Family of human oxysterol binding protein homologues: ORP2 is a new regulator of cellular lipid metabolism

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ORIGINAL PUBLICATIONS

This thesis is based on three published original articles and a manuscript, which are referred to in the text by their Roman numerals.

I. Laitinen Saara, Vesa M. Olkkonen, Christian Ehnholm and Elina Ikonen (1999): Family of human oxysterol binding protein (OSBP) homologues: A novel member implicated in brain sterol metabolism. *J. Lipid Res.* 40, 2204-2211.

II. Lehto Markku, **Saara Laitinen**, Giulia Chinetti, Marie Johansson, Christian Ehnholm, Bart Staels, Elina Ikonen and Vesa M. Olkkonen (2001): The OSBP-related protein family in humans. *J. Lipid Res.* (42), 1203-1213.

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IV. Laitinen Saara, Reijo Käkelä, Sari Lusa, Kimmo Tanhuanpää, Christian Ehnholm, Pentti Somerharju, Elina Ikonen and Vesa M. Olkkonen (2002): Constitutive expression of OSBP-related protein 2 (ORP2) induces profound changes in cellular cholesterol and phospholipid metabolism (Manuscript).

ABBREVIATIONS

ABC	ATP binding cassette
ACAT	acyl-CoA:cholesterol acyltransferase
AP-1, -2, -3, -4	adaptor proteins 1, 2, 3 and 4
ApoA-I	apolipoprotein A-I
ARF	ADP-ribosylation factor
CDP	cytidine diphosphate
CPT	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CD	cyclodextrin
CE	cholesterol ester
COP	coat protein
CYP7A	cholesterol 7α-hydroxylase
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMR	detergent resistant membrane
ER	endoplasmic reticulum
FA	fatty acid
FC	free cholesterol
FXR	farnesoid X receptor
HDL	high-density lipoprotein
LCFA	long chain fatty acid
LDL	low-density lipoprotein
LXR	liver X receptor
MDR	multidrug resistance protein
NPC	Niemann-Pick C
24S-OHC	24S-hydroxycholesterol
25-OHC	25-hydroxycholesterol
ORP	OSBP related protein
OSBP	oxysterol binding protein
PA	phosphatidic acid
PC	phosphatidylcholine
PC-TP	phosphatidylcholine transfer protein
PE	phosphatidylethanolamine
PH	pleckstrin homology
PI	phosphatidylinositol
PITP	phosphatidylinositol transfer protein
PL	phospholipid
PLA_2	phospholipase A_2
PLD	phospholipase D
RXR	retinoic acid X receptor
SCAP	SREBP cleavage activating protein
SM	sphingomyelin
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SR-BI	scavenger receptor type B1
SREBP	sterol regulatory element binding protein
StAR	steroidogenic acute regulatory protein
START	StAR-related lipid transfer
TGN	*trans*-Golgi-network

REVIEW OF THE LITERATURE

1. Introduction

Membranes, composed of lipids and proteins, play a key role in the structure and function of the eukaryotic cell. Plasma membrane forms a barrier between the outside environment and the inside of the cell. It functions as a control point for uptake of different ions and nutrients and passes the external signals into the intracellular space. Membranes make up the structure of different organelles, they separate intracellular compartments from each other and they form the routes for rapid delivery of different compounds to various cellular locations.

Membrane bilayers are composed of complex arrays of lipids and proteins. The main groups of lipids in mammalian membranes are glycerolipids, sphingolipids and cholesterol. Proteins are either integrated into the membrane bilayer or are peripherally associated with membranes, where they interact with headgroups of lipids and/or other peripheral or integral proteins. Membrane rigidity can be regulated by the lipid composition of the membrane; here the fatty acid composition of lipids and the amount of cholesterol in the bilayer are important factors. Membranes also contain different laterally organized domains that consist of specific lipids and proteins with distinct characteristics.

For decades a great deal of research has focused on cholesterol. Cholesterol is an important membrane component, however it can be harmful physiologically when an excess amount is obtained from the diet. Over time, excess cholesterol accumulates in the arterial intima and causes the formation of fatty streaks that finally leads to the disease state, atherosclerosis.

Oxygenated derivatives of cholesterol, oxysterols, are suggested to be involved in many biological processes such as cholesterol turnover, atherosclerosis,

apoptosis, inflammation, immunosuppression and the development of gallstones. Oxysterols are known to affect the expression of several critical enzymes in cholesterol biosynthesis, the receptor-mediated uptake of cholesterol, and other aspects of cellular cholesterol homeostasis. Furthermore, they are potent regulators of catabolism of cholesterol to bile acids.

In this thesis I will describe the recently identified (I, II) human oxysterol-binding protein (OSBP)-related protein (ORP) family and characterize one family member named ORP2 and its effects on cellular lipid metabolism (III, IV).

2. Composition of biological membranes

2.1 The major lipid classes and their distribution within the cell

One important feature of membranes is the asymmetric distribution of lipids between the two leaflets of the bilayer which is actively maintained by specific proteins (Sims and Wiedmer, 2001). Another feature is the unequal distribution of different lipids and proteins between various intracellular membranes. All lipid classes, sphingolipids (SLs), glycerolipids (GLs) and sterols (cholesterol in mammals) have distinct intracellular distributions between different cellular membranes and within a single membrane. The structure and the membrane orientation of three lipids studied in this thesis work: cholesterol, sphingomyelin (SM) and phosphatidylcholine (PC) is illustrated in Figure 1.

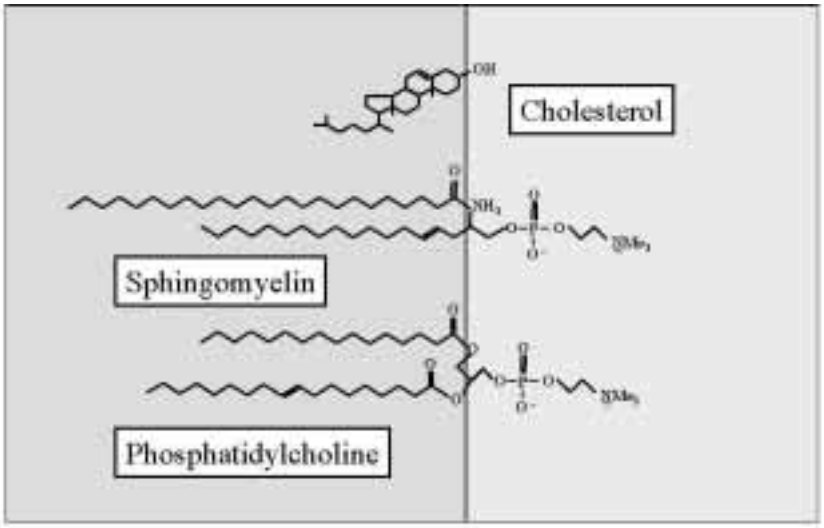

Figure 1. Structure of the three important membrane lipids discussed in this thesis.

Phospholipids (PLs) the most abundant of which are phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA) comprise 80% of all membrane lipids. Their intracellular distributions and functions in cells are largely different. PC is widely spread throughout the cell, while SM and cholesterol are enriched in the plasma membrane (PM) and endosomes (Lange et al., 1989; Slotte et al., 1989; Warnock et al., 1993). In epithelial cells, the apical PM contains more SM compared to the basolateral membrane. Different PL species are asymmetrically distributed in the PM so that most of the PC and SM are found in the outer leaflet and that most of the PE and PS are found in the inner leaflet (Devaux, 1992; Vance, 1996). One can speculate that the shape of a lipid molecule, like in the case of PE, favors its location in the inner leaflet. PE is a cone shaped molecule and its shape can be postulated to prefer curved rather than planar membranes. Localization of PE in the inner leaflet would then shape the PM. This could also be important in vesicle trafficking, as it

would provide a driving force in membrane bending (Devaux, 1992; Sims and Wiedmer, 2001). The head group of PS has a negative charge and this could enhance the interaction of Ca^{2+} ions with the membrane and cytoskeletal elements (Sims and Wiedmer, 2001) as well as with specific enzymes such as protein kinase C (Devaux, 1992; Ochoa et al., 2002). In addition, glycerophospholipids are precursors of many bioactive lipids. For example, PC is hydrolysed to PA and choline by phospholipase D and can also be hydrolyzed to arachinoic acid and lyso-PC by phospholipase A_2 (PLA_2) (Funk, 2001; Ohanian and Ohanian, 2001; Wakelam et al., 1997).

Sphingolipids are found in all eukaryotic cells. They are enriched in the PM, Golgi and endosomal membranes and their metabolites are involved in many cellular processes including differentiation, senescence, apoptosis and proliferation (Ohanian and Ohanian, 2001). Glycosphingolipids are found predominantly in the outer leaflet of the PM and in the lumenal leaflet of intracellular vesicles and in organelles involved in membrane trafficking. Together with the glycoproteins they mediate intercellular interactions, cell to cell communication and have diverse functions in the immune system (Holthuis et al., 2001; Vance, 1996). Sphingolipids are, like glycerolipids, precursors of various intracellular signaling molecules such as sphinganine, sphingosine-1-phosphate and ceramide (Pyne and Pyne, 2000; Ruvolo, 2001; Venkataraman and Futerman, 2000). In polarized cells, sphingolipids are preferentially localized to the apical membrane. Domains rich in sphingolipids and cholesterol are suggested to mediate trafficking to the apical membrane (Maier et al., 2001).

Only a small amount of the cellular cholesterol is found within the ER where it is synthesized and the concentration of cholesterol increases in the membranes along the secretion route towards the PM. For normal cellular processes to function it is essential that the cholesterol concentration in membranes remains under tight control.

This is due to the strong influence of cholesterol on the structure and function of membranes. Cholesterol interacts with the fatty acyl chains of glycerolipids and sphingolipids but it has been shown to prefer the sphingoid base moiety and thus to associate instead with sphingolipids rather than with glycerolipids (Ridgway, 2000). Together with SM and glycosphingolipids cholesterol forms active membrane domains designated as either detergent-resistant membranes (DMRs) or rafts (Simons and Ikonen, 2000). Despite this fact one must not forget that a significant portion of cholesterol is also found in non-raft membranes. The concept of membrane domains will be discussed in the following chapters. Another important function of cholesterol is to regulate the rigidity and permeability of the lipid bilayer. By reducing the leakage of ions through the lipid bilayer, cholestrol acts as a barrier and thereby facilitates sustained membrane function (Haines, 2001). The molar ratio of phospholipids to free cholesterol (PL/FC) is important for determining membrane viscosity. For example, the PL/FC ratio within the ER and mitochondria is low as compared to the PM and this results in lowered rigidity and increased permeability of the intracellular membranes.

2.2 Intracellular transport of lipids

The transport of membrane lipids within a lipid bilayer occurs laterally, however they can also switch sides from the cytosolic to the outer/lumenal leaflet or vice versa. Furthermore lipids are constantly transported between different membranes and organelles, especially when they are synthesized or taken up by the cells. In both cases lipids have to be transported to the site where they are needed. Many of the enzymes involved in phospholipid and cholesterol synthesis are located in the ER and especially at the cytosolic side of the ER membrane. Therefore when synthesis is complete, lipids must somehow translocate to the lumenal side of the bilayer. The

protein factors that facilitate this translocation in the ER remain unknown. What is known, however, are the proteins involved in the translocation of PM phospholipids, such as the P-glycoproteins encoded by the MDR3 (also known as MDR2) genes (Borst et al., 2000).

The lateral and transbilayer movement of lipids occurs spontaneously. A single lipid can also spontaneously diffuse between different membranes, however, experimental data suggests that this is a very slow process. As lipids are hydrophobic compounds, special transport proteins or other mechanisms are required to move them through the hydrophilic environment. It is well established that vesicles transport both membrane bound and secreted proteins and it is possible that lipids use the same or similar routes. Transfer of lipids between different membranes may also occur via fusion or hemifusion of membrane bilayers (Vance, 1996).

Specialized detergent resistant membrane (DRM) domains, rafts, are suggested to have a role in both lipid and protein trafficking. These sphingolipid and cholesterol rich microdomains are apparently involved in the sorting of cargo and the formation of vesicles. In addition to this, more and more evidence supports their role in endocytosis and signaling events (Ikonen and Parton, 2000). In mammals, rafts are formed in the Golgi complex (Simons and Ehehalt, 2002) however in yeast they are already formed in ER membranes (Bagnat et al., 2000; Dickson and Lester, 2002). Rafts may be involved in the transport and recycling of newly synthesized free cholesterol between the TGN, endosomes and the PM (Mukherjee and Maxfield, 2000; Simons and Ehehalt, 2002). There are proteins, especially caveolins, typically enriched in lipid rafts (Fielding and Fielding, 2001a; Zajchowski and Robbins, 2002). Rafts do not necessarily contain marker proteins, instead these membrane domains can arise solely through interactions between lipids (Anderson and Jacobson, 2002).

ABC transporters are a family of proteins whose members are known to function as lipid transporters. One family member, ABCA1 is known to be essential for cholesterol efflux and has also been implicated in the transport of cholesterol via the secretory pathway (Borst et al., 2000). Another ABC transporter, ABCB1 is known to transport cholesterol from the PM to the ER (Schmitz and Kaminski, 2001). In addition, members from ABCG, the white subfamily of the ABC transporters, are also involved in cholesterol trafficking (Schmitz et al., 2001). Another type of lipid transporter, NPC (Niemann-Pick C) protein regulates the transport of LDL-derived cholesterol from lysosomal compartments to other parts in the cell, however, the precise function of this protein is still largely unknown. A dramatic neurovisceral lipid storage disorder, called Niemann-Pick type C disease, arises from genetic defects in the NPC protein (Garver and Heidenreich, 2002). Transport of cholesterol to the inner mitochondrial membrane is mediated by StAR (steroidogenic acute regulatory protein), which is required for steroid hormone synthesis in specific tissues (Christenson and Strauss, 2000; Christenson and Strauss, 2001). StAR-related lipid transfer (START) domains also exist in other proteins that are involved in lipid transport and metabolism like PC transfer protein (PC-TP), MLN64 and a putative acyl-CoA thioesterase (Tsujishita and Hurley, 2000).

Intracellular phospholipid transport, translocation, efflux and uptake are all processes that are connected to each other and to the transport of cholesterol/other lipids (Kent and Carman, 1999; Ridgway, 2000). This provides strict control over lipid homeostasis in the cell. In addition to feedback type regulatory mechanisms of lipid biosynthetic enzymes, lipid transport is also an important way to regulate the distribution and overall homeostasis of lipids.

The phosphatidylinositol transfer protein (PITP) family in mammals consists of two proteins; PITPα and PITPβ, which share 77% identity. They are known to bind

and transfer PI and PC, and PITPβ is also known to transfer SM (Segui et al., 2002). In yeast, Sec 14p is an important PITP protein involved in Golgi vesicle transport. It is postulated that Sec14p is needed for scission of COPI coated vesicles from the Golgi membrane (Simon et al., 1998).

Fatty acid (FA) transport is important for the synthesis of membrane lipids and the intracellular messengers derived from them. Furthermore, unsaturated FAs are the precursors for leukotrienes, prostaglandins and tromboxanes and they are needed for energy production (Eaton, 2002; Hettema and Tabak, 2000). In eukaryotic membranes, long-chain FAs (LCFAs) are taken up as LCFA/albumin complexes and their transport within cells is facilitated by specific proteins (Schaffer, 2002). There are a number of known proteins which putatively bind and transport fatty acids in cells, and they can be divided into three main types (Eaton, 2002): plasma membrane FA binding proteins (FABPpm) (Stremmel et al., 1985), FA translocase (FAT/CD36) (Harmon et al., 1991), and FA transport proteins (Abumrad et al., 1999).

3. Intracellular membrane trafficking

3.1 The intracellular trafficking pathways

There are two main membrane trafficking routes in cells: The secretory or biosynthetic pathway is one in which membrane bound or secreted proteins and many of the lipids travel from the ER to either the PM or to various cellular organelles. The other is the endocytic pathway, which is responsible for the uptake of various compounds from the extracellular milieu (Mellman and Warren, 2000; Olkkonen and Ikonen, 2000).

Membrane bound proteins or proteins that are destined for secretion typically contain a signal sequence that targets the translated protein to the rough ER translocation machinery (Mellman and Warren, 2000). The signal sequence can be cleaved as is the case for most secreted or lumenal proteins or otherwise the signal sequence can anchor the protein to the membrane. Proteins may contain many different signal sequences, and these determine membrane topology and the destination of the protein in cellular compartments (Vance, 1996). After translocation, proteins fold in the ER and glycoproteins acquire sugar moieties. They are then transported from the ER to the Golgi complex (*cis*-, *medial* - and *trans* -Golgi) and then to the major protein sorting station of the cell, the trans-Golgi-network (TGN) (Gu et al., 2001).

In the endocytic pathway vesicles are targeted to their intracellular localization, first to early endosomes from which cargo molecules then move to late endosomes and finally to lysosomes for degradation. The receptors are either recycled back to the PM or degraded. In the endocytic pathway receptors and components of the transport machinery are recycled back to the PM following the release of cargo from the vesicle to the target organelle (Mellman and Warren, 2000). To date most of the studies on endocytosis have focused on the clathrin-mediated pathway (Takei and Haucke, 2001). In addition to this route there are several different endocytic routes that are clathrin-independent (Nichols and Lippincott-Schwartz, 2001).

Secretion and endocytosis are connected at the level of the TGN and early and late endocytic organelles. In the next chapter, 3.2, the machinery of both the secretory and the endocytotic route are described.

3.2 Trafficking machinery

All of the steps involved in the vesicle trafficking in the secretory pathway require the fine tuned interaction of cargo molecules, membrane proteins, cytosolic coat proteins and cytoskeletal elements. All of these components are essential for vesicle budding from the ER, docking and fusing to *cis* Golgi membranes, vesicle traffic between the Golgi membranes, to the TGN and finally to the PM (Aridor et al., 1999; Barlowe et al., 1994; Scales et al., 2000). Coat proteins are needed for vesicles to bud. COPII is required for exit from the ER, COPI in both retro- and anterograde transport between the ER and the Golgi, in the intra-Golgi transport and endosomes. Clathrin mediates in transport routes of the endosomal pathway. The specific adaptor proteins, AP-1, -2, -3 and –4, are needed in the endocytic route including also the transport between endosomal organelles and the TGN (Kreis et al., 1995; Takai et al., 2001). Small GTPases of the ARF (ADP-ribosylation factor) family are considered important for vesicular transport, especially along the secretory and endocytic pathways (Takai et al., 2001). Arf1 and another small GTPase, Sar1, are postulated to have a function in the budding of transport vesicles from the ER and Golgi membranes (Takai et al., 2001; Ward et al., 2001). Transmembrane receptors can bind to either soluble or membrane bound ligands and interact with cytosolic coat protein complexes. Small GTPases are needed not only for assembly of the coat complex and budding of the vesicle, but also for targeting transport vesicles to the correct acceptor compartment. The largest family of small GTPases is the family of Rab proteins. Over 50 members of this family have been isolated in mammals. These proteins function in transport vesicle formation, vesicle movement along the cytoskeletal elements and especially in the specific tethering of vesicles at the target compartments (Pfeffer, 2001; Smythe, 2002; Zerial and McBride, 2001).

For vesicle targeting in the secretory and endocytic pathways, another organelle-specific family of proteins is required. After vesicles are tethered at the acceptor compartment, a more intimate docking and fusion of membranes occurs. This process is mediated by proteins called SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins. There are 36 known mammalian SNARE proteins and many of them have corresponding yeast homologues. For fusion to occur, vesicle SNAREs (v-SNAREs) and the target membrane SNAREs (t-SNAREs) interact. Four α-helixes from v-and t-SNAREs interact to form a coiled coil structure, which is thought to bring membranes of the vesicle and of the target close to each other to facilitate fusion. The exact mechanism of how the fusion pore finally evolves is still under discussion (Bruns and Jahn, 2002; Hay, 2001; Hepp and Langley, 2001).

3.3 The role of membrane lipids in vesicle transport

Vesicle transport of proteins is relatively well characterized, however, the importance of lipids is only beginning to emerge. The lipid composition of vesicles is controlled by lipid modifying enzymes. In the secretory pathway, formation of vesicles in the ER requires the action of phospholipase D (PLD) (Wakelam et al., 1997) to cleave off the polar head group of PC and thereby to produce PA (Howe and McMaster, 2001). The COPI dependent Golgi to ER recycling pathway is stimulated by PA and PtdIns(3,4)-bisphosphate and possibly inhibited by diacylglycerol (DAG) (Roth, 1999). In constitutive secretion, PLD plays the important role of stimulating the production of PtdIns(3,4)-bisphosphate, which is required for vesicle formation (Simonsen et al., 2001). Formation of some Golgi derived vesicle populations appears to require PtdIns(3)-phosphate, and PtdIns(3,4,5)-triphosphate is suggested to stimulate the nucleotide exchange of ARF during regulated secretion. In addition to the enzymes

that modify polar head groups of lipids, other enzymes that modify the FA composition of membrane lipids have an effect on vesicle transport. Acyltransferases like endophilins (Huttner and Schmidt, 2000) and BARS (Weigert et al., 1999) play a role in modifying the FA moieties of lipids. This results in changes in the shape of lipid molecules, which is also important for vesicle transport.

Phosphoinositides, DAG and PA are believed to be important during different secretory and endocytotic steps (Goni and Alonso, 1999; Takenawa and Itoh, 2001). Phosphoinositides are formed in response to PA production (by PLD) (Roth, 1999). In clathrin-dependent endocytosis several proteins (like AP-2) bind to $PI(4,5)P_2$. Association of other proteins with AP-2 initiates assembly of components of the vesicle membranes into clathrin-coated pits. Additional proteins are then required for invagination and scission of the clathrin-coated pits (Takenawa and Itoh, 2001).

4. Fundamental mechanisms responsible for cellular cholesterol homeostasis

Cellular cholesterol exists in two forms. Free cholesterol is the form found in membranes and cholesterol esters are found in cytosolic droplets as a cholesterol reservoir. To maintain cellular cholesterol balance, cells can make cholesterol by de novo synthesis or obtain cholesterol from the external enviroment. When there is an excess of cholesterol inside the cell, the cholesterol can be effluxed to extracellular acceptors or stored as cholesterol esters. Cholesterol is synthesized in the ER, but only a small fraction of the total cellular cholesterol is found in the ER membranes. Part of the cholesterol is transported via the Golgi complex and TGN to the PM where the major fraction of cellular cholesterol is located. The major pathway of cholesterol transport seems to bypass the Golgi. The transport mechanism behind the Golgi bypass route is still under investigation (Simons and Ikonen, 2000). An overview of cellular cholesterol processing and trafficking is given in Figure 2. The way cholesterol is metabolized depends largely on the cell type. In the liver, in adipose tissue, in macrophages or in steroidogenic tissue cholesterol is used for very different purposes.

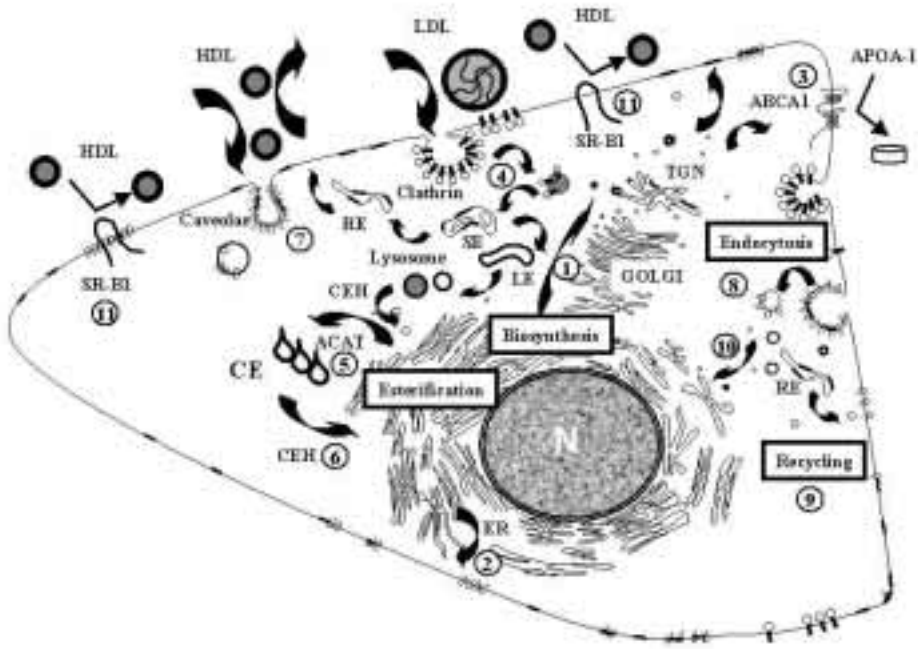

Figure 2. Cellular cholesterol processing and trafficking.
Cholesterol is synthesized in the ER and transported partly via the Golgi complex (**1**) and TGN to the PM. In the PM cholesterol is distributed between raft (black boxes in the membranes) and non-raft microdomains. This can happen already in the Golgi or TGN. A majority of cholesterol, however, bypasses the Golgi and is transported via yet unknown pathway to the PM (**2**). Cholesterol can be internalized back from the PM via endosytosis using either clathrin-coated vesicles or other vesicles, such as those containing caveolin (**8**). Cholesterol can then be recycled back to the PM (**9**) or to the ER (**10**). Cholesterol can be taken up from the external milieu via receptor mediated uptake of LDL particles (**4**). LDL is endocytosed via clathrin-coated pits and cholesterol is transported to sorting endosomes (SE). From there, cholesterol can be recycled back to the PM either directly or via recycling endosomes (RE). The internalized cholesterol is transported to late endosomal compartments (LE) and to lysosomes. In endocytic organelles, cholesterol esters are hydrolyzed by cholesterol ester hydrolases (CEHs) and the free cholesterol is transported to the ER. In the ER, free cholesterol produced *de novo* or taken up by the cells is esterified by the enzyme acyl-coenzyme A:cholesterol acyl-transferase (ACAT) to produce cholesterol esters (**5**). Cholesterol esters are then stored as lipid droplets in the cytosol, however, the cholesterol can again be mobilized by hydrolysis (**6**). Direct cholesterol exchange can also occur between circulating lipoproteins and the PM. Caveolae have been implicated in the uptake of HDL-derived cholesterol esters (**7**) and free cholesterol can be taken up from LDL particles. SR-B1 is involved in cholesterol ester uptake from HDL particles (**11**). During active cholesterol efflux, lipids are removed via the ABCA1 mediated pathway to lipid-poor apoA-I from which nascent HDL particles form (**3**). Cholesterol can be released from cells both from raft or non-raft domains.

4.1 Receptor mediated cholesterol uptake

Cholesterol can be endocytosed from the PM via clathrin-coated vesicles or other types of vesicles such as caveolae. Cholesterol-sphingolipid rafts are found in the PM and in sorting and recycling endosomes. From endosomes, cholesterol can be transported either to the Golgi and then to the ER or recycled to the PM (Fielding and Fielding, 2000; Fielding and Fielding, 2001a). The LDL receptor pathway is the best-described route of cholesterol uptake. LDL particles are internalized together with the receptor in clathrin-coated pits and then transported to sorting endosomes. The receptors recycle to the PM and LDL particles are transported to lysosomes where cholesterol esters are hydrolyzed to free cholesterol by cholesterolester hydrolases, CEH (Belkner et al., 2000; Kraemer et al., 2002).

4.2 Cholesterol esterification

Esterification of free cholesterol is accomplished by acyl-CoA cholesterol acyltransferase (ACAT), which resides in the ER. Free cholesterol is a component of membranes and when an excess amount of cholesterol is produced or internalized by the cell it can be esterified and stored as lipid droplets (Chang et al., 2001). In macrophages, this accumulation of lipid droplets leads to the formation of ‘foam cells’ that are found in atherosclerotic lesions during the early stages of the disease (Kruth et al., 2002). On the other hand, in hepatocytes, CE can be incorporated in apolipoprotein B (apoB)-containing lipoproteins and secreted (Buhman et al., 2000). There are two closely related variants of ACAT; ACAT1 and ACAT2. They are differentially expressed as ACAT1 is more ubiquitous and ACAT2 is tissue-specific and expressed mainly in the liver and intestine. Interestingly in the liver, ACAT2 is localized to hepatocytes whereas ACAT1 is expressed by Kupffer cells (Lee et al.,

2000). The active sites of the two proteins seem to localize to the opposite sides of the ER membrane with ACAT1 positioning at the cytosolic side and ACAT2 at the lumenal side. This may indicate their different functions in cellular cholesterol esterification (Joyce et al., 2000).

4.3 Cholesterol efflux

Cholesterol is constantly effluxed from cells by desorption to circulating lipoproteins. This type of efflux is often denoted as diffusion-mediated or passive. High-density lipoproteins (HDL) are protective against atherosclerosis as they play a major role in cholesterol efflux from specific cell types such as macrophages An active, apoA-I mediated efflux, is dependent on ABCA1. This protein could promote cholesterol translocation from the cytosolic leaflet to the outer leaflet of the PM (Mendez et al., 2001). Interestingly, SR-B1 is postulated to have a role in bidirectional flux of cholesterol between cells and lipoproteins. Overexpression of this protein enhances the efflux of cholesterol from macrophages to lipoproteins, specifically to HDL, and alters the steady state level of both cholesterol and phospholipids in these cells (de la Llera-Moya et al., 1999; Yokoyama, 2000). In the liver, cholesterol from peripheral tissues can be taken up by the receptor mediated pathway or by the SR-B1 pathway (Krieger, 2001). The liver and intestine secrete cholesterol in the form of esters that are complexed with lipoproteins. Another mechanism of cholesterol secretion is also postulated where PM vesicles, possibly enriched with raft lipids are released (Simons and Ikonen, 2000).

4.4 Conversion of cholesterol to bile acids

An important route by which cholesterol is removed from the body is through its catabolism into bile acids by hepatocytes. Bile acids can then be excreted into the lumenal space of the intestine via the gallbladder. The production of bile acids is considered a part of the reverse cholesterol transport process as the cholesterol that is effluxed from peripheral cells is taken from the circulation by the liver and converted into bile acids for excretion (Nofer et al., 2002). The regulation of this process will be discussed in chapter 6.2.

4.5 Cholesterol synthesis

The role of sterol regulatory element-binding proteins (SREBPs) is to regulate the genes of cholesterol synthesis and uptake, and fatty acid synthesis. SREBPs are transcription factors, and the repertoire of their target genes is constantly expanding (Shimano, 2001). SREBPs are localized to ER membranes and are inactive when they are in the full-length membrane bound form. The N-terminal portion of the protein contains the activator and is proteolytically cleaved by site-1 and site-2 proteases in the Golgi. SREBP cleavage activating protein (SCAP) is needed for this cleavage process. When sterol is depleted SCAP undergoes conformational changes and SCAP/SREBP complex is then targeted to the Golgi for the two-step cleavage. After cleavage, SREBP is transported to the nucleus by importin β (Brown et al., 2002; Nagoshi et al., 1999) and SCAP is recycled back to the ER. The sterol sensing process that activates SCAP is still under intense study and is not yet solved. In the nucleus, SREBP binds to specific sequences denoted as sterol-regulatory elements (SREs) or E-boxes present in a number of promotors. There are three isoforms; SREBP-1a, -1c and –2 that are identified to date. SREBP-1a and –1c are products of the same gene

and arise through the use of alternate promotors, whilst a separate gene encodes SREBP-2. The three isoforms have specialized roles in lipid metabolism. SREBP-1 is more likely involved in fatty acid and glucose/insulin metabolism, whereas SREBP-2 plays an important role in the regulation of cholesterol synthesis and uptake (Shimano, 2001).

The regulation of the SREBP-1c gene is interesting. The promoter contains both a SRE element for mature SREBP and a LXR element for the liver X receptor/retinoid acid receptor (LXR/RXR) heterodimer to bind. When oxysterols are present they inhibit maturation of SREBP and no activation of SREBP-1c takes place. On the other hand, oxysterols activate LXR/RXR and binding of this complex to the LXR element in the promoter region activates the gene. This LXR/RXR activation of SREBP-1c seems to form a link between fatty acid and cholesterol metabolism (Repa et al., 2000a; Yoshikawa et al., 2001).

LXRs are cholesterol sensors that regulate many genes responsible for lipid homeostasis (Lu et al., 2001). They are expressed in many tissues. LXRα is expressed in tissues associated with lipid metabolism such as adipose tissue, kidney, intestine, macrophages, lung and adrenals. LXRβ seems to be ubiquitously expressed (Lu et al., 2001). LXRs are activated by naturally occurring oxysterols including 22R-hydroxycholesterol, 24S-hydroxycholesterol, 24S,25-epoxycholesterol and 27-hydroxycholesterol. Regulation of cholesterol homeostasis by LXR/RXR has become increasingly central in the field because these nuclear receptors are not only regulators of the SREBP pathway, but control the expression of a multitude of key genes involved in different aspects of cholesterol metabolism, including ABCA1, ABCG1, ABCG4, ABCG5 and ABCG8 (Repa et al., 2000b) and apoE, LPL, CETP (Chawla et al., 2001; Edwards et al., 2002; Landis et al., 2002). In the liver, the regulation of bile acid synthesis through the classical Cyp7a pathway is mediated by LXRα, FXR and

SREBP. When oxysterols are available LXRα upregulates Cyp7a transcription and at the same time oxysterols suppress the de novo synthesis of cholesterol via SREBP. Bile acids suppress Cyp7a transcription and enhance the transport and intestinal absorption of bile acids via their interaction with FXR (Davis et al., 2002; Russell, 1999).

5. Connections between cholesterol and phospholipid metabolism

Stoichiometric ratios of different membrane lipids are tightly regulated. When this equilibrium is altered, the cells try to adapt so that the ratios between these lipid components remain constant (Ridgway et al., 1999). The mechanisms behind the regulation of cellular phospholipid and cholesterol metabolism seem to be interconnected (Ohvo-Rekilä et al., 2002; Ridgway, 2000). When the PM is depleted of SM, biosynthesis of cholesterol is decreased and its esterification is increased. On the other hand, alterations in cellular cholesterol content may not be equally reflected in SM metabolism (Ridgway, 2000). However, J.P. Slotte and co-workers reported, using cultured human fibroblasts, that when cholesterol is depleted from the PM by cyclodextrin or by apoA-I containing POPC vesicles, the synthesis of SM is increased and the transport of newly synthesized SM is inhibited (Leppimäki et al., 1998; Ohvo-Rekilä et al., 2002). Also, addition of sterols to cultured fibroblasts induced upregulation of PC synthesis (Leppimäki et al., 2000). The well-described regulator of cholesterol biosynthesis, SREBP (see chapter 4.5), also regulates FA synthesis and in this way affects PL synthesis (Shimano, 2001). Furthermore, the rate limiting enzyme in PC biosynthesis, CTP:phosphocholine cytidylyltransferase (Kent, 1997), is subject to SREBP regulation (Kast et al., 2001).

6. Oxysterols

6.1 Structure

Oxysterols are 27-carbon oxygenated derivatives of cholesterol. The nomenclature describes the chemical bondage and active group attached and the number describes the target carbon in the cholesterol backbone. Cholesterol may be oxygenated mainly by hydroxylation, epoxylation or peroxylation. Oxysterols may be formed by autooxidation, by enzymatic activation by monooxygenases, or by lipid peroxidation (Björkhem and Diczfalusy, 2002). Structures of some important oxysterols are depicted in Figure 3.

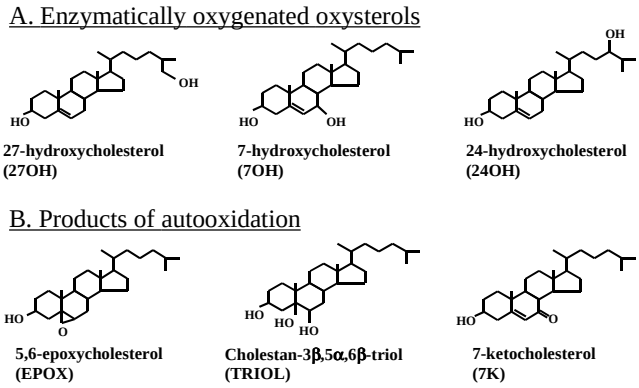

Figure 3. **Examples of biologically important oxysterols. A.** The most abundant oxysterols in the circulation **B.** Important autooxidation products of cholesterol found in LDL and in foam cells/plaques in the arterial wall.

6.2 Biological functions of oxysterols

Oxysterols are biologically active derivatives of cholesterol and have regulatory roles in a variety of cellular processes, such as in the formation of bile acids, cholesterol biosynthesis, uptake and efflux. They also have cytotoxic effects with suggested roles in several pathological processes (Brown and Jessup, 1999; Goldstein and Brown,

1990; Janowski et al., 1996; Repa et al., 2000b; Smith, 1996) (Figure 3). An important feature of oxysterols is their ability to pass membranes much faster than cholesterol. Together with the fact that after oxidation the half-life of the molecule diminishes, oxysterols are useful means of secreting excess sterol. Oxysterols have a number of biological roles, in particular the regulation of cholesterol homeostasis by affecting the synthesis and catabolism of cholesterol to bile acids. Oxysterols are also postulated to have important roles in atherosclerosis, the formation of gallstones, inflammation, immunosupression, apoptosis and necrosis (Björkhem and Diczfalusy, 2002; Brown and Jessup, 1999; O'Callaghan et al., 2002; Panini and Sinensky, 2001; Vaya et al., 2001).

Oxysterols are intermediates in cholesterol excretion pathways and quantitatively the most important oxysterols are those involved in bile acid formation. Classical starting points in bile acid synthesis are the cholesterol hydroxylation products 7α-hydroxycholesterol and 27α-hydroxycholesterol (Javitt, 2002). The most important pathway starts with 7α-hydroxylation of cholesterol by cholesterol 7α-hydroxylase (CYP7A1), a hepatic cytochrome P-450 enzyme operating predominantly in the liver. This pathway is under strict metabolic control and the best known regulatory mechanism is the feedback control by bile acids reabsorbed from the intestine (Repa and Mangelsdorf, 2000). The bile acid sensor FXR, farnesoid X-activated receptor, is a nuclear receptor highly expressed in the liver. Repression of CYP7A1 is mediated by hormone receptors denoted SHP and LRH1 which both have FXRE (FXR response element) in their promotor sequence (Edwards et al., 2002). CYP7A1 is also regulated by LXRα in the mouse and also the rat, however, in humans, regulation via this route is an open question as no LXR-responsive elements are found in the promoter of the human CYP7A1 gene (Björkhem and Diczfalusy, 2002; Edwards et al., 2002).

In addition to the 7α-hydroxylase pathway, an acidic pathway is also used in the liver to make bile acids. In contrast to CYP7A1, the enzyme catalyzing the first step in this route, CYP27A1, has a broad substrate specificity and is present in many tissues. Also, extrahepatic 27-oxygenated cholesterol is taken up by the liver and can be converted to bile acids via this route. It is estimated that 5-10% of the bile acids produced by this pathway are derived from extrahepatic 27-hydroxylation (Lund et al., 1996).

As oxysterols can pass the blood-brain barrier, they are considered important in brain cholesterol metabolism. In the brain a high rate of cholesterol turnover occurs, possibly resulting from an extensive, highly active membrane trafficking in the nervous system (Lange et al., 1995; Lütjohann et al., 1996). The 24-hydroxylation of cholesterol to 24S-hydroxycholesterol is specific for the brain and the gene encoding this cytochrome P450 enzyme is CYP46. The expression of this gene in the brain is 100-fold greater than in other tissues (Lund et al., 1999). The importance of this pathway lies most probably in the transport of cholesterol out of the central nervous system as 24S-hydroxycholesterol has the ability to pass the blood-brain barrier in contrast to cholesterol itself. This hydroxysterol is then taken up by the liver and half of it is converted to bile acids while the other half is conjugated to sulfuric acid and/or glucuronic acid before excretion (Björkhem et al., 2001).

The potency of 25-hydroxycholesterol (25-OHC) as an inhibitor of cholesterol synthesis has been known for a long time (Kandutsch et al., 1978), yet only recently the human cDNA of the enzyme responsible of its formation, CH25H, was described (Lund et al., 1998). This enzyme is expressed in many tissues and does not belong to heme iron containing proteins, as are the previous enzymes described in this chapter (Lund et al., 1998). The 25-hydroxylation product of cholesterol has no significant

role in bile acid synthesis and the amounts detected in the circulation are minor (Björkhem and Diczfalusy, 2002).

Not only the oxysterols that are produced enzymatically, but also the ones formed by autooxidation are potent regulators of cellular cholesterol homeostasis. Many of these are highly cytotoxic, they are present in oxidized LDL, and could therefore contribute significantly towards the development of atherosclerosis (Panini and Sinensky, 2001; Vaya et al., 2001).

There is mounting evidence for the proapoptotic activity of oxysterols and this includes inflammatory responses (Landis et al., 2002; Rosklint et al., 2002), foam cell formation (Hayden et al., 2002) and apoptosis (Panini and Sinensky, 2001). The two known apoptotic pathways are the extrinsic (death receptor pathway, Fas pathway) (Lee and Chau, 2001) and the intrinsic (mitochondrial pathway) (Lizard et al., 1998; Yang and Sinensky, 2000). In the Fas/Fas ligand-mediated pathway, death mediators like p53, FAS and FAS ligand are upregulated when smooth muscle cells (SMCs) or vascular epithelial cells are treated with oxidized LDL. Oxysterols like 7-OH or 25-OH cholesterol were found to induce a similar response (Lee and Chau, 2001). The mitochondrial route involves the release of the cytochrome c from the mitochondria and the activation of caspase-9 (Walter et al., 1998). The work of Yang and Sinensky 2000 and Lizard et al 1998 has shown that 25-OHC activates the cytochrome c release-mediated caspase cascade (Yang and Sinensky, 2000) and that 7-ketocholesterol induces apoptosis using this same pathway (Lizard et al., 1998).

7. Oxysterol binding protein, OSBP

In the early 1980s the launched hypothesis was that cholesterol synthesis is regulated via an oxysterol binding protein; a cytosolic component that bound to oxysterols (Taylor and Kandutsch, 1985). Today two protein families that are known to bind oxysterols have been described: The liver X receptors (LXRs) (Chawla et al., 2001) and the protein family described as cytoplasmic oxysterol receptors, termed oxysterol binding proteins (OSBPs) or OSBP related proteins, ORPs (see Results and discussion, chapters 1. –3. in this study; (Jaworski et al., 2001). The cDNA of the human OSBP protein was first described in 1990 by Levanon and others. In addition, there is a high degree of homology between OSBP proteins from human and other mammals (Levanon et al., 1990).

The OSBP gene is located in the long arm of human chromosome 11 and the corresponding mouse gene is at the proximal end of mouse chromosome 19. OSBP is a cytosolic protein of 809 amino acids (Ridgway et al., 1992). Human and mouse OSBP cDNAs are 94% identical and the encoded proteins share 98% identity between their predicted amino acid sequences (Levanon et al., 1990). In SDS-PAGE the protein migrates as a doublet of 96 and 101 kDa. The slower migrating band represents a phosphorylated form. The phosphorylation is inhibited by the Golgi dissociating toxin brefeldin A because the rapid phosphorylation / dephosphorylation of OSBP requires interaction with Golgi membranes and an associated kinase (Ridgway et al., 1998a). OSBP is also rapidly dephosphorylated when plasma membrane SM is hydrolyzed by exogenous bacterial sphingomyelinase (Ridgway et al., 1998b). The structure of OSBP consists of two main parts: the N-terminal part of the protein which contains a pleckstrin homology (PH) domain (residues 87-185) and the C-terminal domain (residues 417-750) containing a sterol-binding (SB) domain

(Levine and Munro, 1998; Ridgway et al., 1992). The protein binds 25-OHC with high affinity, but it also interacts with many other oxysterols (Taylor et al., 1984).

When OSBP is overexpressed in Chinese Hamster Ovary (CHO) cells it is partly soluble and partly bound to small perinuclear vesicles. Upon binding to its ligand the protein redistributes to the Golgi area. The PH domain is needed for this shift in localization as shown by using a truncated protein lacking the N-terminal region (Ridgway et al., 1992). Many of the proteins containing PH domains are involved in intracellular signaling and cytoskeletal organization, like protein kinases (Btk, β-ARK and Akt), phospholipase C (PLC) isoforms, insulin receptor substrates (IRS-1 and –2), the phosphoinositide 3-kinase (PI3-kinase) p110γ subunit, the guanine nucleotide release factor SOS, the GTPase-activating protein rasGAP and the dynamin GTPase. Four proteins, pleckstrin, CDC25, PLC-γ and Tiam1 contain two copies of a PH domain (Bottomley et al., 1998). Sequence similarity between different PH domains is low (10-20%). This could implicate that their function in cells is diverse in accordance with the fact that they bind a variety of different phosphoinositides as well as proteins. Lefkowitz and co-workers showed that the C-terminal portion of the PH domain and the sequences flanking the PH domain in several proteins bind to the βγ-subunit of G proteins (Touhara et al., 1994). The results of Kawakami and co-workers suggest that the PH domains of several proteins bind to filamentous but not to monomeric actin (Yao et al., 1999). Models for prediction of the ligand binding characteristics of PH domains are under study and some classification of proteins containing PH domains has been done. In one classification Maffucci and Falasca (2001) divide the domains into three groups. In the first group there are PH domains that bind with very high affinity to specific phosphoinositides like $PI(4,5)P_2$ (PLC-δ1), $PI(3,4,5)P_3$ (Btk and Grp1), and these proteins are targeted to the PM. In the second group the PH domains bind with lower

affinity/or specificity to phosphoinositides and are unlikely to be sufficient to drive the translocation of proteins to the PM (Gap1, Dbl, IRS-1). The third group includes PH domains that bind non-specifically to phosphoinositides such as in pleckstrin, DAGK-δ and dynamin (Maffucci and Falasca, 2001).

Levine and Munro have studied the targeting of the PH domain of OSBP (PH^{OSBP}) and other related PH domains in yeast using GFP-PH domain fusion proteins. There are several proteins that have a PH domain highly related to that of OSBP. These include Goodpasture antigen binding protein (GPBP) in human and PtdIns 4-phosphate adaptor protein-1 (FAPP1) in several mammals. The function of these proteins is not known, but the similarity in PH sequence to that of OSBP raises the possibility of similarity in subcellular targeting (Levine and Munro, 2002). Previously, PH^{OSBP} was shown *in vitro* to bind both PI(4)P and PI(4,5)P_2 and the cleavage of the headgroup of the phosphoinositides by phospholipase δ1 blocked PH^{OSBP} targeting to Golgi membranes (Levine and Munro, 1998). It may be that the targeting determinant for Golgi membranes is PI(4)P, although it is unlikely that the localization of PI(4)P is strictly restricted only to Golgi membranes. In addition, analysis of a mutant version of PH^{OSBP}, which does not bind to phosphoinositides, shows some Golgi targeting that is dependent on Arf1p but not on PtdIns 4-kinase1 (Pik1) activity. So it seems that Golgi targeting of PH^{OSBP} involves both Pik1-dependent and –independent components (Levine and Munro, 2002).

Neale Ridgway and co-workers have studied the connection of the OSBP protein to cholesterol and sphingomyelin (SM) metabolism. Using CHO-K1 cells stably overexpressing OSBP, they show that cholesterol synthesis is upregulated by the protein. When mock cells and cells overexpressing OSBP are treated with 25-OHC the synthesis of cholesterol decreases in both cells (Lagace et al., 1997). They have also shown that OSBP stimulates the upregulatory effects of 25-OHC on SM

synthesis (Lagace et al., 1999). It appears that the transition of OSBP from the cytosol to the Golgi upon binding to 25-OHC stimulates the conversion of ceramide to SM. Upregulation of cholesterol esterification after 25-OHC treatment is of the same magnitude in both the control cell line and in the OSBP overxpressing cells, which implicates that OSBP does not activate ACAT (Storey et al., 1998). The protein seems to be involved in both cholesterol and SM metabolism, but its precise function is still unrevealed (Lagace et al., 1997; Lagace et al., 1999). Further, there is evidence that OSBP affects the trafficking of both proteins and ceramide from the ER to the Golgi (Wyles et al., 2002). These latest results together with the proposed function of an OSBP related protein from yeast, Kes1, in Golgi vesicle trafficking (Fang et al., 1996; Li et al., 2002), suggest that OSBP and related proteins may have an important role in intracellular transport of lipids and proteins.

8. OSBP-related proteins

Since the discovery of the human OSBP protein there has been a tremendous step forward in finding new homologues in different organisms. In the human family there are now 12 proteins (OSBP and ORP1-11; in this study) and the corresponding genes are also found in the mouse (Anniss et al., 2002),. In yeast there are 7 proteins (Osh1p-7p) see Results section and (Beh et al., 2001; Jiang et al., 1994). In addition to these, other eukaryotes like *Drosophila melanogaster* (OSBP-Dm) (Alphey et al., 1998) and *Caenorhabditis elegans* (Sugawara et al., 2001) are known to have OSBP homologues. The yeast OSBP homologues can be divided into two groups: the short homologues containing only the oxysterol binding domain (Osh4p-7p) and the long homologues containing also an N-terminal stretch with the PH domain (Osh1p-3p). In addition, Osh1p and Osh2p contain ankyrin repeats in the N-terminal sequence.

Ankyrin repeats mediate protein-protein interactions and are found in many cytoskeletal proteins and in some transcription factors (Sedgwick and Smerdon, 1999). There is no cholesterol in yeast, but instead there is ergosterol, a major component of yeast cell membranes (Zinser et al., 1991). Beh and co-workers have studied the functions of all seven OSBP homologues in yeast. They created disruptions of each gene either alone or in different combinations and found that none of the single disruptions were lethal. Only when all of the seven genes were disrupted, yeast cells no longer survived (Beh et al., 2001). The intracellular localization of Osh1p, Osh2p and Osh3p has been studied in yeast using N-terminal GFP fusion constructs (Levine and Munro, 2001). Under normal growth conditions Osh1p localizes both to the Golgi and the nucleus-vacuole (NV) junction. The domain responsible for the NV junction localization is the ankyrin domain of the protein. Even when the PH domain of Osh1p is replaced with that of spectrin (this PH domain does not show any specific localization alone) the protein localizes to the NV junction but no longer to the Golgi region. Thus, the PH domain contains Golgi targeting information. Although Osh2p has three ankyrin repeats in the N-terminus, it does not target to the NV junction, nor does the fusion protein containing Osh2p ankyrin region and Osh1p PH and OSBP domains. Osh2p localizes to the PM, especially to the budding area of G1 phase cells and to the bud-neck in S-phase cells. Osh3p, the Osh protein that contains an N-terminal PH domain but no ankyrin repeats, seems to have more diffuse localization throughout the cytoplasm (Levine and Munro, 2002). Recent results from two-hybrid analysis of Osh3p interaction partners show that this protein interacts with the DEAD-box RNA helicase called Rok1p that is involved in nuclear fusion during yeast mating. It is also possible that the protein may function either parallel to or downstream of the STE12 (STE = “sterility genes”) transcription factor and be involved in the regulation of filamentous growth of yeast (Park et al.,

2002). Osh4p, more commonly known as Kes1p is implicated in Golgi vesicle trafficking. It is known that in yeast the phospholipid transfer protein Sec14p is required for biogenesis of Golgi-derived transport vesicles (Bankaitis et al., 1990). The Sec14p defect can be bypassed by Kes1p defect. The dysfunction of this protein leads to reduced metabolic flux through the CDP-choline pathway of PC synthesis and in that way diminishes the toxic effect of the deletion of Sec14p (Fang et al., 1996). Recent data indicate that Kes1p could be a phosphoinositide binding protein and that specific phosphoinositides, especially PI(4)P are required for Golgi localization of Kes1p. The Golgi localization is necessary for the function of the protein. Kes1p function is also connected to that of ARF in yeast (Li et al., 2002).

The detailed description of the family of 12 human OSBP homologues is presented later in the Results and discussion section. In the following paragraph I will give an overview of the present status of the OSBP homologues.

According to Xu and collagues (Xu et al., 2001) the human homologues of OSBP, ORP1 (short variant) and ORP2, bind phospholipids *in vitro*. ORP1 and ORP2 seem to bind PA in a dose dependent manner. The proteins were also reported to bind PI(3)P and cardiolipin with apparently lower affinity. ORP2 localizes mainly to the Golgi (Xu et al., 2001) and intracellular localization of the GFP-ORP1 is cytosolic. Recent data by Marie Johansson and co-workers, however, indicates that the intracellular localization of the long ORP1 variant is not in the Golgi, but rather in endosomal membranes (Johansson et al., 2002). ORP3 is differentially expressed during differentiation of specific hematopoetic cell populations and may have a role in mediating oxysterol effects on hematopoiesis (Gregorio-King et al., 2001). ORP4 (OSBP2) is shown to be highly expressed in the retina and it binds both 7-ketocholesterol and 25-hydroxycholesterol (Moreira et al., 2001; Wang et al., 2002). Results from the group of N. Ridgway show that a longer form of the ORP4 protein

containing the PH domain binds efficiently 25-hydroxycholesterol and to a lesser extent 7-ketocholesterol. A shorter form without the PH-domain shows poor binding of both oxysterols. The shorter form of the protein, however, binds to vimentin intermediate filaments and overexpression of the protein decreases the esterification of LDL-derived cholesterol (Wang et al., 2002). Further, ORP4 is suggested to be a marker protein for the detection of metastatic tumor cells derived from solid tumors in the peripheral blood (Fournier et al., 1999).

AIMS OF THE STUDY

1. **To identify new human proteins related to oxysterol binding protein, OSBP, and to analyze their expression patterns.**
2. **To determine the tissue expression pattern and the cellular localization of one family member named OSBP Related Protein 2 (ORP2).**
3. **To characterize the functional role of this protein in cellular lipid metabolism.**

METHODS

All the methods used in the original articles are summarized in the following table:

Method	**Publication**
Generation of cDNA fragments corresponding to EST contigs	I, II
Cloning of ORP cDNAs	II
Cell culture	I - IV
Transfections and selection of stably transfected CHO cells	III
RNA extraction and Northern blot hybridization	I
Measurement of ORP mRNA levels by semiquantitative RT-PCR	II
Determination of the ORP2 mRNA tissue expression pattern	I, II
In situ hybridization	III
Antibodies	III
Western blotting	III
Flotation of cellular membranes in sucrose gradients	III
Immunofluorescence microscopy	III
Analysis of cholesterol synthesis and transport	IV
Analysis of phospholipid synthesis	IV
Analysis of cholesterol efflux and esterification	III
Preparation of PC vesicles	III
ACAT activity assay	III
Analysis of cellular cholesterol content	III
Analysis of cellular phospholipid content	IV
Analysis of VSVG-GFP transport	III
Mass spectrometric analysis of cellular phospholipids	IV

RESULTS AND DISCUSSION

1. The search for human cDNAs encoding OSBP-related proteins, ORPs (I,II)

To gain insight into the cellular effects of oxysterols we began searching for potential cellular receptors specific for oxysterols. For the first search (I), we used the tBLASTn program to scan the National Center for Biotechnology Information (NCBI) dbEST database for human cDNA sequences that were homologous to the sterol-binding region of OSBP (amino acid residues 297-807). The reason for using the sterol-binding domain only was that use of the full-length sequence containing the PH domain would most probably have resulted in finding numerous PH-domain proteins not belonging to the OSBP-family. In order to exclude extremely short and/or poor quality sequences we set the *P* value cutoff level to 0.0001. The search yielded 40 human ESTs, seven of which represented OSBP itself. The remaining ones were grouped to contigs using the University of Wisconsin Genetics Computer Group (GCG) software. As a result, 8 new sequences were found, that had clear homology to OSBP. They were named OSBP-Related-Proteins, ORPs, number 1-8. Contigs 7 and 8 were so short that they were omitted from further analysis. The next step was to combine cDNA cloning techniques and computational analysis for determining the full-length ORP cDNA sequences (II). During the cloning process, it became evident that the previously described ORP 7 and 8 ESTs were derived from ORP4 and OSBP. In addition to ORP1-6, two novel ORP gene products closely related to ORP3 or 5 were identified. These genes were named ORP7 and 8, respectively. During the

database analysis of the full-length cDNAs of ORP1-8, we discovered three additional cDNAs and these were designated ORP9-11 (II).

2. Functional domains and structural relationships of ORPs (II)

The ORP amino acid sequences were deduced from cDNA sequence information. There are two major structural features of OSBP (the founder member of the protein family). Near the N-terminal end is the PH domain and at the C-terminal end is the sterol-binding (SB) domain (Lagace et al., 1997). By computational analysis, nearly all of the novel ORP family members have been found to contain the same structural features. The most conserved region, the SB domain, consists of approximately 400 amino acids. Interestingly, within the SB domain there is an eight amino acid motif (EQVSHHPP) that is fully conserved across all of the human family members. A PH domain is also found in all members except for ORP2 (after publication of article II, a PH domain in ORP9 was also discovered). Additional features in the structure of some of the proteins were also found. Three ankyrin-like repeats were identified at the N-terminal end of ORP1. Ankyrin repeats are also present at the N-terminal end of the yeast OSBP homologues, Osh1 and Osh2. In addition, ORP5 and ORP8 contain potential membrane spanning segments in the C-terminal ends. According to amino acid sequence and intron-exon structure, the ORP genes can be devided to six subfamilies. Compared to the yeast family of OSBP homologues, ORP2 resembles the four short category of ORPs called Osh4/Kes1p, Osh5/Hes1p, Osh6/Yhg1p and Osh7/Yky3p. Functional data available from Kes1p indicates that this protein is involved in Sec14p-dependent Golgi vesicle trafficking in yeast (Li et al., 2002).

3. Tissue expression patterns of ORPs (I,II)

For the first article it was crucial to verify that the novel sequences were expressed and that they represented distinct genes. In the third article a detailed analysis of ORP2 tissue expression, both at the mRNA and the protein level, was also included.

3.1 Analysis of ORP mRNA expression by Norhthern Blotting (I)

The cDNA probes for each fragment were designed as similar in length as possible to obtain comparable signals for the different mRNAs, and were labeled to a specific activity of 1.5-3 x 10^8 cpm/μg. Commercially available human multiple tissue Northern blots were hybridized according to the manufacturer's instructions. OSBP, the expression of which is reported to be ubiquitous (Dawson et al., 1989; Ridgway et al., 1992) was included in this analysis for comparison. We found that the OSBP mRNA was indeed ubiquitously expressed and that the signal was strongest in the liver and kidney. ORP1, also expressed ubiquitously, was most abundant in the brain, heart, skeletal muscle and kidney. Another new sequence that gave rise to a relatively strong signal was ORP2. With ORP2, we noted a number of differently sized mRNAs suggesting a high degree of differential splicing. The main forms of ORP2 mRNA were 4.4 and 2.8 kb in length, were highly expressed in the brain, heart and kidney and were also present in the liver and placenta. In the heart and in skeletal muscle we detected two additional forms at 6.6 and 1.2 kb in length. In the brain a minor 6.0 kb mRNA was also seen. The hybridization signals from the remaining ORPs were weaker yet clearly tissue-specific. Various sizes of ORP3 mRNAs (7.0, 4.5 and 3.5 kb) were detected at very low levels, the most prominent signals being in spleen and white blood cells. The ORP4 mRNAs were most abundant in the brain (4.8 and 3.8

kb) and heart (3.5 kb mRNA species) tissues. The size of ORP5 mRNA was 4.0 kb and this was equally expressed at low levels in all of the tissues studied. However, the signal was slightly higher in thymus and peripheral blood leukocytes. The weakest signals were obtained with the ORP6 probe. These signals were clearest in brain (7.2 kb) and skeletal muscle (4.0 and 3.5 kb).

The expression of ORP1 mRNA in different regions of the brain was studied further by Northern blotting; The 3.6 kb mRNA was most abundant in the cortical area as compared to the cerebellum and spinal cord. The next step was to study the possible effects of sterol status in neuronal cells on ORP1 mRNA. The human neuroblastoma cell line, SHEP, was grown either in the presence of 10% fetal bovine serum or in 5% lipoprotein deficient serum. After 2 days delipidation the cells were either loaded with LDL for 24 hours or treated with 25-OHC for 4 hours. LDL loading upregulated ORP1 mRNA approximately 2-fold, whereas treatment with 25-OHC gave a minor, 1.5-fold upregulation. The concentration of 25-OHC that was used has been reported in CHO cells to cause a translocation of OSBP from the cytosol to the Golgi (Ridgway et al., 1992). We carried out a similar experiment using the human hepatoma cell line HuH7, however, no significant alterations in ORP1 mRNA level were found. These results implied a specific role for ORP1 in brain sterol balance. In the brain the major oxysterol produced is 24S-OHC (Bjorkhem et al., 1998). One could presume that treatment with 24S-OHC would induce a more significant change in ORP1 expression. Unfortunately, at the time of the experiments, 24S-OHC was not available, and so it was not possible to test the connection between 24S-OHC and ORP1 expression.

3.2 Detailed analysis of ORP2 mRNA expression in human tissues (III)

For a more detailed analysis of the tissue distribution of ORP2 mRNA, a human MTE (Multiple Tissue Expression) filter array was hybridized using the radioactively labeled full-length ORP2 cDNA as a probe. The ORP2 message was detected in all tissues/cells studied and the signal was quantitated using the phosphorimager technique. The quantitation revealed that the highest levels of ORP2 mRNA were in specific parts of the central nervous system (cerebellum, pituitary gland, pons, and putamen) as well as in leukocytes, placenta, and pancreas. The strongest signal was from the cerebellum and this was 10-fold higher than the weakest signal detected in the ovary. These results verified the previous finding that ORP2 has a ubiquitous expression pattern in human tissues and that the highest expression is found in the brain.

3.3 *In situ* hybridization analysis of ORP2 mRNA in mouse embryonic tissues (III)

To determine if the expression of ORP2 mRNA in mammalian tissues is concentrated in specific cell types, *in situ* hybridization of sections from 12-day old mouse embryos was performed. This analysis showed that the ORP2 mRNA was ubiquitous and evenly distributed, however, the expression in certain tissues, such as parts of the spinal cord, dorsal root ganglia and the inferior ganglion of the vagus nerve, was more prominent. This ubiquitous distribution of the message suggests that ORP2 may have a housekeeping function in mammalian cells and that in the brain and in other parts of the nervous system, whose cholesterol content is the highest of all tissues (Björkhem et al., 1998; Dietschy and Turley, 2001), the expression of the gene is elevated.

3.4 ORP2 protein expression in mouse tissues (III)

To analyse ORP2 distribution in different tissues at the protein level, various mouse tissues were homogenized and equal amounts of total protein (15 μg) were analyzed by Western blotting. A rabbit antibody was generated against a GST-ORP2 fusion protein expressed in *E. coli*. The antibodies were affinity purified using the GST-ORP2 fusion protein, and in Western blots of CHO cell lysates they recognized a major endogenous protein band of 56 kDa as well as additional 60 kDa and 51 kDa bands. The 56 kDa band corresponds to the protein encoded by the cloned cDNA. In the majority of mouse tissues this antibody revealed two major bands with apparent molecular masses of 56 and 51 kDa. An expression pattern similar to that seen in the Northern blots or by *in situ* hybridization was also seen in the Western analysis of mouse tissues. ORP2 is ubiquitously expressed and the brain tissue contains the highest amount of this protein. Interestingly, both forms of the protein have slightly different distributions among the mouse tissues. The higher molecular mass (56 kDa) band is absent in liver and kidney whereas skeletal muscle lacks the 51 kDa band. Variation between the relative abundance of these bands is also apparent across different tissues. It is possible that these two immunoreactive forms of the protein represent variants translated from differently spliced mRNAs or that the 51 kDa form is proteolytically cleaved from the larger form. Taken together all of the expression data obtained thus far supports the suggestion that ORP2 may have a housekeeping function in cells, and in certain specialized cells, like in the nervous system, higher expression of this protein may be needed.

4. Intracellular distribution of the ORP2 protein (III)

The intracellular protein distribution of ORP2 was studied by cell fractionation and by indirect immunofluorescence microscopy. At the time, published data on the subcellular localization of any other ORPs was unavailable. Fortunately, our antibody recognized the endogenous ORP2 protein in CHO cells, and thus enabled us to study the localization of ORP2 without overexpressing the protein. The post-nuclear supernatant of CHO cells was fractionated by ultracentrifugation in sucrose step gradients and was followed by Western blotting. Syntaxin 2, an integral membrane protein, was used as a control for the total membrane fraction. The 56 and 51 kDa forms of the protein distributed between membrane (20%) and soluble (80%) fractions. Interestingly, the 60 kDa band was entirely membrane associated. The affinity-purified antibody was used for immunofluorescense microscopy and both the endogenous and stably overexpressed ORP2 protein were found to localize to the cytosol and to the perinuclear region in CHO cells. The co-localization with different cellular markers was studied and the bright perinuclear aspect of the staining coincided partially with a Golgi marker, FITC-lentil lectin. It is obvious that another mechanism than that of the PH domain is responsible for ORP2 protein association with membranes. A detailed analysis of the localization of the yeast ORP proteins, Osh1p, 2p and 3p, was recently published (Levine and Munro, 2001). All of the proteins contain a PH domain but only Osh1 is localized to the Golgi and in addition, it localizes to the nuclear-vacuolar junction via its ankyrin repeat sequence. There are also indications that sequences flanking the PH domain are important for the targeting specificity of Osh proteins and it is possible that this could be a more general feature in the OSBP family (Levine and Munro, 2002). In Osh4p (Kes1p), a short ORP protein, the Golgi localization and binding of phosphoinositides are needed for its

function in vesicular trafficking. It is postulated that the ligand binding domain of Osh4p has a PH domain like structure (Li et al., 2002). Therefore, the lipid binding properties of possible PH domain-like structures within the ORP ligand-binding domain, may play a role in targeting these proteins to specific locations. At the time of these studies Xu and others reported that a variant of ORP2 lacking amino acid residues 13-24 does not bind oxysterols (Xu et al., 2001). Instead, ORP2 was reported to interact with phosphatidic acid (PA) and weakly with inositol-3-phosphate.

5. The effect of ORP2 overexpression on marker protein trafficking through the secretory pathway (III)

By transiently overexpressing ORP2 in CHO cells, we were able to study the effect that this may have on the trafficking of GFP-tagged vesicular stomatitis virus G (VSVG) protein through the secretory pathway. The VSVG that was used carries the temperature sensitive ts045 mutation that causes its arrest to the ER at 40^{o}C. CHO cells were double transfected either with a control plasmid (pcDNA3.1/CAT) or with pcDNA3.1/ORP2 together with the VSVG-GFP expression vector. Cells were then grown at 40^{o}C for 20 hours during which time the VSVG-GFP accumulated in the ER. The temperature block was then released in by lowering the temperature to 32^{o}C and incubating the cells for increasing time periods. In cells transfected with the control plasmid, VSVG-GFP was in the Golgi after 1 h, and after 4 h the marker had reached the PM. In cells overexpressing ORP2, transport to the Golgi was unaffected. However, transport from the Golgi to PM was inhibited as evidenced by the fact that even after 4 hours at 32^{o}C, Golgi structures displayed a strong fluorescence. This was observed specially in the cells with extensive overexpression of ORP2.

The yeast OSBP homologue, Kes1p (Osh4p), is implicated in Golgi vesicle transport (Fang et al., 1996; Li et al., 2002). In addition to the finding that inactivation of Kes1 bypasses the Sec14p defect (Fang et al., 1996), new data revealed that Kes1p could regulate the ARF cycle in Golgi vesicle transport (Li et al., 2002). Xu and co-workers have shown that when human ORP2 is overexpressed in yeast, trafficking of carboxypeptidase Y to the vacuole is disturbed (Xu et al., 2001). The above findings, together with the functional data on OSBP (Wyles et al., 2002), support the notion that ORP2 may have a role in vesicular trafficking.

6. Lipid metabolism in stably transfected CHO cells overexpressing ORP2 (III,IV)

To determine whether ORP2 is involved in the control of cellular lipid metabolism, we created stably transfected cell lines overexpressing the protein. All of the results we obtained from the ORP2 expressing cells (ORP2/CHO) were compared to a mock transfected cell line containing the empty vector.

6.1 Cholesterol efflux (III)

We first studied the effects of ORP2 expression on the cholesterol efflux process by labeling the cells with [^{14}C]cholesterol for 36 hours. The cells were then washed and efflux was performed to various acceptors. Three different acceptors were used. Small unilamellar PC vesicles (0.3mg PC/ ml) were used for studying non-specific diffusion, human apoA-I (20μg/ml) for possible effects on the more specific ABCA1 mediated pathway and human serum (20 %) was also used as it is a physiological acceptor that represents a combination of both efflux routes. ORP2 enhanced the

efflux of [^{14}C]cholesterol to a similar extent to all of the acceptors. The change in efflux may be caused by changes in the trafficking machinery involved in the process. Another possibility could be a change in the lipid composition in the membranes, especially the PM. Alterations in the synthesis, uptake or esterification of cholesterol could also affect the cholesterol efflux. More rapid or slow recruitment of cholesterol from the stores could also have an effect on the efflux. According to the results obtained it seems that the ORP2 effect was not specific for any single efflux route studied (Fielding and Fielding, 2001b).

6.2 Cholesterol biosynthesis (IV)

To see if the stable expression of ORP2 affects cholesterol synthesis the cells were grown in either complete medium or in delipidated medium. The cells were then pulse-labeled with [^{3}H]acetate for 15 minutes, followed by a 2 hour chase in the presence of mevalonate and lovastatin to inhibit further cholesterol synthesis, and the amount of the synthesized [^{3}H]cholesterol was measured. In these experiments cholesterol synthesis was upregulated approximately 3 fold when cultured in lipoprotein deficient medium as compared to cells grown in complete medium and this was observed in both control and ORP2 cell lines. In both growth conditions, the ORP2 cells displayed 40 to 60 % down regulation of the cholesterol biosynthesis as compared to the control. Thus overexpression of the ORP2 protein decreased cholesterol synthesis in CHO cells. The effect was opposite to that obtained with OSBP; In CHO cells overexpressing OSBP, cholesterol synthesis was enhanced. The overexpression of OSBP together with the addition of 25-OHC decreased cholesterol synthesis in the same way as the addition of oxysterol alone indicating that other mechanisms are mediating this oxysterol induced effect (Lagace et al., 1997).

6.3 Transport of newly synthesized cholesterol to the cell surface (IV)

The finding that ORP2 affected the transport of the marker protein via the secretory route, as described above, led us to examine the transport of newly synthesized cholesterol from the ER to the PM. For this we first cultured the cells in lipoprotein deficient medium to induce cholesterol synthesis and then labeled the cells in the same way as for the biosynthesis study. To detect the cholesterol that had reached the PM, methyl β-cyclodextrin was added to the chase medium for the final 5 minutes and the amount of [^{3}H]cholesterol was measured from both the media and the cells (method described by (Heino et al., 2000)). Approximately 20% of the [^{3}H]cholesterol became accessible to cyclodextrin in the control cells while in the ORP2/CHO cells about 30% of the [^{3}H]cholesterol synthesized had reached the PM and was accessible to cyclodextrin. Therefore, the enhancement observed in ORP2/CHO cells was approximately 50%. The possible mechanism behind this enhanced accessibility of [^{3}H]-cholesterol to cyclodextrin may be that ORP2 affected the PM lipid composition so that cholesterol would more easily transfer to cyclodextrin or that the transport process of the newly synthesized cholesterol between the ER and the PM was enhanced. The first possibility was analyzed by mass spectrometry as described later (Results and discussion paragraph 7). The second possibility, i.e. that the transport of cholesterol was enhanced by ORP2, could in theory be due to an inhibition of the transport via the secretory pathway that could in turn induce an alternative, Golgi independent cholesterol transport route (Heino et al., 2000). ORP2 could also be involved in the formation or in the function of some yet unidentified type of vesicles transporting cholesterol to the PM.

6.4 Cholesterol esterification (III-IV)

In cellular cholesterol metabolism it is important that the balance between free and esterified cholesterol is regulated precisely. According to our results most of the cholesterol in CHO cells is in the free form and only 2-10 % is esterified. This depends on the growth conditions and the physiological status of the cells.

6.4.1 Esterification of [^{14}C]cholesterol (III-IV)

The stably transfected cells were labeled with [^{14}C]cholesterol for 36 hours and to analyze the amount of esterified [^{14}C]cholesterol, the cells were collected and the extracted lipids were separated by thin layer chromatography. Both the free and esterified cholesterol were scraped off the plate and the radioactivity was measured by liquid scintillation counting. The proportion of total [^{14}C]cholesterol found in the esterified form was drastically reduced in the ORP2/CHO cells (40-50 %) as compared to mock transfected cells. The change in the esterification of the [^{14}C]cholesterol could be due to several alternative mechanisms. The transport of cholesterol to the ER where the esterification occurs could be impaired, the transport or the synthesis of the fatty acyl-CoA could be affected or the hydrolysis of cholesterol esters or the ACAT activity itself could be affected.

6.4.2 ACAT activity (III)

To analyze whether the lowering of cholesterol esterification could be due to a decrease in ACAT activity in ORP2/CHO cells, [^{14}C]oleyl-CoA incorporation into cholesterol esters in semi-purified cellular membranes was determined. The assay was performed in the presence of exogenously added cholesterol (20μg / ml final concentration) to compensate for possible differences in the amount of the free

cholesterol pool. The ACAT activity of the ORP2/CHO cells was found to be reduced by approximately 45%. No SRE element has been found in the promotor regions of the ACAT1 and ACAT2 genes and it seems that both genes are regulated at the post-transcriptional level (Buhman et al., 2000; Chang et al., 2001). Regulation may be allosteric since no changes in ACAT1 mRNA or protein expression levels could be seen in cholesterol loaded macrophages, even when cholesterol esterification was increased (Wang et al., 1996). What then are the regulatory mechanisms influenced by ORP2 that are causing the decrease in esterification? It is possible that ORP2 is affecting the transport mechanisms of lipids and cholesterol in the cells and that ORP2 in that way modulates the substrate availability or localization of the ACAT enzyme. It is possible that in the ORP2 expressing cells the membrane environment where ACAT resides is changed. The *in vitro* assay of ACAT activity however, represents a more direct measure of the ORP2 effect and indicates that the enzyme activity is truly reduced. If the decreased ACAT activity were a primary effect of the protein then the previously described enhancement in cholesterol efflux would be secondary. To test this we conducted an experiment where we labeled the cells with [^{14}C]cholesterol, used an ACAT inhibitor to inhibit esterification and then performed the efflux to 20 % serum. The esterification was lowered 99 % in both cell lines. Efflux in ORP2/CHO cells was still enhanced compared to mock cells, but now the difference was approximately one half of that seen without the ACAT inhibitor. This result indicates that the enhancement in cholesterol efflux is partly due to the lowering of esterification, but it does not fully explain the ORP2 effect on cholesterol efflux. Also other mechanisms are likely to be involved that probably reflect changes in the lipid distribution in cellular membranes or in intracellular lipid transport.

6.5 Phospholipid biosynthesis (IV)

To analyze whether ORP2 overexpression results in alterations in phospholipid metabolism, we studied the synthesis of selected phospholipids in the stable cell lines.

6.5.1 Phosphatidylcholine (PC) biosynthesis analyzed by [methyl-^{3}H]-choline labeling (IV)

To determine whether the expression of ORP2 also induces changes in PC synthesis we labeled the cells for two hours with [methyl-^{3}H]choline chloride. Before labeling, the cells were grown in lipoprotein deficient medium for 36 hours. Lipids were extracted and analyzed by HPTLC. There was a significant 30-40 % increase in PC synthesis in ORP2/CHO cells as compared to mock cells. This could be associated with the enhanced efflux of newly synthesized cholesterol to cyclodextrin. Being a major phospholipid in eucaryotic membranes and therefore in intracellular vesicles, an ongoing synthesis of PC is required. Alterations in PC synthesis / turnover may be reflected in membrane trafficking (Howe and McMaster, 2001). This would be in agreement with our results that ORP2 affects trafficking in the secretory pathway. Vesicular transport and PC synthesis must be coordinated and one important factor is the Sec14p, a phosphatidylcholine and/or phosphatidylinositol transfer protein. Xu and co-workers have shown that ORP2 binds the PC metabolite, PA (Xu et al., 2001). The importance of PC, PA, DAG and phosphoinositides in vesicular transport has led to intense study and undoubtedly PC is one of the important connectors between membrane transport and the metabolism of lipids.

6.4.2 Phospholipid biosynthesis analyzed by [^{3}H]-serine labeling

According to previous studies, changes in the cellular or PM sphingomyelin content reflect changes in cholesterol synthesis and efflux (Fukasawa et al., 2000; Kronqvist et al., 1999; Ohvo et al., 1997).

Based on these findings, we analyzed SM synthesis in the ORP2/CHO cells by [^{3}H]-serine labeling. Lipids were extracted and different phospholipids were separated by HPTLC. No significant difference in SM synthesis was observed in ORP2 expressing cells as compared to the mock cells.

7. Phospholipid content of the stably transfected cell line (IV)

One possible explanation for the increased cholesterol efflux in ORP2/CHO cells could be that the phospholipid content or the saturation degree and/or chain length of the FAs in phospholipids was altered in ORP2 overexpressing cells. This could lead to more rapid cholesterol desorption from the membranes. To analyze whether the phospholipid content of the cells was altered cellular lipid extracts were subjected to mass spectrometric analysis. Both ORP2/CHO cells and mock-transfected cells were cultured in either complete medium or in lipoprotein deficient medium for 36 hours before lipid analysis. Differences in the relative proportions of the major phospholipid classes were not observed under either of the growth conditions. Hence, the enhanced PC synthesis observed in the ORP/CHO cells was not reflected by the total cellular amount of PC in the cells, which was the same as in the mock-transfected cells.

Analysis of the FA composition in different PLs revealed that when cells were grown in delipidated medium the PE, PS and PI polyunsaturated species, which are mostly obtained from the serum lipoproteins, were reduced in ORP2/CHO cells and were replaced with mono- and diansaturated species. The loss of the polyunsaturated FAs was compensated for mainly by synthesis of oleic acid (18:1) as evidenced by an increase in the 34:1, 36:1 and 36:2 species. The reason for this phenomenon is still not clear. The polyunsaturated FA species could be more rapidly degraded by β-oxidation in ORP2/CHO cells or they could be lost to the external medium. The loss would then be replaced by using newly synthesized FAs for PL synthesis. The decreased ACAT activity is one possible explanation for the change in FA species. The enhanced loss

of the polyunsaturated species might be connected with the observed defect in ACAT activity. The two ACAT isoforms show differential substrate fatty acyl-CoA preferences as human ACAT1 strongly prefers oleic acid while ACAT2 also utilizes polyunsaturated fatty acids efficiently (Cases et al., 1998; Seo et al., 2001). Therefore, lowering of ACAT2 activity could theoretically lead to reduced storage of polyunsaturated fatty acids in the form of cholesterol esters and thus increase their turnover rate. However, the expression of the two ACAT isoforms in CHO cells and their substrate specificity have not been addressed in detail.

8. Western analysis of SREBP-1 (IV)

We were interested to see if the maturation of SREBPs in ORP2/CHO cells would be affected. For the maturation of SREBP to occur, the precursor must be transported from the ER to the Golgi where proteolytic cleavage takes place. We analyzed both control and ORP2/CHO cells by Western blotting using a mouse monoclonal antibody against SREBP-1a (Wang et al., 1993). We found that the maturation of SREBP was partially inhibited in ORP2/CHO cells. This could provide a plausible explanation for the reduction in the cholesterol de novo synthesis as seen in ORP2/CHO cells.

CONCLUSION

We have identified a new human protein family that is related to the oxysterol binding protein (OSBP). We named the proteins OSBP related proteins, ORPs. The ORP genes are widely distributed in different chromosomes, which implies that they are of considerably old descent. According to their deduced amino acid sequences and intron-exon structures, the ORP genes can be divided into six subfamilies. The mRNA expression data suggests that the ORP genes have a ubiquitous distribution but are subject to tissue-specific transcriptional regulation. The sterol-binding domain is the most conserved part of the proteins and located within this domain is a fingerprint motif: EQVSHHPP, shared by all members of the ORP family. ORP2 is the only member in the family that lacks the N-terminal extension that contains a PH domain. In this respect it resembles the yeast OSBP homologues Osh4p-7p.

We found that ORP2 is ubiquitously expressed in various tissues both at the mRNA and the protein level, suggesting a housekeeping-type function. The highest expression was found in the nervous tissues, which represent the most cholesterol rich tissues in the body. Both the endogenous and the overexpressed protein localize mainly to the cytosol of CHO cells, but a fraction is membrane-associated and localized to the perinuclear region. Transient overexpression of ORP2 partially inhibits the transport of the VSVG protein via the secretory pathway. On the other hand, transport of newly synthesized cholesterol to the plasma membrane is enhanced in cells stably overexpressing ORP2. Studies performed using stably transfected ORP2 overexpressing cells (ORP2/CHO) show that ORP2 has a clear influence on cholesterol and phospholipid metabolism in CHO cells. ORP2 decreases cholesterol synthesis and esterification, but increases cholesterol efflux. The total cholesterol

content in CHO/ORP2 cells was decreased mainly due to a lower amount of cholesterol esters. The enhancement of [^{14}C]cholesterol efflux from ORP2/CHO cells was only partially inhibited under conditions in which ACAT activity was blocked. The lowered cholesterol esterification therefore explains only part of the efflux enhancement, suggesting that the effects of ORP2 overexpression on those two processes may be independent or due to a different common underlying mechanism. The observed increase in transport of newly synthesized cholesterol to the plasma membrane indicates that ORP2 overexpression might affect the intracellular movement of cholesterol, and by this mechanism enhance the efflux.

Since cholesterol and phospholipid metabolisms are subject to integrated regulation, the synthesis and composition of phospholipids were analyzed. Phosphatidylcholine synthesis was markedly enhanced by ORP2 overexpression whereas no effects on the synthesis of other phospholipids could be seen. The molar ratios of the major phospholipids were unchanged in ORP2/CHO cells. Mass-spectrometric analysis of the fatty acid composition of phospholipid classes revealed an accelerated loss of polyunsaturated phospholipid species in ORP2/CHO after they were shifted to lipoprotein deficient medium. This may reflect a change in the intracellular transport of phospholipids, leading to enhanced catabolism or increased release from the cells. It is also possible that the observed decrease in ACAT activity might lead to reduced storage of the polyunsaturated fatty acids in lipid droplets, resulting in accelerated turnover.

The data obtained in my thesis work demonstrate the presence of a human OSBP-related gene family with 12 members and suggest that ORP2 is a new regulator of cellular lipid homeostasis and membrane trafficking.

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