

# Regulatory Aspects of Membrane Microdomain (Raft) Dynamics in Live Cells

*A Biophysical Approach*

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János Matkó and János Szöllösi

## 1. INTRODUCTION

Most vertebrate cells display a considerable microheterogeneity in their plasma membranes, often termed microdomain structure. Some of these microdomains are enriched in glycosphingolipids and cholesterol and are resistant to solubilization with nonionic detergents; they are therefore called detergent-insoluble-glycolipid enriched membrane (DIG) or glycosphingolipid enriched membrane (GEM). These domains, also called “lipid rafts” (Simons and Ikonen, 1997), may form at the plasma membrane (PM) upon external stimuli or may be present in a preassembled form upon vesicular traffic to and fusion with the PM (Simons and Ikonen, 1997; Brown and Rose, 1992). We consider lipid rafts as transient molecular associations between lipid and protein components of the PM, providing a dynamic patchiness and local order in the fluid mosaic membrane (Edidin, 2001). Although the microdomain concept is widely accepted, and the existence of rafts has been confirmed by many lines of experimental evidence (e.g., biochemical data on detergent resistance, resolving membrane patchiness by high-resolution fluorescence and electron microscopies, tracking by videomicroscopy the lipid and protein motions in the membrane, etc.), some basic questions about the microdomains still remain open or highly controversial.

Membrane microdomains are defined in several distinct ways depending on the approach used for their detection: (1) A chemical definition is based on resistance to solubilization with cold nonionic detergents (Triton-X100, Brij, Chaps, and so on), and the microdomains’ composition is usually analyzed by isolating these floating, “light buoyant density,” detergent-resis-

tant membrane fractions (DRMs) with sucrose-gradient ultracentrifugation of cell lysates and subsequent sodium dodecyl sulfate (SDS) gel electrophoresis/immunoblotting (Brown and Rose, 1992; Ilanguamaran et al., 1999); (2) as clusters of proteins and/or lipids detected by optical or electron microscopic techniques, such as confocal laser scanning microscopy (CLSM), fluorescence resonance energy transfer (FRET) microscopy, atomic force microscopy (AFM), or transmission electron microscopy based on immunogold labeling (TEM) on intact cells (Hwang et al., 1998; Vereb et al., 2000); and (3) some microdomains may arise from specific constraints to the lateral movements of diffusible membrane proteins as revealed by tracking molecular motions with videomicroscopy (Saxton and Jacobson, 1997). This confinement may arise from lipid–lipid or lipid–protein interactions (Anderson and Jacobson, 2002), as well as from an “anchoring/capturing” effect of the membrane skeleton (Kusumi and Sako, 1996).

Thus the first important question, arising from the diversity of definitions, is whether the microdomains, detected and defined in the different ways described above, match each other, and if so, to what extent. Second, all the approaches suffer from some specific uncertainties: either due to DRM isolation circumstances (high detergent concentration, cell lysis) or due to the characteristics of the applied labels (antibodies, antibody-coated gold beads), which may be too large in size, or which may initiate protein or lipid crosslinking, thereby perturbing the *in situ* membrane organization. Therefore, circumstances of testing affect both the size and stability of lipid rafts expressed *in situ* at the cell surface and how stable they are. Drawing conclusions from the recent achievements of biophysical approaches, Edidin pointed out in a recent review (Edidin, 2001) that “the domains are now thought to be smaller and less stable” than a decade ago.

In the present overview biophysical aspects of the above questions are discussed with special attention to raft dynamics. The biological and physicochemical factors controlling raft dynamics in cells as well as in specialized, complex biological structures (e.g., the immunological synapse) are also covered.

## **2. BIOPHYSICAL METHODOLOGY TO DETECT AND CHARACTERIZE RAFT MICRODOMAINS: ACHIEVEMENTS, LIMITATIONS, AND CHALLENGES**

Biochemically, detergent insolubility is the main criterion for defining raft domains in the membrane. In fact, the definition of a raft-constituent molecule is that it is recovered in the low-density fraction after cold Triton extraction and sucrose density-gradient centrifugation. This low-density

fraction is the so-called detergent-resistant membrane fraction (Brown and London, 1998). However, such biochemical approaches have provided limited information on the rafts and the mechanism by which they function in the membranes of live cells; researchers would want to know the morphology, lifetime, molecular organization, and dynamics of the raft-constituent molecules and the raft itself in the membrane. In addition, biochemical methods have faced several serious concerns about the capability of detergent extraction to identify *in situ* composition of lipid domains existing in intact cells.

An important question has been whether these domains exist in the plasma membrane prior to detergent extraction, or are created by the extraction procedure itself. This question has not yet been answered convincingly, although several *in vitro* reconstitution studies with model membranes indicate that the extraction itself does not cause significant artificial perturbation of the domain composition (Ahmed et al., 1997; Schroeder et al., 1998). However, problems can arise from the concentration and the composition of the applied detergent. Association of certain membrane proteins with DRM may depend on the concentration and the type of the detergent (Ilangumaran et al., 1999).

Chemical crosslinking was used to show that membrane microdomains of GPI-anchored proteins exist at the cell surface. Clustering of GPI-anchored protein was specific because it was sensitive to cholesterol extraction and did not occur for transmembrane versions of the respective model proteins. In living cells, these GPI-anchored proteins were found to reside in microdomains consisting of at least 15 molecules, which are much smaller than those seen after detergent extraction (Friedrichson and Kurzchalia, 1998).

Results of various biochemical and biophysical studies performed on model membranes have supported the lipid raft theory. A detergent-insoluble fraction was isolated from model membranes with a composition that crudely mimicked that of plasma membranes. This fraction resembled DRM fractions from cells in terms of lipid composition and GPI-anchored protein binding (Schroeder et al., 1994). Similar to liquid ordered-state bilayers, this fraction was enriched in cholesterol and lipid with saturated acyl chains (e.g., sphingolipids). In addition, liquid-ordered DPPC/cholesterol bilayers were found to be detergent insoluble; therefore, it was proposed that lipid rafts are domains of ordered lipid (Schroeder et al., 1994). It should be emphasized, however, that while segregated lipid phases (domains, rafts) are equilibrium structures, membranes of living cells are far from equilibrium. Domains in native membranes are likely to be transient, nonequilibrium structures. Keeping this fact in mind, results of biochemical and biophysical studies performed on model membranes still provide useful information about the structure and dynamics of lipid rafts.

In contrast to biochemical approaches, biophysical studies can be performed on model membranes and on biological membranes so that the structure of the rafts can be studied *in situ*. In addition, depending on the temporal resolution of the biophysical technique, the dynamics of the rafts can also be followed on various time scales. Characterization of membrane rafts depends entirely on the spatial, temporal, and chemical resolution of the method used to detect the rafts. Different methods can give different pictures of the same domain and can be blind to other aspects of raft properties. These biophysical approaches comprise various spectroscopic techniques: electron paramagnetic resonance (EPR), nuclear magnetic resonance (NMR), fluorescence quenching, classical and advanced microscopic techniques (fluorescence microscopy, fluorescence resonance energy transfer microscopy), single-particle tracking (SPT), single-dye tracing (SDT), atomic force microscopy (AFM), optical trapping, and fluorescence correlation spectroscopy (FCS).

The information gained by conventional EPR spectroscopy is based on the reorientational diffusion of the nitroxide moiety placed on lipid probes in the membrane, and is limited to the events that take place in time scales shorter than 10 ns. On this nanosecond timescale, molecules in the microdomain of boundary lipid—that is, lipids adjacent to the transmembrane portion of an integral membrane protein—seem to be immobilized by the protein (Jost et al., 1973). By contrast, NMR measurements, which have a timescale of microseconds, give a different picture of boundary lipids as a layer of mobile and highly disordered molecules (Jost and Griffith, 1980). Similar to NMR, membrane dynamics in longer time-space scale (1–10  $\mu$ s) can be observed in pulse EPR experiments in which the spin-lattice relaxation times ( $T_1$ s) of the nitroxide groups are measured. Using dual-probe pulse EPR, Kusumi et al. were able to determine that tiny, cholesterol-rich domains (containing several molecules) may be continuously forming and dispersing with a lifetime on the order of 1–100 ns in artificial unsaturated phosphatidylcholine-cholesterol bilayers. (Subczynski and Kusumi, 2003).

Fluorescence quenching studies in model membranes have demonstrated that tight packing between sterols and sphingolipids is the driving force for raft formation. In this method, short-range quenchers are used to abolish the fluorescence of directly neighboring fluorescent molecules. Quenchers attached to lipids can detect domains in intact bilayers because the fluorescence intensity of an appropriate membrane-bound fluorescent probe placed in a bilayer containing a random mixture of quencher-containing lipids is quite different from the intensity obtained when the same fluorescent probe is incorporated into a bilayer containing coexisting quencher-enriched and quencher-depleted domains (London, 2002). The short range is sensitive to very small domains, as small as 25–50 lipids. Unfortunately, short-range

quenching requires relatively high levels of quencher-bearing lipid, which limits the types of lipid mixtures that can be investigated and makes it difficult to adopt quenching to studies of cells because such quencher lipids cannot be introduced into cells at high levels.

In contrast to these dynamic methods (EPR, short-range quenching), fluorescence resonance energy transfer (FRET) can sense only somewhat larger domains, and fluorescence microscopy can detect only relatively large domains. FRET microscopy is a very selective and sensitive tool for resolving spatial heterogeneity of molecular interactions within single cells, with a spatial resolution imposed by the inherent diffraction limit of optical microscopy.

FRET microscopy applied to test the distribution of GPI-anchored proteins in lipid rafts has produced controversial results. In one study FRET efficiency measured between donor and acceptor labeled 5'-nucleotidase correlated strongly with the surface density of the acceptor, suggesting that 5'-nucleotidase is at least partially randomly distributed and not constitutively clustered. (Kenworthy and Edidin, 1998).

In another study, the FRET efficiency between fluorescently labeled GPI-anchored folate receptor isoform was density independent, suggesting sub-pixel domains at the surface of living cells. These domains were estimated to be <70 nm in diameter (Varma and Mayor, 1998). To resolve this discrepancy, an extended FRET study was performed using three GPI-anchored proteins and a glycosphingolipid. FRET was detected between glycosphingolipids labeled with the cholera toxin B subunit and GPI-anchored proteins labeled with monoclonal antibody, showing that these raft markers are in submicrometer proximity at the cell surface. However, FRET efficiency correlated with the surface density of lipid marker, suggesting that these proteins were not significantly clustered in microdomains. It was concluded that in plasma membranes, lipid rafts exist only as transiently stabilized structures, or if stable, comprise only a minor fraction of the cell surface (Kenworthy et al., 2000).

Raft localization of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fused to consensus sequences for acylation or prenylation was studied by measuring FRET between these fluorophores. The FRET data was analyzed using an approach that can distinguish FRET in a randomly distributed population of fluorophores from FRET among clustered fluorophores on the basis of the relationship between FRET efficiency and acceptor density. FRET measurements in living cells revealed that acyl but not prenyl modifications promote clustering in lipid rafts. The authors concluded that the nature of the lipid anchored on a protein is sufficient to determine submicroscopic localization within the plasma membrane (Zacharias

et al., 2002). It should be noted, however, that their data were extremely scattered and noisy, probably due to the fact that the version of the FRET method applied here, the so-called acceptor photobleaching method, is inherently error prone as was pointed out in a recent paper (Berney and Danuser, 2003).

Spatial organization and dynamic behavior of lipid domains can also be studied by single particle tracking (SPT). In SPT, nanometer-sized (15–40 nm) colloidal gold particles are coated with specific antibodies against membrane proteins or with ligands to receptor molecules, and then attached to a single (or a small number of) molecule(s). The gold-receptor complexes are monitored by contrast-enhanced video microscopy, and the movements of complexes can be followed with a nanometer-level precision (Sheets et al., 1997; Jacobson and Dietrich, 1999). SPT has shown that the cytoplasmic membrane is compartmentalized with regard to lateral diffusion of transmembrane proteins, and that many proteins undergo “intercompartmental hop” diffusion. These compartments are also termed transient confinement zones (Jacobson and Dietrich, 1999). SPT studies showed that around one third of Thy-1, a GPI-anchored protein, is confined to zones 200–300 nm in diameter (Sheets et al., 1997; Jacobson and Dietrich, 1999). The gold probes used for these SPT experiments may have slightly cross-linked the raft-prefering molecules, which enhances the appearance of the transient confinement zones.

In single-dye tracing (SDT), the lateral motion of single fluorescently labeled lipid molecules can be imaged in native cell membranes on a millisecond timescale with positional accuracy of around 50 nm. Schutz and coworkers applied analogous probe with saturated fatty acid chains to monitor lipid rafts and found that the size of these lipid domains ranged from 0.2 to 2  $\mu\text{m}$  (Schutz et al., 2000).

Using single-molecule techniques (both SPT at a 25  $\mu\text{s}$  resolution and SDT at video rate) the cell membrane movement of CD59, a GPI-anchored protein, was tracked and found almost identical to that of the dioleoyl-palmitoyl-phosphatidyl ethanolamine (DOPE), a typical nonraft phospholipid. Both diffused rapidly while confined in a 110-nm compartment. On average, each hopped to a new compartment every 25 ms. The nearly identical scales of confinement and hop frequencies indicate that CD59 can be associated only with small rafts with lifetimes as short as a few milliseconds (Subczynski and Kusumi, 2003).

Atomic force microscopy (AFM) is a promising approach for detecting rafts at higher resolution than optical microscopy; it is able to discriminate Angstrom-scale height differences between lipid domains. In this technique, a needle with a sharp tip is scanned over the membrane surface at a constant

pressing force, and the height of the needle is recorded at each position, thus generating an image of the terrain of the membrane surface. A recent AFM analysis of a supported bilayer showed partitioning of placental alkaline phosphatase—a GPI-anchored protein—into ordered membranes (Saslowsky et al., 2002). In addition, another AFM study on supported bilayers demonstrated thickening of bilayer upon formation of ordered domains (Rinia et al., 2000). AFM has also been applied to analyze in real time the effects of manipulating cholesterol levels in supported model membranes (Lawrence et al., 2003). Although AFM has superior resolution, the drawback of the approach is that it uses supporting bilayers, which may show perturbed behavior under some condition. (Rinia et al., 2000; Yuan and Johnston 2001; Yuan et al., 2002).

When AFM was applied to living cells, such as erythrocyte membranes, the rafts could not be analyzed because of the rough surface; however, the structure of the erythrocyte membrane skeleton was easily detected (Takeuchi et al., 1998). Protein aggregation in the cell surface can also be monitored by AFM. However, when cell surface distribution of specific membrane proteins was studied by AFM, mostly immunogold labeling was used to provide specificity. This approach was successfully applied to reveal distinct hierarchical levels in cell surface domains of class I and class II MHC molecules as well as the IL-2 receptors in intact lymphoid cells (Damjanovich et al., 1995; Vereb et al., 2000).

Optical trapping has also provided useful information about the local diffusion of single raft proteins, revealing the viscous drag imposed on these proteins. In this novel microscopic method, the proteins to be monitored are labeled with microspheres coated with monoclonal antibodies. The sphere is confined by a laser trap to a small area (diameter less than 100 nm), and the sphere's thermal position fluctuations are tracked with subnanometer and microsecond resolution. Applying this method, researchers found that the viscous drag of raft-associated proteins was independent of the type of anchor and was significantly larger than that of nonraft proteins. The mean radius of the rafts observed on immunocompetent cells was estimated at around 26 nm (Pralle et al., 2000).

Fluorescence correlation spectroscopy (FCS) gives information about the lateral diffusion coefficient as well as the absolute number of molecules that are diffusing. FCS is based on time correlation of temporal fluorescence fluctuation detected in the very small focal volume, which is governed by the dynamic parameters of the system. The power of FCS results from single-molecule sensitivity and the capability of exploring a wide range of dynamic events with high temporal resolution and good statistical accuracy (Korlach et al., 1999; Kahya et al., 2003). Using giant unilamellar vesicles, Kahya



and coworkers demonstrated that cholesterol plays an important role in promoting raft formation and, most importantly, in tuning membrane lipid motility. With the help of FCS the researchers were also able to obtain information about the composition of lipid rafts, allowing mapping of phase diagrams entirely based on dynamic parameters. (Kahya et al., 2003).

### 3. HOW LARGE AND DYNAMIC ARE THE RAFTS?

Several types of specialized sites exist in the plasma membrane of eukaryotic cells that are necessary for proper functioning of the cells. These membrane domains include micrometer-sized domains such as adherens junctions and focal adhesions, 100-nm domains of coated pits and synapses, and also very small complexes like receptor clusters and associated lipids.

Clathrin-coated pits can be identified in thin-section electron micrographs by the presence of a cytoplasmic fuzzy coat and so are easily distinguishable from the surrounding membrane (Kirchhausen, 1999). Although the clathrin-coated pits appear to be large (100 nm) and stable in electron micrographs, their formation and internalization takes several tens of seconds (Gaidarov et al., 1999).

A caveola is a membrane invagination on the surface of endothelial cells. Its membrane coat is composed of caveolin-1, and it contains cholesterol around the rim of the domain. The lower size limit appears to be the diameter of a flask-shaped caveola (50–80 nm). The upper limit is more variable; it ranges from 150 nm to several micrometers in special occasions. (Rothberg et al., 1992)

Noncaveolar lipid rafts are dynamic structures with molecules entering and leaving according to specific rules. Cholesterol-sphingolipid-rich lipid domains must have unique physical features, upper and lower size limits, and functionality, and they represent a system for removing and adding specific molecules.

As mentioned in the previous section, various biochemical and biophysical approaches have resulted in a wide range in the size of the lipid rafts, and no clear consensus about the size, shape and location has emerged (Table 1). Chemical crosslinking has suggested 15 molecules as the rafts' size. Similarly, pulse ESR studies predicted the existence of tiny, cholesterol-rich domains containing only several molecules. According to the short-range fluorescence quenching studies, 25–50 lipid molecules can form functional domains. These approaches predicted very small-sized and very dynamic rafts, whose lifetime ranges from nanoseconds to milliseconds. The size of the lipid raft can be 50–70 nm according to laser trapping and FRET measurement. The transient confinement zone ranges from 110 to 300 nm as



**Table 1**

**Size Distribution of Lipid Rafts as Revealed by Various Biochemical and Biophysical Approaches**

Object	Membrane	Method	Size (nm)	Refs.
GPI-anchored proteins (GH-DAF*)	Cell membrane	Chemical crosslinking	> 4 nm	Friedrichson and Kurzchalia, 1998
Lipids (CSL)	Model bilayer	Pulse EPR	> 5 nm	Jost et al., 1973; Subczynski and Kusumi, 2003
Lipids (Brominated PCs)	Model bilayer	Short-range quenching	> 5 nm	London, 2002
GPI-anchored proteins (5'NT, FR, CD59)	Cell membrane	FRET, classical	0 nm (?)	Kenworthy and Edidin, 1998;
GPI-anchored proteins (FR)	Cell membrane	FRET, anisotropy based	70 nm	Kenworthy et al, 2000 Varma and Mayor, 1998
GPI-anchored proteins (CD59, Thy-1)	Cell membrane	SPT	200–300 nm	Sheets et al., 1997; Jacobson and Dietrich, 1999; Subczynski and Kusumi, 2003
TM proteins (MHC-I)			approx 300 nm	Edidin, 2001
GPI-anchored proteins, (CD59)	Cell membrane	SDT	110 nm	Subczynski and Kusumi, 2003
Lipids (DMPE)			200–2000 nm	Schutz et al., 2000
GPI-anchored proteins (PALP), Lipids (GM1)	Model bilayer	AFM	40–100	Rinia et al.,2000; Yuan and Johnston, 2001;
GPI-anchored proteins (PALP)	Cell membrane	Optical trapping	26 nm	Yuan et al., 2002; Lawrence et al., 2003 Pralle et al., 2000
GM1	Cell membrane	Confocal microscopy	300–1500 nm	Vereb et al., 2000

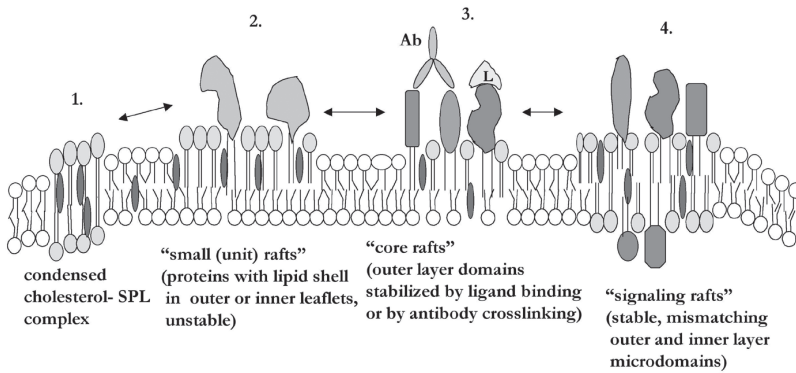
*Abbr:* GH-DAF; growth hormone-decay accelerating factor chimerae; PALP; placental alkaline phosphatase; CSL: cholesterol-type spin label; 5'NT: 5' nucleotidase; FR: folate receptor; PC: phosphatidyl choline; DMPE: 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine; MHC-I: major histocompatibility complex class I; EPR: electron paramagnetic resonance; FRET: fluorescence resonance energy transfer; SPT: single particle tracking; SDT: single dye tracing; AFM: atomic force microscopy.

revealed by SPT and SDT; the lifetime of this transient confinement zone appeared to be more than 200 ms. The size of the rafts detected by confocal microscopy ranges from 250 nm to some micrometers, and the lifetime of this giant lipid raft, which is probably made up of aggregates of originally small-sized rafts, can go up to several minutes. It is clear from these examples that as the virtual size of the lipid rafts increases, the lifetimes of these domains also increase, although this relationship is not a straightforward correlation. Stabilization of these giant lipid rafts is not caused solely by aggregation, but also by the rafts' interaction with cytoskeleton elements.

Subczynski and Kusumi have suggested a very attractive model for explaining the relationships between the small-sized and short-lived dynamic mini-rafts and the larger-sized, more stable functioning rafts and raft aggregates. They name their model the "thermo Lego model," emphasizing the importance of the thermal motion of the constituents in the lipid rafts.

Subczynski and Kusumi suggest that least three types of rafts may be present in the plasma membrane. The first type is a small, unstable kind of raft, which consists of only a few molecules and having a lifetime that may be shorter than 1 ms. The second type of raft is called a "core receptor raft," and it can be created by oligomer formation of GPI-anchored proteins or transmembrane receptors induced by ligand binding. These stabilized oligomers induce the small but stable rafts around them simply because of the slight reduction of thermal motion around the cluster and the subsequent assembly of cholesterol. The lifetime of the core receptor rafts can be in the order of minutes. The third type of raft forms around the core receptor rafts, and is called a "signaling raft," because it is generated by assembling signaling molecules through coalescence of small, unstable rafts containing signaling molecules. The core receptor rafts in the outer leaflet may recruit small, unstable rafts in the inner leaflet beneath them, although the mechanism for the coalescence between outer and inner rafts is not quite clear.

Subczynski and Kusumi hypothesize that transient confinement zones are representative of signaling rafts, because the transient confinement zones closely correlate with the downstream signaling events. The small, unstable rafts are like Lego blocks for building the signaling pathways, the signaling molecules being the central part of the Lego block to which cholesterol, and possibly saturated alkyl chains, are attached as pegs (connecting parts) of the Lego blocks (Fig.1). The advantage of the raft hypothesis is that because lipid interactions are used as the basic mechanism for assembling the rafts, the signaling platforms based on raft assembly could be very versatile, allowing for rapid switching of the downstream signaling pathways and various crosstalks, depending on the cellular environments and history (Subczynski and Kusumi, 2003).



**Fig. 1.** A schematic diagram of raft-dynamics in plasma membranes. Formation of raft domains in live cells likely has a complex molecular background: (1) condensed complexes of cholesterol and sphingolipids; (2) small preformed unit rafts in either the outer or inner leaflets (e.g., unstable GPI microdomains, or proteins with small lipid shells); (3) "core rafts" stabilized by different crosslinking agents (e.g., ligands, antibody, multivalent ligands); and (4) signaling rafts (with mismatched outer and inner layer microdomains) may coexist in the PM in a dynamic fashion (Anderson and Jacobson, 2003; Subczynski and Kusumi, 2003). Rapid conversion of these microdomain forms to each other is regulated by extracellular, membrane, and intracellular factors (e.g., the dynamic actin cytoskeletal machinery).

Another model that tries to encompass the great variety of lipid rafts in terms of size and lifetime in one working hypothesis is the "lipid shell" model (Anderson and Jacobson, 2002). Anderson and Jacobson have suggested that the light buoyant density of so-called raft proteins is caused by the fact that these proteins are encased in a shell of cholesterol and sphingolipid. The estimated diameter of a cholesterol-sphingolipid-rich shell containing 80 lipid molecules is around 7 nm. They suggest that lipid shells are thermodynamically stable structures that have an affinity for preexisting rafts. Hence, lipid shells target the protein they encase specifically to these membrane domains. Condensed complexes recruiting more and more proteins targeted by lipid shells may serve as a functioning raft in the plasma membrane (Anderson and Jacobson, 2002).

#### 4. MODULATION OF PLASMA MEMBRANE RAFTS: STRATEGIES TO STUDY RAFT FUNCTIONS

Since the acceptance of the microdomain concept, many efforts have been made to modulate structural integrity of these domains in model and live cell membranes in order to understand their functional role. Model mem-

brane studies suggested that the basic physicochemical factors inducing lipid segregation into microdomains are mainly the long and saturated fatty acid chains, the glycosylated headgroups of phospholipids, and the extended H-bond network between them (Masserini and Ravasi, 2001). Thus, sphingomyelin (SPM) and glycosphingolipids (GSL) are considered as the major lipid constituents of raft domains, segregating from glycerophospholipids containing unsaturated fatty acyl chains (Ahmed et al., 1997).

Many studies have shown that these lipid molecules are segregated into microdomains in model membranes, even in the absence of proteins (London, 2002). Although this issue is controversial, cholesterol is assumed to be a critical stabilizing component in several types of lipid microdomains, mainly for sterical reasons (Xu and London, 2000). Our current knowledge of the chemical composition and physical properties of raft domains has allowed development of several strategies (Table 2) to manipulate structural integrity of raft domains (Hooper, 1999).

#### ***4.1. Destabilization of Rafts by Manipulating the Membrane Cholesterol Level***

Cholesterol seems to play a pivotal role in stabilizing the SPM/GSL domains occurring at the cell surface, mostly because of favorable steric interaction (mismatch) with the long, saturated fatty chains of GSLs. Therefore, reversible cholesterol depletion or enrichment of the plasma membrane has proved to be a reasonable strategy for studying raft functions. Indeed, depletion of cholesterol by water-soluble methyl- $\beta$ -cyclodextrins (MBCDs) has resulted in microscopically observable dissolution of rafts and a subsequent malfunction of numerous signal transduction activities (Xavier et al., 1998; Ilangumaran et al., 1999; Vereb et al., 2000; Matkó et al., 2002). Interestingly, enrichment of the membrane with cholesterol also destabilized membrane rafts (Bodnar et al., 1996; Xu and London, 2000), similar to enrichment of the plasma membrane with polyunsaturated fatty acids (PUFA) (Stulnig et al., 1998). These effects are likely brought about by the immiscibility/excluded volume effects that arise between the newly incorporated lipid molecules and the raft constituents. Using MBCD derivatives to reversibly modulate membrane cholesterol level thus seems a convenient and reproducible tool for disrupting raft integrity without serious perturbations of the host membrane; however, a side effect of MBCD treatment, the release of some small cholesterol-binding proteins from the membrane, must be considered (Ilangumaran and Hoessli, 1998). In contrast, several other cholesterol-binding agents, such as the polyene compounds filipin or nystatin, have been found to seriously perturb the host membrane by forming large (20–25 nm) pore structures with the selectively bound cholesterol

**Table 2**

**Destabilization of Rafts by Manipulating Membrane Cholesterol and Sphingolipid Levels**

Agent	Effect	Refs.
Compactin Lovastatin	Inhibition of cellular cholesterol biosynthesis (inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase)	Rothberg et al., 1992; Taraboulos et al., 1995
Cholesterol oxidase (enzyme)	Oxidation of cellular cholesterol	Smart et al., 1994
Filipin, Nystatin (polyene antibiotics)	Selectively bind/sequester cholesterol upon incorporation into membranes	Bolard, 1986; Vereb et al., 2000
Methyl- $\beta$ -cyclodextrins [MBCD] (cyclic oligo-glucopyranosides)	Reversibly extracts cholesterol from membranes without incorporation (MBCD preloaded with cholesterol increases membrane cholesterol level)	Ilangumaran and Hoessli, 1988; Ilangumaran et al., 1999
Fumonisin B1 (mycotoxin)	Inhibitor of ceramide synthase	Stevens and Tang, 1997
D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol. HCl	Inhibition of glycosylceramide synthase	Sheets et al., 1997
ISP-1 (myriocin)	Inhibitor of serine Palmitoyl CoA transferase	Cutler et al., 2002 Cutler et al., 2003

molecules sequestered partly from the rafts (Bolard, 1986). Therefore, their application requires special care regarding the experimental conditions. The membrane cholesterol level can also be modulated by deprivation of cellular cholesterol with inhibition of the cholesterol biosynthesis or by cholesterol oxidation (Rothberg et al., 1992; Smart et al., 1994; Taraboulos et al., 1995).

#### ***4.2. Modulation of Raft Domains by Sphingolipid Deprivation***

Manipulation of the sphingolipid biosynthesis/homeostasis may also result in dissolution or disruption of raft microdomains (Sheets et al., 1997; Stevens and Tang, 1997). This modulation is performed mainly by selective inhibition of the synthesis of ceramides and glycosylceramides, basic building blocks of membrane SPM and glycolipids (Table 2). Cellular deprivation of these important lipid intermediates prevents formation and expression of raft microdomains at the cell membrane. Many biochemical and biophysical experiments have convincingly demonstrated so far that these membrane-manipulation strategies, when applied with special care on the problems outlined above, can be successfully used in studying the role of rafts in compartmentation of receptor subunits (Moran and Micelli, 1998; Kabouridis et al., 2000) or signaling molecules (Lin and Weiss, 2000; Alonso and Millan, 2001) in a variety of cell membranes.

### **5. RAFT DYNAMICS IN LIVE CELLS: REGULATION BY EXTERNAL, MEMBRANE, AND CYTOSOLIC FACTORS**

As demonstrated by studies on model membranes, the raft/nonraft structure of the lipid bilayer reflects its complex phase behavior. Raft domains represent a highly ordered liquid phase (Lo), while membrane regions enriched in other phosphoglycerides represent areas of loose lipid packing (Ld) (Brown and London, 1998). It has also been shown that in simple, two- or three-component model membranes, sphingomyelin (SM) requires cholesterol to form stable microdomains, while cerebroside-type glycolipids can spontaneously form ordered domains (Xu and London, 2000; London, 2002). In live cells, the complexity of lipid phases is certainly much higher, mainly due to the large variety (hundreds of combinations) of bulky headgroups and fatty acyl chains of lipid species and the existence of membrane proteins. Thus, we can assume that in live cells a variety of small microdomains with distinct compositions can coexist, reflecting heterogeneities not only in the physical properties but also in the chemical composition. This in turn may result in local segregation phenomena, such as the

selective partitioning of GPI-linked proteins (e.g., Thy-1) or GM1 gangliosides into ordered domains observed in supported bilayers (Dietrich et al., 2001).

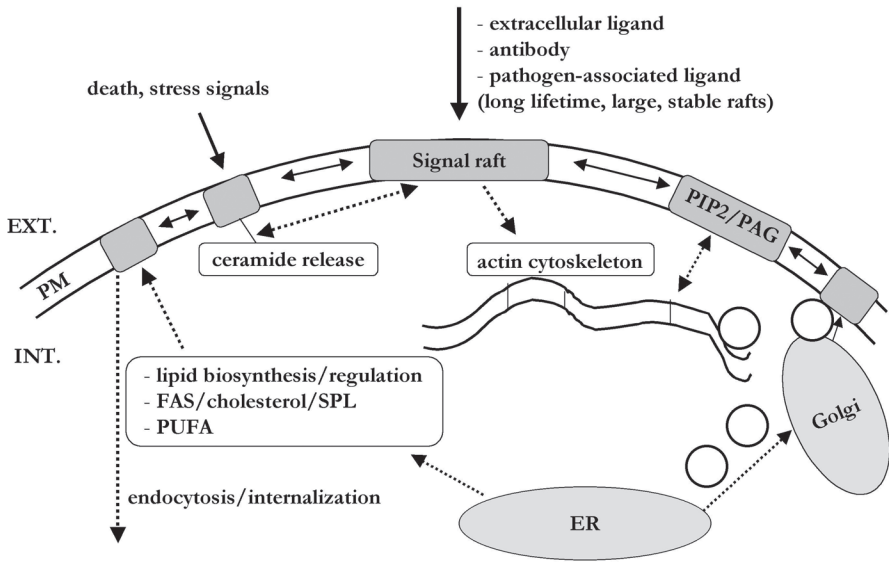
Biophysical studies on raft structure and dynamics (*see* Sections 2 and 3) have shown that lipid probes with saturated fatty acyl chains on average spend tens of milliseconds in one domain (Schutz et al., 2000), and the average lifetime range of stable domains is on the scale of tens of seconds (Dietrich et al., 2002). These observations, together with recent pulse EPR data on the dynamic exchange of lipids between protein-rich areas and the bulk lipid phases (Kawasaki et al., 2001), suggest that the raft domains expressed constitutively at the cell surface are much more dynamic and less stable than we thought earlier. Factors controlling their stability (Fig. 2) are currently under extensive investigation (Anderson and Jacobson, 2002; Harder, 2003).

### ***5.1. External Factors Controlling Raft Dynamics***

Most of the recent physical and functional analyses on rafts suggest that the small and dynamic rafts expressed at the cell surface are themselves unlikely to function as signaling platforms; rather, they are considered as small “preassembled molecular reservoirs” (“signal chips”) that can be modulated (through dynamic assembly and disassembly) via the signals received by the cells. Crosslinking of the lipid or protein constituents (e.g., gangliosides by cholera toxin B and proteins by antibodies) can selectively bring the components into large patches/microdomains observable with microscopic techniques, in support of this idea. Moreover, independently crosslinked DRM-associated molecules may coalesce into common patches, in a process that can be inhibited by cholesterol depletion (Harder et al., 1998). Interestingly, several nonraft proteins (e.g., the transferrin receptor) are always sharply separated from these patches. Therefore, as pointed out by Harder (2003), formation and modulation of microdomains with signaling capacity is likely controlled by an elastic raft/nonraft equilibrium of the plasma membrane constituents.

Thus, the natural antibodies and envelope proteins of viruses or other pathogens recognized by cell surface receptors (e.g., the Human Immunodeficiency Virus by CD4/ chemokine receptors) may all be considered as modulators of raft dynamics through dynamically crosslinking them. That several pathogens use lipid rafts of the target cell plasma membrane as a portal for entry raises another interesting and basic question. It is not clear whether preassembled raft/caveola domains on the host cells are targeted by viruses (or bacteria), or these domains are formed upon binding of pathogen proteins to their receptors, promoting assembly (coalescence) of the unit





**Fig. 2.** Regulation of raft dynamics in the plasma membrane. The dynamic and elastic raft/nonraft equilibrium in the plasma membrane is under a multifaceted regulation: Lipid biosynthesis (and its disorders/accomodations to enviromental signals) may alter lipid composition affecting lipid microdomain distribution or the amount of PM rafts (Section 5.2.). Death, stress, or inflammatory signals can generate membrane ceramide inducing coalescence of rafts, promoting diverse signal transduction processes (e.g. death signaling; Section 5.2.). Cellular signals promoting complexing of the membrane and actin cytoskeleton (involving *in situ* phoshoinositide expression and accumulation in rafts) may regulate (initiate) vesicle motility/ transport and spatial/functional polarization of cells (Section 5.3.). Many extracellular signals (engagement of receptors by ligands, binding of antibodies or pathogen-associated ligands to their receptors) may evoke raft coalescence and stabilization resulting in efficient signaling and/or internalization processes.

rafts. Nevertheless, it is worth noting that a paradoxical bacterial adhesin, FimH, has been recently recognized as a ligand for CD48, a GPI-anchored raft-resident membrane protein of host cells (Rosenberger et al., 2000; Shin et al., 2000). FimH-raft mediated entry of pathogens may represent a novel mechanism, accompanied with raft coalescence, for the pathogens' cellular uptake, allowing their survival (intracellular residence).

A recent flow cytometric analysis has clearly demonstrated that cell surface antigens can be directed into DRMs (rafts) by engagement with direct or indirect antibody labeling (Filatov et al., 2003). Several antigens (e.g., CD20, CD5, sIgM) were reported to translocate into raft domains in the absence of additional crosslinking, whereas others only in the presence of

additional crosslinking (by secondary antibodies). This situation usually mimicks the engagement of cell surface receptors by their extracellular ligands. However, whether ligation (or antibody binding) results in a real translocation (movement) of the protein into preassembled raft domains remains poorly defined.

An alternative hypothesis suggests that engagement of the transmembrane receptors may induce changes in their conformation/position in the membrane, such as changes in tilting or tension/length of the protein molecule (Harder, 2003). This change in turn may remarkably rearrange the immediate lipid microenvironment around the protein to optimize space filling and minimize interaction free energy, resulting in the formation of a raft-like, ordered environment. To decide between these possibilities, further studies on the receptor-ligation induced molecular rearrangements in the PM are necessary.

In addition to extracellular antibodies and soluble and pathogen-associated ligands, intercellular contacts through integrins and adhesion molecules (e.g., CD2/CD58, DC-Sign/ICAM-3 or ICAM-1/LFA-1 interactions) may also generate signals resulting in a dynamic rearrangement of raft domains at the cell surface. This process is of special importance in complex synaptic structures, like the neuronal or immunological synapses (Bromley et al., 1999) and it is discussed in Section 6.

## ***5.2. Control of Raft Dynamics at the Level of the Plasma Membrane***

Dynamics of raft/caveola microdomains within the cell membrane is possibly controlled by a large variety of mechanisms because of the enormous chemical complexity of plasma membranes. First, lipid biosynthesis and metabolism (especially pathological deficiencies in sphingolipid or cholesterol biosynthesis) and membrane-vesicle transport and sorting have a significant impact on formation and stability of domains, by influencing the balance of raft and nonraft lipid constituents. Recently, fatty acid synthase (FAS), a key metabolic enzyme catalyzing synthesis of long-chain saturated fatty acids, has been shown to be rate limiting in production of lipid molecules partitioning into TritonX-100 or Lubrol-resistant membrane microdomains. (The latter may serve as building units of different types of membrane protrusions.) In addition, it has also been pointed out that FAS overexpression is linked to dysregulation of the membrane composition and function in tumor cells (Swinnen et al., 2003).

Second, compared with most of the artificial model membranes, the existence and dynamic recycling of membrane proteins and the protein–lipid interactions further increase the complexity of PM in live cells. An interesting

computer simulation by Gheber and Edidin (1999) has shown that a combination of vesicle trafficking and dynamic barriers to lateral mobility may result in membrane patchiness that is expected to decay upon inhibition of vesicle traffic. Testing the predictions of their model, they analyzed membrane patchiness of class I HLA molecules (Gheber and Edidin, 1999; Tang and Edidin, 2001) by high-resolution scanning near-field optical microscopy (SNOM) or confocal fluorescence microscopy, and found that inhibition of endocytic vesicle traffic from the cell surface, using either hypotonic medium or expression of dominant negative dynamin, resulted in enlarging of HLA patches, while their intensities substantially decreased relative to control cells or cells recovered from the inhibitory treatment. Using selective photobleaching measurements and GPI-GFP or CD59-GFP constructs, Nichols et al. detected a rapid recycling of lipid raft markers between the cell surface (PM) and the Golgi, the mechanism of which (sequence of trafficking events) is currently still unclear (Nichols et al., 2001). This process was found to be raft specific, yet not all raft components follow this pathway. These observations suggest that the PM-Golgi pathway and, in general, the outward and inward vesicular traffic both may have a substantial regulatory influence on lipid raft distribution and function in cells.

A central question about the dynamic feature of membrane microdomains is how the different protein constituents become associated with the raft/caveola microdomains. Some structural motifs as prerequisites for constitutive raft-association of proteins have been recognized in the past few years, such as posttranslationally attached palmitoyl or myristoyl fatty acyl chains or the glycosylphosphatidylinositol (GPI) anchor (van Meer, 2002). It is still not known, however, how the few transmembrane (TM) proteins, found to be constitutively associated with rafts/DRMs and not bearing these directing moieties (e.g., interleukin-2 receptor subunits, CD40, CD44, and so on) accumulate in these microdomains. Except for the receptors extensively crosslinked by their ligands (e.g., B-cell receptor [BcR], FcεRI, or the pattern recognition receptors [PRR; toll-like receptor family] recognizing and binding pathogen surface patterns), many receptors' ligand-engagement induced "translocation" into raft domains still remains a highly controversial and unresolved question.

A very recent model for the molecular organization and dynamics of membrane microdomains, the so-called "lipid shell" model (Anderson and Jacobson, 2002), may partly answer some of these questions. Anderson and Jacobson, going back to the roots, emphasize that the physical, "light buoyant density" (LBD) property of proteins (utilized in isolation of raft/DRM fractions by sucrose density-gradient ultracentrifugation) may also be a critical factor in determining their association with raft/caveola domains.

Protein targeting theories can predict which proteins can accumulate in these microdomains. Several critical intracellular domain motifs (such as tyrosine residues and dileucine motifs) were identified in TM proteins as “molecular addresses” to lipid rafts (Schmid, 1997). However, the exceptions to this rule indicate that intramembrane domains, e.g., in CD40 (Bock and Gulbins, 2003), or the extracellular domains (with a yet unresolved mechanism) may also contribute as molecular addresses. In addition, it has been postulated that the LBD of proteins may target them into microdomains even in the absence of crosslinking.

A basically new viewpoint was introduced into microdomain dynamics by this model, assuming that the LBD of proteins is determined predominantly by their encasing in a lipid shell of sphingolipids and cholesterol. In support of this, the example of Thy-1, often considered as a raft-marker GPI-linked protein, offers a convincing argument. This protein has a density of 1.37 g/mL that would direct it in a sucrose gradient into a bottom fraction of approximately 38% sucrose. Instead, it is always detected in the light, floating fraction. The “lipid shell” model provides a plausible explanation for this apparent discrepancy: Assuming approximately 80 sphingolipid/cholesterol molecules (lipid density: 1.035) around Thy-1, and considering that the detergent molecules interacting with the lipid shell may further decrease the density, it is conceivable that Thy-1 becomes located in the floating fraction.

Concerning the raft dynamics in general, the “lipid shell” model came out with some flexible and widely applicable concepts. Lipid shells surrounding proteins need not show phase separation in PM, which is strongly questioned in live cells, but they can exist as thermodynamically stable structures with autonomic mobility (Anderson and Jacobson, 2003). Thus they may serve as small molecular reservoirs targeting proteins to either condensed sphingolipid/cholesterol complexes (Radhakrishnan et al., 2000) or constitutively expressed, preformed raft/caveola microdomains. This hypothesis, although many details are still waiting for clarification, seems to be consistent with different definitions of rafts, and it may also explain dynamic translocation of TM receptors into rafts upon engagement by ligands on the basis of conformational change-driven alterations in lipid shells.

Several “signal lipids,” such as the phosphoinositides (e.g., PI(4,5)P<sub>2</sub>) or ceramides were also reported to control membrane microdomain dynamics and membrane traffic/vesicle movements. Using GFP-based fluorescent chimera constructs, dynamic concentration gradients and segregated pools of phosphoinositides were detected inside the cells. Phosphoinositide pools transiently accumulated at the active site of phagocytosis/local exocytosis coinciding with PI-kinases and F-actin, consistent with their postulated func-

tion (Botelho et al., 2000; Martin, 2001). The PI(4,5)P<sub>2</sub> was found clustered in sphingolipid/cholesterol-rich raft-like domains lacking Src and caveolin, but dependent on cholesterol.

These phosphoinositide domains may recruit a number of PIP<sub>2</sub>-binding proteins and thereby transiently capture vesicles nearby the PM or induce signals for the accompanying cytoskeletal rearrangements. The PIP<sub>2</sub>-microdomains also exist on trafficking vesicles enriched in lipid raft components; thus these vesicles may be favored sites for actin comet-tail assembly and actin-mediated vesicle motility (Rosella et al., 2000). In general, the dynamic PIP<sub>2</sub>-rich membrane microdomains in the PM, in the *trans*-Golgi network, and in moving vesicles may represent structures critically determining the site where the membrane fission/fusion machineries and the cytoskeletal matrix can act in a coordinated fashion. Mechanistic details and coordination of these processes by a variety of potential PIP<sub>2</sub>-binding proteins still remain unresolved problems.

Sphingomyelin (SM), a phosphosphingolipid, is a membrane constituent critical from both structural and functional viewpoints. Up to 50% of the cellular SM is located in the PM and enriched in the outer leaflet. Together with glycolipids and cholesterol it is a major constituent of raft microdomains (up to 90% of membrane SM is associated with DRMs). Beyond being a structural building block of raft/caveola domains, SM may also serve as a source of signaling molecules for the so called sphingomyelin cycle and may also functionally interact with SM-binding proteins (Shakor et al., 2003). A number of extracellular stimuli (e.g., tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , interleukin-1, stress stimuli, or death signals mediated by Fas/CD95 and CD40 receptors) may activate through an enzymatic pathway the neutral or acidic sphingomyelinases (SMase), resulting in cleavage of SM to form ceramides. Ceramides may then serve intracellularly as multipotential second messengers (Shakor et al., 2003; van Blitterswijk et al., 2003), regulating different signal pathways, the cell-cycle, or initiating a mitochondria-dependent "phospholipid" apoptosis signal pathway.

Ceramides, besides deciding about "death or survival," also have an important structural and physical role in the PM. Because of their tendency to form self-aggregates in a plane, ceramides easily segregate laterally into raft/caveolae microdomains and pack very tightly in association with sphingolipids and cholesterol (van Blitterswijk et al., 2003). In support of this, a substantial amount of ceramide was found accumulated in isolated raft/caveola fractions, even in unstimulated cells. Kinetic data on transbilayer movements (flip-flop) of ceramide convincingly showed that ceramide resides in the layer where it was generated for quite a long time.

Ceramides have been shown to critically regulate membrane raft dynamics essential for efficient death signals. Specifically, the newly formed membrane ceramide was able to induce coalescence of membrane rafts containing the trimeric forms of the engaged death-receptors (Fas/CD95 or CD40) into cap-like structures promoting formation and efficient functioning of death-inducing-signal-complexes (DISC) (Cremestli et al., 2001). Exogenous ceramide was able to rescue the formation of DISC even in SMase-deficient cells. All these observations suggest that membrane ceramide is an important regulator of raft dynamics/death-receptor clustering. These effects of ceramides are thought to be coupled to activation of acidic SMases acting preferentially on the outer membrane leaflet. In a later, effector phase of apoptosis the neutral SMases acting mostly on the inner membrane leaflet become active, resulting in an SM breakdown and a concomitant loss of membrane cholesterol. These events are thought to be involved in membrane blebbing and formation/release of apoptotic bodies, as consequences of a novel SM-related role in membrane dynamics (van Blitterswijk et al., 2003).

### 5.3. Control of Raft Dynamics by Intracellular Factors

The cortical actin cytoskeleton is a critical biological factor that can dynamically control lipid raft distributions and their in-plane rearrangements in the plasma membrane. In addition, the cytoskeletal machinery triggered by different stimuli (e.g., chemoattractive, antigen-specific, integrin, and costimulatory signals) is also involved in development of spatial and functional cell polarization and motility (Ikonen, 2001). Lipid rafts are functionally linked to all of these processes as discussed in this chapter.

We would like to point out that sometimes the quantity of rafts expressed at the cell surface can also control functional consequences of raft-dynamics in the PM. A good example of this is in naïve T lymphocytes, where the primary antigen stimuli have been shown to induce *de novo* sphingolipid synthesis, thereby increasing the amount of cellular raft domains that can direct naïve T cells toward differentiation to effector/memory cells (Tuosto et al., 2001; Viola, 2001). Whether this phenomenon exists in other cell types needs further investigation.

#### 5.3.1. Coupling Between Rafts, Membrane Proteins, and the Actin Cytoskeleton

The cortical cytoskeleton (CSK) may exert physical constraints for lateral mobility of membrane constituents, especially for proteins with long cytoplasmic domains. This action in turn may increase stability of membrane microdomains defined on the basis of restricted diffusion (“fencing

effect”) (Kusumi and Sako, 1996). Many pieces of experimental evidence confirmed this effect using single particle tracking (Tomishige and Kusumi, 1999), and presently it is still considered as an alternative for the “lipid shell” model. Although such membrane-skeleton-mediated constraints on lateral mobility is restricted to several membrane proteins, the basic elements of this model (Kusumi and Sako, 1996) should be taken into account in an integrated model for formation of membrane microdomains.

Rafts, on the other hand, can also influence cytoskeleton function, namely by promoting actin comet formation. Vesicles containing raft markers do induce comet formation essential for vesicle motility, whereas vesicles with nonraft proteins do not induce this formation. It is still unclear, however, how the actin filaments operate in the transport processes: Do they act as cables for myosin motors, or propel vesicles? These questions, together with the potential intracellular regulatory network of actin-myosin motor interactions, remain to be explored. Because the CSK is likely wired to rafts in a complex way, the relationship between the major drivers of CSK rearrangements, the phosphoinositides, rafts, and actin also awaits of further clarification. From studies on lymphocytes we learned that raft domains can be coupled to the CSK through large protein complexes including raft-associated adaptor proteins, such as PAG (protein associated with GEMs) (Brdicka et al., 2000), expressed in both T and B lymphocytes. However, the precise mechanisms of coupling to or decoupling of raft domains from the CSK remains to be elucidated.

Annexin family proteins (II and VI) provide an additional,  $\text{Ca}^{2+}$ -dependent control mechanism on raft domain dynamics, as demonstrated during smooth muscle contraction (Babiyhuk and Draeger, 2000) or in other cell types (Oliferenko et al., 1999). These  $\text{Ca}^{2+}$ -regulated phospholipid binding proteins can promote formation of membrane-cytoskeleton complexes, thereby controlling raft-assembly (Babiyhuk and Draeger, 2000).

### *5.3.2. Raft Dynamics and the Spatial/Functional Polarization of Cells*

Regarding this question, most of the current information is available on lymphoid and myeloid cell types in which these mechanisms are essential to their specialized and dynamic immunological functions. Polarization of these cells is usually initiated by the extracellular chemoattractant environment. Raft microdomains are usually subject to extensive redistribution upon chemoattraction signals. This question, however, is rather controversial. Cancer cells or electrically directed fibroblasts display a leading edge raft accumulation, while lymphocytes or neutrophils show an exclusive uropod raft accumulation (Manes et al., 1999; Millan et al., 2002). This, in support of the idea of microdomain heterogeneity, led to classification of raft domains as



leading edge rafts (L-rafts) enriched in monosialylganglioside GM3 or uropod rafts (U-rafts) enriched in GM1, in polarized cells (Manes et al., 2003). The polarized distribution of rafts in leukocytes persists during chemotaxis and depends heavily on the functional integrity of the cytoskeleton. Association of membrane receptors with distinct raft microdomains can drive their redistribution to the site of their action in polarized cells. A good example of this is a polarized, moving leukocyte where chemosensory receptors and the coupled initiator molecules of cytoskeletal redistribution are concentrated by L-rafts at the leading edge, while long integrin/adhesion molecules suboptimal (inhibitory) for the appropriate scanning and recognition functions are enriched in the uropodal region. Rafts are known to efficiently couple recognition processes to signaling cascades (Matkó and Szöllösi, 2002). In migrating cells, in addition, they are able to restrict and organize signaling to specific areas. This mutual coupling is dynamic, since external signals on these cells may also influence the aggregation state of rafts.

## **6. RAFTS AND THEIR DYNAMICS IN SPECIALIZED COMPLEX BIOLOGICAL STRUCTURES: THE IMMUNOLOGICAL SYNAPSES**

The classical definition of the immunological synapse originates from contacts of antigen-presenting cells (APC) with helper T lymphocytes, as a junctional structure with a 15- to 30-nm-wide cleft formed around the antigen-recognition driven point of contact between these cells (Bromley et al., 2001). Since then, other types of immunological synapses with different fine structure and composition, such as synapses of cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and B cells were also described (Blanchard and Hivroz, 2002; Krummel and Davis, 2002). Lipid rafts, and especially their dynamic rearrangements, seem to play a pivotal role in the function of these synapses.

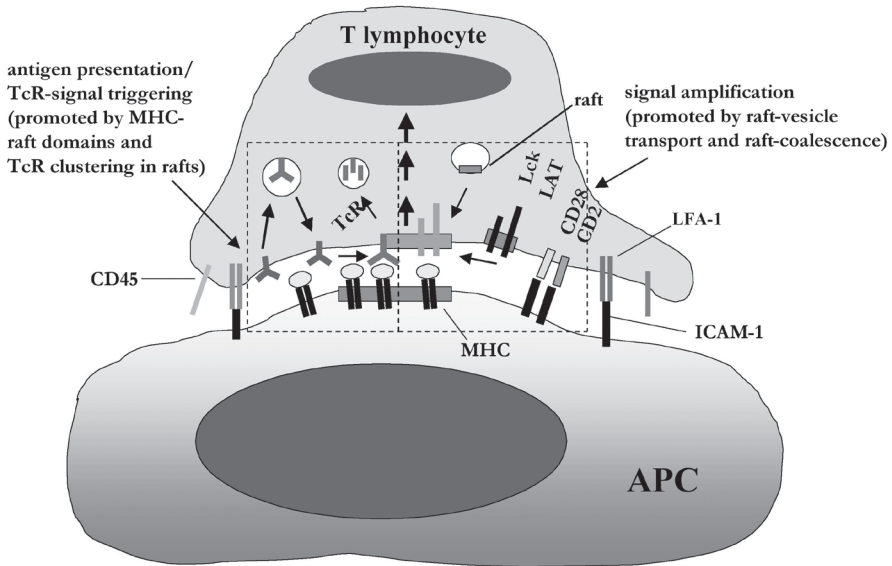
First, elementary (unit) rafts may provide some preassembled “signal chips” (e.g., preassembled Src kinases in the inner leaflet, or GPI-linked proteins in the outer leaflet), while isolating a number of regulatory molecules from rafts, including the major antigen-specific receptors, thereby keeping the cells at rest (Bromley et al., 2001; Blanchard and Hivroz, 2002). According to our present knowledge, organization of mature immunological synapses is preceded by the antigen-specific TCR signaling (Blanchard and Hivroz, 2002; Krummel and Davis, 2002). This initial signal increases the affinity of several pairs of adhesion molecule contacts, thereby stabilizing the intercellular contact.

Real-time microscopy data have shown that in most of T-cell synapses, the next step is the formation of the central supramolecular activation cluster (cSMAC) (Freiberg et al., 2002) in the center of the relatively large contact area (approximately  $50 \mu\text{m}^2$ ), including the occupied TCR molecules, and transiently, the CD4 coreceptors or CD45 (regulatory protein tyrosine phosphatase) molecules, which are excluded from this area later on for a longer time. Rafts are critical building blocks during staging and resetting of SMAC (Freiberg et al., 2002), and they accumulate in a large quantity in cSMAC, but they do not seem responsible for the stability of this structural zone (Burack et al., 2002).

The relationship of adhesion molecules (stabilizing the synaptic contacts) to the lipid raft microdomains remains controversial. Nonetheless, the SMAC is surrounded by a ring-like assembly of adhesion molecules (peripheral SMAC, pSMAC) (Freiberg et al., 2002).

Besides coupling the engaged TCRs to the group of active Src kinases (Lcks) within large rafts in cSMAC, rafts also play a dynamic role in orchestration of the signaling machinery. The hot spot in cSMAC is continuously fueled via transport of raft-enriched vesicles by further Lcks and different adaptor molecules (e.g., LAT, SLP76, and so on) recruiting further downstream signal molecules. In addition, rafts in cSMAC form a stable platform for serial engagement of the rapidly recycled TCR molecules: A few MHC-peptide antigen can trigger hundreds of TCRs in a short time (Valitutti et al., 1995; Viola, 2001). Furthermore, dynamic, actin cytoskeleton-mediated redistribution of costimulatory T-cell rafts may further amplify TCR signaling in the synapse, a prerequisite of sustained signals required for activation of interleukin-2 genes and proliferation (Viola, 2001). Recently, MHC-peptide domains and lipid rafts on APC were also shown to accumulate into the synapse (Hiltbold et al., 2003), consistent with the serial triggering model (Valitutti et al., 1995), and the APC's cytoskeleton also proved to be essential for formation of stable functioning synapses (Trautmann and Valitutti, 2003; Gombos et al. 2004).

The dynamic cytoskeletal machinery, as a hardware of synapses, provides additionally polarized distributions of rafts in T cells contacted by APCs, resulting in enrichment of the signaling machinery (signalosomes) near the contact zone, while dynamically excluding abundant, long inhibitory proteins (e.g., CD43, CD45) from the synapse (Krummel and Davis, 2002; Trautmann and Valitutti, 2003). Thus, these synaptic structures with dynamically redistributing rafts in concert with the cytoskeletal hardware represent a specialized, highly efficient junctional platform with a lifetime of several minutes to several hours, allowing antigen recognition in spite of the occa-



**Fig. 3.** Raft dynamics in specialized complex structures: the T-cell immunological synapse. Rafts on antigen-presenting cells (APC) by clustering the MHC-peptide complexes and on T cells by clustering the engaged T-cell receptors (TCRs) and coupling them to the signal-machinery (inner leaflet domains of Src kinases) may efficiently promote the recognition-triggering phase of T-cell activation. After formation of the mature immunological synapse, cytoskeleton-driven membrane rearrangements, including rafts of costimulatory and adaptor molecules, may serve as amplifiers of the T-cell signal. A polarized transport of vesicles enriched in Src/LAT-rafts also helps to assure a sustained signaling in T cells. Rapid recycling of TCR provides the possibility of repeated visiting of antigen-presenting hot spots by TCRs (serial engagement). (This figure is based on the works and model of Lanzavecchia and coworkers (Valitutti et al., 1995; Viola 2001; Lanzavecchia and Salusto 2001), Bromley et al., 2001, and Hiltbold et al., 2003.)

sionally very low MHC/peptide-TCR affinities and low antigen density on APC, as well as providing a well-organized platform for efficient TCR and costimulation signaling (Fig. 3).

Recently, an interesting physical model for synapse assembly was presented (Lee et al., 2002) on the basis of intermembrane-distance-dependent kinetic data on receptor-ligand or adhesion molecule binding, considering also the length of the interacting molecular partners, the mechanical deformability of the membrane (membrane shape), and thermodynamic considerations. This model may represent a starting framework for quantitative

analysis of the mechanisms underlying assembly of immunological synapses. Although real-time fluorescence videomicroscopy, CLSM, or two-photon microscopy (e.g., Burack et al., 2002; Freiberg et al., 2002; Miller et al., 2002) provide us with improving, detailed insights into formation and function of immunological synapses, even at the level of tissues and organs, several important questions still remain to be explored, such as how the sites of polarized cytokine secretion or the death signal domains relate to the antigen-specific synaptic cleft.

## 7. CONCLUDING REMARKS

Although many studies have investigated the role, size, and dynamics of lipid rafts, much remains to be discovered. Particular attention should be devoted to the following questions: How do proteins recognize appropriate lipid rafts? What are the physical characteristics and lipid composition of rafts in the inner leaflet? What kind of forces and interactions keep the lipid rafts in the outer and inner leaflets together? What is the exact role of cytoskeleton in the migration and coalescence of rafts, and how is it regulated?

The latest developments in modern biophysical techniques provide new methods for addressing these types of questions. Technical advances in imaging and single-molecule detection now allow us to follow the rearrangement and movement of molecules in living cells, and this ability should help to elucidate the molecular mechanisms underlying the physiological functions of lipid rafts.

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