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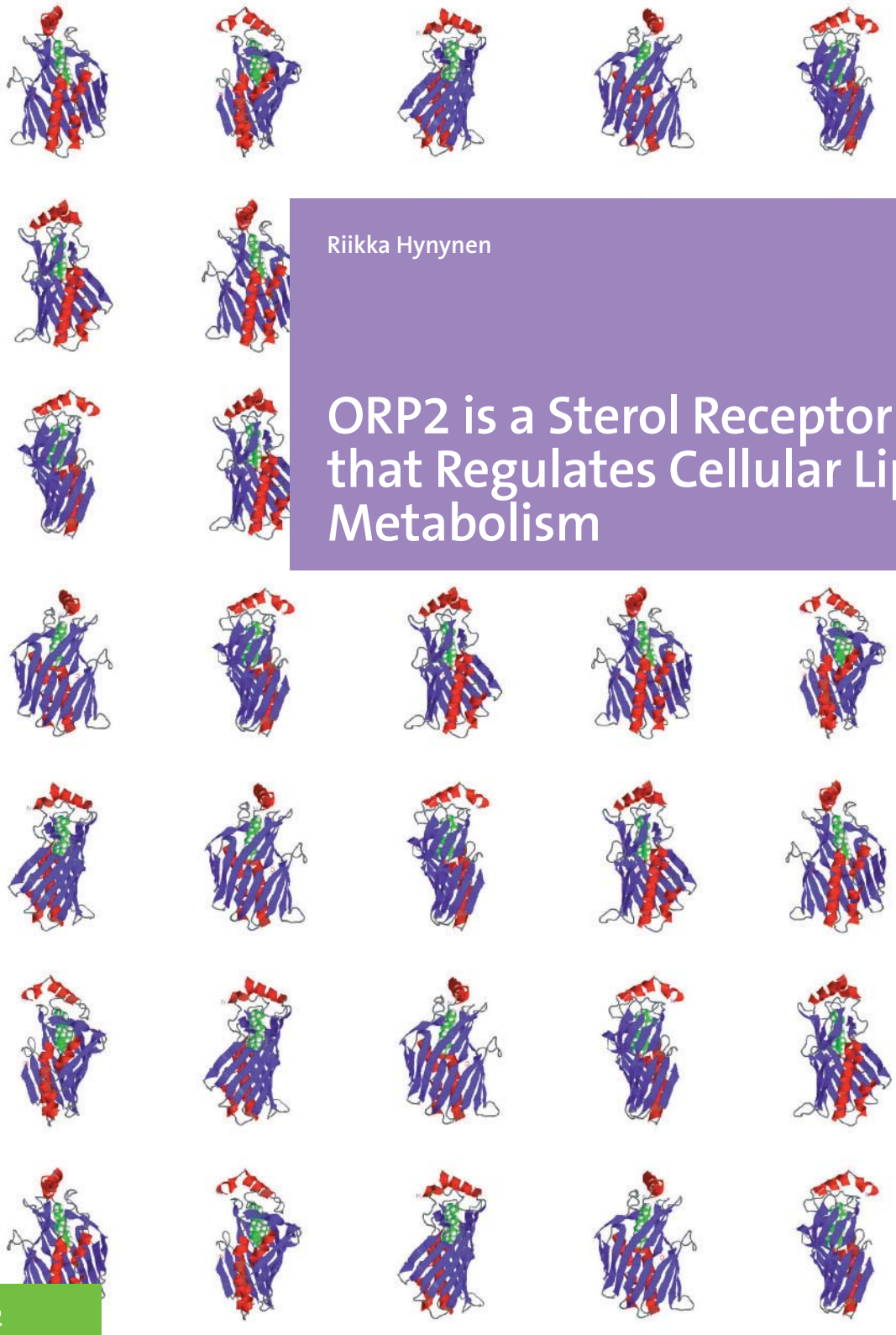

RESEARCH

Riikka Hynynen

ORP2 is a Sterol Receptor that Regulates Cellular Lipid Metabolism

Riikka Hynynen

ORP2 IS A STEROL RECEPTOR THAT REGULATES CELLULAR LIPID METABOLISM

A C A D E M I C D I S S E R T A T I O N

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"Kaikki on hyvin epävarmaa, ja juuri se saa minut levolliseksi"

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ABSTRACT

Oxysterol binding protein-related protein 2 (ORP2) is a member of mammalian oxysterol binding protein (OSBP)-related protein/gene family, which is found in almost every eukaryotic organism. Their function is still unclear, but studies on yeast and mammalian cells have suggested that they are players in cellular lipid metabolism, vesicle trafficking and cellular signaling. ORP2 is a cytosolic protein that is ubiquitously expressed and most abundant in brain. In previous studies employing stable cell lines with constitutive ORP2 overexpression ORP2 was shown to affect cellular cholesterol metabolism.

The aim of this study was to characterize the properties and function of ORP2 further. Since the previous studies have shown that ORPs bind sterols and phosphoinositides, the binding of those lipids was also tested using purified ORP2 in in vitro binding assays. As expected, ORP2 was shown to bind several oxysterols and cholesterol, the highest affinity ligand being 22(R)hydroxycholesterol. In addition, affinity to anionic membrane phospholipids, phosphatidylinositol phosphates was observed, which may assist in the membrane targeting of ORP2.

Intracellular localization of ORP2 was investigated to obtain clues to the function of ORP2. ORP2 was observed on the surface of cytoplasmic lipid droplets, which are storage organelles for neutral lipids. Lipid droplet targeting of ORP2 was inhibited when 22(R)hydroxycholesterol was added to the cells or when the N-terminal FFAT-motif of ORP2 was mutated, suggesting that oxysterols and the N-terminus of ORP2 regulate the localization and thereby also the function of ORP2.

The role of ORP2 in cellular lipid metabolism was studied using HeLa cell lines that can be induced to overexpress ORP2. In that way the acute effects of protein overexpression can be detected. Overexpression of ORP2 was shown to enhance cholesterol efflux from the cells resulting in a decreased amount of cellular free cholesterol. ORP2 overexpressing cells responded to the loss of cholesterol by upregulating the cholesterol synthesis and uptake. Intriguingly, cholesterol esterification was increased in ORP2 overexpressing cells. These results may be

explained by the ability of ORP2 to bind and thus transport cholesterol, which most likely leads to changes in cholesterol metabolism when ORP2 is overexpressed.

ORP2 function was also investigated using an opposite approach, silencing of endogenous ORP2 expression with short interfering RNAs (siRNA) in A431 cells. Silencing of ORP2 led to a delayed break-down of triglycerides under lipolytic conditions and an increased amount of cholesteryl esters in the presence of excess triglycerides. Together these results suggest that ORP2 is a sterol-regulated protein that functions on the surface of cytoplasmic lipid droplets to regulate the metabolism of triglycerides and cholesteryl esters. Although the exact mechanism of ORP2 function still remains unclear, this study serves as a good basis to investigate the molecular mechanisms and possible cell type specific functions of ORP2.

Keywords: oxysterol, oxysterol binding protein, ORP2, cholesterol, triglyceride, lipid droplet

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TIIVISTELMÄ

ORP2 on oksisteroleja sitovan proteiinin (OSBP) sukuisen proteiini- ja geeniperheen jäsen. OSBP:n sukuisia proteiineja (ORP) löytyy lähes kaikista aitotumallisista organismeista. Näiden proteiinen tehtävää ei ole vielä selvitetty, mutta tutkimukset hiiva-ja nisäkässoluilla ovat osoittaneet, että ORP:it osallistuvat solun rasva-aineenvaihdunnan, vesikkelikuljetuksen ja signaalinvälityksen säätelyyn. ORP2 on solulimassa ilmentyvä proteiini. Sitä ekspressoidaan kaikissa kudoksissa, eniten kuitenkin aivoissa. Aiemmissa tutkimuksissa, joissa on käytetty ORP2:a ylituottavia soluja, on havaittu ORP2:n vaikuttavan solunsisäiseen kolesteroliaineenvaihduntaan

Tämän tutkimuksen tarkoituksena oli selvittää tarkemmin ORP2:n ominaisuuksia ja tehtävää solun rasva-aineenvaihdunnassa. Koska useiden ORP:ien oli aiemmin havaittu sitovan steroleja ja fosfoinositideja, määritimme ORP2:n kyvyn sitoa näitä rasvoja käyttäen in vitro sitomiskokeita puhdistetulla ORP2-proteiinilla. ORP2 sitoi eri oksisteroleja ja kolesterolia, sitoutumisen ollessa vahvinta 22(R)hydroksikolesteroliin. ORP2:n havaittiin myös sitovan solun kalvoissa sijaitsevia anionisia fosfolipidejä, fosfatidyyli-inositolifosfaatteja. Tämän sitomisen arvellaan edistävän ORP2:n liittymistä solun kalvoihin.

Tutkimuksessa selvitettiin ORP2:n sijaintia solussa. ORP2 havaittiin solunsisäisten, neutraaleja rasvoja sisältävien rasvapisaroiden pinnalla. Kun soluille lisättiin 22(R)hydroksikolesterolia, ORP2 siirtyi pois rasvapisaroiden pinnalta. Myös ORP2:n aminoterminuksessa sijaitsevan FFAT-motiivin mutatoiminen aiheutti ORP2:n poistumisen rasvapisaroiden pinnalta, viitaten siihen, että oksisterolit ja ORP2:n aminoterminaalinen osa säätelevät ORP2:n sijaintia solussa ja siten myös sen tehtävää.

ORP2:n tehtävää solun rasva-aineenvaihdunnassa tutkittiin HeLa-solulinjoilla, jotka voidaan indusoida tuottamaan ORP2-proteiinia. Tällä metodilla voidaan havaita proteiinin ylituotannon välittömät vaikutukset solun aineenvaihduntaan. ORP2:n ylituotanto lisäsi kolesterolin virtausta ulos solusta. Tämä johti vapaan kolesterolin määrän vähenemiseen soluissa. ORP2:a tuottavat solut pyrkivät lisäämään kolesterolin määrää kiihdyttämällä kolesterolin valmistusta ja sisäänottoa. Yllättäen myös kolesterolin esteröinti oli kiihtynyt ORP2:a tuottavissa soluissa. Näiden

tulosten arveltiin aiheutuvan ORP2:n kyvystä sitoa ja mahdollisesti myös kuljettaa kolesterolia, mikä voi johtaa muutoksiin kolesterolin aineenvaihdunnassa, kun ORP2:a tuotetaan soluissa suuria määriä.

ORP2:n toimintaa tutkittiin myös päinvastaisella lähestymistavalla, hiljentämällä ORP2:n ilmentyminen A431-soluissa käyttäen pieniä häiritseviä RNA-molekyylejä (siRNA). ORP2:n hiljentäminen johti triglyseridien hajotuksen viivästymiseen hajotusta edistävissä olosuhteissa ja kolesteroliesterien määrän lisääntymiseen, kun triglyseridejä oli soluissa runsaasti. Tässä väitöskirjatyössä saadut tulokset viittaavat siihen, että ORP2 on sterolien säätelemä proteiini, joka sijaitsee solun rasvapisaroiden pinnalla, missä ORP2 osallistuu triglyseridien ja kolesteroliesterien aineenvaihduntaan. Vaikka ORP2:n tarkka toimintamekanismi jäi selvittämättä, tämän tutkimuksen pohjalta on hyvä jatkaa ORP2:n molekyylitason toiminnan ja mahdollisten solutyyppispesifisten tehtävien tutkimista.

Avainsanat: Oksisteroli, oksisteroleja sitova proteiini, ORP2, kolesteroli, triglyseridi, rasvapisara

CONTENTS

ABBREVIATIONS

ACAT	Acyl-coenzyme A:cholesterol acyl transferase
ABC	ATP-binding cassette transporter
ARF	ADP-ribosylation factor
ATGL	Adipose triacylglycerol lipase
BSA	Bovine serum albumin
CEH	Cholesteryl ester hydrolase
CERT	Ceramide transport (protein)
CHO	Chinese hamster ovary (cells)
CYP	cytochrome P450
DGAT	acyl-CoA:diacylglycerol acyltransferase
DRM	detergent-resistant membrane
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FFAT	Two phenylalanines in an acidic tract
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HSL	Hormone sensitive lipase
Insig	Insulin-induced gene
LDL	Low-density lipoprotein
LPDS	Lipoprotein deficient serum
LXR	Liver X receptor
MS	Mass spectrometry
OHC	Hydroxycholesterol
ORD	OSBP-related domain
ORP	OSBP-related protein

OSBP	Oxysterol binding protein
OSH/Osh	OSBP homologue
PH	Pleckstrin homology (domain)
PIP	Phosphatidylinositol phosphate
PKA	Protein kinase A
PUFA	Polyunsaturated fatty acid
SREBP	Sterol regulatory element binding protein
Scap	SREBP cleavage activating protein
SMase	sphingomyelinase
VAP	Vesicle-associated membrane protein-associated protein
7KC	7-ketocholesterol
24,25EC	24(S),25-epoxycholesterol

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Hynynen R., Laitinen S., Käkelä R., Tanhuanpää K., Lusa S., Ehnholm C., Somerharju P., Ikonen E. and Olkkonen V.M. Overexpression of OSBP-related protein 2 (ORP2) induces changes in cellular cholesterol metabolism and enhances endocytosis. 2005. Biochemical Journal 390(1): 273-283.

II Suchanek M., Hynynen R., Wohlfahrt G., Lehto M., Johansson M., Saarinen H., Radzikowska A., Thiele C., Olkkonen V.M. The mammalian OSBP-related proteins (ORP) bind 25-hydroxycholesterol in an evolutionarily conserved pocket. 2007. Biochemical Journal 405(3): 473-480.

III Hynynen R.*, Suchanek M.*, Spandl J., Bäck N., Thiele C. and Olkkonen V.M. ORP2 is a sterol receptor on lipid droplets that regulates the metabolism of neutral lipid. 2009. Accepted for publication in Journal of Lipid Research.

* These authors contributed equally to this work

Some unpublished data is also included in this thesis.

Author's contribution to publications

I RH performed the assays employing TREx HeLa cell lines, except for the mass spectrometry analysis that was done by RK. Phosphoinositide binding assay was done by RH. She also participated in the writing of the manuscript.

II RH performed the mutagenesis of ORP2 and the purification of wild-type and mutant proteins. She also carried out in vitro oxysterol pull-down assays of the wild-type and mutant ORP2.

III RH performed the oxysterol and cholesterol binding assays, approximately half of the ORP2 lipid droplet localization studies and assays of the effect of ORP2 silencing on triglyceride break-down and cholesterol esterification. She also wrote the most of the manuscript.

1 INTRODUCTION

Cholesterol is an essential molecule for mammalian cells. It is a precursor of steroid hormones, bile acids and other signaling molecules, but most importantly it is an essential constituent of cell membranes. An appropriate amount of cholesterol is needed for the plasma membrane to function as a barrier between intracellular and extracellular compartments and to relay the extracellular signals inside the cell.

The proper amount of cholesterol in the body – in the cells and in the blood – is important for human health. Excess cholesterol in the blood can accumulate in the vessel walls and together with other factors contribute to the formation of atherosclerosis, a major cause of death in Western societies. Although atherosclerosis is commonly seen as a disease of the extracellular milieu, many ways to prevent or cure it are based on intracellular mechanisms.

Oxysterols are oxidized derivatives of cholesterol, which can form passively by autoxidation or be synthesized by specific enzymes. Because of their reactivity they are difficult to study. In addition, their levels in the body are very low resulting in questioning their relevance in vivo. There is a wide variety of different oxysterols, but their distinct roles have remained mainly unclear. On one hand, they are regarded toxic and harmful, but on the other hand they can inhibit cholesterol biosynthesis and stimulate cholesterol export from the body thus performing beneficial actions, with regard to the prevention of atherosclerosis.

One proof of the importance of oxysterols is the presence of oxysterol binding protein-related protein family (ORP) in eukaryotic organisms. The founding member of the family, oxysterol binding protein (OSBP) was discovered in the 1980s and its precise function is still unknown. It has been shown to be involved in various cellular processes such as signal transduction and sphingomyelin metabolism. OSBP has also been demonstrated to affect the lipid levels in the circulation of genetically modified mice suggesting that the ORPs may play roles in the development of atherosclerosis. Therefore it is important to study their functions more thoroughly.

This study focused on ORP2. It is a ubiquitously expressed protein suggesting that it has an important function in many tissues. ORP2 was shown to bind cholesterol and several oxysterols. It was found to localize on the surface of intracellular lipid droplets, and the localization was inhibited by its ligand binding. ORP2 overexpression influenced cholesterol metabolism in cultured cell lines and ORP2 silencing affected also triglyceride metabolism. Therefore we propose that ORP2 could function to create a regulatory connection between cholesterol and triglyceride metabolism.

2 REVIEW OF THE LITERATURE

2.1 Intracellular cholesterol metabolism

Much is known about the cholesterol circulation in the body – how it is transported between organs in the lipoprotein particles. In contrast, intracellular cholesterol metabolism and trafficking have many fundamental questions that have remained unanswered. The amount of cholesterol in membranes varies a lot between organelles, implying that intracellular cholesterol is not passively distributed around the cell but rather transported inside the cell via carefully controlled routes.

2.1.1 Uptake of cholesterol

Uptake of cholesterol is maybe the best characterized event in cellular cholesterol trafficking. Cholesterol destined to be taken up by peripheral cells is transported in the circulation in low-density lipoprotein (LDL) particles, mostly in an esterified form. Peripheral cells take up the whole LDL particle via the LDL receptors (Brown and Goldstein, 1986). First apo B-100 apolipoprotein in LDL binds to LDL receptor and then the LDL-LDL receptor-complex is internalized by clathrin-mediated endocytosis. During the maturation, endosomes become more acidic and subsequently the LDL receptor is released from the LDL particle and transferred back to the plasma membrane. LDL is transported to the later endosomal compartments, where apo B-100 is degraded and cholesteryl esters are hydrolyzed to free cholesterol and fatty acids by acid lipase. After that the faith of cholesterol is less well known (Ikonen, 2008). Cholesterol is delivered from late endosomal systems to other cellular membranes by processes that involve Niemann-Pick disease type C (NPC) proteins, the defect of which causes accumulation of cholesterol in the lysosomes (Sturley et al., 2004). Possible destinations of the LDL-derived cholesterol are the plasma membrane, endoplasmic reticulum (ER), recycling endosomes and mitochondria.

Uptake of cholesterol is mainly regulated by the amount of LDL receptors in the plasma membrane. The expression of LDL receptor gene is known to be upregulated by sterol regulatory element binding proteins (SREBP), which are active when the level of cellular sterols is low (Goldstein et al., 2006).

LDL in the circulation is also found in oxidized form. In the arterial intima macrophages take up oxidized LDL via scavenger receptors by a process that is not regulated by cellular cholesterol levels (Greaves and Gordon, 2008). This can lead to

a situation, in which macrophages that already contain enough cholesterol still take up oxidized LDL resulting in the formation of macrophage foam cells. Therefore this process contributes to the development of atherosclerosis.

2.1.2 Synthesis of cholesterol

Lipoprotein internalization is not the only way of getting cholesterol into the cell. All nucleated mammalian cells are capable of synthesizing cholesterol from a ubiquitous precursor, acetyl-CoA, which is an activated degradation product of carbohydrates, amino acids and lipids. Biosynthesis of cholesterol (Figure 1) is a long series of reactions by over 40 cytosolic and membrane-bound enzymes that are mainly thought to reside in the ER membranes (Liscum, 2008).

The first reaction of the synthesis pathway is the condensation of acetyl-CoA and acetoacetyl-CoA catalyzed by 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase. HMG-CoA is then reduced to mevalonate by HMG-CoA reductase, which is a rate-limiting enzyme of the pathway. The six-carbon mevalonate is converted to a five carbon isopentenyl pyrophosphate, which is also the precursor of isoprenoids. Six molecules of isopentenyl pyrophosphate are condensed to the thirty-carbon isoprenoid, squalene, which is further made cyclic to form the first sterol of the pathway, lanosterol. Three carbons are then removed in two alternative series of reactions to yield the 27-carbon cholesterol.

Cholesterol synthesis is regulated mainly at the level of HMG-CoA reductase by feedback mechanisms, which affect the expression, phosphorylation and degradation of the enzyme (Espenshade and Hughes, 2007). When the cellular sterol level is low, SREBP transcription factors stimulate the transcription of the HMG-CoA reductase gene. When the sterol level is high, cholesterol and oxysterols induce the inactivation of the SREBP pathway. In addition, sterol intermediates such as oxysterols, lanosterol and 24,25-dihydrolanosterol induce the degradation of HMG-CoA reductase by ubiquitin-proteasome dependent pathway (Ravid et al., 2000; Song and DeBose-Boyd, 2004; Song et al., 2005), which also requires Insig protein (Sever et al., 2003). The activity of HMG-CoA reductase is also regulated by the energy status of the cell. When the supply of nutrients and subsequent ATP levels in the cell are low, AMP-activated kinase phosphorylates and thus inactivates HMG-CoA reductase (Carling et al., 1989). HMG-CoA reductase is a target of cholesterol lowering drugs such as statins that occupy the HMG binding site thus being reversible, competitive inhibitors of the enzyme (Istvan and Deisenhofer, 2001). Statins decrease the amount of cellular cholesterol and subsequently upregulate the LDL receptor thus decreasing the LDL-cholesterol in the plasma.

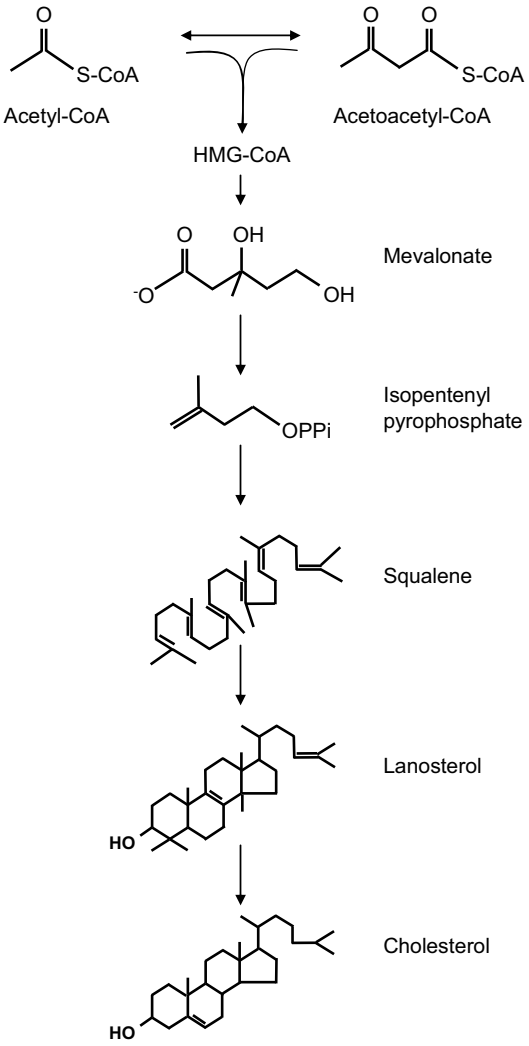

Figure 1. Important intermediates of cholesterol biosynthesis.

2.1.3 Cholesterol efflux

An atherosclerotic lesion contains macrophages that are filled with cholesteryl esters originated from plasma lipoproteins. These cells are called foam cells and they have

a great impact on lesion progression. Therefore the stimulation of macrophage cholesterol efflux is an interesting goal for pharmaceutical research.

Cholesterol efflux is a process in which cholesterol is transferred from the cell surface either to a high-density lipoprotein (HDL) particle or to a lipid-poor apolipoprotein such as apoA-I or apoE. The goal of this process is to transport excess cholesterol from the peripheral cells to the liver, which is the only organ capable of excreting cholesterol out of the body in the bile and faeces. The whole process is called reverse cholesterol transport.

Cholesterol efflux can be either passive or active. In passive diffusion cholesterol is released from the membrane to phospholipid-containing acceptors without need for energy-consuming protein mediators. In active cholesterol efflux proteins facilitate the export of cholesterol to extracellular acceptors (Jessup et al., 2006). Two of the known mediators of cholesterol efflux belong to a family of ATP-binding cassette (ABC) transporters, which are membrane proteins that use the energy of ATP to move substances up the concentration gradient. The first ABC transporter found to be involved in cholesterol efflux was ABCA1, the gene of which is disturbed in a Tangier's disease (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999). This disease leads to an accumulation of intracellular cholesterol and loss of HDL in the plasma due to a defective cholesterol efflux process. The role of ABCA1 is to promote cholesterol and phospholipid efflux to lipid-poor apoA-I particles, which leads to the formation of nascent, lipidated HDL-particles. The precise mechanism of ABCA1 function is not yet known, but it has been postulated that ABCA1 has dual roles in cholesterol efflux. First, it binds to apoA-I and directly promotes the lipidation of apoA-I. Secondly, it affects the lipid distribution in the plasma membrane so that cholesterol is more easily extracted from the membrane by apoA-I (Landry et al., 2006). According to a third model ABCA1 is endocytosed with apoA-I via a clathrin-mediated pathway and recycled back to the plasma membrane (Azuma et al., 2009). This mechanism was found to be important only when cells accumulated lipoprotein-derived cholesterol.

Because ABCA1 deficient cells are still capable of removing cholesterol, other mediators were searched and found (Klucken et al., 2000). ABCG1 is a protein that resides in an intracellular location. When the cells are loaded with cholesterol, it translocates to the plasma membrane and mediates cholesterol efflux to HDL particles (Wang et al., 2006). A sequential model has been proposed, in which ABCA1 first induces the formation of HDL-particle by helping cholesterol and phospholipids lipidate apoA-I, after which ABCG1 can mediate the efflux of cholesterol to HDL (Gelissen et al., 2006). Scavenger Receptor B1 (SR-B1) has also been suggested to participate in cholesterol efflux from peripheral cells to HDL (Ji

et al., 1997). However, the main function of SR-B1 seems to be in the liver, where it mediates the uptake of cholesteryl esters from HDL.

How is cholesterol efflux regulated? The expression of ABCA1 and ABCG1 in macrophages is strongly upregulated by high levels of cholesterol. Both genes contain an element responsive to liver X receptors (LXRs, see below). ABCA1 is also regulated at the posttranslational level. The stability, intracellular trafficking and activity of the ABCA1 protein can be controlled (Oram and Heinecke, 2005).

2.1.4 Intracellular storage of cholesteryl esters and triglycerides

Cell membranes have a limited capacity for free cholesterol. Therefore excess cholesterol has to be stored in an esterified form in the cytoplasmic lipid droplets. Esterified cholesterol can then be released when needed e.g. for steroid hormone synthesis, membrane renewal or signaling purposes. Triglycerides are also stored in lipid droplets. In adipocytes lipid droplets store energy for the whole body, but also other cell types store fatty acids as triglycerides to be used e.g. for phospholipid synthesis and signaling purposes.

Lipid droplets are formed of a lipophilic core consisting mostly of triglycerides and cholesteryl esters (Walther and Farese, 2008). Phospholipid monolayer surrounds the core. Lipid droplets are thought to originate from the ER membranes but the mechanism of lipid droplet formation is still hypothetical. Lipid droplets are not only passive storage sacs of lipids but dynamic organelles that undergo fusions and fissions, interact with other organelles and move along microtubules. A variety of proteins are found on the surface of lipid droplets regulating these events.

Cholesterol esterification

Cholesterol is esterified with long-chain fatty acyl-coenzyme A by Acyl-coenzyme A:cholesterol acyl transferases [ACAT, (Chang et al., 1993; Chang et al., 1997; Chang et al., 2009)]. Two genes encode two ACATs, which are integral membrane proteins residing in the ER (Anderson et al., 1998; Cases et al., 1998a; Oelkers et al., 1998). ACAT1 is expressed in most tissues suggesting a role as a general regulator of free cholesterol homeostasis, whereas ACAT2 is expressed in enterocytes and hepatocytes possibly forming cholesteryl esters for secretion in lipoprotein particles. Also the transcription of ACAT genes is differently regulated. Cholesterol addition seems to increase the expression of ACAT2 by a still unknown mechanism, whereas the expression of ACAT1 was not affected by cholesterol in cultured hepatic cells (Pramfalk et al., 2007). The enzymatic activity of ACAT is regulated by sterols in an allosteric fashion. ACAT seems to have two sterol binding sites – one for the activator and one for the substrate – that differ in their ability to accommodate

different sterols (Zhang et al., 2003). The other ACAT substrates, fatty acids upregulate ACAT activity as well (Seo et al., 2001). The preferred substrate and activator for ACAT1 was found to be oleic acid, whereas ACAT2 had broader substrate specificity. In addition to cholesterol, oxysterols can increase ACAT activity (Brown et al., 1975) and also be esterified by ACAT (Zhang et al., 2003).

Because ACAT is responsible for cholesterol esterification in macrophages hence contributing to the formation of foam cells, it has been studied as a target for pharmaceutical inhibition (Meuwese et al., 2006). Although the animal studies were promising, people treated with ACAT inhibitors did not benefit from the treatment.

Cholesteryl ester hydrolysis

Hydrolysis of cholesteryl esters is less studied than esterification although it is necessary for the removal of cholesterol from the lipid droplet and out of the cell. Cholesteryl esters derived from LDL-particles are hydrolysed by lysosomal acid cholesteryl ester lipase. Cholesteryl esters stored in the lipid droplet are hydrolysed by neutral cholesteryl ester hydrolase (CEH), the human gene of which was cloned in 2000 from monocyte/macrophages (Ghosh, 2000). Liver CEH was later characterized and proposed to participate also in bile acid synthesis (Zhao et al., 2005b). CEH is localized to the surface of lipid drolets in macrophages (Zhao et al., 2005a) and it has been found to be identical with triacylglycerol hydrolase (TGH), (Dolinsky et al., 2004). In addition, hormone sensitive lipase (HSL) can hydrolyze cholesteryl esters suggesting that the same enzymes may be responsible for hydrolyzing cholesteryl esters and triglycerides. A recent study showed that mice lacking HSL had increased level of plasma cholesterol and hepatic cholesteryl esters supporting the fact that HSL is important in hydrolysis of both triglycerides and cholesterol esters (Fernandez et al., 2008). There are also other triglyceride hydrolases that have not yet been characterized and that might also catalyze the hydrolysis of cholesteryl esters.

Triglyceride synthesis

The last step in triglyceride (triacylglycerol) synthesis is acylation of diacylglycerol with fatty acyl-CoA by quite recently identified acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes (Cases et al., 1998b; Cases et al., 2001; Yen et al., 2008). DGAT1 and DGAT2 are expressed from different genes and even belong to different protein families. DGAT1 is a member of a family of membrane-bound O-acyltransferases, in which the ACATs belong as well. DGAT1 is ubiquitously expressed and DGAT2 is also expressed in most tissues. Both genes have the highest expression levels in tissues that synthesize high amounts of triglycerides, such as liver, adipose tissue and mammary gland. DGAT2 has been shown to be more potent than DGAT1 in triglyceride synthesis as shown by in vitro activity assays and

by knock-down mouse model studies (Yen et al., 2008), suggesting that it is responsible for the most of the triglyceride synthesis for long-term storage. In contrast to DGAT2, DGAT1 can acylate other molecules than diacylglycerol (Yen et al., 2005). It can also synthesize triglyceride from monoacylglycerol by two subsequent esterifications, suggesting a role in the reesterification of hydrolyzed triglycerides.

Like the ACATs, the DGAT enzymes are integral membrane proteins residing in ER membranes. Whether they release the synthesized triglycerides in ER lumen or to the cytosolic compartment is not completely understood. It is suggested that the triglycerides destined to be secreted in lipoproteins are synthesized by DGAT1 and are thus released to the ER lumen. Triglycerides that are to be stored in lipid droplets are suggested to be synthesized by DGAT2 and to be released to the cytoplasm as a constituent of lipid droplet. Interestingly, it was recently reported that DGAT2 can also be found on the surface of lipid droplets, where it could synthesize triglyceride directly to the lipid droplet for storage (Kuerschner et al., 2008).

Hydrolysis of triglycerides

Two proteins contributing to the breakdown of triglycerides have been characterized so far, adipose triacylglycerol lipase (ATGL) and HSL (Zimmermann et al., 2004; Fredrikson et al., 1981; Watt and Steinberg, 2008). The removal of the first fatty acid from triglyceride is catalyzed by ATGL. The resulting diacylglycerol is a preferred substrate of HSL. This enzyme hydrolyzes diacylglycerol to monoacylglycerol, which is finally hydrolyzed by monoacylglycerol lipase to yield glycerol and a free fatty acid.

Lipolysis is mainly regulated at the post-translational level (Zimmermann et al., 2008). The classical activation of lipolysis is initiated by catecholamines. They bind to β-adrenergic receptors at the cell surface and stimulate G-protein coupled receptors, which activate adenylase cyclase to produce more cyclic AMP (cAMP). cAMP binds to protein kinase A (PKA), which leads to phosphorylation and subsequent activation of lipolytic proteins. HSL is phosphorylated by PKA. However, its phosphorylation has not been considered as important for hormone stimulated lipolysis as the phosphorylation of the coactivator protein, perilipin. A recent model suggests that in basal, unstimulated conditions perilipin is on the surface of lipid droplets. When it is phosphorylated by PKA in the stimulated condition, it interacts with HSL and promotes the translocation of HSL from the cytosol to lipid droplets and the catalysis of the lipolysis. Unphosphorylated perilipin also interacts with the ATGL activator CGI-58 (comparative gene identification-58; Lass et al., 2006), which was earlier identified as a gene defective in neutral lipid storage disease Chanarin-Dorfman Syndrome (Lefevre et al., 2001).

When perilipin is phosphorylated, CGI-58 is released and is able to activate ATGL. The mechanism of lipolysis is still quite unclear. Although the role of perilipin in adipocytes is much studied, it is not ubiquitously expressed. It is not expressed for example in muscle and liver, where ATGL and CGI-58 play important roles. However, there are many members in the perilipin family of lipid droplet proteins, which may contribute to the control of lipolysis in other cell types (Brasaemle, 2007).

2.1.5 Transcriptional regulation of cellular cholesterol homeostasis

Sterol regulatory element binding protein (SREBP)

The main function of SREBP transcription factors is to maintain the cellular cholesterol and lipid homoeostasis (Brown and Goldstein, 1999). Two SREBP genes encode three isoforms. SREBP-2 regulates mainly the genes involved in cholesterol uptake and synthesis whereas SREBP-1c activates the genes of fatty acid synthesis (Horton et al., 2003). SREBP-1a regulates all SREBP responsive genes.

All the SREBP isoforms function in the same way. SREBP precursors are synthesized in the ER membranes as transmembrane proteins and subsequently bound to SREBP cleavage activating protein (Scap). Scap is an ER membrane protein that has a sterol sensing domain, by which it can sense the level of cholesterol. When the cholesterol level in the ER is low, Scap binds to COPII coat proteins of ER exiting vesicles and the SREBP-Scap complex is transported to the Golgi complex. In Golgi SREBP is cleaved by two proteases to yield a soluble, active fragment that can enter the nucleus and activate genes that have a sterol responsive element (SRE) in their promoter. When the cholesterol level in the ER rises, sterol binding of Scap induces a conformational change that permits the binding to Insig (insulin-induced gene; Yang et al., 2002). This leads to the retention of SREBP-Scap in the ER, because Scap can no longer bind to COPII proteins (Sun et al., 2005).

Recent studies have revealed the importance of Insig proteins in the regulation of cholesterol homeostasis. To date two Insig proteins (Insig-1 and -2) have been identified (Yabe et al., 2002; Yang et al., 2002). They function in the same way, but their transcription is differently regulated. The expression of Insig-1 requires SREBP activation, whereas Insig-2 is constitutively expressed at a low level. Through SREBP pathway sterol depletion increases the Insig-1 mRNA level. On the other hand, sterol depletion decreases the Insig-1 protein level, because when Insig-1 is not bound to Scap (in sterol depleted cells), it becomes ubiquitinated and degraded in proteasomes (Gong et al., 2006). Insig-1 also mediates the effect of

oxysterols on SREBP regulation, because unlike Scap it is able to bind oxysterols (Radhakrishnan et al., 2007). Oxysterol-bound Insig binds to Scap and thus blocks the transport of the SREBP-Scap complex to Golgi apparatus.

Liver X receptors (LXRs)

While the SREBP system aims at increasing the cellular cholesterol content by upregulating cholesterol uptake and synthesis, LXRs activate the genes involved in the stimulation of cholesterol export out of the cells and out of the body (Zelcer and Tontonoz, 2006). Two LXRs have been identified: LXRα is highly expressed in the liver and at low levels in the adrenal glands, intestine, adipose tissue, macrophages, lung and kidney, whereas LXRβ is ubiquitously expressed (Repa and Mangelsdorf, 2000). LXRs form heterodimers with retinoid X receptor (RXR) (Willy et al., 1995). They reside in the nucleus bound to the LXR-responsive elements of target genes and in the inactive state form a complex with corepressors of transcription. LXR/RXR heterodimers can be activated by the ligand of either partner, which leads to the exchange of corepressor to coactivator and subsequent transcription of target genes. Natural ligands of LXRs were unknown for a long time, but finally they were recognized to be oxysterols (Janowski et al., 1996; Lehmann et al., 1997).

The first LXR target gene was identified from Lxra-/-mice (Peet et al., 1998). It was CYP7A1, encoding a rate-limiting enzyme of the classical pathway of bile acid synthesis. The cholesterol efflux-mediating proteins ABCA1 (Repa et al., 2000a) and ABCG1 (Venkateswaran et al., 2000) were later identified as LXR target genes as well as two other ABC transporters ABCG5 and ABCG8 (Repa et al., 2002). ABCG5 and -8 form a heterodimer that appears to limit the absorption of sterols in the intestine and mediate the biliary sterol excretion from the hepatocytes (Repa et al., 2002). Considering these target genes, LXR agonists seemed perfect drug candidates for treating atherosclerosis by stimulating cholesterol export out of the body. However, Schultz et al. found that treating mice and hamster with LXR agonists increased the triglyceride level of plasma and liver (Schultz et al., 2000). This was found to result from LXR-dependent upregulation of SREBP-1c, the gene of which contains an LXR response element (Repa et al., 2000b) and which itself upregulates the transcription of genes involved in fatty acid synthesis. This mechanism is suggested to decrease the amount of free cholesterol by providing oleic acid for cholesterol esterification. Unfortunately it is also an obstacle for using LXR agonists as lipid lowering drugs.

2.2 Oxysterols

Oxysterols are oxygenated cholesterol derivatives that have additional oxygen in the nucleus of the sterol ring structure or in the iso-octyl tail of cholesterol (often referred as side-chain oxysterols) (Figure 2). Because of the additional oxygen they are more hydrophilic than cholesterol, which makes them more accessible to cytosolic oxysterol receptors and thus better signaling molecules of cellular sterol status than cholesterol itself. Because of their greater hydrophilicity and ability to pass lipophilic membranes faster than cholesterol, they are also regarded as transport forms of cholesterol that mediate the excretion of sterol out of the body. However, the physiological concentration of oxysterols in healthy tissue is very low, which makes them more difficult to study and the studies on oxysterol effects often unreliable. Oxysterols are regarded as cytotoxic and increased concentrations of oxysterols are often connected with pathological conditions such as atherosclerosis Brown and Jessup, 1999).

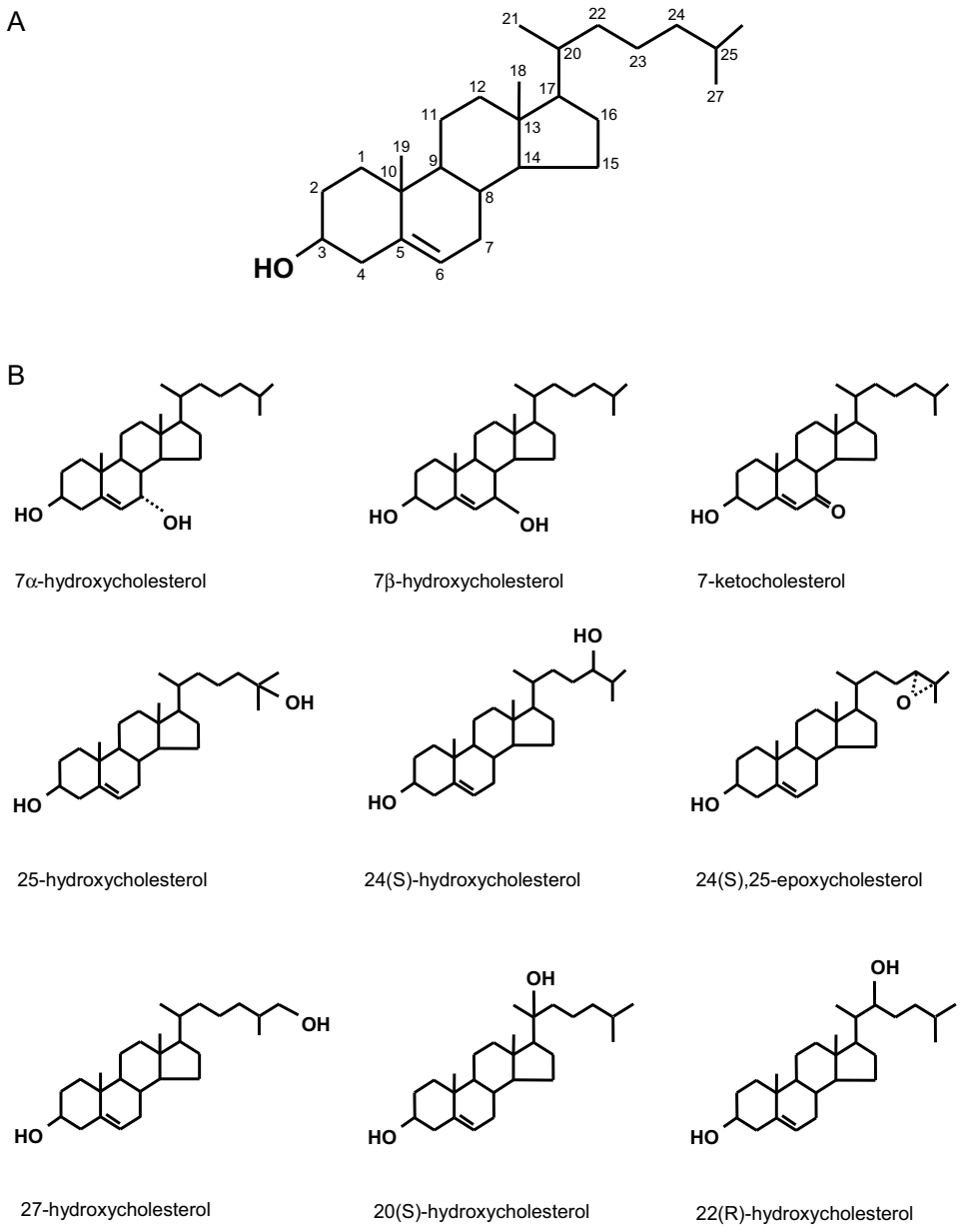

Figure 2. A. The structure of cholesterol with numbered carbon atoms. B. Structures of oxysterols mentioned in the text.

2.2.1 The formation and properties of oxysterols

Oxysterols are formed in tissues by autoxidation or by the action of specific monooxygenases. (van Reyk et al., 2006). They can also be obtained from diet. Autoxidative oxysterols form when radicals attack the cholesterol molecule, most commonly on the 7-carbon, which results in the formation of 7-hydroxycholesterols, mainly the 7β-hydroxycholesterol (7β-OHC), and 7-ketocholesterol (7KC). These oxysterols have been found in very small amounts in the body usually in esterified and thus less harmful form. However, they have been detected in higher levels in sites of cholesterol accumulation, such as macrophage foam cells and atherosclerotic plaques (Brown and Jessup, 1999).

Different enzymes can hydroxylate cholesterol either in the side-chain or in the sterol ring (Russell, 2000). Most of the responsible enzymes are cytochrome P450 (CYP) enzymes that reside in various subcellular locations. 7α-hydroxycholesterol (7α-OHC) is produced by cholesterol 7α-hydroxylase (CYP7A1) in a reaction that is also the first committed step in the classical bile acid synthesis pathway. CYP7A1 can also hydroxylate several oxysterols (Norlin et al., 2000). 20(S)- and 22(R)hydroxycholesterols are formed by cytochrome P450scc as intermediates in the biosynthetic pathway of pregnenolone, which is a precursor of streroid hormones (Hume et al., 1984). Cholesterol 24-hydroxylase (CYP46) produces 24(S)hydroxycholesterol (24OHC), which is relatively abundant in brain, more exactly in the neurons (Lund et al., 1999). 27-hydroxycholesterol (27OHC, previously known as 26-hydroxycholesterol) is produced by sterol 27-hydroxylase (CYP27A1), a mitochondrial enzyme that is expressed in many tissues with the highest expression level in macrophages (Wikvall, 1984; Javitt, 2002a). CYP27A1 has broad substrate specificity; it also hydroxylates 7α-OHC in the 27-carbon in the synthetic pathway of bile acids. Cholesterol 25-hydroxylase differs from the other cholesterol hydroxylases by belonging to a family of non-heme iron containing proteins (Lund et al., 1998). It catalyzes the formation of 25-hydroxycholesterol, which is a minor oxysterol in the body and often regarded as an autoxidation product, formed in the sample processing. 24(S),25-epoxycholesterol (24,25EC) is not a cholesterol derivative, but is formed in a shunt pathway of cholesterol biosynthesis by the action of oxidosqualene cyclase (Nelson et al., 1981). The rate of 24,25EC synthesis has been found to correlate with that of cholesterol (Wong et al., 2007), thus making it an attractive candidate for a feed-back regulator of cholesterol biosynthesis.

2.2.2 Biological functions of oxysterols

Oxysterol effects on cholesterol biosynthesis

Oxysterols first started to gain interest when they were found to inhibit cholesterol biosynthesis in cells (Brown and Goldstein, 1974; Kandutsch and Chen, 1974). Based on these and other studies Kandutsch et al. proposed that oxysterols function as feedback regulators of cholesterol biosynthesis (Kandutsch et al., 1978). In 1984 Taylor et al. discovered a cytosolic protein that binds oxysterols with affinities that correlate with their ability to inhibit cholesterol synthesis (Taylor et al., 1984). Oxysterol binding protein (OSBP) was therefore thought to mediate the oxysterol effects on cholesterol synthesis. However, Nishimura et al. showed much later with gene silencing studies that the cholesterol synthesis inhibition by oxysterols is independent of OSBP (Nishimura et al., 2005). The role of oxysterol binding to OSBP and its later discovered relatives has not yet been solved.

Radhakrishnan et al. found that oxysterols, namely 25OHC, 22(R)OHC, 24(S)OHC, 27OHC and 24,25EC deactivate the SREBP pathway by binding to Insig, which then retains Scap-SREBP in the ER thus inhibiting the cholesterol biosynthesis (Radhakrishnan et al., 2007). Oxysterol binding to Insig also induces the interaction between HMG-CoA reductase and Insig, which then triggers the ubiquitination and subsequent degradation of HMG-CoA reductase (Sever et al., 2003; Song and DeBose-Boyd, 2004). This is in accordance with the finding that 27OHC is necessary for Insig-mediated degradation of HMG-CoA reductase in rapid response to high cholesterol levels (Lange et al., 2008). Based on this study Lange et al. proposed that oxysterols are responsible for the rapid inactivation of HMG-CoA reductase, whereas cholesterol itself takes care of the down-regulation taking place in hours through the SREBP/Scap pathway.

Oxysterols as LXR ligands

In addition to Insigs, oxysterols have been shown to be ligands to Liver X receptors (LXR) in vitro and subsequently activate their function as transcription factors (Janowski et al., 1996; Lehmann et al., 1997). The best ligands were found to be side-chain oxysterols 24(S)OHC, 24,25EC and 22(R)OHC, that bound to LXRs in concentrations comparable to those found in cells (Janowski et al., 1999). 7α- or 7βOHC were not able to bind to or activate LXRs in the same study. It has been uncertain for a long time, if the LXR activation by oxysterols occurs also in vivo. Finally, Chen et al. showed that if the action of oxysterols is blocked by overexpressing a sulfotransferase enzyme that catalyzes the sulfonation of oxysterols in mice, they no longer respond properly to dietary cholesterol by upregulating the LXR target genes (Chen et al., 2007). This was also the case with

mice lacking the functional genes of 24(S)OHC, 25OHC and 27OHC biosynthetic enzymes, proving that oxysterols do activate LXRs also in vivo.

Other roles of oxysterols

Previous results suggest that oxysterols affect cholesterol metabolism by binding to the Insig and LXR proteins. However, Gale et al. recently suggested that also the modulation of membrane properties by side-chain oxysterols can affect SREBP processing and HMG-CoA reductase degradation (Gale et al., 2008). This was based on studies in which enantiomeric 25OHC defective in interacting with protein receptors was able two exert similar effects as 25OHC. The membrane behavior of oxysterols differs from that of cholesterol (Massey, 2006). Oxysterols position themselves in the membranes in a very different way from cholesterol, because it is energetically favorable to position both hydrophilic groups near the water interface. This may lead to a tilted orientation of oxysterols in the membrane (Kauffman et al., 2000). Therefore oxysterols differ from cholesterol and also from each others in their ability to promote the formation of liquid ordered domains depending on the position of the extra hydroxyl group (Massey and Pownall, 2006).

Due to their increased hydrophilicity, oxysterols transfer between membranes much faster than cholesterol (Theunissen et al., 1986). Side-chain oxysterols such as 24OHC and 27OHC were transferred from red blood cells to plasma much faster than cholesterol or oxysterols hydroxylated in the sterol nucleus (Meaney et al., 2002). Indeed, the most abundant oxysterols in human circulation are 27OHC and 24(S)OHC, as determined by isotope dilution-mass spectrometry (Dzeletovic et al., 1995). 24OHC is most abundant in brain (Björkhem, 2007), where the cholesterol 24-hydroxylase is expressed at relatively high level. Knocking down cholesterol 24-hydroxylase gene in mice resulted in reduced cholesterol biosynthesis in brain, while the over-all cholesterol levels were similar to wild-type mice (Lund et al., 2003). This could mean that since the cholesterol is not exported from the brain in the form of 24OHC, the cholesterol synthesis is slowed down. It is thus tempting to speculate that 24OHC is a transport form of excess brain cholesterol, since it can pass lipophilic membranes and probably the blood-brain barrier, which is impermeable to plasma lipoproteins.

27OHC in turn, besides being a ligand of LXRs and Insigs, is a precursor of bile acids in hepatocytes (Javitt, 2002a; Javitt, 2002b). It is also relatively abundant in human atherosclerotic plaques and macrophage-derived foam cells (Brown and Jessup, 1999). The function of 27OHC has been been studied using cells derived from humans suffering from cerebrotendinous xanthomatosis (CTX). This disease is characterized by the lack of functional sterol 27-hydroxylase (Cali et al., 1991). It leads to neurological defects and premature atherosclerosis most probably caused by

abnormal deposition of cholesterol and cholestanol in many tissues, although the cholesterol levels in the circulation are normal (Leitersdorf et al., 1994). Interestingly, mice with disrupted sterol 27-hydroxylase gene displayed less severe defects, although the formation of bile was diminished (Rosen et al., 1998). 27OHC has recently been found to bind to estrogen receptors in vascular wall and thus inhibit estrogen function (Umetani et al., 2007).

The best acknowledged function for oxysterols is their role as precursors of bile acids (Javitt, 2002b). Bile acids are formed in the liver and secreted to the intestine via gallbladder. In intestine they facilitate the absorption and digestion of fats and fat-soluble molecules by acting as detergents. Bile acid synthesis is also the major pathway through which cholesterol is eliminated from the body. The classical pathway of bile acids synthesis takes place in the liver. There cholesterol is first hydroxylated at the 7α-position by CYP7A1. 7α-hydroxylated oxysterols can also form via an alternative pathway, in which oxysterols such as 24OHC, 25OHC and 27OHC that are formed in different organs are 7α-hydroxylated by CYP7B1 and CYP39A1. The latter reactions are common for both pathways and take place in the liver.

It is commonly thought that the most reliable studies on oxysterol action are not the ones, in which exogenous oxysterols are added to cells or to animals, but those employing models in which genes encoding an oxysterol forming or degrading enzymes are knocked out. These studies have usually failed to show dramatic effects of the lack of one oxysterol. This suggests that individual oxysterols can take over the action of others and, on the other hand, cholesterol hydroxylases can add the hydroxyl group also to other positions (Lund et al., 1993). These studies also show that oxysterols affect cholesterol homeostasis, but in a very delicate manner, which is not yet completely understood.

2.3 OSBP

2.3.1 Discovery of OSBP

In 1977 Kandutsch et al. found cytosolic protein fractions capable of binding 25-hydroxycholesterol (Kandutsch et al., 1977). This protein fraction was characterized and purified further by the same group. Oxysterol binding protein (OSBP) was later purified from hamster liver and a monoclonal antibody produced by the group of Brown and Goldstein (Dawson et al., 1989b). Rabbit OSBP cDNA was cloned in 1989 (Dawson et al., 1989a), human cDNA in 1990, and the OSBP gene was

localized to human chromosome 11 (Levanon et al., 1990). By the year 1992 many tools were thus available to study the function of OSBP in the cell.

2.3.2 Structural properties of OSBP

Oxysterol binding region of OSBP was first found to reside between amino acids 296 and 809 (Ridgway et al., 1992). Later the sterol-binding region was narrowed to amino acids 408-459, which contain a CRAC (cholesterol recognition/interaction amino acid consensus) motif, implicated in sterol binding by other proteins (Wang et al., 2008). According to three-dimensional structure of yeast ORP, Osh4p, these amino acids are not in the region of the β-barrel suggested to form the sterol-binding pocket (see figure 4 of Osh4p). Instead these amino acids are thought to reside in the lid region of OSBP. To explain this discrepancy Wang et al. proposed that OSBP binds sterols using the lid region and inserts the sterol to the sterol binding pocket.

The N-terminal part of OSBP was recognized to contain a pleckstrin homology (PH) domain (Musacchio et al., 1993). These domains are found in many proteins with variable functions (Lemmon, 2008). A common feature for many PH domains is that they bind phosphatidylinositol phosphates (PIP) in cellular membranes. PIPs can be phosphorylated at positions 3, 4 and 5 depending on the organelle and the PI-kinases or phosphatases localizing to that specific membrane. Therefore the PIP binding specificity of a PH domain can determine the membrane where it is localizing. In the study of Rameh et al. OSBP was shown to bind PI(4,5)P_2 and PI(3,4,5)P_3 (Rameh et al., 1997), but Levine and Munro later proposed that the binding of PI(4)P is more important regarding the subcellular targeting of the OSBP PH domain (Levine and Munro, 2002).

The first 87 amino acids in the N-terminus of OSBP were identified as a glycine-alanine rich domain (Dawson et al., 1989a). This domain was shown to reduce the cholesterol binding of OSBP without affecting the 25OHC binding (Wang et al., 2008). OSBP was also shown to contain a putative leucine zipper motif at amino acids 209-244, the role of which has remained unsolved (Dawson et al., 1989a). OSBP has also been shown to dimerize (Ridgway et al., 1992), and the responsible protein region was found to reside between amino acids 261-288 (Wyles et al., 2002).

2.3.3 Intracellular localization of OSBP

In standard growth conditions OSBP is cytosolic or localizes to small vesicular structures adjacent to the nucleus. When 25OHC was added to cells OSBP was found to translocate to the Golgi apparatus (Ridgway et al., 1992). The N-terminal

part, which was later found to contain a PH domain, localized constitutively to Golgi apparatus suggesting that the C-terminal oxysterol binding domain, which was identified in the same study, negatively regulates the Golgi localization in the absence of ligand. The studies of Neale Ridgway's group showed that the cholesterol depletion of cells by different manipulations induces a constitutive localization of OSBP to the Golgi apparatus independent of 25OHC addition (Mohammadi et al., 2001; Storey et al., 1998). Wang et al. later proposed that cholesterol binding of OSBP induces the masking of the PH domain resulting in cytosolic localization. In contrast, when oxysterol is bound, PH domain is predisposed resulting in the translocation of OSBP to Golgi apparatus (Wang, P.Y. et al., 2005).

Levine and Munro showed that the PH domain of OSBP is sufficient for the Golgi localization and that the targeting is dependent on $PI(4,5)P_2$ or PI(4)P binding (Levine and Munro, 1998). Other Golgi specific factors were still needed, since $PI(4,5)P_2$ is found also in other membranes. Later Levine and Munro showed using yeast mutant strains that the Golgi localization of the OSBP PH domain is dependent on PI-4-kinase activity and not on PI(4)P 5-kinase activity, proving that the OSBP PH domain binds PI(4)P in the Golgi membranes (Levine and Munro, 2002). However, an OSBP mutant defective in PIP binding still had some affinity towards Golgi membranes and in yeast this affinity required the activity of Arf1p (ADP-ribosylation factor), which is needed for the recruitment of various proteins to Golgi apparatus. Indeed, Godi et al. found that the PH domain of OSBP interacts directly with ARF and even stimulates its Golgi association (Godi et al., 2004), supporting a specific role for OSBP in the Golgi membrane trafficking.

OSBP was later found to also have affinity towards ER-membranes. When the Golgi association was disturbed by mutating the PH domain of OSBP, OSBP was found in the ER compartment with Vesicle-associated membrane protein-associated protein A (VAP-A; Lev et al., 2008), which was recognized as an interaction partner of OSBP in a yeast two-hybrid screen (Wyles et al., 2002). Interestingly, silencing the expression of VAP inhibited also the Golgi targeting of OSBP, even in the presence of 25OHC, which could be explained by a decreased amount of PI(4)P in the Golgi membranes of VAP silenced cells (Peretti et al., 2008). Interaction with VAP-A was mapped to amino acids 351-442 of OSBP (Wyles et al., 2002), which was later shown to contain a FFAT-motif (two phenylalanines in an acidic tract) that mediates the VAP interaction of many lipid binding proteins (Loewen et al., 2003).

2.3.4 The role of OSBP in sphingomyelin metabolism

The study of Storey et al. (1998) hypothesized for the first time that OSBP could be involved in cholesterol or oxysterol dependent regulation of sphingomyelin synthesis, because the stimulatory effect of 25OHC on sphingomyelin synthesis correlated with changes in OSBP localization and phosphorylation status. The link between these two phenomena was still missing. In the next study Ridgway et al. observed that the depletion of plasma membrane sphingomyelin by exogenously added bacterial sphingomyelinase (SMase) induced the dephosphorylation of OSBP (Ridgway et al., 1998). SMase was also shown to induce the Golgi localization of OSBP. The role of OSBP in sphingomyelin metabolism was investigated in the next study, which employed OSBP overexpressing CHO cells (Lagace et al., 1999). In this study OSBP overexpressing cells were able to enhance the 25OHC stimulated sphingomyelin synthesis, although the enzymatic activities of sphingomyelin synthesising enzymes in vitro were not altered. This implied that OSBP could affect the substrate availability of these enzymes, which would make sense since these enzymes reside in Golgi, where OSBP is translocated upon 25OHC treatment. This hypothesis got support from the next study (Wyles et al., 2002). Overexpression of OSBP mutant W174A, which localized constitutively to ER membranes together with VAP-A, was blocking the transport of fluorescent ceramides from ER to Golgi resulting in reduced synthesis of sphingomyelin.

RNA interference studies and the discovery of CERT (ceramide transport) protein (Hanada et al., 2003) helped to produce a mechanistic model of OSBP function in 25OHC mediated stimulation of sphingomyelin synthesis (Perry and Ridgway, 2006). CERT is a ceramide binding protein required for ceramide transport from ER to Golgi apparatus, where sphingomyelin synthesis takes place (reviewed in (Hanada et al., 2007). OSBP is required for the Golgi localization of CERT only in the presence of 25OHC whereas VAP-A is needed for the ER association of CERT. Rescue experiments of OSBP depleted cells by OSBP mutants showed that the PH domain, FFAT-motif and oxysterol binding were all needed for 25OHC mediated stimulation of sphingomyelin synthesis (Perry and Ridgway, 2006). However, OSBP was not required for CERT action in basal sphingomyelin synthesis and neither have the two proteins been shown to interact.

In summary, OSBP functions in the connection between sphingomyelin and cholesterol metabolism. These two have to be connected, because cholesterol and sphingomyelin have a special property of forming liquid ordered domains together in the plasma membrane, which is important for the function of many membrane receptors (Simons and Ikonen, 1997). Although 25OHC is not a good oxysterol for cellular studies because of its low abundance in vivo, it can be thought to reflect the

amount of cellular cholesterol. Therefore, in the presence of 25OHC the synthesis of sphingomyelin could be stimulated in order to maintain the appropriate ratio of sphingomyelin and cholesterol. OSBP binds to 25OHC and translocates to Golgi apparatus to stimulate the transport of ceramide by CERT, which then leads to increased synthesis of sphingomyelin.

2.3.5 The effect of OSBP on cholesterol metabolism

The effect of OSBP on cellular cholesterol homoeostasis has been studied by overexpressing OSBP in CHO cells (Lagace et al., 1997). Cholesterol esterification was reduced in OSBP overexpressing cells compared to mock-transfected cells and also the incorporation of [^{3}H]oleic acid to triglycerides was slightly inhibited. Furthermore, cholesterol biosynthesis and LDL receptor gene expression were upregulated. These results were obtained when the cells were grown in delipidated serum containing medium showing that OSBP overexpressing cells try to increase the cellular free cholesterol levels even more than the control cells. This could be due to enhanced sphingomyelin synthesis reported later (Storey et al., 1998) or due to the sequesteration of cellular sterols by overexpressed OSBP. OSBP overexpression can also lead to altered cholesterol transport, since OSBP was recently demonstrated to be able to transport cholesterol between PI(4)P containing vesicles (Ngo and Ridgway, 2009).

OSBP was also shown to affect cholesterol efflux by regulating the stability of the ABCA1 protein involved in the cholesterol efflux to apoA-I (Bowden and Ridgway, 2008). Silencing of OSBP stabilized ABCA1 protein without affecting its mRNA levels. Concordantly, overexpression of OSBP destabilized ABCA1. This regulation seemed to be independent of the effect of OSBP on sphingomyelin synthesis and to require the functional sterol binding domain.

2.3.6 The role of OSBP in intracellular signaling

Already in the early studies OSBP antibody recognized two bands in the purified sample, 96 kDa and 101 kDa (Dawson et al., 1989a), which were later found to represent the phosphorylated and non-phosphorylated forms of OSBP (Ridgway et al., 1998). OSBP was also found to be dephosphorylated in cells depleted of cholesterol (Storey et al., 1998) and in cells where the plasma membrane sphingomyelin was depleted by exogenously added bacterial SMase (Ridgway et al., 1998). Dephosphorylation of OSBP seemed to result from the action of okadaic acid sensitive phosphatase PP2A, which is activated by ceramides and which later turned

out to be an interaction partner of OSBP (Wang, P.Y. et al., 2005). The phosphorylation sites of OSBP were mapped by Mohammadi et al. (2001).

A new function for OSBP was discovered in 2005, when Wang et al. found that OSBP is an essential part of a protein complex that contains two phosphatases (Wang, P.Y. et al., 2005). Based on their findings they proposed a model in which the cholesterol binding of OSBP induces its interaction with PP2A phosphatase and phosphatase HePTP, which belongs to a family of PTPPBS tyrosine phosphatases. Removal of cellular cholesterol or addition of 25OHC induces the disassembly of this complex, leading to an increase in the amount of phosphorylated extracellular signal-regulated kinase (ERK). Later they localized the HePTP binding to a coiled-coil domain in the C-terminus of OSBP (Wang et al., 2008).

Another potential function for OSBP was found in studies with liver cells (Yan et al., 2007b). Adenoviral overexpression of OSBP in mouse liver was shown to increase the amount of triglycerides in very low density lipoprotein fraction and also in liver. This was accompanied by stimulated expression of SREBP-1c and its target genes involved in triglyceride and fatty acid synthesis. In accordance, OSBP silencing in cultured liver cells inhibited the triglyceride synthesis and the insulin stimulated induction of SREBP-1c responsive genes. These results can be linked to the function of OSBP as a regulator of ERK signaling, because SREBP-1c has been shown to be a target of ERK phosphorylation (Kotzka et al., 1998).

In hypothetical summary, OSBP relays the signal of cholesterol depletion to the phosphatase complex by disassembling it when not bound to cholesterol. The disassembly leads to an increased amount of phosphorylated ERK1/2 kinases, which can then phosphorylate and thus activate nuclear SREBP (Kotzka et al., 2000) to induce the expression of genes involved in the uptake and synthesis of cholesterol.

2.4 OSBP-related proteins

2.4.1 Mammalian ORPs

Human OSBP-related proteins (ORPs) were discovered later than their yeast relatives. Six of them, ORP1-6 were found in the search for cDNA sequences homologous to the sterol-binding domain of OSBP using the EST (expressed sequence tag) sequence database (Laitinen et al., 1999). In the next studies of two independent groups five additional human ORPs were found, cDNAs of all eleven ORPs established and their exon-intron structures and the chromosomal locations determined (Jaworski et al., 2001; Lehto et al., 2001). Functional domains were also predicted (Figure 3). All the human ORPs were found to contain an OSBP-related

domain (ORD) in their C-terminal half. In the middle of this domain they all have a fingerprint sequence EQVSHHPP, which can also be found in the ORPs of other species. Except for ORP2 all the full-length proteins have a PH domain in their N-terminus and ORP5 and ORP8 have a C-terminal transmembrane sequence, structures that predict ORPs to be associated with membranes. In later studies most of the ORP genes were found to encode several protein isoforms due to alternative splicing. Human ORPs were also divided up into six subgroups according to their genomic organization and amino acid homology (Jaworski et al., 2001; Lehto et al., 2001).

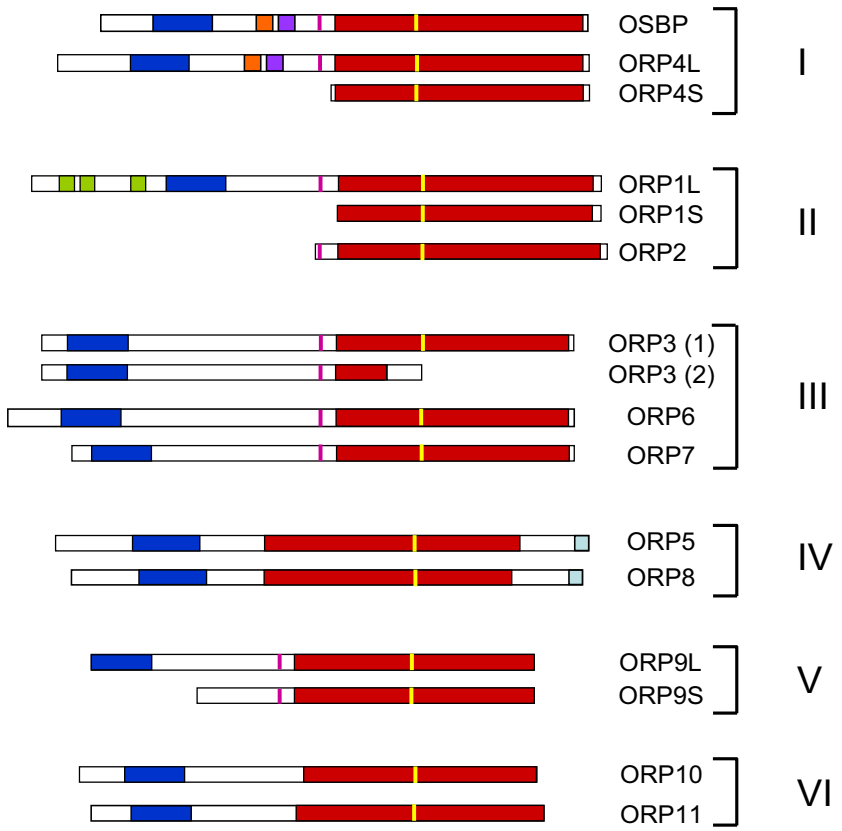

Figure 3. A schematic figure of mammalian ORPs. Different subgroups are specified by Roman numerals. ORD is shown in red, OSBP-fingerprint in yellow, PH domain in blue, FFAT-motif in pink, ankyrin repeats in green, dimerisation domains in orange, leucine repeats in purple and transmembrane domain in light blue.

O R P 1

The ORP1 gene has been shown to encode two isoforms, ORP1Short (ORP1S) and ORP1Long (ORP1L) (Johansson et al., 2003). The size of ORP1S is 50 kDa and it consists only of the OSBP-related domain. ORP1L is 108 kDa in size and it additionally has a PH domain and three ankyrin repeats, which are usually mediating protein-protein interaction in proteins with variable functions (Mosavi et al., 2004). These two isoforms also differ in their tissue expression pattern. ORP1S displays the highest expression in skeletal muscle and heart while ORP1L is most abundant in brain and lung (Johansson et al., 2003). ORP1L expression was also shown to be over 100-fold upregulated during the differentiation of monocytes to macrophages, whereas ORP1S expression displayed only 3-5-fold upregulation (Johansson et al., 2003), suggesting that ORP1L has an important role in this differentiation process or in macrophages.

The subcellular localizations of ORP1S and ORP1L are also different. ORP1S is evenly distributed throughout the cytoplasm and ORP1L localizes to Rab7-positive late endosomes (Johansson et al., 2003). The late endosomal targeting is dependent on the ankyrin repeats. The PH domain of ORP1L was shown to bind several phosphoinositides with low specificity and affinity, but was not necessary for the late endosomal targeting of ORP1L (Johansson et al., 2005), although it was able to strengthen the membrane interaction of ORP1L.

The ankyrin repeat region of ORP1L was shown to mediate the interaction of ORP1L with the active form of the late endosomal protein Rab7 (Johansson et al., 2005). The overexpression of ORP1L in HeLa cells stabilized the active form of Rab7, thus increasing its membrane association. ORP1L overexpression also induced the clustering of late endosomes to the perinuclear area. Rab7 mediated the interaction of ORP1L and RILP, another Rab7 effector protein (Johansson et al., 2007). Together these three proteins form a complex that controls the dynein-driven, minus-end directed microtubular movement of late endosomes. Interestingly, ORP1L also interacts with an adenoviral protein RIDα, which is suggested to mimic the active form of Rab7 in host cells (Shah et al., 2007).

The role of ORP1L in lipid metabolism was studied using ORP1L transgenic mouse macrophages (Yan et al., 2007a). Cholesterol efflux to HDL_2 particles was inhibited in ORP1L expressing macrophages, while the efflux to apoA-I was not changed compared to wild-type cells. In parallel, the expression of ABCG1 and apoE was down-regulated in ORP1L macrophages. These results could at least partly explain the major finding of the study – the transplantation of ORP1L expressing macrophages to LDL receptor knock-out mice resulted in larger atherosclerotic lesions than the transplantation of macrophages from wild-type mice.

ORP1S function has been studied only in yeast cells, in which it was found to affect Golgi-derived vesicle transport (Fairn and McMaster, 2005a; Xu et al., 2001) Despite the lack of a PH domain ORP1S was shown to have affinity to acidic phospholipids, although the specificity has varied between experiments (Fairn and McMaster, 2005a; Xu et al., 2001). ORP1L has been shown to bind 22(R)OHC and 25OHC (Yan et al., 2007a).

O R P 2

The ORP2 gene encodes a 55 kDa protein, which consists only of the OSBP-related domain. It does not have a PH domain, but was still reported to bind acidic phospholipids such as phosphatidic acid and PI(3)P (Xu et al., 2001). According to mRNA and protein expression data from human and mouse tissues, ORP2 is ubiquitously expressed (Laitinen et al., 2002). The expression level varies between tissues and the highest expression can be detected in brain. In mouse tissues polyclonal ORP2 antibody recognizes two bands with the molecular weights of 51 kDa and 56 kDa, which could represent either two splice variants or post-transcriptionally modified forms of ORP2.

In CHO-K1 cells overexpressed ORP2 localized in part to the Golgi apparatus, but also cytosolic staining was observed (Laitinen et al., 2002). Constitutive overexpression of ORP2 in CHO-K1 cell lines had many effects on cellular cholesterol metabolism. Cholesterol efflux to 20% human serum, apoA-I and phosphatidylcholine vesicles was enhanced, and cholesterol esterification inhibited in ORP2 overexpressing cells compared to cells transfected with the control plasmid. Transient overexpression of ORP2 also caused a delay in the secretory pathway from the Golgi to the plasma membrane, which is in accordance with the result from Xu et al. showing the inhibitory effect of ORP2 on Golgi-derived vesicle transport in yeast (Xu et al., 2001). However, attention should be paid to the fact that Xu et al. used an ORP2 construct that is lacking amino acids 13-24.

The effect of constitutive ORP2 overexpression on the cellular lipidome was studied in the CHO cell lines (Käkelä et al., 2005). In ORP2 overexpressing cells cultured in the standard medium the molar percentage of polyunsaturated fatty acids (PUFA) containing (diacyl) phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol was increased. Furthermore, when the cells were cultured in medium containing lipoprotein deficient serum (LPDS), the relative amount of these lipids was decreased compared to control cells that displayed no change between the different culturing conditions. The amount of triglycerides and cholesteryl esters was decreased in ORP2 expressing cells. They also had less PUFAs in neutral lipids than the control cells, when cultured in normal medium. This suggests that ORP2 either inhibits the synthesis of neutral lipids or enhances their degradation. When

cultured in LPDS containing medium, the amount of PUFAs in neutral lipids was not reduced as much in ORP2 overexpressing cells as in control cells, suggesting that PUFA-containing neutral lipids are more stable in ORP2 overexpressing cells.

ORP3

ORP3 was first identified as a gene that had variable expression in hematopoietic cells (Gregorio-King et al., 2001). The ORP3 gene was later found to encode eight isoforms resulting from alternative splicing (Collier et al., 2003). These isoforms displayed distinct expression patterns. The full-length ORP3 was ubiquitously expressed, but substantial differences were found between tissues and cells at distinct stages of differentiation (Collier et al., 2003; Johansson et al., 2003; Lehto et al., 2004). ORP3 has also been found to be upregulated in many cancer tissues (see references in (Lehto et al., 2008)). Similar to most ORPs, ORP3 consists of a PH domain and OSBP-related domain and spaces in between. Four out of the eight ORP3 isoforms have a truncated OSBP-related domain. Full-length ORP3 shows a cytosolic distribution with apparent localization also at the plasma membrane and ER (Lehto et al., 2004). More careful examination revealed that the plasma membrane targeting of ORP3 is dependent on the PH domain, which is able to bind phosphoinositides PI(3,4)P_2 and PI(3,4,5)P_3 in a liposome pull-down assay (Lehto et al., 2005). In Hek293 cells endogenous ORP3 was also detected in the tips of cell membrane protrusions (Lehto et al., 2008). ER-targeting of ORP3 was dependent on the FFAT-motif, but also the flanking regions were required (Lehto et al., 2005). The FFAT-motif was also shown to mediate the VAP-A-binding of ORP3. Overexpression of both ORP3 and VAP-A resulted in the formation of rolled-up structures of ER-membranes, also known as organized smooth ER (OSER) (Snapp et al., 2003). This finding may be an artifact of protein overexpression or support the role of ORPs in membrane contact sites, due to targeting two membranes simultaneously (Olkkonen and Levine, 2004). The N-terminal half of ORP3 was demonstrated to respond to the conformational change induced by ligand binding in the C-terminal domain (Lehto et al., 2005). Since the ligands of the ORP3 ORD are not known, the oxysterol binding domain of ORP3 was replaced by the sterol binding domain of OSBP, which binds 25OHC. This chimeric construct was localizing to ER under normal culture conditions, but when 25OHC was added to the cells, ORP3:OSBP translocated to the plasma membrane, supporting the role of ligand binding in the regulation of ORP3 localization.

More insight into the function of ORP3 was given by the finding that ORP3 interacts with R-Ras, a small GTPase that regulates cell adhesion, spreading and migration (Goldfinger et al., 2007; Lehto et al., 2008). Concordantly, ORP3 gene silencing and overexpression experiments suggested that ORP3 is a negative regulator of cell spreading and integrin activity, thus exhibiting effects opposite to those of active R-

Ras. ORP3 was also shown to be phosphorylated and the phosphorylation was upregulated when the cells had no adhesive contacts (Lehto et al., 2008).

ORP6 and ORP7

ORP6 and ORP7 belong to the same ORP subgroup as ORP3, but they have not been characterized in much detail. ORP6 mRNA displays the highest expression in brain and skeletal muscle and the expression in macrophages is significantly upregulated after loading with acetylated LDL (Lehto et al., 2001). ORP7 displays the highest expression in gastrointestinal tract and fetal lung (Lehto et al., 2004). The localization of overexpressed ORP6 was observed to be similar to ORP3 – association with the nuclear envelope, ER and plasma membrane could be detected (Lehto et al., 2004). ORP7 was detected partially associated with the ER.

ORP4

ORP4 (also referred as OSBP2 and HLM, HeLa metastatic gene) is the closest homologue of OSBP. It was first identified as a gene that was upregulated in circulating tumor cells compared to healthy tissue and was therefore proposed to be a good marker of tumor metastasis (Fournier et al., 1999). ORP4 gene was reported to be highly expressed in retina but also in testis, fetal liver, brain, heart, muscle and kidney (Moreira et al., 2001; Wang et al., 2002). ORP4 gene was found to encode two isoforms, ORP4S (short isoform) and ORP4L (long isoform). ORP4L is similar to OSBP in its protein structure, but ORP4S has only an OSBP-related domain. Both isoforms bind 25OHC and cholesterol (Wyles et al., 2007). Both were also shown to associate with vimentin intermediate filaments. Overexpressed ORP4S caused the bundling and aggregation of these filaments, which was accompanied by inhibited esterification of LDL-derived cholesterol (Wang et al., 2002). The presence of a leucine repeat in ORP4L prevented the vimentin aggregation in ORP4L overexpressing cells (Wyles et al., 2007). ORP4L also has a short sequence that is highly homologous to the dimerisation domain of OSBP. This domain was found to mediate the interaction between ORP4L and OSBP (Wyles et al., 2007), which is the only interaction between different ORPs observed so far.

ORP5 and ORP8

Basic characterization of ORP5 has not yet been done. However, it was recently demonstrated to play a role in the invasion of pancreatic cancer (Koga et al., 2008). Not only was high ORP5 expression connected with high invasiveness of cancer cells, but also overexpression and silencing studies of ORP5 suggested that the protein stimulates cancer cell invasion. Together with ORP8 it forms a subgroup IV. The common structural feature for them is a putative transmembrane domain in their C-terminus. The transmembrane domain mediates the association of ORP8 with ER

membranes and the OSBP-related domain mediates the binding of 25OHC (Yan et al., 2008). ORP8 expression is most abundant in liver, spleen, kidney, brain and adipose tissue. It is also highly expressed in human macrophages and was detected at high levels in human coronary artery atherosclerotic lesion macrophages. Interestingly, ORP8 gene silencing in human macrophage model was demonstrated to upregulate the expression of ABCA1 and enhance the cholesterol efflux to apoA-I suggesting that by inhibiting cholesterol efflux ORP8 could enhance the development of atherosclerotic lesions.

ORP9

Like most other long ORPs, ORP9 is found as two splice variants, ORP9L and ORP9S, which consists only of the OSBP-related domain (Wyles and Ridgway, 2004). Endogenous ORP9L localizes to Golgi membranes in CHO-K1 cells, but was also observed to associate with the ER, when overexpressed (Wyles and Ridgway, 2004). Inducible overexpression of ORP9L in CHO-K1 cells was shown to disrupt the organization of ER and Golgi membranes (Wyles and Ridgway, 2004). Furthermore, the depletion of ORP9 led to the dispersion of Golgi markers and inhibition of ER-Golgi protein transport, suggesting that ORP9 is necessary for the integrity of Golgi in CHO cells (Ngo and Ridgway, 2009). Also the inducible overexpression of ORP9S led to fragmentation of Golgi. This was accompanied by inhibition of cell proliferation. ORP9 was also shown to be able to transport cholesterol from PI(4)P containing donor vesicles to acceptor vesicles (Ngo and Ridgway, 2009).

ORP9 is also participating in PI3-kinase dependent signaling (Lessmann et al., 2007). ORP9S was found to be highly expressed in human bone marrow-derived mast cells, in which it was demonstrated to be phosphorylated in a protein kinase Cβ (PKCβ) dependent way. ORP9 depletion was also shown to activate the phosphorylation of Akt in Hek293 cells and the two proteins were also found to form complexes with PKCβ. Through Akt ORP9 can therefore be linked to signaling cascades controlling the cell survival, cell cycle progression and glucose metabolism.

Common features of ORPs

Although mammalian ORPs have been proposed to have their own distinct functions, they share many common features in addition to their homologous sequences. First of all, many of them interact directly or indirectly with small GTPases - OSBP with ARF, ORP1L with Rab7 and ORP3 with R-Ras. Many ORPs also show associations with the cytoskeleton. ORP1L is involved in the microtubule-dependent transport; ORP3 seems to influence the actin cytoskeleton (Lehto et al., 2008) and ORP4 associates with intermediate vimentin filaments. In addition,

ORP10 has been detected to localize on microtubules (J. Perttilä and V. M. Olkkonen, unpublished observation).

A notable common feature of ORPs is that they have many determinants for intracellular targeting. The PH domain found in the long ORPs or phospholipid binding of the short ones targets them for example to the Golgi apparatus or to the plasma membrane. An FFAT motif or a transmembrane domain (in ORP8) targets the ER. Either the function of these determinants is regulated so that ORPs associate with one organelle at a time or ORPs are targeted to both organelles simultaneously at membrane contact sites. ER is found to form membrane contact sites with other organelles such as trans-Golgi network, plasma membrane, mitochondria, peroxisomes, lipid droplets, endosomes and lysosomes. ER is also a major organelle in which the lipid biosynthesis and the regulation of lipid metabolism take place. Through membrane contact sites lipids could be transported more easily without the need to transfer them over long distances. ORPs have been proposed to assist in the formation of membrane contact sites or in transporting lipids through them (Olkkonen and Levine, 2004)). The possibility that ORPs act as lipid transporter proteins has been studied more intensively in yeast.

2.4.2 Yeast ORPs

Yeast (*Saccharomyces cerevisiae*) does not have cholesterol in cellular membranes. Instead it can synthesize ergosterol, which closely resembles cholesterol in structure and in subcellular distribution (Zinser et al., 1993). Before discovering the mammalian homologues of OSBP, three OSBP-related genes were identified in budding yeast, namely SWH1 (OSH1 in current nomenclature), KES1 (OSH4) and HES1 (OSH5) (Jiang et al., 1994; Schmalix and Bandlow, 1994). The deletion of these genes was shown to affect the levels of membrane ergosterol (Jiang et al., 1994). The whole yeast ORP family was identified and named OSH1-7 in the study of Beh et al. (2001). They also examined the effect of different combinations of OSH gene deletions on cell growth and ergosterol levels. Cells lacking one to six OSH genes were viable, but if all seven OSH genes were deleted, cells had a dramatic increase in their ergosterol levels and they did not survive. Deletion of each one of the OSH genes resulted in changes in gene expression that were distinct for each OSH (Beh et al., 2001). This suggests that the Osh proteins have independent roles, but also a common, essential function that most probably involves ergosterol metabolism.

Beh et al. later proposed that the common role for Osh proteins involves the regulation of endocytosis (Beh and Rine, 2004). Deletion of all the OSH genes resulted in a disturbed ergosterol distribution and accumulation of intracellular lipid

droplets. Golgi-derived protein transport seemed to be unchanged, but endocytosis was severely inhibited. Whether the endocytosis defect resulted from changes in ergosterol distribution or was a direct consequence of OSH depletion remained to be clarified. It was also demonstrated that Osh-proteins are important for the polarized growth of yeast (Kozminski et al., 2006). They were found to be required for the proper polarized localization of small GTPases Cdc42p and Rho1p and also for a specific vesicular secretion pathway.

The possible role of Osh proteins in nonvesicular sterol trafficking was studied using esterification of exogenously added sterol as an indicator of sterol trafficking from the plasma membrane to ER (Raychaudhuri et al., 2006). The yeast strains with Osh deletions displayed inhibited cholesteryl ester synthesis suggesting that Osh proteins transfer cholesterol from the plasma membrane to ER, which could be the common function of the Osh proteins. However, depletion of all OSH genes was found to result in a decreased amount of sterol in the plasma membrane as judged by filipin staining, suggesting the opposite (Beh and Rine, 2004). Osh proteins are so far the only putative nonvesicular sterol transport proteins in yeast. However, their deletion did not completely block the ergosterol transport from ER to the plasma membrane suggesting that there are other sterol transport pathways in yeast as well (Sullivan et al., 2006).

Osh4/Kes1p

Osh4p is the most studied ORP in yeast and it is also the first ORP that was crystallized in complex with different sterols (Im et al., 2005). Osh4p is a short ORP consisting only of the OSBP-related domain. It has a hydrophobic lipid binding pocket that is composed of β-strands, which form a near-complete β-barrel (Figure 4). The N-terminus of Osh4p forms a lid that closes the barrel when a sterol is bound. Sterols are positioned in the barrel so that the 3-OH group is at the bottom and the side-chain is close to the lid. Hydroxyl groups in sterols do not make direct contacts with the amino acid side-chains of Osh4p, the contacts are rather mediated by water molecules. Osh4p could not be crystallized without a ligand. An apo structure of Osh4p without the lid was however obtained reflecting the unbound protein. The apo structure contained two regions that were buried in the ligand-bound state but that were found on the surface of the apo structure making contacts with sulphate ions, which were thought to mimic the phosphate groups of PIPs. A model was thus constructed, in which the apo conformation attaches to the membrane with the help of PIPs and extracts cholesterol from the membrane or alternatively oxysterol from the solution. In the solution the protein-ligand complex is able to relay signals before it translocates to the acceptor membrane and unloads the lipid. This model is supported by the findings that Osh4p is able to transfer

sterols between liposomes in vitro (Raychaudhuri et al., 2006). The transfer was enhanced when PI(4,5)P_2 was included in the liposomes.

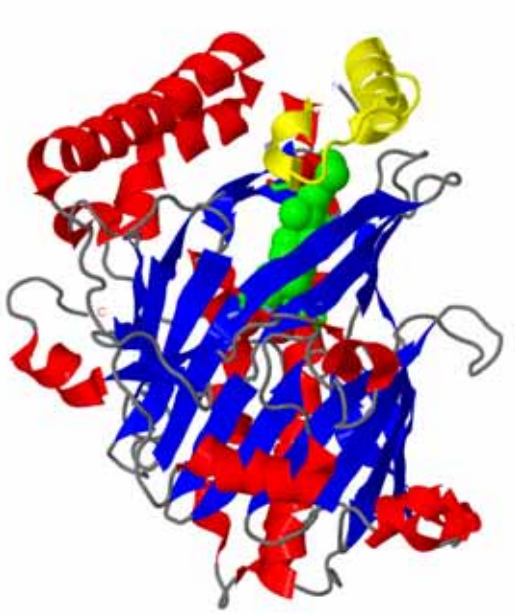

Figure 4. Structure of Osh4p in a complex with 25OHC (Protein Data Bank ID 1zhx). α-helical structures are colored in red and β-strands in blue. 25OHC is colored in green and red, and the lid region (amino acids 1-29) in yellow.

The first study on Osh4p suggested that the protein functions in the Golgi apparatus (Fang et al., 1996). Inactivation of the OSH4 gene resulted in survival of cells lacking the Sec14p function. Sec14p is a PI/PC-transfer protein essential for the transport of secretory proteins from Golgi apparatus. Deletion of any other Osh was not able to bypass the Sec14p deletion effect (Beh et al., 2001). Osh4p was thus proposed to be a negative regulator of Golgi function. Despite the lack of a conventional PH domain, Osh4p binds PI(4,5)P_2. The binding was shown to be mediated by a region outside the ORD, which was proposed to be similar to PH domains (Li et al., 2002). However, based on the three-dimensional structural analysis of Osh4p, lysines 109 and 336 in the ORD were suggested to mediate the interaction with phosphate groups in phosphoinositides (Im et al., 2005), although the mutation in Lys109 did not inhibit the PIP binding in (Li et al., 2002). This mutation still abrogated the Golgi association of Osh4p (Li et al., 2002) supporting

the model of Im et al. Osh4p was also demonstrated to regulate Arf GTPase function (Li et al., 2002), a feature found also for OSBP.

Other Osh-proteins

Localization of Osh1p supports nicely the function of ORPs in membrane contact sites. It localizes to nucleus-vacuole junction, which is a subcompartment of ER between the nuclear envelope and vacuole (Levine and Munro, 2001). This localization is mediated by the FFAT-motif and ankyrin repeats. The FFAT-motif also mediates the interaction of Osh1p, Osh2p and Osh3p with Scs2p, the yeast homologue of VAP-A (Levine and Munro, 2001; Loewen et al., 2003). Osh1p also has a conserved PH domain that mediates the targeting to the Golgi apparatus. In addition, Osh3p has a proposed function in the nucleus (Park et al., 2002). It interacts with a DEAD-box RNA helicase, Rok1p and is proposed to participate in the nuclear fusion and mitotic spindle formation (Park et al., 2002; Yano et al., 2004). Osh6p and Osh7p were demonstrated to interact with Vps4p, a member of AAA (ATPases associated with a variety of cellular activities) protein family (Wang, P. et al., 2005). Deletion of Vps4p resulted in enhanced membrane association of Osh6p and Osh7p suggesting that it mediates the detachment of these proteins from the membrane.

2.4.3 ORPs in other species

Families of OSBP-related proteins can be found in practically all eukaryotic species. Like humans, mice also have twelve ORPs (Anniss et al., 2002). In addition, there are reports of ORPs in fruit fly (*Drosophila melanogaster* (Alphey et al., 1998)), roundworm (*Caenorhabditis elegans* (Sugawara et al., 2001)) and the parasitic protist *Cryptosporidium parvum* (Zeng and Zhu, 2006). Plants such as petunia and *Arabidopsis thaliana* were also demonstrated to have ORPs (Skirpan et al., 2006). The presence of ORPs throughout the eukaryotic kingdom suggests that they are important players in the cellular environment.

3 AIMS OF THE STUDY

1. To find the ligands of ORP2
2. To determine the intracellular localization of ORP2
3. To clarify the role of ORP2 in the intracellular lipid metabolism

4 MATERIALS AND METHODS

4.1 List of published methods

Method	Publication
Recombinant DNA techniques	II, III
Cell culture and transfection	I, III
Expression and purification of recombinant proteins	I, II, III
SDS-PAGE and Western blotting	I, II, III
Lipid extraction and TLC	I, III
Liposome pull-down assay	I
Charcoal-dextran sterol-binding assay	II, III
Cholesterol binding assay	III
Immunofluorescence microscopy	I, III
Assay of cholesterol efflux	I
Isolation of detergent resistant membranes	I
Isolation of plasma membrane fraction using colloidal silica	I
Assay of cholesterol esterification	I, III
Enzymatic analysis of cellular cholesterol	I
Assay of HMG-CoA reductase activity	I
Assays of uptake of endocytic markers	I
Assay of triglyceride hydrolysis	III

4.2 Unpublished methods

4.2.1 In vitro translation and VAP-A pull-down assay

Wild-type ORP2 and the ORP2/FFAT-mutant were translated in vitro using [^{35}S]methionine (>1000 Ci/mmol, GE Healthcare-Amersham) and the TnT-T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's instructions. Twenty microliters out of the 50 µl in vitro reaction was incubated with 12 µg of GST-VAP-A(ΔTM) (A VAP-A construct that lacks the transmembrane domain) or plain GST in 100 µl PBS, 0.5% Triton X-100 for 45 min at room temperature. Glutathione sepharose 4B in PBS, 0.1% Triton X-100 was then added to the samples and incubated 30 min to bind the protein complexes. The matrix was washed three times with PBS, 0.1% Triton X-100 and boiled with SDS-PAGE sample buffer. The samples were resolved on 12.5% SDS-PAGE gels, which were fixed and treated with Amplify (GE Healthcare-Amersham). The dried gels were exposed on X-ray films overnight at -70ºC.

5 RESULTS AND DISCUSSION

5.1 Ligand binding of ORP2

5.1.1 ORP2 binds phosphoinositides (I)

Phosphatidylinositol phosphates (PIPs) are signaling lipids that form in cellular membranes by the action of organelle-specific kinases and phosphatases. Different signals affect the activity of these enzymes resulting in the acute formation of PIPs, which can mediate the targeting of PIP binding proteins to the membranes (Lemmon, 2008). The most common protein domain found to mediate the PIP binding is pleckstrin homology (PH) domain. This domain is found to regulate the membrane targeting of several ORPs.

ORP2 does not have a PH domain. However, it has been shown to bind PI(3)P and phosphatidic acid (Xu et al., 2001). Xu et al. detected PIP binding using an assay, in which PIPs are immobilized on a nitrocellulose membrane and incubated with proteins, which are then recognized by antibodies. Nitrocellulose membrane is however an unnatural platform for lipids, and they may arrange in structures not found in cellular membranes. Therefore we wanted to study the PIP binding using a more natural in vitro vesicle pull-down assay (Schiavo et al., 1996). In this assay PIPs are incorporated into phosphatidylcholine (PC) vesicles containing [^{14}C]PC as a tracer. The vesicles are incubated with GST-fusion proteins bound to glutathione sepharose beads, which are then pulled down and the radioactivity associated with the pellet is measured using liquid scintillation counter. GST was used as a negative control, the results of which were subtracted from the GST-ORP2 results. GST-tagged PH domain of phospholipase Cδ (PLCδ) was used as a positive control that binds PI(4,5)P_2 with high affinity and specificity (Lemmon et al., 1995). [^{14}C]PC vesicles without PIPs were used as control vesicles. Using this assay we observed that GST-ORP2 binds PI(3,4,5)P_3 (I, fig. 9). Some affinity was also observed towards other PIPs probably resulting from non-specific electrostatic interaction between positively charged amino acids and the negative PIPs. The result of PI(3)P or phosphatidic acid binding by ORP2 (Xu et al., 2001) could not be repeated. The affinity of PI(3,4,5)P_3 binding was not high, but may account for the membrane targeting of ORP2. However, PI(3,4,5)P_3 is usually present transiently at low levels in the plasma membrane (Cantley, 2002), which means that PI(3,4,5)P_3 binding proteins must have good affinity for it to mediate efficient downstream signaling.

Four other short ORPs lacking a PH domain, such as ORP1S, ORP9S, ORP10S and Osh4p, have been shown to bind PIPs as well (Fairn and McMaster, 2005b; Li et al., 2002; Xu et al., 2001). This interaction could be mediated by positively charged amino acids on the protein surface. Indeed, based on the three-dimensional structure of Osh4p Im et al. suggested that two lysines in the positions 109 and 336 of Osh4p are buried in the sterol binding conformation, but are exposed to the protein surface when the ligand is not bound (Im et al., 2005). That way the sterol binding could affect the interaction of Osh4p with membranes via PIP binding.

5.1.2 ORP2 binds oxysterols (II, III)

The sterol ligand of ORP2 has been searched for in many ways, unsuccessfully (Xu et al., 2001). We were able to show in Cos7 cells that ORP2 binds [^{3}H]-labeled, photo-activated 25OHC (II, fig. 4A). We wanted to confirm the binding in vitro by a charcoal-dextran sterol-binding assay using purified GST-fusion proteins and [^{3}H]-labeled 25OHC. Also the binding of 7KC, 22(R)OHC, 24(S)OHC and 27OHC was investigated. The best ORP2 ligand of the tested oxysterols was 22(R)OHC (III, fig. 1a), the K_d being 1.4 x 10^{-8} M, which is close to the value of OSBP affinity for 25OHC, 0.8-1.0 x 10^{-8} M (Dawson et al., 1989a; Dawson et al., 1989b). GST-ORP2 showed also binding to 24(S)OHC (unpublished result, K_d was not determined), 7KC (K_d 1.6 x 10^{-7}) and 25OHC (K_d 3.9 x 10^{-6}) (Figure 5; II, fig. 1D-E). The most interesting of these oxysterols is 24(S)OHC. We did not have sufficient amount of [^{3}H]-labeled 24(S)OHC to perform the assay with multiple concentrations, but a single experiment suggested that the affinity of ORP2 towards 24(S)OHC is good. 24(S)OHC is proposed to be important for the cholesterol transport from the brain (Björkhem, 2007). Because ORP2 is highly expressed in the brain, it would be forth studying if ORP2 specifically affects the function of 24(S)OHC.

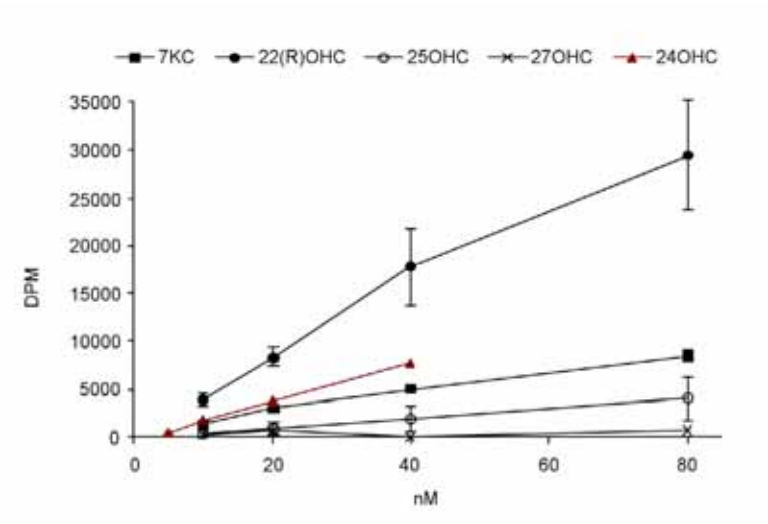

Figure 5. Oxysterol binding of GST-ORP2 was tested using charcoal-dextran sterol-binding assay of [^{3}H]oxysterols. All the results are published in II and III except for the binding of 24(S)OHC (shown in red).

To examine which amino acids of ORP2 participate in the oxysterol binding, mutations were introduced in several positions. Amino acid substitutions were designed based on the analysis of the structure of crystallized yeast ORP-homologue Osh4p (Im et al., 2005) and the three-dimensional model of ORP2 structure with bound 25OHC (II, fig. 2A). Mutations were generated by site-directed mutagenesis; the GST-tagged proteins were expressed in E. coli and purified. Their ability to bind 40 nM 25OHC was tested using the charcoal-dextran sterol-binding assay.

According to the model, nonpolar amino acid methionine 93 resides in the N-terminal lid region of ORP2 close to the hydroxyl-group in the 25-carbon (II, fig. 2B). Substitution of this residue with positively charged lysine was thus expected to modify the sterol binding. However, this mutation resulted in only a small reduction in the 25OHC binding. Hydrophobic phenylalanine 152 corresponds to hydrophobic leucine 111 of Osh4p, which is thought to stabilize the core fold of the protein. L111D mutation in Osh4p abrogated cholesterol binding and biological function of Osh4p. When F152 of ORP2 was mutated to negatively charged aspartic acid, the reduction in oxysterol binding of ORP2 was again not significant, suggesting that F152 is not essential for the oxysterol binding. Interestingly, the purified F152D ORP2-protein was partially fragmented in SDS-PAGE analysis, suggesting that this residue is important for the stability of ORP2 (II, fig. 2C). Phenylalanine 103 is

predicted to be located in the sterol-binding pocket near the B-ring of 25OHC, and introducing bulkier, nonpolar tryptophan to the place of phenylalanine was expected to hinder oxysterol binding to the pocket. However, the oxysterol binding of F103W mutant was only slightly inhibited.

The ORP2 mutants most defective in oxysterol binding were K150A and I249W (II, fig. 2D). According to the ORP2 model, lysine 150 is located near the 25-carbon of the sterol. In the alignment of Osh4p and ORP2 it corresponds to K109 in Osh4p, which is located near the entrance of the binding pocket and suggested to participate in the membrane binding mediated by phosphate groups of PIP. Substitution of lysine by the smaller, nonpolar alanine in ORP2 was expected to create more space and thus affect oxysterol binding specificity. Indeed, 25OHC binding of this mutant was only 70% of the wild-type ORP2 binding, suggesting that K150A mutation affects the local shape of the binding pocket so that oxysterol cannot fit there. Another possibility is that as a positive amino acid K150 forms electrostatic interactions with negative residues of the protein, which could be important for its proper fold. Mutation of lysine to neutral amino acid alanine may compromise these interactions.

Hydrophobic isoleucine 249 is located close to the C and D rings of the sterol. As a bulkier amino acid, tryptophan in the place of isoleucine was hypothesized to interfere with the sterol binding. I249W turned out to be the mutation that most efficiently blocks the oxysterol binding. It was also tested for 22(R)OHC binding, which was reduced 75% compared with the wild-type ORP2 (III, fig. 1A). I249 is not conserved from Osh4p to ORP2 and the mutation was designed based only on the molecular model of ORP2. This finding confirms that the three-dimensional model of ORP2 is at least in part valid. Together these results also confirmed the existence of a sterol-binding pocket in ORP2 and offered a new mutant as a valuable tool to study the effect of ORP2 oxysterol binding on cellular lipid metabolism.

5.1.3 ORP2 binds cholesterol (III)

Cholesterol binding of ORP2 would offer an explanation to the finding that ORP2 overexpression affects the intracellular cholesterol metabolism (Laitinen et al., 2002). Abnormally high amounts of cytosolic protein even with low affinity towards cholesterol could transport cholesterol from the ER to the plasma membrane and thus enhance cholesterol efflux. In addition, other ORPs such as OSBP (Wang, P.Y. et al., 2005), ORP4 (Wyles et al., 2007) and Osh4p (Im et al., 2005) were previously shown to bind cholesterol. OSBP and ORP9 were even demonstrated to transfer cholesterol between vesicles (Ngo and Ridgway, 2009)

In live cells overexpressed ORP2 was not able to bind [^{3}H]photocholesterol (results not shown). However, cholesterol binding of ORP2 was still examined in vitro. Since cholesterol is far less water-soluble than oxysterols, it cannot be used in the charcoal-dextran sterol-binding assay. Therefore we designed an assay, in which [^{3}H]cholesterol is solubilized with methyl-β-cyclodextrin, a cyclic oligosaccharide molecule that in an aqueous environment form a cylindrical shape, which is able to bind a cholesterol molecule inside the cavity, thus making it water-soluble. [^{3}H]cholesterol-cyclodextrin complexes are then incubated with GST-tagged fusion proteins bound to glutathione sepharose beads and the radioactivity associated with the beads is measured. GST was used as a negative control and GST-tagged START (steroidogenic acute regulatory protein related lipid transfer) domain of MLN64 (Metastatic lymph node 64) as a positive one.

In the assay, GST-ORP bound cholesterol approximately twice as much as the negative control GST (III, fig. 1B). Cholesterol binding was still modest compared with that of MLN64 START. When the assay was done in the presence of 40-fold molar excess of unlabeled 22(R)OHC, cholesterol was no longer bound to GST-ORP2. These results indicate that ORP2 is capable of binding cholesterol in the same binding pocket as oxysterols. This is different to OSBP, in which the bound cholesterol could not be replaced by 25OHC (Wang, P.Y. et al., 2005) suggesting two binding sites for sterols.

Together with the fact that ORP2 is a soluble, cytosolic protein, the ability to bind cholesterol makes ORP2 a potential cholesterol transport protein. Although the transport activity might not be relevant in vivo, it could be detected in cells overexpressing ORP2 as changes in cholesterol metabolism.

5.2 Intracellular targeting of ORP2 (III)

5.2.1 ORP2 associates with lipid droplets

In previous studies ORP2 has been shown to localize to the Golgi apparatus (Laitinen et al., 2002; Xu et al., 2001). However, in most cell lines ORP2 has been observed to be soluble throughout the cytoplasm, although it has also been detected in cell membrane fractions. Since ORP2 was found to be a lipid-binding protein and therefore potentially regulated by cellular lipid status, its localization was studied in conditions with different amounts of lipids using the epidermal carcinoma cell line A431. In these cells, under normal culture conditions with 10% FBS (Fetal bovine serum), ORP2 staining was cytosolic, but also punctate structures and small spheres could be detected (III, fig. 2A). Strikingly, when A431 cells were treated with oleic

acid-BSA (bovine serum albumin) to induce the formation of intracellular lipid droplets, the localization of overexpressed ORP2 was changed. ORP2 antibody staining was no longer cytosolic, but formed small circles (III, fig. 2D-F). Interestingly, the interior of these circles was stained with a neutral lipid stain BODIPY 493/503 confirming that these structures were lipid droplets. BODIPY 493/503 staining of cells cultured under normal growth conditions showed that ORP2 localization to lipid droplets was not induced by oleic acid-BSA-loading, since it could be detected also in normal conditions, in which small lipid droplets can be observed.

Lipid droplet localization was proven to be specific by many control experiments. First of all, overexpressed, Xpress-tagged ORP2 was detected in lipid droplets with both anti-Xpress antibody and polyclonal ORP2 antibody (described by Laitinen et al., 2002). Secondly, HcRed-tagged ORP2 localized to lipid droplets also in non-permeabilized cells, showing that the targeting is not an artefact resulting from the treatment of the cells for immunofluorescence microscopy (III, fig. 3D-F). Thirdly, the localization was not cell type specific, because overexpressed ORP2 was detected in lipid droplets also in HeLa, HepG2, Cos7, HuH7 and Hek293 cells (data not published). Finally, also untagged ORP2 targeted to lipid droplets in TREx HeLa cells induced to express ORP2 proving that the tag is not affecting the targeting (III, fig. 3A-C).

ORP2 is endogenously expressed in A431. To determine if also the endogenous ORP2 localizes to lipid droplets, A431 cells were treated overnight with oleic acid-BSA and the cells were stained with ORP2 antibody and BODIPY 493/503. In these conditions, ORP2 staining was concentrated in the area where lipid droplets resided (III, fig. 3G-I). Clear ring-like staining was not observed, as in ORP2 overexpressing cells, which could result from low level of endogenous ORP2 in these cells. The specificity of the ORP2 antibody in this experiment was confirmed by pre-incubating the antibody with purified GST-ORP2 before adding to the cells. The pre-incubation inhibited the ORP2 staining in the area of lipid droplets (III, fig. 3G-I).

5.2.2 22(R)hydroxycholesterol inhibits lipid droplet targeting of ORP2

Because ORP2 targeted to lipid droplets in the standard growth conditions, it was expected that some other growth condition could inhibit the targeting. The Golgi translocation of OSBP is induced by cholesterol depletion or 25OHC treatment. We therefore studied if the addition of oxysterols would inhibit the lipid droplet localization of ORP2. A431 cells transfected with ORP2-expression plasmid were treated overnight with oleic acid-BSA and different oxysterols. The addition of 5

μM 7KC, 24(S)OHC, 25OHC and 27OHC did not significantly affect the targeting. However, when the cells were treated with 5 μM 22(R)OHC, which is the highest affinity ligand of ORP2 so far, the lipid droplet targeting was clearly inhibited (III, fig. 6A-C). In these cells ORP2 staining was dispersed across the cytoplasm, not evenly, but showing a dotty-like appearance.

The effect of 22(R)OHC was also studied in oleic acid-BSA loaded TREx HeLa cells induced to express ORP2. In these cells ORP2 targeting to lipid droplets is somewhat different than in A431 cells. Whereas in A431 cells ORP2 staining surrounds almost every lipid droplet inside one cell, in TREx HeLa cells ORP2 staining is detected only in part of the lipid droplets (III, fig. 3A-C). When 22(R)OHC is added to TREx HeLa cells, the lipid droplet staining of ORP2 vanishes more dramatically and the cytoplasmic staining of ORP2 is more even than in A431 cells (III, fig. 6D-F). In addition, some plasma membrane staining can be seen. It is thus possible that in some cell types 22(R)OHC induces the translocation of ORP2 to other membranes such as the plasma membrane. In hepatic HuH7 cells the plasma membrane staining of overexpressed ORP2 is often detected, also without the addition of 22(R)OHC (data not shown). The possible targeting to plasma membrane requires further investigation since it might offer more evidence for the hypothesis that overexpressed ORP2 can transport cholesterol to the plasma membrane.

The concentration of 5 μM is physiologically high for oxysterols (van Reyk et al., 2006). Therefore we studied the effect of 0.5 μM and 0.05 μM 22(R)OHC on ORP2 lipid droplet targeting in ORP2 transfected A431 cells. The concentration of 0.05 μM was not able to remove ORP2 from the lipid droplets, but 0.5 μM showed a small effect, although the effect was more subtle than with 5 μM 22(R)OHC (III, fig. 6N-O). The fact that the oxysterol effect on ORP2 targeting is dependent on the concentration supports the view that ORP2 localization and thereby also its function is directly regulated by oxysterols.

We then wanted to ensure that the effect of 22(R)OHC was due to its binding to ORP2 and not by a secondary effect. The ORP2 mutant I249W, which is defective in oxysterol binding, was expressed in oleic acid-BSA loaded A431 cells in the presence or absence of 5 μM 22(R)OHC. In the absence of oxysterol ORP2 I249W was targeted to the lipid droplets like the wild-type protein (III, fig. 6G-I). However, also in the presence of 22(R)OHC ORP2 I249W remained associated with lipid droplets (III, fig. 6J-L) unlike the wild-type protein, which lost its lipid droplet association. These results clearly indicate that the ligand binding induces the translocation of ORP2 from the lipid droplet surface to a cytoplasmic location.

5.2.3 The role of FFAT-motif in the lipid droplet targeting of ORP2

Membranes of ER have been regarded as a source of the monolayer surrounding lipid droplets and thus ER can often be detected in the close proximity of lipid droplets (Walther and Farese, 2008). ORP2 has an FFAT-motif, which is found in many lipid binding proteins and mediates their interaction with an ER membrane protein VAP-A (Loewen et al., 2003). Therefore it was possible that ORP2 localizes to the ER membranes surrounding the lipid droplets. To investigate this possibility, we examined the role of the FFAT-motif in the localization of ORP2 and carried out immunoelectron microscopy to visualize the ORP2 localization at the ultrastructural level.

Interaction of ORP2 with VAP-A was previously suggested by Wyles and Ridgway (2004) and we wanted to confirm the result, because the binding seemed rather weak. ORP2 was translated in vitro, labeled with [^{35}S]methionine, and subjected to pull-down with GST-tagged VAP-A bound to glutathione sepharose beads. Complexes associated with the beads were separated in SDS-PAGE and the radioactive, bound protein was detected by autoradiography. In vitro translated ORP2 was clearly bound to GST-VAP-A as compared with GST alone (Figure 5). Next we designed an ORP2 mutant, in which the FFAT-motif was disrupted. Amino acids ^{6}EFFDA^{10} that constitute the FFAT-motif of ORP2 were mutated to ^{6}EVVVA^{10} using site-directed mutagenesis. VAP-A binding of the mutant (ORP2 mFFAT) was determined as previously. ORP2 mFFAT showed no binding to GST-VAP-A (Figure 5) confirming that FFAT-motif of ORP2 was responsible for the VAP-A interaction.

The effect of mFFAT mutation on ORP2 localization was then studied. ORP2 mFFAT was expressed in A431 cells treated with oleic acid-BSA and ORP2 was detected with rabbit ORP2 antibody. In cells expressing ORP2 mFFAT the lipid droplet targeting was inhibited and ORP2 showed a cytosolic localization comparable to that of wild-type ORP2 in 22(R)OHC treated cells (Figure 5). This suggests that the disturbance of VAP-A interaction results in ORP2 targeting to the cytoplasm. The result can also imply that the wild-type ORP2 is not in the surface on the lipid droplets but in the ER membranes surrounding lipid droplets, where VAP-A resides. However, immunoelectron microscopy studies on the localization of ORP2 confirmed that overexpressed ORP2 is on the lipid droplets, and no staining of ORP2 was detected in the ER (III, fig. 4). On the other hand, VAP-A was not detected on the surface of lipid droplets or in the ER membranes surrounding lipid droplets, as judged by immunofluorescence microscopy. These results indicate that either the FFAT-motif mediates an interaction of ORP2 with a still unknown lipid droplet protein, or that the mutation in the FFAT-motif disturbs the fold of ORP2,

which results in a fold reminiscent of the ligand-bound form of ORP2. The FFAT-motif of ORP2 resides near the lid-region of ORP2, which supports the latter explanation.

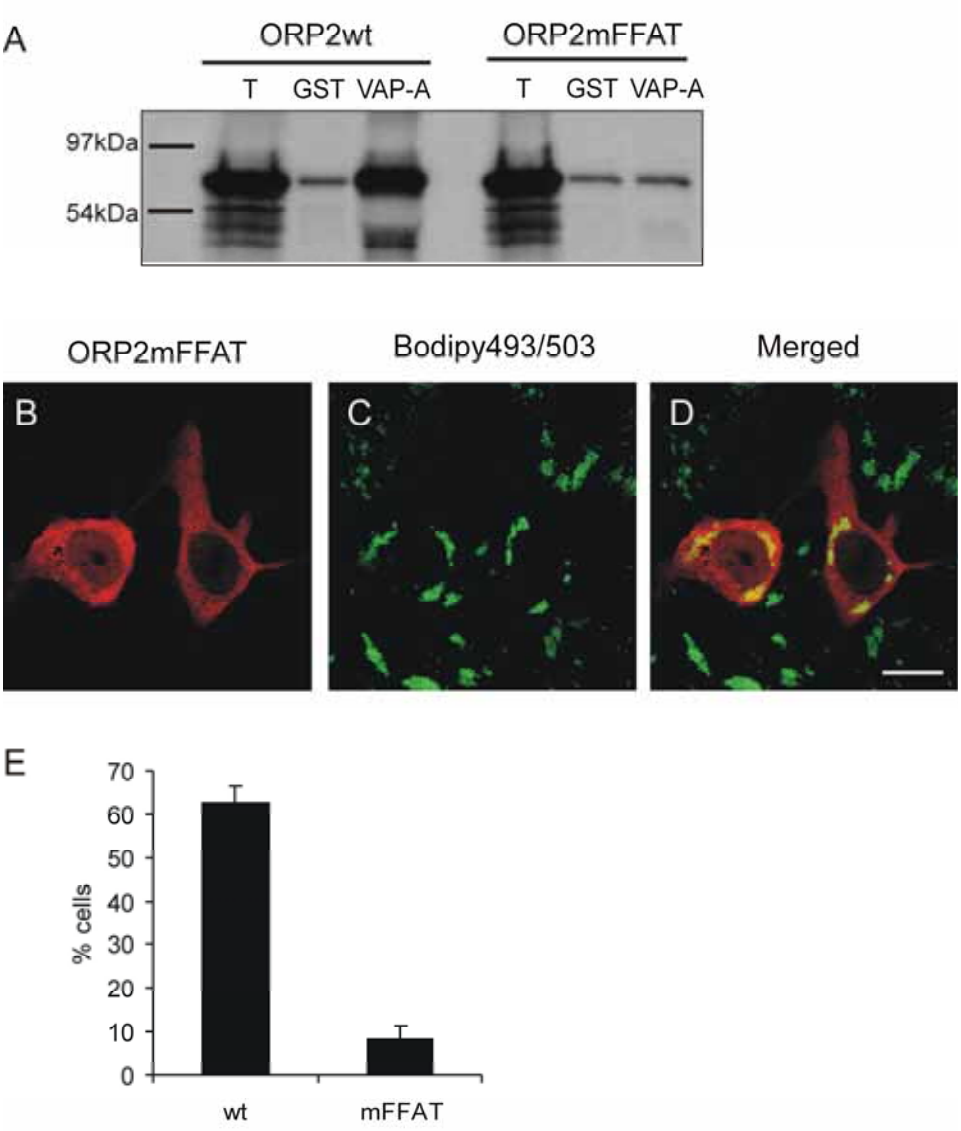

Figure 6. Mutation in the FFAT-motif of ORP2 inhibits the lipid droplet association. A. VAP-A binding of ORP2 was determined by translating ORP2 wild-type or FFAT-mutant (ORP2mFFAT) in vitro and pulling down the translated proteins with GST or GST-VAP-A. T = in vitro translation reaction mixture, 10% of the total amount loaded. B-D. The A431 cells were transfected with ORP2mFFAT and treated with 350 μM oleic acid-BSA. ORP2mFFAT was stained with ORP2 antibody (B) and lipid droplets with BODIPY 493/503 (C). A merge is shown in (D). Bar, 10 μm. The effect was quantified (E) by determining the proportion of cells displaying clear LD association of ORP2 wild-type (wt) or FFAT-mutant (mFFAT). The data represents mean ± S.E.M from three coverslips, 100 transfected cells counted from each.

There are many possible ways by which ORP2 can attach to the surface monolayer of lipid droplets. ORP2 binds phosphoinositides, which can mediate the binding. PIP-binding of ORP2 was not very specific for any particular PIP, suggesting that ORP2 has a positively charged region that may form electrostatic interactions with negatively charged phospholipids. This is supported by the PIP-binding result, in which ORP2 binding seems to be proportional to the amount of phosphate groups in PIP (I, fig. 9).

There is no common amino acid sequence motif in lipid droplet proteins, but the targeting rather seems to depend on larger three-dimensional structures. One way of binding to lipid droplet monolayer is an amphipatic α-helix that forms hydrophobic interactions with membrane lipids. For example, a member of the perilipin protein family TIP47 is suggested to use a bundle of four amphipatic helices as a part of a hydrophobic cleft to associate with the monolayer of lipid droplets (Hickenbottom et al., 2004). However, the interaction of ORP2 with lipid droplets seems rather weak arguing against strong hydrophobic interactions. First, ORP2 has not been found in any of the studies investigating the proteome of the lipid droplets from mammalian cells (Brasaemle et al., 2004; Fujimoto et al., 2004; Liu et al., 2004; Umlauf et al., 2004). Second, we were not able to detect endogenous ORP2 in lipid droplet fractions isolated from A431 cells (unpublished findings). These findings suggest that the lipid droplet targeting of ORP2 would more likely be mediated by an interacting protein. The interaction of ORP2 with TIP47 and triglyceride lipases ATGL and HSL were studied by co-immunoprecipitation, but no interaction was detected (data not published).

5.3 ORP2 regulates cellular cholesterol metabolism

5.3.1 ORP2 inducible TREx HeLa cell lines (I)

The previous studies examining the role of ORP2 in cellular lipid metabolism were done with Chinese hamster ovary-K1 (CHO-K1) cell lines that constitutively expressed ORP2 at relatively high levels (Käkelä et al., 2005; Laitinen et al., 2002). Constitutive expression of a protein can lead to effects that are due to the adaptation of the cells to the primary effect of the protein. Mutations may form in rapidly dividing transformed cell lines and only those cells that can handle the overexpression and its effects survive, and thus they may represent a skewed model. This disadvantage can be partially overcome by using multiple clones of overexpressing cells. Another way is to use stable cell lines that can be induced to overexpress a protein, which makes it possible to observe the acute effects of the overexpression. There are also advantages in using only one cell line, from which both overexpressing and non-expressing control cells can easily be obtained. We created ORP2 HeLa cell lines, in which the expression of ORP2 is under the control of a tetracycline-regulated repressor and can therefore be induced with doxycycline. In the following studies we used three independent cell lines 21f3, 38b10 and 40d5, which were obtained by single cell cloning of transfected cells. In these cell lines ORP2 expression was high already after 20 h induction with doxycyclin, but 42 h was used to detect the changes that ORP2 expression causes. The frequency of ORP2 overexpressing cells was high, over 90%. Uninduced cells of cell lines 21f3 and 38b10 had some leakage of ORP2 expression but were still markedly induced by doxycycline, while the third cell line 40d5 displayed tight regulation (I, fig. 1). Endogenous ORP2 could not be detected by Western blotting in HeLa cells.

5.3.2 Inducible ORP2 expression enhances cholesterol efflux (I)

CHO-cells overexpressing ORP2 previously showed a higher rate of cholesterol efflux to many extracellular acceptors. Using ORP2 inducible HeLa cells we examined if this result can be reproduced in the human cell line. Cells from three independent lines were concomitantly labeled with [^{14}C]cholesterol and treated with doxycycline to induce ORP2 overexpression. After 40 hours of labeling and induction, cholesterol efflux to several acceptors was determined. When using 20% human serum as an extracellular cholesterol acceptor in the medium, [^{14}C]cholesterol efflux was approximately 15% higher in ORP2 induced cells from all three cell lines than in uninduced control cells (I, fig.2). To specifically measure cholesterol efflux employing different mechanisms, apoA-I and HDL_2 were used as

acceptors. Again, ORP2 induced cells effluxed cholesterol more efficiently to both acceptors than the untreated cells, when the efflux time was 4 hours (I, fig.2). Even when only BSA was added to the efflux medium, cholesterol efflux was enhanced in ORP2 induced cells. We then wanted to exclude the possibility that ORP2 expression induces the shedding of plasma membrane or exocytosis of small vesicles containing cholesterol. We measured the efflux to medium with no cholesterol acceptors and again detected slightly more cholesterol in the medium of ORP2 induced cells than in non-induced cells. However, the rate of membrane shedding was so low that it cannot explain the changes in cholesterol efflux to the different acceptors. These results propose that ORP2 does not affect any specific cholesterol efflux mechanism such as those mediated by ABCA1 or ABCG1, but rather influences the distribution or transport of intracellular cholesterol. In addition, these results confirm those obtained with ORP2 overexperssing CHO cells.

The next question to be addressed was if cholesterol in ORP2 overexpressing cells is transported faster from the intracellular pool to the plasma membrane or if it is just more readily diffused from the plasma membrane. This was studied by exposing cells to cyclodextrin, which rapidly extracts cholesterol from the plasma membrane. If the treatment is really short, the intracellular cholesterol trafficking machinery does not have time to replace the cholesterol. TREx HeLa cells were labeled with [^{14}C]cholesterol and simultaneously induced with doxycycline for 42h. Cells were incubated for 5 minutes with 5 mM methyl-β-cyclodextrin, after which the proportion of cholesterol in the medium was determined. Significantly more [^{14}C]cholesterol was extracted by cyclodextrin from ORP2 expressing cells than from uninduced cells (I, fig. 3A). The result implies that there is more cholesterol in the plasma membrane or the specific lipid environment in the plasma membrane facilitates the cholesterol efflux from ORP2 expressing cells.

Cholesterol in the plasma membranes facilitates the formation of special membrane microdomains, lipid rafts, in which cholesterol and sphingolipids are preferentially clustered (Simons and Ikonen, 1997). Some membrane lipids can be isolated based on their resistance to detergents at low temperatures unlike the rest of the membrane. These lipid fractions with a high melting point are called detergent resistant membrane domains, DRMs. In model systems cholesterol is shown to be released more slowly from sphingomyelin-rich membranes (Lund-Katz et al., 1988) indicating that in ORP2 expressing cells more cholesterol is associated with non-raft membranes than in control cells. Therefore we examined the cholesterol distribution between raft and non-raft membranes in ORP2 expressing cells. TREx HeLa cells were labeled with [^{14}C]cholesterol and induced with doxycycline as before. Cells were lysed in ice-cold 1% Triton X-100 to solubilize all but DRMs representing the rafts. DRMs were then isolated by Optiprep-gradient ultracentrifugation, in which

DRMs float in the top two fractions. The distribution of [^{14}C]cholesterol between DRM fractions and the other fractions was similar in ORP2 induced and uninduced cells of all three cell lines thus providing no explanation for the enhanced cholesterol efflux (I, fig. 3C).

Next we compared the lipid composition of the plasma membrane of ORP2 induced and uninduced cells. Plasma membranes of the cells were isolated using colloidal silica method and their lipids were extracted. Cholesterol amount was determined by enzymatic methods and was similar in ORP2 expressing and uninduced cells thus providing no explanation for the efflux difference (I, fig. 3B). The amount of different phospholipids and their fatty acid compositions was determined by liquid chromatography-electrospray ionization mass spectrometry. Again, no difference was observed between ORP2 expressing and uninduced cells.

Recent findings suggest that sterols are located in the cytoplasmic leaflet of plasma membrane bilayer (Mondal et al., 2009). Based on their studies on red cells, Steck et al. (2002) have estimated that the half-time for cholesterol flip-flop movement is relatively rapid, only one second. Although many aspects of plasma membrane lipid content and organization were studied, it is still possible that ORP2 overexpression alters the rate of flip-flop movement of cholesterol from the inner leaflet of the bilayer to the other one. Since ORP2 is occasionally detected in the plasma membrane, ORP2 overexpression could be imagined to increase the proportion of cholesterol in the outer leaflet which could further increase the rate of cholesterol efflux. This could also at least in part explain the result of increased extractability of cholesterol by cyclodextrin from ORP2 overxepressing cells.

Another possible explanation for the enhanced cholesterol efflux could be stimulated cholesterol transport from intracellular membranes to the plasma membrane. Because it is now known that ORP2 is capable of binding cholesterol, it is possible that a high expression level of ORP2 enhances the transport of cholesterol to the plasma membrane. The transport of newly synthesised cholesterol from the ER to the plasma membrane was studied in ORP2 overexpressing CHO-cells, which display more pronounced increment in cholesterol efflux than the ORP2 expressing HeLa cells. When the total cell cholesterol was labeled with [^{14}C]cholesterol and the newly synthesised cholesterol with tritium, the amount of newly synthesised cholesterol in the plasma membrane was higher in ORP2 overexpressing cells than in control cells, also when compared with [^{14}C]cholesterol (I, fig. 4). This suggests that ORP2 enhances specifically the transport of cholesterol from the ER to the plasma membrane.

Of the other ORPs, OSBP, ORP9 and Osh4p have been shown to be able to transfer cholesterol between vesicles (Ngo and Ridgway, 2009; Raychaudhuri et al., 2006).

Taken into account the suggested similarity of the sterol-binding pocket of different ORPs, it seems possible that also ORP2 is able to transfer cholesterol.

5.3.3 The effect of ORP2 on cholesterol esterification

The next question to be addressed was that if cholesterol is cleared more rapidly from the ORP2 expressing cells, where does it originate from? Obvious organelles were lipid droplets, from which cholesterol could be released. Indeed, cholesterol esterification was inhibited in CHO cells overexpressing ORP2 (Laitinen et al., 2002), suggesting that the cells respond to the loss of cholesterol by inhibiting its storage. In addition, even under conditions in which cholesterol esterification was blocked with an ACAT inhibitor, ORP2 expressing CHO cells still showed enhanced cholesterol efflux compared to control cells. This support the fact that enhanced cholesterol efflux leads to reduced esterification and not the other way around (I, fig. 5B).

The effect of inducible ORP2 overexpression (I)

What then happens in ORP2 induced HeLa cells? Do they also inhibit cholesterol esterification in response to enhanced efflux? TREx HeLa cells were labeled with [^{14}C]cholesterol for 42 h in the presence or absence of doxycycline and the amount of [^{14}C]cholesterol incorporated to cholesteryl esters was determined. Interestingly, in ORP2 induced cells from two cell lines (21f3, 40d5) the amount of [^{14}C]cholesterol esters was significantly higher than in uninduced cells, while in the third cell line no change was observed (I, fig. 5A). The discrepancy between ORP2 overexpressing CHO cells and TREx HeLa cells could be explained by the assumption that the CHO cells have adapted to the high rate of cholesterol efflux by inhibiting cholesterol esterification. Maybe only the ORP2 expressing CHO cells that have developed a mutation that leads to inhibited cholesterol esterification have survived the selection of the ORP2 cell lines. In contrast, TREx HeLa cells that have expressed ORP2 only for 42 h have not had time to adapt to the high rate of cholesterol efflux. Therefore the increment in esterification could represent a more direct effect of ORP2 overexpression. How is the increased cholesterol esterification accomplished then? ACAT resides in the ER membranes and is mostly regulated by availability of its substrates, cholesterol and fatty acids (Seo et al., 2001; Zhang et al., 2003). Also oxysterols activate ACAT (Zhang et al., 2003). If ORP2 enhanced the transport of cholesterol from the ER to the plasma membrane, the ER pool would be diminished leading to decreased rate of cholesterol esterification, which is in contrast to our findings. On the other hand, the decreased ER pool of cholesterol can be replenished by newly-synthesized cholesterol or cholesterol from lipoproteins. Indeed, we found later that the amount of unlabeled cholesteryl esters

was not increased in ORP2 overexpressing HeLa cells, when the cells were cultured with lipoprotein deprived serum (see 5.3.4).

The effect of ORP2 silencing (III)

After studying the effects of overexpressed ORP2, we wanted to clarify the role of endogenous ORP2 in cholesterol metabolism, which is possible by using gene silencing with small interfering RNAs (siRNA). However, we had to change the cell line, since the endogenous expression of ORP2 in HeLa cells is below the detection level of Western blotting. The human A431 cell line was chosen, because it has a high endogenous expression level of ORP2 and it was already used in the ORP2 localization studies. Endogenous ORP2 expression was silenced with three siRNAs, which all resulted in at least 80% decrease in the ORP2 protein level (III, fig. 7A). A431 cells were treated with ORP2 and control siRNAs for 48 h and cholesterol esterification was determined as incorporation of [^{14}C]cholesterol to cholesteryl esters. Cholesterol esterification in cells treated with control and ORP2 siRNAs was similar. However, when the cells were treated with oleic acid-BSA to induce the formation of lipid droplets, a significant difference was observed. Cells treated with control siRNA had somewhat increased incorporation of [^{14}C]cholesterol to cholesteryl esters in the presence of oleic acid-BSA, obviously due to a higher amount of ACAT fatty acyl substrate, but the increase in the amount of [^{14}C]cholesterol incorporated was much higher in ORP2 silenced cells. This indicates that endogenous ORP2 functions to decrease the amount of cholesteryl esters, but only in conditions that stimulate the formation of triglycerides and cholesteryl esters. Whether the increased amount of cholesteryl esters in ORP2 silenced cells results from enhanced ACAT activity or inhibited breakdown of cholesteryl esters is not clear. We made efforts to clarify, which alternative could explain the increased amount of cholesteryl esters, but with no success (data not published).

When considering the suggestion that ORP2 stimulates cholesterol hydrolysis on the surface of lipid droplets and the finding that its lipid droplet localization is inhibited by 22(R)OHC, a following scheme can be suggested. Since 22(R)OHC is an intermediate in the synthesis of steroid hormones, it can be hypothesized to accumulate in steroidogenic cells, when the steroid hormone synthesis is active. 22(R)OHC could bind to ORP2 and remove it from the lipid droplets to inhibit the hydrolysis of cholesteryl esters, which is needed to deliberate free cholesterol for the steroid hormone synthesis. By this very hypothetical mechanism ORP2 could mediate the possible negative feed-back effect of 22(R)OHC on steroid hormone synthesis. Supporting the hypothesis, Laitinen et al. showed (2002) that ORP2 protein is well detected in steroidogenic mouse tissues, such as ovary, testis and adrenals.

Taken together, ORP2 overexpression results in increased esterification in HeLa cells and reduced esterification in CHO cells (Laitinen et al., 2002). On the other hand, ORP2 silencing results in increased amount of cholesteryl esters, but only in lipogenic conditions. To compare these results same cell lines and culture conditions should be used. It is possible that the ORP2 effects on cholesterol esterification are dependent on another protein, which is expressed or active only in some cell types or in some culture conditions. ORP2 localization is also slightly different in different cell types, which may partially explain the differences in the results.

5.3.4 Effect of ORP2 overexpression on cellular cholesterol levels (I)

The increased rate of cholesterol efflux and esterification in TREx HeLa cells induced to overexpress ORP2 would most probably lead to a decreased amount of cellular free cholesterol. The amount of unlabeled cholesterol was measured from ORP2 induced and uninduced cells cultured either in normal 10% FBS containing medium or in medium with 5% lipoprotein deficient serum. Total cholesterol and free cholesterol were quantified from cell lysates using two separate enzymatic assays and the amount of cholesteryl esters was calculated by subtracting free cholesterol from the value of total cholesterol. The results supported the findings obtained by radioactive labeling experiments. In the presence of FBS the ORP2 induced cells from cell lines 21f3 and 40d5 had more cholesteryl esters than uninduced cells, and the amount of free cholesterol was decreased so that there was no change in the amount of total cholesterol (I, fig. 6). In cells grown without lipoproteins the amount of cholesteryl esters was not altered in ORP2 expressing cells probably due to lack of cholesterol from the medium and subsequently inhibited cholesterol esterification. The amounts of total and free cholesterol were both slightly decreased in ORP2 induced cells. These results suggest that enhanced cholesterol efflux and esterification leads to a decreased amount of free cholesterol in ORP2 overexpressing cells.

Endoplasmic reticulum is an important organelle for the regulation of cellular cholesterol metabolism. The cholesterol level in the ER determines the activity of SREBP transcription factors, ACAT, and HMG-CoA reductase (Chang et al., 1997; Espenshade and Hughes, 2007; Goldstein et al., 2006). The increased amount of cholesteryl esters in ORP2 induced HeLa cells implies that the amount of cholesterol in an ER pool accessible to ACAT is higher than in uninduced cells.

The activity of HMG-CoA reductase can also be regarded as an indicator of ER cholesterol levels. Therefore the HMG-CoA reductase activity was measured from ORP2 induced and uninduced TREx HeLa cells. In addition, it was important to see, whether the ORP2 expressing cells respond to lower levels of free cholesterol by

upregulating cholesterol biosynthesis. Indeed, ORP2 expression led to higher activity of HMG-CoA reductase in all three cell lines, when it was measured from semipurified membranes (I, fig. 6A), indicating that the ER cholesterol pool accessible to HMG-CoA reductase is diminished.

There seemed to be an apparent discrepancy between the results of cholesterol esterification and HMG-CoA reductase activity. Is it possible that both cholesterol synthesis and esterification are simultaneously stimulated? A recent study suggested that there are at least two distinct cholesterol pools in ER that regulate the SREBP activity and ACAT (Kristiana et al., 2008). These pools respond to increased cholesterol uptake with different kinetics. If also the cholesterol pool regulating the activity of HMG-CoA reductase is distinct from the ACAT substrate pool, the results of ORP2 overexpression can be explained by different effects of ORP2 on different pools. Upregulated cholesterol biosynthesis can also be thought to replenish the ER cholesterol pool to be used as ACAT substrate.

5.3.5 Effect of ORP2 overexpression on LDL internalization (I)

Cholesterol uptake is known to be mainly regulated in the level of LDL receptor expression. The LDL receptor gene has a binding site (SRE) for SREBP, which is upregulated, when the cholesterol level in the ER is low. Therefore it was probable that if the cholesterol pool in the ER regulating the Scap-SREBP transport was diminished in ORP2 expressing cells, LDL receptor expression would be upregulated. We examined the LDL receptor expression level from ORP2 induced and uninduced HeLa cells by Western blot. When the cells were cultured in the standard serum containing medium, LDL receptor expression was clearly upregulated in ORP2 induced cells compared to the control cells (I, fig. 7A). When the cells were cultured in medium containing lipoprotein deficient serum, LDL receptor expression level was similarly increased in both ORP2 induced and uninduced cells. This suggests that the upregulation of LDL receptor by the loss of cholesterol due to blocked availability of cholesterol from the medium is much higher than the upregulation caused by ORP2 overexpression.

We next investigated if the enhanced expression of LDL receptor results in stimulated uptake of LDL particles by endocytosis in ORP2 induced HeLa cells. ORP2 induced and uninduced HeLa cells were incubated with fluorescent DiI-LDL (1,1´-dioctadecyl-3,3,3´,3´-tetramethyl-indocarbocyanineperchlorate-labeled LDL) -particles for 15 minutes, after which the intracellular fluorescence was quantified. The ORP2 induced cells internalized significantly more DiI-LDL than uninduced cells concordantly with the result of upregulated LDL receptor expression (I, fig.

7B-D). In this way ORP2 induced cells can apparently replace the cholesterol that is lost due to enhanced cholesterol efflux.

5.4 Effect of ORP2 overexpression on endocytosis (I)

Yeast Osh-proteins have been suggested to play important roles in endocytosis (Beh and Rine, 2004). Because OPR2 expressing HeLa cells had enhanced rate of LDL uptake, we wanted to investigate if it results, in addition to increased LDL receptor expression, from stimulated endocytosis. LDL in complex with LDL receptor is endocytosed via the clathrin-dependent pathway, which is also used by other complexes such as transferrin-transferrin receptor (Conner and Schmid, 2003). We measured the rate of clathrin-mediated endocytosis from ORP2 induced and uninduced HeLa cells using FITC-conjugated transferrin as a marker. The cells were treated for five minutes with FITC-transferrin, after which the intracellular fluorescence was quantified. ORP2 overexpression led to mild, not significant increase in transferrin internalization (I, fig. 8D-F) supporting the hypothesis that the stimulated uptake of LDL results mostly from upregulated LDL receptor expression.

After internalization transferrin and transferrin receptor enter the endocytic route, where iron is released from transferrin, and recycle back to the plasma membrane (Maxfield and McGraw, 2004). If the recycling of transferrin to plasma membrane was faster in ORP2 cells, the possible effect of ORP2 in endocytosis would not be detected. Therefore we also examined the recycling of transferrin in ORP2 induced and uninduced cells. FITC-transferrin was first internalized for an hour at 16ºC and then chased for 5 to 15 minutes in the presence of excess unlabeled holo-transferrin to prevent the further internalization of FITC-transferrin. The intracellular fluorescence was quantified at two time points and expressed as percentage of the fluorescence before the chase (I, fig. 8G). The rate of transferrin recycling in ORP2-induced cells was similar to uninduced cells supporting the result of ORP2 having only a mild effect on clathrin-dependent endocytosis.

Different molecules and particles can be endocytosed by various mechanisms (Conner and Schmid, 2003). Macrophages can also take up LDL via LDL receptor-independent way by fluid-phase endocytosis (Kruth et al., 2002). Fluorescently labeled dextran can be regarded as a marker for the fluid-phase endocytosis. We used rhodamine-dextran to measure the rate of fluid-phase endocytosis in ORP2-induced and uninduced cells. After 15 minutes internalization intracellular fluorescence was quantified from the cells. Interestingly, ORP2 induced cells internalized significantly more rhodamine-dextran than uninduced cells (I, fig. 8A-C).

Taken together, these results suggest that ORP2 overexpression stimulates fluid-phase endocytosis, but clathrin-mediated endocytosis is mildly enhanced as well. By which mechanism is this achieved? Cholesterol amount on the plasma membrane has been shown to affect endocytosis (Pichler and Riezman, 2004). Also the effects of Osh deletions in yeast were suggested to result from disturbance of ergosterol levels. Although the amount and organization of plasma membrane lipids seemed to be similar in ORP2 induced and uninduced cells, the overall changes in cholesterol metabolism or a hypothetical change in the transbilayer distribution of cholesterol in ORP2 induced cells could have an influence on endocytosis.

5.5 Effect of ORP2 on triglyceride metabolism (III)

When examining the localization of ORP2 in A431 cells treated with oleic acid-BSA, it could be observed that in ORP2 overexpressing cells lipid droplets were organized in a different way than in untransfected cells. In untransfected cells the lipid droplets were clustered together close to the nucleus, whereas in ORP2 expressing cells the lipid droplets were dispersed throughout the cytoplasm and looked smaller (III, fig. 5). This implied that ORP2 could affect the metabolism of triglycerides.

First we investigated if endogenous ORP2 has an effect on the synthesis of triglycerides. A431 cells were treated with control siRNA or ORP2 siRNA for 48 hours, after which [^{3}H]oleic acid-BSA was added to the cells. Cells were harvested at three time-points and the amount of radioactivity incorporated into triglycerides was determined. No difference was detected between ORP2 siRNA and control siRNA treated cells suggesting that ORP2 has no effect on triglyceride synthesis (data not published).

Next we investigated the effect of ORP2 on triglyceride break-down. A431 cells were treated with siRNAs as previously and loaded overnight with oleic acid-BSA including [^{3}H]oleic acid to stimulate the triglyceride formation and to radioactively label triglycerides. After the loading the cells were transferred to delipidated serum –containing medium to induce the hydrolysis of triglycerides. Cells were harvested at different time-points and the incorporation of [^{3}H]oleic acid to triglycerides was measured. Already before the chase the amount of triglycerides was higher in ORP2 siRNA treated cells than in control cells. When the value in zero time-point was set as 100%, it was evident that ORP2 silenced cells hydrolyzed triglycerides more slowly than the control cells (III, fig. 7). This suggests that endogenous ORP2 stimulates the hydrolysis of triglycerides. The result is concordant with the previous finding showing that the amount of triglycerides is decreased in ORP2 overexpressing CHO cells.

The result is also in accordance with the result of altered distribution of lipid droplets in ORP2 overexpressing A431 cells. During stimulated lipolysis in 3T3-L1 adipocytes lipid droplets are fragmented into smaller ones (Marcinkiewicz et al., 2006) probably to increase the lipid droplet surface area and binding region for lipases. It was recently shown that Arf1-COPI vesicle transport machinery is needed for the lipid droplet fragmentation and efficient lipolysis (Guo et al., 2008). In addition, many Rab-proteins, which control vesicular trafficking, are found in the surface of lipid droplets. OSBP has been shown to interact with ARF (Godi et al., 2004) and other ORPs have been shown to bind different small GTPases as well (Johansson et al., 2005; Lehto et al., 2008). Therefore it is tempting to speculate that ORP2 could interact with ARF or other small GTPases, or regulate their activity, and that way affect the lipid droplet structure or mobilization.

ORP2 could also participate directly in triglyceride hydrolysis. The best characterized mechanism of lipolysis is the classical pathway of catecholamine-stimulated triglyceride hydrolysis in adipocytes (Watt and Steinberg, 2008). Adipocytes would thus be a good model to study ORP2 function further. One still poorly investigated area in cellular lipid metabolism is the coregulation of cholesterol and triglyceride metabolism. It could be speculated that ORP2 acts as a sterol sensor that regulates the release of fatty acids from triglycerides, as well as fatty acids and free cholesterol from cholesteryl esters according to the cellular sterol status. Cholesterol in membranes is preferably positioned next to lipids with saturated fatty acids (Simons and Vaz, 2004). Therefore, if the amount of cholesterol in cellular membranes is altered, also the fatty acid composition of membrane phospholipids has to change to preserve the composition and properties of the membranes. Triglycerides in lipid droplets act as a reservoir of fatty acids and ORP2 could function there to control the release of fatty acid for the membrane regeneration.

6 SUMMARY AND CONCLUSIONS

ORP2 is a member of OSBP-related protein family. ORPs have been implicated in various cellular functions such as the regulation of lipid metabolism, vesicle transport and cellular signaling. The studies on ORP2 have concentrated on cellular lipid metabolism. In previous studies the constitutive overexpression of ORP2 in CHO cells was shown to enhance cholesterol efflux, inhibit cholesterol esterification and decrease the amount of cellular triglycerides.

In this study the role of ORP2 in lipid metabolism was investigated using two types of cell lines. Tetracycline-inducible stably transfected HeLa cell lines were employed to study the effects of acute ORP2 overexpression on cholesterol metabolism and endocytosis. A431 cells were employed to study the localization of ORP2 as well as the effect of ORP2 gene silencing on triglyceride and cholesteryl ester metabolism. Furthermore, we performed site-directed mutagenesis and sterol binding assays in vitro to study the ligand interactions of ORP2.

In HeLa cells, inducible ORP2 overexpression led to enhanced cholesterol efflux to all the extracellular acceptors that were studied, suggesting that the effect was not specific to any particular efflux mechanism. The cholesterol amount and distribution in the plasma membrane were investigated to find the explanation for the enhanced cholesterol efflux. The only explanation that was not ruled out was the possibility that ORP2 could affect the distribution of cholesterol between the two leaflets of the plasma membrane bilayer. The other explanation for enhanced cholesterol efflux was suggested to be the stimulated cholesterol transport from intracellular membrane compartments to the plasma membrane. This was supported by the finding that ORP2 binds cholesterol and could thus induce changes in cholesterol trafficking when overexpressed.

The enhancement of cholesterol efflux resulted in a decreased amount of free cholesterol in ORP2 induced HeLa cells. The cells were able to compensate for the loss of cholesterol by stimulating cholesterol biosynthesis and uptake. Upregulated LDL receptor expression was accompanied by stimulated uptake of LDL. In addition, fluid-phase endocytosis was stimulated in ORP2 induced HeLa cells indicating that excess ORP2 has a more general effect on endocytosis and not just on LDL receptor expression.

Whereas cells usually respond to decreased amount of free cholesterol by inhibiting the storage of cholesterol as cholesteryl esters, ORP2 induced HeLa cells had more cholesteryl esters than the uninduced control cells. This result suggests that in HeLa cells the ER cholesterol pool that is used by the cholesterol esterifying enzyme

ACAT is affected differently from the pool that regulates the activity of SREBP-transcription factors and HMG-CoA reductase. On the other hand, stimulated uptake and synthesis of cholesterol were able to provide enough cholesterol for cholesterol esterification.

Cholesteryl esters are stored in cytoplasmic lipid droplets, where also ORP2 was found to localize. Ligand binding was shown to play a role in the lipid droplet targeting of ORP2. The ORP2 ligand with the highest affinity was found to be 22(R)OHC, but the protein also binds other oxysterols and phosphoinositides. Treatment of cells with 22(R)OHC was demonstrated to inhibit the localization of ORP2 on the surface of the lipid droplets, suggesting that ORP2 localization and subsequently the function are regulated by the ligand binding.

The function of endogenous ORP2 was investigated by silencing its expression in A431 cells. Silencing of ORP2 led to delayed hydrolysis of triglycerides, when the cells were shifted from lipogenic to lipolytic conditions. This suggests that ORP2 stimulates the hydrolysis of triglycerides. When the formation of triglycerides was induced, ORP2 silencing also resulted in increased amount of cholesteryl esters, but this effect was not observed under normal cell culture conditions. This suggests that the amount of cellular triglycerides affects the activity of ORP2 in the regulation of cholesteryl ester metabolism.

Taken these results together, we propose that ORP2 is a sterol-regulated protein localizing to the surface of lipid droplets, where it participates in the regulation of triglyceride and cholesteryl ester metabolism. Through cholesterol binding it can also have effects on cholesterol metabolism in other cellular locations. The possible cell type-specific functions of ORP2 and its role in metabolic disorders such as obesity or atherosclerosis remain to be clarified.

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