USING FLUORESCENT AND NON-FLUORESCENT STEROLS TO STUDY RAFTS AND INTRACELLULAR CHOLESTEROL TRANSPORT IN MAMMALIAN CELLS

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ORIGINAL PUBLICATIONS

This thesis is based on the following original research articles, which are referred to in the text by their assigned Roman numerals.

I. Vainio S, Jansen M, Koivusalo M, Rog T, Karttunen M, Vattulainen I, Ikonen E. Significance of sterol structural specificity. Desmosterol cannot replace cholesterol in lipid rafts. *J Biol Chem* 2006, 281:348-55.

II. Jansen M*, Pietiäinen VM*, Pölönen H, Rasilainen L, Koivusalo M, Ruotsalainen U, Jokitalo E, Ikonen E. Cholesterol substitution increases the structural heterogeneity of caveolae. *J Biol Chem* 2008, 283:14610-8. (*, shared first authors)

III. Jansen M, Ohsaki Y, Rega L, Bittman R, Olkkonen VM, Ikonen E. Role of ORPs in sterol transport from plasma membrane to ER and lipid droplets in mammalian cells. *Traffic* 2010. [Epub ahead of print]

ABBREVIATIONS

12-SLPC	1-palmitoyl-2-(12-doxyl)stearoylphosphatidylcholine
20,25-DAC	20,25-diazacholesterol
ABCA1	ATP-binding cassette transporter A-I
ACAT	acyl-coenzyme A: cholesterol acyltransferase
ADRP	adipocyte differentiation-related protein
Ag^{+}-HPLC	silver ion high performance liquid chromatography
APOA-I	apolipoprotein A-I
ATP	adenosine triphosphate
BODIPY	boron dipyrromethene difluoride
BSA	bovine serum albumin
CERT	ceramide transporter
CHO	Chinese hamster ovary
CoA	coenzyme A
DHCR24	24-dehydrocholesterol reductase
DOPC	dioleoyl phosphatidylcholine
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPC	dipalmitoyl phosphatidylcholine
DRM	detergent resistant membrane
ER	endoplasmic reticulum
ERC	endocytic recycling compartment
FFAT	two phenylalanines in an acidic tract
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
HMG	3-hydroxy-3-methylglutaryl
HMGR	3-hydroxy-3-methylglutaryl reductase
HPTLC	high performance thin layer chromatography
HDL	high density lipoprotein
L_d	liquid-disordered
LDL	low density lipoprotein
L_o	liquid-ordered
LPDS	lipoprotein depleted serum
LTP	lipid transfer protein
LXR	liver X receptor
MβCD	methyl-β-cyclodextrin
MLV	multilamellar vesicle
NBD	7-nitrobenz-2-oxa-1,3-diazol-4-yl
NPC	Niemann-Pick type C

ORD	OSBP related domain
ORP	OSBP related protein
OSBP	oxysterol binding protein
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PH	pleckstrin homology
PI	phosphatidylinositol
SCAP	SREBP cleavage activating protein
SCP-2	sterol carrier protein-2
S_o	solid-ordered
SR-AI	scavenger receptor class A type 1
SREBP	sterol recognition element binding protein
START	StAR related lipid transfer
STED	stimulated emission depletion
TIRF	total internal reflection
TLC	thin layer chromatography
T_m	melting temperature
TMA-DPH	N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium
UV	ultra violet
VAMP	vesicle associated membrane protein
VAP	VAMP associating protein

ABSTRACT

Cholesterol is an essential component in the membranes of most eukaryotic cells, in which it mediates many functions including membrane fluidity, permeability and the formation of ordered membrane domains. In this work a fluorescent and a non-fluorescent cholesterol analog were characterized as tools to study cholesterol. Next, these analogs were used to study two specific cell biological processes that involve cholesterol, i.e. the structure and function of ordered membrane domains/rafts and intracellular cholesterol transport.

The most common method for studying ordered membrane domains is by disrupting them by cholesterol depletion. Because cholesterol depletion affects many cellular functions besides those mediated by membrane domains, this procedure is highly unspecific. The cellular exchange of cholesterol by desmosterol as a tool to study ordered membrane domains was characterized. It turned out that the ability of desmosterol to form and stabilize membrane domains *in vitro* was weaker compared to cholesterol. This result was reinforced by atomistic scale simulations that indicated that desmosterol has a lower ordering effect on phospholipid acyl chains. Three procedures were established for exchanging cellular cholesterol by desmosterol. In cells in which desmosterol was the main sterol, insulin signaling was attenuated. The results suggest that this was caused by desmosterol destabilizing membrane rafts. Contrary to its effect on ordered membrane domains it was found that replacing cholesterol by desmosterol does not change cell growth/viability, subcellular sterol distribution, Golgi integrity, secretory pathway, phospholipid composition and membrane fluidity. Together these results suggest that exchanging cellular cholesterol by desmosterol provides a selective tool for perturbing rafts. Next, the importance of cholesterol for the structure and function of caveolae was analyzed by exchanging the cellular cholesterol by desmosterol. The sterol exchange reduced the stability of caveolae as determined by detergent resistance of caveolin-1 and heat resistance of caveolin-1 oligomers. In addition, the sterol exchange led to aberrations in the caveolar structure; the morphology of caveolae was altered and there was a larger variation in the amount of caveolin-1 molecules per caveola. These results demonstrate that cholesterol is important for caveolar stability and structural homogeneity.

In the second part of this work a fluorescent cholesterol analog was characterized as a tool to study cholesterol transport. Tight control of the intracellular cholesterol distribution is essential for many cellular processes. An important mechanism by which cells regulate their membrane cholesterol content is cholesterol traffic, mostly from the plasma membrane to lipid droplets. The fluorescent sterol probe BODIPY-cholesterol was characterized as a tool to analyze cholesterol transport between the plasma membrane, the endoplasmic reticulum (ER) and lipid droplets. The behavior of BODIPY-cholesterol was compared to that of natural sterols, using both biochemical and live-cell microcopy assays. The results show that the transport kinetics of BODIPY-cholesterol between the plasma membrane, the ER and lipid droplets is similar to that of unesterified cholesterol. Next, BODIPY-cholesterol was utilized

to analyze the importance of oxysterol binding protein related proteins (ORPs) for cholesterol transport between the plasma membrane, the ER, and lipid droplets in mammalian cells. By overexpressing all human ORPs it turned out that especially ORP1S and ORP2 enhanced sterol transport from the plasma membrane to lipid droplets. Our results suggest that the increased sterol transport takes place between the plasma membrane and ER and not between the ER and lipid droplets. Simultaneous knockdown of ORP1S and ORP2 resulted in a moderate but significant inhibition of sterol traffic from the plasma membrane to ER and lipid droplets, suggesting a physiological role for these ORPs in this process. The two phenylalanines in an acidic tract (FFAT) motif in ORPs, which mediates interaction with vesicle associated membrane protein associated proteins (VAPs) in the ER, was not necessary for mediating sterol transport. However, VAP silencing slowed down sterol transport, most likely by destabilizing ORPs containing a FFAT motif.

INTRODUCTION

Since its discovery as the main component of gallstones, cholesterol (chol: gall and stereos: solid) has remained a molecule with a dubious reputation. By the public, cholesterol is probably best known for its involvement in cardiovascular disease, which is the main cause of death in the Western world (Lewington et al., 2007). Other cholesterol related disorders are Alzheimer's disease, Niemann-Pick type C (NPC) disease, Familial hypercholesterolemia, Tangier disease and several biosynthetic disorders including Desmosterolosis and Smith-Lemli-Opitz Syndrome. It is interesting to point out that clinical treatment of cholesterol related disorders is a profitable enterprise. For example, a cholesterol lowering drug from Pfizer called Lipitor (Atorvastatin) is at the moment the best selling drug of all time.

It is generally less well known that cholesterol is required for the growth and development of nearly all animals and eukaryotic cells. Cholesterol has been found to be one of the most functionally diverse molecules in biology. For instance, it serves as a precursor for many types of biologically active compounds including, steroid hormones, bile acids, oxysterols and vitamin D. However, the most general function of cholesterol among eukaryotic species is as a structural component of biological membranes. In cellular membranes, cholesterol is a unique constituent that regulates diverse properties including membrane permeability, fluidity, and ordered domain formation.

Because cholesterol is involved in many aspects of biology it is not surprising that much research has been focused on this small molecule. Several Nobel prizes have been awarded to researchers that studied cholesterol (http://nobelprize.org): Windaus and Wieland for solving its molecular structure, Bloch and Lynen for elucidating its biosynthetic pathway, and Brown and Goldstein for discovering receptor mediated uptake of cholesterol by cells. Despite these key discoveries there are many aspects of the biology of cholesterol which remain unclear. For example, it is poorly understood how cholesterol, after uptake by the cell, is transported between different cellular organelles. Another example is the role of cholesterol in ordered membrane domains. These cholesterol enriched membrane assemblies have been suggested to be important for many cellular functions but detailed understanding regarding their structure and function is still lacking.

Relatively little is known about the cellular behavior of cholesterol and other lipids compared to the knowledge of proteins and nucleic acids. An important reason for this difference is the lack of good tools to study the cellular behavior of lipids. For example, the discovery of green fluorescent protein (GFP) enabled the visualization of proteins in living cells and caused a breakthrough in cell-biology (Chalfie et al., 1994). Unfortunately, this technology does not apply to lipids and the lack of good fluorescent cholesterol probes has hampered biological research on cholesterol. In this work, a fluorescent and a non-fluorescent cholesterol analog were characterized as tools to study specific cell biological aspects of cholesterol, i.e. the structure and function of ordered membrane domains/rafts

and intracellular cholesterol transport. Furthermore, this work revealed the importance of cholesterol for the stability of caveolae and uncovered roles for ORPs and VAPs in cholesterol transport to the ER and lipid droplets in mammalian cells.

REVIEW OF THE LITERATURE

1. Cell biology of cholesterol

1.1 Cholesterol in biological membranes

Biological membranes

Biological membranes are continuous barriers both around and within cells. They are around 5 nm thick and define enclosed compartments; either the cell itself or intracellular membrane bound organelles (Figure 1A). The membranes are structurally composed of a bilayer of amphiphathic lipids. These lipids contain a polar head group and a hydrophobic tail region. Lipid bilayers can form spontaneously upon dispersion of certain lipids in water. Lipids reduce the energetically unfavorable interaction between hydrophilic water and their hydrophobic tails by aligning their tails towards the center of the bilayer, leaving only their polar headgroup to interact with the water molecules (Figure 1B). Due to their hydrophobic interior, lipid bilayers are largely impermeable to polar compounds. This enables them to function as selective permeability barriers. Other important membrane functions are signal transduction, energy transduction, vesicular transport, cell adhesion and cell motility. These functions are mediated by membrane proteins which can make up to 50% of the total membrane weight. In 1972 Singer and Nicolson proposed the influential fluid mosaic model which stated that lipids and proteins can diffuse freely within the membrane (Singer and Nicolson, 1972). Although some aspects of this model are still valid today, it is now known that within the fluid membrane there are stable structures including protein complexes, cytoskeletal barriers and ordered membrane domains.

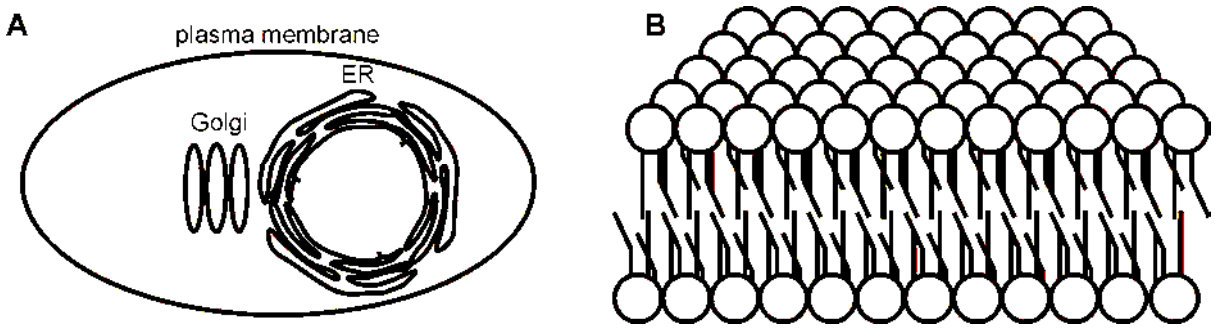

Figure 1. Biological membranes. A) Schematic drawing of a cell indicating the membranes that define the cell itself (plasma membrane) the Golgi complex and the ER. For simplicity other organelles are omitted. B) Schematic drawing of a lipid bilayer. Circles represent the hydrophilic headgroups and the lines represent the hydrophobic tail regions.

Membrane lipids

The major classes of lipids in biological membranes are glycerophospholipids, sphingolipids and cholesterol (Figure 2). Glycerophospholipids have a glycerol backbone which is linked by oxy-ester bonds to two fatty acids (hydrophobic tail region) and one phosphate or a phosphate attached to an alcohol (polar head group). Glycerophospholipids are subdivided in types depending on their head group. The major types are phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylglycerol (PG). Sphingolipids consist of a sphingosine backbone which is attached to a fatty acid and a polar headgroup. The head group can either be phospho-choline making up sphingomyelin (the most prominent sphingolipid in mammalian membranes) or one or more sugar groups making up the glycolipids. The different types of glycerophospho- and sphingolipids are further subdivided by the length and degree of unsaturation of their acyl chains. Due to the permutations of these structural features the cellular lipidome is theoretically composed of thousands of glycerophospholipids and more than 100,000 species of sphingolipids (Yetukuri et al., 2008). Although it is not exactly known why there are so many lipids, certain structural features have been linked to membrane functions. For instance, lipids with long and saturated acyl chains form closely packed membranes. On the other hand, unsaturated acyl chains are more kinked, resulting in weaker van der Waals interactions and more fluid membranes. Regulation of membrane thickness and fluidity has been shown to be important for the functioning of specific proteins and the membrane barrier function. Another example how lipids influence membrane function is illustrated by the concept of hydrophobic matching described by the mattress model (Mouritsen and Bloom, 1984); the thickness of a membrane with respect to the length of the hydrophobic part of an integral membrane protein is important for the lipid-protein interaction and can influence protein function and localization. However, despite the progress made over the last decades, we are only beginning to understand how lipids and proteins work in concert to enable biological membranes to function.

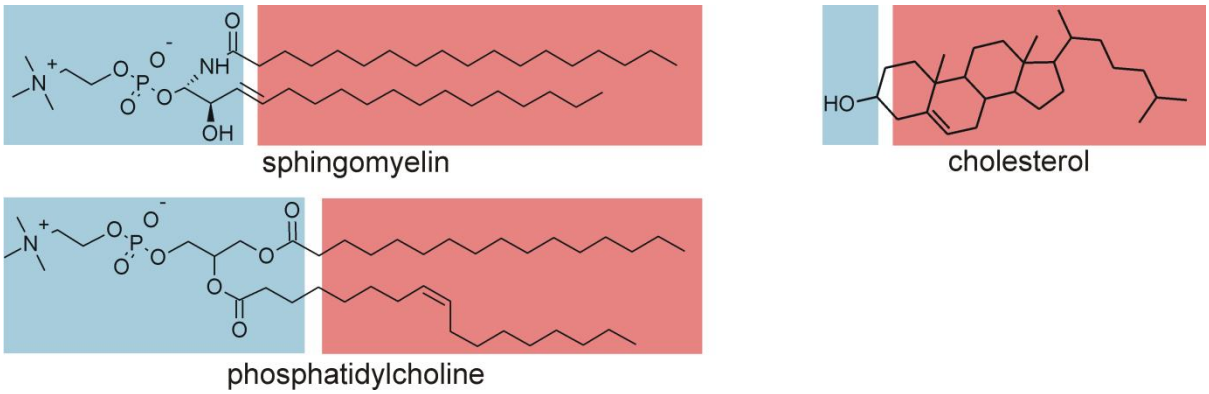

Figure 2. Structures of phosphatidylcholine, sphingomyelin and cholesterol. Blue areas indicate the hydrophilic headgroup and the red areas indicated the hydrophobic tail region.

Membrane properties of cholesterol

Cholesterol is a unique lipid that is present in large amounts in the membranes of eukaryotic cells. The presence of cholesterol drastically alters the biophysical properties of biological membranes. One of the major effects of cholesterol is the promotion of acyl chain order. In solution, acyl chains are very flexible structures; since the carbon-carbon single bonds can freely rotate, acyl chains can bend in all directions. In a membrane, acyl chains are more ordered since they are oriented parallel to each other by the forces that drive bilayer formation. Cholesterol within a membrane will position itself between acyl chains, thereby causing tighter packing and increased order. This phenomenon is clearly illustrated by the cholesterol condensing effect; the average area occupied per molecule in a cholesterol phospholipid bilayer is smaller than the sum of areas of the individual components (Stockton and Smith, 1976).

There are several models that attempt to explain the physical origin of the effect of cholesterol on acyl chain order. The *condensed complex model* proposes a chemical interaction between cholesterol and the acyl chains (McConnell and Radhakrishnan, 2003). This interaction forces the acyl chains to straighten and form more ordered and tightly packed membranes. The *umbrella model*, which is related to the more general *superlattice model* (Somerharju et al., 2009), postulates that hydrophobic cholesterol relies on polar phospholipid head group coverage to avoid the unfavorable free energy of cholesterol contact with water (Huang and Feigenson, 1999). Because cholesterol now occupies the limited space under the phospholipid head groups, the acyl chains are forced together.

Besides its influence on acyl chain order, cholesterol has various other effects on biophysical membrane properties. Cholesterol is known to decrease membrane permeability (Corvera et al., 1992). This phenomenon is partly caused by the increase membrane thickness that results from the straightening of the acyl chains. Other effects of cholesterol on membranes include: thermomechanics, lateral diffusion and binding of solutes. These aspects are reviewed in more detail in (Mouritsen and Zuckermann, 2004). Arguably, the most distinctive feature of cholesterol in biological membranes is its ability to form ordered domains within a liquid bilayer. This unique property of cholesterol will be discussed in more detail in the chapter on ordered membrane domains.

1.2 Cellular cholesterol homeostasis

Because cholesterol is involved in various essential cellular processes and because excess free-cholesterol is toxic for cells, regulation of cholesterol levels is of vital importance. This regulation is a highly dynamic process since cells continually exchange cholesterol with their environment and constantly need cholesterol for cell division. The level of cellular cholesterol follows from several processes including cholesterol biosynthesis, uptake, efflux

and subcellular storage. In order to coordinate these different processes, cells have evolved feedback mechanisms that tightly regulate cholesterol homeostasis.

Biosynthesis

The biosynthesis of cholesterol is a process consisting of more than 30 enzymatic reactions which mostly take place in the ER. The biosynthetic pathway (Figure 3) was elucidated mainly by the work of Konrad Bloch (Bloch, 1965), who received the Nobel Prize in medicine in 1964 for these efforts. The synthesis starts with acetyl-coenzyme A (acetyl-CoA), which is also important for the biosynthesis of fatty acids, amongst other compounds. The first step of the synthesis is a condensation reaction of acetyl-CoA and its derivative acetoacetyl-CoA. This gives 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is subsequently reduced by an enzyme called HMG-CoA reductase (HMGR). This step is rate limiting for cholesterol biosynthesis and produces mevalonate. Statins, the pharmacological compounds that lower blood cholesterol levels, inhibit this reaction. Mevalonate is further converted into the five-carbon isopentyl pyrophosphate. This is the basic unit used for the synthesis of all isoprenoids, which comprises a large class of molecules that includes menthol and cannabinoids. On a side note, because statins inhibit cholesterol biosynthesis at such an early step, they not only inhibit the production of cholesterol but also disrupt the biosynthesis of all other isoprenoids. In the next step of the biosynthesis, the 5-carbon isopentylpyrophosphate condenses with itself in multiple steps to form the 30-carbon squalene. Finally squalene is converted to lanosterol, which contains the tetracyclic ring structure that is characteristic for all sterols. After the synthesis of lanosterol the biosynthesis of cholesterol proceeds with a 19-step process requiring 9 different enzymes (Gaylor, 2002). The different steps include the formation, removal and isomerization of carbon-carbon double bonds and the removal of methyl groups. Two routes involving the same enzymes have been proposed, differing only in the timing of the reduction of the carbon-24 double bond. Consequently, cholesterol biosynthesis can proceed with either desmosterol (Bloch pathway) or 7-dehydrocholesterol (Kandutsch-Russell pathway) as the penultimate precursor of cholesterol. Even though specific cell types prefer one pathway over the other, the functional relevance of having two pathways is unknown.

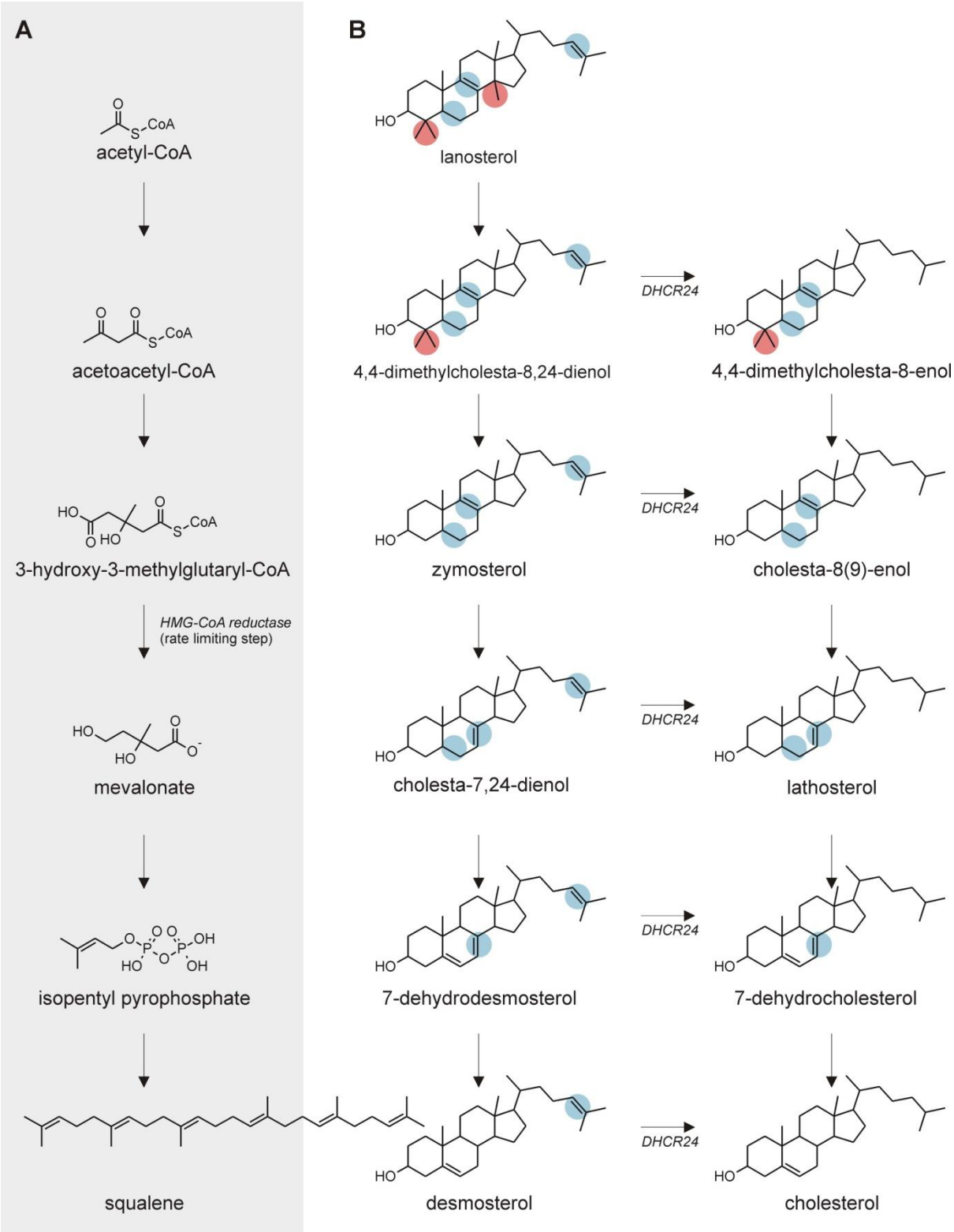

Figure 3. Biosynthesis of cholesterol. A) The biosynthesis of squalene from acetyl-CoA. B) After the conversion of squalene to lanosterol the biosynthesis of cholesterol can proceed either through the Bloch pathway (left) or Kandutsch-Russell pathway (right). Structural differences from cholesterol are indicated with red (additional methyl group) and blue dots (carbon-carbon bond saturation).

Uptake

Besides biosynthesis, mammalian cells can acquire cholesterol, mostly in its esterified form, through uptake of lipoprotein particles from the extracellular environment. This process, called *forward cholesterol transport*, is mainly facilitated by receptor mediated uptake of low density lipoprotein (LDL) particles , which has been characterized mostly by the work of Brown and Goldstein (Brown and Goldstein, 1986). After extracellular LDL binds its receptor on the cell surface, the complex relocalizes to clathrin coated pits that pinch off from the plasma membrane to form an endocytic vesicle. In most familial hypercholesterolemia patients this process is disrupted because of mutations in the LDL receptor; this causes blood LDL levels to rise which increases the risk of developing cardiovascular disease via the atherosclerotic process. After internalization, the complex travels down the endocytic route. Meanwhile, the pH within the endosomes decreases, causing LDL to dissociate from its receptor. After this, the receptor can either be transported back to the plasma membrane or be targeted for degradation. The cholesterol esters continue to move down the endosomal route and are hydrolyzed by a protein called lysosomal acid lipase. Exit of cholesterol from the endosomal compartment is mediated by the proteins NPC1 and NPC2. It is not entirely clear how the NPC proteins function but there is evidence that soluble NPC2 extracts cholesterol from LDL and delivers it to membrane-associated NPC1 that incorporates the sterol in the late-endosomal/lysosomal membrane (Kwon et al., 2009). The cholesterol is then mixed with the preexisting pool of cellular cholesterol by an unknown mechanism. It is worth noting that the internalization of oxidized LDL by macrophages, a process important for the development of atherosclerosis, is mediated through a different route involving scavenger receptor class A type 1 (SR-AI) and CD36. Furthermore, in adrenocortical cells, SR-BI also facilitates uptake of LDL.

Efflux

Because mammalian cells are unable to break down cholesterol, cells need to secrete cholesterol in order to remove it. The cellular secretion of cholesterol by non-hepatic cells is often referred to as *reverse cholesterol transport* and involves the delivery of cholesterol to extracellular acceptors. An important acceptor is apolipoprotein A-I (apoA-I) which accepts cholesterol through the action of ATP binding cassette transporter A1 (ABCA1), the protein defective in Tangier disease. By accumulating lipids, nascent apoA-I containing particles mature to spherical high density lipoprotein (HDL) particles. In turn, spherical HDL also acts as a cholesterol acceptor. This type of efflux is mediated by two other ABC–transporters, namely ABCG1 and ABCG4 (Wang et al., 2004). After cholesterol is secreted to the blood in HDL particles it can be taken up by liver cells via the function of SR-BI that facilitates selective cholesterol ester uptake from HDL (Acton et al., 1996). In the liver, ABCG5/ABCG8 are involved in the secretion of cholesterol into the bile (Graf et al., 2003).

Cholesterol storage in lipid droplets

Cells can regulate their subcellular cholesterol levels by deposition of cholesterol in lipid droplets. Lipid droplets are cellular organelles that function as storage sites for cholesterol and fatty acids, thereby controlling the fat energy reserves of cells. Lipid droplets are also essential for regulating the levels of cholesterol and fatty acids that are present in cellular membranes. This is done by rapidly enabling cholesterol and fatty acids to be deposited and extracted in periods of excess and deficiency (Martin and Parton, 2005). The amount of cholesterol stored in lipid droplets varies among cell types and depends on the metabolic state of the cell. The lipids are stored in a hydrophobic core which is surrounded by a lipid monolayer (Tauchi-Sato et al., 2002). Although lipid droplets contain some free cholesterol, generally most cholesterol is stored in esterified form. The enzyme acyl-CoA: cholesterol acyltransferase (ACAT), which is present in the ER, catalyzes the esterification of cholesterol with a fatty acid. For unknown reasons, adipocytes, which are specialized in cholesterol and triacylglyceride storage, store cholesterol in its unesterified form (Schreibman and Dell, 1975).

Regulation of cholesterol homeostasis

In order to maintain cholesterol homeostasis, the processes that mediate cholesterol biosynthesis, uptake, efflux, and storage, are tightly regulated by several feedback mechanisms. An important transcriptional regulatory mechanism is mediated by sterol regulatory element binding protein (SREBP) (Brown and Goldstein, 1997). SREBP is a transmembrane protein which is kept in the ER by binding to SREBP cleavage activating protein (SCAP). When cholesterol levels are high, the sterol sensing domain in SCAP binds cholesterol. This causes SCAP to bind a protein called Insig, resulting in retention of the entire complex in the ER. When cholesterol levels are low, a conformational change in the sterol sensing domain of SCAP abrogates its binding with Insig. As a result the SCAP SREBP complex is transported to the Golgi complex in COPII vesicles. Upon arrival in the Golgi, SREBP gets cleaved releasing a transcription factor that activates the expression of genes containing a sterol regulatory element promoter. Many of these genes encode proteins that are involved in increasing the cellular cholesterol content, including several enzymes involved in cholesterol biosynthesis and the LDL-receptor. Another transcriptional regulatory mechanism is mediated by Liver X receptors (LXRs) that sense cellular sterol levels by binding to oxidized derivatives of cholesterol. Upon activation, LXRs alter the transcription of proteins involved in reverse cholesterol transport (Tontonoz and Mangelsdorf, 2003). Finally, it should be noted that cholesterol homeostasis can also be regulated by post-transcriptional mechanisms. For instance, a rise in cholesterol levels causes HMGR to bind Insig, resulting in the degradation of HMGR by the proteasome (Sever et al., 2003).

1.3 Subcellular distribution of cholesterol

Cholesterol content of cellular organelles

It has become clear that cholesterol is unevenly distributed over the membranes of different cellular compartments. The ER, where cholesterol is synthesized and its levels are regulated, is relatively poor in cholesterol. Yvonne Lange and colleagues analyzed in cell homogenates the pool of cholesterol that could be acylated by ACAT, a protein that is present in the ER (Lange and Steck, 1997). They found that, depending on growth conditions, 0.1-2% of total cellular cholesterol could react with ACAT. The authors suggested that this fraction represents the pool of ER cholesterol. The lab of Brown and Goldstein isolated ER membranes in a 4-step process and found that cholesterol constitutes 5% of all ER lipids (Radhakrishnan et al., 2008). Even though these results are not directly comparable, they both agree on low ER cholesterol content. It seems likely that this low concentration facilitates the SCAP-SREBP complex to sense small changes in cholesterol levels. Plasma membrane cholesterol can be analyzed by cholesterol oxidase treatment. When added to the cells, this enzyme converts cholesterol in the plasma membrane to cholestenone without modifying intracellular cholesterol (Slotte et al., 1989). Cholesterol oxidase treatment and subcellular fractionation studies agree that 65-80% of cellular cholesterol resides in the plasma membrane and that around 1 out of 3 plasma membrane lipids is cholesterol (Liscum and Munn, 1999; Warnock et al., 1993). In the Golgi the cholesterol concentration lies in between that of the ER and plasma membrane (van Meer, 1998). Furthermore, the concentration appears to increase from the *cis* to *trans* direction (Coxey et al., 1993). Taken together, it appears that the cholesterol concentration in cellular membranes increases along the secretory pathway. Other organelles with high cholesterol levels are the endocytic recycling compartment (ERC) (Hao et al., 2002) and internal vesicles of multivesicular bodies (Mobius et al., 2003), whereas mitochondria, which are involved in steroidogenesis, and lysosomes are generally cholesterol poor (Mobius et al., 2003; Severs, 1982).

Underlying theories for cholesterol gradient

It is not clear how the concentration gradient of cholesterol is maintained among the different membranes. One theory suggests that the amount of cholesterol in a particular membrane is determined by its affinity towards that membrane (Lange and Steck, 2008; Phillips et al., 1987). This affinity is proposed to be dependent on the lipid composition of the membrane. Experimental evidence for this theory came from studies analyzing the desorption rate of lipids from lipid monolayers. Cholesterol associated in a sphingomyelin monolayer desorbs more slowly than from a PC monolayer (Ohvo and Slotte, 1996), and furthermore, higher acyl chain unsaturation increases cholesterol desorption (Ramstedt and Slotte, 1999). Indeed the plasma membrane, which is rich in cholesterol, has a high degree of fatty acid saturation and is enriched in sphingomyelin. The affinity/stabilization of cholesterol in various membranes can be explained by the *condensed complex model* (see 1.1 Cholesterol in membranes). The *condensed complex model* argues that cholesterol forms more stable complexes with some lipids compared to others. It has been shown experimentally that the van der Waals interactions between cholesterol and acyl chains are strongest when the hydrocarbon chains are saturated (Chong et al., 1994). It should be noted that alternatively, the cholesterol gradient could be maintained by selective energy driven cholesterol transport. However, there is no convincing evidence for this theory.

1.4 Intracellular cholesterol transport

In order to maintain its subcellular cholesterol distribution, cells have to traffic cholesterol from the sites of biosynthesis and internalization towards other compartments. In general, lipid transport can be divided into vesicular and non-vesicular mechanisms. Although cholesterol has been found in the membranes of both secretory and endocytic vesicles (Brugger et al., 2000; Mobius et al., 2003), there is growing evidence that most cholesterol traffic between cellular compartments is mediated by non-vesicular transport. This transport appears to be facilitated by different families of lipid transfer proteins (LTPs). These are soluble proteins with a hydrophobic binding domain capable of trafficking lipids between membranes *in vitro*. There is also evidence for the involvement of membrane contact sites. The ER has been shown to be in close contact with most other cellular compartments (Loewen et al., 2003). These sites would be ideal for efficient non-vesicular lipid transport and some LTPs have been shown to function at sites of close membrane approximation (Schulz et al., 2009). Finally, it should be noted that although some of the players have been revealed, the precise mechanisms of cellular cholesterol transport remain unknown.

Plasma membrane-ER transport

An important cholesterol transport route is between the ER, where cholesterol is synthesized, and the plasma membrane, where most cholesterol is present. Transport from the ER to the plasma membrane can be analyzed by monitoring the arrival of newly synthesized cholesterol at the plasma membrane. By biochemically isolating plasma membranes, Simoni and colleagues found that cholesterol is transported to the plasma membrane with a halftime of 10-20 min in an energy dependent manner (DeGrella and Simoni, 1982). Yvonne Lange and colleagues detected plasma membrane arrival of cholesterol by analyzing its conversion by extracellular cholesterol oxidase. They found a transport halftime of around 30 min (Lange et al., 1991). Brefeldin A treatment, disrupting the Golgi and secretory transport, has little effect on the transport kinetics (Heino et al., 2000; Urbani and Simoni, 1990). In yeast *SEC* mutants, with conditional defects in proteins required for ER-plasma membrane vesicular transport, there is either no block or a partial block in cholesterol traffic (Baumann et al., 2005; Schnabl et al., 2005). The reverse route, cholesterol transport from the plasma membrane back to the ER, can be analyzed by labeling the plasma membrane with a radioactive-cholesterol isotope and monitoring its esterification in the ER. Also this transport is not inhibited in several yeast *SEC* mutants (Li and Prinz, 2004). Furthermore, this transport was shown to be independent of ATP or other treatments that affect vesicular transport (Skiba et al., 1996). Taken together these results indicate that cholesterol can move between the plasma membrane and ER in a non-vesicular fashion. Insights into the mechanisms of non-vesicular transport have emerged from research on ORPs, a family of LTPs, which will be discussed in more detail in chapter 4.

Transport to lipid droplets

The deposition of cholesterol in lipid droplets is essential for maintaining cholesterol homeostasis. Most cholesterol moving into lipid droplets comes from the plasma membrane (Lange et al., 1993). Using the fluorescent cholesterol probe dehydroergosterol, it was shown that cholesterol moves independent of metabolic energy to lipid droplets with a halftime of 1.5 min (Wustner et al., 2005). It is not known how cholesterol and cholesterol esters move to lipid droplets. There is evidence that cholesterol targeted to lipid droplets moves through the ER. Cholesterol esters are synthesized by ACAT in the ER and are either packed directly in lipoprotein particles (only by specialized cell types) or stored in lipid droplets. Furthermore, lipid droplets have been visualized in close proximity to the ER membrane (Ohsaki et al., 2008). The prevailing theory of lipid droplet biogenesis states that they originate from lipid accumulations between the leaflets of the ER membrane (Murphy and Vance, 1999; Ohsaki et al., 2009). It is possible that after formation, the lipid monolayer that surrounds lipid droplets remains continuous with the ER, ensuring lipid transport through lateral diffusion. Alternatively, membrane contact sites between the ER and lipid

droplets might be involved. Although the ER is probably the direct source for lipid droplet cholesterol, so far no proteins have been implicated in cholesterol transfer between the ER and lipid droplets.

Other routes and transporters

Maxfield and colleagues studied sterol transport from the plasma membrane to the ERC using the dehydroergosterol and radiolabeled-cholesterol (Hao et al., 2002). They found that traffic was fast (halftime of 2.5 min) and independent of ATP, which suggests that the transport is non-vesicular. Surprisingly, dehydroergosterol transport out of the ERC did require metabolic energy and was suggested by the authors to be mediated by a vesicular pathway.

Cholesterol has to be transported to the inner mitochondrial membrane in order to be converted to pregnenolone, the first step of steroid biosynthesis. Cholesterol movement from the outer to the inner mitochondrial membrane has been shown to be mediated by steroidogenic acute regulatory protein (StAR) (Miller, 2007; Rone et al., 2009). In humans, there are at least 14 other StAR related lipid transfer (START) proteins (Alpy and Tomasetto, 2005). An important member of this family is ceramide transporter (CERT), which has been shown to transport ceramide, a precursor of sphingolipid biosynthesis, from the ER to the Golgi. Two other members, STARD3 (MLN64) and STARD5, have been shown to bind cholesterol (Rodriguez-Agudo et al., 2005; Tsujishita and Hurley, 2000). There is evidence that these proteins are involved in cholesterol transport to the outer mitochondrial membrane (Charman et al., 2010; Soccio et al., 2005; Zhang et al., 2002), but a detailed molecular mechanism is lacking.

Another LTP, sterol carrier protein 2 (SCP-2), promotes the exchange of sterols between membranes *in vitro* and its expression affects sterol trafficking in certain tissue culture systems (Baum et al., 1997; Pfeifer et al., 1993; Puglielli et al., 1995; Seedorf et al., 2000). However, the apparent lack of specificity for cholesterol and its predominant localization in peroxisomes argue against a role for SCP-2 as a sterol transporter (Seedorf et al., 2000). This was further corroborated by studies using SCP-2 knockout mice where, rather than a role in cholesterol transport, SCP-2 was related to the peroxisomal oxidation of certain fatty acyl-CoAs (Seedorf et al., 1998). Finally, another protein shown to be involved in cholesterol transport is caveolin, which will be discussed in more detail in section 2.3.

2. Ordered membrane domains

This chapter discusses different types of ordered membrane domains in both cells and model membranes. The most important ordered membrane domains in cells are called rafts and caveolae (a subtype of raft). One feature all these domains have in common is their dependence on cholesterol. Here, I will focus on ordered membrane domain structure, the methods of analysis and the importance of cholesterol.

2.1 Membrane rafts

Although there had been for some time evidence for the existence of lipid domains (Karnovsky et al., 1982) this phenomenon only started to get wider attention when Simons and van Meer found a functional application. They showed that NBD-glucocylceramide gets sorted from the Golgi to the apical membrane of epithelial cells (van Meer et al., 1987) and postulated the involvement of lipid domains enriched in cholesterol and sphingolipids (Simons and van Meer, 1988). These lipid domains, termed rafts, started to get wide recognition outside the lipid field after the publication of a review which described the concept (Simons and Ikonen, 1997). A consensus definition of a membrane raft emerged at a keystone symposium in 2006 on Lipid Rafts and Cell Function (Pike, 2006): "*Membrane rafts are small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize in cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein interaction"*. A wide array of cellular processes has been shown to be mediated by rafts including membrane trafficking, signal transduction, cell adhesion and endocytosis. Below are discussed the main techniques for studying membrane rafts in cells.

Studying rafts: detergent resistance, cholesterol removal and clustering

Detergent resistance of certain lipids and proteins has been an important tool for raft analysis. Detergent resistance was first used to support the raft hypothesis by a study by Brown and Rose. They showed that, *en route* to the apical membrane, GPI anchored proteins become associated with a detergent resistant membrane (DRM) fraction which was enriched in sphingolipids (Brown and Rose, 1992). Since then, detergent resistance has grown to become a useful tool for studying potential raft association of proteins, especially when DRM association can be triggered by a physiologically meaningful event (Lingwood and Simons, 2010). However, it should be noted that DRMs are artifactual entities that do not exist in living cells. Furthermore, detergent resistance does not always indicate raft association of a protein (Shogomori and Brown, 2003).

Another important advance in the raft field came from the discovery that deprivation of cholesterol decreased the detergent resistance of a glycosylphosphatidylinositol (GPI) anchored protein (Hanada et al., 1995). Later it was shown that by selectively depleting cholesterol from the plasma membrane using the sterol carrier methyl-β-cyclodextrin (MβCD) the influenza virus protein hemagglutinin became excluded from DRMs (Scheiffele et al., 1997). Together with DRM isolation, MβCD treatment became a widely used tool to analyze involvement of rafts in cellular processes. This method will be critically discussed in section 2.4.

Another important tool for studying rafts is by clustering raft proteins or lipids by antibodies or toxins. Maxfield and colleagues showed that antibody induced clustering of a GPI-anchored receptor led to relocalization of the receptor to caveolae, a subtype of raft (Mayor et al., 1994). Later the same was shown for sphingomyelin and glycolipids (Fujimoto, 1996). In a similar sort of experiment, simultaneous cross-linking of several membrane proteins led to colocalization of raft proteins and segregation of raft and non-raft proteins (Harder and Simons, 1997). This selective clustering of raft proteins appears to be dependent on cholesterol as determined by MβCD treatment (Scheiffele et al., 1997). Another interesting observation was that clustering of raft proteins can initiate a signaling process that stimulates T-cell activation (Stefanova et al., 1991).

Studying rafts: microscopy and spectroscopy

Until 2003, 15 years after the postulation of the raft hypothesis, researches had not been able to demonstrate ordered membrane domains in unperturbed living cells (Munro, 2003). In other words, there was a lack of definite proof for the existence of rafts. A breakthrough in the raft field was instigated by the emergence of non-invasive microscopic and spectroscopic methods. Important techniques include: (1) stimulated emission depletion (STED) microscopy (Eggeling et al., 2009), (2) fluorescence correlation spectroscopy (FCS) (Lenne et al., 2006), (3) fluorescence resonance energy transfer (FRET)) (Rao and Mayor, 2005), (4) single particle tracking (Suzuki et al., 2007). These studies suggest that clusters of GPI-anchored proteins exist with a size of 10-200 nanometers and a sub-second life time.

2.2 Ordered domains in model membranes

Studying lipid interactions in cells is difficult due to the complexity of cellular membranes; they contain hundreds of different lipids which are constantly influenced by cellular processes like, for instance, membrane transport. For this reason researchers started to utilize simple model membrane systems for studying domain behavior of lipids.

Phase behavior in membranes

Lipids in a bilayer can exist in different phases. Two common phases are the solid-ordered (S_o) and liquid-disordered (L_d) phase. Besides physical parameters such as temperature, the phase behavior of a bilayer depends on structural features of the lipids. One important feature is the melting temperature (T_m) of the lipid. Lipids with long and saturated acyl chains have strong intermolecular van der Waals interactions and therefore a high T_m. On the other hand, lipids with short *cis*-unsaturated acyl chains are more kinked and have a lower T_m. In a bilayer, lipids with a high T_m tend to form a closely packed S_o phase which is characterized by high acyl chain order and low lateral mobility. On the other hand, low-T_m lipids tend to form a L_d phase, characterized by low acyl chain order and high lateral mobility. A single membrane can exist in more than one phase. For instance binary mixtures containing one high and one low-T_m lipid can have S_o domains in an overall L_d membrane (Shimshick and McConnell, 1973). However, for most researchers this phenomenon did not explain the presence of membrane rafts since the existence of a S_o phase was considered incompatible with the view of a fluid cellular membrane.

Cholesterol and the liquid-ordered phase

Before the raft hypothesis was postulated, researchers had shown that the addition of cholesterol to model membranes alters their phase behavior. Cholesterol was found to make the L_d phase more ordered and the S_o phase more fluid. As a consequence, cholesterol enabled the formation of a new type of phase which was named liquid-ordered (L_o) (Ipsen et al., 1987). The L_o phase is characterized by high acyl chain order and high translational mobility. Because the cellular plasma membrane is roughly composed of ⅓ cholesterol, ⅓ high-T_m lipids (mainly sphingolipids) and ⅓ low-T_m lipids (mainly glycerophopholipids), many model membrane studies are performed with tertiary lipid mixtures of this composition. Using fluorescence quenching methods, such mixtures were shown to contain L_o ordered domains in an L_d phase (Ahmed et al., 1997; Silvius et al., 1996). Indeed, addition of detergent to these membranes gave rise to DRMs enriched in cholesterol and high-T_m lipids (Schroeder et al., 1994). During the second half of the 1990s, many researchers believed that the lipid interactions that form L_o domains in model membranes may cause

raft formation in cells (Rietveld and Simons, 1998). Today, it appears that the formation of rafts cannot entirely be explained by L_o phase behavior. For instance, transmembrane proteins that are present in rafts do not partition in L_o domains of model membranes (Shogomori et al., 2005). Also, it was found that clustered rafts in isolated plasma membranes are not in the L_o state (Lingwood et al., 2008). Nevertheless, the work in model membranes has convincingly illustrated that without the help of proteins, lipids are capable of forming ordered platforms within in a liquid phase.

2.3 Caveolae

Caveolae are often considered as a subtype of raft that are also cholesterol/sphingolipid enriched and resistant to detergent treatment. Different from other membrane rafts, the caveolar structure is highly stable and dependent on proteins of the caveolin family. Caveolae are present in many types of cells, and are especially abundant in adipocytes, endothelial cells and muscle cells. They have been implicated in many different functions including endocytosis of certain lipids and virus particles, transcytosis, signaling platforms, adhesion and migration, and lipid regulation.

Structure

Caveolae are small flask shaped invaginations (50-100 nm in diameter) on the plasma membrane that can be visualized by electron microscopy (II, Figure 5A) (Palade, 1953). The main structural component of caveolae is caveolin-1, which is a member of the protein family that also comprises caveolin-2 and caveolin-3. Caveolin-1 is localized to the cytosolic sites of the plasma membrane with a putative hairpin intramembrane domain within the membrane bilayer (Parton and Simons, 2007). The importance of caveolin-1 is illustrated by the findings that caveolin-1 knockout cells are devoid of caveolae (Drab et al., 2001) and that overexpression of caveolin-1 can (but not necessary always does) bring about the formation of caveolae in various cell types (Fra et al., 1995; Lipardi et al., 1998). Caveolin-1 has been shown to oligomerize as caveolae are formed (Monier et al., 1995) and these oligomers have been proposed to make up a filamentous coat which decorates the cytoplasmic surface of caveolae (Fernandez et al., 2002; Rothberg et al., 1992). It has been estimated that the number of caveolin-1 proteins per caveolae is around 150 (Pelkmans and Zerial, 2005). Besides caveolin-1 there are several other proteins that are important for caveolar structure. Caveolin-2 is often co-expressed with caveolin-1 but is not required for caveolar formation (Razani et al., 2002). Instead, caveolin-2 appears to have a modulator role, leading to the formation of more uniform caveolae (Li et al., 1998). Caveolin-3 is specifically found in muscle and appears to be both structurally and functionally similar to caveolin-1 (Tang et al., 1996). Recently, a protein outside the caveolin family was found to

be a structural component of caveolae (Hill et al., 2008). This protein, named cavin, was shown to be recruited to caveolae by caveolin-1 and is required for caveola formation and sequestration of mobile caveolin-1 into immobile caveolae.

Cholesterol and caveolae

As has been shown for membrane rafts, also caveolae depend structurally and functionally on cholesterol. Biochemically isolated caveolae have been shown to be enriched in cholesterol (Ortegren et al., 2004) and caveolin-1 appears to directly bind cholesterol (Murata et al., 1995). This interaction may be mediated by a specific cholesterol binding motif which is also found in other proteins (Li and Papadopoulos, 1998). Furthermore, cholesterol appears to be involved in caveolar biogenesis, since cholesterol stabilizes the formation of caveolin oligomers *in vitro* (Monier et al., 1996) and is necessary for loss of diffusional mobility of caveolins in the Golgi (Hayer et al., 2010). Also, cholesterol appears to be important for caveolar structure, since the addition of filipin, a cholesterol binding polyene, flattens caveolae (Rothberg et al., 1992). Furthermore, cholesterol depletion by MβCD disrupts the close proximity between caveolin-1 and cavin (Hill et al., 2008). It should be noted that these treatments are harsh and might affect the caveolar structure indirectly. Finally, it is interesting to point out that caveolae also affect cholesterol. For instance, overexpression of caveolin-1 leads to an increase in reverse cholesterol transport (Truong et al., 2010) and adipocytes from caveolin-1 knockout mice contain 10-fold reduced levels of cholesterol (Le Lay et al., 2006).

2.4 Studying ordered domains by cholesterol depletion

Removal of cellular cholesterol by cyclodextrins has been one of the most widely used methods for studying whether a process takes place in membrane raft/caveolae. Here, the procedure, side effects and the general cellular response to cholesterol removal will be discussed.

Cholesterol depletion by methyl-β-cyclodextrin

Cyclodextrins are synthetic cyclic oligosaccharides which have a hydrophilic exterior and a hydrophobic core. These characteristics allow cyclodextrins to solubilize certain hydrophobic compounds. The hydrophobic core of the heptamer methyl-β-cyclodextrin (MβCD) is especially suitable for solubilizing cholesterol (Ohtani et al., 1989) (Figure 4). When added to cells, MβCD efficiently removes cholesterol from the plasma membrane. The most common protocol for cholesterol removal prescribes incubation with 10 mM

MβCD for 30 min at 37 ºC. This treatment results in a removal of ~60% of total cellular cholesterol in BHK cells (Keller and Simons, 1998) and ~70% in HeLa cells (Vainio et al., 2006). Although the cholesterol is removed from the plasma membrane, it should be noted that also intracellular compartments will be affected because of continuous transport of cholesterol between cellular organelles (Lange et al., 2004; Rosenbaum et al., 2010).

There are several side effects when using MβCD to remove cellular cholesterol. First, MβCD also extracts certain phospholipids (Ohvo and Slotte, 1996). For instance, a 30 min MβCD treatment of rat cerebellar granule cells resulted in a decrease of sphingomyelin (15%) and glycerophospholipids (17%), besides cholesterol (50%) (Ottico et al., 2003). Second, MβCD has been reported to extract peripheral membrane proteins from cells (Ilangumaran and Hoessli, 1998; Ohtani et al., 1989). Most researchers who use MβCD to study rafts neglect these side effects and therefore do not include proper control samples in which cholesterol levels are replenished after depletion.

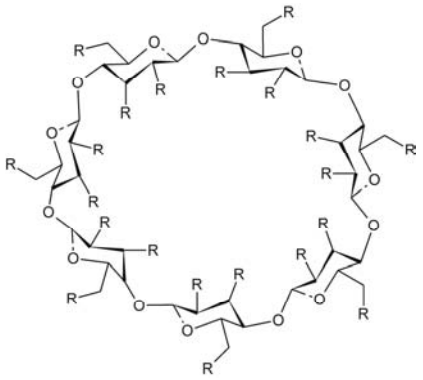

*Figure 4. Structure of methyl-**β**-cyclodextrin. R = -OH or -OCH_3*

Effect of cholesterol depletion on cells

Since cholesterol has many cellular functions apart from forming rafts/caveolae, acute cholesterol removal by MβCD also affects these other processes. For instance, cholesterol has been shown to be important for proper membrane permeability and fluidity. Indeed, cholesterol depletion from cells results in increased plasma membrane permeability to small molecules and ions (Grunze and Deuticke, 1974). Furthermore, membrane cholesterol levels have been shown to also affect the functions of non-raft enzymes. It was shown that Na^+/K^+-ATPase activity decreased when the cholesterol content was altered from native levels (Yeagle et al., 1988). Furthermore, cholesterol depletion inhibits the formation of clathrin-coated vesicles (Rodal et al., 1999). Another difficulty in the interpretation of cholesterol depletion arises from the fact that certain raft mediated processes affect other cellular processes that do not take place in rafts. For instance, cholesterol depletion delocalizes

phosphatidylinositol 4,5-bisphosphate (PIP_2) from the plasma membrane (Pike and Miller, 1998). PIP_2 is involved in cytoskeleton/membrane interaction and actin fiber formation (Caroni, 2001) and thereby affects many cellular processes. Indeed, it has been shown that cholesterol removal affects the lateral mobility of membrane proteins by disrupting the interactions between PIP_2 and actin (Kwik et al., 2003). Taken together, loss of a particular function after cholesterol depletion does not necessarily imply that this process occurs in membrane rafts.

3. Cholesterol analogs as a tool

Cholesterol analogs can function as valuable tools to study the biology of cholesterol. I have made a distinction between fluorescent and non-fluorescent cholesterol analogs since their use lies in distinct areas.

3.1 Fluorescent cholesterol probes

Fluorescent cholesterol analogs can be utilized to analyze the dynamic localization of sterols in living cells. The usefulness of such probes depends on two factors: their fluorescent properties and their functional similarity to cholesterol. The labeling of lipids with a fluorescent group is generally more challenging than the labeling of proteins. Proteins often consist of distinct functional domains. The addition of a fluorescent group outside these functional domains often does not interfere with the protein's activity. On the other hand, most properties of membrane lipids are not mediated by distinct functional domains but instead follow from the physical properties of the entire molecule. Because cholesterol is a small molecule, most minor structural changes will have relatively large effects on the physical properties of cholesterol. In this section the benefits and drawbacks of the most widely used fluorescent cholesterol analogs (Figure 5) will be discussed.

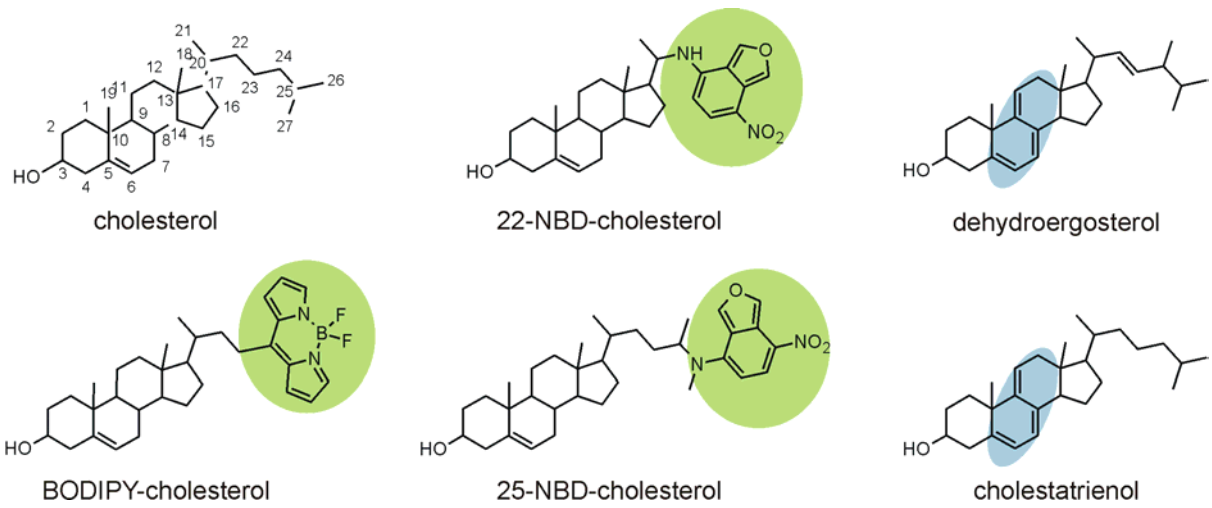

Figure 5. Structures of fluorescent cholesterol analogs. The green areas indicate the added fluorescent groups and the blue areas indicate the conjugated triene moiety. The BODIPY-cholesterol analog shown is also known as BODIPY-cholesterol compound 2 (Li et al., 2006).

NBD-cholesterol

The addition of a fluorescent 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group to cholesterol gives rise to NBD-sterols (Figure 5). There are several NBD-sterols that differ by the positioning of the NBD group. Most common are 22-NBD-cholesterol and 25-NBD-cholesterol. The NBD group has relatively good fluorescent properties; excitation (470 nm) and emission (530 nm) are in the visible region and the quantum yield is good (Chattopadhyay, 1990). 22-NBD-cholesterol has been shown to interact with the sterol binding sites of cholesterol modifying enzymes such as HMGR (Craig et al., 1981) and ACAT. Furthermore, NBD-cholesterol has been used to characterize sterol binding sites of StAR (Petrescu et al., 2001) and SCP-2 (Avdulov et al., 1999). Even though NBD-cholesterol is recognized as cholesterol by many sterol binding enzymes, the biophysical properties of NBD-sterols in lipid bilayers are very different from those of cholesterol. Neither of the NBD-sterols partition in cholesterol rich domains (Loura et al., 2001; Mattjus et al., 1995). Moreover, NBD-sterols were shown to orient upside down in model membranes (Scheidt et al., 2003). Also the behavior of NBD-sterols in cells deviates much from that of cholesterol. For example, 22-NBD-cholesterol and 25-NBD-cholesterol both accumulate in mitochondria (Mukherjee et al., 1998). Altogether, because of their aberrant biophysical behavior and subcellular localization, NBD-sterols are unsuitable for studying the cell biology of cholesterol.

Dehydroergosterol and cholestatrienol

Dehydroergosterol is a naturally fluorescent sterol which is synthesized in certain types of yeast. Structurally, dehydroergosterol closely resembles cholesterol; differing only by three additional double bonds and an additional methyl group (Figure 5). Cholestatrienol, an analog very similar to dehydroergosterol, also contains a fluorescent conjugated triene system but is otherwise identical to cholesterol. Because cholestatrienol is not commercially available, not many researchers use this compound. Therefore, the focus of this section will be on dehydroergosterol. As might be expected from its structural resemblance to cholesterol, dehydroergosterol behaves quite similarly to cholesterol. In model membranes the orientation of dehydroergosterol is correct, with the free hydroxyl group pointing towards the water interphase (Scheidt et al., 2003). Furthermore, dehydroergosterol has affinity for ordered membrane domains (Garvik et al., 2009). However there are small differences between dehydroergosterol and cholesterol regarding certain physical properties. For instance, the sterol induced phospholipid condensation as measured by ^{2}H NMR order parameters was significantly lower for dehydroergosterol (Scheidt et al., 2003). Nevertheless, dehydroergosterol has been used in many studies to analyze the behavior of cholesterol *in vitro*. An interesting example is a study that analyzed sterol/protein interaction by measuring FRET between tryptophan residues and dehydroergosterol (de Almeida et al., 2004).

Dehydroergosterol has also been shown to resemble cholesterol *in vivo*. The worm *Caenorhabditis elegans*, which needs sterol uptake in order to survive, can live and reproduce on dehydroergosterol (Matyash et al., 2001). Also mammalian cells are viable when containing dehydroergosterol as the main sterol (Kavecansky et al., 1994). Furthermore, the intracellular dehydroergosterol distribution resembles that of cholesterol as determined by filipin staining and sucrose density fractionation (Hao et al., 2002). Dehydroergosterol has been used in several studies to analyze intracellular cholesterol transport (see 1.5 Intracellular cholesterol transport). However, it should be noted that in cells dehydroergosterol does not behave identically to cholesterol, e.g. its rate of esterification was shown to be 8 fold higher in fibroblasts (Frolov et al., 2000).

A major drawback in the use of dehydroergosterol and cholestatrienol results from their unfavorable spectroscopic properties. Excitation and emission take place at 376 nm and 422 nm, respectively. This low wavelength region is both phototoxic to cells and requires a special microscopic setup, suitable for UV-imaging. Another disadvantage is the poor image quality obtained with these probes. This is caused by the fluorophore's low quantum yield (0.04 in ethanol) and high bleaching rate. Furthermore, the high sensitivity to photobleaching does not allow continuous imaging of the same field. To summarize, dehydroergosterol and cholestatrienol resemble the biophysical properties of cholesterol better than other fluorescent cholesterol analogs. This makes these probes highly valuable

for studying the cell biology of cholesterol. However, the poor fluorescent properties of these probes severely restrict their use.

BODIPY-cholesterol

BODIPY-sterols are chemically synthesized analogs of cholesterol containing a covalently attached boron dipyrromethene difluoride (BODIPY) group. The BODIPY group has excellent fluorescent properties for live cell imaging; its excitation and emission are very similar to that of GFP, its quantum yield is 0.9 (in membrane bilayers) and its sensitivity to photobleaching is very low. As for NBD-sterols, there are different types of BODIPY-cholesterol. Here I will focus on the recently developed BODIPY-cholesterol, which has the BODIPY group bound to carbon-24 of the sterol side chain (Li et al., 2006) (Figure 5). BODIPY-cholesterol appears to mimic the behavior of cholesterol in different systems. In model membranes, this probe has been shown to partition in cholesterol rich ordered domains (Li et al., 2006; Shaw et al., 2006). This is a good indication for the biophysical similarity to cholesterol because, except for dehydroergosterol and cholestatrienol, none of the other fluorescent sterol analogs mimic this behavior. In an atomistic simulation study, modeling its behavior in lipid bilayers, BODIPY-cholesterol mimicked the effect of cholesterol on acyl chain ordering (Holtta-Vuori et al., 2008). Also in cells, BODIPY-cholesterol resembles cholesterol. It has been shown to partition into DRMs, suggesting its presence in membrane rafts. The subcellular distribution of BODIPY-cholesterol was found to be similar to cholesterol as judged by biochemical fractionation analysis and filipin staining (Holtta-Vuori et al., 2008). Furthermore, BODIPY-cholesterol accumulates in the lysosomes of cells lacking NPC1 in a manner similar to cholesterol (Holtta-Vuori et al., 2008). However, just like the other fluorescent probes, BODIPY-cholesterol does not behave identically to cholesterol. For instance, compared to cholesterol, its esterification is reduced and its secretion enhanced (Holtta-Vuori et al., 2008). Overall, it appears that BODIPY-cholesterol is a useful new tool for the analysis of cholesterol. Because this probe has only been commercially available since 2009 there are only few published studies. Still, it is already apparent that BODIPY-cholesterol behaves more similarly to cholesterol compared to NBD-sterols. One reason for this difference is the higher hydrophobicity of the BODIPY-group, making it more similar to the natural side chain of cholesterol.

3.2 Structure/function analysis using cholesterol analogs

Cholesterol analogs can be used as tools for studying the relationship between cholesterol's structure and function. The cellular functions mediated by cholesterol rely on different structural features. For instance, certain cholesterol modifying enzymes recognize only a specific region of cholesterol. On the other hand, the interactions of cholesterol within lipid bilayers depend more on its overall biophysical characteristics. Knowledge regarding the structure/function relationship of cholesterol has been important for the design of fluorescent cholesterol analogs. Furthermore, because certain cholesterol analogs only affect specific cellular functions of cholesterol, these analogs can be useful tools for studying biological properties of cholesterol.

Types of cholesterol analogs used for structure/function analysis

The term *cholesterol analog* in principle applies to any structural derivative of cholesterol (Ikonen and Jansen, 2008). Here, the different types of analogs that have been used to analyze the structure/function relationship of cholesterol will be discussed. Cholesterol analogs can be divided into natural and synthetic compounds. The most widely used natural analogs are biosynthetic precursors of cholesterol, plant sterols (phytosterols), and ergosterol (the main sterol in yeast) (Figure 6). Compared to other sterol analogs (e.g. oxysterols, sulfonated sterols, steroid hormones, bile acids) these have no additional hydrophilic groups that severely alter the biophysical properties of the sterol. Instead, they differ from cholesterol by additional alkyl groups and/or carbon–carbon double bonds, thereby introducing only minor structural changes. Synthetic cholesterol analogs that have been useful for structure/function analysis are the stereoisomers of cholesterol called 3-epicholesterol and ent-cholesterol (Figure 6). 3-Epicholesterol is an epimer of cholesterol in which the hydroxyl-group is in the α-position instead of the normal β-position. Ent-cholesterol is the enantiomer (mirror image) of cholesterol, i.e. the stereochemistry of all chiral centers is reversed. Ent-cholesterol is a particularly useful tool since its physical properties in lipid bilayers are identical to cholesterol (Mannock et al., 2003). On the other hand, ent-cholesterol is not recognized correctly by many cholesterol interacting proteins (Westover and Covey, 2004). These properties make ent-cholesterol a useful tool for distinguishing sterol-lipid and sterol-protein interactions.

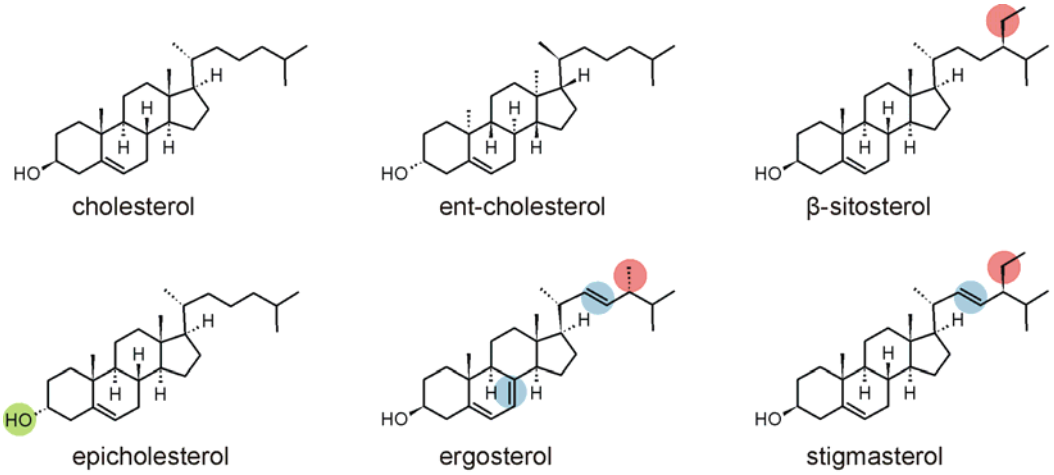

Figure 6. Structures of several cholesterol analogs. Structural differences with cholesterol are indicated with green (stereoisomer), red (additional alkyl group) and blue (carbon-carbon bond unsaturation) dots. Ent-cholesterol is the enantiomer of cholesterol.

Insights from model membrane studies

Studies on the effects of sterol structure on membrane properties have been carried out since the 60's. Most of these studies utilized the sterols described in the previous paragraph and analyzed their effect on acyl chain order, membrane permeability, membrane thickness, and raft domain formation (Demel and De Kruyff, 1976; McMullen et al., 1996; Miao et al., 2002; Ohvo and Slotte, 1996; Xu and London, 2000; Yeagle et al., 1988). Most studies found that the effect of the sterol modification was highly dependent on experimental conditions such as sterol concentration and the type of matrix lipids used. Therefore, it is difficult to compare data from different studies. However, in general it was found that structural modifications of cholesterol reduced the sterol mediated ordering effect on phospholipid acyl chains. The degree of this reduction depends on the type of modification. One structural feature of cholesterol that was found to be important for mediating its ordering effect is the smoothness of the ring structure. Lanosterol, a precursor of cholesterol, has three additional methyl groups attached to the sterol nucleus, making the sterol more bulky and less streamlined. Lanosterol was found to be poor in promoting order in phospholipid bilayers (Miao et al., 2002). It has been suggested that the additional methyl groups disrupt the van der Waals interactions between the sterol and the acyl chains. Other essential structural features are: the hydroxyl group on carbon-3, a rigid planar ring system, and a flexible hydrocarbon side chain. The degree of unsaturation of the ring system and alkylations and unsaturations in the sterol side chain were found to be less important (Mannock et al., 2010). Finally, it is interesting to point out that sterol structure can also affect membrane curvature. It was found that cholesterol promotes domains with positive curvature (bulging out), whereas other sterols, like lanosterol, facilitate negative curvature (Bacia et al., 2005).

Insights from cell studies

The effects of sterol structure have been extensively analyzed in different types of cells. The most popular model systems have been *Mycoplasma capriolum* and *Saccharomyces cerevisiae*. The prokaryote *M. capriolum* is a sterol auxotroph. This microorganism is useful for analyzing the effect of sterol structure since internalized sterols are not metabolically modified. Most of the sterol structure analysis in the yeast *S. cerevisiae* have been performed in the GL7 strain, which is defective in 2,3-oxidosqualene-lanosterol cyclase activity (Gollub et al., 1977). Other *S. cerevisiae* strains with defects in ergosterol biosynthesis enzymes downstream of the production of lanosterol have also been used. These strains are able to synthesize sterols but accumulate specific biosynthetic precursors of ergosterol (Parks et al., 1999). Finally, there are several studies using mutated mammalian cell lines that are auxotrophic for cholesterol (Rujanavech and Silbert, 1986; Xu et al., 2005).

Studies in these different model systems have led to numerous insights regarding the effects of sterol structure on cellular functions. It was observed that sterol modifications led to adaptive changes in the phospholipid composition (Low et al., 1985). This was also found in the mammalian LM cell line (Rujanavech and Silbert, 1986). These results suggest that cells are able to adapt to biophysical membrane changes resulting from foreign sterols by modifying its fatty acid composition (Bloch, 1983). Furthermore, sterol structure was shown to be important for specific processes like endocytosis (Heese-Peck et al., 2002) and yeast cell shape and cell fusion (Aguilar et al., 2010). There are several studies that analyzed the ability of cholesterol analogs to support growth in cells auxotrophic for cholesterol. One study, using mammalian cells, showed that small modifications of the sterol side chain supported cell growth but that more complex modifications in the sterol nucleus or 3-hydroxy group did not (Xu et al., 2005). Overall, the use of cell systems reinforced the structure/function relationship found from artificial membranes studies; in general, sterols more downstream of the cholesterol biosynthetic pathway have a larger order promoting effect. Based on these results, and the fact that in cyanobacteria cholesterol biosynthesis stops at the stage of lanosterol, Konrad Bloch argued that the further metabolism towards cholesterol was evolved to perfect the biological functions of the sterol (Bloch, 1989).

4. Oxysterol binding protein related proteins

4.1 A conserved family of lipid transfer proteins

Oxysterol binding protein related proteins (ORPs) are a family of lipid transfer proteins that are conserved from humans to yeast. Their founding member, oxysterol binding protein (OSBP), was identified in a search for proteins that bind oxysterols (Taylor et al., 1984). DNA sequence analysis revealed that OSBP is a member of a larger family of proteins present in all eukaryotes (Lehto and Olkkonen, 2003). The human genome encodes, in addition to OSBP, 11 ORPs which are named ORP 1-11 (Lehto et al., 2001) (Figure 7). Because of differential splicing several ORP genes encode for a full length (L) and a truncated (S) splice variant. Based on sequence similarity the human ORP family can be divided in 6 subgroups (Lehto et al., 2001). Yeast, which contains 7 ORPs named Osh1-7 (Beh et al., 2001), has been an important model for the functional analysis of ORPs. A comprehensive study, in which all 127 possible permutations of Osh deletion mutants were created, showed that deletion of all Osh genes is lethal but that expression of any individual gene ensures viability (Beh et al., 2001). This implies that the Osh proteins share a common essential function. Although ORPs have been implicated in a wide range of cellular processes including cholesterol homeostasis and transport, this essential function in yeast has not yet been discovered. Nonetheless, recent functional and structural studies have provided some mechanistic insights into the cellular functions of ORPs.

Oxysterols

Oxysterols are oxidized derivatives of cholesterol that can be formed either by auto-oxidation or by an enzymatic reaction. This derivation can be a hydroxyl, keton, epoxy, or carboxyl group. Like cholesterol, oxysterols mainly partition in lipid bilayers but, because of their more hydrophilic nature, move more rapidly between membranes (Yan and Olkkonen, 2008). They are present at concentrations usually around 1000 times lower than cholesterol (Brown and Jessup, 2009). Oxysterols have been shown to be involved in feedback regulation of cholesterol homeostasis by taking part in the SREBP signaling. 25-hydroxycholesterol (25-HC) directly binds Insig, causing Insig to bind SCAP and retain the SREBP complex in the ER (Radhakrishnan et al., 2007). Oxysterols also regulate cholesterol homeostasis by acting as natural ligands of LXR (Janowski et al., 1996). In addition, oxysterols have been shown to effect ORP proteins. For instance, 25-HC triggers OSBP localization to the Golgi (Ridgway et al., 1992) and 22R-HC inhibits the lipid droplet localization of ORP2 (Hynynen et al., 2009). Although it is clear that oxysterols influence ORPs and *vice versa*, the functional relevance of this interaction is not understood.

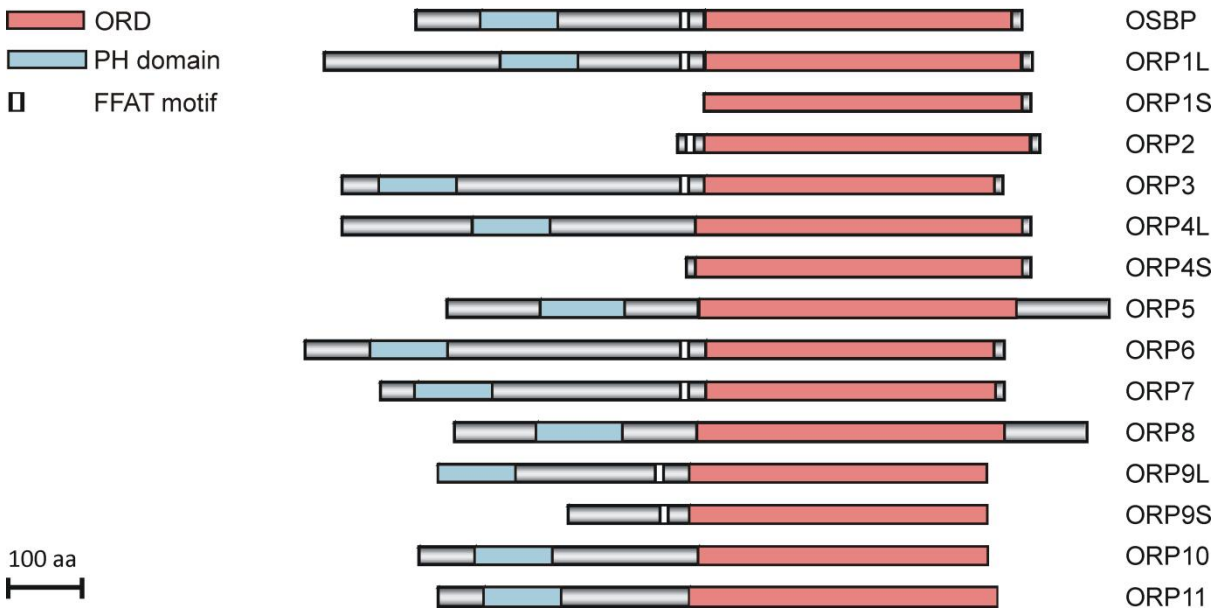

Figure 7. Human OSBP related proteins. Key structural motifs are indicated. OSBP related domain (ORD), pleckstrin homology (PH) domain and two phenylalanines in an acidic tract (FFAT) motif.

Functional analysis

After its discovery, OSBP was thought to be involved in 25-HC mediated HMGR downregulation (Dawson et al., 1989). However, more in-depth analysis led to rejection of this theory (Nishimura et al., 2005). Since then, ORPs have been implicated in various cellular processes including vesicular transport, cell signaling, lipid metabolism and non-vesicular sterol transport. For example, Osh4 is a negative regulator of Golgi derived vesicular transport (Fang et al., 1996), OSBP regulates extracellular signal-regulated kinase (ERK) phosphorylation (Wang et al., 2005b) and OSBP is required for cholesterol and 25-HC dependent stimulation of sphingomyelin synthesis (Perry and Ridgway, 2006). In a recent review, Raychaudhuri and Prinz propose that ORPs might mediate at least four types of mechanisms (Raychaudhuri and Prinz, 2010): (1) non-vesicular sterol transport between membranes, (2) creating transient changes in the lipid composition of membranes, (3) acting as lipid sensors, (4) regulating access of certain lipids to other lipid binding proteins. These different mechanisms are not mutually exclusive and even a single ORP protein might employ more than one. Recently, several studies have added weight to the proposed function of ORPs as direct sterol transporters (see 4.3 Role of ORPs in sterol transport).

4.2 ORP protein domains

OSBP related domain

Sequence analysis revealed that the ORP family of proteins contains various functional domains. The family is defined by the presence of an OSBP related domain (ORD) which is the part of the protein that has been implicated in sterol binding. The crystal structure of the ORD domain of Osh4 has been solved, showing that a single sterol molecule binds within a hydrophobic tunnel which is covered by a flexible lid (Im et al., 2005). Because measuring dissociation constants requires protein purification, binding affinities have been determined just for a handful of ORPs. So far, these results indicate that different ORPs bind cholesterol and oxysterols with different affinities. For instance, OSBP has a preference for 25-HC (K_d = 37 nM) over cholesterol (K_d = 173 nM) (Wang et al., 2008) and ORP2 prefers 22R-HC (K_d = 14 nM) over 25-HC (K_d = 3.9 µM) (Hynynen et al., 2009; Suchanek et al., 2007). A study using photo-cross-linking with photo-sterols suggest that most human ORPs can bind sterols (Suchanek et al., 2007). Besides sterols, the ORD has been shown to bind different types of phospholipids at opposite surfaces of the domain (Schulz et al., 2009). This enables the ORD to bind two membranes simultaneously.

Pleckstrin homology domain

Many ORPs have been shown to contain a pleckstrin homology (PH) domain. This domain occurs in a wide range of proteins and has been shown to bind phosphorylated derivatives of PI. The head group of PI can be phosphorylated on various positions to form in total 7 different subspecies which are enriched in specific cellular compartments. Because PIs are localized at the cytosolic side of biological membranes they can be used as docking sites for proteins containing a PH domain. For instance, OSBP is localized to the Golgi in a PI(4)P dependent manner (Levine and Munro, 2002). For most other ORPs the specific membrane targeting by the PH domain is poorly understood. Partly, this can be explained by the observation that several ORPs need additional factors for PH-mediated membrane targeting (Lehto et al., 2005; Levine and Munro, 2002). Finally, it should be noted that several ORPs lacking a PH domain have been shown to bind to phosphorylated PIs with their ORD (Li et al., 2002; Wang et al., 2005a).

FFAT motif

In the human ORP family, 11 splice variants have been shown to contain a two phenylalanines in an acidic tract (FFAT) motif. Besides in ORPs, FFAT motifs are present in other proteins, most of which are involved in lipid metabolism (Loewen 2003). This motif has been shown to bind vesicle associated membrane protein (VAMP) association proteins (VAPs) (Loewen et al., 2003). VAPs are conserved transmembrane proteins in the ER. The FFAT motif has been suggested to function as an ER targeting domain (Loewen et al., 2003). This has been shown for the yeast proteins containing a FFAT motif (Loewen et al., 2003). In mammalian cells, a FFAT motif dependency for ER targeting has been shown for ORP3 (Lehto et al., 2005) and CERT (Kawano et al., 2006). However, it should be noted that in mammalian cells not all proteins with a FFAT motif have been localized to the ER. Some studies have shown the importance of the FFAT motif for protein function. The FFAT motif in CERT is important for efficient traffic of ceramide from the ER to the Golgi (Kawano et al., 2006). Furthermore, it has been shown that an intact FFAT motif is important for ORP1L mediated positioning of late endosomes (Vihervaara et al., 2010). However, it is unknown whether the FFAT domain is important for ORP mediated sterol transport. For instance, ORPs lacking a FFAT motif have been shown to be efficient sterol transporters (Raychaudhuri et al., 2006).

4.3 Role of ORPs in sterol transport

Yeast

The most important advances regarding the role of ORPs in sterol transport have come from studies in yeast. The genetic study that revealed a functional redundancy in the yeast ORP family showed that many deletion mutants exhibited specific sterol-related defects (Beh et al., 2001). Another study by the same group showed that removal of all Osh genes gives a 3.5-fold increase in cellular ergosterol and alters the cellular sterol distribution as judged by filipin staining (Beh and Rine, 2004). The first evidence for a direct role of ORPs in sterol transport was that Osh4 contains a hydrophobic pocket capable of binding sterols (Im et al., 2005). Further evidence was provided in a functional study by the group of William Prinz. They found that Osh proteins are required for sterol transport between the plasma membrane and ER (Raychaudhuri et al., 2006). In a later publication, the same group showed that all Osh proteins can transfer sterols between membranes *in vitro* (Schulz et al., 2009). Based on these structural and functional observations the authors proposed that ORPs are vehicles that are able to shuttle cholesterol between two membranes. More information about the mechanism of ORP mediated sterol transport was provided by showing that Osh4p mediated sterol trafficking is dependent on the ability of Osh4p to simultaneously bind two apposed membranes (Schulz et al., 2009). Furthermore, this study

shows that most of the yeast ORPs are enriched in regions of the ER that are closely associated with the plasma membrane. This interaction was found not to require a FFAT domain or Scs2p, the yeast equivalent of VAP. The authors suggest the localization of ORPs at membrane contact sites could explain how Osh proteins could transfer sterols rapidly enough to contribute to the bulk sterol transfer. However, the authors also argue that these observations do not exclude functional mechanisms other than direct sterol transport.

Mammals

Not much is known about the role of ORPs in mammalian cholesterol transport. One difficulty in the functional analysis of mammalian ORPs results from the lack of tools to disrupt expression/function of all ORPs simultaneously. Single knockdown experiments would not be very informative if mammalian ORPs are functionally redundant, as Osh proteins in yeast are. Despite this, several studies have implicated human ORPs in sterol traffic. The role of ORP2 in relation to cholesterol homeostasis has been extensively studied by the group of Vesa Olkkonen. They made four key observations: (1) ORP2 overexpression increased transport of newly synthesized cholesterol to extracellular acceptors (Hynynen et al., 2005), (2) ORP2 silencing increased the amount of cholesterol esters (Hynynen et al., 2009), (3) ORP2 was shown to bind cholesterol and several oxysterols (Hynynen et al., 2009; Suchanek et al., 2007), (4) ORP2 localized to lipid droplets, the plasma membrane, and cytosol (Hynynen et al., 2009). Together these observations could be explained by a role for ORP2 in sterol transport from the ER to the plasma membrane.

OSBP and ORP9L have also been implicated in sterol transport. Both proteins transfer cholesterol between membranes *in vitro*, in a PI(4)P dependent manner (Ngo and Ridgway, 2009). These proteins were shown to localize to the Golgi and the ER, which was dependent on a functional PH domain and FFAT motif, respectively (Wyles and Ridgway, 2004). Furthermore, OSBP overexpression results in increased cholesterol biosynthesis and a reduction of cholesterol esterification (Lagace et al., 1997) and ORP9 silencing leads to Golgi fragmentation and reduced filipin staining of late endosomes (Ngo and Ridgway, 2009). Based on these results the authors suggest that both proteins might be involved in cholesterol transport from ER to Golgi. However, it remains to be determined whether the primary function of OSBP and ORP9 is ER to Golgi sterol transport.

AIMS OF THE STUDY

In this work the aim was to characterize both fluorescent and non-fluorescent cholesterol analogs as tools to study membrane rafts and intracellular cholesterol transport. Furthermore, the aim was to use these new tools along with more conventional ones to address the importance of cholesterol for ordered membrane domains and intracellular cholesterol transport.

The specific aims of this study were:

1. To characterize desmosterol as a tool to study ordered membrane domains
2. To study the importance of cholesterol for caveolae by substituting cellular cholesterol by desmosterol
3. To characterize BODIPY-cholesterol as a tool to analyze cholesterol transport between the plasma membrane, the ER and lipid droplets
4. To utilize BODIPY-cholesterol to analyze the importance of ORPs for cholesterol transport between the plasma membrane, the ER, and lipid droplets in mammalian cells

METHODS

Details regarding the materials and methods are provided in the original articles (I – III).

1. Model membrane experiments

Multilamellar vesicle (MLV) preparation (I)

Lipids from stock solutions were pipetted in a glass tube (Table 1). The lipid mixtures were dried by heating under a soft flow of nitrogen. In order to remove most organic solvent molecules, the mixture was further dried for 1 h in a speed vac. Phosphate buffered saline (PBS) was added to the dried lipid film and MLVs were generated by vortexing each sample 4 times 30 s at 55 °C.

Table 1. Lipid content of multilamellar vesicles

Procedure	Molar ratio[1]			DPH[2]	Lipid concentration (µM)
	DPPC	DOPC	12-SLPC		
Detergent resistance	1	1		-	500
DPH quenching					
$F_{12-SLPC}^{DPPC}$	1		1	yes	50
F_{DOPC}^{DPPC}	1	1		yes	50
$F_{12-SLPC}^{DOPC}$		1	1	yes	50
F_{DOPC}^{DOPC}		1		yes	50
DPH polarization	1	1		yes	50

1, MLVs were prepared with and without 15 mol % of cholesterol or desmosterol
2, 1,6-diphenyl-1,3,5-hexatriene (DPH) was present at 0.5 mol %

Detergent resistance of MLVs (I)

Detergent solubilization was assayed by measuring the loss of light scattering as determined by optical density (OD) at 400 nm as done in (Xu et al., 2001). The OD_{400} was measured before and after 2 h incubation with 0.5% Triton X-100 at room temperature. The detergent resistance (% OD) was determined by dividing the OD_{400} after the incubation by the OD_{400} before addition of the detergent.

1,6-diphenyl-1,3,5-hexatriene (DPH) quenching (I)

The procedure was performed essentially as described in (Xu and London, 2000). Temperature dependent DPH quenching was measured by heating the MLVs continuously from 21 to 56 °C at 1 °C per min while stirring the suspension. A single quenching experiment was performed by acquiring temperature dependent quenching curves of four different MLV preparations (Table 1). The 1-palmitoyl-2-(12-doxyl)stearoylphosphatidyl-choline (12-SLPC) dependent quenching of DPH ($F_{12-SLPC}^{DPPC}$) was corrected for intensity changes unrelated to the quencher lipid 12-SLPC (divide by F_{DOPC}^{DPPC}). Next, this value was corrected for un-quenching unrelated to domain formation by subtracting $\frac{F_{12-SLPC}^{DOPC}}{F_{DOPC}^{DOPC}}$. Altogether, $\Delta\frac{F}{F_0}$ was calculated as follows.

$$\Delta\frac{F}{F_0} = \frac{F_{12-SLPC}^{DPPC}}{F_{DOPC}^{DPPC}} - \frac{F_{12-SLPC}^{DOPC}}{F_{DOPC}^{DOPC}}$$

The quenching curves, $\Delta\frac{F}{F_0}$ as a function of temperature, were fitted with sigmoidal Boltzmann equation as in (Wenz and Barrantes, 2003). The T_m was derived from the halfpoint of the curves and the domain formation coefficient *a* was determined by subtracting the lower from the higher asymptote.

Polarization (I)

The fluorophore was excited with polarized light. Emission of parallel ($I_{\parallel}$) and perpendicular polarization ($I_{\perp}$) was detected. Polarization was calculated as: $P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$

2. Biochemical procedures

Lipid extraction (I, II, III)

Lipids were extracted by a modified method of (Bligh and Dyer, 1959). Cells were scraped in 1.6 ml of PBS and transferred to a glass tube. Lipids were dissolved by adding 2 ml chloroform and 4 ml methanol. Aggregated proteins were removed by centrifugation. The solution formed two separate phases after the addition of 2 ml 2% NaCl solution and 2 ml chloroform. The lower organic phase containing the lipids was collected and dried by heating under a soft flow of N_2.

Sterol determination (I, II, III)

Total sterols and sterol esters were analyzed by high performance thin layer chromatography (HPTLC) using a running buffer of hexane/diethyl ether/acetic acid (80:20:1

v/v/v). BODIPY-sterols were analyzed with a buffer of petroleum ether/diethyl ether/acetic acid (60:40:1 v/v/v). In order to resolve cholesterol biosynthetic precursors, the sterol fractions purified by thin layer chromatography (TLC) were further separated by silver ion high performance liquid chromatography (Ag^+-HPLC).

Replacing cholesterol for desmosterol using MβCD/sterol complexes (I)

Cholesterol was depleted from Huh7 cells by 30 min incubation with 10 mM MβCD at 37 °C while shaking. Next, the cells were replenished with either cholesterol or desmosterol by incubating for 1 h with a MβCD/sterol complex (8.7:1 mol/mol) at a sterol concentration of 50 µg/ml.

Subcellular membrane fractionation (I, II)

Cells were collected in PBS and the samples were kept at 0-4 °C for the remainder of the procedure. After pelleting, the cells were resuspended in a hypotonic lysis buffer supplemented with protease inhibitors. The cells were lysed by passing them 60 times through a 25-gauge needle. The nuclei were removed by centrifugation and 400 µl of the post nuclear supernatant was collected in a SW28Ti ultracentrifuge tube. The supernatant was mixed with 1600 µl of lysis buffer containing 2.4 M sucrose. The sample was overlaid with 7 parts of 2 ml of lysis buffer containing 1.8, 1.6, 1.4, 1.2, 1.0, 0.8, and 0.6 M sucrose. After centrifugation at 70,000 x *g* for 24 h, the fractions were collected.

Detergent resistant membrane isolation (II)

Cells were collected in PBS and the samples were kept at 0-4 °C for the remainder of the procedure. The pelleted cells were resuspended in lysis buffer containing 1% triton X-100. Optiprep™ was added to give a final concentration of 40% and the mixture was transferred to a SW60Ti ultracentrifuge tube. A step gradient of 35, 30, 25, 20, and 0% Optiprep™ was pipetted on top of the sample. After centrifugation at 40,000 x *g* for 4 h, the fractions were collected. DRMs were present in the top fraction.

Immunoisolation of caveolar membranes (II)

Adipocytes were prepared from epididymal fat pads of male Wistar rats. The fat pads were digested in Krebs-Ringer-HEPES buffer containing collagenase at 37 °C for around 1 h. The cells were filtered through a 200-µm nylon mesh and washed in Krebs buffer. Cells were incubated for 2 days in the presence of [^{14}C]cholesterol, [^{3}H]desmosterol and 20,25-DAC. Subsequent steps were carried out at 0-4 °C. Cells were collected, resuspended in lysis

buffer and broken by 10 strokes with a dounce homogenizer. Nuclei and unlysed cells were removed by centrifugation at 1000 x *g* and a plasma membrane containing pellet was obtained by centrifugation at 16,000 x *g*. The pellet was resuspended in 2 ml of 500 mM sodium carbonate (pH 11) and membranes were disrupted by sonication. The homogenate was transferred to a SW40Ti tube and sucrose was added to a final concentration of 45% sucrose. The mixture was overlaid with 4 ml of lysis buffer containing 35% and 5% sucrose and centrifuged for 24 h at 39,000 x *g*. The caveolar enriched fraction at the 5-35% sucrose interphase was divided and incubated overnight with and without caveolin-1 antibodies. The antibody complexes were precipitated using Dynal beads linked to anti-mouse IgG. The amount of radioactivity that co-precipitated was corrected for the unspecific pulldown in the samples without caveolin-1 antibodies.

Lipid droplet isolation (III)

Lipid droplets were isolated as described in (Brasaemle and Wolins, 2006). Oleate loaded HeLa cells were collected and kept at 0-4 °C for the remainder of the procedure. The cell pellet was resuspended in a hypotonic lysis buffer containing protease inhibitors. Nuclei were removed by centrifugation for 10 min at 1000 x *g*. The post nuclear supernatant was transferred to a SW40Ti ultracentrifuge tube and adjusted to 20% sucrose. The samples were overlaid with 5% sucrose and 0% sucrose in lysis buffer. The samples were spun for 2 h at 28,000 x *g*. The lipid droplet fraction, which was visible at the top of the gradient as a milky white layer, was collected with a tube slicer.

N,N,N-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium (TMA-DPH) anisotropy (II)

The plasma membrane fluidity of living cells was analyzed as in (Kuhry et al., 1985). A suspension of trypsinized cells was mixed with the fluorescent probe TMA-DPH, which selectively localizes to plasma membranes. The fluorophore was excited with polarized light. Emission of parallel ($I_{\parallel}$) and perpendicular polarization ($I_{\perp}$) was detected. Anisotropy was calculated as: $A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$

[^{3}H]cholesterol esterification (III)

This procedure was performed as reported (Huang et al., 2003). The plasma membranes of oleate loaded HeLa cells were labeled with [^{3}H]cholesterol from a bovine serum albumin (BSA) complex for 30 min at 15 °C. Next, the cells were incubated for different times at 37 °C after which the lipids were isolated. [^{3}H]cholesterol and [^{3}H]cholesterol esters were analyzed by TLC and scintillation counting.

3. Fluorescence microscopy assays

The following microscopy assays were performed either on fixed or living cells. For fixation, cells were grown on coverslips, incubated with paraformaldehyde and mounted on a glass slide. The live-cell microscopy experiments were performed at 37 °C in CO_2 independent medium, which kept the cells at physiological pH outside a CO_2 rich atmosphere of five to ten percent.

Quantification of caveolin-1-GFP mobility by TIRF microscopy (II)

The transport of caveolae to and from the plasma membrane was measured as reported (Tagawa et al., 2005). HeLa cells expressing caveolin-1-GFP were analyzed by TIRF microscopy. Mobile caveolae are visible as appearing and disappearing dots in the TIRF plane. Movies (4 frames per min) were made of the caveolar fluorescence in the TIRF plane. The Pearson's correlations of consecutive images were calculated and averaged. Lower correlation indicates more change between images and therefore higher mobility.

Caveolin-1 composition of caveolae (II)

High resolution TIRF microscopy images were taken of HeLa cells stably overexpressing caveolin-1 GFP. Image quality was improved by deconvolution, using Huygens image restoration software. The intensities of the dots were determined using automated spot detection and fitting their intensity as a function of *x* and *y*, with a normal distribution. The intensity histogram of the dots revealed Gaussian shaped intensity groups representing the intensities of individual caveolae (first group) and caveolar clusters. The first three intensity groups were fitted with a mixture of three univariate Gaussian distributions. The width of the first peak was taken as a measure of variation in the amount of caveolin-1 molecules per caveolae.

BODIPY-cholesterol labeling of cells (III)

Cells were incubated with a MβCD/BODIPY-cholesterol complex for 1 min at 37 °C in serum free medium. It was determined by HPTLC that this procedure labeled the cells with 1 molecule of BODIPY-cholesterol for every 1000 molecules of natural cholesterol. The complex was prepared by adding a 370 mM MβCD solution to solid BODIPY-cholesterol to reach a 100:1 molar ratio. The complexes were formed by sonicating the mixture 6 times 2 min on ice. Uncomplexed BODIPY-cholesterol crystals were spun down by centrifugation.

BODIPY-cholesterol pulse chase experiments (III)

HeLa cells were pre-labeled with either BODIPY-C_{12} (lipid droplet marker) or ER-tracker. Next, the cells were labeled with BODIPY-cholesterol followed directly by live-cell imaging using a confocal microscope. Three channel images were taken by sequential scanning using three different laser lines: BODIPY-cholesterol 488 nm, the organelle markers 561 nm, HcRed (marker for transfected cells) 633 nm. The focal plane was aimed ~1 µm above the basal plasma membrane. Images were taken continuously for up to 3 h to analyze the kinetics of transfer. In experiments where treatments were compared to controls, 6-9 images were taken at 15-25 min after chase and 6-9 images after 180 min. Colocalization, as expressed by the Pearson's correlation, was calculated between BODIPY-cholesterol and the organelle marker. This value was used as a measure of the relative amount of BODIPY-cholesterol in the organelle.

Dehydroergosterol pulse chase (III)

Huh7 cells were pre-labeled with the lipid droplet marker BODIPY-C_{12}, and then labeled with a MβCD/dehydroergosterol complex (molar ratio 8:1) for 3 min at 37 °C as described (Wustner et al., 2002). The label was chased for 45 min before imaging with a wide field microscope, equipped with filters and a dichroic mirror suitable for dehydroergosterol imaging.

Fluorescence recovery after photobleaching of BODIPY-cholesterol in lipid droplets (III)

HeLa cells, pre-labeled with the lipid droplet marker BODIPY-C_{12}, were labeled with BODIPY–cholesterol, which was equilibrated by chasing for 2 h. The BODIPY fluorescence in a cluster of immobile lipid droplets was bleached with 5 short pulses (1.5 sec in total) of the 488 nm argon laser line at full power. Before and after bleaching, this area was imaged at low intensity illumination with scanning settings that favor fast acquisition: high zoom factor (6), low resolution (256 x 256 pixels) and high scanning speed (1400 Hz).

Endocytosis of BODIPY-lactosylceramide (II)

The endocytosis of BODIPY-lactosylceramide was analyzed as described (Singh et al., 2003). For this experiment, primary human fibroblasts were used because of their high caveolae content. Cell were labeled with BODIPY-lactosylceramide at 0 °C and imaged with a wide-field microscope to determine labeling efficiency. Next, caveolae were allowed to internalize the label by incubating the cells for 5 min at 37 °C. Most uninternalized label was removed

by washing the cells extensively with PBS containing BSA at 0 °C. The cells were again imaged by fluorescence microscopy. Internalized BODIPY-lactosylceramide was visible as dots. The homogenous intensity of the BODIPY-lactosylceramide that remained on the plasma membrane was removed by image processing using a rolling ball background subtraction.

Endocytosis of integrins (II)

This procedure was performed as published (Upla et al., 2004). HeLa cells expressing caveolin-1-GFP were incubated with antibodies against α2 integrin for 1 h on ice. Cells were subsequently incubated with Alexa Fluor 568-conjugated secondary antibodies resulting in clustering of α2 integrin. The clustered integrins were allowed to internalize by incubating the samples for 5 and 15 min at 37 °C. The samples were fixed and analyzed by confocal microscopy. Caveolar internalization of clustered integrins was assessed by determining the Pearson's correlation coefficient between caveolin-GFP and the Alexa Fluor 568-conjugated antibodies.

RESULTS AND DISCUSSION

1. Desmosterol as a tool to study ordered membrane domains

1.1 Desmosterol cannot fully replace cholesterol in ordered membrane domains

Ordered domains in model membranes

Three biophysical experiments were performed in order to compare the ordered membrane domain stabilizing properties of desmosterol with those of cholesterol. Multilamellar vesicles (MLVs) were used, composed of DPPC and DOPC in a 1:1 molar ratio with or without 15 mol % sterol. When high-T_m DPPC (42 ºC) and low-T_m DOPC (-20 ºC) are mixed, DPPC-enriched ordered domains coexist with DOPC-enriched disordered domains. In the following three experiments the effect of sterol addition on ordered domain formation and stabilization was measured.

(1) The detergent resistance of the MLVs was determined by measuring the light scattering of the vesicles before and after addition of Triton X-100. A previous study has shown that the percentage of remaining light scattering roughly approximates the amount of DRMs in the vesicles (Xu and London, 2000). Addition of cholesterol to the MLVs resulted in a fivefold increase of the remaining light scattering (I, Figure 1A), in agreement with a previous study (Xu and London, 2000). Compared to cholesterol, desmosterol was 75% less efficient in promoting detergent insolubility. These results indicate that MLVs that contain desmosterol have a reduced fraction of DRMs.

(2) The potential of both cholesterol and desmosterol to promote membrane ordering was analyzed by measuring the polarization of the fluorescent probe DPH, which was added in trace amounts to the MLVs. The degree of DPH polarization is positively related to the ordering of the lipids within the MLVs. The addition of both cholesterol and desmosterol increased DPH polarization in the MLVs (I, Figure 1B). However, the effect of desmosterol was significantly weaker compared to cholesterol.

(3) A DPH fluorescence quenching assay was used to determine the effect of cholesterol on the formation and stability of ordered domains. The MLVs used for this experiment contained trace amounts of DPH, and DOPC was replaced by 12-SLPC. The phase behavior of 12-SLPC is similar to DOPC, but its additional nitroxide group is capable of quenching DPH fluorescence. At room temperature, these vesicles laterally segregate in a DPPC enriched ordered phase and a 12-SLPC enriched disordered phase. DPH has a similar affinity for both phases. The DPH present in the ordered phase, which is depleted of 12-SLPC, is not quenched. Raising the ambient temperature causes the ordered DPPC domains to melt and mix with the disordered 12-SLPC phase. Consequently, the probability of an interaction between DPH and 12-SLPC increases, resulting in higher quenching (I, Figure 1C). Addition of cholesterol to the membranes increased the degree of domain formation as expressed by *a*

(I, Figure 1D). The ability of desmosterol to increase unquenching was significantly lower. Next, the T_m of the ordered domains in MLVs containing cholesterol, desmosterol, or no sterol was determined (I, Figure 1E). Addition of cholesterol increased the melting temperature by 3 °C, indicating higher ordered domain stability. On the contrary, desmosterol did not significantly increase the T_m.

Atomic scale molecular dynamics simulations

To better understand why the biophysical properties of desmosterol in membranes differ from those of cholesterol, atomic scale molecular dynamics simulations were performed. The properties of DPPC bilayers in the fluid phase with and without 20 mol % cholesterol or desmosterol were studied. The sterol-dependent ordering of lipid hydrocarbon chains was assessed by determining the molecular order parameter, $-S_{CD}$ (I, Figure 6A). Whereas both sterols were found to increase acyl chain order, the effect of desmosterol was 50% lower as compared to cholesterol. Analysis of the orientation of the lipids showed that desmosterol did not straighten the acyl chains as much as cholesterol (I, Figure 6B). Furthermore, desmosterol itself was more tilted in the membrane, as compared to cholesterol (I, Table 2; I, Figure 7). To study the molecular mechanisms responsible for the difference of desmosterol, experiments were focused on the conformation of the sterol tail and its interactions with the acyl chains. The molecular order was lower in the entire tail region of desmosterol (I, Figure 6C). This was associated with a 120° difference in the probabilities of torsion angles of the bond between carbons 17-20 (I, Figure 6D). It thus appears that the double bond in the tail of desmosterol changes the shape and flexibility of the tail region as compared to cholesterol. Consequently, the van der Waals interactions of the desmosterol tail with the hydrocarbon chains of the DPPCs should be different from those of cholesterol. Surprisingly, the van der Waals interactions of the last four atoms of the tail with DPPC acyl chains were found to be stronger for desmosterol. However, at the beginning of the tail the effect was opposite, as the acyl chains packed better around that part of the tail of cholesterol than desmosterol.

Replacing cholesterol with desmosterol in cellular membranes

In order to investigate the effect of desmosterol on ordered membrane domains in cells, three different protocols were used to exchange cellular cholesterol by desmosterol.

(1) For the first method, Chinese hamster ovary (CHO) cells were used. These cells naturally accumulate some desmosterol while synthesizing cholesterol. When grown in medium containing lipoprotein depleted serum (LPDS), these cells can accumulate 10% of desmosterol relative to the total unesterified sterol content. This method provides a natural way to study desmosterol in cells.

(2) In the second method, the cellular cholesterol was removed with MβCD and was replaced with desmosterol from a MβCD complex (I, Figure 4A). By incubating Huh7 hepatoma cells for 30 min with 10 mM MβCD at 37 ºC, 70% of the cellular free cholesterol was depleted. Immediately thereafter, the cells were replenished with either desmosterol or cholesterol from a MβCD complex, restoring the cellular sterol content to the starting level. This method allows for the acute exchange of most cellular cholesterol by desmosterol. It should be noted that the replaced desmosterol is metabolized by the cell; 1 h after the exchange ~10% of the desmosterol was converted to cholesterol.

(3) In the third method, cellular cholesterol was exchanged for desmosterol by pharmacological inhibition of 24-dehydrocholesterol reductase (DHCR24), the enzyme that converts desmosterol to cholesterol. HeLa cells were cultivated for 1 week in medium containing LPDS with and without 10 nM 20,25-diazacholesterol (20,25-DAC), an inhibitor of DHCR24. This treatment resulted in a cellular desmosterol content of 90% (II, Figure 1B) without changing total sterol levels or leading to detectable accumulation of other sterols (II, Figure 1A). If the cells were grown in normal serum (containing cholesterol), 20,25-DAC gave hardly any accumulation of desmosterol, indicating that under these conditions HeLa cells acquire most of their sterol from the medium.

Membrane rafts

It was analyzed whether desmosterol perturbs the structure and function of ordered membrane domains in cells. In CHO cells, the detergent solubility of desmosterol and cholesterol in a plasma membrane enriched fraction (membranes with the density of 0.7-0.9 M sucrose) was determined (I, Figure 3A). This fraction was treated with 0.5% Triton X-100, and the low density DRMs were separated from the solubilized membranes on a discontinuous sucrose density gradient as described (Arreaza et al., 1994). The amount of cholesterol and desmosterol was analyzed from the DRM and solubilized membrane fraction by Ag^{+}-HPLC. The desmosterol/cholesterol ratio was found to be more than three

times higher in the soluble membranes than in DRMs (I, Figure 3B). This result suggests that the affinity of desmosterol for membrane rafts is lower as compared to cholesterol.

Next, it was analyzed whether raft dependent signaling was effective in Huh7 cells in which cholesterol was replaced by desmosterol using MβCD. It has been shown before that in Huh7 cells, insulin treatment triggers insulin receptor association with DRMs and that receptor autophosphorylation is dependent on rafts (Vainio et al., 2002). In cells containing desmosterol, the insulin receptor did not get associated with DRMs and its tyrosine phosphorylation, as determined by immunoblotting, was ~60% decreased (I, Figure 5A,C). It was analyzed whether this result could be explained by decreased plasma membrane localization of insulin receptors. This appeared not to be the case, since total [^{125}I]insulin binding was unaltered by the sterol exchange (I, Figure 5B). Therefore, these results suggest that the defective insulin receptor activation is related to the weaker membrane ordering effect of desmosterol.

1.2 Desmosterol can replace most cellular functions of cholesterol

The effect of replacing cholesterol by desmosterol on cellular functions unrelated to ordered membrane domains was analyzed. Initially, it was observed that the viability and growth rate of 20,25-DAC treated HeLa cells was not altered, as compared to control cells. This suggests that desmosterol can replace, at least partly, the essential functions of cholesterol in cells. Next, the effects of sterol replacement on more specific functions mediated by cholesterol were analyzed.

Subcellular sterol distribution

The specific distribution of cholesterol over different cellular membranes is known to be essential for the cellular functions relying on cholesterol. In order to analyze whether the subcellular distribution of desmosterol is similar to that of cholesterol, the fluorescent cholesterol binding antibiotic filipin was utilized. Both the MβCD and the 20,25-DAC mediated sterol exchange did not change the filipin staining pattern (I, Figure 4B; Figure 8). As an alternative method, the subcellular sterol distribution was determined by sucrose density fractionation analysis. As expected, most sterols were found to be present in low density fractions that also contained caveolin-1, a protein enriched in the plasma membrane. Only a small amount of cholesterol was found in high density fractions together with calnexin, an ER marker. No difference was found between the relative density distribution of desmosterol and cholesterol (I, Figure 3A; II, Figure 2B). Together, these results demonstrate that the subcellular distribution of desmosterol is similar to that of cholesterol.

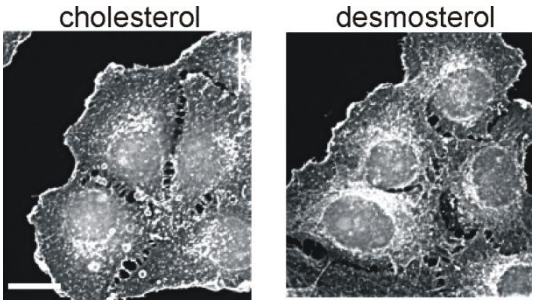

Figure 8. Epifluorescence images of HeLa cells stained with filipin. Shown are control cells (cholesterol) and cells treated with 20,25-DAC (desmosterol). The images were deconvolved with Huygens deconvolution software (SVI). Scale bar: 10 µm.

The Golgi and the secretory pathway

The structure and function of the Golgi apparatus have been shown to be dependent on cholesterol levels (Grimmer et al., 2005). Therefore, the effects of the sterol exchange on Golgi morphology and albumin secretion were analyzed. MβCD mediated sterol depletion of Huh7 cells perturbed the morphology of the Golgi complex as judged by fluorescein isothiocyanate-lentil lectin staining (I, Figure 4B). Replenishing the cells with either cholesterol or desmosterol restored the Golgi staining pattern.

To determine whether the secretory function of the Golgi was affected by the sterol exchange, albumin secretion was analyzed. Immediately after performing the MβCD mediated sterol exchange, the cells were incubated for 1 h in fresh medium. The secreted albumin was analyzed by Western blotting (I, Figure 4C). The results indicated that similar amounts of albumin were secreted from cells containing cholesterol or desmosterol.

Phospholipid composition and membrane fluidity

Various studies have shown that sterol structure can modify the cellular phospholipid composition (Low et al., 1985; Rujanavech and Silbert, 1986). In 20,25-DAC treated HeLa cells, the cellular composition of PC, PE, PS and sphingomyelin was analyzed by mass spectroscopy (Figure 9). Desmosterol did not alter the cellular amounts of these lipids nor their acyl chain length and unsaturation.

Plasma membrane fluidity is important for proper functioning of the cell and is known to be dependent on cholesterol. To analyze plasma membrane fluidity, TMA-DPH fluorescence anisotropy (similar to fluorescence polarization) was measured in HeLa cells. When added to cells, the probe TMA-DPH localizes selectively to the plasma membrane (Kuhry et al., 1985). Hela cells containing either desmosterol or cholesterol gave identical anisotropy values

(0.32±0.01 and 0.32±0.01, mean±S.D., *n* = 3), suggesting that the overall plasma membrane fluidity was not altered. In comparison, inhibition of sterol biosynthesis (by 10 µM lovastatin, 100 µM mevalonate in LPDS for 3 days) resulted in increased membrane fluidity, as indicated by lower anisotropy (0.29±0.01).

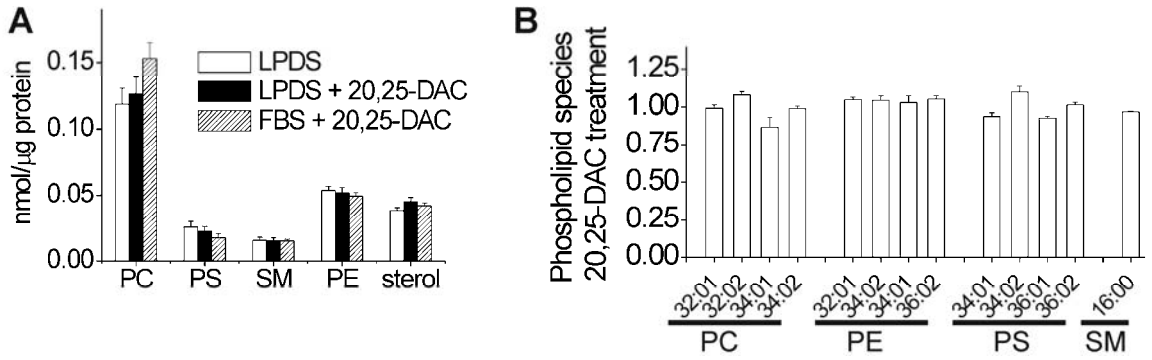

Figure 9. Phospholipid composition of 20,25-DAC treated HeLa cells. A) Quantification of the major classes of membrane lipids in HeLa cells grown for 8 days in LPDS or FBS with or without 10 nM 20,25-DAC (mean±S.E.; PC, PE, PS, sphingomyelin (SM), n = *3; sterol,* n = *5). B) Quantification of phospholipid molecular species in cells grown as in (*A*). The most abundant species constituting at least 70% of each phospholipid class are shown, the numbering indicates the total number of carbon atoms and unsaturations in the acyl chains (mean±S.E.; PC, PE, and PS,* n = *3; SM,* n = *2).*

1.3 Using desmosterol to analyze caveolae

Desmosterol has reduced affinity for caveolae

Sterol association with caveolar membranes was determined by labeling cells with [^{14}C]cholesterol and [^{3}H]desmosterol followed by immunoisolation of caveolar membranes. For this experiment, primary adipocytes were used because of their high caveolar content (Parton and Simons, 2007). Cells were labeled for 2 days with both [^{3}H]desmosterol and [^{14}C]cholesterol in the presence of 10 nM 20,25-DAC to prevent the conversion of [^{3}H]desmosterol. The cellular membranes were disrupted by sonication and subjected to sucrose density gradient fractionation (II, Figure 3A). The fraction enriched in plasma membrane, was used for isolation of caveolar membranes by immunoprecipitation using caveolin-1 antibodies. Both [^{3}H]desmosterol and [^{14}C]cholesterol specifically precipitated with caveolin-1 antibodies (II, Figure 3B). However, the amount of co-isolated [^{3}H]desmosterol was 35% lower than that of [^{14}C]cholesterol. These results indicate lower affinity of desmosterol than cholesterol for caveolar membranes.

Caveolar stability

The effect of desmosterol on the stability of caveolae was determined by analyzing the detergent and heat resistance of caveolin-1 oligomers. In HeLa cells, in which cholesterol was replaced by desmosterol by 20,25-DAC treatment, the amount of caveolin-1 in DRMs was decreased by 50% (II, Figure 3C). This effect was reversed when the cells were grown in FBS indicating that 20,25-DAC did not act unrelated to the sterol exchange.

Caveolae are structurally composed of caveolin-1 oligomers (Monier et al., 1995; Rothberg et al., 1992) that can be analyzed as high molecular weight protein complexes by Western blotting using caveolin-1 antibodies. Their stability can be assessed by analyzing the fraction of oligomers remaining after boiling the samples in the presence of sodium dodecylsulfate. It was found that 8±8% (mean±S.D.) of caveolin-1 immunoreactivity remained as heat-resistant oligomers in desmosterol cells as compared to 43±6% in cholesterol cells (II, Figure 3D).

It is known that the detergent resistance and the oligomerization of caveolin-1 are related to its plasma membrane localization. Consequently, these results could be explained by an altered subcellular distribution of caveolin-1. It was analyzed whether the sterol exchange effects the subcellular distribution of caveolin-1 using immunofluorescence microscopy and sucrose density fractionation (II, Figure 2A,B). The sterol exchange did not affect the caveolin-1 distribution. Therefore, it seems likely that the decrease in detergent resistance and the oligomer stability of caveolin-1 results from the lower affinity of desmosterol for caveolae.

Caveolar morphology

The morphology of caveolae was analyzed by immunoelectron microscopy using antibodies against caveolin-1. As expected, this antibody detected uncoated invaginations in the plasma membrane (II, Figure 5A). The morphology of uncoated membrane invaginations was determined in primary human fibroblasts. In desmosterol-containing cells, uncoated plasma membrane invaginations with small openings (20–45 nm) were less abundant (II, Figure 5A; II, Table 1). Furthermore, there was an increase in the average width and depth of the invaginations. The most prominent difference in desmosterol cells was an increased variation in all the measured dimensions. This difference was not observed if the cells were allowed to take up cholesterol from the medium, indicating that the drug was not directly responsible for the altered morphology. These results imply that the sterol exchange results in larger invaginations with higher morphological variation.

Caveolin-1 composition of caveolae

In a previous study, the caveolin-1 composition of caveolae was analyzed in HeLa cells expressing caveolin-1-GFP (Pelkmans and Zerial, 2005). The cells were imaged by total internal reflection fluorescence (TIRF) microscopy, a technique that allows selective visualization of fluorescence on the plasma membrane. The authors found that fluorescence intensity of the imaged dots was distributed in quantum units of 144 Caveolin-1 molecules; 1 unit structures were identified as individual caveolae and structures composed of multiple units as caveolar clusters. Using the same cell line and procedure, the clustering behavior of caveolae was analyzed in cells in which cholesterol was replaced by desmosterol using 20,25-DAC (II, Figure 4). Caveolae clustered similarly in cholesterol and desmosterol containing cells. However, the range of fluorescence within individual caveolae was found to be ~30% larger in desmosterol cells (II, Figure 4C). This can be explained by a higher variation in the number of caveolin-1-GFP molecules per individual caveola.

Caveolar endocytosis

Caveolae continuously pinch off and fuse with the plasma membrane. This phenomenon can be analyzed in HeLa cells stably expressing caveolin-1-GFP. As visualized by TIRF microscopy, dots appear and disappear on the plasma membrane (II, Figure 6A). Without any stimulation, the rate of appearance and disappearance of dots was similar to the rate in cells containing desmosterol. The caveolar mobility could clearly be stimulated by the dephosphorylation inhibitor vanadate, which increased tyrosine-14 phosphorylation of caveolin-1. After vanadate stimulation, the caveolar mobility was also similar in desmosterol and cholesterol containing cells.

Next, the effect of desmosterol on the endocytosis of a ligand was analyzed. Previous studies have shown that the fluorescent sphingolipid BODIPY-lactosylceramide is selectively internalized by caveolar endocytosis (Singh et al., 2003). Fibroblasts were labeled with BODIPY-lactocylceramide, which was allowed to internalize for 5 min at 37 °C. Uninternalized label was removed from the plasma membrane by BSA washing and the remaining intensity quantified (II, Figure 6B). Desmosterol cells showed a moderate but significant reduction in BODIPY-lactocylceramide uptake.

In an alternative approach to analyze caveolar endocytosis, the caveolar internalization of crosslinked integrins was determined. This was measured by determining the time dependent colocalization between caveolin-1 and croslinked α2-integrin as described (Upla et al., 2004). The sterol exchange resulted in a slight but significant reduction in the colocalization, suggesting a decrease in caveolar endocytosis of integrins (II, Figure 7A). In comparison, no inhibition was detected in the internalization of the fluid-phase tracer dextran or transferrin, a marker of clathrin-mediated endocytosis (II, Figure 6C).

Caveolin phosphorylation

Integrin cross-linking is known to induce caveolin-1 phosphorylation at tyrosine-14, which is required for caveolar endocytosis (Nomura and Fujimoto, 1999). Contrary to our expectations, caveolin-1 phosphorylation upon integrin cross-linking was more pronounced in desmosterol cells (II, Figure 7B). The mechanism underlying the increased caveolin-1 phosphorylation in desmosterol cells was addressed using an inducible MDCK cell line overexpressing Src, the kinase that phosphorylates caveolin-1. Both in the basal and Src-induced states, the caveolin-1 tyrosine-14 phosphorylation was found to be more pronounced in desmosterol cells (II, Figure 8). This increase appeared to be selective for caveolin-1 since the overall degree of tyrosine phosphorylation was not significantly altered.

1.4 Discussion

Desmosterol cannot fully replace cholesterol in ordered membrane domains

This study provides the first evidence that the potential of desmosterol to support ordered domains in both model membranes and in cells is significantly lower than that of cholesterol. Three model membrane experiments showed that the potential of desmosterol to form and stabilize ordered domains is weaker than that of cholesterol. This result was reinforced by atomic scale simulations that indicated that desmosterol has a lower ordering effect in a DPPC membrane. Furthermore, the simulations showed that the double bond in the hydrocarbon tail of desmosterol gives rise to an additional stress in the tail, thus changing its conformation at the beginning of the tail, as compared with cholesterol. This seemingly minor difference had large implications for various structural properties of the bilayer, in particular membrane order. Several months after the publication of these results, another study confirmed the present observation that desmosterol is weaker than cholesterol in stabilizing ordered domains (Megha et al., 2006).

The present data show that the sterol exchange also affects the structure and function of ordered membrane domains in cells. A relatively low desmosterol content was observed in DRMs suggesting a low affinity of desmosterol for membrane rafts. Furthermore, cholesterol replacement was found to perturb both raft association and phosphorylation of the activated insulin receptor. Since insulin signaling has been shown to be dependent on rafts (Vainio et al., 2002), these results suggest that desmosterol perturbs insulin signaling by destabilizing membrane rafts.

Desmosterol can replace most cellular functions of cholesterol

Cells containing desmosterol as the main sterol behave normally with respect to cell growth/viability, subcellular sterol distribution, Golgi integrity, secretory pathway, phospholipid composition, and membrane fluidity. Because all these processes are affected by MβCD cholesterol depletion, our results indicate that desmosterol can replace most non-raft functions of cholesterol. This is in agreement with earlier studies: (1) DHCR24 knockout mice that contain desmosterol as the main sterol are viable (Wechsler et al., 2003) and (2) in mutant CHO cells desmosterol supports growth equally well as cholesterol (Xu et al., 2005).

It is important to point out that the present results do not imply that desmosterol can mediate all cellular functions of cholesterol besides those in ordered membrane domains. However, it is clear that alternative techniques used to disrupt ordered membrane domains, including MβCD cholesterol depletion, are far more harmful for cells. Together, the results in this thesis show that exchanging cholesterol for desmosterol provides a gentle and selective procedure for disrupting ordered membrane domains in cells.

Using desmosterol to analyze caveolae

The importance of cholesterol for the structure and function of caveolae was investigated by exchanging cellular cholesterol by desmosterol. The data demonstrated that the double bond in desmosterol reduced its affinity for isolated caveolae. It is important to note that this result does not inform about the actual caveolar sterol content in cells that contain primarily desmosterol. The sterol exchange reduced the stability of caveolae as determined by detergent resistance of caveolin-1 and heat resistance of caveolin-1 oligomers. Also, the sterol exchange led to aberrations in the caveolar structure; the morphology of caveolae was severely altered and there was a larger variation in the amount of caveolin-1 molecules per caveola. Because caveolin-1 has been shown to be involved in membranes bending (Fra et al., 1995) the larger variation of caveolin-1 might cause the altered morphology. Alternatively, sterols themselves have been implicated in membrane curvature (Bacia et al., 2005), and therefore the double bond in desmosterol or an altered caveolar sterol content might have caused the altered shape of the invaginations.

Analysis of caveolar endocytosis suggested that the sterol exchange led to a moderate defect in the selective uptake of both BODIPY-lactocylceramide and cross-linked integrins. It is unlikely that this decreased uptake resulted from an inability of caveolae to pinch off from the plasma membrane, since both the steady state and the vanadate stimulated caveolar mobility were found to be unaffected by the sterol exchange. The decreased uptake was neither explained by a reduction of caveolin-1 phosphorylation. Instead, the phosphorylation of caveolin-1 was found to be increased in the desmosterol cells, perhaps in order to preserve caveolar endocytosis. Taking all this into account, a likely explanation

for the decrease in caveolar uptake is a reduced affinity of BODIPY-lactocylceramide and cross-linked integrins for caveolae. This would be in line with the previous observation that activated insulin receptors had a reduced affinity for membrane rafts in cells containing desmosterol (Vainio et al., 2006).

2. Using BODIPY-cholesterol to study intracellular sterol transport

2.1 Characterizing BODIPY-cholesterol as a tool for studying cholesterol transport

The second part of this study aimed to resolve whether the fluorescent sterol probe BODIPY-cholesterol can be used as a tool to study cholesterol transport from the plasma membrane to the ER and lipid droplets. Three transport assays based on live cell microscopy were developed. For all experiments HeLa cells were used that were incubated overnight with 0.35 to 0.5 mM oleate. The oleate load increased the cellular triacylglyceride content from ~5 to ~30 µg/mg cellular protein, which facilitated lipid droplet visualization.

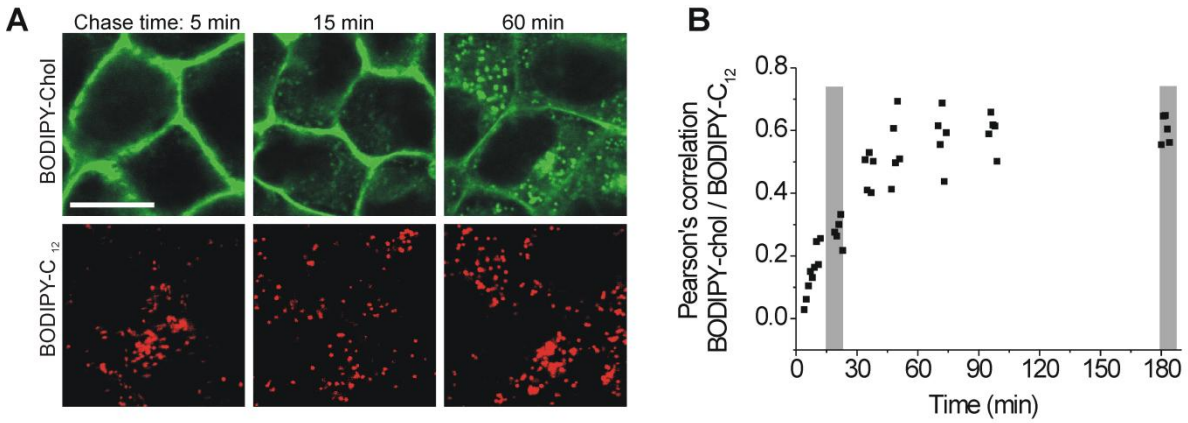

Figure 10. BODIPY-cholesterol transport from the plasma membrane to lipid droplets. (A) Hela cells were prelabeled with BODIPY-C_{12} to stain lipid droplets. The plasma membrane was pulse labeled with BODIPY-cholesterol (BODIPY-chol). Shown are confocal images at different chase times. Scale bar: 15 µm. (B) Pearson's correlation of BODIPY-cholesterol and BODIPY-C_{12} as a function of time. Combined data from three independent experiments. Gray areas indicate time windows used for quantification in later experiments.

Transport assay I: BODIPY-cholesterol transport from plasma membrane to lipid droplets

To study sterol transport from the plasma membrane to lipid droplets a live cell microscopy pulse chase assay was developed. Lipid droplets were visualized by labeling with the fluorescent fatty acid analog BODIPY-C_{12}. This label is preferentially incorporated in di- and triacylglycerides at the time points analyzed (Kasurinen, 1992). Next, the plasma membrane was pulse labeled with trace amounts of BODIPY-cholesterol. The cells were imaged continuously for up to 3 h by confocal microscopy while keeping the temperature at 37 °C. Within the first 10 min after labeling some of the BODIPY-cholesterol started to localize to lipid droplets (Figure 10A). The transfer of BODIPY-cholesterol to lipid droplets proceeded for about 1 h, at which time a steady state appeared to be reached. The kinetics of transport was quantified by determining the time-dependent colocalization between BODIPY-cholesterol and BODIPY-C_{12}. The colocalization, as expressed by the Pearson's correlation coefficient, was plotted as a function of time (Figure 10B). This curve revealed that BODIPY-cholesterol moved from the plasma membrane to lipid droplets with a halftime ($t_{½}$) of ~30 min.

Transport assay II: BODIPY-cholesterol transport from plasma membrane to ER

An assay identical to "*transport assay I"* was used except that an ER-tracker was utilized instead of the lipid droplet marker (III, Figure 6A). The plot of the Pearson's correlation between BODIPY-cholesterol and the ER-tracker as a function of time revealed transport $t_{½}$ of ~30 min (III, Figure 6B), which is similar to the kinetics found for plasma membrane to lipid droplet transport of BODIPY-cholesterol.

Transport assay III: FRAP of BODIPY-cholesterol in lipid droplets

Cells were labeled with BODIPY-cholesterol, which was allowed to equilibrate during a 3 h incubation period. Next, the BODIPY-cholesterol in a cluster of immobile lipid droplets was photobleached (III, Figure 5). The fluorescence recovery within those lipid droplets was measured as a function of time. The $t_{½}$ of recovery was found to be ~1 min.

Biochemical analysis of [^{3}H]cholesterol and BODIPY-cholesterol transport

Transport of [^{3}H]cholesterol and BODIPY-cholesterol from the plasma membrane to lipid droplets was analyzed with a biochemical pulse chase assay. The plasma membrane was pulse labeled with [^{3}H]cholesterol from a BSA complex or with BODIPY-cholesterol from a MβCD complex. Next, the lipid droplets were isolated by sucrose density gradient

fractionation after different chase times. The purified lipid droplet fraction was, as expected, enriched in triacylglycerides and adipocyte differentiation-related protein (ADRP) (Martin and Parton, 2005). Furthermore, the lipid droplet fraction contained no or little plasma membrane and ER contamination, as shown by Na^+/K^+-ATPase and calnexin Western blotting (III, Figure 1C). The time dependent lipid droplet association of unesterified [^{3}H]cholesterol and BODIPY-cholesterol was determined, indicating transport halftimes of ~45 min and ~30 min, respectively (III, Figure 1D, S2). It is of note that, at steady state, the BODIPY-cholesterol lipid droplet content was ~4 times higher compared to unesterified natural cholesterol.

Effects of pharmacological treatments on BODIPY-cholesterol transport.

Next, the effects of several pharmacological treatments on BODIPY-cholesterol transport from the plasma membrane to lipid droplets were investigated (III, Figure 2). In order to make the assay time-efficient, confocal images were acquired only at two chase times: 15-25 min after labeling, at which time there is a net transport of BODIPY-cholesterol to lipid droplets, and 180 min after labeling, when a steady state is reached (Figure 10B). The transport kinetics of BODIPY-cholesterol was not altered by ATP depletion as judged by unaltered colocalization between the sterol probe and the lipid droplet marker after both 15-25 min and 180 min chase. On the other hand, hydrolyzing the sphingomyelin on the plasma membrane using sphingomyelinase increased the colocalization after 15-25 min chase but not at steady state. This indicates that sphingomyelinase treatment increases BODIPY-cholesterol transport to lipid droplets but has no effect on the total amount of sterol transported. Brefeldin A, on the other hand, did not affect transport at either time point.

BODIPY-cholesterol esterification

The degree of BODIPY-cholesterol esterification was analyzed by TLC and compared to that of [^{3}H]cholesterol. After a 3 h chase, 7% of the [^{3}H]cholesterol was esterified (III, Figure 1D) whereas no BODIPY-cholesterol esters could be detected. In order to determine whether BODIPY-cholesterol can get esterified in HeLa cells, the cells were labeled for 24 h in medium containing 1 µM BODIPY-cholesterol. This procedure results in 10 times more BODIPY-cholesterol moving into the cells compared to the MβCD labeling protocol that was used in all other experiments. In order to stimulate the formation of cholesteryl esters, the cells were loaded with cholesterol from a MβCD/cholesterol complex (6:1; 2 mM MβCD). Under these conditions, 2.5% of the BODIPY-cholesterol got esterified. This was 20 times lower compared to the fraction of esterified cholesterol in the same cells (Figure 11).

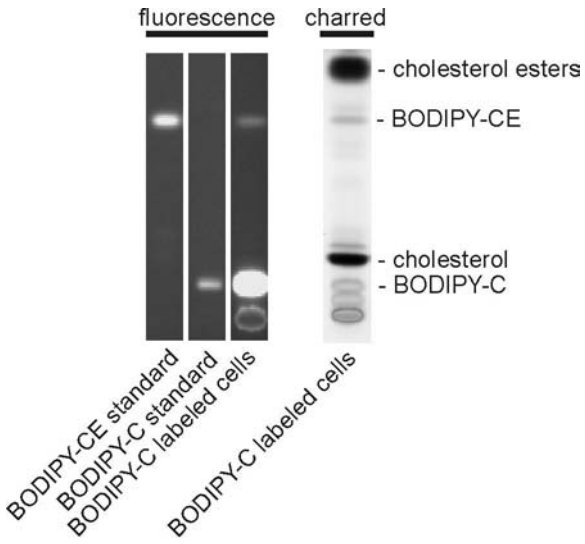

Figure 11. Esterification of BODIPY-cholesterol. HeLa cells were labeled overnight with 1 µM BODIPY-cholesterol (BODIPY-C) in the presence of a MβCD/cholesterol complex (6:1; 2 mM MβCD). Lipids were extracted and analyzed by HPTLC. BODIPY-cholesterol and BODIPY-cholesterol esters (BODIPY-CE) were visualized with a fluorescent plate reader.

2.2 ORP dependent transport of cholesterol to the ER and lipid droplets

Screening of ORPs in BODIPY-cholesterol transport from plasma membrane to lipid droplets

The BODIPY-cholesterol plasma membrane to lipid droplet transport assay was used to analyze the effect of ORP overexpression on sterol traffic. HeLa cells were transiently transfected with cDNAs of all human ORP splice variants containing an N-terminal Xpress tag. Xpress-ORP protein levels were analyzed by Western blotting showing that all were expressed albeit at varying levels (III, Figure 3B). In order to identify the ORP overexpressing cells by fluorescence microscopy, the ORP constructs were co-overexpressed with a plasmid coding for the fluorescent protein HcRed. As judged by α-Xpress staining, ~90% of the cells expressing HcRed also expressed the Xpress-ORP (III, Figure S1). Confocal images were taken at 15-25 min after BODIPY-cholesterol labeling of fields in which more than half of the cells were expressing HcRed. Overexpression of several ORPs increased the colocalization between BODIPY-cholesterol and lipid droplets (III, Figure 3C; Figure 12). Out of these, ORP1S and ORP2 gave the largest increase. Because overexpression of ORP1S and ORP2 did not alter the colocalization at steady state (180 min chase) (III, Figure 3C; Figure 12), these results suggest that ORP1S and ORP2 can increase the plasma membrane to lipid droplet transport kinetics of BODIPY-cholesterol. The protein levels of overexpressed ORP1S and ORP2 were roughly similar to those of OSBP, ORP1L, ORP3, ORP5, ORP7 and ORP9S. Therefore, out of these ORPs, ORP1S and ORP2 appear to be most efficient in enhancing BODIPY-cholesterol transport to lipid droplets.

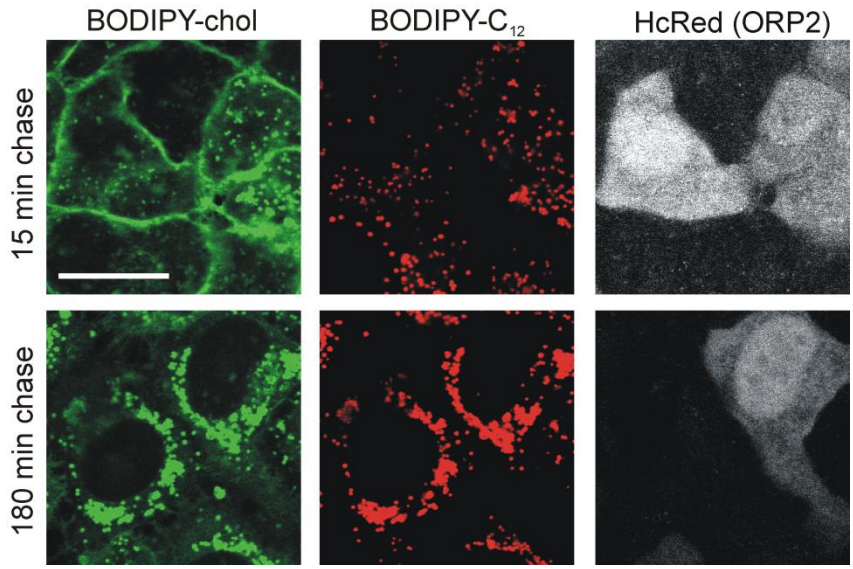

Figure 12. ORP2 overexpression increases BODIPY-cholesterol transport to lipid droplets. HeLa cells coexpressing ORP2 and HcRed were prelabeled with BODIPY-C_{12} and pulse labeled with BODIPY-cholesterol (BODIPY-chol). Shown are confocal images after 15 min and 180 min chase. Scale bar: 15 µm.

ORP2 mediated transport of natural sterols

It was next clarified whether the ORP2 mediated sterol transport also applies to natural sterols. The effect of ORP2 overexpression on the plasma membrane to lipid droplet transport of dehydroergosterol was analyzed by epifluorescent microscopy. In this experiment, Huh7 cells were used because their large lipid droplets facilitate the visualization of dehydroergosterol in lipid droplets. As was shown for BODIPY-cholesterol, dehydroergosterol transport from the plasma membrane to lipid droplets was stimulated by ORP2 overexpression (III, Figure S5).

Next, the effect of ORP2 overexpression on the esterification of plasma membrane derived [^{3}H]cholesterol was analyzed. Because cholesterol is esterified by ACAT in the ER, this assay indirectly measures cholesterol transport from the plasma membrane to the ER. ORP2 overexpression significantly increased the fraction of [^{3}H]cholesterol esters after a 30 min chase time (III, Figure 4D). ORP2 overexpression did not affect the ability of ACAT to esterify sterols, as determined by an *in vitro* activity assay. Therefore, it is likely that the higher esterification resulted from increased cholesterol transport to the ER. This result is in agreement with findings from the BODIPY-cholesterol plasma membrane to ER transport assay that showed a similar ORP2 dependency (III, Figure 6C,D). Interestingly, the FRAP of BODIPY-cholesterol in lipid droplets was shown to be unaffected by ORP2 overexpression (III, Figure 5B).

Physiological role for ORP1 and ORP2 in sterol transport

We determined the endogenous levels of ORP1S and ORP2 in HeLa cells by Western blotting using purified recombinant proteins as calibrators. Both proteins were found to be present at around one million copies per cell (our unpublished results). Next, the effect of simultaneous knockdown of ORP1 and ORP2 on BODIPY-cholesterol transport from the plasma membrane to lipid droplets was measured. On a side note, because the ORP1S splice variant cannot be selectively silenced, both ORP1L and ORP1S were downregulated. Knockdown resulted in a moderate but significant reduction of sterol transport kinetics (III, Figure 4B). Of note, single knockdown of either ORP1 or ORP2 had no effect on sterol transport (our unpublished observations). In order to analyze whether ORP1 and ORP2 double knockdown effects the transport of natural sterols, the esterification of plasma membrane derived [^{3}H]cholesterol was analyzed. Knockdown decreased the esterification of [^{3}H]cholesterol without altering ACAT activity, suggesting a reduction of cholesterol transport from the plasma membrane to the ER (III, Figure 4C).

ORP dependent in vitro sterol transfer

To assess more directly whether ORP1S and ORP2 can function as cholesterol transporters, cholesterol transfer was studied *in vitro.* Recombinant Glutathione-S-transferase (GST) fusion proteins of ORP1S, ORP2, and the MLN64 START domain were produced in *E. coli* and purified using Glutathione-Sepharose. The ability of these proteins to stimulate [^{3}H]cholesterol transfer from large unilamellar vesicles to a 50-fold molar excess of acceptor vesicles was analyzed. ORP1S significantly increased sterol transfer to acceptor vesicles (III, Figure 4E). A similar tendency was found for ORP2. These results suggest that ORP1S and ORP2 function as sterol transporters. However, the transfer activity of both ORPs was substantially lower than that of MLN64 START domain which was used as a positive control.

Role of conserved domains in sterol transport

Next, the importance of two conserved domains present in ORP2, the ORD and FFAT motif was analyzed for BODIPY-cholesterol transport from the plasma membrane to lipid droplets (III, Figure 4F). Overexpression of an ORP2 I249W mutant (ORP2mORD), which is defective in binding sterols, was unable to stimulate BODIPY-cholesterol transport. On the other hand, overexpression of an ORP2 mutant with a defective FFAT-motif (ORP2mFFAT) increased BODIPY-cholesterol transport to lipid droplets to the same extent as wild-type ORP2. Western blot analysis indicated that the lack of transport activity of the ORD mutant could not be explained by reduced expression levels (III, Figure 4G). Therefore, these results

indicate that a functional ORD, capable of cholesterol binding, is necessary for ORP2 mediated sterol transfer activity.

2.3 VAP proteins are involved in cholesterol transport by stabilizing ORPs

VAP knockdown decreases ORP2 levels and sterol transport

The effect of VAP-A and/or VAP-B knockdown on BODIPY-cholesterol transport from the plasma membrane to lipid droplets was investigated. Knockdown of either VAP-A or VAP-B alone did not affect this transport (Jansen and Ikonen, unpublished observations), but simultaneous knockdown of both proteins resulted in slower BODIPY-cholesterol trafficking from the plasma membrane to lipid droplets, as suggested by reduced colocalization of BODIPY-cholesterol and lipid droplets at 15-25 min of chase (III, Figure 7A).

To assess whether ORP2 can facilitate sterol transfer in the absence of VAPs, ORP2 was overexpressed in control and VAP-A/B knockdown cells and the delivery of BODIPY-cholesterol from the plasma membrane to lipid droplets was analyzed. ORP2 overexpression increased BODIPY-cholesterol transport kinetics in a similar extent in both control and VAP-A/B knockdown cells (III, Figure 7A). This demonstrates that ORP2 can facilitate sterol transport independently of VAPs.

VAPs stabilize endogenous ORPs by interacting with their FFAT motif

To investigate whether ORPs are involved in the effect of VAP knockdown on sterol transport, ORP2 levels were analyzed by Western blotting. A significant reduction in the amount of ORP2 was observed in the VAP-A/B silenced cells (III, Figure 7B). Conversely, VAP-A overexpression resulted in increased levels of the endogenous ORP2 protein (III, Figure 7D). By using [^{35}S]methionine pulse-chase experiments followed by immunoprecipitation, it was found that ORP2 degrades more rapidly in VAP-A/B silenced cells (III, Figure 7C), suggesting the need of VAPs for ORP2 stability.

To study whether the FFAT motif is important for VAP dependent ORP2 stability, the effect of VAP-A/B knockdown on the level of overexpressed ORP2mFFAT was analyzed (III, Figure 7E). Overexpressed ORP2 wild-type was downregulated whereas the ORP2mFATT was not, indicating the importance of the FFAT domain for ORP stability. Consistently with this, endogenous levels of the FFAT motif containing ORPs, ORP1L and ORP3, were downregulated by VAP-A/B silencing, whereas ORP1S and ORP11 that lack a FFAT motif were not (III, Figure 7F). Still, not all FFAT motif containing ORPs were downregulated by VAP-A/B silencing, as indicated by ORP4 and ORP9.

2.4 Discussion

Characterizing BODIPY-cholesterol as a tool for studying cholesterol transport

BODIPY-cholesterol was characterized as a tool for studying cholesterol transport between the plasma membrane, the ER and lipid droplets by comparing the transport kinetics of BODIPY-cholesterol with the kinetics of natural sterols. BODIPY-cholesterol moved from the plasma membrane to lipid droplets with a $t_{½}$ of ~30 min, which is roughly similar to the kinetics observed for unesterified [^{3}H]cholesterol. These results are also in agreement with a previous study that analyzed the transport kinetics of dehydroergosterol for the same route (Wustner et al., 2005). The transport of BODIPY-cholesterol from the plasma membrane to the ER also occurred with a $t_{½}$ of ~30 min, which is very similar to the kinetics found for ER to plasma membrane transport as determined by the time dependent oxidation of newly synthesized cholesterol (Lange et al., 1991). Furthermore, the recovery of BODIPY-cholesterol fluorescence in lipid droplets occurred with a $t_{½}$ ~1 min, as also observed for dehydroergosterol ($t_{½}$: ~1.5 min) (Wustner et al., 2005). BODIPY-cholesterol was further characterized by analyzing the effects of pharmacological treatments on BODIPY-cholesterol transport. BODIPY-cholesterol traffic to lipid droplets was unaffected by ATP depletion, as was shown previously for dehydroergosterol (Wustner et al., 2005). Furthermore, the present observations that plasma membrane to lipid droplet transport of BODIPY-cholesterol is affected by SMase but insensitive to BFA treatment, share similarities with cholesterol traffic between the plasma membrane and the ER, which is affected by sphingomyelinase (Okwu et al., 1994) but mostly insensitive to brefeldin A (Heino et al., 2000). Together, these results show that BODIPY-cholesterol resembles natural cholesterol regarding transport from the plasma membrane to the ER and lipid droplets.

Considering the observation that an additional double bond in the sterol side chain has pronounced effects on ordered membrane domains, it is remarkable that the addition of the bulky BODIPY-group does not radically change the behavior of the sterol. This can partly be explained by the low concentration of BODIPY-cholesterol within the labeled cells. The presence of trace amounts of BODIPY-cholesterol probably has no major effect on membrane properties. Furthermore, present results show that BODIPY-cholesterol does not mimic all cell biological behavior of natural cholesterol. At steady state the cellular fraction of BODIPY-cholesterol in lipid droplets was around four times higher compared to that of natural cholesterol. This might be explained by the affinity of the BODIPY-group for lipid droplets (Listenberger and Brown, 2007). Furthermore, the rate of BODIPY-cholesterol esterification was found to be significantly lower compared to that of natural cholesterol. In most cell types, lipid droplet sterols are predominantly present in esterified form. Therefore, BODIPY-cholesterol transport kinetics to lipid droplets only mimics the transfer of free cholesterol, not of cholesterol esters. Nevertheless, the study of free sterol transport to lipid droplets is informative since lipid droplets do contain small amounts of unesterified

cholesterol. Notably, in the lipid droplets of human adipocytes, 90% of the cholesterol is present in unesterified form (Schreibman and Dell, 1975).

ORP dependent transport of cholesterol to the ER and lipid droplets

In the overexpression screen, ORP1S and ORP2 were found to be most efficient in facilitating sterol transport from the plasma membrane to lipid droplets. Interestingly, these two ORPs are part of the same subfamily based on sequence similarity. Another structural feature that ORP1S and ORP2 have in common is the absence of a PH domain (Raychaudhuri et al., 2006). This lack is relatively rare in mammalian ORPs since twelve of the sixteen splice variants tested in our screen do contain a PH domain. In yeast studies, it has been shown that out of the three ORPs most effective in mediating sterol transport, two are missing a PH domain. Together, these findings show that a PH domain is not necessary for ORP mediated sterol transport. Furthermore, they suggest that absence of this domain might improve its efficiency in mediating sterol transport. Possibly, interactions between PI containing membranes and the PH domain hinder the sterol transfer activity of ORPs.

To obtain more insight into the transport route(s) of sterols moving from the plasma membrane to lipid droplets, the recovery of BODIPY-cholesterol was analyzed in lipid droplets after photobleaching. The $t_{½}$ of BODIPY-cholesterol FRAP was very rapid, ~1 min, and unaffected by ORP2 overexpression. This implies that the majority of BODIPY-cholesterol moving to lipid droplets do not come directly from the plasma membrane, since the $t_{½}$ for plasma membrane to lipid droplet transport is ~30 min. There are several arguments that suggest that most BODIPY-cholesterol moving from the plasma membrane to lipid droplets transfers through the ER (Figure 13): (1) plasma membrane to ER and plasma membrane to lipid droplet transport are stimulated in a similar manner by ORP2 overexpression, (2) the kinetics of sterol transport from plasma membrane to ER (~30 min) + lipid droplet FRAP (~1 min) is similar to the kinetics of plasma membrane to lipid droplet transport (~30 min), (3) The ER has been shown to be in close proximity to both the plasma membrane and lipid droplets, (4) cholesterol esters are synthesized in the ER before deposition in lipid droplets. Although it appears likely that sterols moving to lipid droplets come from the ER, the present results do not exclude transport routes involving other cellular compartments.

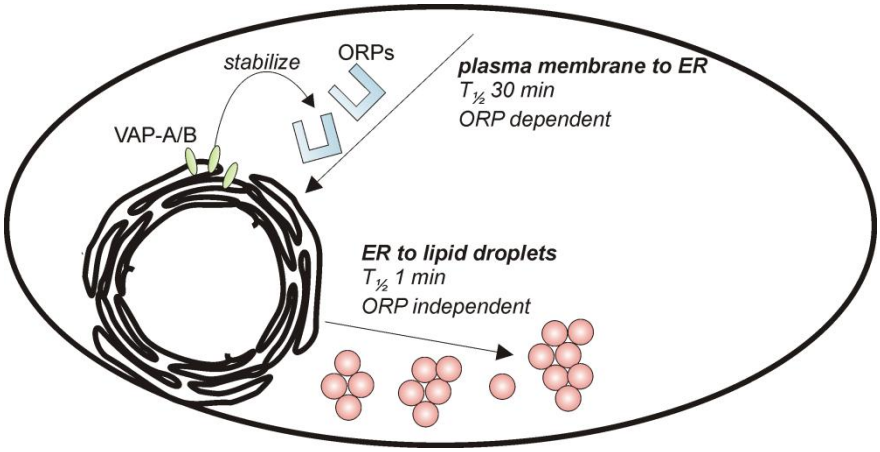

Figure 13. Model of cholesterol transport from the plasma membrane to lipid droplets based on the results of this study. Cholesterol transport is indicated by the straight arrows. The bended arrow indicates the stabilization of ORP's (blue) by VAP-A/B (green). The rationale behind this model is discussed in the text.

In this study, results from the ORP overexpression screen using BODIPY-cholesterol were validated with assays using dehydroergosterol and [^{3}H]cholesterol. These results indicate that the effects of ORPs on sterol transport are similar for the three tested sterols. This implies that the bulky BODIPY group is not hindering binding of BODIPY-cholesterol within the hydrophobic cavity of the ORP proteins. The crystal structure of the yeast ORP Osh4, in complex with cholesterol, indicates that hydroxyl side of cholesterol is buried deep inside the hydrophobic Osh4 tunnel whereas the side chain region is covered by a flexible lid (Im et al., 2005). The same study shows that also 25-HC can be complexed with Osh4. The 25-hydroxyl group of 25-HC was found to bind ordered water molecules close to the flexible lid. The presence of water molecules inside that region of the binding pocket indicates that there is extra space within the part of the tunnel that is expected to harbor the BODIPY-group of BODIPY-cholesterol. This additional space might explain how mammalian ORPs can bind BODIPY-cholesterol and mediate its transfer.

The finding that ORP1S and ORP2 are capable of transferring cholesterol *in vitro* suggests that they might directly transfer cholesterol in cells. It was approximated that ~40,000 cholesterol molecules move every second from the plasma membrane to the ER. This value was calculated with the assumptions that 2% of cellular cholesterol is in the ER (Lange and Steck, 1997), one HeLa cell contains 5 billion cholesterol molecules, the $t_{½}$ of cholesterol transport to the ER is 1800 s and that transport occurs by first-order kinetics: $0.02 \cdot 5 \cdot 109 \cdot \frac{\mathrm{Ln}(2)}{1800}$ = ~40,000. Taking into account that ORP1S and ORP2 are each present at 1 million copies per cell, this means that these ORPs are present at an abundance that would allow them to significantly contribute to the plasma membrane to ER cholesterol transport by acting as sterol carriers. Nevertheless, these results do not allow us to draw

conclusions regarding the mechanism by which ORPs facilitate sterol transport from the plasma membrane to the ER.

Even though double knockdown of ORP1 and ORP2 reduced sterol transport from the plasma membrane to the ER and lipid droplets, the decrease was only moderate. This mild affect could be explained by a functional redundancy within the human ORP family regarding sterol transport. Such an explanation appears likely since the present ORP overexpression screen indicated that other ORPs can also stimulate sterol transport, albeit less efficiently. A functional redundancy in the human ORP family would also be in line with results from yeast, which suggest that all Osh proteins are involved in sterol transport from the plasma membrane to the ER (Raychaudhuri et al., 2006). It is also possible that there are ORP independent sterol transport mechanisms between the plasma membrane and the ER. Such mechanisms might be mediated by other LTPs, such as SCP-2 or members of the START family (Puglielli et al., 1995; Soccio et al., 2005).

VAP proteins are involved in cholesterol transport by stabilizing ORPs

VAPs have been implicated in lipid transport (Kawano et al., 2006; Peretti et al., 2008) and interact with the FFAT domain present in several ORPs. In the present study VAP-A/B double knockdown reduces BODIPY-cholesterol transport from plasma membrane to lipid droplets. VAPs stabilize ORP2 protein levels in a FFAT domain dependent manner and the protein levels of several FFAT domain containing ORPs were downregulated by VAP-A/B silencing. Therefore, it seems likely that the reduction in sterol transport is caused by the effect of VAPs on the stability of FFAT domain containing ORPs. It should be noted that VAP interacting proteins include a number of lipid binding/transport proteins, such as CERT and Nir1-3 (Loewen et al., 2003). Therefore, silencing of VAPs might affect sterol transport by ORP unrelated mechanisms. Yet another cause for the decrease in sterol transport upon VAP-A/B knockdown could be a requirement of VAPs for the sterol transfer activity of ORPs. This would be in line with a study that suggests that VAP dependent targeting to the ER is important for the activity of the FFAT domain containing CERT (Kawano et al., 2006). However, the present results provide evidence that interaction between the ORP FFAT domain and VAPs is not directly involved in the sterol transport function of ORPs: (1) ORP2 overexpression stimulated sterol transport in the absence of VAPs, (2) overexpression of ORP2 with a defective FFAT motif induced sterol transport to a similar extent compared to overexpression of wild-type ORP2, (3) overexpression of ORP1S, which does not contain a FFAT motif, gave the highest increase in sterol transport out of all screened ORPs. Studies by other laboratories also suggest that the FFAT-VAP interaction is not relevant for ORP mediated sterol transport. In yeast, the ORPs that were found to be most important for sterol transfer from the plasma membrane to the ER did not contain a FFAT motif (Raychaudhuri et al., 2006). It has also been shown that ORPs in yeast can use their ORD to

target the ER, illustrating that a FFAT motif is not necessary for localization to this organelle (Schulz et al., 2009). However, despite this evidence, it is possible that the ORP-VAP interactions may under physiologic conditions be functionally important for sterol transport; upon overexpression, activity of CERT was shown to be independent of VAPs, whereas at lower expression levels, VAP interaction was shown to be important (Kawano et al., 2006).

CONCLUSIONS

Desmosterol as a tool to study ordered membrane domains

1. Desmosterol cannot functionally replace cholesterol in ordered membrane domains
2. Exchanging cellular cholesterol for desmosterol provides a selective tool for perturbing ordered membrane domains
3. Cholesterol is important for the stability and structural homogeneity of caveolae

Using BODIPY-cholesterol to study intracellular cholesterol transport

1. BODIPY-cholesterol behaves similarly to natural cholesterol regarding transport between the plasma membrane, the ER and lipid droplets
2. ORP1S and ORP2 facilitate cholesterol transport from the plasma membrane to the ER
3. ORP1S and ORP2 can transfer cholesterol *in vitro*
4. VAP proteins are involved in cholesterol transport from the plasma membrane to the ER by stabilizing ORPs

PERSPECTIVES

In this work, cholesterol analogs were used to analyze cell biological functions of cholesterol. Sterol substitution has in the past been used to analyze the role of cholesterol for biological processes including endocytosis (Heese-Peck et al., 2002), cell growth (Rujanavech and Silbert, 1986), lipid adaptation (Low et al., 1985) and receptor binding affinity (Klein et al., 1995). In the present study, this procedure was used for the first time to analyze the importance of cholesterol for ordered membrane domains in cells. The most common procedure to disrupt ordered membranes is by cholesterol removal or sequestration. These techniques lack selectivity as they affect general membrane properties outside ordered domains. Exchanging cholesterol for desmosterol was found to perturb ordered membrane domains without significantly altering other membrane functions as indicated by unchanged cell growth/viability, subcellular sterol distribution, Golgi integrity, secretory pathway, phospholipid composition and membrane fluidity. Furthermore, the sterol substitution perturbed caveolae without totally abolishing them. This can be an advantage since it allows the analysis of the role of cholesterol for caveolar structure. On the other hand, the lack of total disruption can also be a drawback, since the effect of the sterol substitution might be small or even absent. For instance, even though the sterol substitution altered the caveolar structure, the endocytic function of caveolae was only moderately affected. For this reason it would be interesting to screen the effects of other cholesterol precursors on ordered membrane domains in cells. It is likely that inhibition of an enzyme more upstream of cholesterol biosynthesis would have more pronounced effects on ordered membrane domains in cells. However, cholesterol precursors more upstream are also more likely to affect cellular functions unrelated to ordered membrane domains. In the end, it would be probably best to use a combination of sterol manipulations, ranging in severity, to analyze ordered membrane domains in cells in a prudent manner.

Cholesterol transport from the plasma membrane to the ER and lipid droplets is an important mechanism by which cells regulate their membrane cholesterol content. By comparing transport kinetics and the effect of drug treatments, BODIPY-cholesterol resembles cholesterol regarding its transport from the plasma membrane to the ER and lipid droplets. By using BODIPY-cholesterol, this study uncovered a role for ORPs and VAPs in the transport of sterols from the plasma membrane to the ER in mammalian cells. Even though our results are in line with a sterol transport function of ORPs, they do not exclude other mechanisms of transfer. In order to address this question, yeast would be a better model since it allows for multiple knockouts. One could screen whether overexpression of mammalian proteins, capable of *in vitro* sterol transfer (e.g. the MLN64 START domain), can restore viability in the yeast strain lacking all Osh proteins. In case a sterol transporter could substitute for ORPs in yeast this would strongly suggest that ORPs function as sterol transporters in cells. Another unresolved issue is the importance of ORP mediated sterol transport from the plasma membrane to the ER in mammalian cells. Even though ORP1 and ORP2 were efficient in mediating sterol transport upon overexpression, their knockdown

gave only a moderate reduction in sterol transfer. This issue could be addressed by disrupting the function all mammalian ORPs simultaneously. The development of pharmacological ORP inhibitors would be helpful for this purpose.

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Maurice Jansen

LITERATURE

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