FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 43, No. 1, 2005
pp. 3-10

Review article

Rafts - the current picture

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Abstract: Although evidences that cell membrane contains microdomains are accumulating, the exact properties, diversity and levels of organization of small lipid patches built mainly of cholesterol and sphingomyelin, termed rafts, remain to be elucidated. Our understanding of the cell membrane is increasing with each new raft feature discovered. Nowadays rafts are suggested to act as sites of cell signaling events, to be a part of protein sorting machinery but also they are used by several pathogens as gates into the cells. It is still unclear how rafts are connected to the membrane skeleton and cytoskeleton and with how many different types of rafts are we actually dealing with. This review summarizes some of the most recent discoveries trying to make a view of the complex raft properties.

Key words: Rafts - Detergent-resistant membranes - Membrane skeleton - Immunological synapse

Introduction

Plasma membrane is no longer seen as a lipid sea with embedded protein islands. The discovery of membrane domains both, caveolae-containing and flat rafts, prompted new research efforts towards cell membrane biology. Huge resources are engaged into identification of the components and the role of lipid rafts in various cell types [23, 26, 32, 38, 57]. After several years of studies it is generally assumed that eukaryotic cell plasma membrane is subdivided into lipid raft and non-raft regions. However, the term "raft" is not unique. One can find terms as: DRMs - detergent resistant membranes [48, 62]; DIGs - detergent insoluble glycolipid rich complexes [46]; GEMs - glycolipid enriched membranes [42]; LDMs - low density membranes [24] *etc*. Each of these to some extent characterizes the features of lipid domains *i.e.* rafts are membrane parts that are resistant to cold detergent extraction (usually Triton X-100 but also other detergents are used - see below) [48, 55], which are enriched in cholesterol, glycosphingolipids and phospholipids with saturated acyl chains and are isolated by sucrose gradient centrifugation with the low density fraction. Apart from lipids that are characteristic to rafts, there is also a specific subset of proteins that localize preferentially to the rafts. These are the GPI-anchored proteins [11] and proteins attached to the membrane via its other lipid components [4, 20].

An enormous number of sophisticated techniques such as single particle tracking [21], single dye tracing [49], fluorescence microscopy [27], FRET [59], ESR [56], have been involved in studies aiming at the explanation of the function of raft components but also at the observation of the behavior of rafts *per se*. The picture that is emerging from all these studies shows rafts as membrane platforms that are spatio-temporally organizing the signaling events in the cell [22, 29]. The lipid domains are, however, also sites of various pathogen entries into the cell and some viruses are known to preferentially bud from the raft regions [4, 34].

In this paper we try to draw a picture of lipid rafts, present some evidence for their role in the cell, and show some problems that urgently need to be solved before one can clearly demonstrate a complete view on the raft concept.

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Abbreviations used in the text: GPI - glycosylphosphatidylinositol, FRET - fluorescence resonance energy transfer, ESR - electron spin resonance, PC - phosphatidylcholine, DOPC - dioleoylphosphatidylcholine, SM - sphingomyelin, Chol - cholesterol, l_d - liquid disordered state, l_o - liquid ordered state, DRM - detergent resistant membrane, IS - immunological synapse, TCR - T cell receptor, BCR - B cell receptor

Rafts - isolation artifact or natural necessity?

It is generally known that hydrated pure lipids may exist in different phases. The solid-like state (or gel phase) describes lipids that posses tightly packed lipid acyl chains, parallel to one another (all *trans* conformation), whereas the liquid disordered (l_d) state characterizes acyl chains (in *trans-gauche* conformation) with high mobility, tumbling around the axis perpendicular to the membrane surface. Even in artificial membrane both phases can coexist [19]. Between those two there is a middle phase. The so called liquid ordered (l_o) state describes lipids that are tightly packed but show rather high mobility [15]. Many experiments demonstrate that the l_o state is crucial for raft existence [13, 47]. Certain lipids have the propensity to associate with one another, therefore abolishing the l_d state in model membranes, including liposomes [31]. Sphingolipids, cholesterol and saturated glycerophospholipids can easily associate forming domains [47]. Sphingolipids contain mostly long saturated acyl chains, which allow them to pack tightly together - a property that makes their T_m (melting temperature) higher compared to glycerophospholipids, which contain more unsaturated acyl chains. Sphingolipids would normally exist in a gel-phase, but the presence of cholesterol prevents them from entering the gel-phase, changing it instead into the l_o state. The l_d phase is usually abolished in favor to the l_o phase if the content of cholesterol reaches ~30 mol% [15]. And so, vesicles composed of DOPC:SM:Chol undergo phase separations, exhibiting the coexistence of l_o and l_d phases at varying compositions [3]. It is then likely that the l_o phases exist in a cell membrane with a sufficient content of cholesterol and sphingolipids [6]. The l_o state is thought to form discrete microdomains (rafts) interspersed in the continuous l_d phase.

Rafts are thought to be thicker than the rest of the membrane. This is due to the presence of sphingomyelin. The molecule contains long sphingosine moiety and a long saturated fatty acid chain. Therefore SM:Chol patches are thought to be thicker than the surrounding lipid matrix that contains more unsaturated phospholipids. The X-ray diffraction shows that DRMs are ~0.9 nm (~30%) thicker than the rest of the bilayer [31].

Another relevant feature of SM:Chol bilayers is that they have a much larger area compressibility modulus than do unsaturated phosphatidylcholine bilayers [33]. Since the bilayer bending modulus is proportional to the compressibility modulus times the square of the bilayer thickness [12], the bending modulus of SM:Chol bilayer would also be larger than that of unsaturated PC membrane. Therefore, to accommodate proteins or peptides of a given hydrophobic length it should take more energy to separate or deform adjacent lipid molecules in raft bilayers compared to non-raft regions with lower compressibility and bending modulus. This implies that for a given extent of hydrophobic mismatch, the l_o SM:Chol bilayer would provide a more energetically unfavorable enviroment for a protein than would a l_d phospholipid bilayer [31].

Some facts, however, put doubts about the raft theory - the presence of Triton X-100 can induce or even promote domain formation even in an initially homogenous, fluid PC:SM:Chol membrane and it is also possible to isolate raft domains from raft-free membranes [13]. One immediately asks a question whether rafts really exists in living cell membranes, or whether they are just an artifact created during extraction.

It has been suggested [47] that DRMs isolated from living cell membranes arise from native raft regions. Therefore many groups either use detergents other than Triton [48] or extract raft domains without detergents [10] in identifying the composition of natural rafts. These studies showed that DRMs vary depending on the way of extraction not only in the quantity of extracted proteins but also in the quality. Detergent is said to disrupt most of the lipid-lipid and lipid-protein interactions, solubilizing most of the membrane proteins. However, the l_o state is said to be sufficient to avoid detergent extraction [39]. The GPI-anchored proteins that are mostly thought to reside in natural raft domains were shown to resist the Triton X-100 extraction, due to the strong associations of their lipid tail with SM:Chol patches [47]. The difference in raft composition isolated with various detergents is suggested to be due to different association strength between protein and lipids. The ability to isolate a peptide with the DRM fraction means only that its interactions with the surrounding lipids were strong enough to resist the solubilization. A protein that is not present with the raft fraction may still be present in native raft regions, but its interactions are too weak to resist the solubilization process [48]. The distribution of cholesterol and peptides containing a single hydrophobic α-helix has been reported to be different at 37˚C and at 4˚C, a temperature at which most detergent solubilization experiments are performed on biological membranes [31]. It has been shown, however, that DRM solubilization proceeds upon heating and the membrane solubilization is expected to proceed upon cooling [13]. This indicates that detergent solubility experiments performed on biological membranes at low temperatures may not give a completely accurate insight into the concentrations of specific proteins and lipids in raft membranes at physiological temperatures [31]. It does not mean that rafts do not exist, but that some common assumptions are likely to be wrong.

When a raft becomes a platform

Rafts in biological bilayers containing a large variety of molecules [22, 32, 62] should not be considered as stable areas, the size and number of which depends only on the

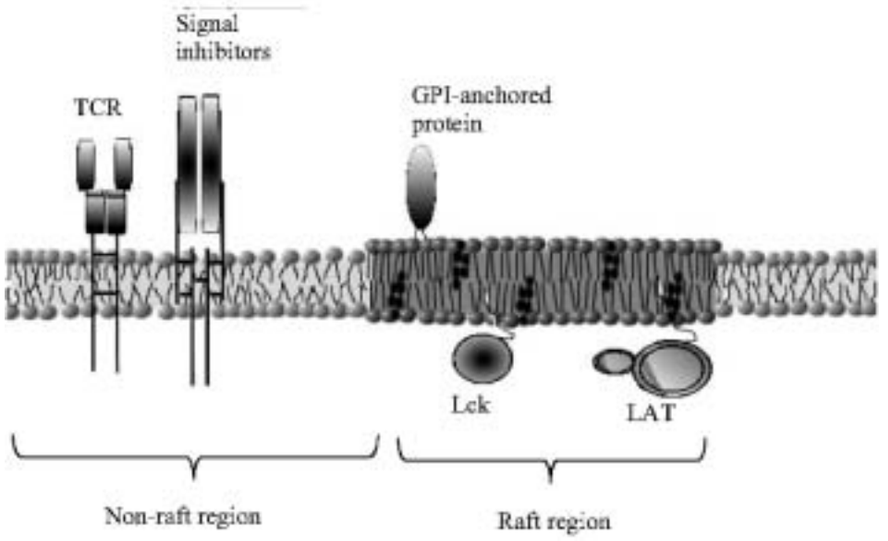

Fig. 1. Model of protein organisation in the cell membrane of resting T cell. TCR is localized outside lipid microdomains in the vicinity of negative regulators of signal transduction (CD43/CD45 etc.), whereas the signal propagators (LAT, Lck, CD28 etc.) are located in the raft region of the cell membrane. In this way rafts act as sites for the spatio-temporal organization of molecules engaged in signaling. The raft region is probably thicker than the rest of the membrane, containing most of the GPI-anchored proteins. The picture is highly schematic, therefore the relation of size of lipids and proteins are neglected.

SM and Cholesterol content and the temperature. Instead, they form and disappear, grow or shrink and cluster or break up triggered by small amounts of other compounds or other effect such as structural changes of proteins interfering with lipid packaging [13].

In T cells, TCR and negative regulators of signal transduction like CD43 and CD45 reside outside lipid microdomains [7, 16, 42], whereas the positive signal transducers like LAT, Fyn, Lck and CD48, all localize preferentially to raft regions in the membrane [7, 65] (Fig. 1). Such localization of proteins is normally observed in resting cells and prevents unnecessary signaling. Upon stimulation, the small dynamic rafts aggregate into bigger platforms sustaining signal transduction [17, 38, 61]. As the small dynamic microdomains coalesce into bigger ones, new signal propagator proteins are recruited to raft regions, forming the so-called Immunological Synapse (IS) (reviewed in [29]), a domain that may be observable even under light microscope. The redistribution of several receptors into IS is correlated with their gained resistance to solubility in Triton solutions [57]. All those changes lead to the creation of a signalling center which "informs" the cell about the contact it has been engaged into. The cell response depends on which costimulators (apart from the TCR) have been cross-linked, *e.g.* CD28/TCR or CD48/TCR costimulation leads to raft coalescence [38], while CD45/TCR cross-linking strongly downregulates the signaling [17]. Protein-protein and protein lipid interactions which are strongly enhanced upon raft aggregation act as an impediment to the mobility of raft localized molecules [57].

The recruitment of signal transduction regulators to rafts is not only restricted to T cells, although it is most widely studied. The critical signal-competent Lyn (an essential protein tyrosine kinase in T lymphocytes) is constitutively expressed and resides in raft region, but the BCR relocates to lipid rafts only upon antigen cross-linking [20]. Other signal propagators accompany the same domain after they are phosphorylated [8]. Activation of the IGF-I also leads to translocation in the lipid rafts [26] and the Fas-induced apoptosis leads to extensive raft reconstruction [51].

The presence of some proteins inside and others outside rafts has its implications on the cell itself. Rafts are said to constitute about 4% of the total membrane proteins [44]. The close proximity of proteins residing in rafts allows their interactions. Membranes contain both receptors for ligands and the regulators for signal transduction. It seems very useful for the cell to contain both receptors and regulators in the membrane in close proximity in order to quickly respond to the incoming signal. But the true masterpiece is to separate one from another or at least to put the receptor in the vicinity of negative regulators only as long as no outside signal reaches the cell membrane. Then those proteins that were normally excluded from raft regions would be recruited into rafts in order to transduce a signal into the cell. The small size of rafts in resting cells may suggest that each contains only a few proteins randomly distributed between different rafts. This would explain the reasons for the formation of IS in T cells [17]. Although this hypothesis is very attractive, further studies are needed to confirm it. Also, the molecular mechanism responsible for the rearrangement of small domains into big IS needs further studies.

Rafts - gates into the cell

The presence of so many different molecules that participate in signaling processes highlights the great importance of rafts for the cell. However, different

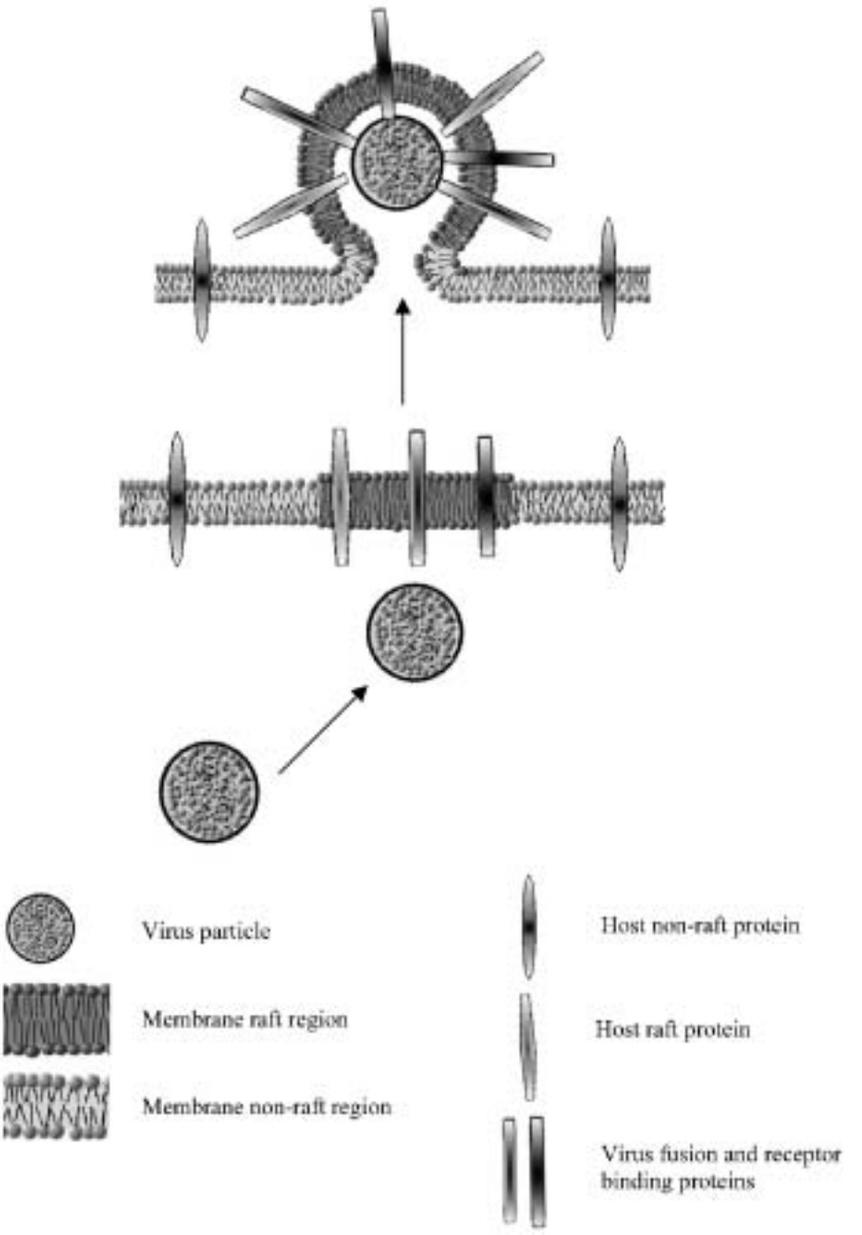

Fig. 2. The viral proteins are preferentially localized in the raft region of the host cell. The complete virus buds from this region of plasma membrane. Due to the very small area of the raft and the proximity of viral proteins in it, the chances for budding a complete virus increase. During the budding process the virion is encapsulated by plasma membrane with incorporated host cell proteins, which may act as a sort of camouflage against immune system. The picture is highly schematic, therefore the relations of size of lipids and proteins are neglected.

receptors that are present in raft regions also play a role in some pathogen entries. Both CD4 and CCR5 were found to associate with the raft region [28, 63]. For example, HIV-1 enters the cell by binding to CD4 [25], whereas CCR5 strongly promotes the virus entry [9]. A similar situation concerns Coxackievirus A9. The MHC class I, which is required for virus internalization [60], and the integrin $\alpha v\beta 3$, which is a coreceptor, both reside in DRMs [59]. But it is not only viruses that use rafts for their entry. The raft disruption by methyl-β-cyclodextrin, a potent cholesterol binding molecule both on erythrocyte membrane and parasite vacuolar membrane, inhibits *Plasmodium falciparum* infection of the erythrocytes [44]. Some of the GPI-anchored proteins (alkaline phosphatase, carboxypeptidase M) associated with DRMs have been identified, and it has been suggested that rafts function by concentrating parasite ligands for interaction with erythrocyte receptors, thereby speeding up the binding process and thus increasing the binding avidity [51].

The doors that lead into the cell, lead also out of it. The HIV proteins co-localize with the DRM's markers and HIV-1 virions, although possessing many proteins and lipids of host cells, lack CD45, a protein that covers

from 10% to 25% of lymphocyte surface [2]. It has been proved that this characteristic feature is due to the fact that HIV-1 proteins are preferentially targeted to lipid rafts and that the virus assembly and budding proceed in raft regions [34]. HIV-1 is not the only virus to use rafts for those processes. Filoviruses, Ebola and Marburg are two of the most deadly viruses. It has been demonstrated that their envelopes incorporate raft-associated GM1 and exclude transferrin receptor, a protein that is commonly used as non-raft marker [4]. Also the influenza virus hemagglutinin preferentially localizes to lipid rafts [45]. It does not mean, however, that all viruses use lipid rafts for their assembly and budding processes. The VSV (vesicular stomatitis virus) and SFV (Semliki Forest virus) do not posses high amounts of detergent-insoluble complexes in their envelopes [46].

The process of virion assembly and budding from the plasma membrane is quite complicated and requires that viral nucleocapsid, matrix and glycoprotein envelope are put together in an orchestrated manner. Thus, the compartmentalization of the process in a special membrane microdomain may provide the required coordination and may increase the virus budding efficiency and decrease the release of defective non-infectious particles [4]. The presence of several receptors, normally occurring in lipid rafts, in the viral envelopes may act as a camouflage for the host immunity system, but also may efficiently increase the ability to infect new cells because of the enrichment of virus envelope with certain adhesion molecules [59].

Sorting and trafficking - how does a protein know where to go?

The association of raft proteins with raft regions usually takes place in the Golgi apparatus [55]. The GPI anchor as well as palmitoylation of proteins seems to be a hallmark of raft-associated proteins. The acyl chains of GPI anchor of proteins are largely saturated [30]. It is proposed, then, that such GPI anchors are sufficient for l_o domains localization [47]. Also the studies on some of the Ebola virus glycoproteins that were mutated at specific cysteine residues (the putative palmitoylation sites) showed that the proteins that normally participate in DRMs failed to localize to raft regions [4]. N-terminal mirystoylation is required for Lyn kinase anchoring to the plasma membrane and raft partitioning [20].

Another feature that has been proposed as a regulator of protein sorting between detergent-soluble and detergent-insoluble membrane regions is the bilayer thickness [53]. The DRMs are supposed to be thicker than the non-raft regions, therefore they would incorporate proteins with relatively longer transmembrane domains. Some experiments with synthetic transbilayer peptides supported this hypothesis [31].

Rafts play also important role in apical trafficking of proteins in polarized cells. The transport of proteins to the apical membrane may occur directly from the TGN (Trans Golgi Network) or indirectly, by sending the protein first to the basolateral membrane and then *via* transcytosis to the apical compartment. The cholesterol depletion in HepG2 cells has been shown to strongly affect trafficking, causing mislocalization of newly synthesized MDR1 protein to the basolateral surface [55].

Caveolae are flask-shaped rafts that are known to operate as transcytotic carriers [35]. The cholesterol depletion has been shown to affect caveolae causing gradual disappearance of these structures and diffusion of caveolae-associated proteins [58].

Cytoskeleton - raft's anchor

Even at the time the Singer's and Nicolson's model was published [54], the problem of non-uniform lateral organisation of the biological membrane was known; *e.g.* large membrane domains such as mitochondrial cytochrome oxidase or purple membrane of *Halobacterium halobium*. Restriction of integral membrane protein mobility by membrane skeletal or cytoskeletal elements suggested by the authors of the model and many other researchers [18, 36, 37] led Sheetz to propose "corrals" (membrane skeleton) and "fence posts" (transmembrane proteins associated with skeletal elements) as interpretation of membrane lateral organization [52]. This model was further developed by others (reviewed in [40, 41]). The "fence posts" which are the immobilized by membrane skeleton proteins contain lipid of restricted mobility as well, therefore they could function as the organizing centres of at least one kind of protein-lipid rafts. Indeed, Nebl *et al.* [32] isolated from neutrophil plasma membrane so-called DRM-H which is rich in membrane skeleton components.

Membrane rafts which are involved in so many different cellular functions and processes such as signaling, protein transport, cell adhesion and movement need to be connected with the cytoskeleton. Raft proteomic analysis reveals that many proteins of membrane cytoskeleton co-isolate with DRM fraction. Raft-associated cytoskeletal proteins from Jurkat cells and neutrophils show many similarities. Fodrin (non-erythroid spectrin), actin, myosin IIa, supervillin, flotillin are just a subset of those [32, 62]. Also, the DRMs isolated from erythrocytes are enriched in those proteins [43].

The formation of immunological synapse seems crucial for the signal sustaining and propagation [17, 38]. Although the molecular basis of synapse formation remains to be elucidated, the role of cytoskeleton cannot be omitted when discussing dynamic changes in rafts (reviewed in [50]). The coalescence of small patches and the formation of immunological synapse require dynamic membrane changes and therefore involvement of

the membrane skeleton [61]. DRMs in Jurkat cells have been shown to include many cytoskeleton-associated proteins [62] which may be involved in the IS formation and preservation of its integrity. The proposed cytoskeletal reorganization coordinated with raft clustering could be responsible for the mobility restriction of molecules localized to aggregated rafts [57]. Some evidence is brought by the fact that EBP50, an ERM family protein that actively binds to actin cytoskeleton, is also a binding partner for the PAG protein (raft-associated protein). This interaction seems to be crucial in connecting membrane rafts to actin cytoskeleton, which in turn may be essential for re-distribution of rafts during immunoreceptor signaling [5].

The transmembrane CD99 molecule is abundantly present on plasma membrane of T cells. CD99 appeared to be incorporated into lipid rafts in a regulated manner. It has been shown that upon engagement of CD99, the molecule becomes associated with the cytoskeleton as well as lipid rafts. After the engagement, CD99 elicits export of several transmembrane proteins and GM1 and the association of CD99 with the cytoskeletal compartment occurs in a lipid-dependent manner [64]. In neuronal cells, all major NCAMs promote incorporation of spectrin into DRMs. It has been demonstrated that spectrin-mediated coordination between NCAM and $PKC\beta_2$ is required to trigger NCAM-mediated neurite outgrowth [23]. It has also been demonstrated that lipid rafts are intrinsically linked to cytoskeleton and it may be hypothesized whether cytoskeleton molecules are required for the stabilization and/or localization of rafts within the membrane [32]. However, some of the lipid-binding proteins (annexins, proteins containing pleckstrin homology domain) have been suggested to act as a linker between the rafts and the cytoskeleton and contribute for the raft formation and stabilization [1].

Questions to be answered

The interest in rafts has put new energy into a broad range of research fields such as cell biology, membrane biophysics or signal transduction. And as the raft hypothesis is enriched with new exciting data, each answer results in the appearance of several new problems. Those that are recently under investigation include:

- The three-dimensional structure of lipid rafts. The specific lipid-lipid and lipid-protein interactions are still one of the most urgent problems when discussing rafts. The problem seems even more complicated in view of the recently demonstrated lack of specific interactions between cholesterol and sphingomyelin [14]. It would be also of great interest to obtain a clear picture of the cytoplasmic face of the rafts.
- The molecular mechanism determining the stability and size of lipid rafts is still unknown. What is the role of membrane skeleton in localization of raft domains within the biological membranes?
- What are the exact relationships between lipid rafts, caveolae and smooth invaginations free of caveolin and clathrin? Are there different subclasses of rafts?

Although much has already been said, and a lot has been discovered, no one can assure that rafts really exist. Anyway, they are still an exciting phenomenon to investigate.

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Received: September 28, 2004
Accepted after revision: November 25, 2004