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Sterol Carrier Protein-2: New roles in regulating lipid rafts and signaling

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Abstract

Sterol carrier protein-2 (SCP-2) was independently discovered as a soluble protein that binds and transfers cholesterol as well as phospholipids (nonspecific lipid transfer protein, nsLTP) *in vitro*. Physiological functions of this protein are only now beginning to be resolved. The gene encoding SCP-2 also encodes sterol carrier protein-x (SCP-x) arising from an alternate transcription site. *In vitro* and *in vivo* SCP-x serves as a peroxisomal 3-ketoacyl-CoA thiolase in oxidation of branched-chain lipids (cholesterol to form bile acids; branched-chain fatty acid for detoxification). While peroxisomal SCP-2 facilitates branched-chain lipid oxidation, the role(s) of extraperoxisomal (up to 50% of total) are less clear. Studies using transfected fibroblasts overexpressing SCP-2 and hepatocytes from SCP-2/SCP-x gene-ablated mice reveal that SCP-2 selectively remodels the lipid composition, structure, and function of lipid rafts/caveolae. Studies of purified SCP-2 and in cells show that SCP-2 has high affinity for and selectively transfers many lipid species involved in intracellular signaling: fatty acids, fatty acyl CoAs, lysophosphatidic acid, phosphatidylinositols, and sphingolipids (sphingomyelin, ceramide, mono- di- and multi-hexosylceramides, gangliosides). SCP-2 selectively redistributes these signaling lipids between lipid rafts/caveolae and intracellular sites. These findings suggest SCP-2 serves not only in cholesterol and phospholipid transfer, but also in regulating multiple lipid signaling pathways in lipid raft/caveolae microdomains of the plasma membrane.

Keywords

Caveolae; lipid rafts; sterol carrier protein-2; SCP-2; phosphatidylinositol; sphingolipids; cholesterol; fatty acyl CoA; fatty acid

INTRODUCTION

Sterol carrier protein-2 was originally discovered in 1970 by Scallen and associates as one of two proteins in the rat liver soluble fraction which was required for cholesterol biosynthesis

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(rev. in [1]. Subsequent studies from the Scallen and other labs revealed that the respective purified proteins exhibit very different specific roles in cholesterol biosynthesis: (i) Sterol carrier protein-1 (SCP-1, also called supernatant protein factor or SPF) is a 47 kDa acidic protein originally discovered to enhance the enzymatic conversion of squalene to lanosterol (rev. in [1]. (ii) Sterol carrier protein-2 (SCP-2) is a13 kDa, basic protein with multiple roles in isoprenoid and cholesterol metabolism (rev. in [1,2].

SPF has been cloned and its x-ray crystal structure solved [3]. It is a member of the CRAL_Trio family of proteins. Supernatant protein factor (SPF) promotes the epoxidation of squalene catalyzed by microsomes. Several studies suggest its *in vivo* role in the cholesterol biosynthetic pathway by a yet unknown mechanism. SPF belongs to a family of lipid binding proteins called CRAL_TRIO, which include yeast phosphatidylinositol transfer protein Sec14 and tocopherol transfer protein TTP. The crystal structure of human SPF at a resolution of 1.9 A reveals a two domain topology. The N-terminal 275 residues form a Sec14-like domain, while the C-terminal 115 residues consist of an eight-stranded jelly-roll barrel similar to that found in many viral protein structures. The ligand binding cavity has a peculiar horseshoe-like shape. Contrary to the Sec14 crystal structure, the lipid-exchange loop is in a closed conformation, suggesting a mechanism for lipid exchange. SPF forms a complex with RRR-alpha-tocopherylquinone, suggesting a link between oxidized Vitamin E and cholesterol biosynthesis [4].

SCP-2 has multiple roles in isoprenoid metabolism including stimulating cis-prenyltransferase to form longer polyisopreoids common to both the dolichol and cholesterol biosynthetic pathways [2] as well as enhancing the final enzymatic steps involved in conversion of lanosterol to cholesterol (rev. in [1]. With a high affinity for cholesterol and phospholipids, sterol carrier protein-2 (SCP-2), also called non-specific lipid transfer protein (nsLTP), most research was initially focused on determining the role(s) of this protein in cholesterol and phospholipid metabolism. *In vitro*, SCP-2 transfers cholesterol [5] and phospholipids (rev. in [6] between membranes or between membranes and serum lipoproteins [1,7–11]. In keeping with these findings, studies with transfected cells overexpressing SCP-2 demonstrate that this protein increases cholesterol uptake [12], intracellular cholesterol cycling [13,14], cholesterol oxidation (rev. in [1,15], cholesterol esterification [12,16], and phospholipid formation [17, 18]. In contrast, SCP-2 overexpression inhibits HDL-mediated cholesterol efflux through lipid rafts/caveolae [19]. *In vivo* studies with liver from wild-type mice, rats treated with antisense SCP-2 RNA, and mice wherein the SCP-2/SCP-x gene is ablated indicate that SCP-2 stimulates cholesterol esterification in liver [20], cholesterol hypersecretion into bile [21,22], delivery of newly synthesized cholesterol from the endoplasmic reticulum (ER) for secretion into bile [23], and oxidation of branched-side chain lipids not only *in vitro* [24–26] but also in mice [20,27–30]. Specifically, SCP-x oxidizes the branched-side chain of cholesterol (to form bile acids) as well as the branched-chains of fatty acids (to detoxify) [24,27–29]. In summary, while compelling evidence indicates important direct roles for SCP-2 in mediating cholesterol, phospholipid, and fatty acid metabolism, exciting recent data indicate that this protein may have additional physiological functions, especially in intracellular lipid signaling.

Gene structure

One gene encodes SCP-2 and SCP-x, with the latter arising from an alternate transcription site (rev. in [30,31]. Separate mRNAs are transcribed for full-length 58 kDa SCP-x and a precursor 15 kDa pro-SCP-2 proteins which share the same C-terminus structure. Partial posttranslational modification cleaves the 58 kDa SCP-x into a 46 kDa protein and fully functional 13 kDa SCP-2. Only the 13 kDa SCP-2, but not the 15 kDa pro-SCP-2, is detected in intact cells/tissues indicating that pro-SCP-2 undergoes complete post-translational cleavage to fully functional 13 kDa SCP-2 and a 2 kDa peptide. I*n vitro* and *in vivo* studies have shown that 58 kDa SCP-x as well as its 46 kDa post-translational cleavage product are peroxisomal 3-ketoacyl-CoA

thiolases functioning in oxidation of branched-chain lipids (cholesterol to form bile acids; branched-chain fatty acid detoxification) (rev. in [30,31]. *In vitro* SCP-2 enhances cholesterol transfer from other intracellular membranes to mitochondria as well as from the outer to the inner mitochondrial membrane for oxidation, albeit less effectively than steroidogenic acute regulatory protein (StAR) [1,32–35]. The function, if any, of the 2 kDa post-translational cleavage product of the 15 kDa pro-SCP-2 is unknown (rev. in [31].

Protein Structure

The structure of SCP-2 has been determined by time-resolved fluorescence spectroscopy, circular dichroism, NMR, and x-ray crystallography as recently reviewed in detail [36]. Therefore, only those aspects relating structure to function will be briefly covered here. SCP-x/SCP-2 is comprised of at least four functionally important domains:

First, while almost nothing is known about the tertiary or secondary structure of SCP-x, its functional significance has become the subject of intense interest. The N-terminal 46 kDa is a 3-ketoacyl-CoA thiolase enzyme, involved not only in peroxisomal oxidation of straight-chain fatty acids, but is the only known 3-ketoacyl-CoA thiolase enzyme specific for oxidizing the branched-chain fatty acids and the branched-side chain of cholesterol. In contrast to mitochondrial fatty acid oxidation (net production of ATP), SCP-x does not mediated oxidations resulting in net energy production, but enhances detoxification and metabolic remodeling of branched-chain lipids. Branched-chain and straight-chain fatty acids differ markedly in their origin (isoprenoid vs classic fatty acid biosynthesis pathway), toxicity (highly toxic branched-chain vs much less toxic straight-chain), and oxidative pathway (peroxisomal vs mitochondrial) (rev. in [20,24,30,37–44]. Peroxisomal SCP-x mediated oxidation of the branched-side chain of cholesterol is essential for bile acid biosynthesis [27,29,45]. Interestingly, SCP-x expression in mice is gender dependent (female $\ll$ male) and female mice are much more susceptible to the toxic actions of dietary branched-chain fatty acids [40]. The first report of a spontaneous mutation in human SCP-x indicated a lipid metabolic phenotype reflecting loss of 3-ketoacyl-CoA thiolytic activity with branched-chain fatty acids and pathology similar to that noted in SCP-x/SCP-2 gene ablated mice [46].

Second, the N-terminal 20 amino acid presequence of 15 kDa pro-SCP-2 plays a very important role in intracellular targeting of SCP-2 (rev. in [31]. While examination of the amino acid and cDNA sequence of SCP-x/SCP-2 suggests that the 15 kDa pro-SCP-2 is product of an alternate transcription site and/or C-terminal cleavage of SCP-x (rev. in [31], ablation of the SCP-x gene component (but not SCP-2 gene expression) in mice did not reduce expression of SCP-2 in primary cultured hepatocytes, indicating that the SCP-x transcript is not essential for production of SCP-2 [47]. Comparison of the structures (by circular dichroism) and accessibility of the C-terminal AKL peroxisomal targeting sequence (by mass spectrometry) indicated that the C-terminal AKL peroxisomal targeting sequence of the 13 kDa SCP-2 is only slightly exposed to the aqueous buffer [48]. In contrast, presence of the 20 kDa N-terminal presequence in 15 kDa pro-SCP-2 alters the conformation of the protein to increase aqueous exposure of this C-terminal AKL peroxisomal targeting sequence [48]. The following section on intracellular targeting further details the functional consequence of the structural modification elicited by the presence of the N-terminal 20 amino acid presequence.

Third, the N-terminal 32 amino acids of the mature 13 kDa SCP-2 represents an amphipathic α-helical domain required for interaction of the protein with membranes to elicit ligand binding/transfer (rev. in [36]. This amphipathic α-helix directly interacts with the polar head groups of anionic phospholipids [49,50] and, as shown recently, represents the interaction domain for SCP-2 binding to caveolin-1 at the plasma membrane cytofacial leaflet [51,52]. Anionic phospholipids and caveolin-1 are highly enriched in the cytofacial leaflet of caveolae/lipid rafts

—plasma membrane microdomains wherein multiple proteins involved in signaling are localized (rev. in [53].

Fourth, the β-strands 4,5 and helix D along with the hydrophobic faces of the N-terminal amphipathic α helix in the mature 13 kDa SCP-2 form a very large ligand binding cavity suitable for accommodating multiple types of ligands—see below (rev. in [36]. Although molecular details comprising the fatty acid binding site in SCP-2 have been established (rev. in [36], nothing is known regarding the specific amino acid residues that interact with other bound ligands in the SCP-2 binding pocket.

Tissue and intracellular distribution

The tissue and intracellular distribution of SCP-x and SCP-2 were previously reviewed (rev. in [7,31,54,55]. Briefly, SCP-x/SCP-2 gene products are: (i) highly expressed in tissues involved in cholesterol trafficking and oxidation: liver, intestine, adrenal, testis, and ovary [7]. (ii) SCP-x is almost exclusively peroxisomal [1,56], (iii) SCP-2 is present at highest concentration in peroxisomes, but half or more is extraperoxisomal in cytoplasm [1,56–58]. The molecular basis for SCP-2 being present in both peroxisomes and cytoplasm was recently resolved by examining structure (see above) and distribution of SCP-2/SCP-x gene products in transfected cells overexpressing the 15 kDa pro-SCP-2 and 13 kDa SCP-2 [31,59]. Overexpression of the 15 kDa pro-SCP-2 resulted in significant localization in peroxisomes, but with nearly 50% localized in extraperoxisomal sites [48]. In contrast, overexpression of the mature 13 kDa SCP-2 resulted in SCP-2 being largely extraperoxisomal [48]. The differential distribution of these proteins was due weak exposure of the C-terminal AKL peroxisomal targeting sequence of the 13 kDa SCP-2 to the aqueous buffer while that of 15 kDa pro-SCP-2 was more exposed (see above). Thus, the 15 kDa pro-SCP-2 (gene product of the second promoter region) may better interact with the peroxisomal membrane AKL receptor and/or peroxisomal membrane proteins involved in 15 kDa pro-SCP-2 translocation into the peroxisomal matrix [48]. These findings suggest that the relative proportion of SCP-2 appearing in peroxisomes vs cytoplasm is likely attributed to the extent of N-terminal 20 amino acid cleavage of the 15 kDa pro-SCP-2 prior to entry into the peroxisomal matrix. However, the specific protease which accomplishes this cleavage and the intracellular localization of this protease are not yet known.

Lipids involved in intracellular signaling

Although lipids were originally construed primarily as components of membrane bilayers and energy storage, more recent data indicate that many lipid molecules also participate in intracellular signaling. Table 1 provides a summary of lipid classes involved in intracellular signaling and some selected examples of physiological responses elicited by these lipids. Because of space limitations in this review, it is not possible to provide a detailed, exhaustive listing of all signaling pathways in which these lipids participate. However, the reader is encouraged to the cited articles and specialized reviews noted in Table 1. Although structures of these lipids are diverse, all include a hydrophobic acyl chain (fatty acids, fatty acyl CoAs, lysophosphatidic acid, phosphatidylinositides, sphingolipids) or a hydrophobic side chain (cholesterol). While there are many other lipidic molecules involved in signaling (isoprenoids, prostaglandins, leukotrienes, thromboxanes, etc), only molecules with evidence of interaction with SCP-2 are listed. For example, by interacting with isoprenoids SCP-2 may influence signaling pathways in both the cytoplasm and nucleus. SCP-2 stimulates cis-prenyltransferase to form longer polyisopreoids [2]. The finding that SCP-2 has high affinity for isoprenyl pyrophosphates [e.g. dimethylallyl pyrophosphate, farnesyl pyrophosphate, geranyl pyrophosphate, geranyl geranyl pyrophosphate [60]] suggests potential roles in regulating the activity of isoprenyl transferase enzymes that covalently modify (e.g. farnesylate, geranylate) proteins—resulting in targeting of these protein to caveolae/lipid rafts, plasma membrane

microdomains highly involved in cell signaling. However, there have been no reports further investigating this interesting possibility. Finally, SCP-2 binds [60] and enhances the peroxisomal metabolism of branched-chain fatty acids in cultured cells [61], and mice [20]. Branched-chain fatty acids, isoprenoids derived from cleavage of chlorophyll side chain (rev. in [20,61], are nuclear signaling molecules representing the most potent endogenous inducers of peroxisome proliferator activated receptors known [62–67]. Finally, SCP-2 also facilitates the peroxisomal oxidation of the branched-side chain of cholesterol to form bile acids [1,20, 68]. Bile acids are also potent ligand signaling molecules in the nucleus where they interact with and regulate transcriptional activity of nuclear receptors, e.g. FXR (rev. in [31,69,70]. Although these findings suggest that by binding these branched-chain isoprenoids or facilitating their formation SCP-2 may influence their trafficking/availability to the nucleus for nuclear signaling, these possibilities remain to be investigated.

Lipid rafts/caveolae: a conceptual framework forming the basis for lipid signaling at the plasma membrane

Signaling lipid substrates/precursors (e.g. phospholipids containing arachidonic acid, phosphatidylinositols, sphingolipids, etc) and the enzymes that produce/release them (kinases, lipases) both reside in the plasma membrane, yet do not interact to produce signaling cascades until induced by receptor activation at the cell surface. It has been hypothesized that cholesterol-rich lipid rafts/caveolae provide a conceptual organizing framework for these transmembrane signaling pathways (rev. in [53,71–75]. It is thought that at least two types of these cholesterol-rich microdomains exist: (i) lipid rafts, and (ii) caveolae, a subset of lipid rafts (Fig. 1). These plasma membrane microcomains are believed to be enriched not only in signaling proteins, but as indicated in Figure 1 also in signaling lipids.

While lipid rafts/caveolae provide an attractive conceptual framework for organizing lipid substrates and proteins, the precise definition and even the existence of cholesterol-rich lipid rafts/caveolae is a continuing subject of discussion (rev. in [53,71,75–85]. The idea of lipid domains (rafts?, caveolae?) is based on early model membrane studies which demonstrate the physical immiscibility of different lipid classes into multiple coexisting phases: liquid crystalline (most fluid), liquid ordered (intermediate, detected in biochemically isolated lipid rafts/caveolae), and gel (least fluid) (rev. in [86–88]. Depending on the specific marker used, the size of lipid rafts obtained through morphological studies of model membranes ranges from 260–330 nm for fluorescent tagged GM1 and 230–2000 nm for fluorescent tagged PE (rev. in [53,81,82,89]. Since cholesterol induces formation of phase separated cholesterol-rich and –poor domains in simple two component model membranes, it has been proposed that cholesterol forms the organizing principle of lipid ‘domain’ or ‘microdomain’ formation in membranes (rev. in [90]. The finding that plasma membranes of mammalian cells are highly enriched in cholesterol (70–90% of cell total cholesterol) suggested the potential existence of cholesterol-rich and –poor regions therein. Indeed, early biochemical subfractionation studies resolved cholesterol-rich and –poor plasma membrane subfractions (apical/serosal/sinusoidal, basolateral/contiguous, microvillar/canalicular plasma membrane subfractions) differing in lipid composition, protein distribution, and structure from polarized cells (liver, intestine, kidney) (rev. in [91]. It is important to note that microvillar/brush border/canalicular subfractions represent highly dynamic regions of the plasma membrane where absorption (intestinal microvilli) and secretion (kidney brush border/hepatic canaliculi) of lipids such as cholesterol occur. These microvillar/brush border/canalicular subfractions are (i) highly enriched in cholesterol and sphingomyelin (rev. in [92], (ii) less fluid (rev. in [92], and (iii) rich in proteins involved in reverse cholesterol transport (i.e. SRB1, caveolin-1, ABCA1) which are also enriched in ‘lipid rafts/caveolae’ [85,93–97]. Likewise, spicules/pseudopodia blebbing off the surface of deoxygenated red blood cells are enriched in sterol and exhibit high sterol dynamics [98]. Affinity chromatography, electrophoresis, ionic lysis, and gradient

centrifugation also resolve plasma membranes from non-polarized cells into subfractions differing in lipid composition and/or protein distribution [92,99–110]. Concurrent with these studies over three decades ago, morphologically distinct flask-like (60–90 nm diameter) structures were observed in plasma membranes and called ‘caveolae’ or little caves (rev. in [106]. Again, depending on the specific marker used, the size of lipid rafts obtained through morphological studies of biological membranes ranged from 26–330 nm for GPI-anchored proteins and 500–2000 for MHC-1 (rev. in [53,81,82]. This indicates that some lipid rafts may be so small as to constitute an ‘annulus’ surrounding a specific protein, a suggestion supported by studies showing cholesterol forming an annulus around the oxytocin receptor [111–113]. Alternately, other lipid rafts may be sufficiently large (i.e. 230–2000 nm) to be visible by optical microscopy (limit of resolution near 200 nm).

Despite the above findings, morphological studies did not address whether the caveolae were cholesterol-rich or –poor. To begin to resolve these questions, a variety of biochemical techniques were used to isolate cholesterol-rich fractions from cholesterol-containing model membranes and from biological membranes through use of detergents, high-pH carbonate buffer, density gradient centrifugation, and affinity-chromatography (rev. in [53]. These biochemically isolated fractions were selectively lipid rich (especially in cholesterol and sphingomyelin), exhibited a liquid-ordered structure (i.e. less fluid), and were subsequently termed detergent resistant membranes (DRM), lipid rafts, caveolae, etc. Whether caveolin-1 is present in these fractions is cell type dependent [e.g. hepatocytes do not contain caveolin-1 [114]]—indicating that caveolin-1 is not required for formation of these cholesterol- and sphingomyelin-rich fractions (rev. in [53]. Finally, since the existence of cholesterol-rich and –poor domains in plasma membranes of living cells had not been demonstrated, it was unclear whether the biochemically isolated lipid rafts/caveolae, DRM, etc represented artifactually induced enrichments of cholesterol in these fractions. This issue was resolved recently by real-time multiphoton imaging of a naturally-occurring fluorescent sterol (dehdyroergosterol) in the plasma membrane of living cells [53,115–118]. The multiphoton imaging studies showed that sterol was not uniformly distributed in the plasma membrane of L-cell fibroblasts, but instead into sterol-rich and sterol-poor regions in plasma membranes of living cells. The size range of sterol-rich domains was estimated to be from 200 nm (limit of optical microscopy) to 565 nm [116]. This size range was generally smaller than or, at the high range, overlapped the lower range of sizes typically observed for microvilli which were in the range of 500–1000 nm as determined by electron microscopy [110,119–121]. While video imaging of HepG2 cells confirmed the sterol-rich (especially canalicular microvilli) and –poor distribution in the plasma membrane of polarized cells [97]. However, the video imaging studies resolved regions in the size range of 2000–3000 nm and it was concluded that this represented microvilli and that there were no ‘sterol-rich’ vs ‘sterol-poor’ regions [97]. This conclusion is difficult to reconcile with existing data showing that: (i) microvilli (rev. in [92] and pseudopodia [98] isolated by biochemical fractional are sterol-rich; (ii) morphological (electron microscopy, immunofluorescence) studies of fixed cells and biochemical fractionation studies using the non-cytolytic perfringolysin O (highly selective for cholesterol-rich regions) confirmed the existence of cholesterol-rich and –poor domains in microvilli/filopodia in lymphoblastoid cells and platelets [122,123]; (iii) morphological (electron microscopy, immunofluorescence) of fixed cells and biochemical fractionation studies using the non-cytolytic perfringolysin O confirmed the existence of cholesterol-rich and –poor domains in lipid rafts of erythrocytes [123]; (iv) Morphological studies with filipin (disrupts cholesterol-rich lipid rafts) indiscriminately labeled cholesterol (both cholesterol-rich and –poor) regions of the plasma membrane and could not differentiate between them [122,123]. The reasons for the apparent discrepancy with results obtained by video imaging vs multiphoton imaging (as well as morphological studies with the perfringolysin O) is not clear, but may be due in part to video imaging resulting in strong photobleaching of the fluorescent sterol, the use of methyl-beta cyclodextrin to incorporate the fluorescent sterol at high amounts for video imaging, saturation

of all the plasma plasma membrane sterol with fluorescent sterol for video imaging, and video imaging's weak resolution in the Z-axis. In contrast, the multiphoton imaging studies used bioincorporation (small phospholipid/dehydroergosterol vesicles) of small amounts of dehydroergosterol, multiphoton excitation results in much less photobleaching of the fluorescent sterol, and multiphoton imaging has resolution in the Z-axis only slightly less than confocal imaging. Taken together, the bulk of data suggest that cholesterol-rich and poor 'domains' exist in plasma membranes of living cells. Microvilli, plasma membrane regions associated with high cholesterol flux, are also enriched in cholesterol, sphingomyelin, and protein markers typically also associated with 'lipid rafts/caveolae'. The upper range of sizes obtained with multiphoton imaging for some sterol-rich 'domains' appears to overlap with the lower range of microvilli/filipodia, suggesting that some cholesterol-rich lipid rafts/caveolae may cluster in microvilli/filipodia. However, the resolution of optical microscopy (200 nm) is insufficient to resolve this issue.

In summary, there is as yet no universal definition of lipid rafts/caveolae is lacking. Thus, for the sake of simplicity and to avoid semantic issues in the current review we will use the term 'lipid rafts/caveolae' to refer to cholesterol-rich, specific marker-rich, domains isolated from purified plasma membranes without the use of detergents or high pH carbonate buffers. It is not completely clear whether DRMs are equivalent to lipid rafts/caveolae (rev. in [53].

Lipid rafts/caveolae microdomains involved in lipid signaling: Preparations based on detergents or high pH carbonate buffers

Early biochemical fractionation studies initially suggested that plasma membrane lipid rafts/caveolar microdomains were lipid-rich, especially in cholesterol and sphingolipids, but relatively poor in other structural lipids such as phospholipids (especially phosphatidylcholine) such that the cholesterol/phospholipid molar ratio therein was several-fold higher than the non-raft component (rev. in [124]. The relative proportion of lipid raft/caveolae vs non-raft domains in plasma membranes varied from a few % to nearly all the membrane, depending on the type of detergent used, temperature, pH, and other conditions (rev. in [124]. Thus, it was not clear whether lipid rafts/caveolae represented a minor or a major part of the plasma membrane. Significant to the current review, such detergent- and high pH carbonate- based lipid raft/caveolae preparations were also enriched in high levels of signaling proteins (rev. in [124] and signaling lipids [phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), phosphatidylinositol-4,5-bisphosphate (PIP2), sphingomyelin, gangliosides, ceramide, and diacylglycerol] (rev. in [72,125–132]. However, detergent or high pH carbonate based lipid raft/caveolae preparations are increasingly being criticized as producing artifactual distributions of proteins and/or lipids into lipid rafts/caveolae, i.e. not reflecting the physiological distribution of these molecules in plasma membranes (rev. in [133–140]. One recent proteomics/mass spectrometry study comparing the purity of detergent- and high pH carbonate-based lipid raft/caveolae preparations showed that almost 33% and 75% of proteins, respectively, were non-specific contaminant copurifying proteins [141].

Lipid rafts/caveolae microdomains involved in lipid signaling: Preparations based on density gradients and affinity chromatography

Density gradients [53,61,114,140,142–149] or density gradients/affinity chromatography methods [53,61,110,114,140,149] do not use detergents or high pH carbonate buffer. Biochemical analysis of marker protein distribution in lipid rafts/caveolae and non-rafts isolated from purified plasma membranes from cultured cells (e..g. L-cell fibroblasts, MDCK cells, endothelial cells, primary hepatocytes) by density gradient centrifugation and affinity chromatography demonstrated that lipid rafts/caveolae were enriched (on the basis/mg protein) in appropriate caveolae/lipid raft protein (caveolin-1, except in hepatocytes; flotillin; SRB1; eNOS in endothelial cells only) and lipid (GM1, cholesterol, sphingomyelin, total lipid)

markers while concomitantly being relatively poor in non-raft protein (Na^+,K^+-ATPase; transferrin in endothelial cells) and poor lipid markers (GM1, cholesterol, sphingomyelin, total lipid) [61,115,140,147]. Furthermore, neither fraction was enriched in intracellular protein markers, e.g. calnexin—an endoplasmic reticulum marker [61]. The presence of caveolin-1 was not required for the existence of lipid rafts, as shown by lipid rafts isolated from cultured mouse primary hepatocytes and Fisher rat thyroid (FRT) cells—which do not contain caveolin-1 [114,150–152]. Lipid markers (cholesterol, phospholipid, sphingomyelin, GM1) were also enriched in lipid rafts/caveolae and did not require the presence of caveolin-1 [114]. Again the latter enrichments did not require the presence of caveolin-1 [114]. A typical biochemical analysis of lipid distribution in lipid rafts/caveolae and non-rafts isolated from plasma membranes purified from cultured fibroblasts is shown in Table 2. In control fibroblasts, lipid rafts/caveolae were enriched 2.5-fold in cholesterol, 3.7-fold in phospholipid, and 3-fold in total lipid as compared to non-rafts (Table 2). Phospholipids, especially phosphatidylcholine, were not depleted in lipid rafts/caveolae isolated from fibroblasts [61] or in lipid rafts isolated from cultured primary hepatocytes [114]. Consequently, the cholesterol/phospholipid molar ratio in lipid rafts/caveolae and lipid rafts did not differ significantly from non-rafts in fibroblasts (Table 2) or in cultured primary hepatocytes [114]—in agreement with other methods not using detergents or high pH carbonate buffers for isolation (rev. in [53]. Taken together, these and earlier findings with non-detergent, non-high pH carbonate buffer based isolation procedures suggest that benchmark markers for the caveolae/lipid raft preparations should include enrichment in caveolin-1, GM1, cholesterol, and sphingomyelin (all based on/mg protein) as compared to non-rafts. As a negative control, the caveolae/lipid rafts should be deficient in non-raft (e.g. Na^+,K^+-ATPase; clathrin; transferring in some cell types) and intracellular membrane (e.g. calnexin for ER; golgin 97 and giantin for Golgi) markers.

Lipid rafts/caveolae isolated without detergents/high pH carbonate buffer (see below) were enriched in both signaling proteins and in signaling lipids (rev. in [53]. Taken together, these data alleviated concerns that, in case of select signaling proteins [141] and select signaling lipids such as sphingomyelin and phosphatidylinositol [146], the fundamental qualitative (but not quantitative) conclusions about lipid rafts/caveolae being enriched in signaling proteins and signaling lipids remained valid—regardless of the method of lipid raft/caveolae isolation [61]. This conclusion is underscored by the fact that disruption of cholesterol-rich microdomains inhibits these signaling activities [124].

Signaling lipids are enriched in lipid rafts/caveolae

The enrichment of signaling lipids in caveolae and non-caveolar rafts and their putative intracellular trafficking pathways mediated by SCP-2 are schematically illustrated in Fig. 1. Multiple classes of signaling lipids are enriched in lipid rafts/caveolae isolated without detergents or high pH carbonate buffer (Table 3). For example, fatty acids are enriched 1.6-fold in lipid rafts/caveolae as compared to non-rafts isolated by affinity chromatography from purified plasma membranes derived from cultured fibroblasts (data not shown). Likewise, fatty acids are enriched 4.6-fold in lipid rafts as compared to non-rafts isolated by affinity chromatography from plasma membranes purified from cultured primary hepatocytes (Table 3 and [114]. These findings were consistent with the fact that fatty acid uptake across the plasma membrane into cells occurs through cholesterol-rich, sphingolipid-rich lipid rafts/caveolae [153]. Plasma membranes contain several proteins that bind and transport fatty acids: caveolin-1 [154], FAT/CD36 (rev. in [155–157], and FATP [158]. Within plasma membranes, all these fatty acid transport/translocase proteins are enriched in lipid raft/caveolae microdomains: caveolin-1 [53,61,114,140,142–146,149], FAT/CD36 (rev. in [74,159–161], and FATP (rev. in [160–162].

Lipid rafts/caveolae are highly enriched (3–16 fold, Table 3) in phosphatidylinositol (PI) [61, 114], phosphatidylinositol-4-phosphate (PIP) [125], and phosphatidylinositol-4-5-bisphosphate (PIP2) [125]. PI serves as a substrate for kinases that produce phosphorylated forms of PI (PIP, PIP2) while PIP and PIP2 in turn serve as substrates for phospholipase enzymes present in lipid rafts/caveolae resulting in release of phosphoinositide messengers and intracellular signaling (Table 1).

Sphingolipids represent a complex group of subclasses involved in lipid signaling and many are highly enriched in lipid rafts/caveolae, 1.7 to >100-fold (Table 3). Sphingomyelin, ceramide, neutral glycosphingolipids (monohexoside, dihexoside, globoside), and acidic glycosphingolipids (gangliosides) are enriched 2–20+ fold (Table 3) in lipid rafts/caveolae as compared to non-raft domains of fibroblasts, primary hepatocytes, and/or MDCK cells [53, 61,114,140,149]. Interestingly, incorporation of a photoactivatable ganglioside GM1 into lipid rafts/caveolae, followed by photocross-linking demonstrated that ganglioside cross-links to caveolin-1 in plasma membrane caveolae [163]. Thus, caveolin-1 may represent a close neighbor of gangliosides, a ganglioside binding protein within lipid rafts/caveolae, and/or may possibly transport/target gangliosides to caveolae through vesicular or chaperone-complexed caveolin-1 docking/interaction with plasma membrane caveolae [163]. Since caveolin-1 is not present in hepatocytes, caveolin-1 may be important for regulating the distribution and/or trafficking of signaling lipids to caveolae in tissues/cells (e.g. fibroblasts, endothelial cells) highly enriched in caveolin [78,79,164]. Although SCP-2 is not normally present in significant amounts in fibroblasts and endothelial cells, in fibroblasts overexpressing SCP-2 this protein is detected in caveolae in very close proximity to caveolin-1 [51,165]. These data suggest that both of caveolin-1 and SCP-2 may in part regulate the distribution of signaling lipids within caveolae (Fig. 1). Although caveolin-1 is not present in plasma membranes or plasma membrane lipid rafts isolated from cultured primary hepatocytes, hepatocytes contain high level of SCP-2 and signaling lipids (PI, GM1) are enriched in plasma membrane lipid rafts [114]. Thus, depending on the cell type SCP-2 and/or caveolin-1 may contribute to retention, binding, or trafficking of signaling lipids to lipid rafts (Fig. 1).

SCP-2 exhibits high affinity for lipids participating in intracellular signaling

Lipid signaling molecules are in general highly membrane-bound, since these lipophilic molecules are relatively insoluble in the aqueous environment of cytoplasm. Cells have evolved multiple mechanisms (Fig. 1) to transfer lipid signaling molecules between intracellular sites of synthesis (ER) and utilization (lipid rafts/caveolae) (rev. in [53,71,79,124,166,167]. Newly synthesized cholesterol and signaling lipids (or their substrates) move between Golgi and plasma membrane by one or more mechanisms: (i) relatively slowly (10–20 min) as vesicles from golgi to lipid rafts (Fig. 1, Vesicle) or caveolae (Fig. 1, Caveolar Vesicle), or (ii) more rapidly (1–2 min) by protein-mediated molecular transport (Fig. 1, SCP-2/signaling lipid complexes; Fig. 2A) (rev. in [19,168]. Lipid binding proteins may also regulate vesicular trafficking [169–173], by binding signaling molecules involved in vesicle budding such as fatty acyl CoA and phosphatidylinositides (Fig. 1, SCP-2/LCFA-CoA and SCP-2/PI mediating vesicle budding from golgi). Consistent with these possibilities, overexpression of SCP-2 in cultured fibroblasts enhanced molecular transfer of a fluorescent cholesterol (i.e. SCP-2/sterol complex), while concomitantly decreasing vesicular transfer of cholesterol to lipid rafts/caveolae for efflux to HDL—likely by binding fatty acyl CoA and/or phosphatidylinositides necessary for vesicle budding [19].

A fundamental requirement for protein-mediated lipid transport is that the protein binds the signaling lipid with high affinity. Mammalian cells express not only specific proteins that directly interact with select, signaling lipids in select tissues/cells (fatty acid binding proteins, phosphatidylinositol binding protein, glycolipid binding protein, etc), but also the more

promiscuous SCP-2 which binds multiple signaling lipids, is ubiquitously distributed in all tissues, and is expressed at highest level in liver, steroidogenic tissues (adrenal, testis, ovary), and intestine [31]. Sterol carrier protein-2 (SCP-2) exhibits high affinity for many lipid classes involved in intracellular signaling (Table 4). Its broad range of affinities for several of these ligands reflects the influence of acyl chain composition as well as from use of fundamentally different assays. Classical radioligand binding and separation assays typically yield much weaker affinities (i.e. higher Kds) than those obtained with fluorescence and isothermal titration calorimetry techniques that do not require separation of free from bound ligand (rev. in [174–179]. Some of these signaling lipid binding proteins are highlighted below:

SCP-2 exhibits high affinity for fatty acids, with Kds as low as 234 nM (Table 4). While SCP-2 has not classically been thought of as a fatty acid binding protein, its affinity for these ligands is in the same range as exhibited by members of the large family of fatty acid binding proteins (rev. in [177]. SCP-2 also has high affinity for fatty acyl CoAs, as low as 3 nM Kds (Table 4). These affinities are in the same range as acyl CoA binding protein (ACBP) (rev. in [176,180] and PITP [181]. SCP-2 shows high affinity for phosphatidylinositol (rev. in [165]. The affinities of SCP-2 for phosphorylated PI species are in the order PIP2>PIP>PI (Table 4), where SCP-2 has highest affinity for the most negatively-charged PI species. These affinities/functions of SCP-2 in PI binding are in the same range as for phosphatidylinositol transfer proteins such as PITPα and PITPβ (rev. in [165]. Although PITPα and less so PITPβ are both cytoplasmic, significant amounts are detected in perinuclear Golgi (rev. in [165]. PITP is hypothesized to directly present bound substrate to phospholipase C and interact with phosphoinositide kinase signaling complex in plasma membrane caveolae (rev. in [165]. As indicated above, nearly half of total SCP-2 is extraperoxisomal in cytoplasm, ER, and/or mitochondria (rev. in [15, 48,57]. The finding that some SCP-2, but not PITP, is present at the plasma membrane and directly interacts with caveolin-1 in caveolae [51,165], suggests that in tissues rich in SCP-2 this protein may contribute significantly to protein-mediated PI transfer. SCP-2 is one of the few proteins known to exhibit high affinity for sphingolipids including sphingomyelin, ceramide, neutral glycosphingolipids (monohexosides, dihexosides, globosides), and acidic glycosphingolipids (gangliosides) (Table 4). For example, human recombinant SCP-2 has high affinity (K_ds of 5–215 nM) for all sphingolipid classes tested and this affinity was selective, decreasing in the order ceramide > acidic glycosphingolipid (ganglioside) > neutral glycosphingolipid (monohexoside, globoside) > sphingomyelin (Table 4) [149].

In summary, as shown in Table 3, SCP-2 binds a variety of lipids that could serve as second messengers in several intracellular signaling pathways. To date, very little is known regarding specific signaling pathways wherein SCP-2 may function. However, at least two examples indicate that SCP-2 regulates signaling in both cytoplasm and nucleus. For example, SCP-2 overexpression stimulates insulin-mediated inositol-triphosphate production [165]. Likewise, SCP-2/SCP-x gene ablation enhances transcriptional activity of PPARα to induce formation of fatty acid oxidative enzymes [20,47]. However, it remains to be determined what is the specific (or predominant) intracellular signaling pathway(s) that SCP-2 regulates.

SCP-2 transfers intracellular signaling lipids *in vitro* and intact cells

SCP-2 mediates intracellular transport/targeting (Fig. 1, Fig. 2A) of many lipid classes involved in intracellular signaling (Table 5). For example, SCP-2 enhances not only cellular uptake of signaling lipids such as fatty acids [37,38,177,182], but also enhances fatty acid intracellular diffusion/transport [177,182] and fatty acid targeting to ER for incorporation into select phospholipid classes [18] or peroxisomes for oxidation [38]. Whether SCP-2 also regulates transport/presentation of fatty acids released by phospholipase A2 activity in the lipid rafts caveolae (Fig. 2C, arachidonic acid) to intracellular sites where such fatty acids (e.g. arachidonic, docosahexaenoic, eicosapentaenoic) are converted to signaling lipids (e.g.

prostaglandins, leucotrienes, and/or thromboxanes) is not known. Precedent for possible involvement of SCP-2 in this process is based on studies *in vitro* demonstrating that a fatty acid binding protein with high affinity for linoleic acid and arachidonic acid inhibits 15-lipoxygenase induced oxygenation of these fatty acids to signaling lipids [183].

SCP-2 also binds fatty acyl CoAs with high affinity (Table 4) and enhances their transport and/or presentation to microsomal enzymes involved in fatty acyl CoA transacylation. For example, SCP-2 stimulates microsomal fatty acyl CoA incorporation into phosphatidic acid *in vitro* [17] and in intact cells [18]. Since fatty acyl CoAs regulate vesicle budding at the Golgi (see above) by binding acyl CoAs the SCP-2 may potentially be involved in regulating vesicular trafficking of signaling lipids as well as in their molecular transfer to the plasma membrane lipid rafts/caveolae (Fig. 1, Vesicle and Caveolar Vesicle budding from Golgi). Overexpression of SCP-2 in cultured fibroblasts not only enhances molecular sterol transfer to plasma membrane lipid rafts/caveolae for HDL-mediated efflux but simultaneously decreases vesicular sterol transfer to plasma membrane lipid rafts/caveolae to inhibit efflux to HDL [19]. Since vesicular sterol transfer predominates over molecular sterol transfer in L-cell fibroblasts, the net effect of these opposing influences was that SCP-2 overexpression inhibited HDL-mediated sterol efflux [19].

SCP-2 may also mediates phosphatidylinositol molecular transport from its site of synthesis in ER to plasma membrane lipid rafts/caveolae (rev. in [53]. *In vitro* studies [165] show that SCP-2 stimulates PI transport from microsomal donor membranes to acceptor membranes by 13-fold (Fig. 2A). Direct comparisons of SCP-2 mediated enhancement of PI transfer with that of another phosphatidylinositol transfer protein, PITP, revealed that the fold-stimulation of these proteins was not significantly different—suggesting that both proteins are highly effective in transferring PI from microsomes [165]. Both PITP isoforms (PITPα and PITPβ) are well known to enhance PI transfer (rev. in [165]. Further, SCP-2 overexpression significantly redistributes PI from intracellular membranes (mitochondria, ER) to plasma membranes and concomitantly downregulates expression of the other major PI binding protein (PITP) nearly 6-fold [165]. The finding that SCP-2 stimulates insulin-mediated inositol-triphosphate production [165] suggests that SCP-2 either: (i) binds PI and PIP to present these molecules as substrates for kinase enzymes to produce more phosphorylated PI in the lipid rafts/caveolae (Fig. 2B), or (ii) binds PI, PIP, and PIP2 to present these bound substrates for phospholipase C in the lipid rafts/caveolae (Fig. 2C). Finally, since phosphoinositides regulate vesicle budding at the golgi (see above), by binding PI, PIP, and PIP2 the SCP-2 may potentially be involved in regulating vesicular trafficking of signaling lipid substrates as well as in molecular transfer of these lipids (Fig. 1).

Early studies with soluble fractions from liver homogenates suggested that SCP-2 enhances molecular transfer of sphingomyelin (Fig. 1, 2A), neutral (globoside) glycosphingolipids (Fig. 1), and acidic (ganglioside) glycosphingolipids (Fig. 1) *in vitro* [184,185]. However, these supernatants contain not only SCP-2 but also several other lipid transfer proteins (PITP, L-FABP, etc) [186]. PITP also binds/transfers sphingomyelin (rev. in [169,170]. Likewise, it is not known if SCP-2/sphingomyelin complexes are better substrates for sphingomyelinase to release ceramide in lipid rafts/caveolae (Fig. 2C). Thus, it remains to be shown if highly purified recombinant SCP-2 enhances sphingolipid transfer and/or alters sphingomyelinase activity (see below).

SCP-2 not only enhances the cellular uptake of cholesterol [12,19], but also increases cholesterol transport/trafficking to ER for esterification *in vitro* [1,187] and in intact cells [16]. Cholesterol delivery to ER regulates release of sterol regulatory element binding protein (SREBP), which in turn trafficks to the nucleus and regulates transcription of the gene encoding HMG-CoA reductase—the rate limiting enzyme in cholesterol synthesis [188–193]. The

finding that SCP-2 overexpression in hepatoma cells enhances uptake of exogenous cholesterol hydroperoxide and elicits hypersensitivity of mitochondria to its peroxidative damage, suggests that SCP-2 binds and targets this cholesterol oxidation product to mitochondria [194]. These data, plus the fact that many oxidized forms of cholesterol are potent inhibitors of cholesterol synthesis [195], suggests that SCP-2 may also regulate intracellular cholesterol synthesis by transporting these potent regulatory lipids to sites of inhibition.

SCP-2 expression regulates the distribution of signaling lipids to caveolae/lipid rafts

Significant amounts of SCP-2 are associated with lipid rafts/caveolae in plasma membranes. Cell fractionation of fibroblasts overexpressing SCP-2 and western blotting detected SCP-2 in purified plasma membranes, especially in caveolae/lipid rafts as compared to the non-raft fraction [51]. Dual immunolabeling immunofluorescence microscopy showed SCP-2 at the plasma membrane was significantly colocalized (37–72%) with caveolin-1 in multiple cell types [165]. Fluorescence resonance energy transfer (FRET) demonstrated that at the plasma membrane SCP-2, but not PITP, was located in close proximity (48±4 Å) to caveolin-1 [51]. Similar findings were confirmed by double immunogold labeling and electron microscopy [51]. Interaction of SCP-2 with caveolin-1 was specific since FRET showed PITP further away from SCP-2, not close to caveolin-1 and not coimmunoprecipitated with caveolin-1 [51]. Yeast two hybrid analysis showed SCP-2 directly interacting with caveolin-1 in cell culture. Thus, data has now shown for the first time that SCP-2 (but not PITP) selectively interacts with caveolin-1, both within cytoplasm and at the plasma membrane [51]. SCP-2 overexpression in transfected fibroblasts significantly altered intracellular distribution of signaling lipids to plasma membrane lipid rafts/caveolae (Table 6). These data are consistent with SCP-2 mediating trafficking of bound lipids between lipid rafts/caveolae and intracellular sites.

Lipid raft/caveolae content of unesterified fatty acids is cell-type and SCP-2 expression dependent (Table 6). In cultured primary hepatocytes from SCP-2/SCP-x gene-ablated mice (complete loss of SCP-2) there was no effect on fatty acid content of lipid rafts [114]. This observation may be due hepatocytes having a high content of plasma membrane lipid raft proteins (FAT/CD36, FATP) that bind/transport fatty acids into the hepatocyte. In contrast, increased level of SCP-2 as in transfected L-cell fibroblasts resulted in a 2.9-fold decreased fatty acid level in lipid rafts/caveolae (data not shown). The latter finding may be due to fibroblast plasma membrane lipid rafts/caveolae expressing lower levels of fatty acid binding/transport proteins. Once fatty acids are taken up into cells, SCP-2 binds and stimulates their intracellular diffusion/transport (rev. in [177,182] and enhances their transacylation to complex lipids (phosphatidic acid, cholesteryl ester) (rev. in [1].

SCP-2 altered intracellular distribution of phosphatidylinositol to plasma membranes and lipid rafts/caveolae therein (Table 6). SCP-2 overexpression in cultured fibroblasts significantly redistributed phosphatidylinositol away from intracellular sites (ER, mitochondria, lysosomes) to plasma membranes such that plasma membrane mass of PI was increased by 32% [165]. However, this increase was not reflected in the fibroblast plasma membrane lipid rafts/caveolae [61]. This observation may be explained in part by the fact that SCP-2 overexpression also enhanced insulin-stimulated inositol triphosphate production—an activity localized in lipid rafts/caveolae [165]. Conversely, ablation of the SCP-2/SCP-x gene completely abolished the synthesis of SCP-2 and increased the content of PI by 2.5-fold in lipid rafts isolated from plasma membranes of cultured primary hepatocytes [114]. These data suggest that SCP-2 enhances distribution of PI to the plasma membrane and lipid rafts/caveolae therein, while concomitantly enhancing PI utilization by enzymes involved in PI signaling in lipid rafts/caveolae. An analogous function has been proposed for another PI transfer protein, PITP (rev. in [170, 172].

SCP-2 overexpression in cultured L-cells further enriched the content several sphingolipid classes in lipid rafts/caveolae (Table 6). Neutral sphingolipids such as monohexosides and globosides were increased 2- and 2.4-fold, respectively (Table 6) [61]. Although SCP-2 overexpression decreased GM1 content, the mass of total gangliosides increased slightly [61]. SCP-2 overexpression decreased the lipid raft/caveolae content of sphingomyelin by nearly 50% [61,149] but increased ceramide content nearly 2-fold [149]. The reduced sphingomyelin concomitant with increased ceramide in lipid rafts/caveolae of SCP-2 overexpressing cells may reflect increased signaling activity, i.e. SCP-2/sphingomyelin facilitates sphingomyelinase to release ceramide (Fig. 2C). Since ceramide is both an intermediate in the synthesis of multiple sphingolipids and the product of sphingomyelinase-mediated sphingomyelin hydrolysis, SCP-2 may influence production/utilization of this sphingolipid intermediate during signaling reactions in lipid rafts/caveolae. Thus the reduced sphingomyelin in lipid rafts/caveolae of SCP-2 overexpressing cells may arise from: (i) SCP-2 stimulating sphingomyelin hydrolysis to ceramide, (ii) SCP-2 facilitating removal of ceramide from lipid rafts/caveolae to intracellular sites, or (iii) SCP-2 stimulating utilization of ceramide by enzymes that produce more complex sphingolipids. Consistent with the latter interpretation, SCP-2 overexpression significantly increased the cell content of neutral glycosphingolipids (1.7-fold higher lactosylceramide and neutral glycosyl ceramide) and acidic total gangliosides [196]. This effect was specific since the levels of one of the gangliosides (GM1) as well as glucosylceramide were decreased [196]. These findings suggest that SCP-2 may enhance the utilization of sphingomyelin for signaling in lipid rafts, analogous to SCP-2-enhanced insulin stimulation of inositol triphosphate production from phosphorylated PI [165], a process which depletes lipid rafts/caveolae of PI [61].

Since SCP-2 transfers both cholesterol and bulk phospholipids between intracellular sites and plasma membrane lipid rafts/caveolae, it may change the absolute level of these lipids at the plasma membrane. However, SCP-2 affects these lipids similarly in plasma membrane lipid rafts/caveolae such that neither SCP-2 overexpression in cultured fibroblasts nor SCP-2/SCP-x gene ablation in cultured primary hepatocytes significantly altered the cholesterol/phospholipid ratio (Table 2) [61,114].

Studies with SCP-2 overexpression fibroblasts and SCP-2/SCP-x gene ablated mice

SCP-2 plays a role in modulating cellular content, distribution, and metabolism of several classes of signaling lipids including cholesterol, fatty acids, phosphatidylinositol, and sphingolipids: (i) SCP-2 regulated cholesterol uptake, intracellular trafficking and esterification in SCP-2 overexpressing cultured cells [1,12,13,16], transgenic mice overexpressing SCP-2 [14], and rats treated with SCP-2 antisense RNA [23]; (ii) SCP-2 enhanced the uptake and metabolism of fatty acids, especially branched-chain fatty acids in cultured cells [38] and primary hepatocytes from SCP-2/SCP-x gene ablated mice [41]; (iii) SCP-2 overexpression significantly altered the distribution and content of phosphatidylinositol and sphingolipids in lipid rafts/caveolae as well as altered the structure and cholesterol dynamics in lipid rafts/caveolae [61,118,140,147,149]; (iv) SCP-2 may have important roles in providing SCP-2-bound signaling lipids as substrates to enzymes that metabolize these lipids during signaling reactions in lipid rafts/caveolae. Precedent for the latter possibilities is based in part on the fact that SCP-2-bound fatty acyl CoA is a better substrate than free fatty acyl CoA for transacylase reactions [1,16,17,197]. Furthermore, SCP-2 overexpression in cultured fibroblasts stimulated insulin-mediated inositol-triphosphate production [165]. Thus, cultured cell data as well as studies with SCP-2 overexpression and siRNA SCP-2 knockdown in animals suggest that SCP-2 may have important roles in intracellular trafficking of signaling lipids, remodeling lipid raft/caveolae lipid composition, and providing SCP-2-bound signaling lipids as substrates to enzymes that metabolize these lipids during signaling reactions in lipid rafts/caveolae. Nevertheless, the full physiological significance of SCP-2 in intracellular

signaling is yet to be resolved *in vivo*. Part of the challenge is that gene-targeted mice may not necessarily exhibit a strong phenotype under control-chow and otherwise unchallenged conditions. For example, despite the apparent promiscuity as a lipid binding and regulatory protein, SCP-2/SCP-x gene ablated mice generated by disruption of Exon 14 have yielded phenotypic changes that are mostly related to defects in branched chain lipid (i.e. fatty acid, cholesterol) metabolism [20,43]. Unfortunately, there have been no studies to date examining any of the known signaling pathways in these mice. Moreover, it must be noted that in this specific SCP-2/SCP-x ablation of Exon 14 concomitantly resulted in 5–6-fold upregulation of another promiscuous lipid binding protein (L-FABP) which also binds many signaling lipids (rev. in [177,178,183,198]. Whether additional proteins that bind/transfer signaling molecules (e.g. phosphatidylinositol binding protein, glycolipid transfer protein) or other component proteins of the signaling cascades were also upregulated to compensate for loss of SCP-2 in these mice is not known. In contrast, another study generated SCP-2/SCP-x gene ablated mice through targeted disruption of exon 16 [114]. The resultant knockout mice exhibited very little change in L-FABP expression [114]. Furthermore, cultured primary hepatocytes from these SCP-2/SCP-x gene ablated mice (i.e. exon 16 knockout) confirmed that ablating SCP-2 regulates lipid raft properties as evidenced by: (i) Increased proportion of lipid rafts 1.5-fold; (ii) Increased raft total phospholipid; (iii) Decreased fluorescence polarization indicating a decreased liquid disordered phase and a lesser transbilayer fluidity gradient in hepatocyte lipid rafts. Thus, lipid rafts present in primary cultured mouse hepatocyte plasma membranes displayed unique protein, lipid, signaling lipid, structure, and biophysical properties which were differentially regulated by SCP-2 expression. The availability of mouse models overexpressing SCP-2 [23,199], ablated in SCP-x [47], or ablated in SCP-2/SCP-x [20,114] now provides the means to examine these possibilities in more physiologically relevant systems. However, dietary or other stress (age, exercise, etc) challenge may have to be performed to overcome compensatory upregulation of other proteins that may prevent observation of specific signaling phenotypes under control conditions.

SUMMARY AND CONCLUSIONS

Although SCP-2 was established as a protein that transfers cholesterol and phospholipids decades ago, recent findings with lipid rafts/caveolae and SCP-2 suggest that they may provide a conceptual link to metabolic processes that might be regulated through the respective signaling pathways. The molecular basis for this possibility resides in three major factors:

First, SCP-2's lipid binding pocket exhibits high affinity for a large variety of lipid molecules that are directly or indirectly involved in signaling: (i) fatty acids which are precursors of prostaglandins, leukotrienes, and thromboxanes (i.e. arachidonic, docosahexaenoic, and eicosapentaenoic acids), (ii) fatty acids which when transported into nuclei regulate transcriptional activity of receptors (e.g. peroxisome proliferator activated receptors); (iii) fatty acyl CoAs which regulate vesicular budding from the Golgi, (iv) fatty acyl CoAs when transported into nuclei regulate transcriptional activity of receptors (e.g. peroxisome proliferator activated receptors, hepatocyte nuclear factor 4α); (v) phosphoinositides (PI, PIP, PIP2) which regulate vesicular budding or as a result of phospholipase C action yield second messengers (IP, IP2, IP3); (vi) sphingolipids (sphingomyelin, ceramides, globosides, gangliosides) which are involved in multiple signaling pathways, and (vii) cholesterol which regulates production of SREBP at the endoplasmic reticulum and in turn trafficks to the nuclei to regulate transcription.

Second, cholesterol-rich lipid rafts/caveolae exist in part to compartmentalize signaling lipids with select proteins that use them as substrates. Although early studies suggested that lipid rafts/caveolae isolated with detergents or high pH carbonate buffers are highly enriched in proteins and lipid involved in signaling, increasing concern that these fractionation methods

may induce artifacts has introduced uncertainty. This issue was resolved by development of new methods that do not use detergents or high pH carbonate buffers to isolate lipid rafts/caveolae. Biochemical analysis of the proteins and lipids isolated by the latter methods confirmed that many of the basic conclusions obtained by the earlier detergent and high pH carbonate buffer based methods were often qualitatively, if not quantitatively, correct. Furthermore, newly developed fluorescence imaging technologies for the first time provided real-time data indicating that plasma membranes of living cells contain sterol-rich and –poor domains.

Third, SCP-2 provides a conceptual link between physico-chemical data and metabolic processes that might be regulated through multiple signaling pathways. SCP-2 may exhibit new roles in lipid signaling mediated through plasma membrane lipid rafts/caveolae by mechanisms that involve molecular transfer of signaling lipids (Fig. 1, 2A), vesicular transfer of signaling lipids (Fig. 1, Vesicle and Caveolar Vesicle), presentation of lipid substrates to enzymes that produce more complex signaling molecules/substrates (Fig. 2B), and presentation of signaling lipid substrates to enzymes for hydrolysis and release of product signaling molecules (Fig. 2C).

In summary, despite the fact that SCP-2 was known for nearly thirty years for its ability to bind and transfer cholesterol and phospholipids, SCP-2's apparent promiscuity in binding other classes of lipids (especially lipids involved in intracellular signaling) suggest more complex physiological function(s). The challenge of future studies will be to begin to resolve the physiological function of SCP-2 in mediating these lipid signaling pathways. Because of the likelihood of compensatory upregulation of other proteins (e.g. PITP, etc) with overlapping functions in the signaling cascades, studies with SCP-2/SCP-x gene-targeted mice will require additional challenges (nutritional, endocrine, exercise, etc. stress). To further elucidate mechanistic details, it is highly likely that these findings will need to be supplemented/complemented by studies with cultured cells and *in vitro* systems.

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Abbreviations

SCP-2 sterol carrier protein-2

PITP phosphatidylinositol transfer protein

L-FABP liver fatty acid binding protein

HDL high density lipoprotein

SRB1 scavenger receptor B1

P-gp P- glycoprotein

ABCA1 ABCA1 transporter

conA concanavalin A

SL Sphingolipid

SM sphingomyelin

Chol cholesterol (C)

Cer ceramide

Glo globosides

GlcCer glucosylceramide

Gal galactose

GalCer galactosylceramide

CGT ceramide glucosyltransferase

LacCer lactosylceramide

GSL glycosphingolipid

LBPA
lysobisphosphatidic acid

NANA
N- acetylneuraminic acid

LCFA
long chain fatty acid

LCFA-CoA
long chain fatty acyl CoA

OlLyso
oleoyl lysophosphatidic acid

PI
phosphatidylinositol

PIP
phosphatidylinositolphosphate

PIP2
phosphatidylinositolbisphosphate

MHC
monohexosylceramide

DHC
dihexosylceramide

G
globoside

GM1
ganglioside M1

PC
phosphatidylcholine

PS
phosphatidylserine

PE
phosphatidylethanolamine

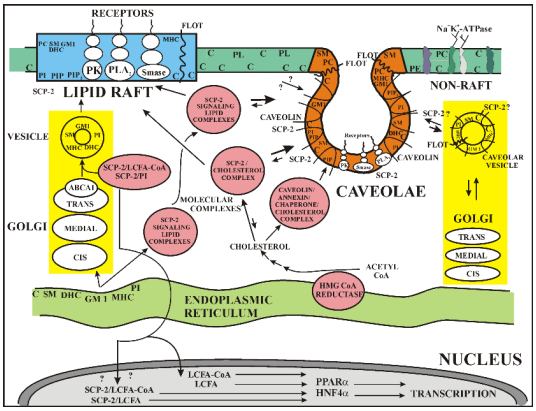

Fig 1. SCP-2 mediates signaling and lipid trafficking to plasma membrane lipid rafts and caveolae

At the plasma membrane, blue shading refers to lipid rafts, red shading refers to caveolae (a lipid raft subfraction), and dark green shading refers to non-rafts. Within the cell, yellow shading refers to vesicular pathways, salmon (dark) pink shading refers to molecular complexes of SCP-2 with SM, DHC, GM1, MHC, PI, cholesterol. Light pink shading refers to molecular complexes of SCP-2 with fatty acyl-CoA or PI. Caveolin (∩), flotillin (FLOT), sterol carrier protein-2 (SCP-2), protein kinase (PK), phospholipase A2 (PLA-A2), phospholipase C (PL-C), peroxisome proliferator activated receptor α (PPARα), hepatocyte nuclear factor 4α (HNF4α), cholesterol (C), phosphatidylinositol (PI), phosphatidylinositolphosphate (PIP), phosphatidylinositolbisphosphate (PIP2), sphingomyelin (SM), ceramide (CER), monohexosylceramide (MHC), dihexosylceramide (DHC), globoside (G), ganglioside M1 (GM1), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE).

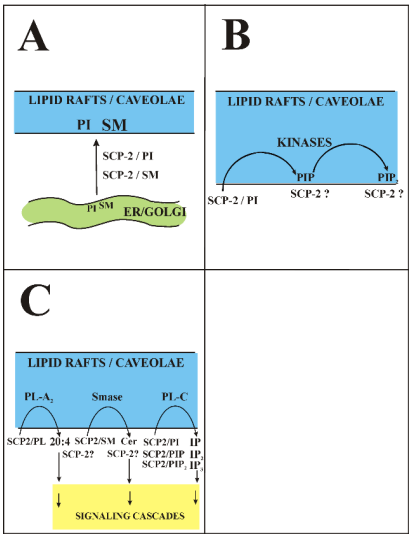

Fig 2. Putative mechanisms whereby SCP-2 mediates signaling and lipid trafficking to plasma membrane lipid rafts/caveolae

Panel A: Complexes of SCP-2/PI or SCP-2/SM transport signaling lipids and/or signaling lipid precursors (i.e. PI, SM) from intracellular sites of synthesis (ER, Golgi) to plasma membrane lipid rafts/caveolae. Panel B: Complexes of SCP-2/PI or SCP-2/PIP are better substrates than the unbound PI or PIP for kinases that produces PIP and PIP2, respectively. Panel C: Complexes of SCP-2/PL, SCP-2/SM, SCP-2/PI, SCP-2/PIP, and SCP-2/PIP2 are better substrates than the unbound lipids for lipases that release signaling lipids (arachidonic acid, ceramide, IP, IP2, IP3). Phospholipase A2 (PLA-A2), sphingomyelinase (SMase), phospholipase C (PLA-C), phospholipid (PL), phosphatidylinositol (PI), phosphatidylinositolphosphate (PIP), phosphatidylinositolbisphosphate (PIP2), inositol phosphate (IP), inositol bisphosphate (IP2), inositol triphosphate (IP3), sphingomyelin (SM), ceramide (CER).

Table 1

Lipids involved in intracellular signaling.

Lipid Class	Responses	References
Straight-chain fatty acids	Regulate diacylglycerol kinase; sodium channel regulator; Tyr phosphorylation; neutrophil function; cell-cell signaling; stimulate transcriptional activity of nuclear receptors such as PPARα	[200–209]
Branched-chain fatty acids	Stimulate transcriptional activity of nuclear receptors such as PPARα	[62,62–67]
Fatty Acyl CoAs	Activate K+ channel; Stimulate Ca++ release; differentially regulate PKC subtypes; enhance vesicle budding, release and transport; protein transport; stimulate transcriptional activity of nuclear receptors such as PPARα and HNF4α.	[208–223]
Lysophosphatidic acid	Membrane fission; vesicle trafficking	[224,225]
Phosphatidylinositols	Phospholipase C signaling	[51,165,169,170,172]
Sphingomyelin	Substrate for ceramide formation	[149]
Ceramide	Enhances cholesterol efflux to apo-A1; Activates eNOS;	[75,226,227]
Glucosylceramide	Modulates endocytic trafficking	[228]
Galactosylceramide	Ca++ flux; oligodendrocyte development	[229]
Lactosylceramide	Activates Ras and MAP kinase phosphorylation of Raf ; simulates endocytosis	[229–231]
Globoside	P determinant in the P system of blood groups	[229]
Ganglioside GM1	Tyr phosphorylation; PI turnover; Ca++ flux; Inhibits beta-adrenergic receptor-induced cAMP formation	[149,229]
Farnesyl-PP Geranyl-PP	Isoprenylate or geranylate signaling proteins targeted to caveolae/rafts?????	[60]
Cholesterol	Required for caveolae/raft; stimulates caveolar endocytosis; Intracellular cholesterol sensor pool regulating release of SREBP and transcriptional activity in nucleus.	[75,127,144,230]

Table 2

Structural lipids enriched in lipid rafts/caveolae of cultured fibroblasts[a].

Lipid Class	Control		SCP-2 Overexpressor	
	Non-raft	Lipid raft/caveolae	Non-raft	Lipid raft/Caveolae
Cholesterol	380±96	963±82*	442±99	857±128*,+
Phospholipid	330±55	1221±150*	681±116	871±136+
C/PL Ratio	1.1±0.3	0.6±0.1	0.7±0.1	1.0±0.2
Total Lipid	1016±126	3046±196*	1270±158	1910±186*,+

[a]Lipid raft/caveolae and non-raft domains were isolated from purified plasma membranes of cultured L-cell fibroblasts using density gradient centrifugation and concanavalin-A affinity chromatography [61]. Values represent nmol/mg protein ± SD (n=3–4). Asterisk = $p<0.05$ vs control; dagger = $p<0.05$ vs non-raft.

Table 3

Signaling lipids are enriched in lipid rafts/caveolae.

Lipid Class	Fold-Enrichment Relative to Non-Rafts	References
Fatty acids	4–12	[114]
PI, PIP, PIP2	3–16	[61,71,124,232,233]
Total sphingolipid	2 to >100	[149]
Sphingomyelin	1.7 to >100	[61,131,149]
Ceramide	Yes/No	[72,149]
Monohexosides	7	[149]
Dihexosides	Yes/No	[149]
Globoside	5	[149]
Ganglioside GM1	2–5	[53,61,114,149]
Total gangliosides	>20	[131,149]
Cholesterol	2–3	[53,61,114,131,142,143,146,149,234]

Table 4

SCP-2 exhibits high affinity for lipids participating in intracellular signaling.

Signaling Lipid Ligand	Kd or Ki (nM)[a]	References
Fatty acids	234–650	[174,235–238]
Fatty acyl CoAs	3–250	[20,149,175,237]
Lysophosphatidic acid	114±16	[149]
Phosphatidylinositols	PIP2>PIP>PI	[51,181]
Sphingomyelin	101±16	[149]
Ceramide	5±1	[149]
Glucosylceramide	101±16	[149]
Galactosylceramide	110±10	[149]
Lactosylceramide	116±9	[149]
Globoside	121±2	[149]
Ganglioside	43±6	[149]
Cholesterol	4–2600	[15,20,149,187,236–238]

[a] Affinities depend on specific acyl chain esterified to the lipid and on type of binding assay used.

Table 5

SCP-2 stimulates transfer of intracellular signaling lipids.

Lipid Class	References
Fatty acids	[17,177,182]
Fatty acyl CoAs	[17,239]
Phosphatidylinositol (PI>PIP>PIP2)	[51,165,181]
Sphingomyelin	[184,185]
Monoheoxosylceramide	[184]
Dihexoxylceramide	[184]
Globoside	[184,185]
Ganglioside GM1	[184,185]
Cholesterol	(rev. in [31,240–242]

Table 6

Effect of SCP-2 expression on distribution of signaling lipids to caveolae/lipid rafts.

Cell type	SCP-2 Level	Lipid Class	Response	References
Fibroblasts	Overexpression	Fatty acids	Decrease	Current data
Hepatocyte	Ablation	Fatty acids	No effect	[114]
Fibroblasts	Overexpression	PI	Decrease	[61,149]
Hepatocyte	Ablation	PI	Increase	[114]
Fibroblasts	Overexpression	Total sphingolipid	Increase	[149]
Fibroblasts	Overexpression	Sphingomyelin	Decrease	[61,149]
Hepatocyte	Ablation	Sphingomyelin	Small Increase	[114]
Fibroblasts	Overexpression	Ceramide	Increase	[149]
Fibroblasts	Overexpression	Glucosylceramide	Increase	[149]
Fibroblasts	Overexpression	Galactosylceramide	No change	[149]
Fibroblasts	Overexpression	Lactosylceramide	No change	[149]
Fibroblasts	Overexpression	Globoside	Increase	[149]
Fibroblasts	Overexpression	Ganglioside GM1	Decrease	[61]
Fibroblasts	Overexpression	Total gangliosides	No effect	[149]
Hepatocyte	Ablation	Ganglioside GM1	Small Decrease	[114]
Fibroblasts	Overexpression	Cholesterol	No change	[61,149]
Hepatocyte	Ablation	Cholesterol	No change	[114]