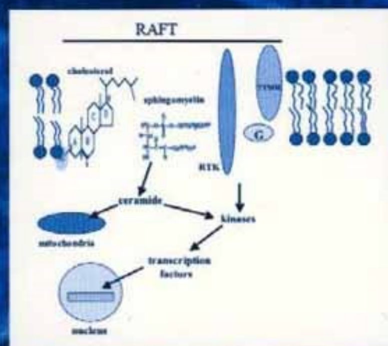
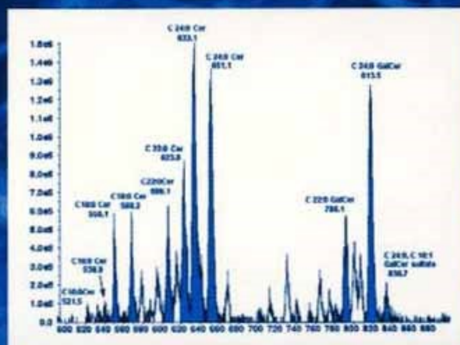
Membrane Microdomain Signaling

Lipid Rafts in Biology and Medicine

Edited by

Mark P. Mattson





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Cover: Lipid rafts, regions of the cell membrane that are enriched in cholesterol, ceramides, and sphingolipids, contain receptors for various growth factors (RTK, receptor tyrosine kinase), cytokines and GTP-binding protein (G) coupled seven *trans*-membrane receptors (7TMR). Kinase cascades activated by these signaling pathways, and by lipid biomediators such as ceramide that are generated from raft lipids, influence a variety of cellular processes.

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Preface

Membrane Microdomain Signaling not only explores a fascinating aspect of cell biology that is emerging as pivotal for a variety of signaling processes in cells throughout the body, but also focuses on abnormalities in lipid rafts for various pathological conditions. Membranes of most—if not all—cells contain discrete regions that are rich in cholesterol and sphingolipids and function as sites where various receptors, cell adhesion proteins, signal transduction molecules, and cytoskeletal elements are concentrated. These microdomains, called lipid rafts, are now recognized as playing fundamental roles in the regulation of a range of cellular processes from proliferation and differentiation to signal transduction responses to a variety of stimuli.

In *Membrane Microdomain Signaling*, experts in the field of lipid rafts and signal transduction provide detailed reviews on the current state of understanding these membrane microdomains and their physiological and pathophysiological roles. The first chapter by Ephraim Yavin and Annette Brand reviews the lipid structure of membranes and how asymmetries in the membrane are formed, maintained, and modified. The reader is introduced to the various lipids and their organization within membranes.

János Matkó and János Szöllosi next describe recent findings related to dynamic aspects of lipid rafts, how they change in cells over time, and how they respond to various environmental signals. Elizabeth Luna and colleagues review the structural interactions between lipid rafts, the extracellular matrix, and the cell cytoskeleton.

Chris Fielding focuses on cholesterol, a fundamental component of lipid rafts, and describes how this ancient and critical lipid modulates the signaling functions of lipid rafts. Arnold van der Luit and colleagues discuss the role of lipid rafts as portals for endocytic uptake of an anti-cancer and anti-apoptotic alkyl-lysophospholipid. Furthermore, they describe how cellular resistance to this agent is associated with a defect in sphingomyelin synthesis, new raft formation, and raft-mediated endocytosis. Markus Delling and Melitta Schachner describe emerging evidence of critical roles for lipid rafts as well as signaling pathways in lipid rafts in the regulation of synaptic function in the nervous system. It appears that lipid rafts are concentrated in synapses and play major roles in such processes as learning and memory. Wu Ou and Jonathan Silver review the evidence suggesting that lipid rafts are sites at which viruses bind and enter cells, focusing on this aspect of

diseases. My colleagues and I then describe emerging evidence implicating alterations in raft lipid metabolism in the pathogenesis of such neurodegenerative disorders as Alzheimer's disease and amyotrophic lateral sclerosis. Mordechai Liscovitch and colleagues then review information concerning the roles of lipid rafts in cancer. In particular, the raft associated protein caveolin-1 has been linked to various cancers. Finally, the importance of dietary lipids in modifying function and affecting risk for disease in the context of lipid rafts is briefly considered. It is clear that lipid components of rafts can be modulated by diet, with dietary cholesterol and sphingolipids being examples.

A better understanding of the effects of dietary lipids on raft function may lead to novel prevention and treatment methods for various diseases that involve abnormalities in lipid rafts. Collectively, the information in this book will provide a valuable resource for graduate students, postdoctoral fellows, and established investigators interested in membrane signaling and the lipid and protein components of rafts. There is much to be learned in this emerging and critical area of cell biology, and we hope that *Membrane Microdomain Signaling* will provide a foundation for the development of research projects by various investigators in a broad array of fields in the biological and biomedical sciences.

Mark P. Mattson

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1

From Intramolecular Asymmetries to Raft Assemblies

A Short Guide for the Puzzled in Lipidomics

Ephraim Yavin and Annette Brand

1. INTRODUCTION AND CLASSIFICATION

For the first-time explorer in biology, lipids are perceived as the building blocks of a barrier that living cells must have in order to maintain their internal milieu of organelles, macromolecules, and solutes in a dynamic steady state. Although this view is generally correct, emerging evidence indicates that many lipids fulfill important regulatory roles and participate actively in cellular signaling. Furthermore, studies based on natural and model membranes suggest that transient and long-lived associations between lipids in the plane of the membrane give rise to yet another dynamic feature of these building blocks: the creation of functional domains in the barrier without which cells may not be able to function appropriately. In this context, the asymmetry between the two leaflets of the plasma membrane (Bretscher, 1973) or the lateral segregation into functional domains on the same leaflet (Brown and London, 2000) suggest a high diversity among lipid constituents. The aim of this chapter is to emphasize that the molecular basis for segregation into rafts or related structures stems from the unique intramolecular asymmetry conferred upon the lipid molecule by its various constituents.

It is common knowledge that the same lipid building blocks are shared by prokaryotes and eukaryotes, and yet, the evolutionary pressure leading from single cells to multicellular assemblies and the acquisition of functional specificity has led to further diversification of lipid molecular species to meet novel functions. That diversity is well-reflected in the composition of

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most glycerophospholipids (GPL), glycosphingolipids (GSL), and to a lesser extent, sterol lipids. In accordance with acquisition of function, the lipid composition of various membranes in the same cell (i.e., nuclear, mitochondrial, or lysosomal membrane organelles) has been subject to substantial specialization. According to estimates, a single membrane may have a repertoire of 500 to 1000 different lipids (Edidin, 2003).

Only recently some of the molecular basis for this diversity has been revealed as have the multiple roles of lipids in signal transduction and in specific cell–cell interaction. Because this assortment is crucial to cell function, it is not surprising that most lipids exhibit longer half-lives than many proteins do, and in addition, many of the lipid components are recycled after degradation by specific enzymes rather than being synthesized *de novo*.

Understanding of the basis of this complex repertoire of lipids lies within the unique chemical and physical characteristics of these molecules.

2. INTRAMOLECULAR ASYMMETRY

The first and perhaps the most striking feature for the explorer in the sea of lipids is the amazing asymmetry of molecules with which a lipid acquires its complex structure and identity. The intramolecular asymmetry is primarily a feature of GPL and GSL, but is also apparent in phosphosphingolipids (PSL). Examples of this asymmetry are presented in Fig. 1, which illustrates typical members of the aforementioned classes and cholesterol, in a schematic bilayer configuration.

The intramolecular asymmetry is based on the ability of cells to utilize a variety of long-chain hydrocarbons (LCH) and polar head groups (PHG) in various combinations. Notable in this schematic representation is the bilayer configuration of juxtaposed outer and inner leaflets, containing a wide range of LCHs with varying degrees of unsaturation that constitute the inner hydrophobic domain of the bilayer. The LCHs are primarily associated with the GPL class. GPL, also known as phospholipids or phosphoglycerides, constitute the bulk portion of the polar lipids in eukaryotic cells.

2.1. Long-Chain Hydrocarbons

The vast majority of the aliphatic LCHs, commonly known as fatty acids (FAs), contain a carboxyl group on one end that can either establish an ester bond with the hydroxyl group, as in PGL, or connect via an amide linkage to a nitrogen, as in GSL and PSL classes. Some of the LCHs contain a terminal aldehyde group that gives rise to a vinyl ether (alkenyl) bond, as in plasmalogens. A smaller proportion of LCHs contain an alcohol residue, which upon reaction with hydroxyl groups forms an ether bond. Some typical structures of LCH are presented in Fig. 2.

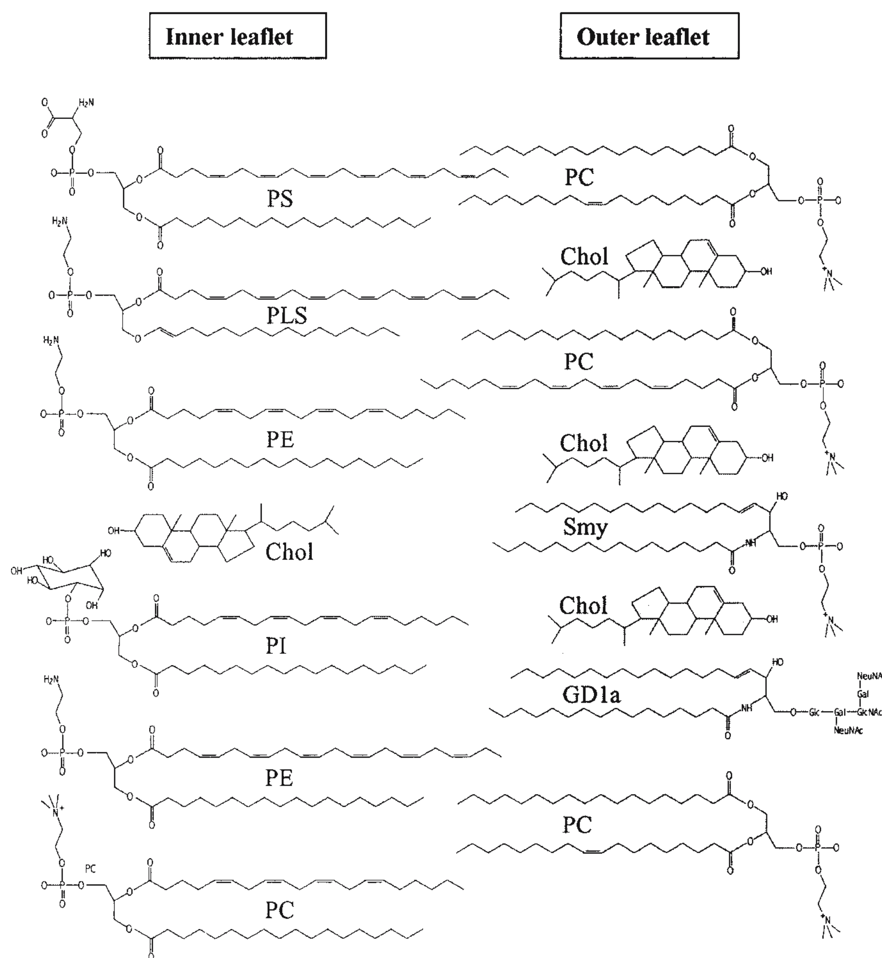

Fig. 1. Molecular representation of selected lipids in the bilayer. Typical molecules of the outer leaflet, including sphingomyelin (Smy), phosphatidylcholine (PC), cholesterol (Chol), and GD1a ganglioside, contain mostly saturated aliphatic molecules. The inner leaflet is composed primarily of phosphatidylethanolamine (PE), phosphatidyl ethanolamine plasmalogen (PLS), and phosphatidylserine (PS), which contain mainly aliphatic mono- and polyunsaturated fatty acids and aldehydes.

The saturated FA (SAFA) are usually linked to the sn-1 position whereas monounsaturated (MUFA) and polyunsaturated (PUFA) acids are linked to the sn-2 position of the glycerol "backbone." In addition to FAs, some of the attached hydrocarbons are long chain alcohols or aldehydes, and as a result ether or vinyl ether bonds are formed. Of these, the most ubiquitous group is

Chain lenght: unsaturation	Chemical name	n-series	Chemical structure
SAFA			
12:0	Lauric		
14:0	Myristic		
16:0	Palmitic		
18:0	Stearic		
20:0	Arachidic		
22:0	Behenic		
24:0	Lignoceric		
MUFA			
16:1	Palmitoleic	n-7	
18:1	Oleic	n-9	
24:1	Nervonic	n-9	
PUFA			
20:3	Mead	n-9	
18:2	Linoleic	n-6	
18:3	γ-Linolenic	n-6	
20:3	Dihomo-γ-Linolenic	n-6	
20:4	Arachidonic	n-6	
18:3	α-Linolenic	n-3	
20:5	Eicosapentaenoic	n-3	
22:6	Docosahexaenoic	n-3	
Plasmalogens			
16:0	Palmitaldehyde		
18:0	Stearaldehyde		
18:1	Oleylaldehyde		
Long-Chain Bases: Sphingolipids			
18:0	Dihydro-sphinghosine		
18:0	Phyto-sphinghosine		
18:1	Sphinghosine		

Fig. 2. Representative long-chain hydrocarbons (LCH) in the mammalian plasma membrane. The lipids are grouped according to saturated (SAFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids. Aliphatic chains are arranged according to the growing chain length and degree of unsaturation.

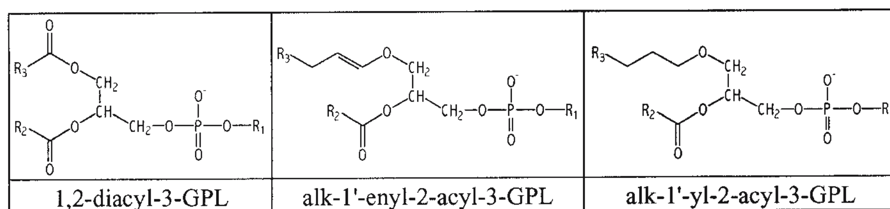

Fig. 3. Scheme of 1,2 diacyl, alk-1-enyl, 2-acyl, and alk-1-yl, 2-acyl bonds. R2 and R3 represent various LCHs, and R1 various polar head groups.

the plasmalogenic GPLs that are usually attached to the *sn-1* position of glycerol. This attachment is illustrated in Fig. 3, showing the sites of linking of the LCH to the glycerol phosphate moiety of the GPL.

The change in degree of unsaturation gives rise to a great variation among FAs. There are three major families of unsaturated FAs, based on the initial double bond established on the growing aliphatic chain. Accordingly, there are three major series of unsaturated FAs consisting of n-9; n-6, and n-3 double bonds, which in mammals, establish three distinct and noninterchangeable families. Surprisingly, we have little information explaining the molecular basis for the wide variety of LCH constituents, let alone to what extent their functions change when, for example, arachidonic acid (C20:4 n-6) and docosahexaenoic acid (22:6 n-3) are substituted by Mead (C20:3 n-9) and docosapentaenoic acid (22:5 n-6), respectively.

The biosynthesis of FAs with multiple double bonds and >C18 chain length is carried out in mammals at the carboxyl end of the FA molecule by two major enzymatic systems that involve desaturation, starting with an Δ6 desaturase, followed by a two carbon elongation by an elongase (Salem, 1989). The metabolic flow of FA conversion by enzymatic desaturation and elongation steps is schematically illustrated in Fig. 4.

In mammalian cells these enzymatic activities are localized within the microsomal fraction (Salem, 1989). Most lipids are synthesized in the endoplasmic reticulum but some are made in the mitochondria. From their site of synthesis, lipids are subsequently transferred by interbilayer movement to various intracellular organelles and plasma membrane using a variety of mechanisms, including vesicle-mediated and protein-mediated transport. However, at the membrane level, further changes are likely to take place before the final configuration of the PGL is attained. As a result of constant interaction of the membrane with the external milieu or following oxidative stress and formation of lipid hydroperoxides, membrane GPL components may be altered (Budowski, 1988). Additionally, it has been shown

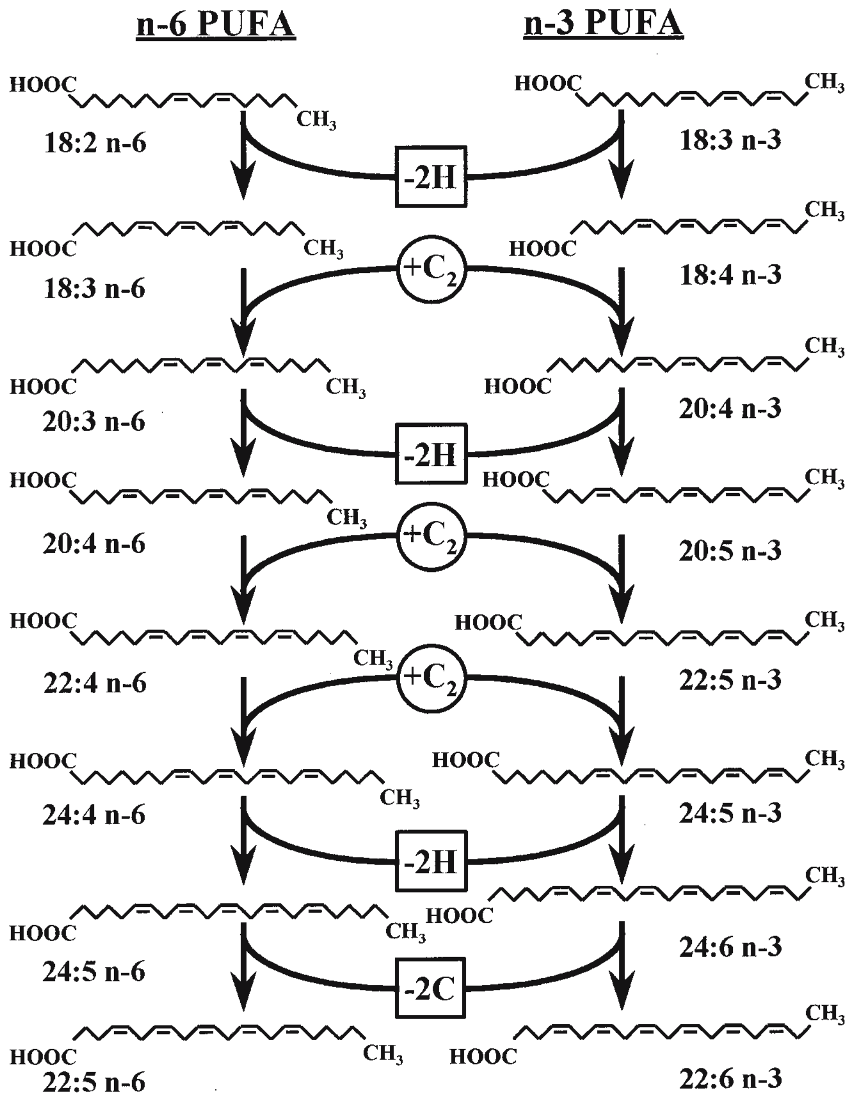

Fig. 4. Elongation and desaturation of n-3 and n-6 fatty acid families. Generation of the n-6 and n-3 families occurs by a sequence of steps involving chain elongation and desaturation. For both families, the first step is catalyzed by a Δ6 desaturase.

that dietary manipulation directly affects membrane lipid composition. Thus the FA composition of the GPL classes, their balance, and cholesterol intake are major variables that could in turn affect ion channels, receptors, transporters, and enzymatic activities.

2.2. Polar Head Groups

The second molecular variable that increases the number of potential molecular species is the polar head group of the GPL classes. Several of these PHGs that give rise to the furthering of the intramolecular asymmetry are depicted in Fig. 1. Basically, there are several groups of PHGs that consist of choline and ethanolamine, together composing the bulk of GPL classes, as well as minor ones that include serine, myo-inositol, and glycerol. PHG exchange is an important mechanism to alter the N-bases and is the major source in mammals for PS formation. Choline is also the predominant PHG moiety of sphingomyelin, the principal member of the PSL group. PHGs are oriented approximately parallel to the aliphatic chains of the bilayer (Seelig and Seelig, 1980) and are also subject to constant dietary alterations. This has been well-documented in cell cultures deprived of PHG or supplemented with PHG analogs (Brand and Yavin, 2000).

2.3. Sphingosine-Derived Lipids

Sphingosine containing lipids comprise a significant portion of the membrane lipids and vary from membrane to membrane. The molecular structure of sphingomyelin, the principal PSL member, and GD1a ganglioside, a most prominent member of the GSL family, is depicted in Fig. 1 (*see also* Chapter 8). Both molecules are predominantly associated with the outer leaflet of the plasma membrane. The basic structure shared by both GSL and PSL classes contains a core molecule of ceramide which is synthesized from either sphingosine, dihydrosphingosine, or phytosphingosine N-base, and a long-chain saturated or hydroxyl-containing FA. The terminal hydroxyl group of the ceramide, when linked via an ester bond to phosphorylcholine, gives rise to sphingomyelin, the only phosphorus-containing sphingoside base in mammals.

Attachment of a sugar residue such as glucose or galactose to the terminal hydroxyl group of the ceramide gives rise to a large group of diverse sugar-containing molecules termed glycosphingolipids. Most common among this class are the gangliosides, which may contain up to five sialic acid residues and which are found both in the invertebrate and vertebrate plasma membranes. To date nearly 100 ganglioside species have been identified. Caveolar membranes have been shown to contain glycosphingolipid-enriched microdomains that may play important roles during cellular signaling (Hakomori et al., 1998; Iwabuchi et al., 2000). Apart from GM1, little is known about the presence of higher order gangliosides in synaptic rafts of synapses and dendritic spines, in spite of their abundance in nerve cells (Hering et al., 2003; Ma et al., 2003). Many GSLs participate in the regulation of cell growth, cell adhesion, binding of viral antigens and toxins, and regulation of cell differentiation (Ledeen et al., 1998).

2.4. Sterols

Sterols appear to be one of the more essential molecules in the organization of the biological membrane as a physical barrier. Perhaps that fact explains why little or no diversification in the molecule has been seen through evolution, and why the basic, highly rigid, four fused planar rings, a β-hydroxyl at the 3 position, and a hydrophobic tail is the prevalent structure in all eukaryotic cells. Furthermore, the presence of the 3β-hydroxyl group is sufficient to confer a parallel alignment of sterols with the LCH as well as polarity with respect to the hydrophilic and hydrophobic plasma membrane domain (Yeagle, 1985). Many physical properties of membranes may be modulated by the amount of sterols present in the membrane, a well-known feature that has been used in synthetic model membranes to attain desired barrier properties (Barenholz and Cevc, 2000).

Cholesterol is the most abundant sterol in the animal kingdom. Limited molecular elasticity has given cholesterol a central role in altering the physical state of the membrane, specifically on short-range order and long-range lateral organization**.** Interestingly, on one end cholesterol lowers the chain order near the PHG (Yeagle, 1993), but it rigidifies the methyl terminals of the LCH on the other end. Furthermore, cholesterol in the bilayer has been shown to reduce the free volume, thereby lowering membrane permeability (Mouritsen and Jorgensen, 1998). Evidence from membrane models has shown that the intercalation of cholesterol between PLs and SLs is the major driving force for the gel-to-liquid crystalline phase transition of the plasma membrane. Cholesterol has been associated with synapse plasticity (Mauch et al., 2001; Hering et al., 2003) and plays a role in neuronal degeneration (Simons et al., 1998; Koudinov and Koudinova, 2001). Additional information on cholesterol is presented in other chapters of this book.

3. MOLECULAR DIVERSITY BETWEEN OUTER AND INNER LEAFLETS

The intramolecular asymmetry of GPL, SGL, and GSL discussed above constitutes the basis for the molecular and functional diversity of the inner and outer leaflets of the membrane, or the bilayer asymmetry—a feature that is characteristic to all cellular plasma membranes (Bretscher, 1973; Boon and Smith, 2002). The overwhelming presence of aminophosphoglycerides (PEs and PSs) in the inner leaflet contributes to the large proportion of PUFAs facing the cytoplasm of the cell. In contrast the presence of PC and sphingomyelin in the outer leaflet establishes a predominantly saturated aliphatic chain composition, which together with cholesterol confers rigid properties to the leaflet. Generation of the bilayer asymmetry and

its active maintenance by specific lipid transporters is a distinct feature of the plasma membrane and is a driving force for cell signaling and cell regulation (Balasubramanian and Schroit, 2003). Conditions leading to the collapse of asymmetry as manifested during platelet aggregation or in Scott syndrome, a bleeding disorder caused by lipid scrambling (Zwaal and Schroit, 1997; Bevers et al., 1999), or in the process of apoptotic cell death (Bratton et al., 1997) may all share a common mechanism related to oxidative stress and possible oxidation of PUFA in the inner leaflet (Brand and Yavin, 2001). Little is known about the lipids that interact with elements of the cytoskeleton or with enzymes or proteins that actively translocate between the cytosol and the plasma membrane. Recent data suggest that lipid asymmetry plays an important role in various signaling cascades; nevertheless many aspects of this biological phenomenon remain unclear (Verkleij and Post, 2000).

4. MOLECULAR DIVERSITY WITHIN THE SINGLE LEAFLET

The concept of membrane domains with an ordered lipid environment mainly driven by cholesterol and sphingomyelin associations, as in rafts and caveolae (Brown and London, 2000), is in essence a third form of molecular diversity which stems from the intramolecular asymmetry and from the unique distribution of lipids between the two leaflets. The presence of lipid domains was first indicated in lipid models (Melchior, 1986). Rafts represent an ordered lipid environment in which certain types of proteins associated with cellular signaling appear to function (Simons and Ikonnen, 1997; Smart et al., 1999; Simons and Toomre, 2000). Membrane domains were originally proposed to account for sorting and trafficking lipids and lipid-anchored proteins in polarized epithelial cells (Simons and van Meer, 1988). Later the hypothesis was extended to other signaling cascades as well, and it has been evaluated for a number of presumed lipid microdomains (Brown and Rose, 1992; Brown and London, 1998; Furuchi and Anderson, 1998; Smart et al., 1999; Anderson and Jacobson, 2002). Protein receptors and cytoskeletal proteins can also contribute to the rapid assembly of lipid domains (Rodgers and Rose, 1996; Sheets et al., 1997) but most of these lipid–protein interactions are presently unknown. At this time little information is available about how the lateral partition of the raft in the outer bilayer is driven by the composition of the inner leaflet (Stulnig et al., 1998) or vice versa, or whether there is a coupling between the two leaflets (Rietved and Simons, 1998). Most recently, the participation of plasmalogens and arachidonic acid in rafts has been indicated (Pike, 2003).

Also notable is that a lack of appropriate probes has hampered the identification of lipid constituents such as polysialogangliosides larger than GM1 in rafts or caveolae of subsynaptic structures.

5. ON PROTEOMICS AND LIPIDOMICS

Evidence accumulated for more than a decade now has rectified the classical fluid mosaic model of the plasma membrane (Nicholson and Singer, 1973) toward a new understanding of its dynamics (Edidin, 2003). Membranes are no longer conceived as barriers with selective pores and spanning integral proteins. Rather they are seen as a continuum of functional grouping and dispersion of both lipids and proteins that perform in concert to convey a cellular signal. Proteins that are membrane associated account for almost one-third of total cellular proteins. As little as a single amino acid mutation may alter the proper function of a cell. Alterations in lipid composition and perturbation of lipid asymmetry arising from supplements of PUFA (Stulnig et al., 1998), changes in cholesterol and sphingolipid content (Hering et al., 2003), increased lipid peroxidation (Halliwell and Gutteridge, 1984; Yavin et al., 2002), or dietary n-3 deficiency may bring about discord in the protein-lipid interplay and often result in deleterious consequences and severe pathologies (Bazan and Rodriguez de Turco, 1994; Martinez and Vazquez, 1998; Anderson et al., 1999; Freedman et al., 1999). Such changes may arise from changes in the regulation of gene expression caused by dietary lipids (Price et al., 2000). It is well-established that diet is a principal source for the membrane lipid repertoire. Changes in dietary conditions over the past century, for example, have been a major determinant of the average increase in the stature of humans. However, they have also caused a rapid rise in cardiovascular mortality, compromised the immune system, and increased certain malignancies (Phylactos et al., 1994; Barker and Clark, 1997; Ghosh and Myers, 1998). Thus, a true molecular dissection of the specific branch of proteomics concerned with integral and membrane-associated protein functions in health and disease will necessitate an equally advanced knowledge in lipidomics to unravel the subtle ways in which lipid components interact with these proteins.

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2

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

A Biophysical Approach

János Matkó and János Szöllősi

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-

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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 that 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 μs) can be observed in pulse EPR experiments in which the spin-lattice relaxation times (T_1s) 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 subpixel 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-preferring 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 μm (Schutz et al., 2000).

Using single-molecule techniques (both SPT at a 25 μs 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 dioleoclpalmitoyl-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 immuncompetent 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 be to 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 Kurzcha1ia, 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; Kenworthy et al, 2000
GPI-anchored proteins (FR)	Cell membrane	FRET, anisotropy based	70 nm	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; Yuan et al., 2002; Lawrence et al., 2003
GPI-anchored proteins (PALP)	Cell membrane	Optical trapping	26 nm	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-phsophoethanolamine; 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).

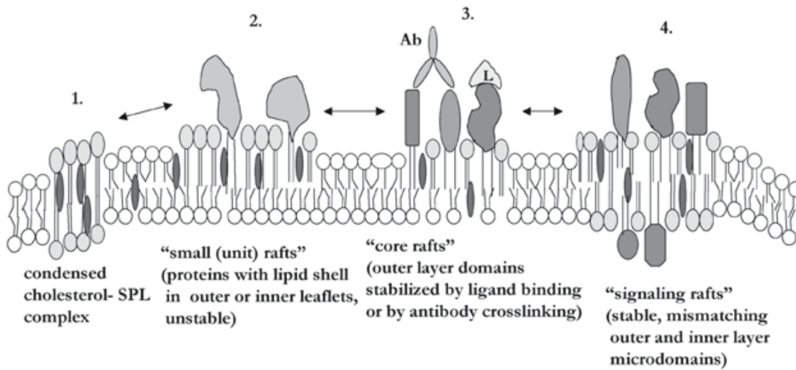

Fig. 1. A schematic diagram of raft-dynamics in plasma membranes. Formation of raft domains in live cells likely has a complex molecular background: (1) condensed complexes of cholesterol and sphingolipids; (2) small preformed unit rafts in either the outer or inner leaflets (e.g., unstable GPI microdomains, 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 occuring 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-β-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-β–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-manipulaton 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

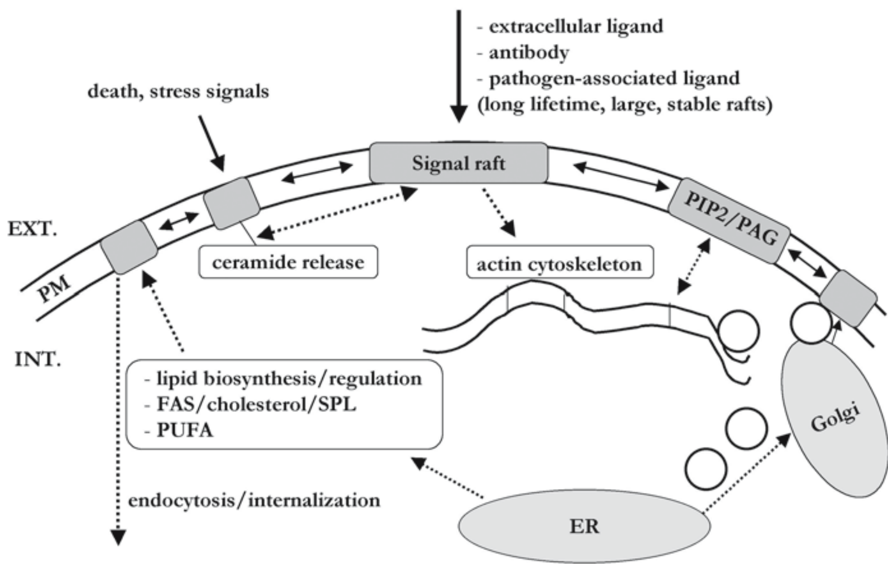

Fig. 2. Regulation of raft dynamics in the plasma membrane. The dynamic and elastic raft/nonraft equilibrium in the plasma membrane is under a multifaceted regulation: Lipid biosynthesis (and its disorders/accomodations to enviromental signals) may alter lipid composition affecting lipid microdomain 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 neccessary.

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 treament. 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 trafficiking 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 recogniton receptors [PRR; toll-like receptor family] recognizing and binding pathogen surface patterns), many receptors' ligand-engagement inducted "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)P2) 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)P2 was found clustered in sphingolipid/cholesterol-rich raft-like domains lacking Src and caveolin, but dependent on cholesterol.

These phophoinositide domains may recruit a number of PIP2-binding proteins and thereby transiently capture vesicles nearby the PM or induce signals for the accompanying cytoskeletal rearrangements. The PIP2-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 PIP2-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 PIP2-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-α, interferon-γ, 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, Ca^{2+}-dependent control mechanism on raft domain dynamics, as demonstrated during smooth muscle contraction (Babiychuk and Draeger, 2000) or in other cell types (Oliferenko et al., 1999). These Ca^{2+}-regulated phopholipid binding proteins can promote formation of membrane-cytoskeleton complexes, thereby controlling raft-assembly (Babiychuk 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 intitated by the extracellular chemoattractant environment. Raft microdomains are usualy 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 Szöllősi, 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 preceeded 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 μm^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 additon, 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 funtioning 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-

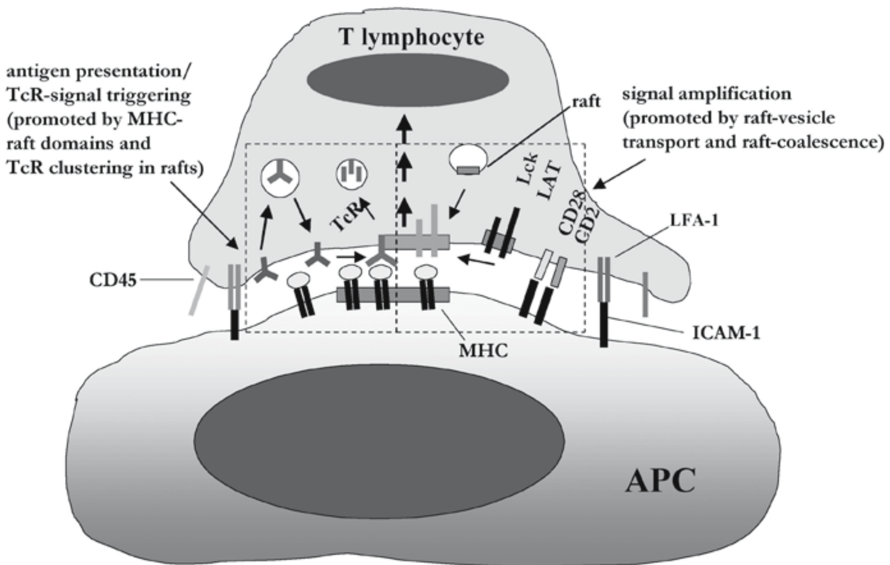

Fig. 3. Raft dynamics in specialized complex structures: the T-cell immunological synapse. Rafts on antigen-presenting cells (APC) by clustering the MHC-peptide complexes and on T cells by clustering the engaged T-cell receptors (TCRs) and coupling them to the signal-machinery (inner leaflet domains 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 immunologial 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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3

Lipid Raft Membrane Skeletons

Elizabeth J. Luna, Thomas Nebl, Norio Takizawa, and Jessica L. Crowley

1. INTRODUCTION

Most cellular plasma membranes are organized into specialized domains that are defined by extracellular cues. For instance, contact with specific extracellular matrix components triggers the formation of focal adhesions and hemidesmosomes at basal cell surfaces (Hahn and Labouesse, 2001; Wehrle-Haller and Imhof, 2002). Similarly, homophilic interactions between cell–cell adhesion molecules create junctional complexes that integrate tissues (Green and Gaudry, 2000; Vasioukhin and Fuchs, 2001; Braga, 2002; Jamora and Fuchs, 2002). Adaptor proteins—which bind simultaneously to cytoplasmic sequences in adhesion molecules, ion channels, and cytoskeletal and signaling proteins—then create protein scaffolds that constitute sites of localized signal transduction. Although the components of these membrane domains can be dynamic, the domains themselves are intra-membrane "landmarks," some lasting for the duration of the cell cycle. Stabilized by multiple strong protein–protein interactions, these "membrane skeletons" resist extraction with nonionic detergents and can, in many cases, be purified to homogeneity (Yu et al., 1973; Skerrow and Matoltsy, 1974; Tsukita, 1989; Slusarewicz et al., 1994).

But, how do highly motile cells—cells that lack stable associations with the substrate or with other cells—create order out of chaotic, or at least transient, signals from the external world? In the fluid mosaic model of membrane structure (Singer and Nicolson, 1972), most of the lipids and proteins

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in the membranes of fast-moving cells (e.g., lymphocytes and neutrophils) would be expected to float free in the membrane "sea." In this model, the primary constraints on lipid and protein distributions are intermittently distributed transmembrane proteins with strong attachments to underlying cytoskeletal proteins. In fact, measurements of lateral diffusion coefficients do support the existence of zones in migrating cells in which free diffusion is restricted by the presence of transmembrane protein "fence posts" associated with underlying membrane skeleton "corrals" (Jacobson et al., 1981; Edidin, 1993; Ritchie et al., 2003).

One approach to establishing organization in the absence of stable external stimuli is the assembly of relatively stable membrane domains through protein–protein interactions that assemble in response to transient or stochastic interactions. For instance, activation of G protein-coupled chemotactic receptors leads to the directional localization of signaling proteins at the plasma membrane (Devreotes and Janetopoulos, 2003). After about 30 minutes of a unidirectional chemotactic signal, these localizations result in differential amplification of subsequent chemoattractant signaling at the "front" vs the "rear" of the migrating cell. Clathrin-coated pits and the membranes of cell surface extensions, such as microvilli and filopodia, provide other examples of dynamic, specialized membrane domains (Bretscher, 1991; Brodsky et al., 2001; Vasioukhin and Fuchs, 2001; Adams, 2002). Additional identifiable, transient domains include those associated with focal contacts and podosomes, actin-containing structures at basal cell surfaces that are implicated in rapid cycles of cell-substrate attachment and detachment (Wehrle-Haller and Imhof, 2002; Destaing et al., 2003; Evans et al., 2003).

Another way to generate transient membrane microdomains is through localized lateral phase partitioning of membrane lipids. As is described extensively elsewhere in this volume (Fielding, 2004; Matkó and Szöll, 2004; Yavin and Brand, 2004), membranes that are enriched in cholesterol, (glyco)sphingolipids, and saturated long-chain glycerolipids have been shown by biophysical methods to contain small areas with a gel-like, liquid-ordered character (Edidin, 2003). Such membrane domains are often called "lipid rafts." Although liquid-ordered bilayers can be assembled in vitro from appropriate mixtures of these lipids (Ahmed et al., 1997; London and Brown, 2000; Dietrich et al., 2001), the organization of such domains in vivo are likely to be controlled by cholesterol-binding proteins (Galbiati et al., 2001; Anderson and Jacobson, 2002; Pike, 2003).

Many families of cholesterol-binding, raft-organizing proteins are known. For instance, the human genome is predicted to encode three caveolins, four stomatins, two flotillins, and podocin. Stomatin is probably the evolutionarily most ancient of these four protein families, with representatives in flies

(*D. melanogaster*), worms (*C. elegans*), yeast (*S. pombe*), fungi (*N. crassa*), and plants (*Z. mays, A. thaliana*). The prohibitins, molecular chaperones in mitochondria and prokaryotes, may have been the ancestral members of all these proteins (Nijtmans et al., 2002). Although different raft-organizing proteins can co-oligomerize or associate closely within the same lipid microdomain (Volonte et al., 1999), cup-shaped, caveolin-enriched caveolae and planar, flotillin-enriched rafts can localize to separate, nonoverlapping regions of the plasma membrane (Ilangumaran et al., 1998).

No one knows how many types of lipid rafts exist. On the one hand, if each human raft-organizing protein assembles a characteristic type of raft, there could be ten different types of rafts. Add to that the possibilities of diversity as a result of alternative splicing and combinatorial effects in rafts containing multiple types of raft-organizing proteins (Galbiati et al., 2001), and at least theoretically, there may be hundreds of distinct types of membrane microdomains. On the other hand, many of the raft-organizing proteins exhibit specific tissue distributions, suggesting a level of functional redundancy. Even mice that lack detectable expression of any of the three caveolins are viable and fertile, although they develop a severe cardiomyopathy (Park et al., 2002).

In vivo, liquid-ordered membrane domains are thought to be quite small (25–75 nm) and consequently below the level of resolution of the light microscope (Jacobson and Dietrich, 1999). Thus, they are optimally investigated with biophysical techniques that can detect short-range interactions. Less technically sophisticated approaches include the induction of clustering of raft components within the plane of the membrane (e.g., with polyclonal antibodies or by receptor activation) and the visualization of membrane components that are resistant to extraction with nonionic detergents, especially Triton X-100 (Simons and Toomre, 2000; Maxfield, 2002; Pike, 2003).

Although alternative strategies have been described (Schnitzer et al., 1995; Smart et al., 1995; Song et al., 1996), lipid raft components are commonly isolated by extracting cells or isolated cell fractions at 0–4°C with 1% Triton X-100, followed by flotation of Triton-insoluble lipoprotein complexes into sucrose gradients and recovery of fractions with buoyant densities of approximately 1.09–1.12 g/mL, which corresponds to about 10–30% (w/v) sucrose (Brown and Rose, 1992; Hope and Pike, 1996). The resulting detergent-resistant membranes (DRMs) are depleted in transmembrane proteins with hydrophobic alpha-helices and enriched in cholesterol-binding raft-organizing proteins and in proteins modified with long-chain fatty acids, e.g., with two or more palmitate groups. DRM-associated proteins anchored to the external surface of the membrane include those containing glycosylphosphatidylinositol (GPI) linkages with long-chain fatty acids.

Many signaling proteins, e.g., Src family kinases and heterotrimeric G proteins, co-isolate both with DRMs and with low buoyant density membrane domains purified in the absence of detergent (Sargiacomo, et al, 1993; Smart et al., 1995; Song et al., 1996; Li et al., 2001). Thus DRMs and, by extension, liquid-ordered domains in biological membranes are thought to be sites of dynamic signal transduction.

The relative contributions of lipid self-assembly and protein-mediated lipid organization to lipid raft structure are still vigorously debated. Anderson and Jacobson propose that detergents potentiate the coalescence of small "condensed complexes" of proteins that contain large numbers of tightly-associated lipids (Anderson and Jacobson, 2002). Linda Pike (Pike, 2003) extends this idea into an "induced-fit model of raft heterogeneity" that is akin to the "hundreds of rafts" concept outlined above. Finally and of special interest for the topic of this chapter, Sean Munro suggests that a great deal of the diversity observed among "lipid rafts" in vivo may be explained by compartmentalization of raft-associated proteins by the underlying cytoskeleton (Munro, 2003).

2. ACTIN AND LIPID RAFTS

The actin cytoskeleton has long been implicated in dynamic events involving liquid-ordered domains. Clustering of membrane skeleton proteins with lipid raft components occurs during the compartmentalized signaling associated with cell activation (Bourguignon and Bourguignon, 1984; Holowka et al., 2000; Simons and Toomre, 2000; Ayala-Sanmartin et al., 2001; Anderson and Jacobson, 2002; Montoya et al., 2002), and actin filament organization plays a role in internalization of raft-associated receptors (Deckert et al., 1996; Ayala-Sanmartin et al., 2001; Stuart et al., 2002). During the redistribution of activated or crosslinked receptors, the receptors co-localize with F-actin, nonmuscle myosin II, fodrin/spectrin, and associated cytoskeletal proteins (Bourguignon and Bourguignon, 1984). Furthermore, disruption of actin filament integrity interferes with lipid raft-associated processes, such as the capping of EGF and insulin receptors (Bourguignon et al., 1988; Khrebtukova et al., 1991), T lymphocyte polarization and activation (Moran and Miceli, 1998; Gomez-Mouton et al., 2001), and down-regulation of FcεRI-mediated signaling in mast cells (Holowka et al., 2000). Defects in myosin I, spectrin, or fodrin function can inhibit redistribution of raft-associated proteins (Dahl et al., 1994; Kwiatkowska and Sobota, 1999; Durrbach et al., 2000; Bose et al., 2002; Pradhan and Morrow, 2002). Receptor redistributions also are inhibited in cells that either lack myosin II (Pasternak et al., 1989; Aguado-Velasco and Bretscher, 1997) or express a

dominant-negative myosin II mutant (Fukui et al., 1990; Burns et al., 1995; Arhets et al., 1998). Taken together, there is a wealth of evidence implicating actin filaments in both the coalescence of lipid rafts and subsequent downstream events during lipid raft-mediated signaling.

While little is known about the biochemical bases for cytoskeletal participation in lipid raft-mediated signaling processes, the involvement of myosins I and II suggest ATP-based movements along tracks of actin filaments. Especially intriguing in this context is the observation that Lyn kinase down-regulation during mast cell signaling is accompanied by the segregation of Lyn along linear actin tracks emanating from central osmophilic patches of aggregated FcεRI receptors (Wilson et al., 2000). The raft-associated GLUT4 glucose transporter also is transported along actin filament tracks (Patki et al., 2001), and insulin-stimulated translocation of GLUT4 to the cell surface depends on the unconventional myosin 1c (Bose et al., 2002). Thus, regulated engagement of myosin motors may function both in moving lipid raft components within the plane of the plasma membrane and in recruitment of signaling proteins to and from internal membrane stores.

3. "HEAVY" DETERGENT-RESISTANT MEMBRANES (DRM-H)

As part of our ongoing studies of actin-based membrane skeletons in highly motile cells, our laboratory recently identified a group of cytoskeletal, signaling, and raft-organizing proteins that co-isolate after extraction of neutrophil plasma membranes with Triton X-100 (Nebl et al., 2002). We used antibodies against fodrin (nonerythrocyte spectrin) and the actin- and myosin II-binding protein, supervillin, to follow this subset of the neutrophil membrane skeleton during fractionation on Optiprep™ and sucrose gradients. We found that fodrin and supervillin exhibited a buoyant density of approximately 1.15–1.18 g/mL during both flotation and sedimentation. Because this membrane skeleton fraction is significantly "heavier" than the "light" detergent-resistant membrane fraction with a buoyant density of approximately 1.09–1.12 that is resolved on the sucrose gradient, we refer to these fractions as "DRM-H" and "DRM-L," respectively.

The DRM-H and DRM-L fractions are both enriched in cholesterol, heterotrimeric $G\alpha_{i\text{-}2}$ proteins, flotillin 2, and Lyn tyrosine kinase—all of which are characteristic lipid raft components (Nebl et al., 2002). Other major raft-associated proteins abundant in the DRM-H fraction include flotillin 1, stomatin, the GPI-anchored membrane type 6 matrix metalloproteinase (MT-6-MMP, leukolysin), and Golgi-associated plant pathogen-

esis-related protein-1 (Salzer and Prohaska, 2001). Fc receptors (Grewal et al., 1978; Worku et al., 1994) also are inferred to be present, based on the tight binding of surface immunoglobulin (Ig) M. While these proteins are also found at DRM-L densities, only DRM-H contains large amounts of cytoskeletal proteins. In addition to actin, fodrin, and supervillin, major cytoskeletal DRM-H proteins are α-actinins 1 and 4, vimentin, nonmuscle myosin II, and myosin 1G, which is a class I unconventional myosin structurally similar to Myo1c. Thus, to a first approximation, the DRM-H fraction can be thought of as a DRM-L fraction attached to a membrane skeleton, or as a "lipid raft membrane skeleton."

The buoyant density of the DRM-H fraction (approximately 1.16 g/mL) is intermediate between that of typical DRM fractions (1.09–1.12; ≤30% sucrose) and the densities (1.25–1.3; 50–64% sucrose) of the well-characterized Triton-insoluble membrane skeletons from human erythrocytes (Sheetz, 1979) and *Dictyostelium discoideum* amoebae (Goodloe-Holland and Luna, 1987). This intermediate density is reasonable, given the large amounts of tightly bound cytoskeletal proteins, which increase the DRM-H buoyant density to that observed for membrane domains with comparable protein-to-lipid ratios.

The intermediate buoyant density of the DRM-H fraction precludes its recovery using standard protocols for purification of DRMs (Brown and Rose, 1992; Schnitzer et al., 1995; Smart et al., 1995; Hope and Pike, 1996; Song et al., 1996). DRMs are usually obtained by floating Triton extracts from cells or purified organelles up through a sucrose step of 30–35% sucrose and collecting the material that bands on top of this sucrose solution, between it and an overlying layer of 5–10% sucrose (Hope and Pike, 1996). Under these conditions, DRMs with buoyant densities of approximately 1.16 g/mL would be either concentrated near the bottom of the centrifuge tube (if using 30% sucrose) or spread throughout a 35% sucrose layer. Thus, gradients of approximately 20% to 45% sucrose solutions are probably required for the identification and recovery of DRMs containing appreciable amounts of associated membrane skeleton proteins. Alternative procedures for generating light membrane fractions involve cell sonication and/or carbonate extraction before flotation into sucrose gradients (Smart et al., 1995). These treatments might release DRM-H lipoprotein components as low buoyant density membranes, but most endogenously associated membrane skeleton proteins would be released by the sonication and/or carbonate pretreatments.

4. DRM-H MEMBRANE SKELETON ARCHITECTURE

As a first step toward understanding the nature of the interactions among DRM-H proteins, we have extracted isolated DRM-H membrane fragments with octylglucoside (Nebl et al., 2002), a nonionic detergent that efficiently solubilizes most liquid-ordered membrane domains (Melkonian et al., 1995; Smart et al., 1995). Except for a small percentage of Lyn kinase, none of the major DRM-H polypeptides are significantly extracted under these conditions. These results indicate that the DRM-H membrane skeleton is stabilized by strong protein–protein interactions in addition to the hydrophobic interactions that presumably also exist.

To determine which proteins are the most proximal to the DRM-H bilayer, we have extracted purified DRM-H membranes with 0.1 *M* sodium carbonate, pH 11.5 (Nebl et al., 2002). This solution disrupts electrostatic interactions and effectively removes most proteins that are peripherally bound to membranes (Hubbard and Ma, 1983). Carbonate extraction removes essentially all actin and α-actinin from DRM-H membranes, approximately 50% of the associated fodrin, and approximately 20–25% of the myosin II and myosin 1G.

Virtually no supervillin or integral membrane DRM-H proteins can be extracted with carbonate. These results suggest that supervillin is the most proximal to the bilayer of the major peripheral DRM-H membrane proteins and that both myosin isoforms can associate with membranes independent of any indirect binding through membrane-associated actin (Fig. 1A).

Although it is not known how supervillin or either of the myosins binds to the DRM-H membrane, it is possible that supervillin is a linker protein for the attachment of both myosin II and F-actin. The amino-terminal 174 amino acids of supervillin bind directly to the regulatory S2 domain of myosin II, and supervillin residues 171–830 contain at least three binding sites for F-actin (Chen et al., 2003). Supervillin sequences promote actin filament bundling and crosslinking, suggesting that supervillin may promote the assembly and/or organization of actin and myosin filaments at liquid-ordered membrane domains. Other regions of supervillin exhibit high degrees of sequence similarity to polyphosphoinositide-binding sequences in the gelsolin/villin family of actin-binding proteins (Pestonjamasp, K. N. et al., 1997; Kwiatkowski, 1999), sequences that mediate gelsolin association with bilayer lipids (Hartwig et al., 1989; Ahmed et al., 1997; He et al., 1997). Regardless of the membrane attachment mechanism(s), all the major DRM-H proteins co-immunoprecipitate with supervillin from neutrophil plasma

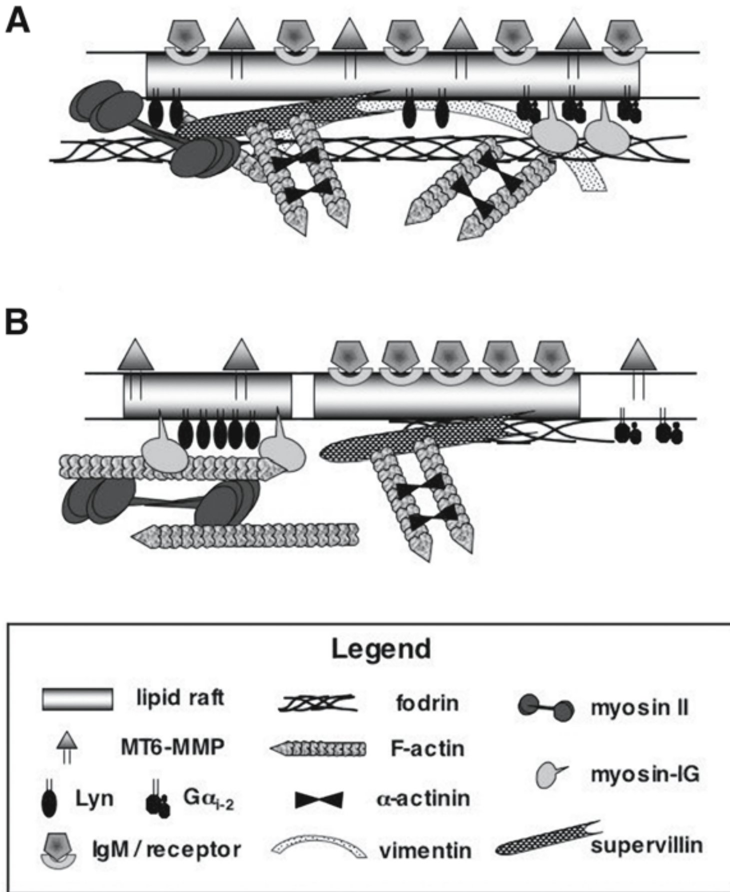

Fig. 1. (**A**) Working model of the DRM-H membrane skeleton. One possible organization of the DRM-H membrane skeleton in neutrophil plasma membranes based on currently available proteomic and biochemical data (Pestonjamasp, K. et al., 1995; Pestonjamasp, K. N. et al., 1997; Nebl et al., 2002; Chen et al., 2003). DRM-H lipoprotein complexes are enriched in cholesterol and contain the integral membrane raft-organizing proteins, stomatin and flotillins 1 and 2 (lipid raft). Additional proteins characteristically found in liquid-ordered domains that are also present include an extracellular GPI-anchored matrix metalloproteinase (MT6-MMP), the intracellular dually acylated non-receptor tyrosine kinase (Lyn), and the transmembrane heterotrimeric G protein ($G\alpha_{i-2}$). Extracellular surface-bound IgM is probably attached though the similarly sized Fc receptor (IgM/receptor). The relatively high buoyant density characteristic of the DRM-H fraction is attributed to the co-isolating membrane skeleton proteins (fodrin, F-actin, α-actinin, vimentin, myosin II, myosin-1G, supervillin). Fodrin, F-actin, and α-actinin are

membranes solubilized with either Triton X-100 or octylglucoside (Nebl et al., 2002). Thus, supervillin is an excellent candidate marker protein for the DRM-H membrane skeleton, because it apparently binds, directly or indirectly, to the other major DRM-H polypeptides through protein–protein interactions. These interactions may be representative of more than one type of DRM-H complex and may be a mixture of stable and transient associations that are regulated during cell signaling.

The buoyant density of the DRM-H fraction and the presence of fodrin, actin, and $G_{i\text{-}2}$ proteins are highly reminiscent of previous work documenting a fodrin-containing membrane skeleton in human neutrophils (Boyles and Bainton, 1979; Jesaitis et al., 1984; Stevenson et al., 1989). This neutrophil membrane skeleton is apparently involved in the lateral organization of superoxide-generating proteins following cell activation by phorbol esters (Quinn et al., 1989) and in the segregation of receptors and $G_{i\text{-}2}$ proteins during chemotactic signaling (Klotz and Jesaitis, 1994). The juxtaposition of these signaling proteins and myosins in the same or overlapping liquid-ordered membrane domains suggests that active motor-driven processes may participate in one or more of these signaling events (Fig. 1B).

The composition of the DRM-H signaling and membrane skeleton proteins suggests that the endogenous region(s) of the plasma membrane from which it derives are involved in lipid raft-based signaling and motility. The presence of Lyn kinase and the (inferred) presence of an Fc receptor are

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Fig. 1. (*continued*) easily extracted from these membranes; myosin II and myosin IG are less extractable, and supervillin apparently binds directly to an integral component of the bilayer. Supervillin also binds directly to F-actin and myosin II. (**B**) Changing membrane skeleton interactions with lipids and/or integral proteins in liquid-ordered domains may potentiate lateral phase separations during signaling processes. In this very hypothetical model, regulated interactions with the membrane skeleton, and/or activation of myosin-mediated translocations of raft components, potentiate re-distribution of rafts or raft components during cell activation and subsequent receptor down-regulation. Redistributions may be translocations of membrane components to or from internal membrane stores, as well as the lateral movements within the plane of the membrane shown here. Translocation of Lyn along F-actin tracks and the release of activated G_i proteins from detergent-insoluble complexes are based on available information (Jahangeer and Rodbell, 1993; Sargiacomo et al., 1993; Huang et al., 1997; Wilson et al., 2000; Li et al., 2001). Although not shown here, signaling events at rafts also may be accompanied by tyrosine kinase-regulated, calpain-mediated cleavage of raft-associated cytoskeletal proteins (Siman et al., 1984; Babiychuk et al., 2002; Nicolas et al., 2002).

reminiscent of the role of similar proteins in IgE signaling during mast-cell activation (Sheets et al., 1999; Draber and Draberova, 2002). Because secreted IgM can bind simultaneously to as many as five Fc receptors (Feinstein and Munn, 1969), additional crosslinking may not be required for initial activation. Signaling downstream of IgM binding to bovine neutrophils is unexplored, but it may resemble signaling cascades present in mast cells and in T (and B) lymphocytes (Cheng et al., 2001; Babiychuk et al., 2002; Sedwick and Altman, 2002; Werlen and Palmer, 2002). As currently understood, these cascades are domino-like sequences of events in which a nonreceptor tyrosine kinase such as Lyn (or Lck or Fyn) is recruited to the raft, which then mediates the tyrosine phosphorylation of receptor subunits and induces the recruitment of Syk/ZAP-70 family tyrosine kinases, which phosphorylate additional raft components and lead to the activation of raft-associated adaptor proteins, phospholipase C, and protein kinase C. T-cell signaling requires actin filament polymerization and integrity for optimal activity (Valensin et al., 2002; Tskvitaria-Fuller et al., 2003), whereas actin filaments promote down-regulation in mast-cell signaling, presumably by promoting the lateral separation of Lyn from the Fc receptor substrate (Holowka et al., 2000; Wilson et al., 2000). Down-regulation of signaling in T cells is thought to involve the binding of Csk kinase to raft-associated Cbp (Csk-binding protein), followed by Csk-mediated inactivation of Lck or Fyn (Kawabuchi et al., 2000).

5. "DRM-H-LIKE" MEMBRANE SKELETONS IN OTHER CELL TYPES

As discussed above, the composition of the neutrophil DRM-H fraction strongly suggests similarities with lipid raft-mediated signaling processes in mast cells and T lymphocytes. This hypothesis is supported by proteomic analyses of total DRMs from Jurkat T lymphoma cells (von Haller et al., 2001), monocytes (Burkart et al., 2003), and Raji B lymphoma cells (Saeki et al., 2003). Fodrin, supervillin, myosin II, vimentin, and actin co-isolate with T-cell DRMs (von Haller et al., 2001); α-actinin 4, vimentin, and actin with monocyte DRMs (Burkart et al., 2003); and myosin II, myosin 1G, myosin 1β, and actin with B-cell DRMs (Saeki et al., 2003). The absence of various DRM-H proteins from these DRM fractions is not surprising, because the isolation procedures were optimized for recovery of DRMs with lower buoyant densities. Thus, available evidence is consistent with a general role for components of the neutrophil DRM-H membrane skeleton in the structure and/or functioning of hematopoietic lipid rafts.

Lipid raft-associated membrane skeletons containing neutrophil DRM-H proteins also may function in nonhematopoietic cells. Myosin II, vimentin, and actin are recovered as components of light DRM fractions from HeLa cells (Foster et al., 2003); erythrocyte DRMs contain spectrin and actin in addition to stomatin, flotillin 1, and flotillin 2 (Salzer and Prohaska, 2001); and low-density membrane fractions from skeletal muscle contain archvillin, a muscle-specific isoform of supervillin (Chen et al., 2003). In fact, if the distribution of supervillin/archvillin can be used as an indicator of the presence of a "DRM-H like" membrane skeleton, then such domains should be abundant in muscle tissues, carcinoma cell lines, and secretory epithelia (Pestonjamasp, K. N. et al., 1997; Pope et al., 1998; Anderson and Jacobson, 2002; Chen et al., 2003).

6. OTHER DYNAMIC LIPID RAFT-ASSOCIATED MEMBRANE SKELETONS

Consistent with the multitude of lipid raft organizing proteins, many types of "lipid raft membrane skeletons" may exist. It is currently unclear whether some membrane skeleton proteins preferentially associate with particular types of lipid rafts, or whether tissue-specific differences in protein expression have led to the diversity of known interactions. For instance, members of the band 4.1/ezrin/radixin/moesin family of actin-binding membrane-skeleton proteins co-purify with DRMs from erythrocytes (Salzer and Prohaska, 2001), B cells (Saeki et al., 2003), T cells (Babiychuk et al., 2002; Tomas et al., 2002), monocytes (Burkart et al., 2003), and HeLa cells (Foster et al., 2003). A potential mechanism for this interaction is through binding of proteins in this family to the NHERF/EBP50 adaptor protein (Na^+/H^+ exchanger regulatory factor/ezrin-binding phosphoprotein of 50 kDa), which in turn binds to raft-associated Cbp (Brdickova et al., 2001; Itoh et al., 2002). The observation that neutrophil ezrin and moesin segregate preferentially with DRM-L, rather than with DRM-H (Pestonjamasp, K. N. et al., 1997), suggests that at least two types of raft-associated membrane skeletons can co-exist in neutrophils.

Annexin II/calpactin 1 may be a marker protein for a third type of lipid raft-associated membrane skeleton, or this protein may be a currently unappreciated component of the DRM-L or DRM-H membrane skeleton. Annexin II, which has been reported in DRM-L fractions (Burkart et al., 2003; Foster et al., 2003), binds directly to F-actin, fodrin, and anionic phospholipids (Gerke and Weber, 1984; Hubaishy et al., 1995; Filipenko and Waisman, 2001). Most annexin binding interactions are potentiated by high [Ca^{2+}] and inhibited by tyrosine phosphorylation, but binding to cholesterol-

rich membranes can occur in the absence of Ca^{2+} (Harder et al., 1997; Ayala-Sanmartin et al., 2001). Treatment of low-density BHK cell membranes with cholesterol-sequestering agents in the absence of Ca^{2+} releases a co-immunoprecipitating complex of annexin II, α-actinin, ezrin, moesin, and actin (Harder et al., 1997). Annexin II also is reported to co-immunoprecipitate with caveolin (Uittenbogaard et al., 2002), the small GTPase Rac1 (Hansen et al., 2002), the tyrosine phosphatase SHP-2 (Burkart et al., 2003), and CD44/H-CAM, the major cell surface transmembrane receptor for hyaluronic acid (Oliferenko et al., 1999). CD44 partitions into low-density membrane fractions (Ilangumaran et al., 1998) and binds directly to members of the ezrin-radixin-moesin family (Hirao et al., 1996; Legg and Isacke, 1998). Annexin II is required for regulated vesicle budding from early endosomes (Mayran et al., 2003), and both annexin II and splice forms of CD44 have been implicated in the control of cell growth and migration (Chiang et al., 1999; Orian-Rousseau et al., 2002; Burkart et al., 2003). Thus, annexin II and CD44 may function in a type of lipid raft that mediates regulated cycles of cell-substrate attachment and plasma membrane recycling during dynamic cell attachment and migration.

A fourth type of raft-associated membrane skeleton may potentiate cellular responses to mechanosensory forces, including changes in extracellular tonicity. GAP-43, a membrane-bound neuronal protein that modulates actin assembly (Meiri and Gordon-Weeks, 1990; Aguado-Velasco and Bretscher, 1997), enhances increases in intracellular calcium ion concentrations ($[Ca^{2+}]_i$) triggered by hypotonic media, at least after overexpression in HEK293 cells (Caprini et al., 2003). Mutation of the palmitoylation sites on GAP-43 that anchor it to lipid rafts abrogates the observed increases in $[Ca^{2+}]_i$. The mechanism apparently includes GAP-43 binding and activation of PLC-δ1, which leads to increased concentrations of diacylglycerol and intracellular inositol-1,4,5-trisphosphate, which in turn causes release of Ca^{2+} from the endoplasmic reticulum. Although it is unclear whether endogenous levels of GAP-43 perform similar functions in neuronal cells, this protein has long been known to be important for axonal growth and synaptic plasticity (Skene, 1989).

Membrane skeletons associated with lipid raft components also may play a role in dynamic cell–cell interactions. In a process that appears to be similar to early stages in the formation of at least some vertebrate adhesion complexes (Vasioukhin et al., 2000), *Dictyostelium discoideum* amoebae initiate cell attachment through what may be a fifth type of lipid raft membrane skeleton. An 80-kDa GPI-linked adhesion protein (gp80), which is sequestered in sterol-rich membrane domains at the tips of cell surface projections, binds through homophilic interactions to an adjacent cell (Harris and Siu,

2002). Binding induces a reorganization of the cell surface, with a flattening of surface extensions into large areas of cell-cell contact (Choi and Siu, 1987; Vasioukhin et al., 2000). In *Dictyostelium*, these mature cell-cell contact regions then recruit the approximately 17-kDa actin-binding and nucleating protein, ponticulin (Wuestehube and Luna, 1987; Chia et al., 1991; Hitt et al., 1994; Harris et al., 2003), which is attached to the membrane through both transmembrane domains and a GPI-anchor (Hitt et al., 1994). Because any direct interaction between gp80 and ponticulin is too weak to detect (Harris et al., 2003), ponticulin may be targeted to rafts through the affinity of its GPI-anchor for sterol-rich membrane domains. In this theory, ponticulin's transmembrane sequences, which are redundant from the perspective of membrane attachment, are required to juxtaposition the F-actin binding site on the cytoplasmic surface of the membrane (Hitt et al., 1994). The high-affinity ponticulin–actin interaction thus constitutes a key linkage between the regions of cell-cell contact and the underlying membrane skeleton, which is rich in myosin II as well as in actin and actin-bundling proteins (Ingalls et al., 1986; Harris et al., 2001).

The *Dictyostelium* contact regions fractionate on sucrose gradients as lipoprotein complexes with densities of 1.16–1.18 g/mL, i.e., approximately that of the neutrophil DRM-H fraction. Similar fractionation behavior is observed regardless of whether the cells are extracted with cold Triton X-100 (Ingalls et al., 1986), or whether low buoyant density membranes are generated by sonication in the absence of detergent (Harris et al., 2001). Although no vertebrate ponticulin sequence is known, a glycosylated protein similar in size to *Dictyostelium* ponticulin has been detected in neutrophil membranes by both immunological approaches (Wuestehube et al., 1989) and direct binding to F-actin (Keresztes and Lajtos, 1997). Because many GPI-anchored proteins participate in lipid raft-based adhesive processes requiring the cytoskeleton (Harris and Siu, 2002), vertebrate membrane skeleton proteins containing both GPI anchors and cytoskeletal attachment sites may yet be discovered.

7. LIPID RAFT MEMBRANE SKELETONS ASSOCIATED WITH "STABLE" MEMBRANE DOMAINS

Many of the morphologically recognizable, presumably more stable plasma membrane domains also may be regulated by lipid raft signaling pathways. For instance, apical surfaces of intestinal epithelial cells contain two types of detergent-resistant lipid rafts (Danielsen and Hansen, 2003). One type localizes to microvilli, where the lectin galectin-4 crosslinks many glycolipids and GPI-anchored extracellular hydrolytic enzymes into stable

"super-rafts." The second type of apically localized lipid raft, which is concentrated in deep apical tubules, is thought to be dynamic and may be associated with a membrane skeleton containing caveolin 1, annexin A2, α-actinin, ezrin, and/or moesin (Danielsen and Hansen, 2003). Thus, the tubule-associated lipid raft may be similar to the annexin II and/or ezrin-associated dynamic rafts discussed above in Section 6.

Cell–cell adhesion and paracellular permeability also may be regulated by lipid raft components. ZO-1 and occludin, two of the major structural proteins in the sealing strands of the tight junctions in polarized epithelial cells, partition in a DRM-L fraction when hyperphosphoryated (Nusrat et al., 2000). Occludin also co-immunoprecipitates with caveolin-1 (Nusrat et al., 2000). Both of these observations are consistent with a role for caveolae and/or rafts in the assembly or function of tight junctions.

Cell–substrate interactions and locomotion in fibroblasts (Shima et al., 2003), as well as in hematopoietic cells (Hogg et al., 2003), are regulated by raft-associated signaling proteins. Furthermore, integrin function at fibroblast focal adhesions is influenced by cholesterol and glycosphingolipid levels (Pande, 2000). Given that GFP-tagged focal adhesion proteins at the rear ends of migrating fibroblasts "slide" along the substrate (Wehrle-Haller and Imhof, 2002), it may be that the major difference between the highly dynamic focal contacts of immune cells and the longer-lived focal adhesions in fibroblasts is the rate at which similar processes occur.

8. CONCLUDING REMARKS

The term "lipid raft membrane skeleton" may at first appear to be paradoxical. "Lipid raft" connotes a dynamic cholesterol-rich membrane domain of low buoyant density, whereas "membrane skeleton" conjures up images of static structures composed of lots of proteins that each bind to multiple other proteins through mostly high-affinity interactions. Yet, the demonstrated association of lipid raft components with cytoskeletal proteins, especially with myosin motors, resonates with the dynamic natures of lipid rafts. Both myosin I and myosin II may participate in lateral movements of lipid raft components within the plane of the plasma membrane. Alternatively, either may function during internalization or trafficking of raft-associated vesicles through the cytoplasm. The binding of myosin II and actin to separable sites on supervillin—which is bound to the DRM bilayer through other, currently unknown sequences—raises the further possibility that supervillin may assemble actin and myosin II at lipid rafts and/or regulate contractility at these membrane domains. Such regulation is presumably controlled by raft-associated signaling events that remain to be investigated. The study of

these signaling processes is inherently complicated by the temporal nature of the interactions, as well as by the potential inherent diversity of raft types. Furthermore, additional types of lipid raft-associated membrane skeletons probably await discovery. These discoveries will be accelerated, though, by appreciating that the attachment of "heavy" cytoskeletal proteins to "light" membranes shifts the buoyant density of the resulting lipid raft membrane skeleton to "heavier" regions of sucrose gradients.

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4

Role of Cholesterol in Membrane Microdomain Signaling

Christopher J. Fielding

1. INTRODUCTION

In monolayers of synthetic mixtures of phospholipids, sphingolipids, and free cholesterol (FC), the spontaneous formation of FC/sphingolipid-rich domains has been shown (Dietrich et al., 2001, 2002). Over the last decade, evidence has accumulated that comparable structures are present in the plasma membranes of living cells (Anderson, 1998; Smart, et al, 1999; Simons and Toomre, 2000; Fielding and Fielding, 2000; Parton, 2003). Because of their lipid composition, FC/sphingolipid-rich membrane domains are insoluble in nonionic detergents (Brown and London, 1998) and on this basis have been purified, together with a characteristic pattern of associated proteins. Flotation of the plasma membrane fraction in the absence of detergent purifies a similar FC/sphingolipid-rich fraction (Smart et al., 1996). More recently, it has become clear that two distinct and nonconvertible kinds of FC/sphingolipid-rich microdomains (lipid rafts and caveolae) (Iwabuchi et al., 1998; Abrami et al., 2001; Sowa et al., 2001) are present in both types of preparations. The two classes are increasingly recognized to play divergent roles at the cell surface.

2. STRUCTURAL PROPERTIES OF LIPID RAFTS

Lipid rafts are planar FC/sphingolipid-rich plasma membrane microdomains. They are enriched in glycosylphosphatidylinositol (GPI)-anchored proteins and lack caveolin, the structural protein of caveolae (see below). Their lifetime, measured by a variety of biophysical techniques in living cells, is of the order of a few seconds or minutes (Table 1). Several studies

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Table 1
Estimates of the Size and Stability of Lipid Rafts

Membrane	Probe	Assay	Diameter	$t_{1/2}$	Reference
Artificial membranes					
PL/FC/GM-1	—	AFM	50–200	—	Yuan et al., 2000
Continuous cell lines					
C3H10T	Thy-1	SPT	100 ± 10	7 ± 1	Sheets et al., 1997
BHK21	HA,PLAP	LOT	270–330	60–120	Pralle et al., 2000
C3H10T1/2	Thy-1	SPT	40–600	5	Dietrich et al., 2002
Jurkat	Lck-GFP	CFM	700	120	Jordan and Rodgers, 2003
T cell	IL-2Rα	CFM	500–700	—	Vereb et al., 2000
Primary cells					
SMC	Lipid	FRAP	600–700	>130	Schutz et al., 2000

Abbr: CFM, confocal microscopy; FRAP, fluorescence resonance; SPT, single particle tracking; FRET, fluorescence resonance; LOT, optical tunneling; HA, influenza virus hemaglutinin; PLAP, placental alkaline phosphatase; AFM, atomic force microscopy; GFP, green fluorescent protein.

have assayed the movement of GPI-anchored proteins or cholera toxin (CT) complexes using continuous cell lines. Raft diameters of 7–150 nm were reported. In some studies, the predicted size of the targeted structure was so small that the existence of rafts could not be unequivocally confirmed. Because of this, some investigators have doubted the existence of lipid rafts in living cells (Kenworthy et al., 2000; Edidin, 2003).

In contrast, Schutz et al. (2000) tagged the Src component of a raft-associated signaling complex in primary smooth muscle cells and reported the presence of relatively large (400 nm diameter) rafts and a more stable association between molecular probe and raft. Structures of similar size have been purified from lymphocytes (Horejsi, 2003). Rafts formed in monolayers of synthetic mixtures of lipids had a diameter comparable to those in living cells (Table 1). The diffusion of individual lipid molecules within the raft was much faster than their transfer out of the raft, and the GPI-anchored protein thy-1 was partitioned into synthetic rafts in much the same way as it was in the membranes of living cells (Dietrich et al., 2001). The lifetime of thy-1 in synthetic lipid rafts has not yet been reported. The present balance of evidence favors the existence of lipid rafts as discrete but transient complexes that may be most developed in primary cells responsive to extracellular signals.

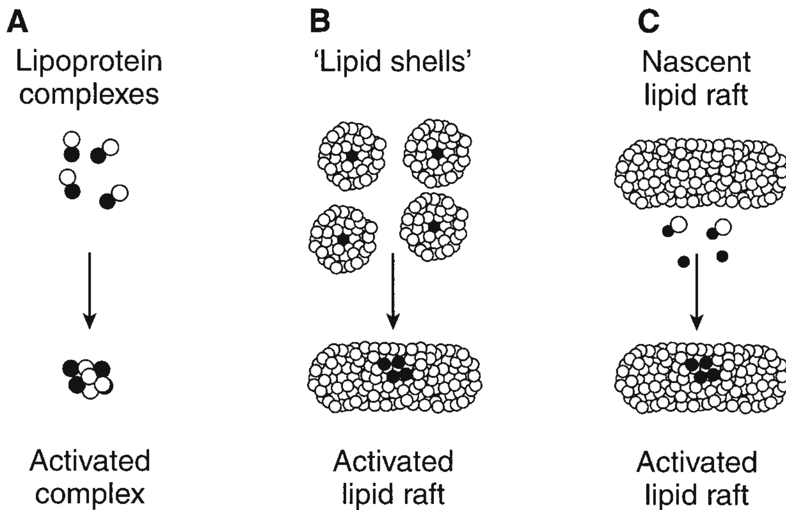

Fig. 1. Lipid–protein relationships in three models of lipid raft. Left, small lipid–protein complexes made up of subunits containing lipids directly bound to protein sites. These complexes would be too small to detect by optical techniques. Center, "lipid shells" made up of proteins associated with shells of approximately 40 lipid molecules which when combined, form rafts of a size comparable to those detected optically (Table 1). Right, large rafts (of a size comparable to those detected optically) to which lipid-free or lipid-poor signaling proteins bind to form signaling complexes. Note that in each model, protein–protein complexes must be formed for effective signal transduction.

Controversy has also arisen about other properties of lipid rafts, including the type of lipid-protein association involved. Broadly, three kinds of structure can be defined (Fig. 1). In the first structure, rafts would be formed from signaling protein molecules to which individual lipid molecules (FC or possibly SPH) remained tightly bound. The temporary association of these proteins in a signaling cassette would be stabilized by both lipid–protein and protein–protein bonds. As a result of kinase activity, the bonds between proteins would weaken and the complex dissociate. Lipid rafts defined in this way would be small (with a diameter of a few protein molecules only) and short-lived (associated for only the time required for signal transduction).

A second model of lipid rafts envisages FC/sphingolipid-enriched membrane patches, stabilized by lipid-lipid bonds and similar in size and composition to those found in FC/sphingolipid-rich lipid mixtures, lymphocytes, and SMC (Table 1). These would interact, within or at the edge of the raft, with lipid-free or lipid-poor proteins. Association between raft lipids and

proteins might also depend on reversible palmitoylation at specific protein sites (Robbins et al., 1995; Acconcia et al., 2003; Fragoso et al., 2003; Hiol et al., 2003). The lifetime of these structures would also be limited by the progress of signal transduction. If structures like this formed a significant proportion of the lipid rafts in living cells, the inability of investigations to detect them by biophysical techniques might be explained by an inability of tagged GPI-anchored proteins to incorporate effectively into rafts in living cells. It is also possible that transformed cell lines, like those used in such studies, contain a preponderance of small rafts, whereas terminally differentiated primary cells (including SMC and lymphocytes) might contain mainly large rafts. Finally, some GPI-anchored proteins might be associated with small rafts, and others with larger structures. An equilibrium of these forms in living cells has not been excluded. Evidently much more information is needed on the properties of lipid rafts, particularly those associated with signaling complexes in primary cells.

A third and intermediate model was recently proposed. The "lipid shell" hypothesis suggests the preferential association of a circumferential, semi-stable band of 40–50 lipid molecules around the periphery of signaling proteins in rafts (Anderson and Jacobson, 2002). This value is both larger than the number likely to bind directly to the proteins in well-characterized signaling complexes, and much smaller than the number of molecules in raft structures of the sizes shown (Table 1). No direct data for this concept appears at the present time, but a contribution of such structures to the total lipid raft population cannot be excluded.

A representative lipid raft-signaling complex, that formed between interleukin 6 (IL-6) and its receptor (IL-6R) in T cells, is shown in Fig. 2. Ligand binding induces dimerization of the receptor. This may involve the induced fusion of discrete rafts containing single subunits. This dimerization is followed by recruitment of linker proteins (such as SH2) and components of the Jak/Stat pathway. Phosphorylation of Stat-3 by the nonreceptor kinase Jak leads to its dissociation from the signaling cassette and transfer to the nucleus, where it acts as a transcription factor in combination with specific enhancers (Darnell et al., 1999; Heim, 1999; Sehgal et al., 2003).

It seems likely that ligand binding would be the major driving force for complex formation within lipid rafts, whereas kinase activity, particularly the phosphorylation of Stat-3, would drive dissociation. If this idea is correct, the mechanism of signal transduction in rafts must depend on a carefully contrived balance between hydrophobic and electrostatic forces.

The structure and properties of lipid rafts are highly dependent on the FC content of the plasma membrane. FC depletion by β-methyl cyclodextrin is generally associated with dissociation of signaling proteins, but this has a

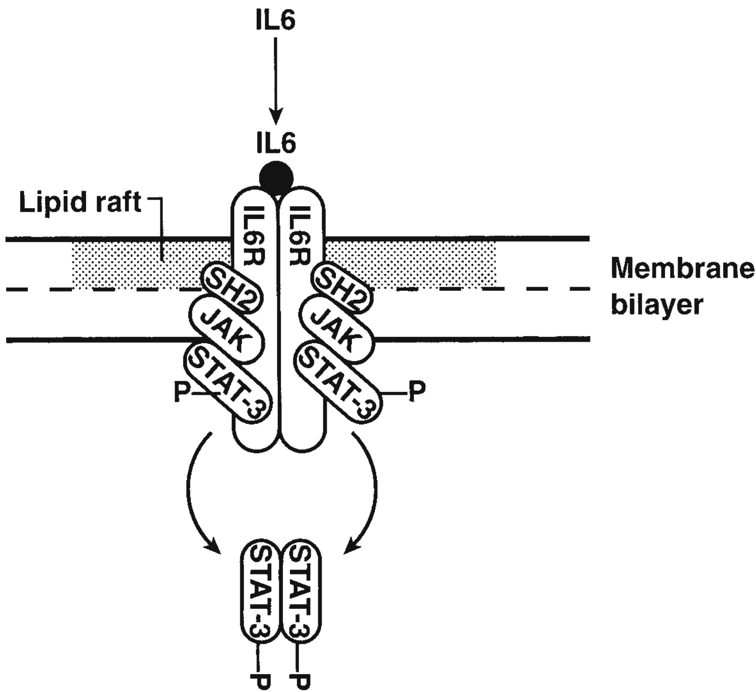

Fig. 2. The IL-6 receptor complex, a signaling complex present in lipid rafts. The FC/sphingolipid-rich domain is located within the exofacial leaflet of the membrane bilayer. The dimerized receptor (IL-6R) is part of a transmembrane complex which includes a nonkinase linker protein (SH2) as well as nonreceptor kinases Jak and Stat-3. Phosphorylated Stat-3 is transported to the nucleus to activate transcription of IL-6 dependent genes.

variable effect on signaling (Table 2). Several mechanisms could contribute. FC may modulate attractive forces between reversibly palmitoylated signaling proteins and lipids in rafts (Moffett et al., 2000; Fragoso et al., 2003). Some raft proteins may contain specific FC binding sites. More generally, FC within rafts may optimize the tertiary structure of raft-binding proteins for effective kinase activity. In support of such a conclusion, FC depletion led directly to IL-6 receptor shedding from its signaling complex (Matthews et al., 2003).

3. STRUCTURAL PROPERTIES OF CAVEOLAE

Caveolae are FC/sphingolipid-rich invaginations of the cell surface (typically with a diameter of 60–90 nm). They are defined by their access to the extracellular space, and by the presence of structural proteins of the caveolin

Table 2
Effects of FC on Signal Transduction from Caveolae and Lipid Rafts[a]

Cell	Cav/raft	Kinase(s)	Effect of FC ↓	Ref.
HEp2	raft	EGFR	act	Ringerike et al., 2002
T cell	raft	LFA-1	inhib	Marwali et al., 2003
Dendrite	raft	AMPAR	inhib	Hering et al., 2003
T cell	raft	LAT[d], I3K, PLCγ	inhib	Inoue et al., 2002
T cell	raft	IL2R	inhib	Matkó et al., 2002
T cell	raft	CD38, Akt	inhib	Zubiaur et al., 2002
HIRcB	cav	IR-dep MEK, MAPK	inhib	Rizzo et al., 2001
Cos	cav	Hh, Ptc	inhib	Karpen et al., 2001
Fibros	cav	PP2A/HePTP/ERK	inhib	Wang et al., 2003
GPAEC	cav	P2Y	inhib	Kaiser et al., 2002
HeLa, Cos	cav	Neu3	inhib	Wang et al., 2002
Rat-1	cav	ERK	act	Furuchi and Anderson, 1998
3T3L1	cav	IR	inhib	Parpal et al., 2001
Fibro	cav	AR	act	Rybin et al., 2000
BAEC	cav	ERK[e]	inhib	Park et al., 1998
SMC	cav	PDGFR(mitogen)	inhib	Stehr et al., 2003
SMC	cav	PDGFR (c-Src)	act	Fielding et al., 2003
SMC	cav	ETHR, VPR[c]	inhib	Dreja et al., 2002
BAEC	cav	VEGFR	act	Labrecque, et al., 2003
SMC	cav	EGFR(AT2)[b]	inhib	Ushio-Fukai et al., 2001

[a]Signaling effects on glucose transport, but not on mitosis.
[b]Signaling effects via angiotensin-2 (AT2) but not via protein kinase B.
[c]ETHR, endotheliin-1; VPR, vasopressin.
[d]Linker activation.
[e]In the same study, FC depletion had no effect on JNK activation.

family (Smart et al., 1999). Unlike lipid rafts, caveolae are depleted of GPI-binding proteins (Abrami et al., 2001; Sowa et al., 2001). Caveolins are well-conserved, low-molecular-weight proteins (18–22 kDa) with a hydrophobic central domain that includes both protein and lipid binding sites. Caveolin is recovered from detergent-extracted cells in the form of polymeric aggregates (Sargiacomo et al., 1995; Monier et al., 1996). It is unclear if these represent intact protein skeletons of caveolae, or extraction artifacts. The C-terminus of the protein plays the major role in the formation of oligomeric caveolin aggregates (Schlegel and Lisanti, 2000).

The recovery of caveolin in a membrane vesicle need not imply it originated from caveolae. Caveolins are also present in other cell fractions. These

fractions include weakly acidic recycling endosomes (Gagescu et al., 2000), trans-Golgi associated vesicles (TGN) (Fielding and Fielding, 1996), and lipid droplets (Ostermeyer et al., 2001). Each is FC rich (Bretscher and Monroe, 1983; Gagescu et al., 2000; Tauchi-Sato et al., 2002). The distribution of caveolin may mainly reflect this property. Alternatively, caveolin vesicles may play a more active role in FC redistribution and transport (Smart et al., 1996). A complex with FC consisting of caveolin and chaperone proteins has also been described (Uittenbogaard et al., 1998), and the suggestion has been made that this complex actively transports newly synthesized FC from the endoplasmic reticulum either to the TGN (at which point it would enter a vesicular transport pathway) or directly to cell surface caveolae. Caveolin-containing vesicles are formed via GTP-dependent fission during membrane fractionation (Oh et al., 1998); but whether or not these are essential transport pathways in the living cell is uncertain.

Like lipid rafts, caveolae are enriched in signaling proteins. Kinases involved in transmembrane signaling in response to growth factors, and integrins contributing to cell attachment are both enriched in caveolae, consistent with the role proposed for these structures. Also consistent with this view, caveolae are reduced or absent in transformed or immortalized cell lines and many cancer cells, where growth is uncontrolled and the cells are poorly adherent and relatively independent of extracellular growth factors (Lavie et al., 1998; Lee et al., 1998). In contrast, cytokine and chemokine receptors are commonly present in lipid rafts (Horejsi, 2003).

Recent data indicate caveolae, unlike lipid rafts, to be stable components of the plasma membrane with a half-life of hours or days (Thomsen et al., 2002). This finding also argues against a major role for caveolae in endocytosis. The stability of caveolae at the cell surface may be related both to the palmitoylation of caveolin (Lee et al., 2001) and to caveolin–caveolin interactions. The irreversibility of caveolin palmitoylation (Parat and Fox, 2001) thus argues against caveolin recycling between internal compartments and the cell surface.

Many caveola-associated signaling proteins (including caveolin itself) contain a "scaffold" motif in the primary sequence ($\varphi xxxx\varphi xx\varphi$, where φ is an aromatic amino acid—W, F, or Y—and x is any amino acid) (Smart et al., 1999) although some caveola-associated proteins lack this motif (Fielding and Fielding, 2000). The extent to which the FC and protein binding sites of caveolin are independent entities, or reflect linked and possibly overlapping domains mediating the changes in both lipid and protein binding that occur in the course of signal transduction, remains to be determined. In caveolae, the caveolin scaffold motif and acylation combine to regulate the protein composition. A reduction of palmitoylation in senescent cells mediates the

transfer of caveolin (and caveolae) from the cell surface to intracellular vesicles (Wheaton et al., 2001).

Caveolae, like lipid rafts, are very sensitive to membrane FC levels. Loss of FC from caveolae occurs by diffusion, rather than by active transport. It may be facilitated by caveola-associated lipid-exchange proteins such as SR-BI or MR-1 (Gu et al., 2000; Garrigues et al., 2002). The major physiological FC acceptor is probably plasma high-density lipoprotein (HDL). Recent research suggests HDL can bind directly to caveolae (Chao et al., 2003). However, FC transfer to other acceptors, including β–methyl-cyclodextrin and the lipid-free HDL protein apolipoprotein A-1 (apo A-1) has also been demonstrated (Fielding et al., 2000). Chronic loss of FC from caveolae is associated with flattening of cell surface and the phosphorylation (at Y_{14}) and internalization of some or all of their caveolin to intracellular FC-rich membranes (Nomura and Fujimoto, 1999). It has not yet been established whether similar change accompanies the transient loss of FC from caveolae observed during signal transduction (Fielding et al., 2002) (*see* Section 6). When cell-surface FC was increased using low-density lipoprotein or FC-rich lipid vesicles, recruitment of additional caveolin to the cell surface from intracellular pools occurred. At the same time, the structure and biological activity of bound signaling complexes was modified. The possibilities of regulated interaction between different signaling proteins within caveolae, between these proteins and caveolin, and between both and lipids, offers considerable scope for increased biological control and selectivity, compared to lipid rafts.

A model for the structure of caveolin in a representative signaling module is shown in Fig. 3. Propinquity between the binding sites of FC and signaling proteins (aa 82–102) and phosphorylation by bound signaling proteins of caveolin Y_{14} suggests significant tertiary structure in the N-terminal half of the molecule.

4. CROSSTALK BETWEEN CAVEOLAE AND LIPID RAFTS

Data reviewed above suggests that while lipid rafts and caveolae are both FC/sphingolipid rich, and whereas both are involved in transmembrane signaling, they normally mediate different signals. There is no evidence that caveolae and lipid rafts are interconvertible. The much greater stability of caveolae seems to preclude this. Nevertheless, functional interaction between caveolae and rafts has been demonstrated. In 3T3 cells, the distribution of PDGF and EGF receptors between caveolar and noncaveolar membrane fractions depended on the FC level of the plasma membrane (Matveev

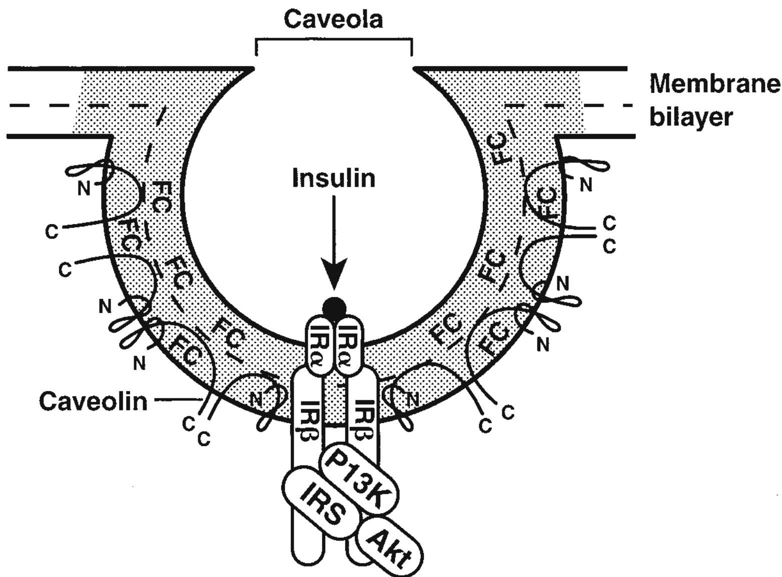

Fig. 3. The insulin receptor complex, a signaling complex localized to caveolae. The complex is located within the caveola. Interactions of signaling proteins both with each other and with caveolin are indicated. The insulin receptor (IR) protein is cleaved post-translationally into external (α) and internal (β) chains crosslinked by cystine bridges. Also shown are the insulin receptor substrate (IRS), phosphatidylinositol-3-kinase (PI3K) and AkT (protein kinase B/AkT), components of the insulin signaling pathway.

and Smart, 2002). Finally in CHO-K1 cells, an inverse relationship was identified between caveolin-1 levels and the expression of GPI-anchored proteins in lipid rafts. Downregulation of one class was associated with an upregulation of the other, reflecting crosstalk between the two classes of FC-enriched domains (Abrami et al., 2001). These data show that differences in the properties of lipid rafts and caveolae may in some cases be exploited to regulate signal transduction. They also show that albeit imperfectly, lipid rafts can substitute for caveolae as signaling platforms in caveolin-deficient cells.

5. CHOLESTEROL DEPENDENCE OF SIGNALING FROM CAVEOLAE

It was originally thought that caveolae served mainly as reservoirs of inactive signaling proteins to be released and activated on demand. According to this hypothesis, an increase in membrane FC should promote the movement

of such proteins into caveolae, whereas FC depletion would release them and activate signal transduction. It is now clear that a much more complex situation exists (Table 2). FC activates some signaling pathways and inhibits others. The content of FC in caveolae is also a major determinant both of the composition of signaling complexes in caveolae, and of their effectiveness in signal transduction. Where branching pathways in the cell are activated by the same transmembrane receptor kinase, FC may differentially promote one pathway at the expense of others. In addition, FC can influence the recruitment of signaling proteins from rafts to caveolae.

In most studies, depletion of caveolar FC was carried out using the synthetic sterol acceptor, β-methyl cyclodextrin (Table 2). In a few, apo A-1 or HDL itself were utilized. Aqueous diffusion is likely to represent the only mechanism of transfer to cyclodextrins, which do not bind to the cell surface. The reaction of HDL with caveolae (and possibly also lipid rafts) probably involves ATP-independent facilitated transfer via FC-transfer proteins such as SR-BI or MDR-1 (Gu et al., 2000; Garrigues et al., 2002) as well as simple diffusion. In the case of lipid rafts, except for one study, FC depletion led to inactivation of signal transduction. In contrast, in the case of caveolae, the response was much more variable. In some cases, the same signaling pathway was activated or inhibited by FC in different cell lines (Table 2). In other studies, using the same cell line, FC activated or inhibited the response of the same receptor kinase to different downstream signaling proteins. For example, FC depletion stimulated the effect of PDGF on SMC to activate c-Src, while inhibiting mitosis directly by the ERK1/2 pathway. FC reduced IR signaling to glucose transport, but was without effect on growth. Finally, EGFR signaling to PK-B was unaffected by FC, whereas signal transduction dependent on AT-2 was inhibited.

In a limited number of studies, the FC content of caveolae and lipid rafts was increased beyond normal physiological levels, either physiologically in vascular cells from hypercholesterolemic animals, or in vitro via FC-loaded liposomes, or low-density lipoprotein (LDL). FC-rich liposomes increased the activity of ICAM-1 in HUVEC via the activity of transcription factor AP-1 (Yuan et al., 2001). The same cells increased cell membrane FC levels and the activity of H-Ras in response to LDL (Zhu et al., 2000). In SMC, FC inhibited PGDF-dependent MAP kinase activity but increased G-protein dependent PGI2 synthesis in SMC (Pomerantz et al., 1997 a,b) even though both pathways are localized to caveolae in SMC (Spinsi et al., 2001). FC loading increased the activity of nitric oxide synthase in BAEC (Peterson et al., 1999), whereas FC depeletion had the opposite effect (Shaul, 2003). However, hypercholesterolemic human plasma or purified LDL decreased the production of e-nos from BAEC and promoted formation of an inactive complex

of the enzyme with caveolin. These effects were probably mediated by LDL oxidation products (oxysterols and/or oxidized phospholipids), which displace FC from caveolae.

6. DYNAMIC REGULATION OF FC IN CAVEOLAE

The relative stability of caveolae at the cell surface, in addition to their content of FC transfer proteins, raised the possibility that in these structures (unlike lipid rafts) the level of FC could be dynamically regulated; that is, that during sequential steps, the level of FC could be transiently raised or lowered to refine the magnitude, duration, and selectivity of signal transduction. Effects of this kind have recently been described for the activities of PDGF and EFG within caveolae.

The reaction of PDGF with its cell surface receptor (PDGFR) within caveolae stimulates multiple pathways regulating growth and apoptosis via signaling pathways including PI3K, c-Src, phospholipase Cγ, and others. As in the case of the insulin receptor, ligand binding to PDGFR stimulates dimerization, autophosphorylation, and PDGFR kinase activities with multiple substrates. In 3T3 cells, PDGF receptors (PDGFR) in the absence of ligand were located in a lipid raft fraction characterized by the presence of GPI-anchored proteins and absence of caveolin. In the presence of ligand, dimerization was associated with a transfer to caveolae (Matveev and Smart, 2002). The early steps of activation of PDGF in SMC were accompanied by a rapid but transient loss of a major part of caveolin-associated FC (Fielding et al., 2002). Within 7.5–15 min, almost the whole of caveolin-associated FC was transferred to noncaveolar pools or (in the presence of an extracellular acceptor) out of the cell (Fielding et al., 2002). This change was associated with a stimulation of PDGF-dependent protein kinase activity that depended upon FC depletion. Following PDGFR-mediated kinase activity and the phosphorylation of downstream kinases, the activated dimer was transferred out of the caveolae for recycling and degradation via the coated pit mechanism (Sorkin et al., 1991) (Fig. 4). If the loss of FC was inhibited, the signal did not proceed beyond the activation of PDGFR. On the other hand, FC was without effect on later stages of signal transduction. Together these data suggest that transient and reversible FC delipidation played a key role in effective signaling from caveolae by PDGFR.

In the case of EGF, loss of FC increased the level of accessible EFGR and promoted its autophosphorylation (Pike and Casey, 2002). However, FC was without effect on the dephosphorylation of the activated receptor, whereas EGFR-dependent phosphatidylinositol turnover (reflecting activity of the PI3K pathway) was inhibited (Pike and Miller, 1998). These changes were

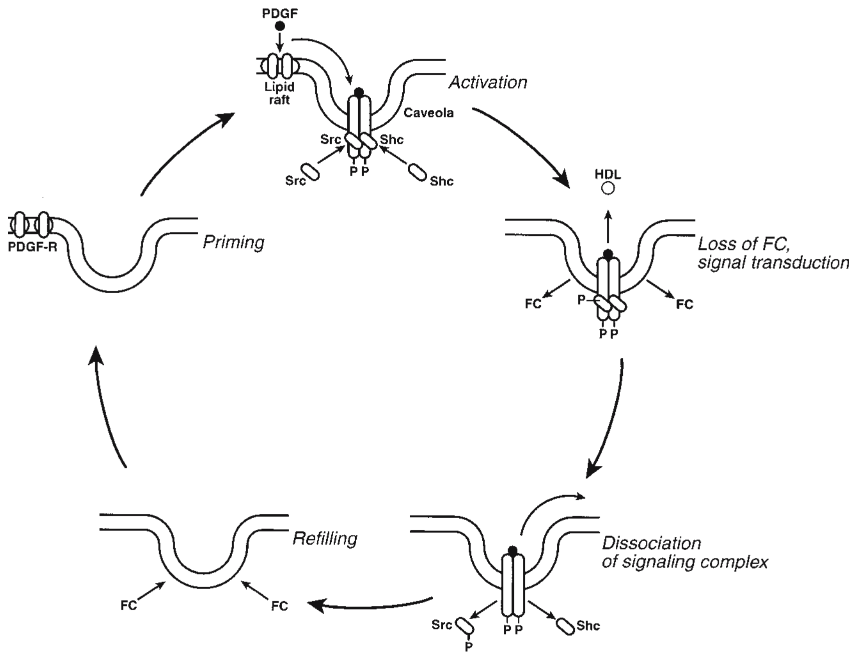

Fig. 4. A model of changes in caveola FC content associated with signal transduction via the PDGF receptor. The figure shows recruitment of PDGF units from lipid rafts, dimerization and activation within caveolae associated with loss of FC, signal transduction and loss of the PDGF receptor from the caveola, and recovery of FC to the caveola.

induced by cyclodextrin. It would be of interest to determine whether spontaneous changes in the level of EGFR-associated FC could be measured under the same conditions.

7. SUMMARY

Newly available data from a limited number of signaling cassettes in caveolae and possibly lipid rafts suggest complex interplay between local FC levels and the rate and selectivity of component kinase reactions, which may contribute significantly to the fine-tuning of cellular responses from extracellular receptor kinases.

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5

Raft Lipid Metabolism in Relation to Alkyl-Lysophospholipid-Induced Apoptosis

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1. INTRODUCTION

Lipid rafts are membrane microdomains occurring mainly (but not exclusively) in the plasma membrane. They show a distinct lipid packing and fluidity compared to the bulk of the plasma membrane. Membrane fluidity is inversely related to the degree of packing of the various apolar acyl and sphingoid chains of phospho- and (glyco)sphingolipids with cholesterol (van Blitterswijk et al., 1987). Lipid rafts have low fluidity (high rigidity) and can only exist by virtue of tight physical interactions between sphingolipids and cholesterol. It has long been recognized that membrane fluidity affects critical cellular processes such as ligand–receptor interactions, endocytosis, antigen presentation, and functional coupling of occupied receptor via G-proteins to effector enzymes.

Lipid rafts are assembled in the trans-Golgi network (TGN). To ensure correct raft assembly, the metabolism and spatial recruitment of the typical raft lipids, sphingolipids and cholesterol, together with inclusion the proper proteins at these sites, must be tightly coordinated. This creates a microenvironment for these raft-associated proteins to ensure spatial cellular control.

Synthetic alkyl-lysophospholipids such as 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (Et-18-OCH_3; Edelfosine; ALP), hexadecylphosphocholine (HePC; Miltefosine), and its piperidine analog D-21266 (Perifosine) induce apoptosis in many types of tumor cells and are used as anti-cancer agents in the clinic. Unlike other anti-cancer treatments, this new

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class of lipid-based anti-cancer agents acts at the level of cell membranes rather than at DNA, interfering with signal transduction (Ruiter et al., 1999, 2002, 2003) and phospholipid metabolism (Wieder et al., 1993; Boggs et al., 1995; Posse de Chaves et al., 1995; van der Luit et al., 2002, 2003).

This chapter discusses the preferred accommodation of exogenous ALP in lipid rafts and the requirement of internalization of these microdomains in order to exert the anti-cancer action of ALP inside the cell. In addition, we discuss the intriguing finding that tumor cells that develop resistance to ALP show a cross-resistance to other types of apoptotic stresses. This cellular phenotype may be causally related to an altered lipid composition and functioning of the lipid rafts.

2. BIOPHYSICAL BASIS OF LIPID INTERACTIONS IN LIPID RAFTS

Based on a systematic steady-state fluorescence polarization study using the probe diphenylhexatriene on a large variety of liposome compositions, we previously defined the mutual interactions (affinities) between individual lipids in biological membranes in quantitative terms (van Blitterswijk et al., 1987). The principal determinant of membrane fluidity is the content of cholesterol. In general, cholesterol increases the structural order (molecular packing) of the membrane glycerophospholipids, causing membrane rigidization (decreased fluidity). This condensing effect of cholesterol has been shown to depend on the molecular species of the lipids involved as well as the degree of unsaturation in the fatty acyl chains, particularly those of the glycerophospholipids. The condensing effect of cholesterol is highest in association with a high content of sphingomyelin (van Blitterswijk et al., 1987).

By nature, sphingolipids confer a high degree of structural order to a membrane because they contain a rigid sphingoid backbone and an acyl chain that is typically saturated. On sphingolipids proper, the effect of cholesterol is opposite to that on acylated glycerophospholipids: It has a small fluidizing effect and a “dissolving” function, creating a liquid-ordered (still relatively rigid) phase, with sufficient plasticity to still allow some degree of lateral mobility of the individual molecules, in comparison with sphingolipid clusters without cholesterol. The preferred association of cholesterol with sphingolipids is mainly based on the high mutual affinity of their rigid hydrophobic regions, strengthened by hydrogen bonding between the 3'-OH group of the sterol and the amide function of the sphingolipid ceramide backbone. Cholesterol partitions preferentially into this liquid-ordered phase (lipid rafts), as compared to the liquid-disordered phase of the bulk of the membrane, and it is essential for preservation of the two

different lipid phases in cellular membranes (Brown and London, 2000; Simons and Ikonen, 2000).

To create raft-like microdomains in cell membranes, both sphingolipids and cholesterol are required. Extraction of cholesterol from lipid rafts stiffens these domains (converting into a solid gel phase) and perturbs raft-associated (protein) functions. Disruption of lipid rafts can be achieved by cholesterol extraction using cholesterol sequestering agents such as methyl-β-cyclodextrin (MβCD) or by cholesterol chelators such as filipin or nystatin. Yet another technique to disorder lipid rafts is to break down sphingomyelin by exogenous (bacterial) sphingomyelinase (SMase), which impedes the physical association of SM with cholesterol and may result in the release of cholesterol from the membrane toward an extracellular acceptor (e.g., MβCD). The ceramide that is formed by SM hydrolysis associates less well with cholesterol, which is therefore more readily extractable by MβCD (Tepper et al., 2000). This effect may have consequences for the physical properties and functioning of the lipid raft.

2.1. Alkyl-Lysophospholipid (ALP) but Not Acyl-Lysophospholipid Partitions in Lipid Rafts

The presence of an alkyl group, with an ether linkage instead of an ester linkage at position *sn*-1 or *sn*-2 (or both) of a glycerophospholipid molecule considerably changes the behavior of these lipids in monolayers. Such lipids have a great tendency to aggregate, and mixing with cholesterol leads to condensed, sterol-enriched domains. These domains are less sensitive to cholesterol oxidation (Mattjus et al., 1996) and exhibit the physical properties of lipid rafts. The alkyl-lysophospholipid, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (Et-18-OCH_3; ALP), possessing two ether bonds, readily incorporates into existing lipid rafts (van der Luit et al., 2002, 2003). Contrary to ALP, and despite the high structural similarity to ALP, lyso-phosphatidylcholine (lysoPC) is excluded from lipid rafts. This finding implies the importance of ether bonds in lyso-phospholipids, such as in ALP, for partitioning in lipid rafts. Albumin back-extraction experiments suggest that lysoPC added exogenously to cells readily inserts in the plasma membrane outer leaflet and then undergoes transbilayer movement (flipping) in membrane regions outside of lipid rafts prior to acylation to PC (van der Luit et al., 2003).

3. INTRACELLULAR TARGETS OF ALP

ALP differs from conventional cytotoxic drugs with respect to its intracellular targets. The ether bonds in ALP are resistant to hydrolysis and acyla-

tion by phospholipases and acylases, respectively. This results in a persistent accumulation of ALP in cellular membranes, which in turn interferes with the rapid and continuous phospholipid turnover that is essential for cell survival (Wieder et al., 1993; Zhou and Arthur, 1995). This interference affects cell signal transduction pathways directly: Et-18-OCH_3 and HePC inhibit phosphoinositide-specific phospholipase C and consequent formation of the second messengers diacylglycerol and inositol 1,4,5-triphosphate (Seewald et al., 1990; Überall et al., 1991; Powis et al., 1992). Furthermore, Et-18-OCH_3 and HePC have been shown to inhibit phosphatidylcholine (PC) turnover at the level of PC degradation as well as PC resynthesis (Wieder et al., 1993; Boggs et al., 1995; Posse de Chaves et al., 1995; Van der Luit et al., 2002, 2003). We and others have shown that the latter inhibition occurs at the level of CTP:phosphocholine cytidylyltransferase (CT), the rate-determining step in PC biosynthesis (Geilen et al., 1992; Baburina and Jackowski, 1998; Van der Luit et al., 2002; Figs. 1 and 2).

These disturbing effects of ALP on lipid metabolism have further consequences for downstream signaling events, including inhibition of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway (Powis, 1995; Zhou et al., 1996), activation of the pro-apoptotic, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling pathway (Mollinedo et al., 1994; Ruiter et al., 1999) and, as we reported more recently, inhibition of the Akt/protein kinase B (PKB) survival pathway (Ruiter et al., 2003). These effects likely contribute to a change in the balance between pro- and anti-apoptotic signaling (Ruiter et al., 2001). Indeed, ALPs are potent inducers of apoptosis in a variety of tumor cell lines (Mollinedo et al., 1993; Diomede et al., 1994; Mollinedo et al., 1997). In addition, ALPs enhance radiation- and chemotherapy-induced cytotoxicity in a synergistic fashion (Stekar et al., 1995; Pauig and Daniel, 1996; Berkovic et al., 1997; Ruiter et al., 1999; Ruiter et al., 2001). As ALPs readily accommodate into lipid rafts, and because many signaling events are determined by proteins that reside in or are recruited to these microdomains, ALPs may well affect their mode of action.

4. MECHANISM OF ALP INTERNALIZATION: ENDOCYTOSIS VIA LIPID RAFTS

PC biosynthesis occurs predominantly via the Kennedy pathway, in which the conversion of phosphocholine to CDP-choline, catalyzed by CTP: phosphocholine cytidylyltransferase (CT), is the rate-limiting step, and the condensation of CDP-choline with diacylglycerol by choline phosphotransferase constitutes the final step (Fig. 2). Upon activation, CT translocates from

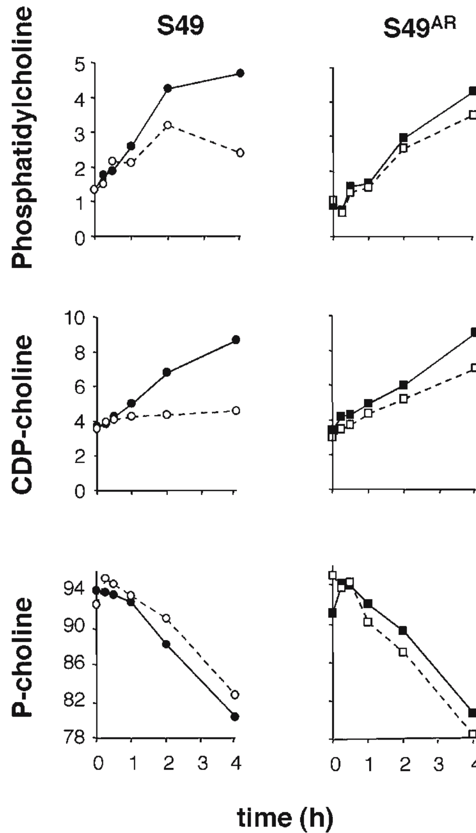

Fig. 1. Effect of ALP on the incorporation of ^{14}C-choline in the *de novo* biosynthetic pathway of phosphatidylcholine (PC) of ALP-sensitive S49 cells and ALP-resistant S49^{AR} cells. ^{14}C-choline incorporation into phosphatidylcholine (upper graph), CDP-choline (middle graph), and phosphocholine (lower graph) was determined as a function of time after chasing with 100 μ*M* unlabeled choline HCl, in S49 (circles) and S49^{AR} lymphoma cells (squares), in the absence (closed symbols) or presence (open symbols) of 15 μ*M* ALP (Et-18-OCH_3). Values in the upper panel are in ^{14}C-arbitrary (PhosphorImager) units as fold increase, whereas values in the lower two panels are expressed as percentage of total ^{14}C partitioning in the inorganic phase of cell extracts.

the cytoplasm to the ER (Clement and Kent, 1999). Brefeldin A treatment of chinese hamster ovary cells expressing a tagged cholinephosphotransferase resulted in a redistribution of this enzyme from a large punctate region to a more diffuse region (Henneberry et al., 2002) indicative for a Golgi local-

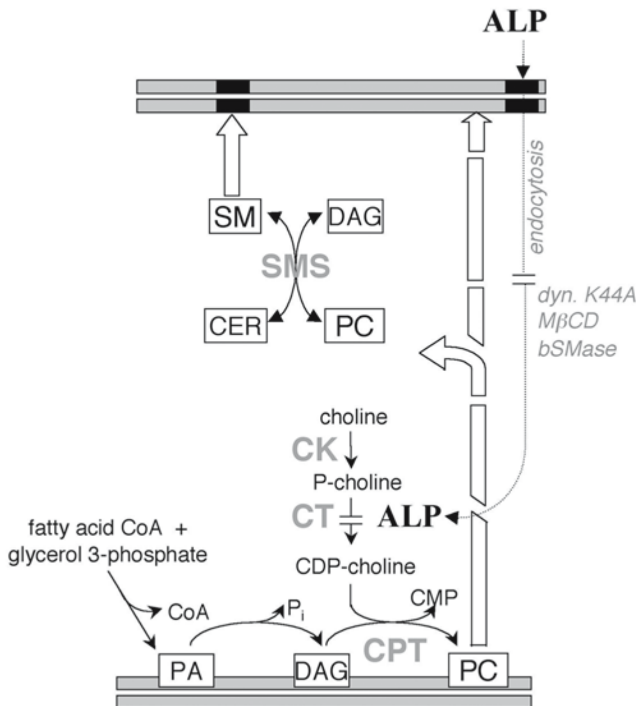

Fig. 2. The Kennedy pathway of PC synthesis, the fate of choline phospholipids, and the molecular target of ALP. ALP is internalized via lipid rafts and targeted toward the ER/Golgi, where it specifically interferes with cytidylyltransferase (CT) to inhibit PC synthesis. Part of the PC is used to synthesize SM at the trans-Golgi network (TGN) for assembly of new lipid rafts. The internalization of ALP, inhibition of PC synthesis, and induction of apoptosis is dependent on intact lipid rafts and dynamin-dependent endocytosis. Methyl-β-cyclodextrin (MβCD), bacterial sphingomyelinase (bSMase) (both disrupting lipid rafts), or expression of a dominant-negative mutant of dynamin (dyn. K44A) abrogate raft-dependent endocytosis of ALP.

ization of this enzyme. In contrast to the earlier belief, this result would imply that, whereas the rate-limiting step is at the ER, the final step in this pathway and hence PC formation occurs at the Golgi apparatus.

In order to inhibit CT in the ER, ALP needs to be internalized. Although the correlation between ALP uptake and apoptosis has been reported (Zoeller et al., 1995; Mollinedo et al., 1997), the mechanism by which ALP is internalized has remained controversial for some time. ALP is not taken up via a specific receptor (such as for the structurally related platelet-activating fac-

tor) (Bazill and Dexter, 1990; Mollinedo et al., 1997; Kabarowski et al., 2001; Ruiter et al., 2002). Various reports described that endocytosis is not involved in ALP internalization (Kelley et al., 1993; Zoeller et al., 1995; Mollinedo et al., 1997), whereas others reached the opposite conclusion (Bazill and Dexter, 1990; Small et al., 1997). We recently resolved this issue (van der Luit et al., 2002, 2003): We found that ALP internalization is inhibited by monensin, by expression of a dominant-negative mutant (K44A) of the GTPase dynamin, and in the cold, which is clearly indicative of endocytosis. In addition, ALP was internalized by a mechanism that involves lipid rafts. Disruption of rafts by different means, i.e., treatment with MβCD, filipin, or bacterial sphingomyelinase, prevented ALP internalization in mouse S49 lymphoma and human HeLa cells. Furthermore, ALP partitioning in rafts appeared to be a very rapid event that could be disturbed by MβCD pretreatment, resulting in a redistribution of ALP to nonraft fractions without altering the initial overall binding to the plasma membrane.

Whereas the classical routes of endocytosis are usually directed toward late endosomes/lysosomes, raft-mediated endocytosis does not target the lysosomes, but rather is directed towards a rapid cycling pathway, via the Golgi back to the plasma membrane (Ikonen, 2001; Nichols and Lippincott-Schwartz, 2001). This route may contribute to correct raft protein/lipid sorting and trafficking (Gagescu et al., 2000). Lipid raft internalization depends on dynamin function as expression of a dominant-negative K44A mutant of dynamin prevented ALP internalization and alleviated ALP-mediated inhibition of PC biosynthesis and ALP-induced apoptosis (Fig. 2; van der Luit et al., 2003). Pagano's group showed that the raft-associated fluorescent lipid, BODIPY-lactosylceramide, is exclusively internalized by a pathway that is dynamin-dependent and clathrin-independent, to reach the Golgi apparatus (Puri et al., 2001). Accordingly, ALP is likely to follow the same internalization pathway to inhibit CT.

5. ALP-RESISTANT CELLS HAVE ABERRANT LIPID RAFTS

ALP-resistant S49 cells (designated $S49^{AR}$) have been generated by application of a selection pressure, i.e., two selection rounds of growth in 15 μ*M* ALP (Et-18-OCH_3) for 72 h, followed by plating in semisolid medium and isolation of colonies of surviving cells, as described by Smets et al. (1999).

5.1. Cross-Resistance to Other Stress Stimuli

Interestingly, ALP-resistance associated with cross-resistance to other apoptotic stimuli, including cold shock, heat shock, H_2O_2, dimethylsulfox-

ide, and ionizing radiation (Smets et al., 1999). The induced phenotype is not caused by gene mutation but is the consequence of a reversible selection pressure, as continuous cell culturing in the absence of the drug results in a regained sensitivity to ALP and loss of cross-resistance (van der Luit et al., unpublished). To address this phenomenon in more detail, we investigated ALP uptake, and the effect on phospholipid synthesis/composition of the ALP-resistant variant cell line $S49^{AR}$ in comparison to the parental S49 cells.

5.2. ALP Resistance Is Associated With Defective Raft-Mediated Endocytosis and Lack of Sphingomyelin Synthesis

We found that the ALP-resistant variant cell, $S49^{AR}$, showed a severe deficiency in ALP internalization, which could be entirely attributed to a defect in endocytosis (van der Luit et al., 2002). In addition, although PC synthesis in these cells appeared normal (also in the presence of ALP), the synthesis to SM was abrogated (van Blitterswijk et al., 2001). This finding must undoubtedly have consequences for the physico-chemical properties of the plasma membrane, particularly for lipid raft formation. A defective SM synthesis likely impairs correct raft assembly at the Golgi, with consequent aberrant rafts appearing at the plasma membrane. We therefore reasoned that this might be causally linked to the defect in ALP internalization, thus converting resistance to apoptosis. Indeed, artificial disruption of lipid rafts in (ALP-sensitive) S49 cells, e.g., by cholesterol depletion with MβCD (Simons and Toomre, 2000), inhibited ALP uptake and ALP-induced apoptosis. We hypothesize that intact membrane rafts are the "sensors" for ALP and possibly other stress agents that require raft-mediated endocytosis to be functional in order to induce apoptosis, and that cells can escape from toxic agents by shutting down the raft-mediated endocytic pathway through a block in SM synthesis and functional raft formation at the Golgi.

It may be questioned whether cells that lack SM still contain lipid rafts and, if so, why their normal function (e.g., in endocytosis) is affected. We found that lipid rafts could still be isolated from SM-deficient $S49^{AR}$ cells as a detergent-insoluble fraction on sucrose gradients. Despite the lack of SM, ALP remained inserted into these domains (van der Luit et al., 2002). Could a defect in SM synthesis and raft assembly prevent ALP internalization by blocking vesicular trafficking originating at these domains? Indeed, artificial disruption of lipid rafts in S49 cells (e.g. by cholesterol depletion with MβCD or by SM hydrolysis using exogenously applied bacterial SMase) inhibited ALP uptake and alleviated PC inhibition and the induction of apoptosis (van der Luit et al., 2002).

6. THE ASSEMBLY OF A LIPID RAFT

6.1. Sphingomyelin and Glycosphingolipid Biosynthesis Require Trans-Bilayer Movement (Flipping) of Ceramide and Glucosylceramide Over the Golgi Membrane

The hydrophobic core of all sphingolipids, ceramide, is produced at the ER (Mandon et al., 1992) and, in some cell types, part of it is converted to galactosylceramide within the ER lumen (Burger et al., 1996). For advanced sphingolipid biosynthesis, ceramide is carried to the Golgi apparatus in either a vesicular (Puoti et al., 1991) or a nonvesicular transport step (Collins and Warren, 1992). At the Golgi, Cer is metabolized into GlcCer at the cytosolic face of the Golgi membrane. Because synthesis of SM and complex glycosphingolipids occur at the lumenal side of the Golgi, Cer and GlcCer, respectively, must first traverse the Golgi membrane. As spontaneous flip-flop of Cer and GlcCer is very slow (Venkataraman and Futerman, 2000; Sprong et al., 2001), the translocation of Cer and GlcCer across the Golgi membrane is likely facilitated by a (yet unknown) protein/translocator (Sprong et al., 2001).

6.2. Necessity of Cholesterol Recruitment for Raft Formation at the Golgi

Cholesterol in association with sphingolipids (in nascent lipid rafts) has been postulated to facilitate post-Golgi protein sorting by regulating the thickness of the lipid bilayer (Bretscher and Munro, 1993). Lipid rafts function to segregate and concentrate membrane proteins that play an important role in signal transduction and in generating cell surface polarity (Brown and London, 1998; Simons and Toomre, 2000). To support these functions, the trafficking of cholesterol must be tightly coordinated. Cholesterol is not uniformly distributed among subcellular membranes (Lange, 1991). Evidence exists for an intracellular cholesterol gradient across the Golgi, from a relative cholesterol-poor *cis*-Golgi to a cholesterol-enriched *trans*-Golgi network and plasma membrane. This gradient results in a higher SM content and degree of fatty acid saturation at these sites.

Although spontaneous diffusion is partly responsible for the intracellular movement of cholesterol, active transport is also important in controlling cholesterol levels (Liscum and Dahl, 1992). Evidence for regulated intracellular cholesterol transport comes from pharmacological studies with energy poisons (DeGrella and Simoni, 1982) and hydrophobic amines, such as U18666A (3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one) (Liscum and

Faust, 1989; Liscum and Collins, 1991) and imipramine (Rodriguez-Lafrasse et al., 1990). Mounting genetic evidence suggests that cellular factors that mediate intracellular cholesterol trafficking directly affect sphingolipid biosynthesis and raft protein assembly at the Golgi. LDL deprivation reduced the cell surface expression of the GPI-anchored gD1-DAF in MDCK cells (Hannan and Edidin, 1996). The endocytic retention of the GPI-anchored folate receptor is regulated by the level of cholesterol in cell membranes (Rothberg et al., 1990; Mayor et al., 1998).

In yeast, many lipid metabolic genes are transcriptionally regulated in response to changes in sterol levels. Long-chain sphingoid-base hydroxylase and long-chain sphingoid-base serine palmitoyltransferase, both involved in sphingolipid metabolism, are affected by sterol levels (Swain et al., 2002a). Yeast cells lacking the *Arv1* gene, involved in sterol trafficking, harbor defects in sphingolipid metabolism that can be complemented by human *Arv1* (Swain et al., 2002b). Yeast mutants defective in long-chain fatty acid synthesis, *Elo3,* have truncated ceramides, which results in an absolute requirement of ergosterol to maintain functional raft domains (Eisenkolb et al., 2002). In higher eukaryotes, the inhibition of *de novo* cholesterol biosynthesis by the addition of HMG-CoA reductase inhibitor lovastatin has been shown to block SM and Cer biosynthesis (Storey et al., 1998). In auxotrophic, sterol regulatory-defective chinese hamster ovary cells (SRD 6 cells), SM synthesis was stimulated twofold by 25-hydroxycholesterol. Slotte and Bierman (1988) showed that sterol esterification rapidly occurs in response to membrane depletion of SM and is accompanied by down-regulation of *de novo* sterol biosynthesis.

6.2.1. Steering Function of NPC Proteins in Cholesterol Trafficking/Recruitment

Niemann-Pick disease, type C (NPC) (Liscum and Faust, 1987; Liscum et al., 1989) and Niemann-Pick type D (NPD) (Butler et al., 1987) are lipid storage diseases caused by defective transport of cholesterol from late endosomes to other cellular sites. NPC is caused by mutations in NPC1 or NPC2 proteins. The NPC1 protein is a protein-motive-force-driven lipid transporter (Davies et al., 2000), which has a typical sterol-sensing domain consisting of five transmembrane helices. NPC1-deficient cells accumulate LDL-derived cholesterol and sphingolipids in late endosomes (Kobayashi, 1999). NPC2 (or HE1) is a small cholesterol-binding protein found in the lysosomal lumen, and it might be involved in transport of cholesterol to the endosomal/lysosomal outer membrane. Cholesterol derived from lysosomes is routed towards the Golgi/ER. Whether a defect in cholesterol recycling from these organelles affect sphingolipid metabolism and correct lipid raft assembly needs to be established.

The raft lipid BODIPY-lactosylceramide is exclusively internalized by a clathrin-independent, raft/caveolae-dependent pathway. In sphingolipid-storage-disease fibroblasts or in normal fibroblasts with elevated intracellular cholesterol levels, the internalization via the raft/caveolae-dependent pathway towards the Golgi is perturbed, and instead, sphingolipid is routed towards the lysosomes (Puri et al., 2001). This implies a sorting role for cholesterol in directing lipids towards different intracellular compartments. Impaired exit of cholesterol from lysosomes in sphingolipid storage disease, as in NPC, might trap sphingolipids there and prevent correct raft assembly at the Golgi.

6.2.2. Cholesterol May Stabilize Newly Formed Sphingomyelin in Lipid Rafts

SM is formed by SM synthase, an enzyme not yet cloned and only poorly been characterized to date. This enzyme is unique in that it not only synthesizes SM by transferring a phosphorylcholine moiety from PC onto ceramide, but also performs the reverse reaction, i.e., releasing ceramide and PC from SM and DAG (Fig. 2), at least in vitro. PC is thought to block vesicle formation at the Golgi, whereas DAG and phosphatidic acid are thought to stimulate vesicle budding at the Golgi (Bi et al., 1997; Kearns et al., 1997; Schmidt et al., 1999; Weigert et al., 1999; Siddhanta et al., 2000; Henneberry et al., 2001; Bankaitis, 2002; Baron and Malhotra, 2002). SM synthase might therefore be a key enzyme in regulating vesicular trafficking from the Golgi towards the plasma membrane. Newly formed SM will be stabilized (shielded from the reverse reaction) by available cholesterol (through their mutual high affinity) at the Golgi. Thus, local control of cholesterol levels might push the equilibrium reaction in the direction of SM and DAG formation, and hence stimulate raft formation and vesicular trafficking toward the plasma membrane. In fact, our finding that MβCD treatment of cells to extract cholesterol results in hydrolysis of SM supports this notion (van der Luit et al., unpublished). The question remains whether this treatment will block vesicular trafficking between Golgi and plasma membrane and have consequences for correct lipid raft formation.

6.2.3. Possible Role of ABC Transporters in Lipid Raft Assembly

ATP-binding cassette (ABC) transporters form a large family of proteins that mediate the ATP-dependent unidirectional transmembrane transport of a multitude of specific hydrophilic and xenobiotic substrates (Borst and Oude Elferink, 2002). Increasing evidence exists that some of these transporters may also function as "flippases" for endogenous membrane lipids (Schmitz et al., 2000) and, as such, play a key role in the regulation of lipid transport. The MDR1 P-glycoprotein that causes multidrug resistance (MDR) acts at the plasma membrane in lipid rafts (Liscovitch and Lavie,

2000), but a substantial amount is also found at the Golgi. MDR1 can translocate short-chain choline-phospholipids and monohexosylceramides (e.g., fluorescent NBD-GlcCer) (van Helvoort et al., 1996), and indirect evidence suggests that MDR1 may also translocate natural (long-chain) GlcCer (Lala et al., 2000; Sprong et al., 2001). In addition, MDR1 has been implicated in controlling the levels of cholesterol esterification (Debry et al., 1997; Luker et al., 1999), suggesting that under normal conditions MDR activity may have an effect on intracellular cholesterol transport to influence sphingolipid biosynthesis and correct raft-protein assembly at the Golgi. On the other hand, P-glycoproteins have been suggested as actively mediating the relocation of cholesterol from the cytosolic leaflet to the outer leaflet of the plasma membrane and the lumenal side of the Golgi, as its expression and basal ATPase activity appear directly regulated by cholesterol levels (Garrigues, 2002). In this way, it could facilitate co-translocation of Cer and GlcCer to the Golgi lumen. Thus, P-glycoprotein may directly contribute in stabilizing the cholesterol-rich microdomains, rafts, and caveolae, by controlling cholesterol (membrane) trafficking and correct lipid raft assembly.

7. OTHER IMPLICATIONS OF LIPID RAFTS IN APOPTOTIC SIGNALING

7.1. Ceramide Facilitates Death-Receptor Clustering in Lipid Rafts

Of all sphingolipids, ceramide (Cer) has received the most attention because it is almost universally generated during cellular stress and apoptosis. The observation that synthetic, short-chain Cer (C2-Cer, C6-Cer) can induce apoptosis has led to the extrapolation that a rapid Cer formation is an obligatory step in the apoptotic process. Because short-chain Cer differs dramatically from natural ceramide in biophysical properties and behavior in membranes and cells, we have questioned the direct regulatory (second messenger) role of endogenous Cer in initiating apoptosis (Tepper et al., 2000; van Blitterswijk et al., 2003). Gulbins and Kolesnick and their co-workers have discovered a novel function for Cer formation during apoptosis. This function is of membrane-structural rather than of second-messenger nature: Rapid Cer formation occurs on the surface of T and B cells in response to activation of the TNF receptor family members CD95/Fas and CD40, respectively. This Cer was generated from lipid rafts by acid SMase and facilitated ligand-induced clustering of these receptors, located in lipid rafts (Cremesti et al., 2001; Grassmé et al., 2001, 2002). This facilitation of receptor clustering was also achieved by nanomolar doses of exogenous natural Cer that apparently mimicked the acid SMase-generated endogenous Cer in

this respect. Receptor clustering and activation were associated with the recruitment to these rafts of downstream signaling proteins: FADD (Fas-associated death domain) and caspase-8 in the case of CD95/Fas (Hueber et al., 2002), and TRAF (TNF receptor-associated factor) in the case of CD40 (Vidalain et al., 2000). Furthermore, CD40 signaling toward extracellular-signal-regulated kinase (ERK) activation and cytokine production in dendritic cells is initiated within lipid rafts by the activation of Src family kinases such as Lyn (Vidalain et al., 2000).

7.2. ALP Induces CD95/Fas Death-Receptor Clustering

Gajate et al., (2000) reported that ALP-induced apoptosis is dependent on CD95/Fas expression in cells. ALP induced the co-capping of CD95/Fas and membrane raft markers before the onset of apoptosis in human leukemic cells. This enhanced lateral redistribution of CD95/Fas by ALP, similar to that induced by natural ceramide (Section 7.1.), provides a mechanism to amplify CD95/Fas signaling by reorganization (coalescence) of membrane microdomains (rafts). Disruption of lipid rafts by MβCD or filipin inhibited both ALP-induced apoptosis and CD95/Fas aggregation in Jurkat cells (Gajate and Mollinedo, 2001). Since CD95/Fas clustering and activation were associated with the recruitment to these rafts of downstream signaling proteins FADD and caspase-8 (Hueber et al., 2002), it was suggested that ALP could activate this pathway independent from ligation of the receptor. However, when we disrupted CD95/Fas signaling in Jurkat or in S49 cells by expression of dominant-negative FADD or FLIP, ALP-induced apoptosis remained unaffected. This result argues against a role of CD95/Fas signaling in ALP-induced apoptosis (van der Luit et al., unpublished). Rather, ALP is internalized in these cells via lipid rafts to inhibit PC synthesis and induce apoptosis as described above. Interference with novel raft formation at the Golgi may likely affect the extent of raft coalescence induction at the plasma membrane.

7.3. Ganglioside GD3 Implicated as a Messenger in Mitochondrion-Dependent Apoptosis

Accumulating evidence suggests that the disialoganglioside GD3, a glycosylated ceramide, could mediate apoptosis at the mitochondrial level (De Maria, et al,. 1997; Tomassini and Testi, 2002; Melchiorri et al., 2002; Giammarioli et al., 2001; Colell et al., 2002). GD3 is synthesized in the Golgi and becomes enriched on the cell surface in lipid rafts/caveolar microdomains. Activation of death receptors (CD95/Fas, TNF receptor) induces an intracellular flow of GD3, probably entirely carried by raft-containing vesicular transport (Ikonen, 2001): GD3 synthesis increased, and

the lipid disappeared from the cell surface via raft-dependent endocytosis and moved via vesicular transport towards the mitochondria (Garcia-Ruiz et al., 2002). Overexpression of GD3 synthase induced apoptosis (De Maria et al., 1997), whereas suppression of GD3 synthase expression inhibited apoptosis induction (Melchiorri et al., 2002). Whereas translocating from the plasma membrane to the mitochondrion, the apolar GD3 remains membrane (raft) bound and co-localizes with early and late endosomal markers, Rab5 and Rab7 (Garcia-Ruiz et al., 2002). The induction of raft coalescence, local negative membrane curvature, and vesicle budding by ceramide formation may facilitate this vesicular transport of GD3 (van Blitterswijk et al., 2003). Perhaps GD3 itself or together with ceramide and a member of the pro-apoptotic Bcl-2 family, such as tBid, dephosphorylated Bad (Basu et al., 1998), or Bax (von Haefen et al., 2002), may serve to specifically guide/direct this vesicular apoptotic signal toward the mitochondrial target. Interestingly in this regard, Bad is attached to lipid rafts in IL-4-stimulated T cells, but dissociates from rafts and associates with mitochondria in IL-4-deprived cells that then die by apoptosis (Ayllon et al., 2002). It may therefore be possible that the apoptotic signal from the cell surface towards the mitochondrion involves vesicular transport of lipid raft-bound GD3 and Bad or another pro-apoptotic Bcl-2 family member, which is facilitated by ceramide formation (van Blitterswijk et al., 2003).

8. CONCLUSIONS AND PERSPECTIVES

In this chapter, we described at least part of the mechanism by which the anti-cancer agent ALP is taken up by cells and induces apoptosis. Prolonged exposure to ALP generates cellular resistance and, most intriguingly, cross-resistance to other stress stimuli. The phenotype of these stress-resistant cells is characterized by a defect in raft-mediated endocytosis. In this way, the cells protect themselves from the deleterious effects of ALP, and apparently also of other stresses, by shutting off this particular type of endocytosis, whereas other (classical) modes of endocytosis, i.e., via clathrin-coated pits or fluid phase, remain unaffected.

The block in raft-mediated endocytosis that characterizes the cellular resistance to ALP and other stresses is associated with a defect in SM synthesis. Obviously, this must have consequences for new raft assembly and the biophysical properties (perhaps the plasticity) of the lipid rafts. How this relates to the defect in endocytosis remains unknown. Is it the raft SM content per se that determines its internalization, probably in concert with cytoskeletal rearrangements? Or is it the impediment of nascent raft formation through lack of SM production at the Golgi that precludes raft-contain-

ing vesicular trafficking toward and from the plasma membrane? New raft formation is a very complex process in which newly made (glyco)-sphingolipids, cholesterol, and specific proteins have to be spatially recruited in a tightly coordinated fashion. Unraveling this mechanism is an enormous challenge for future investigation.

It is conceptually a new development that an anti-cancer agent should enter the tumor cell via lipid rafts. As a consequence, lipid rafts may potentially be considered as new drug targets. ALP is not alone in using this gateway to induce apoptosis; other toxins such as cholera toxin, shiga toxin, and anthrax toxin also use these portals to exert their function (Nichols et al., 2001; Pelkmans and Helenius, 2002; Abrami et al., 2003). A wide variety of pathogens hijack lipid rafts, including bacteria (e.g., *Pseudomonas aeruginosa*), viruses (e.g., simian virus and HIV), and protozoa (e.g., *Plasmodium falciparum*) (Lauer et al., 2000). If tumor cells display a greater need for lipid raft internalization, e.g., for enhanced phospholipid turnover and consequent formation of lipid second messengers, or to control their subcellular cholesterol homeostasis, these microdomains might constitute novel promising targets in anti-cancer research and pathology.

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6

The Role of Lipid Rafts in Signal Transduction and Synaptic Plasticity of Neural Cells

Markus Delling and Melitta Schachner

1. INTRODUCTION

Compartmentalization of proteins within the cell plays a fundamental role in the spatial and temporal organization of intracellular signaling systems. Although protein phosphorylation has long been known to be involved in this process, lipid microdomains enriched in sphingolipids and cholesterol, also known as lipid rafts, have recently been identified as regions within plasma membranes that are important for numerous cellular processes, including signal transduction, membrane trafficking, molecular sorting, and cell adhesion (Harder et al., 1998; Dermine et al., 2001). The unique lipid composition of rafts creates a more ordered lipid environment than is found in the rest of the plasma membrane (Simons and Ikonen, 1997; Brown and London, 2000), conferring to these specialized structures resistance to nonionic detergent extraction at 4°C using Triton X-100 and giving rise to their alternative name of detergent-resistant membranes (DRMs). However, one should be cautious in assuming that lipid rafts can be isolated in their native state and that the relationship between their operational definition, namely detergent-insolubility at 4°C, flotation at a certain buoyancy, and cholesterol-dependency, fully reflects their state in vivo (for review, *see* Lai, 2003). A difficulty with the study of lipid rafts is that they may be too small (a few tens to hundreds of nanometers in diameter) (Brown and London, 2000; Abrami et al., 2001; Brown, 2001; Pierini and Maxfield, 2001) and too

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highly dispersed to be directly observed in unperturbed living cells. However, upon stimulation of raft-inserted receptors or antibody clustering, disperse rafts can aggregate to form large domains of several micrometers (Pierini and Maxfield, 2001), like flotillas (Harder et al., 1998; Brown and London, 2000; Dermine et al., 2001; Pierini and Maxfield, 2001), thereby inducing clustering of membrane components as a prerequisite for signal transduction.

The fundamental principle by which lipid rafts exert their functions is a segregation and/or concentration of specific membrane proteins and lipids in membrane microdomains, which are important for the generation of intracellular signals by recruiting downstream effector molecules (Harder et al., 1998). Thus, lipid rafts may participate in a subset of transmembrane signaling events (Parolini et al., 1999; Niethammer et al., 2002). One of the earliest observations that pointed to lipid rafts as important structures for signal transduction was their enrichment with potent intracellular signaling molecules (Smart et al., 1999), such as trimeric and small GTPases, Src family kinases, lipid second messengers, and the growth-associated protein 43 (GAP-43) (Simons and Ikonen, 1997; Anderson, 1998). The activation of these signaling cascades depends to a large extent on the ability of lipid rafts to serve as docking platforms for extracellular ligands (Brown and London, 2000).

It has become evident that palmitoylation of proteins on cysteine residues is both necessary and sufficient to target them to rafts (Arni et al., 1998; Guzzi et al., 2001; for review, *see* Patterson, 2002). The Src-family kinase Fyn is myristoylated cotranslationally and associates rapidly with membranes, but does not partition into the lipid rafts until it is palmitoylated later on (van't Hof and Resh, 1997). This hiatus between synthesis and palmitoylation is also seen with the synaptic Q-SNARE protein SNAP-25, which does not undergo palmitoylation unless transported intracellularly to the plasma membrane (Gonzalo and Linder, 1998), where it partitions into rafts (Braun and Madison, 2000). Substituting palmitate with unsaturated palmitate analogs reduces raft targeting and signal transduction through the Src family kinase Fyn (Liang et al., 2001). Additionally, ablation of the palmitoylation site by mutating a cysteine to a serine residue reduces the raft localizations of Fyn (van't Hof and Resh, 1997), the integral membrane molecule linker for activation of T cells (LAT) (Zhang et al., 1998), the 140-kDa isoform of the neural cell adhesion molecule NCAM (Niethammer et al., 2002), and CD4 (Fragoso et al., 2003). From these and other examples, it may generally be concluded that dual fatty acylation, or more specifically, multiple palmitoylation predisposes proteins to localize to rafts (Zacharias et al., 2002).

2. ROLE OF LIPID RAFTS IN NEURAL CELL INTERACTIONS

Formation of stable contacts between neurons and their targets is crucial for nervous system functions. The regulation of these contacts by short- and long-distance acting trophic factors is vital for cell interactions during development and in the adult (Schachner, 1997; Walsh and Doherty, 1997; Van Vactor, 1999). Growing evidence supports an important function of lipid rafts in these events. This is suggested by the observation that many cell adhesion molecules are located in rafts: The GPI-anchored molecules TAG-1 (transiently expressed axonal glycoprotein-1), the small major isoform of NCAM, NCAM120, Thy-1, and F3/contactin are well-accepted lipid raft constituents (Kramer et al., 1999; Faivre-Sarrailh et al., 2000; Kasahara et al., 2000;). There is increasing evidence that cell adhesion molecules with a transmembrane domain, such as NCAM140 and NCAM180, can also be found in lipid rafts (He and Meiri, 2002; Niethammer et al., 2002). For example, the two splice variants NCAM140 and NCAM180 can be palmitoylated at four intracellular cysteines adjacent to the plasma membrane. Furthermore, both isoforms have been shown to be present in the lipid raft fraction of growth cones and of brain or cell lysates (He and Meiri, 2002; Niethammer et al., 2002). Ablation of the intracellular palmitoylation sites by mutation of cysteine to serine residues abolishes palmitoylation of NCAM (Little et al., 1998) and removes NCAM140 and NCAM180 from the lipid raft fraction (Niethammer et al., 2002).

Increasing evidence suggests that the presence of both GPI-anchored and transmembrane adhesion molecules in lipid rafts is essential for their role as cell adhesion receptors and thus to transmit signaling events to the cell interior. For example, antibody cross-linking of TAG-1 promotes tyrosine phosphorylation within lipid rafts, for instance by activation of the Src family kinase Lyn (Stefanova et al., 1991; Henke et al., 1997; Kasahara et al., 2000). Antibody cross-linking of F3/contactin activates Fyn in oligodendrocytes (Kramer et al., 1999). Conversely, depletion of TAG-1 from lipid rafts, by blocking the synthesis of glycosphingolipids, inhibits the ability of TAG-1 to promote phosphotyrosine signaling (Kasahara et al., 2000). It is presently not fully understood how cross-linking of GPI-anchored proteins, which do not traverse the inner leaflet of the plasma membrane, influences signaling events such as the activation of Lyn and Fyn. It is conceivable that the accumulation of several GPI-linked molecules induce productive interactions with individual Fyn molecules via yet unknown transmembrane signaling mechanisms, as discussed elsewhere (Kramer et al., 1999; Crossin and Krushel, 2000). Alternatively, activation of intracellular kinases by GPI-

anchored molecules may depend on cis-interactions with other transmembrane molecules inside of lipid rafts, as has been suggested for molecules such as F3/contactin (Zeng et al., 1999). Although GPI-anchored cell adhesion molecules are highly enriched in lipid rafts, and thus depleted in the nonraft compartment (we use this term to refer to the environment in the plasma membrane outside of cholesterol-rich domains, because we cannot rule out that there are other, yet unidentified microdomains in the plasma membrane), the principle that transmembranous cell adhesion molecules can be in both compartments introduces a novel concept in the view of how the triggering of cell interactions by recognition at the cell surface orchestrates signaling cascades. An example is the function of NCAM140 in stimulating neurite outgrowth (Fig. 1).

Upon exposure of NCAM-expressing cultured neurons to soluble NCAM (NCAM-Fc, a fusion protein combining the extracellular domain of NCAM with the Fc domain of human IgG) or to anti-NCAM antibodies, NCAM140 triggers two distinct signaling cascades within and outside of lipid rafts. Outside of lipid rafts, NCAM140 activates the fibroblast growth factor receptor (FGF receptor), most probably via its interaction with the extracellular domain of this receptor (Doherty et al., 1996; Doherty and Walsh, 1996; Cavallaro et al., 2001). Triggering of NCAM140 in lipid rafts leads to the activation of the Fyn-FAK kinase pathway. Transfection of cultured hippocampal neurons derived from NCAM deficient mice (NCAM$^{-/-}$ mice) either with NCAM140 or a palmitoylation-deficient NCAM140 mutant that is not capable of associating with lipid rafts, but still capable of activating the FGF receptor, supported the hypothesis that the separate and independent activation of two pathways is essential for NCAM140-mediated neurite outgrowth. Although NCAM140 transfected NCAM$^{-/-}$ neurons showed NCAM-dependent neurite outgrowth in a manner similar to that of wild-type neurons, NCAM$^{-/-}$ neurons transfected with the NCAM140 mutant did not exhibit neurite outgrowth upon NCAM stimulation. This result shows that both pathways have to be activated simultaneously, suggesting a cosignaling mechanism of NCAM140 via the lipid raft and the nonraft compartments. NCAM180, although present in lipid rafts, is not capable of promoting neurite outgrowth, most likely because it lacks the ability to activate the Fyn kinase pathway because of the additional 268 amino acids contributed by exon 18, which may alter the conformation of the intracellular domain of NCAM180 in a yet unknown manner. It remains to be elucidated which signaling cascades NCAM180 may activate.

Another principle of signaling via lipid rafts is the relocation of receptor from the nonraft compartment to the lipid raft compartment after ligand binding to initiate cellular responses (Simons and Toomre, 2000). An example of

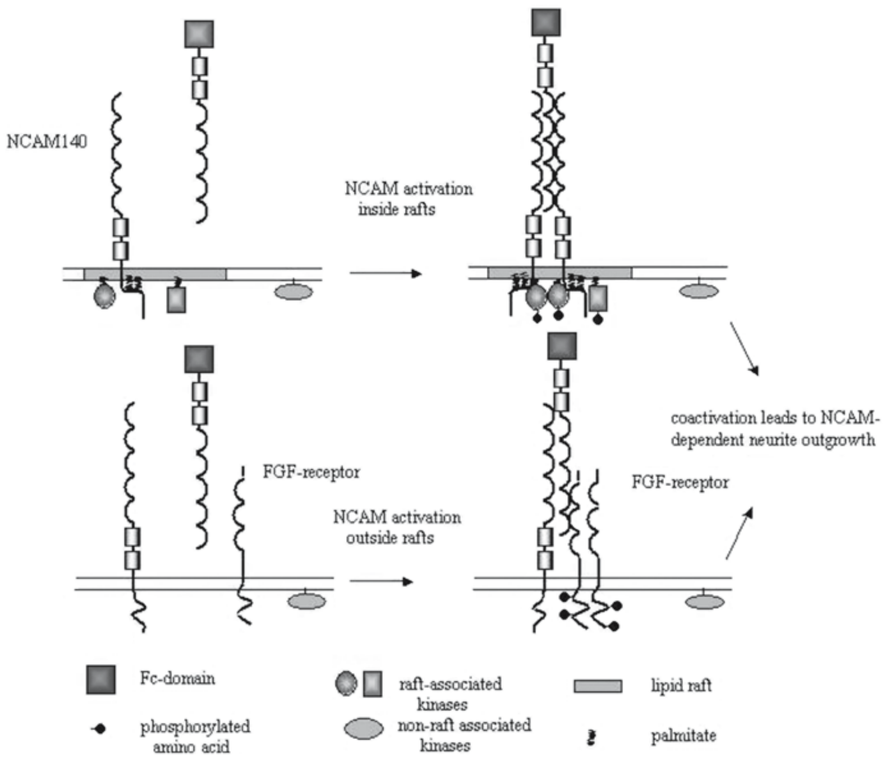

Fig. 1. Hypothetical diagram of NCAM140 co-signaling platforms.Within the plasma membrane, NCAM140 is located both in lipid rafts (top) and outside of rafts (bottom), thus defining two membrane subcompartments in which NCAM140 is present. The presence of NCAM140 in lipid rafts is regulated by palmitoylation of intracellular cysteines by yet unknown mechanisms. Upon homophilic activation, NCAM triggers distinct signal transduction pathways in the two compartments: In lipid rafts NCAM140 activates raft-associated kinases like Fyn, while in the nonraft compartment NCAM140 facilitates FGF receptor-activated downstream signaling. Co-activation of both pathways is necessary for NCAM140 to function as a neuritogenic receptor.

such a mechanism is the glia-derived neurotrophic factor (GDNF)-mediated activation of the Ret receptor tyrosine kinase (RTK) (Tansey et al., 2000). GDNF acts via a receptor complex consisting of the Ret and a GPI-anchored ligand-binding subunit, GFRα1. In the unstimulated state, Ret is outside of lipid rafts and GFRα1 is located inside rafts due to its GPI-anchor (Poteryaev et al., 1999; Trupp et al., 1999). In the absence of GDNF, GFRα1 and Ret do not associate with each other and thus Ret is not present in lipid rafts. Upon

ligand stimulation, GFRα1-GDNF complexes recruit Ret into lipid rafts (Tansey et al., 2000). This translocation to rafts is essential for Ret function, because GFRα1 mutants incapable of recruiting Ret into rafts (e.g., use of a transmembrane-anchored GFRα1 chimera), or the disruption of lipid rafts (e.g., by cholesterol depletion) affect downstream signaling, survival, and differentiation (Tansey et al., 2000). Recently Paratcha and colleagues (Paratcha et al., 2003) unified the two categories of short- and long-distance acting trophic factors by identifying NCAM140 as an alternative receptor for GDNF: In cells that express GFRα1 but lack RET, the GFRα1-GDNF complex recruits NCAM140 as a transmembrane receptor for further downstream signaling. Interestingly, the GFRα1-GDNF-NCAM140 complex appears to signal via lipid rafts, since NCAM140 is enriched in the lipid raft fraction after GDNF stimulation.

3. THE ROLE OF LIPID RAFTS IN NEURONA EXCITABILITY

Recent studies have provided evidence that lipid rafts contribute to the regulation of neuronal excitability, namely in the clustering and regulation of ion channels and neurotransmitter receptors and in the exocytotic process of neurotransmitter release. Although some neurotransmitter receptors, such as the ionotropic glutamate receptor subunits NR1A of the NMDA receptor (Wu et al., 1997) and GluR1 of the AMPA receptor (Ledesma et al., 1998), are not located in lipid rafts, other channels such as the voltage-gated K^+ channel Kv2.1 (Martens et al., 2000), the nicotinic acetylcholine receptor a7 (Bruses et al., 2001), the GABAB receptor (Becher et al., 2001) and the inwardly rectifiying K^+ channels Kir3.1/3.2 (Delling et al., 2002) are located in rafts.

What might be the role of lipid rafts harboring ion channels? In addition to their role as signaling platforms, lipid rafts have also been suggested as being a localization signal for the targeting of proteins to axonal, but not to somatodendritic, membranes (Simons and Ikonen, 1997; Brown and London, 1998). Initially, the view that lipid rafts are involved in intracellular sorting processes stems from the observation that lipid rafts are not only present in the plasma membrane, but also in the late secretory pathway, including the Golgi apparatus and the endocytotic compartments (Brown and London, 2000; Dermine et al., 2001). These localizations support a role for lipid rafts in membrane trafficking (Simons and Ikonen, 1997).

In addition, raft assembly as defined by sphingolipid production and assembly first occurs in the Golgi complex, whereas cholesterol is synthesized in the endoplasmic reticulum (ER) (Simons and Ikonen, 1997). Recent

observations have demonstrated a relationship between the palmitoylation motifs and intracellular targeting in polarized cells. The growth-associated protein GAP-43 and scaffolding protein PSD-95 are selectively transported to the axonal (Goslin et al., 1990) and dendritic (Craven et al., 1999) compartments, respectively, in cultured hippocampal neurons. Both proteins are palmitoylated near the N-terminus, and this palmitoylation motif appears necessary, although not sufficient for PSD-95, to determine their subcellular localization (Liu et al., 1991; El-Husseini Ael et al., 2001). Addition of the GAP-43 palmitoylation motif onto PSD-95 resulted in a protein that distributed to both the dendritic and axonal compartments, with some preference for axons. Adding basic residues close to the palmitoylation site of PSD-95, as in GAP-43, favored the distribution to axons, whereas removing nearby basic residues from the GAP-43 sequence reduced its axonal targeting. The two palmitoylated cysteines in GAP-43 are adjacent to each other, while in PSD-95 they are separated by a leucine. Eliminating this leucine also reduced dendritic targeting of PSD-95 similar to the basic residue mutation, and adding a spacing amino acid in between the two cysteines of the GAP-43 sequence increased its dendritic targeting (El-Husseini Ael et al., 2001).

Thus, there seems to be a complex interplay between the molecular topology of palmitoylated residues and nearby basic amino acids and axo-dendritic targeting of neuronal proteins, which indicates that targeting of proteins to lipid rafts cannot by itself be an exclusive axonal-targeting signal (*see also* Winckler and Mellman, 1999). This view is also supported by the fact that several ion channels, including the α7nAChR and the Kv2.1 and Kir3 channels, as well as intracellular postsynaptic proteins such as GRIP, although partly located in rafts, are subcellularly located in dendrites, particularly spines (Bruckner et al., 1997; Drake et al., 1997; Martens et al., 2000; Bruses et al., 2001).

Targeting of ion channels to the plasma membrane may not only depend on their localization in rafts, but may also be controlled by the raft presence of other molecules. For example, the presence of NCAM140 and NCAM180 in lipid rafts reduces the cell surface localization of Kir3.1/3.2 ion channels in transfected CHO cells and hippocampal neurons and leads to an intracellular accumulation of the channel (Delling et al., 2002). Either removal of NCAM140 from lipid rafts or disruption of lipid rafts by cholesterol depletion fully recovers transport to the plasma membrane. Although Kir3.1/3.2 channels may not be palmitoylated, they are detectable in isolated lipid raft fractions. The intracellular retention of the Kir3 channel is neither dependent on direct interaction of NCAM with the K^+ channel, nor modulated by inhibitors of known NCAM-activated signaling cascades such as the FGF

receptor or Fyn kinase (Niethammer et al., 2002). Therefore, NCAM140 and NCAM180 may regulate the intracellular trafficking of Kir3 channels either by activation of yet uncharacterized lipid raft-dependent signaling cascades, or via some yet unknown productive interactions of NCAM and Kir3.1/3.2 inside of lipid rafts. Whatever the mechanisms of this unconventional influence between functionally interconnected molecules may be, the elucidation of the signals underlying the trafficking of that K^+ channel may unravel new venues in intracellular trafficking modes.

In addition to their potential importance for the localization and clustering of ion channels, lipid rafts are also required for intrinsic channel properties. For example, removal of Kv2.1 from lipid rafts by cholesterol depletion significantly shifts the steady state inactivation of Kv2.1, without altering activation kinetics or voltage sensitivity (Martens et al., 2000). Lipid rafts may therefore modulate voltage-gated and neurotransmitter receptor activity in a manner that has pronounced effects on neuronal excitability. Whether the modulation of the channel properties of Kv2.1 in lipid rafts is caused by specific lipid–protein interactions or by phosphorylation events mediated by proteins concentrated in rafts is currently unknown.

Lipid rafts are also involved in neurotransmitter release, since several of the key regulatory proteins mediating synaptic vesicle fusion (e.g., syntaxin1A, syntaxin3, SNAP-25 and VAMPs, constituting the "core" membrane fusion machinery) are biochemically located in rafts (Chamberlain et al., 2001) or raft-like clusters (Lang et al., 2001). However, other proteins that associate with this core complex and are required for membrane fusion (e.g., aSNAP and nSec1) are not enriched in rafts (Chamberlain et al., 2001). The precise roles of lipid rafts in the interplay of raft and nonraft associated proteins mediating pivotal membrane fusion events or synaptic vesicle trafficking are currently unknown. Nevertheless, cholesterol depletion decreases the amount of exocytosis and evoked dopamine release from PC12 cells, supporting the importance of lipid rafts in regulated exocytosis (Chamberlain et al., 2001; Lang et al., 2001).

4. OUTLOOK

There is growing evidence for the importance of lipid rafts in signal transduction and synaptic transmission of neural cells. For instance, palmitoylation is crucial for neurite outgrowth, since reduction in palmitoylation of growth cone proteins is sufficient to stop neurite extension (Hess et al., 1993; Patterson and Skene, 1994). However, major questions remain regarding the exact roles of rafts in the functioning of the nervous system: What are the mechanisms controlling palmitoylation of pro-

teins? Identification of the palmitoyltransferases would yield further insights into the regulated raft localization of signaling molecules and thus into the regulation of neuritogenesis and excitability of a cell. Using a variety of proteins as substrates, palmitoyltransferase activities have been characterized biochemically, but their molecular entities remain elusive. Because there is considerable heterogeneity in the protein motifs that direct palmitoylation, and because this modification occurs both on intracellular membranes and at the plasma membrane, multiple enzymes are likely to be required.

In the case of axon guidance, what specific functions do rafts subserve? Are rafts involved in attractive and/or repulsive guidance decisions? If so, which downstream signaling molecules located in rafts are required, and are lipid rafts involved in the local influx of Ca^{2+} in growth cones? Which cell adhesion molecules are associated with lipid rafts, and how does raft localization modify their functions? With regard to synaptic transmission, a crucial issue to be addressed is whether lipid rafts have a "morphogenetic" role, for instance, providing a scaffolding platform for the formation of the synaptic cleft, or have a more dynamic role as a specialized subdomain that receptors move into and out of, thus modifying their signaling properties. An intriguing possibility is the influence of rafts on protein folding, since it has been shown that lipid rafts favor the conversion of the cellular prion protein into the scrapie isoform (Naslavsky et al., 1997). It is expected that insights into the functional roles of lipid rafts will yield significant and most likely surprising advances in understanding the modifications in neural cell functions during development and, in the adult, during regeneration after trauma and synaptic plasticity underlying learning and memory.

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7

Role of Rafts in Virus Fusion and Budding

Wu Ou and Jonathan Silver

1. INTRODUCTION

The discovery that cell membranes are a mosaic of at least two domains (raft and nonraft) differing in lipid composition has led to an explosion of studies investigating the domain localization of a large number of individual proteins, including proteins involved in the cellular entry and egress of viruses and other pathogens. The driving force behind these studies is the assumption that the partitioning between domains has functional consequences, and will shed light on fundamental processes in cell biology such as signaling, trafficking, macromolecular assembly, regulation of protein interactions, and membrane fission and fusion.

Although the prospects are exciting, several caveats are in order. Some of the methods for determining raft localization are not very robust and have led to conflicting results for the same protein in different labs. Because raft domains may constitute on the order of 15–20% of the plasma membrane (Schutz et al., 2000), a large number of surface proteins are likely to be found in rafts. Given that membrane proteins interact with lipids, it is not surprising that they partition unequally between different lipid domains, or that the lipid composition of a domain may influence the conformation, and hence function, of a protein (Gimpl et al., 1997). Some methods for evaluating the significance of raft-localization, such as globally disrupting rafts with cholesterol-depleting reagents, have complicated effects on cells, and it should not be concluded based on such experiments alone that the raft localization of a protein is important to its function. The more proteins with known functions that are found to be raft-associated, the longer the list of

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possible functions mediated by rafts. As the field progresses, more sophisticated ways will be found for assessing the functional consequence of raft-association in particular cases, such as analysis of mutations that alter raft association of individual proteins. In this review, we will try to point out the methods used to determine raft association—and to evaluate the significance of that association—for proteins involved in virus entry, assembly, and budding. For another recent review *see* Chazal and Gerlier, 2003.

Two methods, primarily, have been used to determine raft-association: resistance to solubilization by detergents (usually approximately 1% Triton X-100 at 4°C), indicated by cosedimentation with undissolved, low density lipids on a sucrose gradient, and confocal microscopy to determine if a protein co-localizes with a known raft protein or raft lipid aggregated on a cell surface. The latter method seems less robust, given that unmodified rafts are too small and too close together to be resolved by light microscopy (Pralle et al., 2000; Schutz et al., 2000); hence, co-localization is based on the empirical observation that raft proteins tend to aggregate and co-patch when any one of them is cross-linked by antibody (Harder et al., 1998). However, co-localization is rarely evaluated quantitatively, is sensitive to detector sensitivity settings, and is subject to artifact in regions where the cell membrane is damaged (both proteins absent), or concentrated by ruffles (both proteins present) (Singer et al., 2001). Although the first method, detergent lysis and sedimentation analysis, is usually reproducible between labs, many proteins do not segregate cleanly between raft and nonraft fractions, and their distribution may be only partially changed by raft-disrupting agents such as methyl-β-cyclodextrin (M βCD). As is frequently noted, resistance to detergent solubilization is only a surrogate for raft localization in living cells, since lipids and proteins may rearrange during lysis.

The most common method for assessing the significance of raft association for a particular protein is to see whether a function associated with that protein (such as virus entry or budding) is affected by acutely depleting cholesterol with a drug like MβCD. These drugs presumably extract cholesterol from nonraft as well as raft regions, and they alter many fundamental (raft-associated and nonraft-associated) cell processes and structures, such as signaling, endocytosis (Rodal et al., 1999; Subtil et al., 1999) and the cytoskeleton (Edidin 2003), with numerous downstream consequences. Therefore, altered function of a protein after cholesterol depletion could be due to its being affected by cholesterol outside of rafts or changes in other signaling, endocytic or cytoskeletal proteins, rather than a direct effect of rafts on the protein.

One final caveat: As in all complex systems, two things may appear to be associated with one another when, in fact, each is really associated with a

third factor. This situation may arise, for example, if a protein binds a lipid such as cholesterol or sphingomyelin that is concentrated in rafts; extraction of the lipid may affect rafts and the protein function even if the protein does not require rafts *per se*.

2. ROLE OF RAFTS IN VIRUS ENTRY

Several of these caveats are illustrated by studies of fusion caused by the prototype alphavirus, Semliki Forest virus (SFV). SFV fusion is mediated by the E1 envelope protein, which undergoes a low pH-triggered conformational change in endosomes, causing it to dissociate from a companion envelope protein, homotrimerize, and bind to target membranes. SFV fusion requires cholesterol and sphingolipid in the target membrane, highly suggestive of a need for rafts. Cholesterol depletion, which can be long-term and essentially complete for insect cells, a natural target for SFV, eliminates rafts and reduces fusion of wild-type SFV by over 5 orders of magnitude (Chatterjee et al., 2002). The lipid requirements for binding and fusion have been investigated using synthetic liposomes, which bind the E1 ectodomain (E1*) and fuse with low pH-activated virus. It turns out the lipid requirements for binding and fusion are different from those of rafts (Ahn et al., 2002; Waarts et al., 2002). For example, binding requires a 3' OH group of cholesterol in the beta configuration: Liposomes made with epicholesterol, the alpha stereoisomer, contained rafts by a fluorescence assay (Xu and London, 2000) or a detergent insoluble flotation assay (Ahn et al., 2002) but did not bind E1* (Ahn et al., 2002). Similarly, liposomes made with dipalmitoylphosphatidylcholine instead of sphingomyelin contained rafts but did not bind E1*. Conversely, liposomes made with sphingomyelins with unsaturated acyl chains fused with SFV but did not form rafts. Thus, one can have rafts that do not bind SFV and SFV fusion without rafts. In addition, point mutations in E1 have been selected that allow virus entry in the absence of cholesterol or sphingomyelin, and hence of rafts (Chatterjee et al., 2002). Although these experiments do not rule out a role for rafts in SFV fusion in vivo, a simpler interpretation is that the viral fusion protein binds stereospecifically to certain lipid headgroup configurations (*see also* Moseby et al., 1995; Samsonov et al., 2002) rather than to rafts *per se*.

For HIV-1, several steps related to its entry into target cells have been proposed to involve rafts, although there is still considerable controversy. The primary receptor for HIV-1 is the cell surface molecule CD4. Multiple groups have reported that CD4 is located in rafts using detergent lysis-sedimentation methods. Several groups also find that disrupting rafts by extracting cholesterol and blocking its synthesis inhibits HIV-1 entry and

syncytium formation, consistent with (but not proving) a role for rafts in HIV-1 entry (Liao et al., 2001; Popik et al., 2002). Disagreement exists, however, concerning the effect of mutations in CD4 that alter its raft location. Raft-targeting determinants reside in the transmembrane and cytoplasmic domains of CD4, possibly in peri-membrane cysteines that become palmitoylated and in cysteines in the cytoplasmic tail that are involved in binding $p56^{LCK}$, a raft-associated tyrosine kinase. One group reported that replacing the transmembrane and cytoplasmic tail domains of CD4 with portions of other proteins that maintained raft localization retained susceptibility to HIV, whereas replacement with a portion of the low-density lipoprotein receptor (LDLR) that abrogated raft localization inhibited HIV-1 entry (Del Real et al., 2002). In contrast, another group reported that mutating the raft-targeting cysteines of CD4 or treating with 2-bromo palmitate, both of which abrogated raft association, had no effect on susceptibility to HIV infection (Percherancier et al., 2003).

The possible role of rafts in HIV fusion is complicated by observations concerning the location of chemokine receptors CXCR4 and CCR5, which function as HIV coreceptors. Binding to CD4 induces a conformational change in the HIV-1 envelope glycoprotein gp120, which exposes a variable region of gp120 that is involved in binding CCR5 or CXCR4 (Trkola et al., 1996; Wu et al., 1996; Kwong et al., 1998). Coreceptor binding further changes the conformation of envelope, exposing a fusion peptide located at the N-terminus of the transmembrane envelope protein gp41 that inserts into target cell plasma membrane, and leading downstream regions of gp41 to form a "coiled-coil" of α-helices that are believed to pull virus and host membranes together (Eckert and Kim, 2001). CCR5 was reported by one group to be associated with rafts (Manes et al., 1999), but another group found it not to be raft-associated (Percherancier et al., 2003). There is also disagreement about whether CXCR4 is found in rafts, although the majority opinion seems to be that it is not raft-associated (Manes et al., 2000; Kozak et al., 2002; Popiket et al., 2002). If the coreceptors are not in rafts but CD4 is, does HIV bind simultaneously to raft and nonraft proteins? It has been proposed that forced mixing of raft and nonraft domains by HIV might destabilize the membrane in some way that promotes fusion (Kozak et al., 2002).

The pathway for HIV infection is potentially more complex in vivo than in vitro due to the need to pass through a variety of barrier tissues. Rafts have been implicated in several of these steps. For example, HIV is believed to use transcytosis to get through the protective epithelial barrier lining the genital and gastrointestinal tract (Bomsel, 1997; Meng et al., 2002). Transcytosis is initiated by interaction between HIV-1 envelope with glycosphingolipid galactosyl ceramide (Gal Cer), an alternative receptor for

HIV-1 in epithelial cells (Yahi et al., 1992; Bhat et al., 1993). Gal Cer is markedly enriched in rafts at the apical surface of epithelial cells (Simons and van Meer, 1988), and disruption of rafts inhibited HIV-1 transcytosis (Alfsen et al., 2001). To penetrate the blood–brain barrier, HIV-1 is thought to cross microvascular endothelial cells via a related process, macropinocytosis, also dependent on lipid-raft integrity (Liu et al., 2002). Glycosphingolipids like Gb3, GM3, and α-hydroxylated galactosylceramide (GalCer-HFA), which typically have long saturated acyl chains and associate with cholesterol to form rafts, can also work as HIV-1 entry cofactors by organizing gp120-gp41, CD4, and an appropriate chemokine receptor into a membrane fusion complex (Hammache et al., 1998; Hug et al., 2000).

Another approach with HIV has focused on the possible significance of the raft-like composition of HIV virions for their fusion competence. HIV virions are enriched in cholesterol and sphingolipids compared to plasma membrane (Aloia, 1993). This could be a result of budding through raft domains. To see whether this lipid composition was important for infectivity, HIV virions were treated with MBCD. The cholesterol-depleted virions had similar structure, protein content, and binding capacity as untreated virions, but could not be internalized (Guyader et al., 2002). Virion incorporation of signaling molecules derived from raft budding sites was also proposed possibly to promote HIV-1 entry by influencing cellular signaling (Poon et al., 2000; Vereb et al., 2000).

Studies with avian leucosis virus (ALV), an enveloped type C retrovirus, suggest another way in which receptor localization may affect the virus entry pathway. For many years, ALV was thought to enter cells by fusing at the plasma membrane, like HIV, rather than via a low pH-triggered conformational change in endosomes. Consistent with fusion at the plasma membrane, drugs that raise the pH in endosomes did not inhibit infection by ALV, whereas they did block infection of classically endocytosed viruses like SFV and vesicular stomatitis virus (VSV). However, later experiments showed that the endosome-alkalinizing drugs did prevent ALV fusion while they were present, but unlike other viruses that use the endosome pathway, ALV was stable in endosomes, so that when the drugs were washed out (which was necessary to prevent toxicity), ALV infection resumed, explaining the previous failure to detect pH-dependent entry for ALV (Mothes et al., 2000). Further, it was found that the conformational changes in the ALV envelope associated with fusion required two signals—binding to receptor plus acidification—whereas for other viruses, receptor binding or acidification alone were sufficient. For ALV, the receptor is a raft-associated protein, Tva, which is related to the LDL receptor but is attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) linkage. Thus, ALV seems to pro-

vide a model for a new class of viral fusion mechanism involving binding to a raft protein, endocytosis via a non-clathrin pathway, and low pH-triggered fusion in the endosome. (These results remain controversial, however; for another view *see* Earp et al., 2003).

This picture was recently complicated (or enriched!) by studies in which the GPI-linkage-determining portion of Tva was replaced with the transmembrane domain of a related, nonraft protein (Narayan et al., 2003). This change resulted in ALV being endocytosed via the classical clathrin-mediated endocytic pathway. Virus infectivity was only minimally affected. Thus, for ALV, although the raft versus nonraft location of the receptor may determine the endocytic route, it does not strongly affect efficiency of entry.

A similar variation in internalization route was reported for the nonenveloped echovirus 11, a member of the enterovirus family (Stuart et al., 2002). Several enteroviruses use a complement regulatory protein, decay accelerating factor (DAF), as receptor. Like Tva, DAF is anchored to the plasma membrane via a GPI-linkage, and like other GPI-linked proteins, DAF is raft-associated. Several GPI-linked proteins have been shown to undergo endocytosis following antibody-crosslinking in a microtubule- and cholesterol-dependent manner (Deckert et al., 1996). Infection with a DAF-binding strain of echovirus 11 was inhibited by the microtubule inhibitor nocodazole and by the cholesterol binding agent nystatin. In contrast, a tissue culture-adapted strain of the same virus that did not bind DAF was relatively resistant to nocodozole and nystatin, but susceptible to doses of chlorpromazine that inhibited clathrin-mediated endocytosis. Thus, closely related strains of enterovirus appear to infect cells via different (clathrin-independent versus clathrin-dependent) endocytic pathways, depending on whether the cell-surface protein to which they bind is raft-associated or nonraft-associated.

The entry pathway for SV40, a nonenveloped DNA virus, begins with binding to the MHC Class I molecule, followed by translocation to caveolae, specialized cup-shaped invaginations on the surface of many cells that mediate a clathrin-independent form of endocytosis (Stang et al., 1997; Anderson et al., 1998). Caveolae are rich in the raft protein caveolin, which forms a cage around nascent endocytic vesicles similar to clathrin. Dominant-negative amino-terminal truncation mutants of caveolin inhibited SV40 infection. Elegant studies following fluorescently labeled SV40 virions in live cells have illuminated numerous steps in this raft-dependent pathway that couples movement after endocytosis to cytoskeletal elements (Pelkmans et al., 2001).

There is limited experimental support for the use of rafts as a platform for entry by other enveloped viruses, including murine leukemia virus (MLV),

Ebola virus, and Marburg virus. The cellular receptor for the ecotropic class of MLV is physically associated with caveolin in membrane rafts. Disruption of rafts with MβCD inhibited an early step of ecotropic MLV infection. Cholesterol seems to play an asymmetric role in MLV fusion, since fusion was more extensively inhibited by cholesterol depletion of receptor-bearing membrane than of envelope-bearing membrane (Lu et al., 2000b, 2002). Disruption of rafts by depleting cholesterol in the target cells inhibited entry of Ebola and Marburg viruses at a post-binding step (Bavari et al., 2002; Empig and Goldsmith, 2002).

Rafts in the target membrane do not seem to be important for the entry of VSV. Hence VSV, or pseudotype viruses bearing the VSV-G envelope, are often used as negative controls in studies of raft-dependent entry.

3. ROLE OF RAFTS IN THE ENTRY OF BACTERIAL PATHOGENS AND TOXINS

Several bacterial pathogens and toxins use rafts as platforms for entry into mammalian cells. These include enteric bacteria that bind DAF (Selvarangan et al., 2000); bacteria that bind the GPI-linked protein CD38 (Shin et al., 2000); campylobacter that enter via caveolae (Wooldridge et al., 1996); cholera toxin, the B moiety of which binds ganglioside GM1 (Orlandi and Fishman, 1998); Shiga toxin, the 1B subunit of which binds to the raft-associated neutral glycolipid globotriaosylceramide Gb3 (Kovbasnjuk et al., 2001); tetanus toxin, which binds GPI-linked Thy-1 on neuronal cells (Herreros et al., 2001); and anthrax toxin (protective factor), which undergoes post-binding cleavage and oligomerization associated with translocation to rafts (Abrami et al., 2003).

4. ROLE OF RAFTS IN VIRUS ASSEMBLY AND BUDDING

Several kinds of evidence suggest that rafts play a role in the assembly and/or budding of a variety of viruses. The lipid composition of some viruses is richer in cholesterol and sphingolipids than are the membranes of the cells that produce them (Aloia et al., 1992); for some viruses, however, such as VSV and SFV, this difference is not a reliable indicator of raft association. Some viruses preferentially incorporate GPI-linked raft proteins as they bud from cells, suggesting budding from rafts (Pickl et al., 2001). Structural proteins of several enveloped viruses have been shown to associate with detergent-resistant membranes. In some cases the effect of cholesterol depletion on virus production or infectivity has been studied. Future experi-

ments should investigate the effect on assembly of mutations that abrogate raft association.

One of the best studied systems is influenza virus. The hemagglutinin (HA) and neuraminidase (NA) envelope genes of influenza virus are raft-associated (Scheiffele et al., 1997, 1999; Barman et al., 2001) and targeted to the apical membrane of polarized epithelial cells, although these phenotypes do not completely correlate. Raft-targeting determinants were identified in the transmembrane domains of HA and NA. In the case of one influenza virus strain, raft targeting motifs for HA include three palmitoylated cysteines in the TM and cytoplasmic tail, mutation of which reduced raft association without altering fusogenicity (Steinhauser et al., 1991). This suggests that raft association may not be important for entry (but *see* Sakai et al., 2002 for conflicting results). When wild-type HA or NA were coexpressed with matrix protein M1, the detergent resistance of M1 was increased, consistent with raft-association of a virus assembly complex (Ali et al., 2000; Zhang et al., 2000). The existence of mutations in HA and NA that abrogate raft association should provide a way to evaluate whether raft association is required for assembly or budding of influenza virus, but so far no studies of this sort have been reported.

For HIV, the major core structural protein (Pr55gag) is made in the cytosol but concentrated on the inner surface of the plasma membrane due to the presence of membrane targeting motifs at its amino terminus (Lindwasser and Resh, 2002). About 50% of membrane-associated Pr55gag stays membrane-associated after lysis with 0.25% Triton X-100 at 4°C, indicating that it is raft-associated (Ono and Freed, 2001). Pulse chase studies showed that the fraction of wild-type gag that was raft-associated increased over time, which argues that raft association is not an artifact of protein-lipid rearrangement during detergent extraction, since this would not change over time, but rather reflects a post-translational change in gag. Further, C-terminally truncated forms of gag lacking motifs involved in gag–gag interactions were slower to associate with rafts, suggesting that raft association is promoted by gag multimerization. Cholesterol depletion inhibited the release of wild-type HIV, consistent with rafts promoting assembly or release. Interestingly, cholesterol depletion did not impair the release of HIV with a mutation in a C-terminal portion of gag that binds a cellular enzyme complex (ESCRT-I) thought to promote budding (Pornillos et al., 2002). This result raises the possibility that HIV's cholesterol requirement is mediated through a need for cholesterol by the cellular budding machinery "hijacked" by HIV. Recent reports confirm the association of HIV gag with rafts, but indicate that these rafts may be atypical in some respects (Ding et al., 2003; Halwani et al., 2003; Holm et al., 2003). (For other views on the role of rafts in HIV assembly *see* Nguyen and Hildreth, 2000; Rousso et al., 2000; Wang

et al., 2000; Campbell et al., 2001; Zheng et al., 2001; Raulin, 2002; Saez-Cirion et al., 2002; Yang and Ratner, 2002)

Moloney murine leukemia virus envelope has a palmitoylated cysteine near the cytosolic end of its transmembrane domain. Mutation of this cysteine abrogated raft association and slightly reduced the surface expression of MLV env, but had little effect on fusion activity (Li et al., 2002); this result suggests—as for influenza virus—that raft association of envelope may be more important for assembly than for entry.

Rafts have been implicated in the assembly of several paramyxoviruses. Studies report raft association by detergent lysis-flotation analysis of envelope and matrix proteins from Sendai virus (Ali and Nayak, 2000), respiratory syncytial virus (Brown et al., 2002a, 2002b; Henderson et al., 2002), and viral ribonucleoprotein as well as matrix and envelope proteins for measles virus (Manie et al., 2000; Vincent et al., 2000). Membrane and matrix-like tegument proteins from the DNA herpes virus have also recently been reported to be located in rafts (Koshizuka et al., 2002; Lee et al., 2003).

Studies of budding of alphaviruses have shown a requirement for cholesterol and sphingomyelin. Interestingly, the same mutations in envelope that reduce dependence on cholesterol for fusion also reduce dependence on cholesterol for budding (Lu et al., 2000a). This is surprising, because the conformation of envelope for budding is very different than for fusion. The fact that point mutations in envelope promote budding in the absence of cholesterol shows that envelope is involved in budding as well as fusion, which is consistent with the fact that for α–viruses, budding requires envelope as well as nucleocapsid. Presumably, the membrane lipid composition affects the conformation of envelope, and cholesterol-independent mutants have nearly "normal" conformations in the absence of cholesterol for both budding and fusion.

5. ROLE OF RAFTS IN VIRAL REPLICATION AND PATHOGENESIS

Given the level of interest in membrane rafts, it is not surprising that other viral functions are being found to involve raft proteins. For example, replication complexes of hepatitis C virus were recently reported to associate with intracellular membranes with raft properties using detergent lysis methods and confocal microscopy (Shi et al., 2003). Many RNA viruses replicate in cytoplasmic membrane complexes, so it will be of interest to see whether this is a general property. Some viral pathogenesis pathways have recently been tied to rafts, such as a signaling membrane protein from Epstein-Barr virus that immortalizes B cells (Dykstra et al., 2001; Coffin et al., 2003). Because many viral effects on cells involve co-opting of normal

signaling pathways, and many signaling pathways involve rafts, it is likely that many viral pathogenesis pathways will be tied to rafts.

6. WHY RAFTS?

Why do so many viruses appear to use rafts in their entry and exit processes? This question cannot at present be answered. One possibility is that there is no special predilection for rafts—they just constitute a large portion of cell surface molecules used by viruses to get into and out of cells. Other speculative hypotheses are that rafts are specialized domains for endocytosis, a required first step for viruses that do not penetrate or fuse at the plasma membrane; that entry mechanisms require the concerted action of several envelope or receptor molecules and that rafts facilitate this process by concentrating these molecules; that entry requires signaling, and rafts are specialized signaling locales; that budding requires cell enzymes that concentrate in rafts; and that the biophysical properties of rafts or raft-nonraft boundaries help overcome barriers to membrane fusion and fission. Lipid composition affects membrane flexibility, intrinsic curvature, and viscosity, properties relevant to fusion (Huettner et al., 2001). Domains with different lipid composition are predicted to bud out of lipid bilayers (Lipowsky, 1992). Coupling between membrane heterogeneity, protein binding, and protein-induced shape changes likely lies at the heart of biological budding and fusion phenomena—processes that, so far, have eluded detailed molecular explanation.

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8

Alterations in Raft Lipid Metabolism in Aging and Neurodegenerative Disorders

Mark P. Mattson, Roy G. Cutler, and Norman J. Haughey

1. INTRODUCTION

Sphingolipids are a prominent type of membrane phospholipid in eukaryotic cells and are particularly abundant in the nervous systems of mammals. They consist of a glycerol backbone with a phosphocholine zwitterionic hydrophilic headgroup and two long hydrocarbon chains that form a hydrophobic domain (Fig. 1). The hydrocarbon chains of sphingomyelin are relatively long (>20 carbons) and contain more saturated bonds than are present in phosphatidylcholine. Sphingomyelin contributes prominently to the biophysical properties of membranes, and in particular, the large disparity in the lengths of the two chains of sphingomyelin may allow for interdigitation between the hydrocarbons in the two opposing monolayers of the phospholipid bilayer, thereby providing a means for coupling-phase separation with the marked curvature of cell membranes. Sphingolipids also have a Tm near body temperature (37°C). The collective biophysical properties of sphingolipids suggest that they play important roles in the formation of specialized domains in membranes such as lipid rafts. Sphingolipid synthesis is initiated by serine palmitoyltransferase (SPT), which catalyzes the condensation of palmitoyl-CoA (acyl) with serine to form 3-dihydrosphinganine, and additional biosynthetic steps result in formation of sphingosine, which is then acylated to form ceramide. Choline can then be added to ceramide by choline-phosphotransferase, which results in the synthesis of sphingomyelin.

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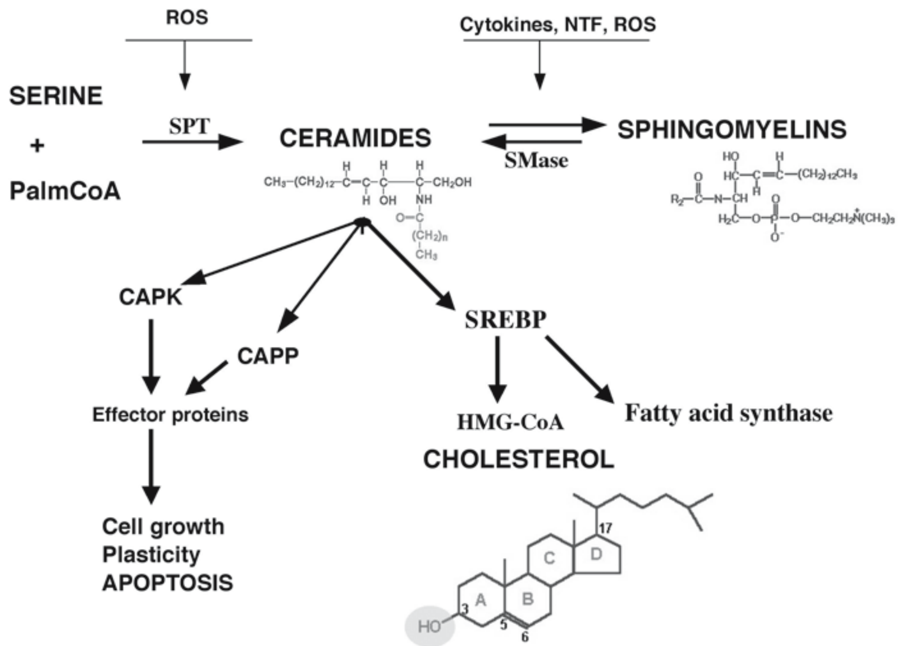

Fig. 1. Pathways of sphingomyelin and cholesterol metabolism relevant to lipid raft biology. Ceramides are produced from serine and palmitoyl CoA, a reaction catalyzed by serine palmitoyl CoA transferase (SPT). Ceramides are incorporated into sphingomyelin, a prominent raft lipid. In response to signals such as cytokines, neurotrophic factors, and reactive oxygen species (ROS), sphingomyelinases (SMase) are activated and hydrolyze sphingomyelin, resulting in the release of ceramide. Ceramide can activate specific protein kinases (ceramide activated protein kinase [CAPK]) and protein phosphatases (ceramide activated protein phosphatase [CAPP]), which modify the phosphorylation of protein substrates involved in a variety of physiological cellular responses; ceramides can also trigger apoptosis. Cholesterol is a major component of lipid rafts. The formation of cholesterol and cholesterol esters can be induced by ceramides through the activation of (SREBP).

Although it was initially thought that sphingolipids and other membrane phospholipids functioned only as structural components of membranes, it has become evident that sphingomyelin, phosphatidylcholine and related membrane phospholipids serve important roles in cellular signal transduction (Alessenko, 2000). A variety of stimuli can induce enzymatic, or in some cases nonenzymatic, cleavage of one or more membrane phospholipids resulting in the release of bioactive messenger molecules within the cell. For example, membrane receptors for neurotransmitters and hormones that are coupled to certain GTP-binding proteins can activate phospholipase C,

resulting in the cleavage of phosphatidyl inositol bis-phosphate and the release of diacylglycerol and inositol trisphosphate; diacylglycerol activates protein kinase C, while inositol trisphosphate induces calcium release from intracellular stores. Phospholipase-A2 hydrolyzes the acyl group of glycerophospholipids at the sn-2 position, resulting in the release of free fatty acids and lysophospholipids, which can be further metabolized to bioactive lipid mediators including platelet activating factor, eicosanoids, and lysophosphatidic acid. Sphingomyelin can be cleaved by several different sphingomyelinases, among which acidic and neutral sphingomyelinases have been most intensively studied. Cleavage of sphingomyelin by sphingomyelinase results in the release of ceramide, a bioactive lipid mediator which has been shown to exert profound effects on various cellular processes. In neurons, ceramide may play particularly important signaling roles in synapses (Yang, 2000).

A deficiency of acidic sphingomyelinase causes Niemann–Pick disease in humans. Mice lacking acidic sphingomyelinase develop normally for approximately 4 months, after which they begin to exhibit signs of ataxia that is associated with degenerative changes in the cerebellum (Horinouchi et al., 1995). Radiation-induced damage to lung cells is markedly reduced in acidic sphingomyelinase-deficient mice, and this resistance to cell death is associated with lack of an increase in ceramide levels in lung tissue after irradiation (Santana et al., 1996). Neutral sphingomyelinase is associated with plasma membranes and is activated in response to a variety of stimuli, including tumor necrosis factor-alpha (TNFα), Fas ligand, and vitamin D (Hannun and Obeid, 1997). Presumably, activation of neutral sphingomyelinase results in local production of ceramide at that membrane site. The regulation of neutral sphingomyelinase is not yet fully understood, but may require arachidonic acid and soluble phopsholipase-A2. Interestingly, sphingomyelinase activities are also modulated by cellular redox state, with oxidative stress increasing enzyme activity, and glutathione and other antioxidants decreasing enzyme activity (Liu and Hannun, 1997).

Cholesterol is highly concentrated in lipid rafts, where it may play roles in maintaining raft stability, in the clustering of receptors, and in modulating signal transduction (Legler et al., 2003; Silvius, 2003). Cholesterol associates with sphingomyelin via hydrogen bonding, and this association appears critical for the formation and maintenance of raft domains; a specific ratio of sphingomyelin to cholesterol is required for raft formation. In neurons, such cholesterol-rich lipid rafts maybe concentrated in synaptic terminals, regions where receptors for neurotransmitters, growth factors, and cytokines are also concentrated. The vast majority of cholesterol in the brain is synthesized by brain cells rather than being transferred from the blood.

Membrane cholesterol is present in its free form, but can also be esterified and sequestered in subcellular compartments. Remarkably little is know about the functions of membrane cholesterol in neurons, although recent studies have provided evidence that cholesterol plays important roles in regulating growth cone behaviors and synaptogenesis and in signaling processes that mediate synaptic plasticity (Goritz et al., 2002; Tsui-Pierchala et al., 2002). A simplified diagram of sphingolipid and cholesterol metabolism in neural cells is shown in Fig. 1.

Several lines of evidence suggest that changes in the formation and/or metabolism of membrane sphingolipids and cholesterol occur during normal aging and in neurodegenerative disorders on the one hand, and contribute to the dysfunction and degeneration of neurons on the other hand. Recent reviews on these and related aspects of raft lipid metabolism in aging and disease have been published (Shinitzky 1987; Dietschy and Turley, 2001; Wood et al., 2002) and we will not duplicate the information in those articles. Instead, we focus on our own studies in which the various raft lipids have been measured in samples from human patients and in animal and cell culture models, and studies in which manipulations of metabolism of the lipids have been shown to affect neuronal vulnerability in disease models.

2. AGING

During normal aging, levels of membrane-associated oxidative stress increase in brain cells as indicated by increased levels of lipid peroxidation products such as 4-hydroxynonenal (Cutler et al., 2003) and of oxidatively modified membrane proteins such as the Na^+/K^+-ATPase and the mitochondrial F1F0 ATP synthase (Davies et al., 2001; Chakraborty et al., 2003). Membrane-associated oxidative stress may compromise the function of an array of signaling pathways, membrane transporters, ion channels, metabolic enzymes, transcription factors, and so on (Kelly et al., 1996; Blanc et al., 1997; Mark et al., 1997; Mattson et al., 1997; Lu et al., 2001). Measurements of concentrations of sphingomyelins, ceramides, and cholesterol in brain tissue samples from mice of different ages, from young to old, revealed highly significant increases in the amounts of long-chain ceramides and free cholesterol during aging (Cutler et al., 2003). Increases in ceramide levels during aging have also been reported to occur in liver cells (Lightle et al., 2000). Particularly large increases in C24:0 ceramide and galactosylceramide occurred in brain cells during normal aging (Fig. 2). Experiments in which sphingolipid and cholesterol metabolism were manipulated provide further evidence for roles of altered raft lipid metabolism in normal aging. When cultured fibroblasts were exposed to ceramide, the level of beta-

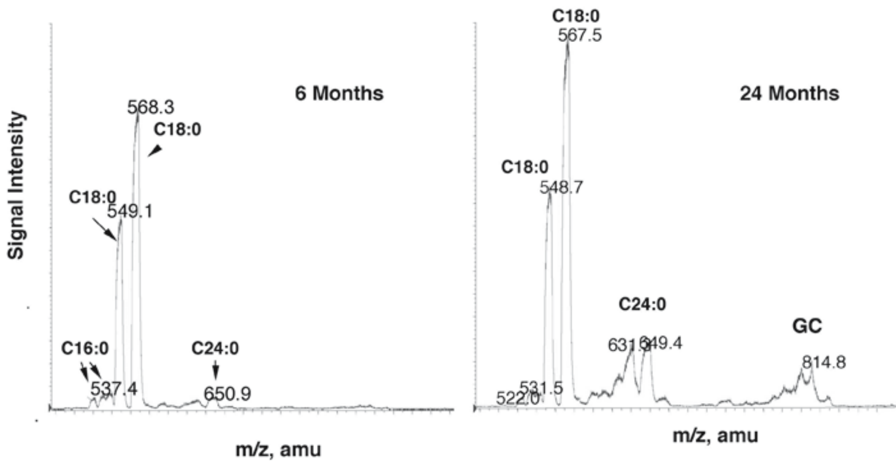

Fig. 2. Mass spectrograms showing a side-by-side comparison of levels of various ceramides in brain tissue samples from the cerebral cortex of a young (6-month-old) mouse *(left)* and an old (24-month-old) mouse *(right)*. Note that levels of C24:0 ceramide and galactosylceramide (GC) are increased in the sample from the old mouse. Modified from Cutler, et al. (2003).

galactosidase, a histochemical marker of senescence, increased greatly (Mouton and Venable, 2000). The kinds of data just described suggest that alterations in lipids that play important roles in raft biology may occur during aging, and these alterations could contribute to the dysfunction of cells in various organ systems during aging and to the pathogenesis of age-related diseases.

3. ISCHEMIC STROKE

Stroke, a major cause of long-term disability and of mortality worldwide, is most commonly caused by occlusion of a blood vessel in the brain as the result of atherosclerotic disease and clot formation in the affected vessel. Transient occlusion of the middle cerebral artery in mice or rats is a well-established model of focal ischemic stroke that has been widely used to elucidate the cellular and molecular mechanisms underlying neuronal damage following a stroke, and to develop novel therapeutic interventions that reduce such damage (McAuley, 1995). Among the mechanisms believed to play major roles in the damage and death of neurons after a stroke are increased oxidative stress, energy deprivation, excitotoxicity/cellular calcium overload, and a form of programmed cell death called apoptosis (Dirnagl et al., 1999; Mattson et al., 2001; Mattson, 2003).

Many different abnormalities in membranes of ischemic neurons have been identified, including lipid peroxidation, protein modifications, and alterations in receptors, ion channels, and transporter proteins. The signaling cascades activated by cerebral ischemia that may either promote or protect against neuronal death are not well understood, although glutamate and proinflammatory cytokines are believed to promote cell death, whereas neurotrophic factors and stress resistance pathways may protect neurons against death (Mattson et al., 2000).

A signaling pathway activated by oxidative stress and cell inury that has recently been characterized in studies of non-neural cells involves hydrolysis of membrane sphingomyelin by acidic and/or neutral sphingomyelinase, resulting in generation of the second messenger ceramide. We critically tested the involvement of the latter sphingolipid signaling pathway in focal ischemic brain injury by comparing cellular and molecular responses to middle cerebral artery occlusion in acidic sphingomyelinase knockout mice with responses in wild-type mice (Yu et al., 2000). Transient focal cerebral ischemia in wild-type mice induced a large increase in acidic sphingomyelinase activity and a corresponding increase in the amount of ceramide in the ischemic brain tissue. Little or no increase in ceramide levels occurred in the ischemic brain tissue of acidic sphingomyelinase-deficient mice (Yu et al., 2000). Interestingly, we found that the expression of pro-inflammatory cytokines (TNFα, interleukin-1β, and interleukin-6) increased greatly in response to focal cerebral ischemia in wild-type mice, but not in acidic sphingomyelinase-deficient mice (Yu et al., 2000). Thus, sphingomyelinase activity, and presumably ceramide production, is an event that plays a critical role in ischemia-induced cytokine producion. The extent of brain tissue damage was significantly decreased and behavioral outcome was significantly improved in acidic sphingomyelinase-deficient mice.

In order to further establish the involvement of ceramide in ischemic neuronal injury, we treated wild-type mice with D-609, an agent that inhibits ceramide production. Ischemia-induced ceramide production and inflammatory cytokine expression were decreased, extent of neuronal damage (infarct volume) reduced, and behavioural outcome was improved in mice receiving D-609 (Yu et al., 2000).

To determine the role of ceramide production specifically in neurons in ischemic neuronal death, we cultured hippocampal neurons from wild-type and acidic sphingomyelinase-deficient mice. Neurons from acidic sphingomyelinase-deficient mice exhibited decreased vulnerability to excitotoxicity and hypoxia, which was associated with decreased levels of intracellular calcium and oxyradicals (Yu et al., 2000). Pretreatment of cul-

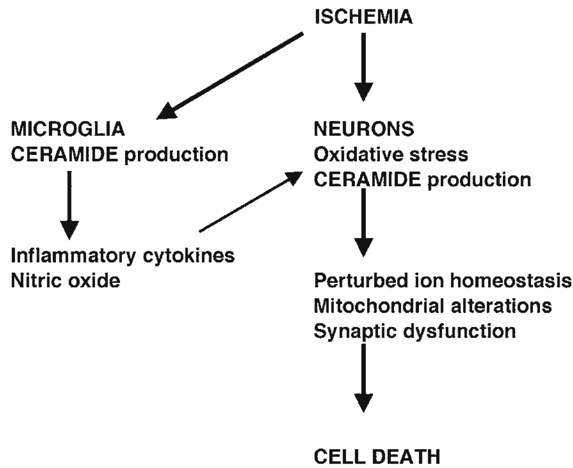

Fig. 3. Roles of ceramide in the pathogenesis of neuronal dysfunction and cell death after an ischemic stroke. Ischemia induces oxidative stress and the activation of sphingomyelinases in neurons and microglia. In neurons, ceramide disrupts cellular ion homeostasis and mitochondrial function resulting in calcium overload, synaptic dysfunction, and cell death. Ceramide production in microglia stimulates these cells to produce proinflammatory cytokines and nitric oxide, which may exacerbate ischemic damage to neurons. Based upon data in Yu et al. (2000).

tured neurons from wild-type mice with D-609 decreased their vulnerability to excitotoxicity and hypoxia, suggesting a key role for ceramide production in ischemic neuronal death. Collectively, the available data suggest that, by generating ceramide, sphingomyelin hydrolysis plays an important role in ischemic neuronal injury, by direct actions in neurons and by indirect, microglia-mediated effects (Fig. 3). Ceramide may induce cell death by triggering apoptotic pathways and mitochondrial dysfunction (Pettus et al., 2002). The specific mechanism(s) whereby ceramide triggers apoptosis is not known, but studies have implicated the involvement of several proteins. For example, Willaime et al. (2001) provided evidence that the protein kinase p38 is activated by ceramide in cortical neurons, and that p38 activation is a pivotal event in ceramide-induced apoptosis. Ceramide might also promote apoptosis by inhibiting cell survival-promoting proteins such as Akt kinase (Zhou et al., 1998). Our findings suggest that drugs that inhibit ceramide production, or that block its cytotoxic actions, may prove beneficial in the treatment of stroke patients. Additional evidence for a key role for ceramide in ischemic neuronal death comes from a study showing that the drug FK506 suppresses stroke-induced generation of ceramide and apoptosis (Herr et al., 1999).

While increases in ceramide production have been linked to apoptosis, ceramide can also activate cell survival-promoting pathways. For example, pretreatment of cultured rat hippocampal neurons with membrane-permeant forms of ceramide protected them from death induced by glutamate, oxidative stress, and amyloid β-peptide (Goodman and Mattson, 1996). A more recent study showed that ceramide can protect neurons against hypoxic-ischemic injury by a mechanism involving upregulation of Bcl-2 (Chen et al., 2001). Ceramide may mediate cell survival-promoting effects of some neurotrophic factors and cytokines, including nerve growth factor (Brann et al., 1999) and TNFα (Mattson and Camandola, 2001).

4. ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a common disease in the elderly that is characterized by the progressive degeneration of neurons in brain regions involved in learning and memory and affective behaviors; more than 4 million Americans are currently living with AD. AD involves the progressive deposition of amyloid β-peptide (Aβ) and associated degeneration of neurons in brain regions involved in learning and memory (DeKosky, 2001). Two factors that are believed to contribute to neuronal dysfunction and degeneration in AD are increased oxidative stress and increased production of neurotoxic forms of Aβ (Mattson, 1997). Aβ may damage and kill neurons by inducing membrane-associated oxidative stress resulting in the impairment of membrane transporters and disruption of ion homeostasis (Mattson, 1997). Interestingly, Aβ has a sphingolipid-binding domain that may promote its interaction with lipid rafts (Mahfoud et al., 2002). The latter finding suggests that Aβ may induce a particularly high level of oxidative stress in lipid rafts and impair raft-associated signaling pathways.

Emerging evidence suggests that alterations in lipid metabolism also contribute to the pathogenesis of AD. For example, the risk of AD is affected by inheritance of different isoforms of the cholesterol transport protein apolipoprotein E (Smith, 2000), and experimentally induced changes in cholesterol metabolism can affect Aβ production in cell culture and in vivo (Puglielli et al., 2001). Moreover, drugs that lower cholesterol levels may reduce the risk of AD. We have begun studies aimed at understanding how alterations in the metabolism of cholesterol and sphingolipids might contribute to synaptic dysfunction and neuronal degeneration in AD.

We measured concentrations of sphingomyelins, ceramides, and different forms of cholesterol in tissue homogenates and isolated membranes taken from vulnerable (middle frontal gyrus and superior frontal gyrus) and nonvulnerable (cerebellum) brain regions of AD patients and age-matched

control subjects. The concentrations of long-chain ceramides and free cholesterol were significantly increased in samples from AD patients compared to controls (Cutler et al., 2004). In one set of samples taken from subjects in the religious orders study in Chicago, the AD patients were classified as having either mild, moderate, or severe AD. In the latter patients the concentrations of ceramide and free cholesterol levels in membrane samples increased with increasing disease severity (Cutler et al., 2004). Other investigators have also reported that ceramide levels are increased in brain tissue samples from AD patients compared to samples from age-matched control subjects (Han et al., 2002). Membrane-associated oxidative stress increased in association with the lipid alterations, and exposure of hippocampal neurons to Aβ induced membrane oxidative stress and the accumulation of ceramides and free cholesterol, thereby mimicking the changes observed in the patient tissue samples (Cutler et al., 2004). Treatment of neurons with an inhibitor of sphingomyelin synthesis called ISP-1 reduced levels of membrane-associated oxidative stress, prevented accumulation of ceramides and free cholesterol, and protected the neurons against death induced by Aβ, suggesting a pivotal role for altered sphingolipid metabolism in the pathogenesis of AD (Fig. 4; Cutler et al., 2004). The available data therefore suggest a scenario in which the oxidative stress associated with aging and Aβ aggregation enhance ceramide production and cholesterol accumulation, resulting in synaptic dysfunction and activation of cell-death cascades (Fig. 5).

5. AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is characterized by degeneration of motor neurons in the spinal cord, resulting in progressive paralysis and death. The pathogenic mechanism of in ALS is unknown, but may involve increased oxidative stress, overactivation of glutamate receptors, and apoptosis. Although the cause of most cases of ALS is unknown, a small number of families have been identified in which the disease is inherited in an autosomal dominant manner as the result of mutations in the antioxidant enzyme Cu/Zn-SOD (Cudkowicz et al., 1997). Transgenic mice expressing the same Cu/Zn-SOD mutations exhibit histopathological and clinical phenotypes remarkably similar to ALS patients (Del Canto et al., 1995). We have obtained data from studies of ALS patients, Cu/Zn-SOD mutant mice, and cultured motor neurons that suggest the pathogenic mechanism responsible for motor neuron degeneration involves oxidative stress (Pedersen et al., 1998, 1999), overactivation of glutamate receptors (Kruman et al., 1999), and a form of programmed cell death called apoptosis (Pedersen et al., 2000).

	Abeta	ISP-1	ISP-1+Abeta
4-hydroxynonenal	Increase	n.c.	attenuation
SM C18:0	n.c.	decrease	n.c
SM C24:0	Increase	decrease	attenuation
Ceramide C18:0	Increase	n.c.	attenuation
Ceramide C24:0	Increase	n.c.	attenuation
Gal-Cer C24:0	Increase	n.c.	attenuation
Cholesterol	Increase	n.c.	attenuation
Chol ester C18:1	n.c.	n.c.	n.c.

Fig. 4. Effects of amyloid beta-peptide on levels of membrane lipid peroxidation products, sphingomyelins, ceramides, and cholesterols in cultured neocortical neurons. Levels of the lipid peroxidation product 4-hydroxynonenal, sphingomyelin C24:0, ceramides C18:0 and C24:0, galactosylceramide C24:0, and free cholesterol were significantly increased in neurons exposed to amyloid beta (Aβ)-peptide. Treatment of neurons with ISP-1, an inhibitor of serine palmitoyl CoA transferase, attenuated the effects of Aβ-peptide on the raft lipids. Based upon data in Cutler, et al. (2004).

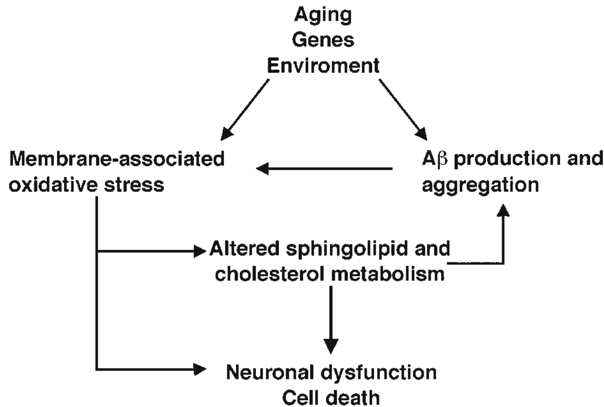

Fig. 5. Roles of membrane-associated oxidative stress and abnormalities in raft lipid metabolism in the pathogenesis of Alzheimer's disease. The aging process, and genetic (e.g., Apo E4 allele, or APP or presenilin mutations) and environmental factors (e.g., high-calorie diet or a folate-deficient diet) promote oxidative stress and perturbed APP metabolism, resulting in increased production and aggregation of Aβ-peptide. Aβ-peptide exacerbates membrane-associated oxidative stress, resulting in alterations in sphingolipid and cholesterol metabolism, which may, in turn, contribute to altered APP processing, neuronal degeneration, and cell death.

We have documented abnormalities in sphingolipid and cholesterol metabolism in the spinal cords of ALS patients and in a transgenic mouse model (Cu/Zn-SOD mutant mice), which manifest increased levels of sphingomyelin, long-chain ceramides, and cholesterol esters; in the ALS mice these abnormalities precede the clinical phenotype (Cutler et al., 2002). Increases in the amount of cholesterol esters in spinal cords of ALS patient and ALS mice were particularly striking (Fig. 6). Increased oxidative stress occurs in association with the lipid alterations in ALS patients and mice, and exposure of cultured motor neurons to oxidative stress increases the accumulation of sphingomyelin, ceramides and cholesterol esters. Pharmacological inhibition of sphingomyelin synthesis prevents accumulation of ceramides and cholesterol esters and protects motor neurons against death induced by oxidative and excitotoxic insults, suggesting a pivotal role for altered sphingomyelin metabolism in the pathogenesis of ALS.

6. HIV DEMENTIA

Acquired immune deficiency syndrome (AIDS), caused by infection with the HIV-1 virus, is responsible for millions of deaths worldwide each year. In addition to its adverse effects on the immune system, HIV-1 can infect the brain, and, as a consequence, many AIDS patients develop a neurodegenerative disorder called HIV dementia. Analyses of brain tissue samples from patients with HIV dementia have demonstrated evidence of oxidative damage including increased amounts of peroxynitrite, 4-hydroxynonenal, and protein carbonyls (Boven et al., 1999; Turchan et al., 2003). The HIV-1 virus produces at least two proteins called gp120 and Tat that are neurotoxic by a mechanism involving increased oxidative stress (Foga et al., 1997; Kruman et al., 1998). In addition, proinflammatory cytokines that are increased in the brains of HIV dementia patients can induce ceramide production in non-neural cells by increasing the activity of sphingomylinases (Shi et al., 1998). CNS inflammation in HIV dementia may be initiated by the influx of HIV-infected macrophages into the brain parenchyma; the macrophages release HIV products such as Tat and gp120 that may then promote a cytokine imbalance. Consistent with the latter scenario, successful antiretroviral treatment can slow cognitive decline and restore the cytokine balance (McArthur et al., 2003).

To determine whether alterations in membrane sphingolipid metabolism play a role in the pathogenesis of HIV dementia, we measured levels of oxidative stress, sphingolipids, and cholesterol in brain tissue samples from HIV dementia patients, HIV-infected patients without dementia, and neurologically normal control subjects. We found that levels of long-chain

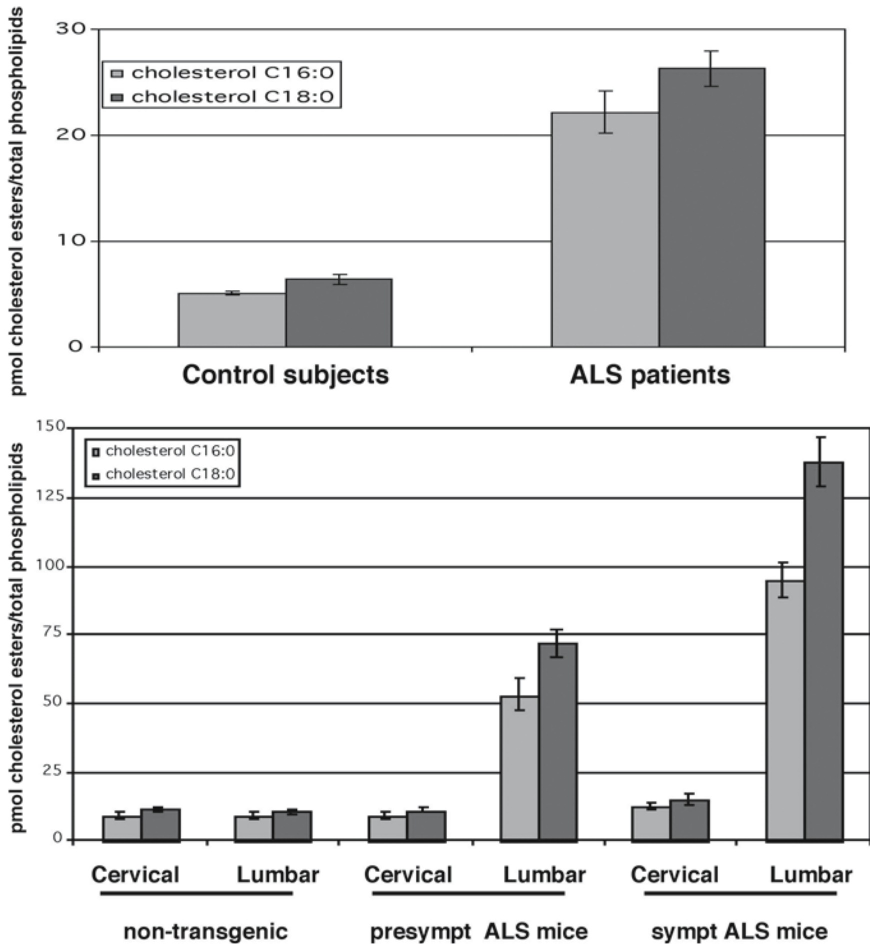

Fig. 6. Levels of cholesterol esters are increased in spinal cords of patients with amyotrophic lateral sclerosis (ALS) and in a mouse model of ALS. Concentration comparisons of C16:0 and C18:0 cholesterol esters were made in lumbar spinal cord tissue samples from ALS patients *(upper graph)*, and in cervical and lumbar spinal cord tissue samples from presymptomatic and symptomatic ALS mice (Cu/Zn-SOD mutant mice), and age-matched nontransgenic control mice *(lower graph)*. Modified from Cutler, et al. (2002).

ceramides and sphingomyelin are significantly increased in brain tissue samples from HIV dementia patients compared to nondemented HIV-infected patients and control subjects (Haughey et al., 2004). We also found that levels of ceramides and sphingomyelin are significantly increased in cerebrospinal

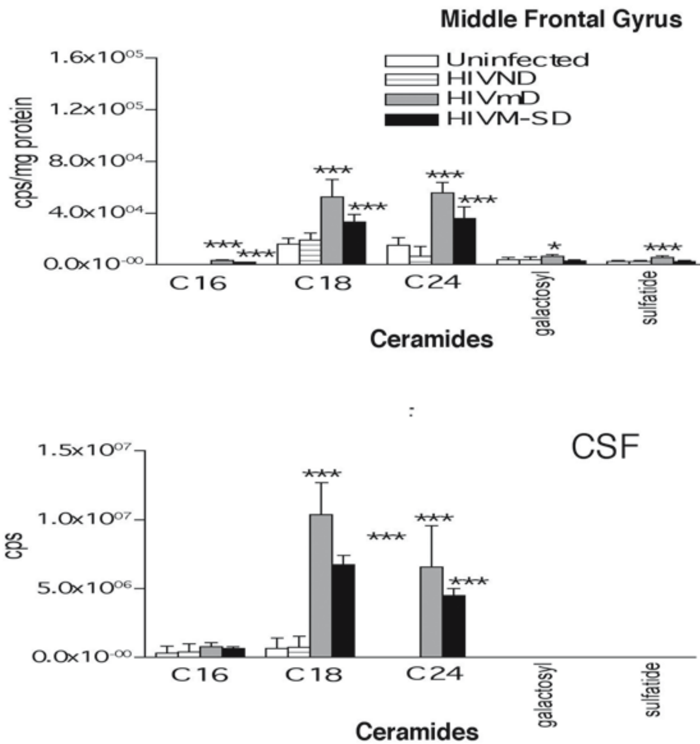

Fig. 7. HIV dementia patients exhibit increased amounts of long-chain ceramides in brain cells and cerebrospinal fluid. Concentrations of the indicated lipids were measured in tissue samples from the middle frontal gyrus, and in the cerebrospinal fluid (CSF) of uninfected control patients, nondemented HIV-infected patients (HIVND), and HIV-infected patients with mild (HIVmD) and severe (HIVSD) dementia. Modified from Haughey et al. (2004).

fluid samples from HIV dementia patients (Fig. 7). When cultured rat cortical neurons were exposed to the HIV proteins, gp120 and Tat levels of ceramide were increased (Haughey et al., 2004). The ceramide precursor palmitoyl-CoA sensitized neurons to Tat and gp120 toxicity, while an inhibitor of ceramide production (ISP-1) protected against Tat- and gp120-induced cell death. Collectively, our findings suggest that HIV-1 infection may promote a lipid imbalance in neural cells, resulting in an overproduction of ceramide and consequent neuronal dysfunction and death that results in dementia (Fig. 8).

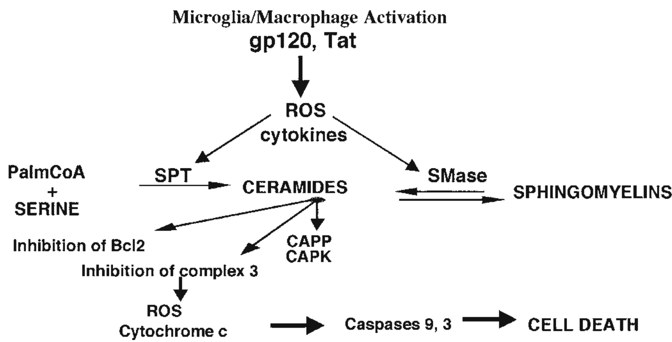

Fig. 8. Working model for the role of perturbed membrane lipid metabolism in the pathogenesis of HIV dementia. Microglia/brain macrophages infected with HIV-1 release neurotoxic HIV-1 proteins (gp120 and Tat), which induce oxidative stress in neurons. Membrane-associated oxidative stress and cytokines induce ceramide production and thereby trigger neuronal apoptosis.

7. SUMMARY

Membrane sphingolipids are a source of ceramides, intracellular messengers that regulate the activities of kinases, phosphatases, and transcription factors. Production of ceramides occurs in response to activation of various cell surface receptors coupled to the activation of neutral or acidic sphingomyelinases, but it can also occur when cells are subjected to oxidative stress. Physiological roles of ceramide include the regulation of cell proliferation, differentiation, and survival. However, high levels of ceramide can trigger a cell-death cascade called apoptosis, which is implicated in a number of different pathological conditions. The present article focuses on the normal functions of sphingomyelin-ceramide signaling in the nervous system, and the contributions of perturbed sphingomyelin metabolism in the pathogenesis of age-related neurodegenerative disorders. Ceramide mediates effects of several neuronal growth factors on neurite outgrowth and cell survival and may also play a role in regulating synaptic plasticity in the mature nervous systems. Excessive production of ceramide occurs in several different neurodegenerative conditions, including ischemic stroke, AD, amyotrophic lateral sclerosis, and HIV dementia. Studies in which ceramide production is blocked by knockout of sphingomyelinase genes or by pharmacological agents suggest a pivotal role for ceramide in neuronal dysfunction and death in these disorders. Interestingly, sphingolipid and cholesterol metabolism can also be modified by dietary manipulation (*see* Chapter 10).

Therefore, dietary and pharmacological manipulations of cholesterol and sphingolipid metabolism might prove effective in extending healthspan and/ or treating various age-related diseases.

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9

Caveolin and Cancer

A Complex Relationship

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1. INTRODUCTION

Caveolin-1 was first identified as an approximately 22-kDa tyrosine-phosphorylated protein in Rous sarcoma virus-transformed cells (Glenney, 1989; Glenney and Soppet, 1992). Caveolin-1 has subsequently attracted much attention because it serves as a major coat protein of caveolae (Rothberg, et al., 1992; Smart, et al., 1999). The caveolar membrane system, comprising plasma membrane invaginations and juxtamembrane uncoated vesicles, mediates certain protein transport processes, including transcytosis, potocytosis, and clathrin-independent endocytosis (Anderson, 1998). Caveolin-1 and caveolae are also involved in mediating cellular cholesterol efflux (Smart et al., 1999; Fielding and Fielding, 2001), and a large body of evidence implicates caveolin-1 in regulating intracellular signaling pathways.

Caveolin-1 is a member of a gene family that also includes caveolin-2, which is co-expressed with caveolin-1 in mesenchymal, endothelial, epithelial, neuronal, and glial cells (Scherer et al., 1996; Scherer et al., 1997). Caveolin-2 assembles with caveolin-1 into hetero-oligomers that are part of the caveolar filamentous coat structure (Scherer et al., 1997; Scheiffele et al., 1998). Caveolin-3, a third member of this gene family, is selectively expressed in skeletal and heart muscle cells (Song et al., 1996; Galbiati et al., 2001a). Caveolin-1 binds characteristic raft lipids such as cholesterol and sphingolipids (Liu et al., 2002). In addition, caveolin-1 interacts with numerous proteins via a juxtamembrane “scaffolding” domain that binds short sequence motifs rich in aromatic amino acids (Liu et al., 2002). The ability of caveolin-1 to interact with many raft-localized signaling proteins

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indicates that its actions include regulation of cell signaling and that its expression may have a profound effect on cell function and cell fate.

The biology of caveolin-1 was reviewed recently (Smart et al., 1999; Liu et al., 2002; Razani et al., 2002). In the present chapter we shall focus on the phenomenology and actions of caveolin-1 in cancer cells and tumors, and we shall critically evaluate the different roles it may play during cancer progression and metastasis.

2. UPREGULATION OF CAVEOLIN EXPRESSION DURING CELL DIFFERENTIATION

Caveolar invaginations are abundant in the plasma membrane of differentiated epithelial and mesenchymal cells, e.g., pneumocytes, endothelial cells, fibroblasts, smooth muscle cells, and adipocytes. Caveolin-1 mRNA and protein are strongly induced upon exposure of 3T3-L1 pre-adipocytes to differentiation-inducing agents (Scherer et al., 1994; Scherer et al., 1997). Previously, little or no caveolin expression was reported in neuronal cells, but more recent work has demonstrated that caveolin-1 and -2 expression is upregulated in differentiating PC-12 cells and dorsal root ganglion neurons (Galbiati et al., 1998b). Caveolin-1 expression is also upregulated upon induction of alveolar type I-like and type II-like phenotypes by progenitor human lung cells in vitro (Campbell et al., 1999; Fuchs et al., 2003). Caveolin-1 expression is likewise increased during differentiation of keratinocytes induced by in vitro growth on a collagen matrix (Li et al., 2001b). In yet another example, differentiated Schwann cells exhibit high caveolin-1 levels, whereas its expression is downregulated when the cells revert to a premyelinating phenotype (Mikol et al., 1999). Induction of human breast- and colon-carcinoma cell differentiation can be accomplished by natural and synthetic ligands of peroxisome-proliferator-activated receptor-γ (PPARγ), and such ligands induce transcription of caveolin-1 and caveolin-2 mRNA and elevate expression of caveolin-1 and -2 proteins (Burgermeister et al., 2003). In summary, it appears that, in a wide variety of cells, acquisition of a differentiated phenotype is associated with elevated expression of caveolin proteins and caveolae organelles.

3. CHANGES IN CAVEOLIN EXPRESSION IN ONCOGENE-TRANSFORMED CELLS, CANCER CELL LINES, AND TUMOR SAMPLES

The induction of caveolin-1 expression during cell differentiation suggests a possible anti-proliferative action of caveolin-1. Consistent with this idea, results from early studies have shown that the protein is downregulated

upon transformation of fibroblasts by oncogenes such as Bcr-Abl, v-Abl, H-Ras, *Polyoma virus* middle T, Crk1 (Koleske et al., 1995), or Neu-T, c-Src^{Y52F}, and Myc (Engelman et al., 1998b). However, the analysis of accumulated studies that compared caveolin-1 (and in some cases, caveolin-2) expression in many different cancer cell lines and tumor samples reveals a more complex picture. Transcriptional profiling of the human cancer cell lines included in the NCI60 panel by DNA microarray analysis (Ross et al., 2000) exemplifies the variability of caveolin-1 expression in human tumor-derived cells (Fig. 1). For example, this analysis revealed that all renal cancer cell lines have high levels of caveolin-1 mRNA (Ross et al., 2000). In contrast, all leukemia and all melanoma cell lines examined exhibit low-level expression of caveolin-1 (Ross et al., 2000). Cancer cell lines derived from other tissues are less uniform in terms of caveolin-1 expression, as described in Sections 3.1.–3.11. A cDNA array-based expression analysis of caveolin-1 using a set of tumor samples and their matched normal tissue controls showed that in kidney, prostate, and stomach tumors caveolin-1 is elevated, whereas in breast, ovary, colon, and lung tumors caveolin-1 is downregulated (Wiechen et al., 2001a). In tumors from the uterus and rectum the changes are variable. As we shall see, the expression of caveolin-1 seem to depend not only on the tumor's origin but also on its stage and grade.

3.1. Breast Cancer

In an early study of gene expression in breast cancer cells, caveolin-1 was identified as one of several genes whose expression is downregulated in human mammary carcinoma cell lines, as compared with normal mammary epithelial cells (Sager et al., 1994). Similarly, caveolin-1 mRNA and protein are reduced or absent in most mammary tumor cell lines examined (Lee et al., 1998; Hurlstone et al., 1999). However, caveolin-1 is highly expressed in other human breast cancer cell lines—such as MDA-MB-231, HS578T, and BT-549 (Hurlstone et al., 1999; Ross et al., 2000). Additionally, high caveolin-1 expression was demonstrated in a metastatic rat mammary cell line (MTPa) but not in a nonmetastatic cell line (MTLy) (Nestl et al., 2001). Thus, caveolin-1 apparently is differentially expressed in different breast cancer cell lines, possibly reflecting the variable cellular origin of the source tumors (e.g., epithelial, myoepithelial) and its pathological stage (e.g., primary, metastatic).

Immunohistochemical examination of breast tumor samples revealed low levels of caveolin-1 in normal mammary epithelium and greatly increased expression in breast carcinomas of all grades (Yang et al., 1998b). In this study, 78–93% of malignant human breast tissues, including intraductal carcinoma, infiltrating ductal carcinoma, and lymph node metastasis, were posi-

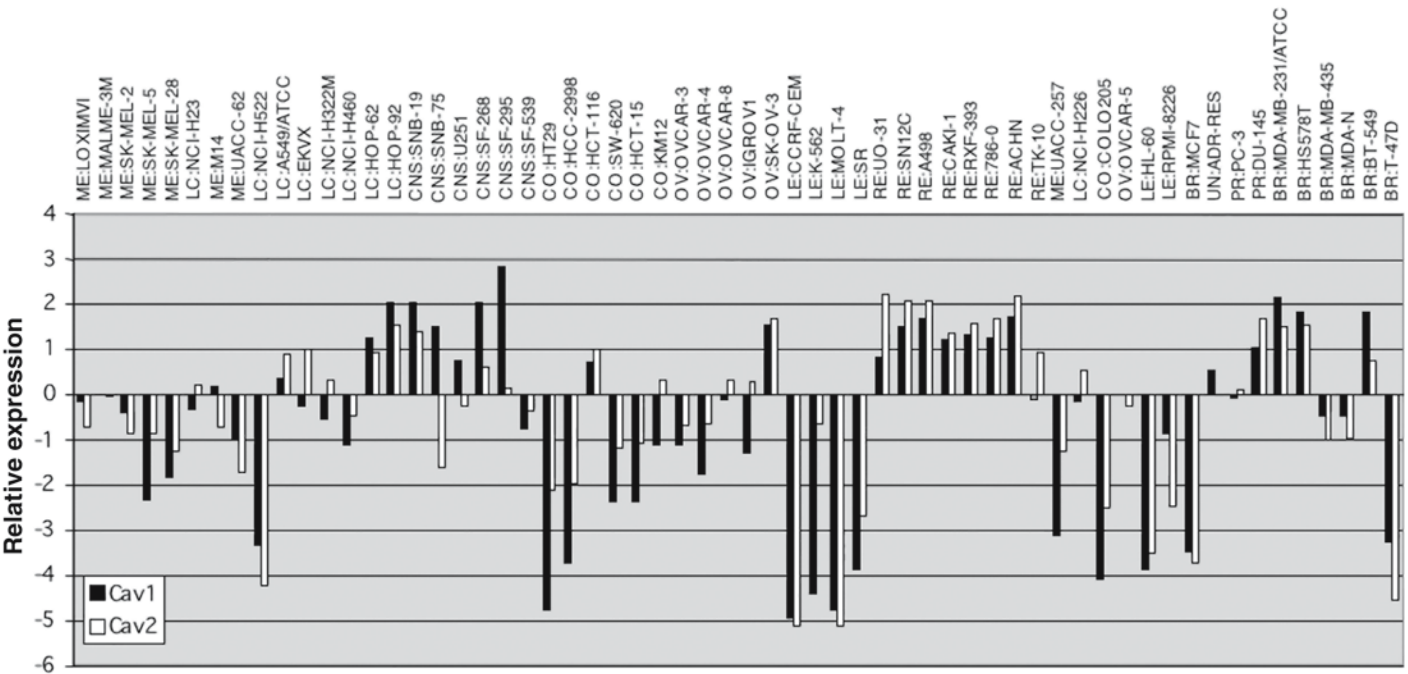

Fig. 1. Relative expression of caveolin-1 and caveolin-2 in 60 human cancer cell lines. Caveolin-1 and caveolin-2 expression in the 60 cancer cell lines included in the NCI60 panel was determined by utilizing cDNA microarrays representing about 8000 unique human genes (Ross et al., 2000). The log ratio of caveolin expression in each cell line relative to a reference sample consisting of a pool of 12 cell lines is presented. The figure was prepared from the caveolin-1 and caveolin-2 data available on the Internet at http://genome-www.stanford.edu/nci60/. Types of cancer: ME, melanoma; LC, lung carcinoma; CNS, central nervous system; CO, colon; OV, ovarian; LE, leukemia; RE, renal; PR, prostate; BR, breast; UN, unidentified.

tively immunostained with a caveolin-1 antibody (Yang et al., 1998b). However, the picture that emerges from DNA microarray analysis of 40 human breast tumors is quite different. According to that analysis, caveolin-1 is highest in normal breast tissue samples, whereas its expression in the tumors is lower but very variable, ranging from near normal in a minority of samples to a very low level in others (Perou et al., 2000). A similar picture is seen in arrays of breast tumor tissue cDNA, where caveolin-1 was more or less suppressed in all tumor samples matched with corresponding normal tissue (Wiechen et al., 2001a).

3.2. Lung Cancer

Examination of human lung adenocarcinoma and small cell carcinoma cell lines revealed little or no caveolin-1, whereas a lung squamous cell carcinoma line (Calu-1) expressed high levels of caveolin-1 (Racine et al., 1999). However, four other lung squamous cell carcinoma lines expressed reduced levels of caveolin-1 compared with normal human lung epithelia (Razani et al., 2000). Another analysis similarly found low-level caveolin-1 expression in two lung adenocarcinoma, a bronchioalveolar cell carcinoma, and a non-small cell lung carcinoma cell lines, and high caveolin-1 expression in two other non-small cell lung carcinoma cell lines (Hop-62, Hop-92) (Ross et al., 2000). Ho et al. (2002) reported that caveolin-1 expression is positively correlated with metastatic potential in a series of lung adenocarcinoma cell lines established by selection for increasing invasiveness. In addition, while primary lung adenocarcinoma tumors were largely caveolin-1 negative, there was a statistically significant trend toward higher expression of caveolin-1 in lung tumors with nodal metastases and in the lymph nodes metastases proper (Ho et al., 2002). DNA microarray analysis of primary lung adenocarcinomas identified caveolin-1 as one of the most prominent downregulated genes (Wiechen et al., 2001a; Wikman et al., 2002). Together, the above data are consistent with a suppression of caveolin-1 gene expression in primary lung tumors (in particular, adenocarcinomas). However, as in mammary tumors, some cell lines are caveolin-1 positive, and a positive correlation seems to exist between its expression and the metastatic potential of the cells or tumors.

3.3. Prostate Cancer

The positive correlation and functional relation between tumor progression and metastasis and caveolin-1 expression was first demonstrated in prostate cancer cell lines and tumor specimens (Yang et al., 1998b). Caveolin-1 was found to be higher in metastatic site-derived mouse prostate

cell lines than in the primary tumor-derived cells; also, three of four metastasis-derived human cell lines examined (Du-145, PC-3, and ND-1) expressed caveolin-1. Somewhat conflicting data were reported by Pflug et al. (1999), in which caveolin-1 was reduced in LNCaP-derived androgen-independent tumorigenic cell clones grown continuously for 6–42 months in castrated nude mice. In prostate cancer tissue sections, the percentage of caveolin-1 positive samples was 29% in T_3N_1-stage primary cancer, and it increased to 56% in lymph node metastasis, compared to 8% and 18% in normal and hyperplastic epithelia, respectively (Yang et al., 1998b). Further studies revealed a significant positive correlation of caveolin-1 expression with the Gleason score (i.e., tumor cell grade), with lymph node involvement and with positive surgical margins (i.e., lack of cancer cell-free margins in the resected tumor) (Yang et al., 1999; Satoh et al., 2003). The expression of caveolin-1 in primary confined prostate tumors correlated also with decreased probability of recurrence-free survival (Yang et al., 1999; Satoh et al., 2003).

3.4. Bladder Cancer

Analysis of three bladder cancer cell lines (A1698, A1698OR, and J82) has shown that all were caveolin-1 positive (Hurlstone et al., 1999). An apparent association of caveolin-1 with the tumor grade and stage is strongly exemplified in bladder cancer specimens. Immunohistochemical determination revealed that although most bladder cancer samples are caveolin-1 negative, the percent of positive tumors increased from 0 to 21% in G1 to G3 graded tumors (Rajjayabun et al., 2001). Another study has shown that caveolin-1 expression occurs only in grade 3 urothelial carcinoma tumors and that it is positively correlated with squamous differentiation of the tumor cells (Fong et al., 2003). Largely similar results were obtained using transcriptional profiling by DNA microarrays in another set of bladder cancer samples, namely a statistically significant association of caveolin-1 expression with tumor grade and stage (Sanchez-Carbayo et al., 2002).

3.5. Pancreatic Cancer

In an early study, only one of five pancreatic tumor cell lines (TMSG) was found to be caveolin-1 positive (Hurlstone et al., 1999). In contrast, fine-needle aspirates that were 95% positive for the pancreatic tumor marker cytokeratin 10 exhibited elevated expression of caveolin-1 compared to normal pancreas (Crnogorac-Jurcevic et al., 2001). In a comparison of a series of increasingly metastatic rat pancreatic cell lines, caveolin-1 was found only in the most metastatic cell line (Nestl et al., 2001). The results from cell

lines are supported by an immunohistochemical study on pancreatic ductal adenocarcinoma that found a statistically significant positive correlation of caveolin-1 expression with tumor cell grade and with poor prognosis (reduced survival time after tumor resection) (Suzuoki et al., 2002). DNA microarray analysis of pancreatic intraductal papillary-mucinous tumors revealed that although on average caveolin-1 was downregulated in the tumors versus the normal tissue, it was one of several genes that were highly expressed in invasive tumors but not in the noninvasive tumors (Terris et al., 2002).

3.6. Ovarian Cancer

DNA microarray-based transcriptional profiling of the NCI human cancer cell line panel revealed that only one of five ovarian cell lines examined (SKOV-3) had high caveolin-1 levels (Ross et al., 2000). Similarly, in three other studies in which partially overlapping sets of ovarian carcinoma lines were examined by Western blot, most cell lines exhibited low or no caveolin-1 expression, although a few (SKOV-3, ES-2, SW626) showed high expression levels (Hurlstone et al., 1999; Bagnoli et al., 2000; Wiechen et al., 2001a). DNA array analysis of three ovarian tumors and their matched normal controls showed reduced caveolin-1 expression in all three (Wiechen et al., 2001a). These data are largely confirmed by immunohistochemical analysis in which caveolin-1 staining seen in nonneoplastic lesions was higher than in primary ovarian tumors and metastases (Davidson et al., 2001).

3.7. Astrogliomas and Glioblastomas

The presence of caveolin-1 and caveolae in the rat C6 glioma cell line was reported by Silva et al. (1999). DNA microarray-based transcriptional profiling of the NCI cancer cell line panel then demonstrated that five of six human glioma and glioblastoma cell lines examined had high caveolin-1 levels (Ross et al., 2000). In a different set of five human glioblastoma cells lines, high caveolin-1 expression was observed in all lines, and immunocytochemistry revealed a caveolae-like punctate staining pattern, confirmed by transmission electron microscopic identification of invaginated plasma membrane caveolae (Cameron et al., 2002). The upregulation of caveolin-1 was confirmed by cDNA microarray analysis of grade II–IV gliomas, where caveolin-1 expression was elevated 5.2-fold in glioblastoma multiformis tumor specimens compared to normal brain (Sallinen et al., 2000). Thus, expression of caveolin-1 in association with an abundance of morphological caveolae seem to be the norm in the glioma family of malignancies.

3.8. Colon Cancer

All human colon cancer cell lines included in the NCI60 panel express low levels of caveolin-1 mRNA (Ross et al., 2000). Similar results were obtained in a partially overlapping set of lines analyzed by Western blotting (Bender et al., 2000). The latter study examined caveolin-1 immunoreactivity in tumor tissues, reporting a decrease in caveolin-1 presence in tumor mucosa and tumor stroma as compared with normal mucosa and stroma (Bender et al., 2000). Likewise, DNA array analysis of 11 colon tumors and their matched normal controls showed dramatically reduced caveolin-1 mRNA in nearly all samples (Wiechen et al., 2001a). However, conflicting results were reported according to which caveolin-1 immunostaining was elevated in most colon adenocarcinoma cases while normal colon epithelium and most adenomas were caveolin-1-negative (Fine et al., 2001).

3.9. Various Sarcomas

Wiechen et al. (2001b) examined benign and malignant mesenchymal tumors utilizing caveolin-1 immunohistochemistry and reported that its expression in benign tumors (fibromatoses, leiomyomas, hemangiomas, and lipomas) was as high or higher than in normal mesenchymal tissues. However, expression of caveolin-1 was lost or strongly reduced in most or all of the malignant sarcomas examined, including fibrosarcomas, leiomyosarcomas, liposarcomas, angiosarcomas, histiocytomas, and synovial sarcomas (Wiechen et al., 2001b). Another study of dermal vascular tumors also showed a significant reduction of caveolin-1 immunostaining in certain forms of malignant sarcomas as compared with benign hemangiomas (Morgan et al., 2001). However, in another report it was found that both benign and malignant smooth muscle tumors and adipose tissue tumors expressed caveolin-1, suggesting that downregulation of caveolin-1 does not contribute to pathogenesis of liposarcomas and leiomyosarcomas (Bayer-Garner et al., 2002).

3.10. Other Forms of Cancer

Ito et al. (2002) reported that normal follicular cells of the thyroid did not express caveolin-1 and that all cases of follicular adenoma and carcinoma were likewise caveolin-1 negative, whereas papillary thyroid carcinoma cells were caveolin-1 positive. These results were confirmed in a subsequent study (Aldred et al., 2003). In addition, expression profiling of sporadic follicular thyroid carcinoma in comparison with normal thyroid tissue revealed that caveolin-1 and caveolin-2 were significantly downregulated in the tumor tissue (Aldred et al., 2003). Caveolin-1 was likewise greatly reduced in four cervical squamous cell carcinoma lines compared with nor-

mal human cervical squamous epithelia (Razani et al., 2000). However, Kato et al. (2002) reported that overexpression of caveolin-1 in esophageal squamous cell carcinoma is correlated with lymph node metastasis and with worse prognosis after surgery.

3.11. Multidrug-Resistant Cancer Cells

Multidrug-resistant (MDR) cancer cells are characterized by either intrinsic or acquired resistance to chemotherapeutic anti-cancer drugs. There are multiple MDR mechanisms, often involving the overexpression of a cell surface drug transporter such as P-gp/MDR1, usually together with changes in one or more proteins that affect drug metabolism and/or drug response (Gottesman 1993; El-Deiry 1997). Acquisition of the MDR phenotype is accompanied by marked changes in lipid constituents of lipid rafts and caveolar membranes such as glucosylceramide (GlcCer) (Lavie et al., 1996) and cholesterol (reviewed by Lavie and Liscovitch, 2000). The elevated GlcCer and cholesterol, independently reported in different types of MDR cells, was consistent with the possibility that cancer MDR is associated with an increase in plasma membrane rafts and caveolae. Indeed, plasma membrane caveolae and expression of caveolin-1 are upregulated in numerous human MDR cancer cells (Lavie et al., 1998; Yang et al., 1998a; Bender et al., 2000; Belanger et al., 2003). In HT-29-MDR human multidrug-resistant colon cancer cells, the upregulation of caveolin-1 is accompanied by a five-fold increase in the density of juxtamembrane, 50- to 100-nm noncoated invaginations that exhibit the characteristic flask-shaped morphology of caveolae. A comparable surface density of caveolae was also observed in NCI-AdrR human multidrug-resistant cells (Lavie et al., 1998). In addition, caveolin-1 is relocalized from the cytosol to the plasma membrane in DC-3F/ADX multidrug resistant Chinese hamster lung fibroblasts (Garrigues et al., 2002). Caveolin-1 expression may be directly regulated by exposure to cytotoxic drugs, although the mechanism(s) involved in this induction are still obscure (Yang et al., 1998a; Belanger et al., 2003).

The results discussed above are summarized in Table 1. The accumulated evidence is clearly consistent with the conclusion that the changes in caveolin-1 expression in cancer cells depend, to a major extent, on the type of cancer. Furthermore, results from tumor-derived cell lines (usually Northern and/or Western blot) and from tumor specimens (usually immunostaining and/or DNA array-based expression profiling) are largely consistent with each other. Thus, expression of caveolin-1 is most often downregulated in breast, ovarian, colon, lung, and pancreatic cancers. In kidney cancer, prostate cancer, and gliomas, caveolin-1 is usually upregulated. Another conclusion from this survey of the literature is that a

Table 1
Changes in Caveolin-1 Expression in Tumor-Derived Cell Lines and Tumor Specimens*

Cancer	Sample		Correlation with tumor
	Cell lines	Tumors	grade/stage
Breast	↓↑	↓↑	Positive
Kidney	↑	↑	—
Leukemia	↓↑	—	—
Melanoma	↓	—	—
Prostate	↑↓	↑	Positive
Stomach	–	↑	—
Ovary	↓↑	↓	—
Colon	↓	↓↑	—
Lung	↓↑	↓	Positive
Uterus	–	↓↑	–
Rectum	–	↓↑	–
Bladder	↑	↓↑	Positive
Glioma	↑	↑	–
Pancreas	↓↑	↓	Positive
Sarcoma	–	↓↑	–
Thyroid	–	↓↑	–

*Summary of results obtained by immunoblotting, immunohistochemistry and DNA microarrays. See text for details and references. ↑, increase; ↓, decrease; ↑↓, mostly increase; ↓↑, mostly decrease; ↓↑, variable change; —, not determined.

positive correlation was found whenever the relationship of caveolin-1 expression with the tumor cell grade and/or stage of progression was examined, and in some cases the expression of caveolin-1 was shown to be an independent predictor of poor disease prognosis.

The variable changes in caveolin-1 seen in various cancers likely reflect its multiple cellular actions, especially those actions related to signaling cell growth and survival. In the following paragraphs we shall review the effects of experimental manipulations of caveolin-1 expression on cell physiology and their possible relationship with its role in cancer.

4. EFFECTS OF CAVEOLIN-1 EXPRESSION AND SUPPRESSION ON CANCER CELL BIOLOGY

To understand the role(s) of caveolin-1 in the pathophysiology of cancer it is necessary to experimentally manipulate its cellular expression and observe the resultant phenotype(s). Heterologous expression of caveolin-1 in

oncogenically transformed NIH-3T3 cells abrogates anchorage-independent growth, measured by colony formation in soft agar, thus depriving the transformed cells of a critical tumorigenic ability (Engelman et al., 1997). Likewise, transfection of T47D human mammary cancer cells with caveolin-1 cDNA results in substantial growth inhibition as demonstrated by a 50% decrease in growth rate and a 3- to 10-fold reduction in soft agar colony formation (Lee et al., 1998). Caveolin-1-mediated inhibition of in vitro anchorage-independent growth, a parameter that is highly correlated with in vivo tumorigenesis (Shin et al., 1975), was similarly seen in other cancer cell lines, including MTLn3 rat mammary tumor cells (Zhang et al., 2000), SiHa human cervical squamous cell carcinoma cells (Razani et al., 2000) and MCF-7 human breast adenocarcinoma cells (Fiucci et al., 2002). Consistent with these data, heterologous expression of caveolin-1 attenuates in vivo tumor development by HT-29 human colon adenocarcinoma cells (Bender et al., 2000).

Caveolin-1-induced inhibition of cancer cell anchorage-independent growth indicates that it blocks a matrix-independent (i.e., intrinsic) growth or survival signal (e.g., a signal that emanates from an activated oncogene). Indeed, transient transfection with caveolin-1 reduces growth rates in human mammary tumor cells (Lee et al., 1998; Fiucci et al., 2002) and causes delayed entry into mitosis in human skin fibroblasts (Fielding et al., 1999). Transgenic overexpression of caveolin-1 in mouse embryo fibroblasts results in cell cycle arrest at the G_0/G_1 phase, by a mechanism that requires sequential activation of p53 and $p21^{WAF1/Cip1}$ (Galbiati et al., 2001b). Furthermore, overexpression of caveolin-1 in these cells advances their senescence—reducing their proliferative lifespan, inducing a senescent morphology, and increasing senescence-associated β-galactosidase activity (Volonte et al., 2002).

In addition to these growth-inhibitory actions of caveolin-1, caveolin-1 may exert also pro-apoptotic actions. Such is the case in OVCAR-3, an ovarian carcinoma cell line (Wiechen et al., 2001a), in T24 bladder cancer cells (Liu et al., 2001), and in mouse embryo fibroblasts (MEFs) that transgenically overexpress caveolin-1 (Galbiati et al., 2001b) (see detailed discussion below). Caveolin-1 overexpression studies thus show that it may exert antiproliferative as well as pro-apoptotic actions. This conclusion is supported by studies in which caveolin-1 expression or action were suppressed by caveolin-1 antisense DNA or by a dominant-negative caveolin-1 mutant, respectively. A full-length caveolin-1 antisense construct was found sufficient to induce morphological transformation of stably-transfected NIH-3T3 cells (i.e., formation of foci in monolayer cultures), that was associated with dramatically increased anchorage-independent growth in vitro and

high-frequency of tumorigenesis in vivo (Galbiati et al., 1998a). These cells were also characterized as having a significantly reduced fraction of cells at G_0/G_1 and increased number of cells in the S phase of the cell cycle, indicating that loss of caveolin-1 accelerates exit from the G_0/G_1 phase (Galbiati et al., 2001b). Furthermore, these caveolin-1 antisense-suppressed NIH 3T3 cells were reported to be more resistant to staurosporine-induced apoptosis (Liu et al., 2001).

Functional antagonism of normal caveolin-1 function can be accomplished by use of mutant proteins that act in a dominant-negative manner. Hayashi et al. (2001) first identified a caveolin-1 mutation (P132L) in about 16% of primary human breast cancer specimens. The caveolin-1^{P132L} mutant protein induces morphological transformation in NIH 3T3 cells and supports anchorage-independent growth of mutant-transfected cells (Hayashi et al., 2001). The mutant protein appears to act by causing missorting of normal caveolin-1, leading to its retention at a perinuclear compartment that is probably the Golgi apparatus (Lee et al., 2002). In conclusion, antagonism of caveolin-1 expression or function implicates it as an important antiproliferative protein. However, further insight into the complex functions of caveolin-1 in regulating cell growth and survival awaited the development of caveolin-1 null mice.

5. EFFECTS OF CAVEOLIN-1 GENE KNOCKOUT: IS CAVEOLIN-1 A TUMOR SUPPRESSOR PROTEIN?

The caveolin-1 gene is localized on human chromosome 7 (7q31.1/D7S522) (Engelman et al., 1998c; Engelman et al., 1999; Fra et al., 1999; Fra et al., 2000). The locus 7q31 is a suspected tumor suppressor that is deleted in several forms of cancer, raising the possibility that caveolin-1 may be the tumor suppressor gene responsible for tumorigenicity associated with loss of this locus. This suggestion remains controversial (Hurlstone et al., 1999; Tobias et al., 2001; Zenklusen et al., 2001). However, the presumed tumor-suppressor action of caveolin-1 could be examined directly once caveolin-1 gene-disrupted mice had been generated and their phenotype analyzed (Drab et al., 2001; Razani et al., 2001; Zhao et al., 2002).

These initial studies confirmed the essential requirement for caveolin-1 in the biogenesis of caveolae as these plasma membrane invaginations were absent in caveolin-1 null mice. Furthermore, a potential role of caveolin-1 as a regulator of endothelial cell proliferation was suggested by the observed hyperplasia of endothelial cells in pulmonary alveolar septa (Drab et al., 2001;

Razani et al., 2001). Mouse embryo fibroblasts derived from caveolin-1 null mice exhibit a hyperproliferative phenotype, i.e., a faster proliferation rate, a higher fraction of cells in S phase, and an increased [^{3}H]-thymidine incorporation into DNA, compared with the wild type MEFs (Razani et al., 2001). In an additional study, genetic knock-out of the caveolin-1 gene was shown to result in mammary intraductal epithelial cell hyperplasia as early as 6 weeks after birth, while at 9 months of age the mammary glands of caveolin-1 null mice showed increased lobular development, hyperplasia of the epithelial lining and evidence of fibrosis (Lee et al., 2002).

Whereas loss of the caveolin-1 gene clearly results in hyperplasia of certain epithelial cells and fibroblasts, spontaneous development of mammary or other tumors was not evident in caveolin-1 null mice (Razani et al., 2001; Lee et al., 2002). However, more recent work has demonstrated that loss of caveolin-1 enhances tumorigenesis when present together with another carcinogenic stimulus. This transformation-suppressor activity of caveolin-1 was revealed when caveolin-1 null mice were interbred with mammary tumor prone MMTV-PyMT mice. In PyMT/Cav1–/– mice, development of multifocal dysplastic lesions in the mammary gland is greatly accelerated, resulting in increased number, area, and grade of the dysplastic foci, compared with PyMT/Cav1+/+ mice (Williams et al., 2003). Genetic knock-out of the caveolin-1 also dramatically sensitizes mice to 7,12-dimethylbenzanthracene (DMBA)-induced skin carcinogenesis. While caveolin-1 null mice exhibit normal skin morphology, DMBA treatment induces massive epidermal hyperplasia, subsequently leading to a highly significant increase in skin tumorigenesis (both fraction of mice with tumors and number of tumors per mouse), compared with wild-type DMBA-treated animals (Capozza et al., 2003). These data combined indicate that loss of caveolin-1 gene alone is neither necessary nor sufficient for induction of tumors in the skin or the mammary gland in vivo, but it may promote tumorigenesis when combined with another tumorigenic stimulus, either intrinsic (e.g., activated oncogene) or extrinsic (e.g., chemical carcinogen). Thus, in mice, caveolin-1 may be considered as a tumor susceptibility gene for skin and mammary tumors. In humans, the loss of the caveolin-1 gene (e.g., when 7q31 is deleted) or its functional inactivation (e.g., through the P132L or another yet to be identified mutation) may predispose normal epithelia to tumor formation by sensitizing the cells to a host of cocarcinogenic stimuli. The loss or dysfunction of caveolin-1 is likely to act by disinhibition of mitogenic pathways and/or cell cycle progression mechanisms, thus promoting epithelial cell hyperplasia and leading, subsequently, to tumor development.

6. MECHANISMS OF ANTIPROLIFERATIVE ACTION OF CAVEOLIN-1

The proposed action of caveolin-1 as a tumor susceptibility gene is supported by copious data showing that expression of caveolin-1 inhibits mitogenic signaling pathways and cell cycle control mechanisms. Caveolin-1 likely inhibits cell proliferation by virtue of its diverse interactions with mitogenic signaling molecules. The development of biochemical methods for isolation of caveolin-rich membranes domains revealed that many signaling molecules are co-localized with caveolin-1 (Lisanti et al., 1994; Smart et al., 1995; Liu et al., 1997a). Many of these molecules were shown to physically interact with caveolin-1 (reviewed by Smart et al., 1999; Liu et al., 2002). Caveolin-1 was hypothesized to form a scaffold onto which the signaling molecules can assemble and thus co-localize within distinct domains of the plasma membrane (i.e., caveolae), from which signaling cascades might be launched upon proper stimulation (Okamoto et al., 1998). The binding of caveolin-1 to these molecules (mainly via the peptide sequence designated the caveolin scaffolding domain) may regulate their function, as described below.

Components of the mitogenic pathway involving Ras-Erk1/2-Elk1, activated by receptor tyrosine kinases such as ErbB1 (EGF-R), ErbB2 (c-Neu), and PDGF-R, are localized, at least in part, within caveolin-rich membrane domains (Mineo et al., 1996; Liu et al., 1997b). This localization may have important functional consequences because caveolin-1 inhibits the activation of the mitogen-activated protein (MAP) kinase-responsive transcription factor Elk1 in vivo when cotransfected with ErbB1, Raf1, Mek1, or Erk2 (Engelman et al., 1998a). Caveolin-1 exerts a similar effect on ErbB2-induced activation of Elk1-dependent transcription (Engelman et al., 1998b). The inhibitory action of caveolin-1 resides in its N-terminal 95 amino acids. In addition, a caveolin-1-derived peptide that corresponds to the caveolin scaffolding domain (caveolin-1_{82-101}) inhibits the kinase activity of Mek1 and Erk2 in vitro toward their respective substrates, Erk2 and Elk1 (Engelman et al., 1998a).

The hypothesis that caveolin-1 normally attenuates signaling via the Erk1/2 pathway is supported by the fact that gene-specific antisense suppression of its expression results in constitutive activation of Mek1 and Erk1/2 in resting unstimulated cells (Galbiati et al., 1998a). Reduction of caveolin-1 expression in senescent human diploid fibroblasts by small interfering (si)RNA restored normal growth factor-induced activation of Erk1/2, its nuclear translocation, and activation of Elk1 (Cho et al., 2003). Likewise, a dominant-negative mutant, caveolin-1^{P132L}, activates Erk1/2 when expressed in fibroblasts (Hayashi et al., 2001). A negative regulatory effect of caveolin-

1 in the Erk1/2 pathway is also supported by the hyperactivation of the Erk1/2 pathway seen in cardiac tissue and in isolated cardiac fibroblasts derived from caveolin-1 null mice (Cohen et al., 2003). In addition, the Erk1/2 pathway appears to be overstimulated in caveolin-1 null epidermal keratinocytes upon DMBA-induced hyperproliferation (Capozza et al., 2003). It should be noted, however, that a higher activation state of Erk1/2 is not always observed in caveolin-1 null cells, as in mouse embryo fibroblasts (Razani et al., 2001) and in dysplastic mammary lesions (Williams et al., 2003).

Another important mitogenic pathway that is negatively regulated by caveolin-1 is the Wnt1-β-catenin-Lef1 pathway. Transient overexpression of caveolin-1 inhibits Lef1-driven expression of a luciferase reporter gene induced by cotransfection with either Wnt1 or β-catenin in NIH 3T3 fibroblasts, and stable re-expression of caveolin-1 in caveolin-1-negative U251 cells attenuated constitutive Lef1-dependent transcription in these cells (Galbiati et al., 2000). The effect of caveolin-1 would appear to involve its ability to form an immunoprecipitable complex with β-catenin and thus sequester it within caveolin-rich membrane domains. This segregates it from its regulating kinase GSK3-β and prevents its nuclear translocation and interaction with Lef1 (Galbiati et al., 2000).

The negative regulation of β-catenin seems to play an important role in caveolin-1-induced suppression of cyclin D1. Antisense inhibition of caveolin-1 expression upregulates cyclin D1 levels, while overexpression of caveolin-1 inhibits transcription driven by the cyclin D1 promoter (Hulit et al., 2000). Cyclin D1 is upregulated at sites of epidermal hyperplasia induced by exposure to the carcinogen DMBA in the skin of caveolin-1 null mice (Capozza et al., 2003). Cyclin D1 expression is similarly and dramatically elevated in dysplastic mammary lesions that form in caveolin-1 null mice crossed with mammary tumor-prone MMTV-PyMT mice (Williams et al., 2003). Mutation of the β-catenin-Lef1 site in the cyclin D1 promoter significantly reduced the inhibitory effect of caveolin-1 and, furthermore, the β-catenin-Lef1 site was sufficient for caveolin-1-induced repression of a luciferase reporter (Hulit et al., 2000). However, cyclin D1 expression is also regulated by the Jak2/Stat5 pathway (Matsumura et al., 1999) and caveolin-1 may thus suppress cyclin D1 expression also via its inhibitory action on Jak2/Stat5-dependent transcription (Park et al., 2002). Cyclin D1 forms complexes with the cyclin-dependent kinases, Cdk4 and Cdk6, thereby activating them and stimulating cell cycle progression through the G_1 phase, and its amplification or overexpression has been implicated in various forms of cancer (Stacey, 2003). Therefore, the inhibitory effect of caveolin-1 on cyclin D1 expression is potentially a major mechanism explaining its antiproliferative actions.

7. MODULATION OF CELL DEATH AND SURVIVAL SIGNALS BY CAVEOLIN-1

Subcellular fractionation and immunocytochemical methods have indicated that many constituents of various apoptotic signaling pathways are localized to caveolin-rich membranes, suggesting that caveolin-1 may regulate programmed cell death (Liu and Anderson, 1995; Bilderback et al., 1997; Ko et al., 1999; Veldman et al., 2001; Czarny et al., 2003). However, while caveolin-1 does modulate apoptotic and survival signals in both normal and cancer cells, its effects may vary depending on the type of cell and on the physiological context. Transient transfection of caveolin-1 sensitizes Rat-1 fibroblasts to two different apoptotic stimuli, i.e., C2-ceramide and γ-irradiation (Zundel et al., 2000). Likewise, stable expression of caveolin-1 in T24 bladder carcinoma cells (that normally do not express the protein) potentiates staurosporine-induced activation of caspase-3 and increases the number of cells undergoing apoptosis. Conversely, antisense downregulation of caveolin-1 in NIH 3T3 cells reduces their sensitivity to staurosporine-induced apoptosis (Liu et al., 2001). Sensitization to staurosporine was observed also in MEFs that overexpress a wild type caveolin-1 transgene (Galbiati et al., 2001b). In addition, transfection with caveolin-1 directly increased apoptotic cell death in OVCAR-3 ovarian carcinoma cells (Wiechen et al., 2001a) and increased the sensitivity of human HEK and HeLa cells to sodium arsenite toxicity (Shack et al., 2003). Together, these data show that caveolin-1 can act as a pro-apoptotic protein.

Parallel studies have indicated, however, that caveolin-1 can also act as an anti-apoptotic protein. Antisense suppression of the elevated caveolin-1 expression, found in androgen-insensitive mouse prostate cancer cells, reduces their survival upon androgen deprivation, indicating that the presence of caveolin-1 protects the cells from deprivation-induced apoptosis (Nasu et al., 1998). Additional studies showed that induction of caveolin-1 by testosterone mediates the survival effects of this hormone in androgen-sensitive prostate cancer cells (Li et al., 2001a). Intriguingly, caveolin-1 seems to exert its pro-survival actions in this system through an autocrine/paracrine mechanism. Testosterone stimulates caveolin-1 release into the medium, and the pro-survival actions of the conditioned medium are abrogated by a caveolin-1 antibody (Tahir et al., 2001). Adenoviral expression of caveolin-1 protects LNCaP human prostate cancer cells from c-Myc-induced apoptosis, and similar results were seen in LNCaP cells that stably express caveolin-1, further supporting the anti-apoptotic role of caveolin-1 in prostate cancer cells (Timme et al., 2000).

More recent work has extended the scope of cell types and pathophysiological contexts whereby caveolin-1 may have pro-survival action(s). In MCF-7 human breast cancer cells, stable expression of caveolin-1 results in inhibition of anoikis (i.e,. cell detachment-induced apoptosis), even though MCF-7/Cav1 cells are anchorage-dependent, thus showing that within the same cells caveolin-1 can simultaneously mediate antiproliferative and anti-apoptotic actions (Fiucci et al., 2002). An anti-apoptotic action was observed also in NIH 3T3 cells, in which antisense suppression of caveolin-1 expression increased their sensitivity to hydrogen peroxide-induced cell death (Volonte et al., 2002). It is of interest that these same cells previously were found less sensitive to another apoptotic agent, staurosporine (Liu et al., 2001). Thus, the role of caveolin-1 in regulating apoptosis seems to depend on the nature and perhaps potency of the death signal.

8. MECHANISMS OF ACTION OF CAVEOLIN-1 IN APOPTOSIS AND SURVIVAL SIGNALING PATHWAYS

The pathway involving activation of phosphoinositide 3-kinase (PI3K) and its downstream kinase Akt is a major survival pathway in eukaryotic cells that plays an important role in tumorigenesis (Cantley and Neel, 1999). Induction of apoptosis by ceramide in Rat-1 cells is associated with a dramatic, early formation of a complex containing caveolin-1, growth factor receptors, and p85, the regulatory subunit of PI3K (Zundel et al., 2000). The translocation of PI3K to a caveolin-1-containing complex is observed also in γ-irradiated wild type cells, but not in γ-irradiated acid sphingomyelinase (SMase) null cells. The translocation of PI3K to the vicinity of caveolin-1 results in reduced PI3K activity that is caveolin-1-dependent and can be mimicked by overexpression of caveolin-1, implicating it as a pro-apoptotic protein (Zundel et al., 2000). However, caveolin-1 seems to play an opposite role in regulating the PI3K/Akt pathway in multiple myeloma cells. In these human cancer cells, IL-6 and IGF-1 are survival factors that trigger the recruitment of PI3K into a complex with caveolin-1 and stimulate PI3K and Akt activity (Podar et al., 2003). The recruitment of PI3K is accompanied by Src-mediated phosphorylation of caveolin-1 on Tyr-14, and both phenomena are dependent on cholesterol as indicated by their sensitivity to methyl-β-cyclodextrin. Finally, cholesterol depletion by methyl-β-cyclodextrin was found to abrogate IL-6- and IGF-1-induced cell survival, indirectly implicating caveolin-1 in regulation of PI3K/Akt-mediated survival (Podar et al., 2003).

The phosphorylation of caveolin-1 on tyrosine 14 appears to be an important functional switch of caveolin-1 action. Tyr-14 is phosphorylated consti-

tutively in Src- and Abl-transformed NIH 3T3 cells, and its phosphorylation is stimulated by insulin in 3T3-L1 adipocytes and by EGF in A431 cells (Lee et al., 2000; Kim et al., 2002). Phosphorylated caveolin-1 binds the adaptor protein Grb7, while cotransfection of caveolin-1, c-Src, and Grb7 results in significant stimulation of anchorage-independent growth that requires a phosphorylatable tyrosine at position 14 of caveolin-1 (Lee et al., 2000). Additional studies imply a positive regulatory role of caveolin-1 on survival signaling by showing that caveolin-1 interacts with a component of the TNF-α-NFκB pathway (Feng et al., 2001), and that the caveolin scaffolding domain peptide inhibits a caveolar neutral SMase (Veldman et al., 2001). In conclusion, the diverse interactions of caveolin-1 with a major apoptotic pathway involving activation of SMase and a major survival pathway involving PI3K may explain its pro-apoptotic and pro-survival actions as observed in different cell types. Clearly, the rules governing these differential actions of caveolin-1 remain to be elucidated.

9. ROLE OF ENDOTHELIAL CAVEOLIN-1 IN TUMOR ANGIOGENESIS AND TUMOR VASCULAR PERMEABILITY

The importance of angiogenesis in supporting solid tumor growth is well established. Angiogenesis depends on release from tumor cells of angiogenic factors that stimulate endothelial cell migration and proliferation, and these processes are mediated by nitric oxide produced by endothelial nitric oxide synthase (eNOS) (Fukumura et al., 2001). Caveolin-1 plays a major role in regulation of eNOS, acting as a negative regulator of its activity (reviewed by Goligorsky et al., 2002). Consistent with much previous work, analysis of caveolin-1 null mice revealed that stimulus-induced activation of eNOS in aortic rings is upregulated (Razani et al., 2001) and the systemic levels of nitric oxide are about fivefold higher than in the wild-type animals (Zhao et al., 2002). Furthermore, caveolin-1 gene disruption dramatically blunts the angiogenic response to bFGF in vivo and reduces tumor size and tumor vascular density, suggesting a defective angiogenic response to tumor-derived factors (Woodman et al., 2003). It has been shown recently that a cell-permeable peptide derivative of the caveolin scaffolding domain reduces the elevated permeability of tumor microvessels to macromolecules in a mechanism that depends on the presence of eNOS, and that this effect causes delayed tumor progression in mice (Gratton et al., 2003). In conclusion, endothelial caveolin-1 seems to play an important role in the regulation of the tumor microvasculature formation and function through its interaction with eNOS, thus indirectly affecting tumor progression.

10. CONCLUDING REMARKS

In summary, the studies reviewed herein indicate that changes in caveolin-1 expression in tumor-derived cells and tumor specimens depend on the type of cancer and that the expression of caveolin-1 is positively correlated with the tumor cell grade and with the tumor's stage of progression (Fig. 2). How can we explain the complex, cell type-dependent and tumor grade/stage-dependent changes in caveolin-1?

Overexpression and gene-specific suppression studies suggest that caveolin-1 can exert both antiproliferative and pro-survival effects. The differential expression of caveolin-1 in various tumor cells and specimens may thus be explained by these different actions even if their molecular basis is still far from being elucidated.

Low caveolin-1 expression levels seen in many cancer types likely reflect a transcriptional change that occurs when a cancer cell reverts to a dedifferentiated state. Alternatively, it may result from a direct inhibitory effect of an activated oncogene. Genetic knockout of caveolin-1 results in tissue-specific hyperplasia and confers hypersensitivity to carcinogenic stimuli, indicating that caveolin-1 may be a tumor susceptibility gene. Therefore, given the inhibitory action of caveolin-1 on mitogenic signaling, the downregulation of caveolin-1 may promote the proliferative potential of the cancer cell during early stages of tumor progression, and this in turn is likely to increase the susceptibility of the initiated cells to other carcinogenic stimuli. At these early stages of progression the cells are hypersensitive to stress-induced apoptosis, and therefore are more susceptible to chemotherapy and radiotherapy (reviewed by Benhar et al., (2002). Tumor cells and specimens that exhibit low-level expression of caveolin-1 are therefore representative of low grade/early stage cancers (Fig. 2).

It should be noted that in some cancer cells, caveolin-1 expression may be maintained even in the face of oncogenic transformation. The high expression of caveolin-1 in such cells may reflect the inability of caveolin-1 to inhibit cell proliferation in the specific cellular context. However, as we have seen, caveolin-1 may also be able to promote cancer cell survival, and because of this ability it would be positively selected (along with other proteins) during later stages of cancer progression when survival, rather than rapid proliferation, is critical (Benhar et al., 2002). Such might be the case in high-grade cancer cells that evolve metastatic abilities and in cancer cells that are exposed to chemotherapeutic drugs (Fig. 2). An analogy may therefore be drawn between caveolin-1-induced pro-survival function in metastatic cancer cells and in multidrug resistant cancer cells. One possibility that requires further study is that metastatic cancer cells that

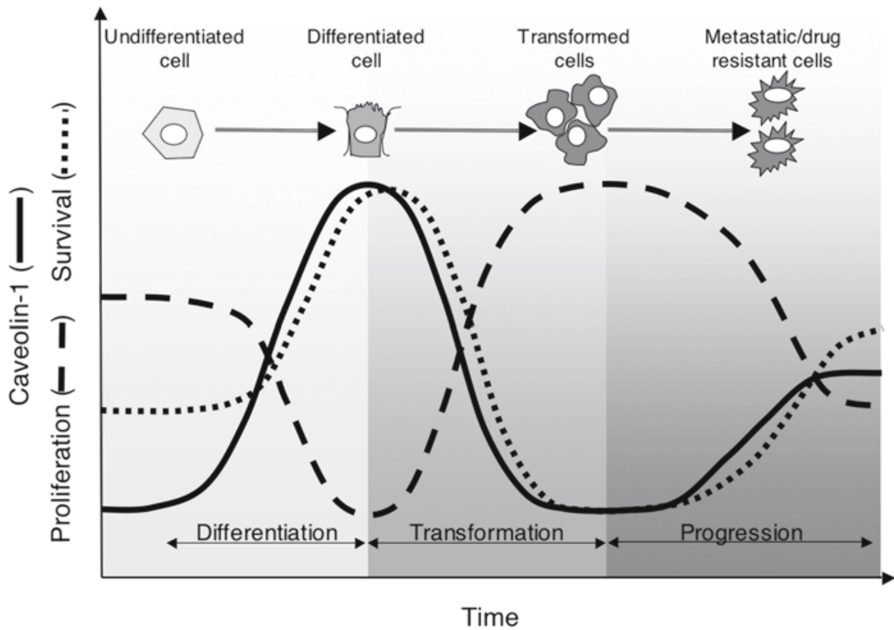

Fig. 2. Dynamic changes in caveolin-1 expression during cell differentiation, tumor initiation, and tumor progression. The expression of caveolin-1 is low in undifferentiated epithelial cells and is upregulated upon induction of differentiation. When epithelial cells are initially transformed by an oncogene, caveolin-1 expression is downregulated, thus allowing rapid proliferation due to disinhibition of mitogenic signaling. This effect leads to hyperplasia, and subsequently, through selective clonal expansion, to formation of preneoplastic lesions. The low expression of caveolin-1 sensitizes the initiated cells to cocarcinogenic stimuli, such as other oncogenes or environmental carcinogens, resulting in formation of tumors. The progression of early-stage cancer cells to malignancy depends upon further accumulation of genetic changes, due in part to increasing genomic instability, ultimately leading to formation of malignant tumors. At this stage, expression of caveolin-1 may contribute to cell survival, supporting positive selection of caveolin-1-expressing clonal lines that dominate high-grade and metastatic tumors. See text for a more detailed discussion.

express caveolin-1 are intrinsically more resistant to chemotherapeutic drugs. Such resistance may obviously be related to the caveolin-dependent suppression of apoptosis mechanisms.

The complex relationship between caveolin-1 and cancer is clearly derived from the multiple cellular functions of this fascinating protein and its multifarious interactions with so many cellular proteins. Understanding how caveolin-1 mediates different phenotypic changes in different cellular con-

texts is obviously a major problem for future research. The regulation of caveolin-1 expression is similarly complicated and is just beginning to be understood. Elucidating these issues will not only shed light on the functions of caveolin-1 in normal cells, but will also determine the potential of caveolin-1 and the signaling pathways it regulates as cancer drug targets.

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10

Dietary Modulation of Lipid Rafts

Implications for Disease Prevention and Treatment

Mark P. Mattson

1. INTRODUCTION

Other chapters in this book describe emerging findings concerning the roles of lipid rafts in physiological and pathological processes. The lipid components of rafts not only define their structure, but also influence the various signaling pathways associated with the rafts. It is well known that the lipid composition of cell membranes can be modified by various dietary lipids (Field et al., 1989). If raft lipids are subject to modification by diet, then presumably the signaling functions of the rafts are also affected by the diets. Although there is considerable evidence that dietary cholesterol and (to a lesser extent) sphingolipids can affect the functions of a variety of cell types, there have been few if any studies specifically addressing the effects of dietary lipids on raft-based signaling. However, given the multitude of signaling processes that involve lipid rafts (Fig. 1), it is important for investigators in a diverse array of fields to design studies aimed at establishing how dietary factors affect raft-based signaling in various cell types. The data obtained in such studies will have important implications for human health. In this chapter, I therefore consider a few ways in which dietary lipids and other dietary factors might affect lipid rafts in the context of cell types and signal transduction pathways involved in several prominent diseases.

2. DIETARY CHOLESTEROL

The cholesterol present in lipid rafts presumably comes from two sources: cholesterol synthesized by cells, and dietary cholesterol (Lutton, 1991), although the relative contributions of these two sources under various

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G-protein coupled receptors (m-2 acetylcholine, β1, β2 adrenergic, adenosine)
G-proteins (αs, αi, o, $\beta\gamma$, q)
Insulin receptor
EGF receptor
bFGF receptor
PDGF receptor
Trk A, p75
C-Ret
Tyrosine kinases (Src, Fyn, Lck, JAK2, STAT3)
Ser/Thr kinases (PKA, Raf, MEK, ERK, PI3 kinase, GRK, PKCα, δ, ε)
PIP, PIP2
IRS-1
Adenylyl cyclase
nNOS, eNOS
Phospholipase-D2
Cyclooxygenase-2

E-cadherin, β-catenin
NCAM, VCAM
GAP-43
Calreticulin, calsequestrin

GLUT4
Ca2+-ATPase, H+-ATPase
L-type VDCC
Kv1.5 potassium channel

Actin, annexins II and IV
SNARE, SNAP, dynamin

APP
Presenilin-1
beta-secretase
Parkin
CASK/Lin-2
Prion protein

T cell receptor
CD4, CD45
interleukin-2 receptor
Raftilin
Flotillins

Fig. 1. Examples of lipid raft-associated proteins involved in signaling pathways that are relevant to physiological and pathophysiological processes that may be subject to modification by diet.

physiological and pathological conditions remains to be determined. Hydroxy-3-methylglutaryl (HMG) CoA reductase, the rate-limiting enzyme in cholesterol production, is subject to feedback inhibition by dietary cholesterol, but may also be modulated by changes in cholesterol metabolism, such as formation of cholesterol esters. There are clear links between the amount of cholesterol in the diet and cardiovascular and cerebrovascular diseases. Cardiovascular disease and stroke result from atherosclerosis, a complex process involving multiple cell types including vascular endothelial cells, smooth muscle cells, and macrophages (Libby, 2000). Lipid rafts are believed to be

sites where cholesterol enters and exits cells (Smart et al., 1996) and so may influence both circulating and intracellular levels of cholesterol. Importantly, lipid rafts are also presumptive sites of receptors and downstream signaling cascades implicated in the process of atherosclerosis. Examples of such signaling pathways include those activated by inflammatory cytokines and cell adhesion molecules (Libby, 2000). The raft-associated protein caveolin may play a particularly important role in the alterations in macrophage lipid metabolism that are involved in the process of atherosclerosis (Gargalovic and Dory, 2003). There is certainly strong evidence that high amounts of dietary cholesterol increase the amount of oxidized low density lipoproteins (LDLs), resulting in damage to endothelial cells and ensuing events in the atherosclerotic process. Dietary cholesterol has been shown to stimulate the activation of monocytes and macrophages, and may thereby promote or exacerbate a variety of disorders, including cardiovascular and kidney disease (Pawluczyk and Harris, 2000). Aortic smooth muscle exhibited an enhanced contractile response to depolarization in rabbits fed a high-cholesterol diet (Wong, 1996), which may be a result of increased amounts of cholesterol in the raft environment of membrane calcium channels.

In addition to affecting cellular ion homeostasis by modulating the activity of ion channels, dietary cholesterol can affect the activities of ion pumps. For example, the activity of the plasma membrane Na^+/K^+-ATPase was increased in erythrocytes of rabbits fed a high-cholesterol diet (Makarov et al., 1995). Given the impact of cholesterol on lipid raft structure and function, it seems very likely that high levels of dietary cholesterol may also perturb raft-based signaling in ways that promote atherosclerosis.

Interactive effects of cholesterol and other dietary lipids on raft structure and function may influence disease processes. It is now well established that the initiation and propagation of the signaling events that activate immune cells occur in lipid rafts (van Laethem and Leo, 2002). The access and translocation of immune receptors to lipid rafts are developmentally regulated and sensitive to pharmacological agents and dietary factors. Lipid rafts are therefore important targets for modulation of immune function in health and disease. Signal transduction pathways in lipid rafts in lymphocytes have been shown to be very sensitive to levels of cholesterol and sphingolipids (Baumruker and Prieschl, 2002; Pierce, 2002). Considerable evidence indicates that dietary lipids can affect immune function; for example, high-cholesterol diets suppress immune function and may thereby increase the risk of certain types of cancer (Vitale and Broitman, 1981). The effects of dietary fat on immune function may be mediated though its component parts, including cholesterol, linoleic, linolenic, and arachidonic acids. The dietary fats may

affect lymphocyte signaling by altering the lipid composition of the cell membrane, which may affect rafts and the signaling proteins in those rafts.

Dietary cholesterol led to an increase in the cholesterol content of liver microsomes, an increase in the cholesterol/phospholipid and PC/PE (phosphatidylethanolamine) ratios, and depletion of saturated fatty acids. Dietary olive and primrose oils increase the amount of oleic and arachidonic acids in rat liver microsome membranes, suggesting increased d 9 and d 6 desaturation of n-6 essential fatty acids (Muriana et al., 1992). In a rat model of diabetes, the animals were maintained on diets with low or high cholesterol levels, with or without fish oil supplementation. Feeding a high-cholesterol diet increased the activity of intestinal brush-border microsomal-membrane activity of d-5 and d-9-desaturases when fed with fish oil in non-diabetic control rats, and increased d-5-desaturase in diabetic rats fed fish oil (Keelan et al., 1994). Thus, intestinal brush-border membrane desaturases are capable of adapting in response to changes in dietary lipids or to diabetes.

Emerging evidence suggests that individuals with a high dietary cholesterol intake and with elevated levels of circulating cholesterol, particularly cholesterol in LDLs, are at increased risk of Alzheimer's disease (Cooper, 2003). Amyloid β (Aβ)-peptide accumulates in the brains of patients with Alzheimer's disease and is believed to damage and kill neurons by a mechanism involving oxidative stress and perturbed cellular calcium homeostasis (Mattson, 1997). The amount of Aβ-peptide produced is increased in cultured cells exposed to high levels of cholesterol and in mice fed a high-cholesterol diet (Puglielli et al., 2001; Runz et al., 2002). It has been shown that the cleavage of the amyloid precursor protein by the γ-secretase enzyme (which generates Aβ-peptide) occurs in lipid rafts and is cholesterol-dependent (Wahrle et al., 2002). The available data therefore suggest the possibility that high-cholesterol diets increase the risk of Alzheimer's disease by enhancing production of Aβ-peptide in lipid rafts. Increases in the amount of membrane cholesterol in brain tissue samples from Alzheimer's patients have been documented (Cutler et al., 2004). The latter study also showed that exposure of cultured neurons to oxidative stress and Aβ-peptide can reproduce the alterations in membrane cholesterol and sphingolipids that occur in Alzheimer's disease, suggesting an important contribution of membrane-associated oxidative stress to perturbed lipid raft function in aging and neurodegenerative disorders. Alterations in cellular cholesterol metabolism may contribute to the pathogenesis of several different neurodegenerative disorders, because analyses of spinal cord tissue from patients with amytrophic lateral sclerosis (ALS), and from a mouse model of ALS, have revealed marked increases in levels of cholesterol esters that precede and accompany the neurodegenerative process (Cutler et al., 2002).

3. SPHINGOLIPIDS

Sphingolipids are a structurally diverse group of membrane lipids produced by all eukaryotic cells. They consist of a long chain sphingoid-base, usually sphingosine, which is acylated at the 2-amino position, forming a ceramide. Sphingolipids are present in all plants and animals and are therefore ingested in essentially any normal diet. In addition, molecules that inhibit sphingolipid metabolism are present in some foods (Merrill et al., 1997). Surprisingly, little is know about how dietary sphingolipids and inhibitors of sphingolipid metabolism affect cell functions and how they might influence the structure and signaling functions of lipid rafts.

Although there is no known nutritional requirement for sphingolipids, animal studies have shown that consumption of sphingolipids inhibits colon carcinogenesis, reduces serum LDL cholesterol, and elevates high-density lipoproteins (HDLs), which suggests that they are functional components of food (Berra et al., 2002). Insight into the specific mechanism whereby sphingolipid consumption suppresses colon carcinogenesis was provided by a study in which cancer-prone mice were fed diets supplemented with ceramide, sphingomyelin, glucosylceramide, lactosylceramide, and ganglioside G(D3). The latter diet reduced the number of tumors in all regions of the intestine, and caused a marked redistribution of β-catenin from a diffuse cytosolic and membrane pattern to a more "normal" concentration at intercellular junctions between intestinal epithelial cells (Schmelz et al., 2001). In cultured colon cancer cells sphingosine and long-chain ceramides reduced cytosolic and nuclear β-catenin, inhibited growth, and induced cell death. Administration of α-galactosylceramide has been shown to be effective in the treatment of experimental metastatic cancers, infections, and autoimmune diseases. A recent study showed that long-term administration of α-galactosylceramide inhibits tumor formation in three different mouse models of cancer (Hayakawa et al., 2003). Thus, the anticancer effect of dietary sphingolipids may result from increased production of ceramide and sphingosine.

Dietary cholesterol can influence the activities of sphingomyelin-metabolizing enzymes. For example, the activities of protein kinase C, phosphatidylcholine:ceramide-phosphocholine transferase, and phosphatidylethanolamine:ceramide-phosphoethanolamine transferase were increased in rat liver plasma membrane (Nikolova-Karakashian et al., 1992). The protein kinase C activation in cholesterol-enriched plasma membranes is likely caused by increased production of diacylglycerol and increased acylation of sphingosine to ceramide. The amounts of cholesterol and sphingomyelin in the blood and in cell membranes, and the activities of sphingomyelinases,

can be influenced by dietary factors. Dietary pectin decreases the amount of cholesterol in the blood and in liver cells of rats, and decreased the amount of sphingomyelin in very low density lipoproteins (VLDLs) while increasing its amount in HDLs (Bladergoren et al., 1999). The latter study also showed that dietary pectin increases the activities of both lysosomal and plasma membrane sphingomyelinases, which would be expected to increase ceramide production.

Abnormalities in sphingolipid metabolism occur in Alzheimer's disease and ALS (Cutler et al., 2002, 2004). In both disorders, levels of long-chain ceramides are increased in association with the neurodegenerative process. Increased amounts of long-chain ceramides occur in cell culture and animal models of Alzheimer's disease and ALS, and in cultured neurons exposed to oxidative stress. The increased levels of ceramide may play a key role in the neurodegenerative process, because treatment of neurons with myriocin, an inhibitor of sphingolipid synthesis, reduces ceramide levels and protects the neurons against death induced by membrane-associated oxidative stress (Cutler et al., 2002, 2003).

4. OTHER DIETARY LIPIDS

Membrane structure and function can be affected by many different lipids in addition to cholesterol and sphingolipids, and it will be of considerable interest to determine how various dietary lipids affect lipid rafts. There are now numerous examples of evidence supporting important effects of specific dietary lipids on the function of cells and/or in disease processes. Docosahexanoic acid (DHA) has been shown to affect the function of several different cell types and organ systems, including neurons in the brain (Yavin et al., 2002). It has been shown that DHA can stimulate phospholipiase D in lymphocytes by a mechanism that may involve exclusion of phospholipase D from lipid rafts (Diaz et al., 2002). Data suggest that n-3 fatty acids, including eicosapentaenoic acid and DHA, may reduce the risk of cardiovascular disease by lowering blood pressure and by interfering with key steps in the formation of the atherosclerotic plaque (Semplicini and Valle, 1994). Dietary n-3 fatty acids can reduce triglyceride levels and may improve cholesterol levels. A specific effect of dietary n-3 fatty acids on lipid rafts is suggested by a study showing that n-3 fatty acids suppress the formation of ceramide in lymphocytes (Jolly et al., 1997). When mice were fed a diet supplemented with n-3 fatty acids, there was a reduction in antigen-specific delayed hypersensitivity reactions and mitogen-induced proliferation of T cells (McMurray et al., 2000). The reduction in proliferative capacity was accompanied by reductions in interleukin (IL)-2 secretion and IL-2 receptor

α-chain expression, and in ceramide production. However, the effects of n-3 fatty acids on the lipid composition and signaling functions of lipid rafts in lymphocytes and cells involved in the atherosclerotic process remain to be determined.

In one study it was shown that dietary supplementation with essential phospholipids reduces the formation of atherosclerotic plaques in rabbits on a high fat diet (Wojcicki et al., 1992). The reduction in plaque formation was associated with decreased levels of circulating cholesterol and increased amounts of cholesterol esterified with polyunsaturated fatty acids. Interestingly, the high-fat diet impaired immune function in the rabbits, and this effect of the diet was largely prevented by dietary supplementation with essential phospholipids (Wojcicki et al., 1992). It was suggested that the beneficial effects of the essential phospholipids were a result of participation of the phospholipids in membrane functions that are otherwise altered by high fat diets.

5. OTHER DIETARY FACTORS

The leading risk factor for cardiovascular disease, stroke, and diabetes in the United States is overeating (Pradhan et al., 2002). Dietary restriction (reduced caloric intake and/or meal frequency) increases the life span of rodents and monkeys and decreases the incidence of cardiovascular disease, cancers, and diabetes (Mattson et al., 2002). Dietary restriction may protect against cardiovascular disease by reducing blood pressure, enhancing insulin sensitivity, and improving cardiovascular stress adaptation (Wan et al., 2003a, 2003b). Dietary restriction suppresses the neurodegenerative process and improves functional outcome in animal models of stroke, Alzheimer’s, Parkinson’s, and Huntington’s diseases (Bruce-Keller et al., 1999; Duan, and Mattson, 1999; Yu and Mattson, 1999; Duan et al., 2003). The mechanism whereby dietary restriction protects against disease may involve reduced levels of oxidative stress and induction of a mild beneficial cellular stress response that results in activation of genes encoding proteins that promote cell survival and plasticity, including heat shock and related protein chaperones and growth factors (Duan and Mattson, 1999; Yu and Mattson, 1999; Lee et al., 2002).

Although there is no direct evidence that dietary restriction affects lipid raft structure or function, some of the signaling pathways that change in response to dietary restriction do involve raft-associated proteins. For example, receptors for insulin and insulin-like growth factor-1 are concentrated in lipid rafts (Vainio et al., 2002; Huo et al., 2003), and it is well known that dietary restriction enhances insulin signaling. BDNF signaling

is enhanced by dietary restriction (Lee et al., 2002; Duan et al., 2003), and it has been shown that receptors for BDNF are located in lipid rafts (Higuchi et al., 2003).

Dietary antioxidants and pro-oxidants might also be expected to affect lipid rafts by reducing or increasing levels of lipid raft-associated oxidative stress, and thereby affecting the metabolism of raft lipids and the function of raft-associated proteins. Indeed, it has been shown that exposure of cells to oxidative stress can increase the amount of membrane cholesterol and can activate sphingomyelinases resulting in the liberation of ceramides from sphingomyelin (Cutler et al., 2002, 2003). Numerous raft-associated proteins have been shown to be modified by oxidative stress in ways that affect their function. Examples include GTP-binding proteins (Blanc et al., 1997), glucose transporters (Mark et al., 1997) and voltage-dependent calcium channel proteins (Lu et al., 2001).

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