Aus1p and Pdr11p [15••,27], are essential for import of sterol under conditions where yeast are auxotrophic for sterol.

The polytopic membrane protein, NPC1, is involved in sterol transport out of late endosomes so that the sterol can be delivered to other organelles [28]. Loss of NPC1 function leads to the accumulation of cholesterol and some other lipids in lysosome-like storage organelles.

Recently, a protein related to NPC1, NPC1L1, has been shown to play an important role in the absorption of cholesterol from the intestines [29••]. This protein has been shown to be the target for ezetimibe, a drug that blocks uptake of dietary cholesterol and is in clinical use [30••]. The mechanisms by which NPC1 and NPC1L1 facilitate sterol delivery into the cytosol remain uncertain. There is evidence that NPC1 can facilitate the transbilayer movement of some nonpolar molecules [28], but it is unclear how this relates to movement of cholesterol, which can spontaneously flip between the leaflets of a membrane bilayer. One possibility is that the flipping of another molecule helps to present sterol in the cytoplasmic leaflet in a way that promotes its delivery to cytoplasmic acceptors. Another possibility is that the NPC1 proteins bind other proteins that facilitate sterol transport into the cytosol. Interestingly, mutations in *dnpc1a*, one of two *Drosophila* genes related to mammalian *NPC1*, also cause a sterol accumulation defect, suggesting a conserved function from flies to mammals [31].

Soluble sterol carriers

Non-vesicular sterol transport between intracellular compartments is presumably mediated by LTPs. Sterol-specific LTPs would be expected to equilibrate sterols such that the chemical potential of sterol in the participating membranes is the same (see next section). An LTP that mediates inter-organelle sterol transport *in vivo* has yet to be identified although various proteins, including caveolin and non-specific lipid transfer protein (SCP-2), have been proposed — but not proven — to perform this

function [4]. Proteins classified as LTPs are functionally characterized by their ability to transfer specific lipids between vesicles *in vitro*. Their role in transporting lipids *in vivo* is less clear, and it is likely that many proteins described as LTPs play a role in aspects of lipid biology that do not directly involve lipid transport. For example, some LTPs are lipid sensors that influence lipid metabolism and lipid-based signaling (see below). The best evidence for LTP-mediated lipid transport *in vivo* comes from studies of proteins containing the lipid-binding START domains that were originally described in the mitochondrial cholesterol-binding StAR protein (now StARD1) [32]. StARD1 transports cholesterol from the mitochondrial outer membrane to the matrix side of the inner membrane, where it is converted to pregnenolone.

Specific exchange among subcellular compartments may require compartment-specific targeting modules within the LTPs, such as the PH domain and FFAT motif, which allow the ceramide-transporting START protein, CERT, to interact with the Golgi (via PI(4)P) and ER (via the ER membrane protein VAP), respectively [32]. LTPs with dual organelle-targeting modules could transfer lipids locally, rather than in a cell-wide fashion through the cytoplasm, by engaging both organelles simultaneously at membrane contact sites (MCSs).

OSBP, originally identified as a cytoplasmic receptor for oxysterols such as 25-hydroxycholesterol, was recently shown to act as a sterol sensor in controlling the activity of two phosphoprotein phosphatases in the ERK signaling pathway [33] and in regulating the CERT-dependent synthesis of sphingomyelin in mammalian cells [34]. Most eukaryotes have a number of OSBP-related proteins (ORPs), and yeast possess a family of seven cytoplasmic proteins (Osh1p–Osh7p) [35] that contain a ~400-residue lipid binding domain resembling that found in OSBP. Deletion of all seven *OSH* genes is lethal, but expression of any individual *OSH* gene product allows cells to remain viable. This suggests that the seven Osh proteins share an essential common function. Genetic analyses indicate that the yeast Osh proteins affect some aspect of cellular sterol distribution, since sterols accumulate intracellularly in the absence of *OSH* function [36]. Yeast lack cytoplasmic START-domain proteins. Like CERT, some OSBPs and Osh proteins contain FFAT motifs and PH domains that permit Golgi and ER membrane association.

Osh4 is a soluble β-barrel protein with a hydrophilic exterior and a central hydrophobic tunnel; the tunnel is closed at one end and gated at the other by a hinged lid [37•]. This general architecture resembles that seen in other LTPs. Structures of Osh4 co-crystallized with ergosterol (as well as with cholesterol or various oxysterols) show that the hydrophobic binding pocket can accommodate a single sterol molecule oriented with its 3-OH group at the bottom and its alkyl side chain interacting with the hinged lid. Sterols and oxysterols bind similarly with a Kd value in the range 55–300 nM. Without sterol, the lid domain appears to be mobile, potentially allowing Osh4 to interact with membranes; on acquisition of sterol, sterol–lid interactions promote lid closing. This provides a sealed hydrophobic environment for the sterol and allows release of the sterol-loaded protein from the membrane.

The structure of Osh4p suggests that ORPs may function as sterol-carrying LTPs. This was tested in a recent study [38••]. *In vitro* assays showed that Osh4p can extract sterols from vesicles and transfer sterol between vesicles, albeit inefficiently, which is consistent with the view that Osh proteins can function as sterol LTPs. The efficiency with which sterol could be transported *in vitro* was improved somewhat by including $PI(4,5)P_2$, a known effector of Osh4p [39], in the vesicles. Most, but not all, mutants of Osh4p that were non-functional in the transport assay also failed to complement *osh*Δ cells.

The role played by Osh proteins *in vivo* was examined by monitoring the transport-dependent conversion in yeast of exogenously supplied cholesterol to cholesteryl ester. Yeast cells bearing a mutant Upc2 transcription factor take up cholesterol from the medium under aerobic conditions; once transported to the ER by a Sec18p-independent pathway, this cholesterol is esterified by the sterol acyl transferases Are1p and Are2p [11]. Conditional elimination of all seven Osh proteins in this strain background reduced the rate of cholesterol esterification approximately seven-fold (compared to transport in an isogenic Osh-replete strain), pointing to a role for Osh proteins in transport and esterification. However, some of the observed decrease can be attributed to the two-fold reduction in sterol acyl transferase activity measured in homogenates of *osh*Δ cells. Thus, it is clear that even in the absence of Osh proteins there is significant sterol transport between the PM and the ER. Complementary assays of the transport of newly synthesized ergosterol from the ER to the PM also showed only a partial decrease in sterol transport rate in *osh*Δ cells [17]. Thus, if Osh proteins and ORPs in general function as sterol LTPs, then other sterol LTPs must also exist. Alternatively, ORPs could be sterol sensors that affect sterol transport only indirectly by, for example, influencing membrane composition and consequently the chemical potential of sterol pools (see below). The results mentioned above [33,34] would appear to support the idea that ORPs may be sterol sensors rather than sterol LTPs.

A different approach to identifying proteins involved in intracellular sterol transport was recently reported [40]. Making use of the fact that yeast are sterol auxotrophs under anaerobic conditions, Reiner *et al.* screened the yeast deletion mutant collection for genes required for anaerobic growth. Of the mutants identified, 17 were

defective in cholesterol uptake. Some of these displayed a defect in cholesterol incorporation into the PM while others showed transport defects. None of the mutants in the latter category could be correlated to an obvious transporter (such as an LTP) or mechanism although many, surprisingly, were affected in mitochondrial functions. More work will be needed to determine the precise nature of the connection between sterol transport and mitochondrial function.

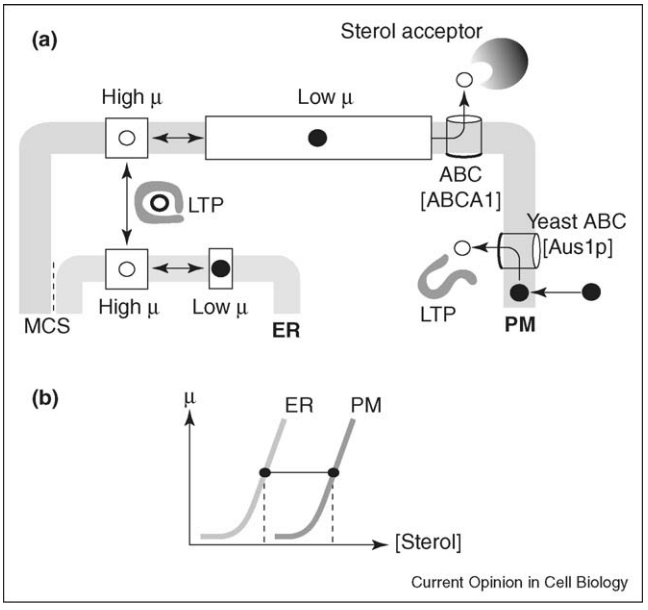
NPC2 is a mammalian cholesterol-binding protein that is found in the lumen of late endosomes and lysosomes [41] and that could play a role as an intra-organelle LTP. Defects in NPC2 lead to cholesterol accumulation in these organelles, and this is responsible for a small percentage of cases of Niemann–Pick C disease. The affinity of NPC2 for sterol is very high, but the release of NPC2-bound cholesterol to liposomes is accelerated at acidic pH values [42•], especially to liposomes that contain bis-monoacylglycerol phosphate (also known as lyso-bis phosphatidic acid, LBPA), a lipid that is found mainly in late endosomes [43]. It appears that NPC2 sequesters cholesterol after hydrolysis of cholesterol esters and delivers it to the limiting membranes of late endosomes and lysosomes. A functionally conserved yeast homolog of NPC2 was recently identified [12].

Non-vesicular transport and the maintenance of intracellular sterol distribution

How do different intracellular organelles, especially the PM and ER, maintain their widely different content of sterols in the face of non-vesicular, bi-directional sterol transport? Tests of cholesterol partitioning between liposomes and isolated intracellular membranes indicate that sterols partition more readily into PM vesicles than into ER vesicles or mitochondria [44]. The affinity of PM vesicles for cholesterol correlates with their lipid composition (sphingomyelin and a higher proportion of phospholipids with saturated acyl chains) rather than with their specific protein content. Similar cholesterol partitioning behavior is seen in model systems using vesicles prepared from defined lipid mixtures [45,46]. If the notion of the affinity of the PM for sterols is supplemented with organelle-specific LTP-mediated sterol mixing (for example, sterol-LTPs may mediate sterol traffic only between the PM and ER), then it is possible to see how different organelles are able to maintain their differences in sterol content under conditions near equilibrium (Figure 1).

These ideas have received new impetus from recent theoretical studies, as well as from experiments in model systems and in cells, which describe the physicochemical behavior of cholesterol in membranes. McConnell and colleagues [47•] suggest that sterols associate reversibly with phospholipids to form 'condensed complexes'. In

Figure 1



A model for sterol equilibration between the plasma membrane (PM) and the endoplasmic reticulum (ER). The sterol/phospholipid ratio is much higher (possibly 10-fold higher) in the PM than in the ER. **(a)** Sterol transport between the ER and PM occurs by a non-vesicular equilibration mechanism, probably involving lipid transfer proteins (LTPs). LTPs may ferry sterols though the cytoplasm as depicted, or facilitate sterol exchange between the PM and ER at a membrane contact site (MCS). A central feature of the model is that a given membrane has a characteristic capacity for sterol because of its particular phospholipid composition. This is because sterols associate with phospholipids in defined ways to form condensed complexes and/or to minimize exposure of the sterol ring system to water. As the mole fraction of sterol exceeds the capacity limit in a particular membrane, the chemical potential of sterol (μ) in that membrane rises rapidly. If we consider that individual sterol molecules are in different environments within a membrane (e.g. with low μ in a condensed complex or high μ as a water-exposed sterol), then the overall chemical potential of sterol is the sum of the chemical potential for each species multiplied by molar abundance of that species ($\mu = n_A\mu_A + n_B\mu_B + n_C\mu_C + \ldots$). The sharp rise in sterol chemical potential occurs at a higher sterol/phospholipid ratio in the PM than in the ER — illustrated graphically in **(b)** — because the PM has a higher content of sterol-interacting phospholipids, such as sphingomyelin. The majority of sterol in the PM has a low μ whereas the majority of ER sterol has a high μ. At equilibrium (which can be kinetically accelerated by LTP-mediated sterol transport), the PM will have more moles of sterol per mole of phospholipid, but the chemical potential of sterol in both membranes will be equal. This is indicated by the horizontal tie-line in (b) that connects the chemical potential profiles of the ER and PM at a fixed chemical potential, corresponding to quite different sterol/phospholipid ratios in the two organelles. Thus the transportable sterol pools in the PM and ER can be readily equilibrated via a bi-directional, non-vesicular mechanism. ABC transporters such as mammalian ABCA1 and yeast Aus1p are proposed to play a role in sterol efflux or influx by increasing the chemical activity of sterol, making it more available for capture by extracellular acceptors such as high density lipoprotein (HDL) or intracellular sterol-LTPs. Sterol molecules are depicted as filled circles (corresponding to sterols with low chemical potential) and open circles (sterols with high chemical potential). Only two chemical potential states (high and low) of sterol are shown to simplify the diagram; in reality, a range of chemical potential states (μ_A, μ_B, μ_C, etc) would be expected.

model systems, condensed complexes form between sterols and phospholipids containing saturated acyl chains, reflecting the membrane-partitioning behavior of sterol. The effect of complex formation is to lower the chemical potential of sterol, or, in other words, to decrease its availability or reactivity. If the mole fraction of sterol exceeds the capacity of membrane phospholipids to form condensed complexes, then the chemical potential of sterol increases more rapidly with increasing sterol concentration. This is reflected experimentally in the increased rate with which sterols can be extracted from membranes with cyclodextrin, or, in the case of cholesterol, react with cholesterol oxidase. In a different but related view, Feigenson and coworkers point out that cholesterol's small polar headgroup is insufficient to shield its hydrophobic ring system from water [48]. Consequently, cholesterol–cholesterol interactions are unfavorable and membrane cholesterol associates with phospholipids, taking cover under the 'umbrella' provided by phospholipid headgroups. Phosphatidylcholine, with its relatively large phosphocholine headgroup, can shield two cholesterol molecules whereas phosphatidylethanolamine with its smaller headgroup can shield only one sterol [48]. Poorly shielded sterol would have a high chemical potential.

A common feature of the 'condensed complex' and 'umbrella' models is that the chemical potential of sterol is expected to increase sharply when membrane sterol levels exceed the capacity of partnering phospholipids to form complexes and/or provide umbrellas. Consistent with this, sterol leaves the PM and is delivered to intracellular organelles more rapidly in cells with elevated sterol levels [49]. Lange *et al.* [5•] showed that the chemical activity of PM cholesterol rises abruptly and linearly as the mole fraction of cholesterol exceeds the sterol-partnering capacity of membrane phospholipids. In light of these ideas it is interesting to consider that the function of ABC transporters (or NPC1) in promoting sterol efflux or influx (Figure 1) may not be to transport sterol across the bilayer (this occurs spontaneously and rapidly) but to increase its chemical activity, thus promoting its extractability by extracellular acceptors or intracellular LTPs [50]. Ceramide and diglyceride resemble sterols in having a hydrophobic portion capped with a small polar headgroup. These molecules would be expected to compete with cholesterol in condensed complex formation and/or for space under phospholipid umbrellas. Introduction of ceramide or diglyceride into sterol-containing membranes should therefore increase the chemical potential of the sterol; this has been recently demonstrated [51•,52••]. The well-documented escape of PM sterol to the ER in response to sphingomyelinase treatment of cultured cells can be explained by the increase in sterol potential resulting not only from a decrease in the number of sphingomyelin partners (the usual explanation) but also from the displacement of cholesterol from its association with other phospholipids by ceramide, a product of sphingomyelinase action.

The majority of sterol in the PM is partnered with phospholipids and consequently has a low chemical potential. Only a small fraction of PM sterol, corresponding to the sterol pool in excess of phospholipids, has a high chemical potential and can, in principle, be extracted and transported by LTPs. Newly synthesized sterol in the ER, lacking suitable phospholipid partners, is similarly predicted to have a high chemical potential and to be readily extractable by sterol-LTPs. Even though the PM content of sterol is much higher than that of the ER, sterol in the two membranes could have a similar chemical potential. Thus the transportable sterol pools in the PM and ER can be readily equilibrated via a bi-directional, non-vesicular mechanism without affecting the difference in sterol content of the two membranes. These ideas are elaborated in a transport model [16••] illustrated in Figure 1.

Conclusions

In the past few years, several proteins that play an important role in intracellular transport of sterols have been identified. There has also been some progress in characterizing the movements of sterols within cells and in understanding the biophysical basis for differences in sterol content of various organelles. However, we still lack a clear mechanistic understanding of the molecular basis for most key aspects of sterol movement between membranes of a cell. This remains a significant challenge for researchers in this field.

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