PART I

CHOLESTEROL REGULATION OF MEMBRANE PROPERTIES

CHAPTER 1

CHOLESTEROL TRAFFICKING AND DISTRIBUTION BETWEEN CELLULAR MEMBRANES

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1.1 CHOLESTEROL — AN ESSENTIAL LIPID FOR NORMAL CELL FUNCTION

Cholesterol is an essential lipid component of cellular membranes. This sterol regulates permeability, fluidity, and bending rigidity of membranes, as well as the activity of several membrane proteins (Maxfield and Tabas, 2005; Wüstner, 2009). Beside this structural function, cholesterol is also the precursor molecule for bile acid and steroid hormones synthesis. The importance of cholesterol for cellular homeostasis is illustrated by its known contribution to development and function of the central nervous system (CNS) and bones (Porter, 2002), to signal transduction and sperm development, and to embryonic morphogenesis (Björkhelm, 2002; Travis and Kopf, 2002). Various human malformation syndromes result from a defect in cholesterol synthesis, such as Smith–Lemli–Opitz syndrome (SLOS), desmosterolosis, Greenberg dysplasia, and Antley–Bixler syndrome (Porter, 2002). Fatal clinical outcomes in these diseases are either a direct consequence of a lack of cholesterol or of accumulation of a synthetic cholesterol precursor. Its very low water solubility makes excess cholesterol also a life-threatening condition (Tabas, 2002). This is well known from the most frequent causes of death in the western world, cardiovascular disease and atherosclerosis (Maxfield and Tabas, 2005). Lysosomal storage disorders such as Niemann–Pick and Wolman diseases are either caused or accompanied by fatal cholesterol accumulation in degradative compartments (Ikonen, 2006). Recent

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research underlines the important role played by deregulated cholesterol trafficking in pathogenesis of Alzheimer's and Parkinson's disease (Liu et al., 2010). In addition to this tremendous biomedical importance, studying cholesterol provides insight into basic aspects of cell biology by deciphering the orchestration of membrane traffic and the interplay between proteins and lipids in living cells.

1.2 CHOLESTEROL METABOLISM, SENSING, AND DISTRIBUTION BETWEEN CELLULAR MEMBRANES

Cholesterol synthesis starts from condensation of acetyl-CoA in the cytoplasm, followed by reduction of the resulting hydroxymethyl-glutaryl-CoA (HMG-CoA) to mevalonate by HMG-CoA reductase. This enzyme has been localized not only to the endoplasmic reticulum (ER) but also to peroxisomes (Liscum and Munn, 1999; Olivier and Krisans, 2000). While most of the subsequent steps of cholesterol biosynthesis take place at the ER, several enzymes of isoprene synthesis contain peroxisomal targeting sequences, such that cholesterol synthesis might be compartmentalized in cytoplasm, ER, and peroxisomes also (Olivier and Krisans, 2000). Importantly, none of these compartments are specifically enriched in cholesterol; in fact, the ER, for example, contains only 1–2% of the total cellular cholesterol (Wüstner, 2009). This might play an important role in sensing of changes in cellular cholesterol levels. For example, when cellular cholesterol increases above the threshold level, a slight rise in ER cholesterol causes inhibition of HMG-CoA reductase (product inhibition) and eventually ubiquitination and degradation. At the transcriptional level, HMG-CoA is regulated via inhibition of the sterol regulatory element-binding protein (SREBP) pathway (Wüstner, 2009). In addition to HMG-CoA reductase, this pathway also regulates transcription of other proteins involved in cholesterol synthesis and the low density lipoprotein (LDL) receptor. At normal cholesterol levels, a membrane-bound complex of SREBP, SREBP cleavage activating protein (SCAP), and insulin-induced proteins (INSIG) reside in the ER. A decrease in cholesterol is sensed by a sterol sensing domain (SSD) in SCAP. This causes INSIG to dissociate from the complex. While INSIG is degraded in proteasomes, the remaining SREBP/SCAP complex is transferred from the ER to the Golgi by incorporation into COPII-coated vesicles. In the Golgi, the SREBP is cleaved by two proteases, releasing a soluble transcription factor. This fragment then enters the nucleus where it promotes transcription of HMG-CoA reductase, LDL receptor, and other proteins involved in cholesterol synthesis. Thus, activation of the SREBP pathway promotes both synthesis of cholesterol in the ER and uptake of cholesterol from plasma LDL. For a detailed review of the SREBP pathway, see Goldstein et al. (2006). Importantly, INSIG was found to control the expression of HMG-CoA reductase via SCAP/SREBP as well as by ubiquitination and degradation of the enzyme. INSIG binds to SCAP or to HMG-CoA reductase, suggesting a competitive mechanism regulated by cholesterol and lanosterol, as well as by oxysterols (Goldstein et al., 2006). Several membrane

proteins being involved in cholesterol trafficking and sensing contain an SSD with five membrane-spanning α-helices. In addition to SCAP, INSIG, and HMG-CoA reductase, an SSD is found, for example, in Niemann–Pick disease C1 (NPC1) protein and in Niemann–Pick disease C1-like 1 (NPC1L1) protein (Goldstein et al., 2006; Jia et al., 2010; Millard et al., 2005), but the exact function of this transmembrane domain in each of these and other proteins remains to be clarified.

Cholesterol can be esterified at its 3′-hydroxy position by acetyl-CoA acyl transferase (ACAT). This enzyme is allosterically activated by high cellular cholesterol levels. There exist two homologs of ACAT in mammals with differing tissue expression: ACAT1 produces cholesteryl esters (CEs) mainly in macrophages, where the enzyme resides in a poorly defined subcompartment of the ER (Khelef et al., 1998, 2000). The CEs are stored in cytoplasmic lipid droplets (LDs) and can be hydrolyzed by a neutral CE hydrolase that is probably associated with or recruited to the LD surface (McGookey and Anderson, 1983). ACAT2 generates CEs to be incorporated into chylomicrons and very low density lipoproteins (VLDLs) in the enterocyte and hepatocyte (Chang et al., 2009; Ikonen, 2006). In addition to esterification, cholesterol can be metabolized to bile acids and oxysterols. Since cholesterol cannot be degraded into noncyclic hydrocarbons, the only way to remove it from the circulation is its secretion into feces. Some cholesterol is directly secreted, mostly from the intestine and, to a minor extent, by the liver into bile. Conversion of cholesterol into bile acids in hepatocytes and their biliary excretion account for approximately half of daily cholesterol elimination.

Oxysterols are oxidized 27-carbon derivatives of cholesterol with diverse biological effects and activities. Addition of hydroxyl groups at various positions, either in the side chain or at the steroid backbone, makes oxysterols much more polar than cholesterol. This facilitates their intermembrane transfer (Massey and Pownall, 2006). Oxysterol synthesis can be mediated by cytochrome P450 and various sterol-specific hydroxylases. In addition, some oxysterols are generated as metabolic by-products because of nonenzymatic autooxidation. An example for the latter process is 7-ketocholesterol (7-KC) being generated during oxidation of LDL (Zhang et al., 2003b). This oxysterol is proatherogenic, for example, macrophages ingesting oxidized LDL accumulate cholesterol and 7-KC in their lysosomes and have impaired cholesterol efflux, eventually leading to apoptosis (Vejux et al., 2005). Two oxysterols, 27-hydroxycholesterol and 7α-hydroxycholesterol, are precursors of bile acid synthesis and initiate the acidic and neutral pathway of bile acid synthesis, respectively. In addition, 27-hydroxycholesterol induces cholesterol efflux in macrophages and endothelial cells. This process requires its binding to liver-X-receptor, a nuclear receptor that stimulates the expression of cholesterol efflux proteins ABCA1 and ABCG1 (see Section 1.6). Suppression of cholesterol synthesis via inactivation of HMG-CoA reductase has also been attributed to 27-hydroxycholesterol (Björkhelm, 2002; Olkkonen, 2009). Another interesting oxysterol is 24-(*S*)-hydroxycholesterol,

which is almost exclusively synthesized in the brain. Serum concentrations of this oxysterol reflect cholesterol turnover in the brain, and alterations in this parameter have been associated with Alzheimer's disease and multiple sclerosis (Björkhelm, 2002; Olkkonen, 2009).

Finally, cholesterol is converted into steroid hormones in adrenals, gonads, placenta, and brain. The first step in this process involves, in all steroidogenic tissues, the cleavage of side chain of cholesterol by P450 side chain cleavage (P450scc) enzyme in the inner mitochondrial membrane producing pregnenolone. Most cholesterol destined for steroidogenesis comes from HDL (high density lipoprotein) and is imported into steroidogenic cells via scavenger receptor B1 (SR-BI; see Section 1.3) (Krieger, 1999).

In typical cell culture models, most cellular cholesterol resides in the plasma membrane (PM; about 50–60% of the total cholesterol) and in the endocytic recycling compartment (ERC; about 30%), which is in continuous exchange with the cell surface due to membrane traffic (Maxfield and McGraw, 2004; Maxfield and Wüstner, 2002; Wüstner, 2009). Accordingly, mitochondria, lysosomes, and most of the Golgi, exclusively the trans-Golgi network (TGN) have relatively low cholesterol content. A well-balanced amount of cholesterol seems to be required for sorting along the secretory pathway (Grimmer et al., 2005; Ridsdale et al., 2006; Runz et al., 2006; Stüven et al., 2003; Ying et al., 2003). The exact proportion of cholesterol in various intracellular membranes is not known with certainty and might depend on the cell type and cell cycle (Wüstner, 2009). In the following, we consider how cells receive cholesterol from their environment and how the very heterogeneous cholesterol distribution between organelle membranes might be established and maintained during continuous intercompartment membrane traffic.

1.3 HOW DOES CHOLESTEROL ENTER MAMMALIAN CELLS?

Owing to its extremely low water solubility, cholesterol is carried in plasma as part of lipoproteins, either as free or as esterified cholesterol in the lipoprotein shell or core, respectively. Cholesterol delivery to peripheral tissues (i.e., adipocytes and muscle cells) occurs predominantly by receptor-mediated endocytosis of LDL. This is called *forward cholesterol transport*. LDL also delivers cholesterol to hepatocytes, an important step in the maintenance of plasma LDL levels. Prolonged circulation of LDL, for example, due to impaired LDL uptake, degradation, or dysregulated LDL formation, causes modification of these particles by acetylation and oxidation in the plasma. This modified LDL can aggregate in the intima of the vessel wall triggering the recruitment of macrophages to these areas. Macrophages try to engulf these particles by binding to scavenger receptors, such as SR-A. Recently, a novel uptake mechanism of these lipoprotein deposits by macrophages has been described (Haka et al., 2009). It involves formation of an acidifed extracellular compartment, *a lysosomal synapse*, in which CEs derived from the atherogenic LDL are hydrolyzed. Thus, free cholesterol

is liberated into the extracellular space and directly inserted into the PM of the involved macrophages. Increased PM cholesterol causes recruitment of actin-binding proteins, cell ruffling, and inhibits cell migration (Nagao et al., 2007; Qin et al., 2006). As a consequence, the affected cells stay in close contact with the LDL aggregates and keep internalizing cholesterol from the atherogenic particles (Buton et al., 1999; Grosheva et al., 2009). Importantly, the receptors for modified LDL, such as SR-A, are not downregulated in response to cellular cholesterol loading via the SREBP pathway, in stark contrast to the LDL receptor. Consequently, macrophages that are in contact with aggregated modified LDL become heavily cholesterol loaded, resulting in massive cholesterol esterification and formation of foam cells, an early sign of atherosclerosis. The reverse cholesterol transport involves formation of HDL by lipidation of apoA1 and shuttling of excess cholesterol via HDL to the liver. In contrast to LDL uptake, internalization of HDL-associated sterols does not require holo-particle uptake, but occurs by a selective uptake process, mainly via SR-BI (Krieger, 1999). Absorption of dietary cholesterol from mixed bile salt micelles takes place in the intestine, which requires the combined action of SR-BI and NPC1L1 protein, as well as other transporters such as aminopeptidase N or even caveolin (Knöpfel et al., 2007; Wang, 2006). The two most relevant uptake pathways for cholesterol and its ester from LDL and HDL are shortly summarized in the following text. For extensive reviews on these subjects, we refer the readers to the literature.

Pioneering studies by Goldstein and Brown in the 1970s to reveal the causes of familial hypercholesterolemia (FH) led to the discovery and characterization of the LDL receptor (Goldstein and Brown, 1974). In FH, patients suffer from extremely elevated plasma LDL concentrations causing atherosclerosis and heart attacks early in life. Excess cholesterol can even be deposited under the skin in so-called xanthomas, a prominent sign of the disease. In early 2009, more than 1100 mutations in the LDL-receptor gene have been described, which helped to clarify the molecular mechanisms underlying import and digestion of LDL in mammalian cells (Goldstein and Brown, 2009). On binding of LDL to the receptor, the ligand–receptor complex is recruited to clathrin-coated pits and internalized by endocytosis. Shortly after, the formed vesicles lose their clathrin coat in an ATP-dependent process and fuse with sorting endosomes (SEs). Within SEs, a slightly decreased pH causes the LDL to dissociate from its receptor. The released LDL follows the degradative pathway toward late endosomes (LEs), which likely form by maturation from SEs (Dunn and Maxfield, 1992; Stoorvogel et al., 1991). LEs are in continuous exchange with lysosomes, and likely, in both compartments, the CEs from the core of LDL are converted to free cholesterol while the apoproteins are digested. At the same time, the unbound receptor molecules are recycled to the cell surface. One cycle takes approximately 10 min, and the average lifespan of an LDL receptor is 20 h (Goldstein and Brown, 2009). Thus, on an average, a receptor molecule is recycled 120 times. As the core of one LDL protein contains about 1600 CE molecules, one receptor molecule transports approximately 200,000 molecules of CEs into the cell. From the late endosomes/lysosomes (LE/LYS), free cholesterol is transferred either directly to

the ER or to the PM (Wüstner, 2009). Transport to ER results in cholesterol targeting to ACAT or the SREBP machinery (Lange et al., 2002; Neufeld et al., 1996; Underwood et al., 1998; Urano et al., 2008). Recent evidence indicates that certain cholesterol released from the LE/LYS is transported to the ER via the TGN bypassing the PM (Urano et al., 2008). The actual mechanism by which cholesterol is released from the LE/LY remains obscure. Again, studying a genetic disease called *Niemann–Pick type C* (*NPC*) disease provided an insight into the molecular mechanisms underlying cholesterol release from these organelles (Lange et al., 2002). NPC disease is a fatal disorder characterized by accumulation of cholesterol, sphingomyelin, and other lipids in endosomes and lysosomes of liver cells, neurons, and fibroblasts of affected patients (Mukherjee and Maxfield, 2004). Recent studies on NPC disease have shed new light on intracellular cholesterol trafficking. NPC patients experience progressive neurodegeneration and hepatosplenomegaly (enlargement of liver and spleen), which is caused by mutations in either of the two genes (Mukherjee and Maxfield, 2004; Storch, 2009). One gene encodes NPC1, a large 1278-amino acid polytopic membrane protein that is localized to the limiting membrane of LE/LYS. The other gene encodes a small protein of 132 amino acids, which resides to some extent in LE/LYS. Current treatments of NPC disease are largely symptomatic, and the life expectancy of affected patients is variable; most patients die in childhood (Mukherjee and Maxfield, 2004). Both NPC1 and NPC2 have been shown to bind cholesterol and other sterols at nano- to micromolecular affinity (Friedland et al., 2003; Infante et al., 2008; Liu et al., 2009; Xu et al., 2007). The crystal structure of NPC2 with bound cholesterol sulfate, the strongest ligand of the protein, reveals one sterol buried with its side chain in a hydrophobic tunnel (Friedland et al., 2003). Similar structural data of the purified N-terminal loop of NPC1 in complex with cholesterol and 25-hydroxycholesterol show the sterol with 3′-hydroxy group in the binding pocket (Kwon et al., 2009). These results together with biochemical data and systematic mutagenesis analysis led to the hypothesis that NPC2 receives first hydrolyzed LDL cholesterol and shuttles it to NPC1 in a kind of hand-off mechanism (Wang et al., 2010). Although this model is very attractive, its validity depends on a definitive proof that both proteins interact within cells, which has not been demonstrated, yet. In fact, several lines of evidence indicate that in normal cells, NPC2 resides mostly in lysosomes, while NPC1 localizes preferentially to a subset of LEs (Chikh et al., 2004; Storch, 2009; Zhang et al., 2003a). Moreover, NPC1 seems to be dispersed throughout cells under cholesterol-depletion conditions and recruited to LEs only on uptake of LDL, while NPC2 is present in lysosomes under both conditions (Naureckiene et al., 2000; Zhang et al., 2003a). In fact, NPC1- but not NPC2-deficient cells have a decreased capability of LE/LYS back-fusion, a process that is required for release of lysosomal cargo (Goldman and Krise, 2010) Thus, any interaction model for both proteins needs to take the dynamic nature of LE/LYS into account. This is further outlined in Section 1.4.

In steroidogenic cells, LDL-derived cholesterol is imported into mitochondria, where the cholesterol is converted into steroid hormones (Stocco, 2000).

Steroidogenic acute regulatoryprotein 1 (StAR1) mediates import of cholesterol from the outer to the inner mitochondrial membrane, and lack of StAR1 causes congenital lipoid adrenal hyperplasia (Rigotti et al., 2010; Stocco, 2000). One of the family members, MLN64 (also known as StARD3), has a START (steroidogenic acute regulatory protein-related lipid transfer) domain at its N-terminus, in addition to a MENTAL domain (MLN64-N-terminal). This MENTAL domain binds cholesterol, and also tethers MLN64 to LEs (Hölttä-Vuori et al., 2005). A recent study demonstrates that MLN64 acts independent of NPC1 in cholesterol egress from LE/LYS toward mitochondria in steroidogenic cells (Charman et al., 2010). Targeted disruption of the MLN64 gene causes dispersion of LE/LYS, while intact MLN64 seems to interact with actin and cause sterol transfer to mitochondria during transient alignment with LEs, where MLN64 colocalizes with NPC1 (Alpy et al., 2001; Hölttä-Vuori et al., 2005). MLN64 shares structural similarities with other members of the StART family, including a hydrophobic pocket limited by α-helices and a flexible lid, which might function as a gate for binding and releasing of a cholesterol molecule (Alpy and Tomasetto, 2005; Murcia et al., 2006). More details on the vesicular trafficking of LDL-derived cholesterol in the endocytic pathway are given in Section 1.4.

Recently, it has been shown that a missense mutation in proprotein convertase subtilisin/kexin type 9 (PCSK9) causes autosomal dominant hypocholesterolemia (Mousavi et al., 2009). These patients have greatly reduced plasma LDL levels and appear to be protected against cardovasicular disease. Importantly, individuals heterozygous for a nonsense mutation in PCSK9 have normal hepatic triglyceride levels and no other sign of abnormalities, making pharmacological inhibition of PSCK9 an attractive strategy against coronary heart disease (Mousavi et al., 2009; Zhao et al., 2006). The molecular mechanisms underlying PCSK9-mediated regulation of plasma LDL remain to be deciphered in detail. After synthesis, PCSK9 undergoes autocatalytic cleavage in the secretory pathway, followed by export into plasma where PCSK9 controls LDL levels. The enzyme probably binds to the LDL receptor at the surface of hepatic cells, where it redirects the receptor from its normal recycling route to LE/LYS for degradation.

SR-BI, an 82 kDa cell surface glycoprotein, has been characterized as the first HDL-receptor importing cholesterol mainly into liver and steriodogenic tissues (Connelly and Williams, 2003; Kozarsky et al., 1997; Krieger, 1999). In these tissues, SR-BI plays a central role in controlling the level of HDL in plasma and in cholesterol stores for steroid synthesis. Different physiological studies have indicated that SR-BI is a key player in reverse cholesterol transport, and that deficiencies in SR-BI increase the risk of cardiovascular diseases. The mechanism by which SR-BI mediates cholesterol transfer from HDL to cells is not known in detail, but a large number of studies demonstrate that lipid uptake is separated from HDL apoprotein uptake after binding of the lipoprotein to SR-BI (Krieger, 1999; Rhainds and Brissette, 2004; Silver, 2004). This process is called *selective lipid uptake*, and is in stark contrast to internalization and processing of LDL-associated lipids via the LDL-receptor pathway (see 2.3.1, above). On binding HDL, SR-BI selectively takes up CEs and HDL-associated phospholipids

through a process that is either entirely restricted to the cell surface or involves rapid HDL endocytosis, lipid release, and recycling of lipid-depleted HDL remnants to the cell surface (Krieger, 1999; Rhainds and Brissette, 2004; Silver, 2004). Rapid internalization and recycling of HDL has been described in human hepatoma HepG2 cells by quantitative fluorescence imaging and by biochemical studies (Sun et al., 2006; Wüstner, 2005b). It is not yet clear how the CE is processed and transferred from the PM or early endosomes to sites of hydrolysis, but basolateral SEs have been implicated in hepatic sorting and recycling of HDL (Wüstner, 2005b, 2006). Inhibition of lysosomal degradation did not affect hydrolysis of HDL-associated CEs, and neutral CE hydrolase has been suggested to mediate extralysosomal degradation of HDL-associated CEs (Connelly et al., 2003; Connelly and Wiliams, 2004). Further studies are required to decipher the detailed intracellular route of HDL lipids.

1.4 VESICULAR CHOLESTEROL TRAFFICKING ALONG THE ENDOCYTIC AND SECRETORY PATHWAY

Cholesterol can be exchanged between intracellular compartments as a constituent of normal membrane flow in vesicles and tubules. For example, fluorescent markers of cholesterol, dehydroergosterol (DHE, a fluorescent cholesterol analog from yeast) and BODIPY-cholesterol, have been shown to be internalized from the PM by clathrin-dependent endocytosis (Wüstner et al., 2011b). Sterol endocytosis seems to be enhanced in cells expressing NPC1L1 protein, a putative intestinal and hepatic sterol transporter (Ge et al., 2008; Hartwig Petersen et al., 2008). Export of DHE from the ERC was shown to depend on the EHD protein, Rme-1 (Hao et al., 2002). Cholesterol esterification and recycling from the ERC requires the rab-GTPase, rab-11 (Hölttä-Vuori et al., 2002). Normal secretory membrane traffic depends on cholesterol in the ER (Ridsdale et al., 2006; Runz et al., 2006), while sterol sensing in ER involves vesicular shuttling of the SCAP/INSIG complex from the ER to the Golgi under cholesterol-depletion conditions (Goldstein et al., 2006). Vesicular transport is probably also involved in cholesterol egress from the LE/LYS after ingestion of LDL, as suggested by dependence of re-esterification of LDL-derived cholesterol on TGN-specific SNARE proteins (Urano et al., 2008). Similarly, degradation of LDL requires functional rab7, while rab7 is involved in correct positioning and movement of LE via its effectors, Rab7-interacting protein (RILP), associating to the motor protein dynactin (Bucci et al., 2000; Rocha et al., 2009). A recent study provided evidence that the oxysterol-binding protein ORP1L senses LE cholesterol levels and triggers the formation of ER-contact sites with LE, thereby releasing the Rab7-RILP complex from associated motors and allowing LEs to move to the plus end of the microtubule (Rocha et al., 2009). Under conditions of cholesterol loading, similar to that found in NPC disease, LEs accumulate at the minus end of the microtubule, since dynein motor activity is not inhibited (Rocha et al., 2009). While providing much molecular detail, this study could

not relate the LE positioning to cholesterol export from LE/LYS and targeting of the liberated cholesterol to ER or PM. Export of hydrolyzed LDL cholesterol from degradative compartments was enhanced by stimulated endocytic recycling via rab8 and Arf6 (Linder et al., 2007; Schweitzer et al., 2009). Lysosome-associated membrane protein 2 (LAMP-2) has been shown to be essential for esterification of endogenous and LDL-derived cholesterol (Schneede et al., 2009). Mutations in the LAMP-2 gene cause Danon disease. This disease is characterized by myopathy and mental retardation and, at the cellular level, by accumulation of late autophagic vacuoles in the heart and skeletal muscles. Mouse embryonic fibroblasts deficient in LAMP-2 show endosomal/lysosomal cholesterol accumulation, similar to that found in the NPC phenotype (Schneede et al., 2009). This defect can be rescued by reexpression of mouse LAMP-2A or rat LAMP-1, indicating that this class of lysosomal proteins is essential for proper trafficking of cholesterol through the endocytic system (Fig. 1.1).

In summary, many trafficking proteins involved in correct intracellular protein targeting seem to play a role in cholesterol trafficking as well. Further studies are required to determine how key regulators of general membrane traffic, motor, and adaptor proteins orchestrate vesicular trafficking of cholesterol as a constituent of intracellular membranes. Moreover, it remains to be clarified how sorting of sterols during vesicle budding, fission, and fusion takes place. Recent progress in fluorescence imaging approaches might pave the way to answer these questions (Wüstner et al., 2011a,b).

1.5 NONVESICULAR CHOLESTEROL TRANSPORT

In addition to the vesicular transport of cholesterol and related sterols between cellular membranes, there is accumulating evidence that a significant portion of cellular sterols move by nonvesicular means. Since the distribution of cholesterol between organelle membranes is very heterogeneous, it remains a major challenge to unravel how cells regulate nonvesicular sterol transport by avoiding passive equilibration of sterol between all intracellular membranes. Experimental evidence for nonvesicular sterol transport, current mechanistic hypotheses, and potential significance of this transport mode for cellular sterol homeostasis are discussed in the following section.

First evidence for involvement of a nonvesicular pathway in intracellular cholesterol transport dates back to investigations of *de novo* cholesterol trafficking almost 30 year ago (DeGrella and Simoni, 1982; Kaplan and Simoni, 1985). In mammalian cells, cholesterol is transferred from the ER to the PM with a halftime ($t_{1/2}$) of about 10 min, which is faster than secretory traffic of typical membrane proteins (DeGrella and Simoni, 1982; Hirschberg et al., 1998; Kaplan and Simoni, 1985). While the arrival of radioactive cholesterol at the PM made from ^{3}H- or ^{14}C-acetate in the ER was ATP-dependent and temperature-sensitive, it bypassed the Golgi suggesting that ER-to-PM cholesterol transport does not follow the secretory route (DeGrella and Simoni, 1982; Heino et al., 2000;

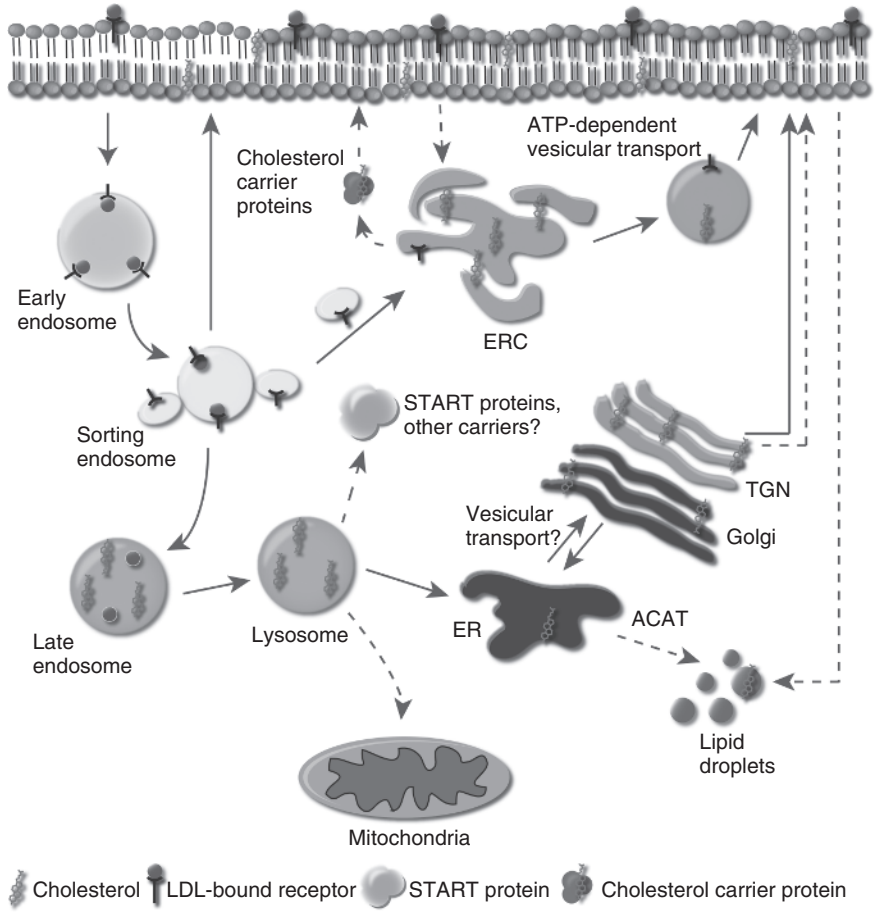

Figure 1.1 Survey of interorganelle cholesterol transport pathways in mammalian cells: LDL binds to its receptor at the cell surface and carries cholesterol and CEs into the cells by clathrin-dependent endocytosis. Cholesterol is liberated in LE/LYS and either directly sent to the ER or to the plasma membrane/endocytic recycling compartment (ERC). Within the ER, cholesterol can be re-esterified by ACAT and stored in lipid droplets (LDs). Plasma membrane cholesterol is in continuous exchange with the sterol pool in the ERC by vesicular and nonvesicular transport. From LE/LYS, cholesterol can be delivered to mitochondria, at least in steroid-producing cells. *De novo* synthesized cholesterol follows vesicular traffic along the secretory pathway only to a minor extent. Vesicular and nonvesicular pathways are indicated by straight and dashed arrows, respectively. Vesicle transport between LEs and TGN is not shown for clarity. Some putative cholesterol carrier proteins are indicated. See main text for further information. *Source*: Adapted from Maxfield and Mondal (2006). (*See insert for color representation of the figure*.)

Kaplan and Simoni, 1985). Once established, the large concentration gradient of cholesterol between PM and ER did not depend on the metabolic energy (Kaplan and Simoni, 1985). Experiments with brefeldin A, which blocks vesicular transport out of the ER, showed only a minor decrease in cholesterol transport from the ER to the PM in BHK (baby hamster kidney) cells (Heino et al., 2000; Urbani and Simoni, 1990). Similarly, experiments with the yeast *Saccharomyces cerevisiae* have shown a $t_{1/2}$ for transport of ergosterol from the ER to the PM of approximately 10 min. This transport was not significantly decreased by mutations in the Sec18p protein, which is required for vesicular trafficking between the ER and the PM (Baumann et al., 2005). While mutations in other SEC proteins showed a decrease of 20–50 %, all of these results still indicate a nonvesicular pathway for cholesterol transport from the ER to the PM.

Nonvesicular transport from the PM to the ER has also been demonstrated. In yeast, nonvesicular transport was inferred from the observation that esterification of cholesterol was not decreased in SEC mutants of *S. cerevisiae* (Baumann et al., 2005). In J774 murine macrophages, ATP depletion did not affect sphingomyelinase-induced transport of cholesterol from the PM to the ER (Skiba et al., 1996). Disruption of vesicular transport routes by addition of *N*-ethylmaleimide and inhibitors of phosphatidylinositol 3-kinase showed no decrease in the rate of cholesterol transport in these experiments (Skiba et al., 1996). Sphingomyelinase triggers ATP-independent endocytosis by locally increasing the ceramide concentration in the PM (Zha et al., 1998). The authors therefore suggested that a novel vesicular pathway for cholesterol trafficking from the PM to the ER exists in these cells. However, their results are also consistent with nonvesicular transfer of sterol from the PM to the ER. In fact, in cholesterol-loaded J774 cells, DHE was rapidly targeted to LDs in an ATP-independent manner, bypassing sphingomyelinase-induced endosomes (Wüstner et al., 2005).

ERC is another organelle with a high concentration level of cholesterol. When the transport of DHE into the ERC was measured by photobleaching recovery in Chinese hamster ovarian (CHO) cells, it was found that the $t_{1/2}$ for refilling the ERC was approximately 2.5 min (Hao et al., 2002). Additionally, it was found that the rate of recovery was not affected by ATP depletion of the cells, and the extent of recovery was only slightly reduced (Hao et al., 2002). Since ATP is required for vesicular transport, this indicates that most of the delivery to the ERC is nonvesicular in CHO cells. In J774 macrophage foam cells, DHE was transported from the PM to the ERC only to a minor degree, while it was rapidly shunted to LDs in an ATP-independent process (Wüstner et al., 2005). It has to be emphasized, however, that similar studies in other mammalian cell types found a lower contribution of nonvesicular transport to total uptake of DHE from the cell surface. For example, in J774 cells with normal cholesterol content as well as in rat and human hepatoma cells, at least 65% of sterol internalization required ATP, and occurs in vesicles moving from the PM to the perinuclear region (Hartwig Petersen et al., 2008; Wüstner and Færgeman, 2008; Wüstner et al., 2002). Trafficking of DHE between the PM domains of polarized human

HepG2 cells via the subapical compartment (SAC), a central sorting organelle in epithelial cells (Hoekstra et al., 2004), occurred almost exclusively in vesicles (Wüstner, 2005a; Wüstner et al., 2002, 2011a). In addition to this pathway, there is a rapid nonvesicular transport mode for sterol between the surface domains of these cells, probably involving flip-flop and lateral sterol diffusion in the PM (Wüstner et al., 2002). In summary, the relative proportion of vesicular and nonvesicular cholesterol transport in mammalian cells seems to be cell-type specific for unknown reasons.

It has been suggested that the rate-limiting step for nonvesicular cholesterol transport is the escape of cholesterol from the membrane (Bar et al., 1989, 1987; Steck et al., 1988). The rate of escape depends on the lipid composition and shape of the membrane, as well as the chemical activity of the cholesterol molecules (Bar et al., 1986, 1987). On the basis of experimental evidence, two models describing how cholesterol is build into membranes have been proposed. The umbrella model predicts that the small, mostly hydrophobic, cholesterol molecules pack closely to the phospholipids under their larger headgroups to hide from the surrounding water (Huang and Feigenson, 1999). In this model, sterol–sterol interactions are disfavored, as shielding a cluster of sterols costs much more free energy than shielding a single sterol molecule. In the condensed complex model, it is suggested that cholesterol associates with the phospholipids, particularly, those with saturated acyl chains (McConnell and Radhakrishnan, 2003). This association lowers the chemical activity of the cholesterol. Both models have in common that there is a critical threshold mole fraction of sterol in the bilayer above which excess cholesterol cannot be dissolved in the membrane, thereby abruptly raising the chemical activity of this cholesterol pool. In the umbrella model, this point will be characterized by a limited ability of the phospholipid head groups to cover the very hydrophobic cholesterol molecule (Huang et al., 1999). In the condensed complex model, the threshold will occur when no suitable phospholipid partners are available for cholesterol to associate with and form complexes. Both models have in common that the threshold cholesterol concentration depends on the phospholipid/sphingolipid composition of the host bilayer, a phenomenon well known from studies on model membranes and cell membranes (Lange and Steck, 2008; Steck and Lange, 2010). Accordingly, two membranes, such as the PM and the ER, can have very different sterol mole fractions at steady state, since the threshold for sterol release is set by the distinct lipid composition of these membranes (Wüstner, 2009). A higher escape propensity of excess cholesterol has also been demonstrated in living cells. Lange and coworkers demonstrated that raising the PM cholesterol level in human skin fibroblasts from 25% below to 25% above the basal level caused a 20-fold decrease in ER 3-hydroxy-3-methylglutaryl-CoA reductase activity independent of SREBP and with a $t_{1/2}$ of 30 min (Lange et al., 1999, 2008). On the basis of these results, the authors argued that the decrease in ER 3-hydroxy-3-methylglutaryl-CoA reductase activity was the result of an increased thermodynamic activity of cholesterol.

Nonvesicular cholesterol transport may occur by different mechanisms and several possibilities will be put forward. Monomeric cholesterol exchange might include spontaneous release of a cholesterol molecule from a donor membrane and transfer to an acceptor membrane via the aqueous phase. Since cholesterol has a very low partition probability into the water phase, it is unlikely that pure sterol diffusion through the aqueous cytoplasm contributes significantly to intracellular sterol trafficking. Cells are filled with internal membranes into which sterols likely will partition, and the cytoplasm is a very structured fluid with up to 40% of the total mass contributed by dissolved macromolecules. This together makes free diffusion of the hydrophobic sterol molecules a very unlikely transport mode. Various sterol transfer proteins (STPs) may therefore facilitate nonvesicular transport between certain organelles. STPs fall into five families: steroidogenic acute regulatory protein (StAR) and START proteins, Niemann–Pick type C class 2 (NPC2), oxysterol-binding protein (OSBP) and OSBP-related proteins (ORPs), sterol carrier protein 2 (SCP-2), and caveolins. X-ray crystallographic structures of a number of STPs [NPC2 (Friedland et al., 2003; Xu et al., 2007), Osh4p (Im et al., 2005), StarD4 (Romanowski et al., 2002), and MLN64-START (Tsujishita and Hurley, 2000)] have revealed a hydrophobic pocket that could harbor a single sterol molecule. Many of the STPs have a lid that could open and close on interaction with the membrane (Murcia et al., 2006).

Membrane contact sites (MCSs) are dynamic structures, which are formed and/or stabilized by protein–protein or protein–lipid interactions. They have been shown to be enriched in proteins involved in synthesis and trafficking of lipids, in particular, in yeast cells (Baumann et al., 2005; Li and Prinz, 2004). Particularly, the ER which is emanating throughout the whole cell with regions of varying curvature, has many MCSs with other organelles including mitochondria, the Golgi apparatus, and lysosomes (Holthuis and Levine, 2005; Levine and Loewen, 2006; Shibata et al., 2010). Thus, it is possible that newly synthesized cholesterol is distributed from the ER via these contact sites, as proposed first for yeast cells (Baumann et al., 2005; Schulz et al., 2009). Owing to the small distance between membranes at MCSs, transport by diffusion will be significantly enhanced. Furthermore, it was found by Schultz and coworkers that transport by the OSBP, Osh4, was greatly enhanced when the donor and acceptor membranes were close together, since Osh4 has two membrane binding sites; one at the sterol binding pocket and one at a distal site that can bind a second membrane (Schulz et al., 2009). OSBP and some ORPs contain a C-terminal two-phenylalanines in an acid-tract (FFA, free fatty acid) motif, which binds to VAMP-associated protein in the ER, and an N-terminal pleckstrin homology (PH) domain, which can recruit the protein to phosphatidylinositol-2,5-bisphopsphate (PIP2) containing Golgi membranes (Levine and Loewen, 2006). By this double-binding mechanism, ORPs could create an MCS and, at the same time, shuttle a sterol molecule via their inherent sterol binding domain. Although this is an attractive mechanistic hypothesis, evidence so far is rather indirect and stems mostly from work in yeast cells. A very recent study links an ER-resident ORP, ORP5, to cholesterol export from LE/LYS in concert with NPC1 (Du et al.,

2011). Silencing of ORP5 created an NPC-like phenotype with free cholesterol accumulation in LE/LYS and impaired cholesterol esterification in the ER. At the same time, ORP5 knockdown resulted in redistribution of TGN markers, such as cation-independent mannose-6-phosphat receptor (CI-MPR), TGN46, and a fusion construct of N-terminal galactosyl transferase, to LEs (Du et al., 2011). Thus, it is possible that ORP5 is involved in cholesterol transport between TGN, ER, and LEs, similar to that suggested for another member of this family, ORP9 (Ngo and Ridgway, 2009).

1.6 CHOLESTEROL EFFLUX FROM CELLS

Research conducted during the last 15 years has revealed a complex and finely tuned machinery for efflux of excess cholesterol from mammalian cells. In this final section, we shortly review the various pathways of cholesterol efflux from cells, either to lipoprotein acceptors or to body fluids such as intestinal juice and bile.

Apolipoprotein A1 (apoA1)-mediated efflux of excess cholesterol from cells is one of the major events in "reverse cholesterol" transport, a process that generates HDL. apoA1 receives cholesterol and phospholipids from peripheral cells in a process depending on the ATP-binding cassette transporter A1 (ABCA1) causing formation of HDL. Within the nascent HDL particles, the FC gets esterified by lecithin-cholesteryl acyl transferase (LCAT) creating a sink for further efflux of cholesterol from cells. HDL circulates back to the liver, where it releases its cholesterol content and CEs in a selective process involving SR-BI (1.3 Linsel-Nitschke and Tall, 2005). Patients with mutated ABCA1, as observed in Tangier disease, have an increased risk of developing cardiovascular diseases due to a strongly impaired cellular lipid efflux to apoA1, and consequently dramatically reduced plasma HDL leves (Wang et al., 2001). Several hypotheses have been put forward to explain the molecular mechanisms underlying ABCA1's function: (i) ABCA1 acts as a receptor for apoA1 on the cell surface, thereby mediating direct transfer of lipids onto apoA1 (Fitzgerald et al., 2004; Wang et al., 2001). (ii) ABCA1 acts as a pump for lipids or other hydrophobic substances, which is supported by its intrinsic ATPase activity (Linsel-Nitschke and Tall, 2005; Tall et al., 2002; Wang et al., 2001). ABCA1 triggers externalization of phosphatidylserine (PS), an aminophospholipid normally located on the cytoplasmic PM leaflet (Alder-Baerens et al., 2005; Zha et al., 2001). This is accompanied by altered inner surface membrane potential and reduced rate of endocytosis (Alder-Baerens et al., 2005; Zarubica et al., 2009; Zha et al., 2001). ABCA1-expressing BHK cells are more susceptible to cholesterol oxidase, an enzyme that modifies cholesterol preferentially in loosely packed membranes (Vaughan and Oram, 2003), probably due to its effect on transbilayer distribution of charged lipids, such as PS (Zarubica et al., 2009). Expression of ABCA1 also triggers outward curvature of the cell surface, which has been shown to enhance apoA1 binding (Vedhachalam et al., 2007). Active,

but not dysfunctional, ABCA1 slows lateral diffusion of the transferrin receptor (Zarubica et al., 2009). How all these observations relate to membrane mobility of sterols remains unclear. (iii) ABCA1, which follows a complex intracellular trafficking scheme (Neufeld et al., 2002, 2004; Zha et al., 2003), mediates lipidation of apoA1 during its passage through the cell, likely by a retroendocytic pathway (Denis et al., 2008). The latter is indicated by ABCA1's localization to LEs and the Golgi apparatus (Neufeld et al., 2004; Zha et al., 2003). Further studies are required to distinguish between these models and to unravel the exact effects of ABCA1 on membrane order and structure. In macrophages, cholesterol efflux not only depends on ABCA1 but also on the related ABCG1 (ATP-binding cassette transporter G1) as well as on SR-BI (Tarr et al., 2009). In primary murine peritoneal macrophages from ABCA1-/-, ABCG1-/-, or SR-BI-/- mice, it was found that 20% of intracellular cholesterol is effluxed by ABCG1, 35% by ABCA1, 10% by SR-BI, and 50% by an unknown mechanism, likely involving aqueous sterol diffusion to lipoprotein acceptors (Adorni et al., 2007).

As exemplified repeatedly in this chapter, elucidation of genetic diseases has helped tremendously to uncover the molecular mechanisms of cellular cholesterol transport. Sitosterolemia (phytosterolemia) is a rare genetic disease characterized by abnormally high plasma levels of plant sterols, such as sitosterol. Plant sterols are part of our diet, but they are in contrast to cholesterol almost not absorbed in the intestine (i.e., about 50% of cholesterol compared to <5% of plant sterols). Mutations in the ABC half transporters ABCG5 or ABCG8 cause highly increased intestinal absorption of plant sterols, a characteristics of sitosterolemia (Yu et al., 2002). On the basis of these observations, ABCG5 and 8 have been proposed to expel plant sterols from the brush border membrane of enterocytes back into the intestinal lumen. ABCG5 and 8 assemble into a heterodimer in the secretory pathway of intestinal and liver cells. In polarized hepatocytes, the dimer is expressed in the apical canalicular membrane (Graf et al., 2002). Overexpression of both proteins leads to a fivefold increase in biliary cholesterol secretion (Yu et al., 2002). These observations have led to the hypothesis that ABCG5 and 8 regulate biliary cholesterol secretion. The transporters ABCG5 and 8 might counteract canalicular NPC1L1, which likely triggers cholesterol reimport into the hepatocyte, thereby preventing excessive cholesterol release into the bile (Jia et al., 2010). The molecular mechanisms underlying ABCG5/8-mediated export of cholesterol are not known, but it has been suggested that these transporters extrude cholesterol partially from the bilayer, followed by pickup by bile salt/phospholipid micelles in the canalicular lumen (Small, 2003). Some direct cholesterol secretion has been measured in the intestine, but the involvement of ABCG5/8 in this process is disputed.

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