The EMBO Journal Vol.17 No.23 pp.6772–6775, 1998

EMBO WORKSHOP REPORT

A kibbutz full of lipids

Lipids: Regulatory Functions in Membrane Traffic and Cell Development Kfar Blum Kibbutz Guest House, Galilee, Israel, May 10–15, 1998

Anthony H.Futerman[1,2], Riccardo Ghidoni[3] and Gerrit van Meer[4]

[1]Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel, [3]Instituto di Scienze Biomediche, Ospedale San Paolo, via A di Rudini 8, Milan, Italy and [4]Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands

[2]Corresponding author
e-mail: bmfuter@weizmann.weizmann.ac.il

Introduction

Readers of this journal may be surprised to read that the development of our thinking about the membrane lipid bilayer parallels the development of the kibbutz, one of Israel's most famous institutions. Whereas the founding fathers of the kibbutz movement (Hebrew word for 'communal settlement') preached 'all for one and one for all', today much greater emphasis is placed on the freedom of expression and needs of the individual kibbutz member. Just so our understanding of the membrane lipid bilayer. In the early days, the bilayer was no more than a collective enterprise deemed necessary to keep the good guys in and the bad guys out. But all this has changed, and it is now apparent that some individual lipid members are 'more equal than others'.

Our thinking about biological membranes has come a long way over the past century since Overton first described their lipidic nature, and since Gorter and Grendel suggested that the lipids are arranged in a bilayer. Although it was clear in those early days that there was some heterogeneity in the lipid components of biological membranes, never in their wildest dreams could these early pioneers have imagined the huge number of possible permutations in both the hydrophobic 'tails' and in the hydrophilic 'heads' of the three major lipid classes (glycerolipids, sphingolipids and sterols), that accounts for the existence of the many hundreds of individual lipids known today. If lipids simply act in a communal manner to maintain the structural integrity of the cell, why are there so many different lipid species? It is now becoming apparent that individual lipids are key players in their own right. For instance, a bewildering variety of lipids are known to turn over in response to various extracellular stimuli, and individual lipid species have been implicated to play pivotal roles in regulating intracellular protein and vesicle traffic. A hundred years after Overton, today's challenge is to elucidate the regulatory functions of individual lipid species and to identify their modes of interaction with other membrane components. In this brief report of a recent EMBO workshop ('Lipids: Regulatory Functions in Membrane Traffic and Cell Development', Kfar Blum Kibbutz Guest House, Galilee, Israel, 10–15 May, 1998), we will summarize progress in these areas. Readers are referred to recent comprehensive reviews for more details.

A recurring theme in the workshop was that new methodologies are required to analyze the properties of lipids in cellular membranes, as opposed to artificial lipid bilayers (liposomes). Although the study of liposomes has provided important biophysical information about the dynamic properties of lipids, most studies have been performed on liposomes of relatively simple composition, and with a symmetric rather than an asymmetric distribution of lipids across the bilayer. As a result, analysis of protein–lipid interactions has not addressed regulatory functions that might derive from the complex, asymmetric lipid distribution found in biological membranes. Similarly, no techniques are available to address ways in which the cytoskeleton might modulate cellular membranes. Together, these technical limitations have led to oversimplified views of the structure and dynamics of lipids in biological membranes. The good news is that new methodologies are on the way.

Metabolism, transport and subcellular localization of lipids

As a prerequisite to understanding the regulatory roles of lipids in membrane traffic and cell development, it is essential to know where and how lipid synthesis is regulated, how lipids are transported within the cell, how their local concentration in a particular membrane is determined, and how they interact with other membrane components.

Where are lipids synthesized and degraded and how are these processes regulated?

Whereas the early years of lipid research were devoted to defining the major pathways of lipid synthesis by biochemical analysis, recombinant DNA technology has recently had a tremendous impact on lipid enzymology and has yielded a number of surprises. For instance, the major structural lipid, phosphatidylcholine (PC), is thought to be synthesized at the endoplasmic reticulum, but unexpectedly, a nuclear localization signal was found in one of the two isoforms of the key regulatory enzyme of PC synthesis, cytidylyltransferase (Wang *et al.*, 1995; S.Jackowski, Memphis, TN). 'Knock-out' (k.o.) technology is also shedding light on problems that could not previously be resolved biochemically. A k.o. mouse of a key gene involved in PC synthesis in the liver, phosphatidylethanolamine (PE) methyltransferase has been generated. Upon choline starvation (to inhibit the CDP-choline pathway of PC synthesis), the k.o. mouse develops a 50% loss in liver PC, and liver failure, demonstrating the importance of the methylation pathway (D.Vance, Edmonton, Canada).

In addition to the cloning of enzymes of glycerolipid synthesis and degradation, such as two phosphatidylserine (PS) synthases (M.Nishijima, Tokyo, Japan) and two phospholipase D forms active in the regulated hydrolysis of phospholipids (M.Liscovitch, Rehovot, Israel), a number of enzymes in sphingolipid synthesis have recently been cloned (Ichikawa and Hirabayashi, 1998). The importance of cloning was evident from the consensus, after repeated and lively discussions, about the location of sphingomyelin (SM) synthesis: 'stop discussing, clone the genes'. Whereas the bulk of SM synthase activity has been assigned to the *cis* and *medial* Golgi apparatus (R.Pagano, Rochester, NY; A.Futerman, Rehovot, Israel; G.van Meer, Amsterdam, The Netherlands; F.Wieland, Heidelberg, Germany), additional activities have been assigned to the plasma membrane (D.Voelker, Denver, CO; A.Futerman and R.Pagano), at both its exoplasmic (G.van Meer) and cytoplasmic surfaces (T.Levade, Toulouse, France), or to the *trans*-Golgi network (TGN; D.Allan, London, UK). Cloning will also resolve whether multiple isoforms exist. Upon infection of erythrocytes, the malaria parasite expresses an SM synthase in its surrounding vacuole which is required for development of the tubulovesicular membrane system and for parasite viability; this enzyme (isoform?) has different properties from the intracellular enzyme (K.Haldar, Stanford, CA). Apart from cloning, generation of mutants in pathways of lipid metabolism may reveal features that were not predicted based on biochemical pathways. For instance, some mutants in lysosomal glycosphingolipid hydrolysis have turned out to be due to defects in activator proteins rather than in the hydrolytic enzymes themselves (K.Sandhoff, Bonn, Germany).

Where are lipids localized in the cell?

Unlike proteins, for which antibodies can easily be raised for determination of subcellular localization, lipids are far less antigenic. Moreover, lipid immunolocalization depends on fixing and permeabilizing cells, which can cause lipid redistribution (Schwarz and Futerman, 1997). Recently, by a combination of microscopy, lipid analysis and subcellular fractionation, the tantalizing observation was made that internal membranes of late endosomes contain the unique antigenic lipid, lysobisphosphatidic acid (Kobayashi *et al*., 1998). This lipid appears to stimulate lysosomal hydrolysis of glucosylceramide (GlcCer) by glucocerebrosidase, which is facilitated by differences in physical properties of the internal vesicles compared with the surrounding membrane (K.Sandhoff) from which they originate (see Hopkins *et al*., 1990). The fact that the related semilysobisphosphatidic acid is enriched in the Golgi apparatus (Cluett *et al*., 1997) lends support to the idea that specific lipids perform specific tasks in different organelles.

New techniques are also needed to determine lipid transbilayer distribution. To date, transbilayer distribution has only been studied in detail at the plasma membrane, and studied for the most part using cell-impermeable phospholipases (e.g. sphingomyelinase; D.Allan; T.Levade; G.van Meer; J.Vance, Edmonton, Canada). The assumption using these enzymes is that they hydrolyze all of the lipid on the outer membrane leaflet, but none on the inner membrane leaflet. This assumption may not be valid in cases where the lipid product does not form a bilayer (such as diacylglycerol or ceramide), or when the lipid substrate constitutes a large fraction of the lipid in the outer leaflet. This may have led to erroneous assignments of the trans-bilayer distribution of particular lipids.

How are lipids transported to various intracellular locations?

Four possible mechanisms have been proposed for how lipids move around cells, namely vesicle transport, monomer diffusion, lipid-transfer proteins (facilitated transport), and transport via direct membrane contacts between organelles. The first of these is the only realistic mechanism to account for the massive amount of lipid transported between intracellular organelles; a vesicle contains thousands of lipid molecules, whereas lipid transfer proteins usually bind lipid stoichiometrically.

Genetic approaches are revealing new insights into lipid transport. Of great impact is APYG ('awesome power of yeast genetics'), which for many provides an attractive means for manipulating lipid transport pathways. Using APYG, a screen for aminoglycerophospholipid transport mutants detected two genes that participate in transport from the ER to the Golgi apparatus and/or vacuole; one gene encodes the phosphatidylinositol (PI)-4-kinase Stt4p, and the second gene encodes a new protein of 43 kDa. Both genes complement the phospholipid transport defects of mutant strains *in vivo*, and can be used to reconstitute lipid transport in permeabilized cells. They do not belong to the class of previously isolated *sec* mutants (D.Voelker).

Can lipids be sorted by formation of lateral domains?

Is there any evidence, apart from biophysical data using liposomes (D.Brown, Stony Brook, USA; Brown and London, 1998), that lateral segregation of lipids in the same bilayer subserves sorting functions in biological membranes? Using fluorescent (BODIPY-labeled) sphingolipid analogs whose fluorescence emission is concentration dependent, SM was shown to internalize into two different populations of endosomes identifiable within the first seconds of endocytosis (R.Pagano). By far most SM appears to be sorted in early endosomes to a recycling pathway. Using other fluorescent lipids (e.g. DiI) with varying hydrocarbon chains, the tubules of early endosomes involved in recycling appear to be more fluid than the vacuolar membrane (S.Mukherjee, New York, NY), suggesting that lipid sorting in the endosomal pathway may indeed depend on lateral segregation. Lipid sorting in the endocytic pathway could also occur by domain formation at the plasma membrane rather than in endosomes. Caveolae (generically termed 'rafts') are plasma membrane domains enriched in (glyco)sphingolipids, cholesterol, and glycosylphosphatidylinositol (GPI)-anchored proteins, according to the criterion of detergent-insolubility (D.Brown) and electron microscopy (Parton, 1994), and have received much attention lately. Does endocytosis of GPI-anchored proteins occur from rafts other than caveolae? Probably. Endocytosis from caveolae (and from clathrin-coated pits) depends on dynamin, but a GPI-anchored diphtheria toxin receptor construct is still internalized in the absence of functional dynamin (G.Skretting and K.Sandvig, Oslo, Norway). During prion infection,

rafts appear to be essential for the conversion of cellular prion proteins to their scrapie form (A.Taraboulos, Jerusalem, Israel).

Lateral heterogeneity was originally proposed to explain the different lipid composition of the two plasma membrane domains of epithelial cells, with lateral segregation in the TGN giving rise to distinct vesicles each destined for a different plasma membrane domain (G.van Meer; Simons and Ikonen, 1997). Exciting new evidence suggests that the lipid composition of a vesicle can differ from the donor membrane from which it originates. Using nano-electrospray ionization tandem mass spectrometry (ESI–MS/MS) to determine lipid composition in the low picomole range (F.Wieland), SM and cholesterol levels are lower in vesicles (COPI-coated) compared with donor membranes. Similar sorting may occur in the ER, where GPI-anchored proteins and ceramides may be sorted into distinct COPII-coated vesicles, while other lipids and proteins are transported in another type of vesicle, which although also coated by COPII, may have a quite different membrane composition (M.Muniz and H.Riezman, Basel, Switzerland). Interestingly, GPI biosynthetic enzymes are segregated into unique subspecializations of the ER and newly synthesized GPIs are consequently heterogeneously distributed in the ER membrane (A.Menon, Madison, WI). In the Golgi apparatus, lateral segregation of lipids may be involved in sorting of membrane spanning proteins by generating domains of increased thickness that prevent resident Golgi proteins from entering forward-moving transport vesicles (S.Munro, Cambridge, UK).

Are rafts lipid-based signaling platforms?

Lateral domain formation could also serve other functions apart from sorting. Because many molecules involved in signaling, like phospholipase D (M.Liscovitch) and a variety of kinases, appear to be associated with rafts according to one or other criterion, the possibility exists that rafts act as lipid-based signaling platforms. Thrilling as this possibility is, it still generates considerable controversy, so much so that participants in this discussion were treated to the soothing sounds of a Beethoven symphony to calm them (and the session chairman) down! The general consensus is that present technology does not permit unambiguous biochemical and functional characterization of rafts. Complicating factors are: (i) the notorious impurity (and low yield) of subcellular fractions, which is especially true for the separation of two domains of a continuous membrane; (ii) the lipid asymmetry of plasma membranes, where one leaflet of the membrane might be resistant to detergent and the other not, resulting in reorientation of the lipids during detergent-dependent isolation procedures; (iii) the temperature-dependence (and detergent-dependence) of lipid partitioning between domains in membranes of complex lipid composition, resulting in changes in domain composition during isolation procedures; and (iv) the size range (nanometers) and time resolution (nanoseconds) of the domains, which define their nature in living cells.

Lipids as regulators of membrane traffic and cell development

Most of the issues discussed above deal with lipid metabolism, sorting and transport *per se*. However, lipids play a variety of additional specific regulatory roles in various aspects of membrane traffic and cell development. One emerging concept is that some lipids may function as 'cellular lipostats', with the local concentration of the lipid somehow reflecting a physiological state of the cell. The local concentration would be the result of a combination of enzymatic activities and lipid transport, and would be monitored by a molecular sensor. The sensor could in turn regulate an effector of either membrane traffic or cell development. Some examples of this developing concept are given below.

Small GTP-binding proteins and signaling lipids

The GTPases, ARF and Rab, may regulate vesicle traffic. ARF–GTP recruits the coat protein COPI to the membrane (F.Wieland), but also stimulates membrane traffic by activating phospholipase D (S.Cockroft, London, UK); the function of phosphatidic acid generated as a result of phospholipase D is not known. A new twist to this story is the finding that ARF, by an independent mechanism, stimulates PI-4- and PIP-5-kinases on Golgi membranes, thus controling the local levels of PIP and PIP_2 and assembly of a spectrin-based Golgi apparatus skeleton (M.A.De Matteis, Santa Maria Imbaro, Italy). PIP_2 by itself may recruit a number of proteins of the vesicle-budding machinery.

Lipid transfer proteins: transporters or modulators?

Lipid transfer proteins do not normally generate net phospholipid transfer, but rather lipid exchange, implying they function to modulate local lipid environments. The best example of this is the yeast PI transfer protein (Sec14p), which appears to control the lipid environment of the Golgi apparatus membrane. In its PI-bound form, Sec14p may stimulate diacylglycerol production from PI in the Golgi apparatus, whereas in its PC-bound form it reduces diacylglycerol consumption by the CDP-choline pathway (V.Bankaitis, Birmingham, AL); the Golgi apparatus pool of diacylglycerol (or of a related lipid, but probably not phosphatidic acid) may regulate vesicle budding. In contrast, the mammalian PI transfer protein apparently supplies PI from its site of synthesis in the ER to the plasma membrane in response to PI consumption via PIP_2 hydrolysis, and carries a PC molecule back to the ER (van Paridon *et al*., 1987; S.Cockcroft).

Diacylglycerol and ceramide

Ceramide and diacylglycerol are similar structurally inasmuch as neither have bulky polar head groups. Both appear to regulate membrane traffic by more than one mechanism. Whereas ceramide synthesis is required for GPI-anchored proteins to reach the Golgi apparatus (Horvath *et al*., 1994), presumably due to a direct requirement for ceramide, intermediates in ceramide metabolism (i.e. sphingoid long chain bases) play a role in endocytosis in yeast, presumably by interaction with the endocytic machinery in a indirect signaling manner, perhaps via a kinase and a long chain base-activated protein phosphatase, whose regulator subunit is Cdc55p (H.Riezman). In contrast, ceramide decreases rates of exocytosis and endocytosis in mammalian cells (Chen *et al*., 1995). Since the diacylglycerol and ceramide pools can be interconverted

by SM synthase (or by IPCeramide synthase in yeast), it will be important to establish the relationship between the size of these two pools.

As mentioned earlier, ceramide also acts as a second messenger in signal transduction pathways unrelated to membrane trafficking. In compartmentalized cultures of sympathetic neurons, ceramide generation in distal axons (by a neutral sphingomyelinase), but not within the cell body, inhibits axon growth (J.Vance). Although the site of ceramide generation by sphingomyelinases is normally assumed to be the plasma membrane, production of signaling ceramide in endo-lysosomes (S.Schütze, Kiel, Germany) or in autophagic lysosomes (R.Ghidoni, Milan, Italy) has been reported. The binding of photoactivatable ceramide analogs to the lysosomal protease, cathepsin D (S.Schütze), suggests that ceramide could also act as a second messenger within endo-lysosomes; interestingly, it has been reported that ceramide generated in lysosomes is unable to exit lysosomes (Chatelut *et al*., 1998). The recent cloning of acidic and neutral (Tomiuk *et al*., 1998) sphingomyelinases will now permit detailed analysis of ceramide generation in signal transduction pathways.

A regulatory pool of GlcCer?

GlcCer is synthesized on the cytosolic surface of the Golgi apparatus. Short acyl-chain sphingolipid analogs, such as C_6-GlcCer, are translocated from the cytoplasmic to the exoplasmic leaflet of the plasma membrane by multidrug transporters like the MDR1 P-glycoprotein and MRP1, which occur in most cells (R.Raggers and G.van Meer). Interestingly, 40% of cellular MDR1 is present in caveolin-enriched membranes obtained by detergent solubilization, and up-regulation of MDR1 expression also results in up-regulation of caveolin-1, phospholipase D and of GlcCer synthesis (M.Liscovitch). This suggests that GlcCer located in the cytosolic leaflet may be involved in a signaling function. For instance, in hippocampal neurons, GlcCer synthesis is required for growth factors to stimulate axonal growth (A.Futerman). Since GlcCer is a relatively minor lipid in these neurons, the requirement for GlcCer synthesis is presumably due to its involvement in a regulatory function, as yet unidentified.

Regulation of proteolytic activity by cholesterol

Elevation of intracellular cholesterol levels activates feedback mechanisms that reduce cholesterol uptake and synthesis. The regulated proteolysis of HMG-CoA reductase in response to mevalonate or sterols is mediated by increased susceptibility of the reductase to ER proteases, rather than induction of a new proteolytic activity (R.Simoni, Stanford, CA). This event is apparently regulated by the cholesterol concentration of the ER membrane, but how this is related to cholesterol concentration in other cellular membranes is unclear. A breakthrough in this field is the cloning of the Niemann-Pick type C (NPC) gene *NPC1* (Liscum and Klansek, 1998). In NPC disease, LDL-cholesterol internalized by endocytosis is unable to exit lysosomes. One interesting possibility is that the NPC1 protein is not involved in directing vesicle traffic, but rather stimulates absorption of cholesterol and possibly other lipids (ceramide, see above) from structures within lysosome into the surrounding membrane.

Perspectives

If there is one message to be gleaned from this brief report, it is that for cell biologists, lipids are no longer the poor second cousins of their illustrious relatives, the proteins, but are themselves key regulatory players in many intracellular processes, be it controlling intracellular traffic or regulating more general properties related to cell development. A challenge for the years ahead is to develop better methodology to visualize lipids in living cells, in particular in sub-domains of defined membranes, and to define their physical behavior and interactions with other membrane components at the molecular level. We can safely assume that the next EMBO meeting on the regulatory roles of lipids, hopefully to be held in three years time, will provide a more detailed insight in the delicate interplay between membrane lipids and proteins in regulating cell physiology.

Abbreviations

GlcCer, glucosylceramide; GPI, glycosylphosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

References

Brown,D.A. and London,E. (1998) Functions of lipid rafts in biological membranes. *Annu. Rev. Cell. Dev. Biol.*, **14**, 111–136.

Chatelut,M., Leruth,M., Harzer,K., Dagan,A., Marchesini,S., Gatt,S., Salvayre,R., Courtoy,P. and Levade,T. (1998) Natural ceramide is unable to escape the lysosome, in contrast to a fluorescent analogue. *FEBS Lett.*, **426**, 102–106.

Chen,C.S., Rosenwald,A.G. and Pagano,R.E. (1995) Ceramide as a modulator of endocytosis. *J. Biol. Chem.*, **270**, 13291–13297.

Cluett,E.B., Kuismanen,E. and Machamer,C.E. (1997) Heterogeneous distribution of the unusual phospholipid semilysobisphosphatidic acid through the Golgi complex. *Mol. Biol. Cell*, **8**, 2233–2240.

Hopkins,C.R., Gibson,A., Shipman,M. and Miller,K. (1990) Movement of internalized ligand–receptor complexes along a continuous endosomal reticulum. *Nature*, **346**, 335–339.

Horvath,A., Sütterlin,C., Manning-Krieg,U., Movva,N.R. and Riezman,H. (1994) Ceramide synthesis enhances transport of GPI-anchored proteins to the Golgi apparatus in yeast. *EMBO J.*, **13**, 3687–3695.

Ichikawa,S. and Hirabayashi,Y. (1998) Glucosylceramide synthase and glycosphingolipid synthesis. *Trends Cell Biol.*, **8**, 198–202.

Kobayashi,T., Stang,E., Fang,K.S., de Moerloose,P., Parton,R.G. and Gruenberg,J. (1998) A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. *Nature*, **392**, 193–197.

Liscum,L. and Klansek,J.J. (1998) Niemann-Pick disease type C. *Curr. Opin. Lipidol.*, **9**, 131–135.

Parton,R.G. (1994) Ultrastructural localization of gangliosides; GM_1 is concentrated in caveolae. *J. Histochem. Cytochem.*, **42**, 155–166.

Schwarz,A. and Futerman,A.H. (1997) Determination of the localization of gangliosides using anti-ganglioside antibodies: comparison of fixation methods. *J. Histochem. Cytochem.*, **45**, 611–618.

Simons,K. and Ikonen,E. (1997) Functional rafts in cell membranes. *Nature*, **387**, 569–572.

Tomiuk,S., Hofmann,K., Nix,M., Zumbansen,M. and Stoffel,W. (1998) Cloned mammalian neutral sphingomyelinase: Functions in sphingolipid signaling? *Proc. Natl Acad. Sci. USA*, **95**, 3638–3643.

van Paridon,P.A., Gadella,T.W.J.,Jr, Somerharju,P.J. and Wirtz,K.W.A. (1987) On the relationship between the dual specificity of the bovine brain phosphatidylinositol transfer protein and membrane phosphatidylinositol levels. *Biochim. Biophys. Acta*, **903**, 68–77.

Wang,Y., MacDonald,J.I. and Kent,C. (1995) Identification of the nuclear localization signal of rat liver CTP: phosphocholine cytidylyltransferase. *J. Biol. Chem.*, **270**, 354–360.