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Lipid rafts : dream or reality for cholesterol transporters ?

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Abstract

As a key constituant of the cell membranes, cholesterol is an endogenous component of mammalian cells of primary importance, and is thus subjected to highly regulated homeostasis at the cellular level as well as at the level of the whole body. This regulation requires adapted mechanisms favoring the handling of cholesterol in aqueous compartments, as well as its transfer into or out of membranes, involving membrane proteins. A membrane exhibits functional properties largely depending on its lipid composition and on its structural organization, which very often involves cholesterol-rich microdomains. Then there is the appealing possibility that cholesterol may regulate its own transmembrane transport at a purely functional level, independently of any transcriptional regulation based on cholesterol-sensitive nuclear factors controling the expression level of lipid transport proteins. Indeed, the

main cholesterol "transporters" presently believed to mediate for instance the intestinal absorption of cholesterol, that are SR-BI, NPC1L1, ABCA1, ABCG1, ABCG5/G8 and even P-glycoprotein, all present privileged functional relationships with membrane cholesterol-containing microdomains. In particular, they all more or less clearly induce membrane disorganization, supposed to facilitate cholesterol exchanges with the close aqueous medium. The actual lipid substrates handled by these transporters are not yet unambiguously determined, but they likely concern the components of membrane microdomains. Conversely, raft alterations may provide specific modulations of the transporter activities, as well as they can induce indirect effects via local perturbations of the membrane. Finally, these cholesterol transporters undergo regulated intracellular trafficking, with presumably some relationships to rafts which remain to be clarified.

Keywords: lipid rafts, cholesterol transport, ABC proteins, SR-BI, NPC1L1, intestinal absorption.

Abbreviations: ABC: ATP-binding cassette; ACAT: acyl-coenzyme A:cholesterol acyltransferase; Cav1: caveolin 1; HDL: high density lipoprotein; HMGCoA: hydroxymethylglutaryl coenzyme A; LDL: low density lipoprotein; LXR: liver X receptor ; (M)βCD: (methyl)β-cyclodextrin; MDR: multidrug resistance; MLN64: metabolic lymph node 64 protein; NPC1(L1): Niemann-Pick C protein 1 (-like 1); NPC2: Niemann-Pick C protein 2; P-gp: P-glycoprotein; RND: resistance-nodulation-division; SCP-2: sterol carrier protein 2; SR-BI: scavenger receptor class B type I; SSD: sterol-sensing domain; SUV: small unilamelar vesicles.

Introduction: cholesterol "transport" versus membrane structure and organization.

Cholesterol, a key constituant of the cell membranes, is an endogenous component of mammalian cells of primary importance, and is thus subjected to highly regulated homeostasis at the cellular and the whole body levels (Ikonen 2006). Its physiological metabolism and the patho-physiological bases for its observed dysregulations are deeply relevant for pharmacological perspectives in the field of cardiovascular diseases.

Cholesterol turn-over and homeostasis in the body involve many pathways: dietary incomes, entero-hepatic cycle with biliary excretion and intestinal reabsorption, reversible fluxes in the bloodstream between liver and peripheral tissues, biosynthesis (mainly) in liver and steroidogenic tissues, and fecal elimination. The intestinal absorption via the enterocytes is the main determinant process for the global input of cholesterol in the organism (Hui and Howles 2005). In the aqueous compartments of the body, cholesterol is "transported" through its association with lipoprotein particles in the plasma and in the lymph or by inclusion in bile salt micelles in the bile and in the digestive lumen, ensuring in every cases its solubility in the biological fluids. At the cellular level in peripheral cells, cholesterol metabolism relies on uptake from LDL, efflux to HDL, neosynthesis involving HMGCoA reductase and esterification involving ACAT for storage inside the cells. Furthermore, intracellular cholesterol trafficking is a complex process involving either vesicle flux or proteolipid assemblies involving various protein partners (e.g. SCP-2, NPC2, MLN64, Cav1/cyclophilins…) shuttling between organelles and the various intracellular membrane systems (Soccio and Breslow 2004). Anyway, the "limiting" step for cholesterol transfer from any aqueous compartment to another is the crossing of the cell membrane which forms the frontier between them. It is not clear whether a cholesterol molecule crossing a membrane

actually mixes with the membrane lipid phase or is only transfered through the membrane without inserting. However, since a membrane exhibits functional properties largely depending on its lipid composition, especially in cholesterol, and on its structural organization which very often involves cholesterol-rich microdomains, there is the appealing possibility that cholesterol may regulate its own transmembrane transport at a purely functional level, independently of any transcriptional regulation based on cholesterol-sensitive nuclear factors controling the expression level of lipid transport proteins.

Indeed, the cell plasma membrane is currently described as composed of the juxtaposition of lateral heterogeneities, forming “membrane microdomains”, relying on local lipid and protein compositions that can be distinguished from the rest of “bulk” membrane phase: this is so viewed as “more mosaic, i.e. crowdy and heterogenous, than fluid” (Engelman 2005; Maxfield 2002). More precisely, these membrane microdomains are generally characterized by an enrichment in cholesterol and sphingolipids, leading to a lipid phase locally more ordered, more compact and thicker than the surrounding membrane, on which these so-called “lipid rafts” are floating (Pike 2004). They are also characterized by the segregation of some proteins, often presenting a GPI anchor, which can favor protein-protein interactions realizing functional regulations (Lucero and Robbins 2004). As a consequence, the lipid rafts are involved in various processes important in cell physiology such as signal transduction, protein and lipid traffic, endocytosis and even cellular entry of pathogens (Simons and Toomre 2000). However, the lipid rafts are presently under lively debates as regards to how they can be unambiguously detected. Indeed, their isolation depends on the protocol used to disrupt the plasma membrane, mostly based on their non-solubilization by a weak, generally non-ionic, detergent or by sonification in a detergent-free medium, and their microscopic observation using fluorescent probes seems to depend on the cell type (Munro 2003). As a matter of fact, these lipid rafts are still badly defined considering their structures

at the molecular level, including their size, their lifetime or their dynamics (Lai 2003). To clarify our topic, and beyond the different sensibilities of the various authors cited, the term "lipid rafts" will be used here to refer to as cholesterol-enriched membrane microdomains creating lateral heterogeneities in the membrane phase; "caveolae", whose definition is also subjected to discussion, will be considered as cholesterol-enriched membrane domains containing caveolin and presenting well-discernable morphological characteristics. Nevertheless, whatever their exact definitions, these lateral heterogeneities in the cell membrane must be taken into account, since the lipid rafts are responsible for biochemical, chemico-physical and biophysical influences on local membrane processes.

In the view of these observations, we will discuss here on the functional relationships between the cholesterol-containing membrane microdomains and the various cholesterol "transporters" present at the cell membrane. We will focus mainly on those expressed at the apical and basal poles of polarized cells such as enterocytes, and proved or suspected to be more or less directly involved in intestinal cholesterol absorption, taken as illustrative of the cellular cholesterol transport processes. Within this frame, the scope of this review will more generally address important questions such as: (i) the intracellular location and traffic of these membrane proteins, (ii) their possible presence in detergent-resistant membrane fractions and their functional modulation by them, (iii) the effects of the cholesterol "transporters" on the membrane structure and organization, (iv) their opportunities for being regulated by protein partners (e.g. caveolin) through possible cosegregation in membrane domains.

SR-BI: a multifunction membrane "scavenger" receptor involved in cholesterol homeostasis.

It is now well established that SR-BI, the scavenger receptor class B type I, is responsible for the selective (i.e. independent of whole HDL endocytosis (Acton et al. 1996)) cholesteryl ester cellular uptake. This occurs in particular at the level of hepatocytes but also in steroidogenic tissues, and for bidirectional free cholesterol exchange with plasma lipoproteins in peripheral tissues, especially in macrophages (Stangl et al. 1999). Binding of the lipoprotein constitutes the first key step in this process (Liu and Krieger 2002; Thuahnai et al. 2003). Actually, SR-BI is able to bind with fair but different affinities HDL and LDL, as well as modified (acetylated or oxidized) LDL, and HDL and LDL appear not to share the same binding sites (Gu et al. 2000). The exact facilitating role of lipoprotein binding to SR-BI on cholesterol exchange with the membrane is still under debate, since the two events are reported to be correlated or not, depending on the experimental system considered (Connelly et al. 2003; de la Llera-Moya et al. 1999; Liu et al. 2002). However, cholesterol transfer is quantitatively determined by the nature of the lipoprotein particle and its lipid composition (Pownall 2006; Thuahnai et al. 2004; Yancey et al. 2000), as well as by the cholesterol cellular content (de La Llera-Moya et al. 2001). Such a property is expected for a facilitated but passive (in the sense of energy-independent) process of transport of the lipid between the lipoprotein and the membrane. In the case of cholesterol transport in the absence of lipoprotein, for example with SUV-type vesicles or mixed biliary micelles as it happens in the intestine lumen, the direct role of SR-BI is strongly suspected in-vitro (Altmann et al. 2002; Hauser et al. 1998), but not yet ascertained in-vivo although it appears involved in the digestive absorption of cholesterol along with other lipids (Altmann et al. 2002; Bietrix et al. 2006; Cai et al. 2004; Hansen et al. 2003; Levy et al. 2004; Mardones et al. 2001), and also liposoluble micronutrients such as vitamin E (Reboul et al. 2006). Anyway, the composition and the organizational structure of the membrane harboring SR-BI is obviously determining in the efficiency of the cholesterol transfer mediated by SR-BI.

As an illustration, since caveolin-1 (Cav1), a protein characterizing the caveolae involved in cellular cholesterol efflux (Simons and Ikonen 2000), has been described to directly participate to cholesterol trafficking in cells (Smart et al. 2004; Uittenbogaard et al. 2002; Uittenbogaard et al. 1998), relationships between SR-BI and Cav1 were investigated and reported. As a matter of fact, murine SR-BI expressed in transfected CHO cells was preferentially found in the low-density caveolin-rich plasma membrane fraction, and this was confirmed by immunofluorescence showing general colocalization of SR-BI with Cav1 in these cells, as well as in the constitutively SR-BI-expressing adrenocortical Y1-BS1 cells (Babitt et al. 1997). SR-BII, the isoform of SR-BI obtained by alternative splicing and truncated on its C-terminal part, displayed the same membrane distribution (Webb et al. 1998). In human THP-1 monocytes, SR-BI was found mainly (55-70%) in the caveolar fraction of the cells, both in undifferentiated and differentiated states (Matveev et al. 1999). Otherwise, SR-BI and Cav1 exhibited closely correlated expression levels in HEK-293T cells transfected with either one of their encoding genes; further immunolocalization in COS-7 cells cotransfected with both genes showed a cellular pattern similar to that of SR-BI-transfected cells (i.e. mainly intracellular) and not that of Cav1-transfected cells (i.e. mainly at the plasma membrane), which suggested a “stabilizing” (in the sense of dragging) role for SR-BI on Cav1 (Frank et al. 2002). Functionally, in SR-BI-transfected CHO cells, cholesterol ether (a non-hydrolyzable analogue of cholesterol ester) was rapidly and selectively uptaken from HDL in the caveolae membrane fraction, where SR-BI was mainly localized; it was also effluxed from caveolae to HDL, showing that caveolae membranes provide a reversible pool of cholesterol ether in the plasma membrane constituting a platform for exchanges with HDL (Graf et al. 1999). However, attempts on various cell types to perform chemical cross-linking or coimmunoprecipitation between SR-BI and Cav1 failed (Matveev et al. 2001), and no coimmunoprecipitation of SR-BI and Cav1 could be evidenced in duodenum detached

epithelial cells or in Caco-2 cells (Levy et al. 2004). Likewise, no colocalization was observed by immunofluorescence on either stably SR-BI-transfected WI38 (human lung fibroblasts) or ACTH-treated Y1-BS1 cells (Peng et al. 2004). It has been subsequently reported that Cav1 expression was not necessary for the SR-BI activity in cholesterol handling. First, in the human adrenal cell line NCI-H295R expressing very low levels of Cav1 and devoid of any morphologically-defined caveolae, the SR-BI-mediated cholesterol ester selective uptake was normally upregulated by a forskolin treatment (Briand et al. 2003). Second, in the two cell lines HEK293 and FRT, both selective uptake of cholesterol ester and cholesterol efflux were similar when measured either in cells constitutively expressing no Cav1 or in Cav1-transfected cells (Wang et al. 2003). Finally, in mice deleted for the Cav1 gene, intestinal absorption of cholesterol was unchanged with respect to the wild-type control mice, and their treatment by ezetimibe, a drug recently discovered to inhibit intestinal cholesterol absorption, was equally efficient in both mice types (Valasek et al. 2005). As a whole, functional relationships between SR-BI and Cav1 appear highly dependent on the cell type considered and the experimental method used for testing them; however, at the intestinal level, no connection could be evidenced.

Independently of these facultative relationships with Cav1, SR-BI was generally present, at least partially, in some cholesterol-rich membrane microdomains. In HepG2 cells, SR-BI was found in majority in the low density membrane fraction isolated on a sucrose gradient after carbonate treatment; interestingly, the unglycosylated non-mature form of SR-BI (≈55 kDa instead of ≈82 kDa) was detected in the non-raft fractions (Rhainds et al. 2004). In primary adipocytes, SR-BI was present in the lowest density membrane sub-fraction isolated by a detergent-free protocol out of three sub-classes of rafts revealed by differences following density gradient ultracentifugation: these “low-density rafts” contain the highest cholesterol concentration (Ortegren et al. 2006). By contrast, in the microvillar fraction of pig

enterocytes treated by 1% Triton X100, SR-BI was completely extracted and found in the non-raft membranes (Hansen et al. 2003). In SR-BI-transfected Cos7 and WI38 cells, SR-BI was found in large majority in the membrane fractions solubilized by 1% Triton X-100, but also appeared to be partially present in the membrane fractions resistant to 1% Lubrol WX (Peng et al. 2004), evidencing the differences in composition of the "Triton-rafts" and the "Lubrol-rafts", consistently with what has been previously demonstrated (Schuck et al. 2003). Similarly, in NCI-H295R cells, SR-BI was mainly present in the low density membrane fraction isolated by a detergent-free protocol, whereas less than 10% was associated to the "Triton-rafts" (Briand et al. 2003).

Whatever its membrane localization with respect to the (various types of) rafts, the presence of SR-BI is however able to induce some kinds of membrane perturbations, likely revealing specific effects on the local structural organization of the plasma membrane. In COS-7 cells, SR-BI expression induced an increased size of the cell membrane cholesterol pool sensitive to metabolisation by the soluble enzyme cholesterol oxidase, whereas the related class B scavenger receptor CD36 did not (de la Llera-Moya et al. 1999); moreover, the human homolog of SR-BI, CLA-1, had the same effect as SR-BI, and this was independent of the total cholesterol cellular content (de La Llera-Moya et al. 2001); also, cholesterol extraction by 2-hydroxypropyl-β-CD revealed the induction by SR-BI of an increased size of the cholesterol pool fastly desorbed from the membrane, whereas CD36 was without any effect (Kellner-Weibel et al. 2000). In addition, SR-BI-transfection of Sf9 insect cells allowed to observe an increased abundance of phosphatidylcholine with longer mono- or polyunsaturated acyl chains (Parathath et al. 2004). Otherwise, the same heterologous expression of SR-BI in insect cells promotes the formation of double-membraned microvillar channels (Reaven et al. 2001); these specific morphological differentiations are strickingly reminiscent of those observed on endocrine cells under trophic hormonal stimulation (Reaven

et al. 2000), but not in adrenal gland of SR-BI-KO mice (Williams et al. 2002), as well as in SR-BI-transfected WI38 cells (Peng et al. 2004). These modifications of plasma membrane shape are indicative of alterations in the lipid phase as regards to its local fluidity and/or its curvature, possibly linked to the modulation of intramembrane cholesterol (and possibly other lipids) distribution. It remains to determine the functional relationship of these effects with the cholesterol exchanging property of SR-BI between membranes and lipoproteins.

Reciprocally, various experimental manipulations of membrane cholesterol induced some alterations in SR-BI functioning. First, in SR-BI-transfected CHO cells, membrane cholesterol depletion by β-CD increased HDL cholesteryl ester selective uptake, while caveolae cholesterol enrichment by overexpressing Cav1 (consistent with previous data reported by the same authors (Uittenbogaard et al. 1998)) induced a decrease of cholesteryl ester uptake (Matveev et al. 2001). Second, in HepG2 cells, disruption of the lipid rafts by treatment with cholesterol oxidase or sphingomyelinase inhibited LDL-mediated cholesteryl ester selective uptake whereas it enhanced HDL_3-mediated cholesteryl ester selective uptake; however, intramembrane cholesterol sequestering by the polyene antibiotic filipin decreased LDL-, but not HDL_3-, mediated cholesteryl ester selective uptake; conversely, cholesterol extraction by β-CD increased HDL_3-, but not LDL-, mediated cholesteryl ester selective uptake (Rhainds et al. 2004). Third, overexpression of Cav1 induced a concommitent decrease of LDL cholesteryl ester selective uptake and an increase of HDL_3 cholesteryl ester selective uptake, correlated with SR-BI dimerization (Truong et al. 2006). Thus, beyond the different handling by SR-BI of the two endogenous ligands, LDL and HDL, as already described (Acton et al. 1996; Gu et al. 2000), membrane cholesterol is clearly involved in SR-BI functioning by subtle modulations involving free intramembrane cholesterol as well as cholesterol constituting the lipid rafts.

In addition, SR-BI function appears to be modulated by some cellular processes which are more or less related to the membrane microdomains. In particular, a cholesterol depletion (by an acute MβCD treatment) of SR-BI-transfected MDCK cells induced the transcytosis of SR-BI from basolateral membrane, where it was colocalized with caveolae, to apical domain by a PKA-dependent process (Burgos et al. 2004). Furthermore, intracellular trafficking of SR-BI and its surface exposure, conditioning its activity, were under the dependence of PI-3-kinase (Shetty et al. 2006). Finally, SR-BI is also described to mediate HDL-cholesterol exchange with the cell by an endocytosis/resecretion mechanism, so-called "retro-endocytosis" (Pagler et al. 2006; Rhainds et al. 2004). The role of cholesterol-rich membrane microdomains on such cellular traffic of SR-BI is still unclear, especially in the enterocytes where it was observed by immunoelectron microscopy at the apical side in the microvilli and in plasma membrane invaginations (Levy et al. 2004). However, SR-BI appeared more associated with clathrin-coated pits than with caveolae (Hansen et al. 2003). Finally, the propency of SR-BI to dimerize or oligomerize (Reaven et al. 2004; Sahoo et al. 2006), as well as its specific interaction with its chaperone-like CLAMP/PDZK1 partner (Assanasen et al. 2005; Ikemoto et al. 2000), may provide additional modulation mechanisms involving membrane microdomains, even if their functional relevancies are still under discussion.

As a whole, SR-BI is largely involved in cholesterol metabolism and homeostasis, at variance with the other scavenger receptors, SR-A or CD36, mostly involved in the interactions with various ligands (including pathogens) and signaling processes (Krieger 2001). Indeed, although CD36 (also known as the fatty acid translocase FAT4) was partially found in CHAPS-insoluble, Cav-rich membrane fractions from adipocytes (Pohl et al. 2004), it contrasts from SR-BI since it displayed a cellular localization under the dependence of Cav1, which dragged CD36 (Frank et al. 2002), and it did not induce any change in the membrane sensitivity to cholesterol oxidase or cyclodextrin (Kellner-Weibel et al. 2000).

Thus, SR-BI appears to establish some crucial "privileged" relationships with lipid microdomains in the surrounding plasma membrane. In this way, SR-BI should be considered as coupling the destabilization of the lipid phase of the membrane to specific interactions with cholesterol donnors or acceptors in the extracellular medium in order to catalyze the passive (i.e. energy-independent) exchange of cholesterol.

NPC1L1 & NPC1: two sterol "transporters" related to the bacterial multidrug transporters of the RND family.

Very recently, bioinformatic technics have pointed out a novel membrane protein, called NPC1L1, as being a fair candidate for playing an important role in intestinal absorption of cholesterol, mainly on the basis of presenting a "sterol sensing domain" (SSD) in its sequence (Altmann et al. 2004). Further, this role was confirmed since the mice deleted for its gene displayed a largely reduced cholesterol and sitosterol absorption, and a resistance to diet-induced hypercholesterolemia, similarly to what was observed on wild-type mice treated with ezetimibe, the new potent drug inhibiting intestinal cholesterol absorption (Davis et al. 2004). Moreover, ezetimibe had no effect on the NPC1L1-deleted mice (Altmann et al. 2004), and the glucuronide derivative of this molecule (its metabolized form) could be shown to specifically bind to NPC1L1 with a high affinity (Garcia-Calvo et al. 2005).

However, very few is presently known on the functionning of NPC1L1 at the molecular level. In particular, its cellular localization, initially predicted to be at the plasma membrane in enterocytes (Altmann et al. 2004), and as actually observed in transiently transfected CHO cells (Iyer et al. 2005), was subsequently reported to be largely intracellular in various cells expressing NPC1L1 either spontaneously (Davies et al. 2005; Sane et al.

2006) or after transient or stable transfection (Davies et al. 2005; Yu et al. 2006). Some light was shed by the use of fibroblasts disrupted for NPC1L1 expression (obtained from NPC1L1-null mice), that showed a marked mislocalization of Cav1 which was exclusively present at the plasma membrane where it appeared trapped, in contrast to the various intracellular structures observed in the wild-type cells; however, this "anti-caveolae effect" of the presence of NPC1L1 was not a consequence of its colocalization with Cav1 (Davies et al. 2005). Nevertheless, the SSD in NPC1L1 sequence suggests the likelyhood of a specific interaction with cholesterol, hence the possibility to sense cholesterol-rich microdomains in its vicinity. This could be consistent with the relative detergent resistance of NPC1L1 evidenced by biochemical fractionation experiments using sequential detergent extraction with taurocholate and digitonin (Iyer et al. 2005). It is worth noting that the measurements of ezetimibe binding on recombinant NPC1L1 was clearly enhanced by the presence of low concentrations of taurocholate and digitonin (Garcia-Calvo et al. 2005). In this context, it was important to observe that a cell cholesterol depletion by MβCD (or to a lesser extent by the cholesterol synthesis inhibitor mevinolin) induced on NPC1L1-overexpressing hepatoma cell line a stricking relocation of NPC1L1 (initially mainly intracellular) at the plasma membrane in a subdomain presenting some characteristics of an apical pole between adjacent cells (Yu et al. 2006). Such an "apical sorting" of a membrane protein induced by cholesterol manipulations is reminiscent of the reported relationship in HepG2 cells between cell cholesterol level and the sorting of various membrane proteins, which suggested the involvement of different types of cholesterol-enriched microdomains (Slimane et al. 2003). In addition, this apical expression of NPC1L1 in hepatocytes appeared functionally relevant since it was accompanied by an increased, ezetimibe-sensitive cellular cholesterol uptake (Yu et al. 2006). Moreover, this polarization is consistent with the presence of NPC1L1 in the brush border membranes of enterocytes (Iyer et al. 2005; Sane et al. 2006).

NPC1L1 has a high sequence homology with NPC1, the main protein whose mutation is responsible for Nieman-Pick type C disease: NPC1 also presents a SSD (Davies and Ioannou 2000), actually binding a photoactivatable cholesterol analog (Ohgami et al. 2004). This should provide some guidelines about NPC1L1 functionning in the cell assuming that both proteins can share common molecular mechanisms. As a matter of fact, NPC1L1 disruption has been shown to provoke an altered intracellullar trafficking of sphingolipids such as lactosylceramide as well as of cholesterol (Davies et al. 2005). This is in line with the well-described abnomal membrane trafficking in NPC1-null cells, regarding cholesterol (Cruz et al. 2000; Millard et al. 2000; Puri et al. 1999) and sphingolipids (Sagiv et al. 2006; te Vruchte et al. 2004; Zhang et al. 2001b), but also sucrose, the weakly basic drug daunorubicin, and 70 kDa-dextran, all markers of fluid phase endocytosis (Gong et al. 2006; Neufeld et al. 1999). However, NPC1 was reported to reside in intracellular membranes, within dynamic compartments related to the late endosomes (Garver et al. 2000; Ko et al. 2001; Neufeld et al. 1999; Zhang et al. 2001a). At that level, NPC1 is predominently present in non-raft membrane fractions (Garver et al. 2000; Lusa et al. 2001), with only partial colocalization with Cav1 (Garver et al. 2000). Nevertheless, when fibroblasts or CHO cells were loaded with LDL-derived cholesterol, the fraction of detergent-resistant NPC1 increased, but it was proposed that the local cholesterol accumulation was the consequence of a defective intracellular traffic rather than of the actual working of NPC1 (Lusa et al. 2001). However, NPC1 deletion in liver homogenates from heterozygous and homozygous mice has been shown to induce an overexpression of Cav1 (Garver et al. 1997) and annexin II (Garver et al. 1999), respectively, which both participate to intracellular cholesterol trafficking (Uittenbogaard et al. 2002). Also, NPC1-deleted hepatocytes have been observed to exhibit increased raft-like microdomains, as reported by a decreased membrane fluidity and an increased resistance to detergent solubilization (Vainio et al. 2005). In addition, cholesterol

depletion induced ubiquitylation (a sorting signal) of NPC1 expressed in transfected COS cells and of endogenous NPC1 in human skin fibroblasts; it also induced the association of NPC1 with SKD1, a component of the endosomal sorting complex, and these events were attributed to cholesterol recognition of the SSD in NPC1 (Ohsaki et al. 2006). Furthermore, point mutations in the NPC1 SSD induced alterations of the intracellular trafficking of LDL-derived cholesterol and of a fluorescent analog of the sphingolipid lactosylceramide (Millard et al. 2005). Otherwise, NPC1 has been shown to present a higher global level of homology with some bacterial multidrug transporters from the RND superfamily (like AcrB or MexD) than with some other SSD-containing eukaryotic proteins (Davies et al. 2000). Indeed, NPC1 could be demonstrated to transport in a proton-dependent maner the cationic amphiphilic drug acriflavine, a substrate of AcrB along with various other lipophilic molecules, in normal fibroblasts and in NPC1-expressing E. coli; interestingly, a membrane transport of oleic acid could also be evidenced but not of cholesterol-oleate or of free cholesterol (Davies et al. 2000). The true relationships between NPC1 and cholesterol are thus still unclear (Ioannou 2005), and the similar question is also open for NPC1L1 (Davies and Ioannou 2006). At this stage, it can be proposed that NPC1L1 would be, actively or indirectly, involved in the vesicular traffic of cholesterol, possibly by using ionic electrochemical gradients as a driving force.

ABCA1: a full-size ABC transporter involved in lipid translocation and transfer

The superfamily of ABC proteins is composed of numerous membrane active transporters which work at the expense of ATP hydrolysis to realize the transmembrane translocation of a great variety of substrates, such as ions, sugars, aminoacids, vitamins,

peptides, proteins, lipids and even drugs (Gottesman and Ambudkar 2001). They are expressed from bacteria to man, and as a rule they are formed by two transmembrane domains and two nucleotide binding domains, and they can be either monomeric or di- or tetrameric by homo- or hetero-association of such domains (Higgins 1992). In higher mammalians, ABCA1 was first considered as a phosphatidylserine translocase involved in macrophage phagocytosis (Hamon et al. 2000). Later on, ABCA1 took a novel status by being involved in Tangier disease when mutated (Remaley et al. 1999), and this suggested that ABCA1 played a role in cholesterol transfer to HDL (Young and Fielding 1999), ensuring the first steps of the physiologically important "cholesterol reverse transport" (Oliver et al. 2001). Subsequently, as the mechanism of HDL formation was progressively analyzed, ABCA1 appeared as responsible for the cellular efflux of phospholipids and cholesterol to lipid-free apoA-I or lipid-poor lipoproteins thanks to a specific interaction with apoA-I (Wang et al. 2000b). However, some controversies remain as regards the detailed molecular processes involved, in particular concerning the actual transported substrate, phospholipid or cholesterol (Vaughan and Oram 2003; Wang et al. 2001), the sequentiality for their handling, in "two steps" or "one step" (Fielding et al. 2000; Smith et al. 2004), the relationship between lipid transfer and apoA-I binding (Chambenoit et al. 2001), and whether the transfer occurs at the cell surface or inside during a "retroendocytosis" process (Cavelier et al. 2006).

Since the membrane microdomains, rafts and caveolae, were believed to mediate cholesterol export from cells in order to maintain its homeostasis (Fielding and Fielding 2000), relationships between ABCA1 and such lipid microdomains have been investigated. It then appeared the generally admited view according to which ABCA1 is not localized in the "classical", Triton X100-resistant membrane domains (Drobnik et al. 2002; Mendez et al. 2001). However, a significant fraction of ABCA1 was found in the "larger", Lubrol WX-resistant membrane domains from human macrophages, but not from fibroblasts (Drobnik et

al. 2002; Bared et al. 2004). In addition, cholesterol efflux from the cells to apoA-I derived from rafts whereas cholesterol efflux to HDL did not (Drobnik et al. 2002; Mendez et al. 2001). Furthermore, relationships with rafts were not straightforward since the lipid acceptor apoA-I was found to interact with lipid rafts in macrophages (Gaus et al. 2004) but not in ABCA1-overexpressing BHK cells (Landry et al. 2006). The composition of the lipids secreted by ABCA1 could also give indications on their origin in the cell membrane: the two types of lipid-poor, nascent HDL particles secreted by macrophages expressing upregulated ABCA1 came respectively from liquid-ordered and disordered membrane domains, and the microparticles released by the cells likely came from raft-like domains (Duong et al. 2006; Liu et al. 2003). This cellular release of lipidic microparticles (devoid of apoA-I) is suggestive of a marked membrane destabilization induced by ABCA1 presence and/or activity; indeed, ABCA1-transfected BHK cells were recently shown to exhibit disrupted cholesterol- and sphingomyelin-rich rafts with redistribution of caveolin, while the cells transfected by an inactive ABCA1 mutant were unperturbed (Landry et al. 2006). In line with these data was the observation of an increased sensitivity to high MβCD concentrations of cells treated by 8Br-cAMP inducing ABCA1 expression, as compared to the non-treated control cells (Smith et al. 2004). Another manisfestation of such a membrane desorganization is the report of an increased sensitivity to cholesterol oxidase of the membrane cholesterol in ABCA1-overexpresing BHK cells; interestingly, this cholesterol pool can either be transfered to extracellular apoA-I (but not HDL) or to intracellular esterifying enzyme ACAT (Vaughan and Oram 2003). ABCA1-mediated lipids redistribution in the cell membrane has been also evidenced by measuring the exofacial exposure of fluorescent phospholipid analogues, showing a preferential outward movement of phosphatidylserine and phosphatidylethanolamine (Alder-Baerens et al. 2005). This translocase activity is likely to be related to the modulation of ABCA1 ATPase activity recently determined on proteoliposomes

reconstituted with purified ABCA1, and showing a preferential stimulation by choline-containing lipids (Takahashi et al. 2006). Also, some cellular mophological alterations, typically echinocyte-like protrusions, observed on ABCA1-transfected HEK293 cells, were highly suggestive of an asymmetry of the cell membrane likely induced by a flip-floppase activity (Wang et al. 2000b). Actually, the exact role of these kinds of membrane disturbances and their relationships with cholesterol-rich rafts and cholesterol traffic remain to be further elucidated (Jessup et al. 2006).

Notably, ABCA1 also displays various cellular effects concerning general membrane trafficking; indeed, Tangier fibroblasts (deficient in ABCA1) display enhanced endocytosis (Zha et al. 2001) and phagocytosis (Bared et al. 2004), while ABCA1 overexpression decreased both receptor-mediated and fluid-phase endocytosis (Alder-Baerens et al. 2005). However, Tangier fibroblasts exhibit altered membrane trafficking, including caveolae, between Golgi and plasma membrane (Orso et al. 2000). More precisely, Tangier fibroblasts accumulated both cholesterol and sphingomyelin in the late endocytosis compartment, and ABCA1 transfection induced a correction of this trafficking defect along with the location of tagged ABCA1 in endosome vesicles (Neufeld et al. 2004). Interestingly, vesicular transport from Golgi to plasma membrane increased during ABCA1-mediated cholesterol efflux from both macrophages and fibroblasts (Zha et al. 2003). This is consistent with the observation of preferential cholesterol efflux from “internal pools” (i.e. late endosomes and lysosomes) fed by LDL endocytosis than from “superficial pools” (i.e. recycling endosomes and trans-Golgi) fed by soluble donnors such as serum or cyclodextrin (Chen et al. 2001). Although ABCA1 has been repeatedly reported to be expressed mainly at the cell surface (Landry et al. 2006; Wang et al. 2000b), these data highlight that the (small) intracellular fraction of ABCA1 is likely to have a clear functional importance (Chen et al. 2005; Neufeld et al. 2001; Neufeld et al. 2004), in particular revealing an unexpected possible functional synergy with NPC1 (Chen

et al. 2001; Neufeld et al. 2004). This would be in line with the regulation of the internal localization of ABCA1 by various protein partners, such as syntrophins (Munehira et al. 2004; Okuhira et al. 2005) and syntaxins (Bared et al. 2004). Finally, cholesterol-induced regulation of ABCA1 intracellular trafficking and its relationships with retroendocytosis still remain to be clarified.

ABCG1: an homodimeric ABC transporter transfering cholesterol to acceptors

Within the frame of cholesterol homeostasis in the body, the HDL-mediated reverse cholesterol transport from peripheral cells to hepatocytes has received much attention, in particular considering the efflux of cholesterol from macrophages, since they are involved in the first steps of atheromatosis when they incorporate uncontrolled amounts of cholesterol leading to foam cells. Indeed, on the basis of their expression regulation providing adapted responses to loading/depletion of cellular cholesterol, three ABC transporters appear to be involved in such an export of cholesterol to ApoAI-containing lipoproteins: A1 (see above), G1 and G4 (Klucken et al. 2000). Cell transfection experiments confirmed that ABCG1 and G4 (a very close homologue to G1) induce cholesterol transfer to HDL (Wang et al. 2004). The use of gene-disrupted and transgenic mice for ABCG1 brought further evidence of its involvement in preventing cholesterol accumulation in tissues (Kennedy et al. 2005). Furthermore, it appears now that ABCG1 displays a complementary role to that of ABCA1, since ABCA1 can transfer cholesterol to lipid-free/poor apoA1 while ABCG1 can transfer cholesterol to “nascent” HDL particles (i.e. discoidal preβ-HDL, formed in medium conditioned by ABCA1-expressing cells), to finally form mature HDL (Baldan et al. 2006; Gelissen et al. 2006).

Under the perspective of possible functionally relevant interactions of ABCG1 with membrane cholesterol, very few is presently known. First, it has been observed that human ABCG1 overexpressed in BHK cells induced an increased amount of cholesterol in the plasma membrane accessible to the soluble enzyme cholesterol oxidase; this cholesterol pool was either removed by HDL or devoted to esterification in internal compartments in the absence of external cholesterol acceptors (Vaughan and Oram 2005). Such an influence of ABCG1 on cellular distribution and trafficking of cholesterol could be related to an alteration of cholesterol-rich membrane microdomains. In addition, in transfected HEK293 cells, ABCG1 has been shown to efflux cholesterol to external acceptors, along with sphingomyelin preferentially to phosphatidylcholine (Kobayashi et al. 2006). Second, concerning its intracellular localization and trafficking, some controversial data have been reported: ABCG1 appears either mainly perinuclear (Gelissen et al. 2006; Lorkowski et al. 2001), or constitutively at the plasma membrane (Kobayashi et al. 2006), or redistributed to the cell surface by LXR activation (Wang et al. 2006b). This leads to two alternative possibilities allowing ABCG1 to redistribute cholesterol in the cell: either it handles cholesterol at the plasma membrane level, or it processes cholesterol in some internal membranes before it becomes exposed at the cell surface thanks to membrane traffic (Baldan et al. 2006). Anyway, it seems likely that this could lead to the formation of cholesterol-enriched microdomains in the cell membrane, which should then favor cholesterol transfer to ApoAI-containing lipoproteins or even other cholesterol acceptors.

ABCG5/G8: an heterodimeric ABC transporter expelling sterols

The recent cloning of two genes responsible for the dyslipidemic disease sitosterolemia has highlighted the ABC family as providing new partners in the control of cholesterol homeostasis in the body (Berge et al. 2000). Indeed, studies on mice bearing a deletion for either of the gene coding for ABCG5 or ABCG8 (Yu et al. 2002a), or overexpressing both of these genes (Wu et al. 2004; Yu et al. 2002b), have shown that these two "half-transporters" are involved in biliary secretion of cholesterol and other sterols, in particular phytosterols like sitosterol. Furthermore, these half-transporters (i.e. each harboring only one nucleotide binding domain) were demonstrated to be necessarily coexpressed as an heterodimer in order to exhibit their full fonctionality (Graf et al. 2003; Wang et al. 2006c). They are present in hepatocytes at the apical canalicular membrane, where they participate to bile formation (along with BSEP = ABCB11 and MDR3 = ABCB4, respectively secreting bile salts and phosphatidylcholine), and also in the intestine where they are believed to limit sterol absorption (mainly in jejunum and ileon) (Duan et al. 2004; Hazard and Patel 2007).

The molecular mechanisms of ABCG5/G8 are still very elusive, in particular regarding its relationships with membrane environment and its modulation by the cellular parameters. Some points can nevertheless be considered as potentially relevant as regards functional links with cholesterol regulating its activity. First, intracellular trafficking of ABCG5/G8 is carefully controled so that each monomer is stabilized by the other one, and only heterodimerization will allow its expression at the cell surface (at the apical pole when the cell is polarized) (Graf et al. 2002), which requires an efficient protein sorting (Okiyoneda et al. 2006). Second, from recent in-vitro data on inside-out vesicles receiving cholesterol from donor liposomes, ABCG5/G8 seems to operate an efficient cholesterol transfer to the luminal leaflet of the vesicle membrane thanks to a floppase mechanism since the vesicle lumen were devoid of any cholesterol acceptor, suggesting that spontaneous cholesterol flip-flop rate would be limiting in the membranes containing ABCG5/G8 (Wang et al. 2006a). If

this could be confirmed under more physiological conditions, it would imply that ABCG5/G8 could play an important role in the intramembrane distribution of cholesterol. Third, from in-vivo experiments on intraveinously infused ABCG5/G8-KO mice with high concentrations of hydrophobic bile salts, the observation of a depletion of cholesterol present in the canalicular membrane, leading to bile duct lesion and cholestasis, led to infer that ABCG5/G8 can have a protective effect against the detergent action of the bile acids (Kosters et al. 2006). Furthermore, the absence of any stimulation of biliary cholesterol secretion under these conditions led the authors to consider that the limiting step should be the intramembrane translocation of cholesterol, and thus to suggest that this transporter acts as a floppase (Kosters et al. 2006). In that case, the observation of the extended selectivity of this transporter to handle various natural sterols, in order to exclude them from the body in spite of their abundance in the diet (Yu et al. 2004), would raise the question of their intramembrane distribution (between the two leaflets as well as regarding the cholesterol-rich microdomains likely to be present). As a mater of fact, it has been observed on model membranes that the phytosterols, sitosterol and stigmasterol, either behave approximately as cholesterol in promoting ordered, detergent-resistant microdomains (Xu et al. 2001), or are less able to favorably interact with phospholipid bilayers and stabilize microdomains (Halling and Slotte 2004). However, no direct investigation of the relationships between ABCG5/G8 and membrane microdomains have been presently reported, and this would thus be desirable.

P-gp: a (possible) model ABC transporter for relationships with cholesterol

P-glycoprotein (P-gp = ABCB1) is well-described for almost three decades for being responsible for the multidrug resistance (MDR) phenotype of some tumor cells (Gerlach et al.

1986). Its gene cloning about twenty years ago has shown that it belongs to the superfamily of ABC active membrane transporters (Bosch and Croop 1996). P-gp works as expelling by an ATP-dependent process various cytotoxic drugs out of the tumor cells which express it in the plasma membrane (Gottesman and Pastan 1993), leading to anticancer chemotherapy failures (Nooter and Herweijer 1991). In the nineties, it became progressively clear that P-gp, which is also expressed in some healthy tissues such as intestine mucosa, blood-brain barrier, biliary canalicule and kidney tubule, can handle a very large number of amphiphilic molecules of pharmaceutical interest, and is consequently involved in their pharmacokinetic characteristics (such as digestive absorption, CNS disposition, biliary and urinary excretion) (Schinkel et al. 1997). P-gp is thus characterized by the rather unique property of multispecific transported substrate recognition, concerning a very much broad spectrum of molecular structures, provided they are amphiphilic, neutral or cationic, and presenting a size ranging between about 250 and 1250 Da (Ambudkar et al. 1999). The underlying molecular mechanisms only begin to be unraveled (Garrigues et al. 2002b; Shilling et al. 2006); in particular, a body of evidences have shown that the various, more or less hydrophobic substrates recognized P-gp at the level of its transmembrane domains after partitioning in the surrounding lipid phase (Ferte 2000; Higgins and Gottesman 1992; Sharom 1997).

Various steroids belong to the number of substrates transported by P-gp, as they notably exibit reduced cellular accumulation in MDR cells and inhibition of P-gp-mediated drug transport, and among them cholesterol behaves similarly (Barnes et al. 1996; Wang et al. 2000a). In addition, cholesterol modulates ATPase activity of P-gp contained in native inside-out membrane vesicles prepared from MDR (selected or transfected) cells (Barakat et al. 2005; Garrigues et al. 2002a; Gayet et al. 2005; Rothnie et al. 2001; Wang et al. 2000a). It has then been demonstrated in-vitro on such P-gp-containing inside-out vesicles that exogenous radiolabelled cholesterol associates in an ATP-dependent manner, and in addition that

endogenous cholesterol becomes less accessible to the exogenously added soluble enzyme cholesterol oxidase also in an ATP-dependent manner (Garrigues et al. 2002a). Taken as a whole, these data provide the evidence that P-gp actively translocates membrane cholesterol from the cytosolic leaflet to the exoplasmic one. Such an intramembrane redistribution of cholesterol, leading to an increased exposure at the cell surface and/or a stabilization of membrane microdomains, is consistent with the observation of alterations of cholesterol interaction with the external medium, including resistance to permeabilization by digitonin (Ramu et al. 1991), and variable efficiency of MβCD extraction (Le Goff et al. 2006; Reungpatthanaphong et al. 2004). However, cellular efflux of cholesterol to MβCD appears rather cell type-dependent since it was increased in stably transfected LLC-MDR1 but not in conditionally transfected HeLa-MDR/Tet cells (Le Goff et al. 2006). Also, cellular cholesterol depletion by MβCD was less efficient in MDR K562 versus parental sensitive cells (Reungpatthanaphong et al. 2004), and in that case an increased recycling of cholesterol from internal stores can be invoked as well as an altered cholesterol distribution/organization within the plasma membrane. Indeed, the whole cellular trafficking of cholesterol has been observed to be stimulated in some MDR cells, with increased biosynthesis (Metherall et al. 1996) and esterification (Debry et al. 1997; Field et al. 1995), although this has been subsequently contested (Issandou and Grand-Perret 2000), and appears once more as depending on the cell type considered (Luker et al. 1999). Anyway, such an increased membrane trafficking can provide a fair explanation to the apparently unexpected observation of an increased influx of cholesterol from mixed micelles to intestinal cells overexpressing P-gp (Tessner and Stenson 2000). Finally, in the double KO, *mdr1a*-/*mdr1b*-disrupted mice, the observation of a decreased cholesterol accumulation with an increased cholesterol esterification in the hepatocytes, but not in the other tissues, when cholesterol was administered orally, but not intraveinously (Luker et al. 2001), illustrates that all the

consequences, both direct and indirect, of P-gp expression on the cellular traffic and metabolism of cholesterol are not yet deciphered.

The involvement of P-gp in cell cholesterol homeostasis is nevertheless indicated by the regulation of its gene expression by the cholesterol loading level of the cell: human macrophages treated by HDL for cholesterol depletion, or by LDL for cholesterol repletion, respectively up-regulated and down-regulated P-gp expression (Klucken et al. 2000). As a mater of fact, MDR cells submitted to cholesterol starvation during culture felt a much smaller cholesterol level decrease than the sensitive counterparts (Mazzoni and Trave 1993). Furthermore, it has been observed that in the MDR cells, in contrast to sensitive tumor cells, cholesterol level is mainly regulated via its efflux to HDL (Liscovitch and Lavie 2000). This could be viewed as consistent with the observation of an up-regulation of Cav1 expression in some MDR cells (Lavie et al. 1998; Pang et al. 2004; Yang et al. 1998), although this could not be considered as a general rule (Lavie et al. 1998).

Reciprocally, P-gp function has been amply shown to be influenced by the nature, the composition and the structural organization of its surrounding membrane. Actually, P-gp often localized (at least partially) in cholesterol-rich, detergent-resistant membrane microdomains (Barakat et al. 2005; Luker et al. 2000; Troost et al. 2004), possibly depending on the detergent used (Hinrichs et al. 2004; Radeva et al. 2005), at variance with a report using a detergent-free method for P-gp isolation (Reungpatthanaphong et al. 2004). Furthermore, P-gp colocalized with some membrane proteins included in rafts, and these protein-protein interactions were shown to functionally regulate P-gp (Demeule et al. 2000; Ghetie et al. 2004; Luciani et al. 2002). However, P-gp function was also modulated by treatments altering rafts (Barakat et al. 2005; Luker et al. 2000; Troost et al. 2004), although these experimental conditions rather targeted the cholesterol presence in the cell membrane. The interpretation of such experimental results are thus not always unambiguous concerning

the respective roles of free cholesterol versus cholesterol composing the rafts in the membrane. In addition, P-gp has also been described in internal membranes (Molinari et al. 1994), where its functional implication has been discussed in relation to intracellular membrane trafficking (Kim et al. 1997; Lee et al. 2001; Rajagopal and Simon 2003; Shapiro et al. 1998); furthermore, intracellular trafficking leading to apical polarized expression of P-gp appeared to be dependent on cholesterol (Slimane et al. 2003). As a whole, it appears that P-gp establishes privilegied relationships with membrane cholesterol, even if its inclusion in the rafts is likely partial and dynamic in essence, and clearly depends on the cell type considered as well as the exact nature of the microdomain involved (e.g. presence of Cav1, detergent-type resistance and MβCD sensitivity) (Orlowski et al. 2006). Thus, even if P-gp could be considered as a (both theoretical and experimental) guideline for other ABC transporters involved in lipid traffic, many points still remain unclear and should be addressed in order to gain the undisputed status of "model ABC transporter". However, it could be envisioned that the different ABC transporters, considered here to be involved in cellular cholesterol transport, all present an active floppase function of rather broad selectivity for various lipids, which may be converted to an (active) "extrudase" function if the transporter specifically interacts with a lipid acceptor (such as a lipoprotein).

Synthesis and perspectives

Cholesterol homeostasis is of primary importance for body health and cell physiology. It involves various membrane proteins which mediate cholesterol transfer from a donnor in an aqueous medium to an acceptor in another aqueous compartment: cell membrane is thus a limiting step in such cholesterol "transport". In this frame, the molecular mechanisms of these

cholesterol “transporters”, whenever they behave as “translocases” or “transferases”, is thus desirable to be analyzed and elucidated, since this determines the resultant biological processes which ultimately are considered as relevant for public health (e.g. hypercholesterolemia leading to atherosclerotic diseases). Indeed, understanding cellular physiology of cholesterol metabolism and traffic needs the description of the lower integration levels, i.e. the membrane and within it the proteins, which are the actual pharmacological targets. As a matter of fact, lipid composition and structural organization of the cell membrane are well-known to be essential for the biological activity of the membrane proteins, especially membrane transporters. The cholesterol-rich microdomains, lipid rafts, are then prominent candidates for regulating cholesterol transporters. They indeed appear to play noticeable roles in the various proteins we discussed above. However, it is not always clear whether this is realized directly by providing either a specific close environment or an alteration of the local biophysical properties of the membrane (curvature, asymmetry, thickness…), or indirectly by modulating the transporter activity within the membrane, for example by altering membrane trafficking or also by recruiting regulatory protein partners: this will need further work in every case.

As a whole, it is noteworthy that the main cholesterol transporters considered here show variable intracellular distribution or presence in the membrane microdomains, often depending on the cell type and on the experimental procedure. Obviously, some methodological pitfalls are always possibly underlying the works cited here, such as: (i) cell and membrane fractionation, generally based on the use of detergents under specific conditions, (ii) cell cholesterol manipulation using cyclodextrin, not always straightforward for preserving the integrity of the cell physiology, and (iii) protein colocalizations using fluorescence microscopy, always subjected to the limitations due to the optical microsopy resolution. However, the whole data presented above fairly suggest that the cholesterol

"transporters" display a highly dynamic behavior with respect to their membrane environment, taking full benefit of the intracellular traffic. This should be considered as an evidence of all the regulation processes these membrane proteins can undergo, and as an illustration of their likely functional versatility when considering the cellular consequences of their molecular working.

The cholesterol "transporters" can be classified in two categories, according to their passive (i.e. acting as facilitators) or active (i.e. enegy-dependent) activity. The second case, mostly concerning the members of the ABC family, raises the interesting question of the relationships between the cholesterol flip-flop in the membrane and the "raft determinism". Since the lipid rafts in plasma membrane are currently described as presenting an asymmetry between the two membrane leaflets, as regards their lipid composition, the actual question is whether these microdomains are at thermodynamic equilibrium. Considering their highly dynamic behavior and their involvement in the whole membrane trafficking, these membrane microdomains are likely out of equilibrium (as is the biological membrane as a whole!). The following question is about the nature of the molecule(s) taken as the primary substrate of an active intramembrane translocation and whose asymmetric distribution provides the driving force for the whole raft formation and then raft stability at the steady state. Although it is difficult to claim that it is the only lipid component to be concerned, cholesterol is indeed outwardly translocated ("flopped") by some ABC transporters, and this may then be at least part of the global mechanism of raft determinism (also involving, among others, sphingomyelin). In addition, this translocation is not necessarily limited within the rafts themselves, since cholesterol may also be actively flopped at any place of the membrane, allowing to passively diffuse laterally in the exoplasmic leaflet up to incorporate a raft domain. As a matter of fact, the representative and long-known ABC transporter P-gp is able to establish an altered cholesterol distribution between the two membrane leaflets when

assayed in-vitro (Garrigues et al, 2002a). This straight experimental observation clearly demonstrates that the active intramembrane flux of cholesterol is faster than its passive back flux in that case, but this could also be achieved by other ABC transporters under different conditions.

Furthermore, for a more general point of view, and given the rather rapid rate at which cholesterol can spontaneously transfer between membrane pools, we can wonder whether the function of a cholesterol "transporter" is to regulate exchange with the extracellular medium or to maintain the disequilibrium of sterol between cholesterol-rich and cholesterol-poor domains. It seems fair to assume that an heterogenous cholesterol abundance between the various membrane pools is necessary to determine cholesterol exchange rates with the extracellular medium as part of the general process of lipid (and membrane proteins) sorting among the intracellular compartments. Thus, the various cholesterol transporters, at least the active ones, would be as well involved in regulating the different intracellular cholesterol pools as in ensuring the actual fluxes of cholesterol with extracellular donnors and acceptors. In any case, these active transporters could be considered as participating in a global process of feeding a continuous cholesterol turn-over within the cells or in the organism, which is inherent to the homeostasis of this important biological component.

Anyway, all these possible aspects of functioning of these cholesterol transporters make them likely to be sensitive to the presence of cholesterol in their membrane environment. In the case of SR-BI, the cellular consequences of a local facilitating effect on the passive exchange of cholesterol between the membrane phase and a soluble complexing agent is obviously dependent on various conditions imposed by the metabolic status of the cell, especially regarding its lipid loading level: this could determine the direction of the mediated cholesterol flux. Also, when cholesterol has to "choose" between different pathways for cellular export, rafts may play a role (Fielding and Fielding 2003; Jessup et al. 2006), in

particular in relation with the cell polarization. In the case of NPC1L1, the strong homology with NPC1 indicates that it could be involved in vesicular trafficking, which is known to depend on cellular rafts (Rajendran and Simons 2005). In the case of members of the ABC family, which most likely mediate an active process in the membrane, the question remains about the relationship between a local interleaflet translocation of cholesterol and the resulting effect in the cell, taking into account the lipid trafficking under the influence of the rafts (Orlowski et al. 2006). In all cases, the effect of the rafts should also depend on the substrate selectivity of the various cholesterol transporters, since they all are known or suspected to handle a more or less broad spectrum of lipids. Finally, the rafts can also well be a factor favoring the "functional harmonization" of the different lipid transporters involved in cholesterol cellular traffic and presenting possible functional redondancy and/or complementation, allowing for subtle coregulations and optimization: this will undoubtly warrant futur investigations in the field. Anyway, cholesterol-rich membrane microdomains appear to provide a novel way, besides the more described genic regulations involving various nuclear factors, for cholesterol transport regulation based on chemico-physical and biophysical properties of the membrane.

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