New EMBO Member's Review

Cellular lipidomics

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The cellular lipidome comprises over 1000 different lipids. Most lipids look similar having a polar head and hydrophobic tails. Still, cells recognize lipids with exquisite specificity. The functionality of lipids is determined by their local concentration, which varies between organelles, between the two leaflets of the lipid bilayer and even within the lateral plane of the membrane. To incorporate function, cellular lipidomics must not only determine which lipids are present but also the concentration of each lipid at each specific intracellular location in time and the lipid's interaction partners. Moreover, cellular lipidomics must include the enzymes of lipid metabolism and transport, their specificity, localization and regulation. Finally, it requires a thorough understanding of the physical properties of lipids and membranes, especially lipid–lipid and lipid–protein interactions. In the context of a cell, the complex relationships between metabolites can only be understood by viewing them as an integrated system. Cellular lipidomics provides a framework for understanding and manipulating the vital role of lipids, especially in membrane transport and sorting and in cell signaling.

The EMBO Journal (2005) **24**, 3159–3165. doi:10.1038/sj.emboj.7600798; Published online 1 September 2005

Subject Categories: membranes & transport

Keywords: lipid asymmetry; lipid rafts; lipid translocators; membrane lipids; sphingolipids

Introduction

After the explosive developments of genomics and proteomics, our analytical methods, mass spectrometry in particular, have advanced to the point where metabolomics is following suit. Thousands of metabolites can be accurately measured and high-throughput technology starts to spawn data in amounts that flood our databases. However, data generation does not make science. While powerful data analysis is a first requirement, the outcome is useful only to the extent that it answers a scientific question. Cell biologists must find out how metabolomics can be applied to further our understanding of the living cell in health and disease.

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Received: 28 February 2005; accepted: 20 July 2005; published online: 1 September 2005

Within metabolomics, lipidomics has its own identity. Cardiovascular disease, obesity and a number of inborn errors of metabolism are frequent lipid-related disorders, but all major human diseases including cancer and Alzheimer's have a lipid component. This warrants the expectation that clinical diagnosis of these diseases will greatly profit from lipid pattern analysis. Moreover, lipids have a characteristic chemical nature that determines their inherent self-assembly into membranes and lipid droplets and supplies a common denominator to the functions that they exert in organizing membranes and orchestrating membrane events like signal transduction. Via glycolipids lipidomics is linked to the field of glycomics, and via the lipid second messengers to the field of signalomics. Notably, in the end all precursors for lipid synthesis and products of lipid degradation are water-soluble metabolites, solidly embedding lipidomics in metabolomics.

Lipidomics is more than chemistry alone. At the level of the cell, lipidomics must quantitatively describe all lipids and their functions. This requires knowledge of the local lipid profile in time and the relevant interacting partners: What is the composition of a certain organelle, each of its bilayer leaflets and any specific location laterally in that leaflet? How is the local concentration regulated in time, and on which other molecules does the lipid act? For example, it is rather meaningless to know the cellular level of the signaling lipid lysophosphatidic acid (LPA). What counts is its concentration at the plasma membrane LPA receptor (Chun *et al*, 2002), or at the cytosolic side of the neck of budding synaptic (Schmidt *et al*, 1999) or Golgi vesicles (Weigert *et al*, 1999). This immediately extends the question to: How do cells separate these pools from LPA in lipid biosynthesis, which freely travels as a cytosolic monomer between endoplasmic reticulum (ER) and peroxisomes (Brites *et al*, 2004)? The example also illustrates the importance of the location and activity regulation of the biosynthetic and hydrolytic enzymes. Finally, the local concentration of the many lipids that do not spontaneously move across or between membranes depends on proteins assisting their transport like translocators and transfer proteins.

Research in lipids is revolutionized by the precipitous developments in methodology offering insights in the astounding range of their biological activities (Wenk, 2005). It is a challenge to extend this revolution to our understanding of lipid dynamical organization and function in cells that we have established over the years by trying to include lipid flip-flop and segregation in lateral domains into a coherent picture (van Meer, 1989; Sprong *et al*, 2001).

Unique lipid compositions of animal cell organelles?

In the recent classification of lipids, meant to serve as an international basis for data storage, lipids are loosely defined

as biological substances generally hydrophobic in nature and in many cases soluble in organic solvents (Fahy *et al.*, 2005). Indeed, the behavior of all hydrophobic substances follows the same physical principles and therefore makes them subject of the present review. In practice, the organization of lipids in cells is determined by the bulk lipid classes, and one can consider the behavior and function of the hundreds of minor lipids as superimposed on the dynamic organization of the major ones.

Which are the major lipids in animal cells (Figure 1)? While triacylglycerols and cholesteryl esters fill the core of lipid droplets in the cytosol and of lipoproteins being secreted or endocytosed (van Meer, 2001), the bulk of the cellular lipids is organized in membranes. The standard membrane lipid is the cylindrical phosphatidylcholine (PC), 50% of the cellular lipids. Unsaturated PC yields fluid bilayers. In this category of glycerophospholipids, phosphatidylethanolamine (PE) constitutes 20 mol% in most membranes, phosphatidylserine (PS) appears on the cell surface during apoptosis and blood coagulation, and phosphatidylinositol (PI) is the basis for the phosphoinositides, phosphorylated derivatives whose signaling functions depend on the number and position of the phosphates on the inositol ring. A second category is formed by the sphingolipids. Sphingomyelin (SM), like PC, contains a phosphocholine head, but has a hydrophobic ceramide backbone consisting of a sphingosine tail and one saturated fatty acid. In glycosphingolipids, ceramide carries carbohydrates, the simplest ones being glucosyl- and galactosylceramide. By themselves sphingolipids form a frozen, solid membrane. They are fluidized by cholesterol, the mammalian sterol, a third lipid category (Fahy *et al.*, 2005).

Figure 1 The structure of the major membrane lipids. The more or less cylindrical glycerophospholipid phosphatidylcholine (PC) carries a zwitterionic phosphocholine headgroup on a glycerol with two fatty acyl chains (diacylglycerol), usually one unsaturated (bent). Phosphatidylethanolamine (PE) has a small headgroup and a conical shape and creates a stress in the bilayer: the PE-containing monolayer has a tendency to adopt a negative curvature. The phosphosphingolipid sphingomyelin (SM) tends to order membranes via its straight chains and its high affinity for the flat ring structure of cholesterol (chol). For chemical structures, see Fahy *et al.* (2005).

Unfortunately we do not know the detailed lipid composition of each organellar membrane. What is the problem? (1) Quantitative compositional analyses have been limited to certain lipid classes, mostly the phosphate-containing glycerol- and sphingolipids. Rarely have glycosphingolipids and cholesterol been included. This should no longer be a problem using mass spectrometrical approaches. (2) 'Purified' organelles are not pure. To illustrate the problem: endosomes purified with a yield of 50% and containing a contamination of only 5% of an endoplasmic reticulum (ER) marker contain roughly 50% ER lipids, due to the 10-fold greater surface area of the ER (Griffiths *et al.*, 1989). (3) Organellar membranes are heterogeneous. Whatever purification step increases purity reduces the yield of the specific organelle with the possibility that specific subfractions of the organelle are lost. The overall lipid composition of an organelle provides only limited useful information for understanding lipid function.

With the caveats above, the compositions established in the 1970s provide a simple picture (Figure 2; van Meer, 1989). The secretory organelles beyond the Golgi and the endocytotic organelles are 10-fold enriched in sphingolipids and cholesterol over the Golgi and ER. Lipid droplets, peroxisomes and mitochondria have ER-like polar lipid compositions. So, what mechanism is responsible for the steep gradient of sphingolipid and cholesterol at the Golgi-TGN junction? A first hint is that SM and glycosphingolipids have been found enriched on the noncytosolic surface. In line with this, the enrichment of sphingolipids on the apical surface of epithelial cells in comparison to the basolateral surface is maintained by the tight junction, a barrier to lipid diffusion in the outer leaflet of the plasma membrane bilayer (Dragsten *et al.*, 1981; van Meer and Simons, 1986; Figure 3). Indeed, glycolipids and SM did not diffuse between the apical and basolateral surface (Spiegel *et al.*, 1985; van Meer *et al.*, 1987). This implied also that the sphingolipids did not translocate across the plasma membrane, as this should have allowed

Figure 2 Lipid organization in animal cells. The cellular membranes are in bidirectional contact with each other via vesicular traffic except for, maybe, the mitochondria (MITO) and peroxisomes. Whereas the endoplasmic reticulum (ER) and Golgi (G) nearly exclusively contain glycerophospholipids (gray), the trans Golgi network (TGN) and endosomes (E) contain >10% sphingolipids and 30–40 mol% cholesterol (red). The internal vesicles of late endosomes (LE) and lysosomes (L) contain the unique lipid lysobisphosphatidic acid, which is locally produced (Matsuo *et al.*, 2004), like cardiolipin in mitochondria (blue).

Figure 3 Lipid sorting by lateral segregation. With a composition of 33% sphingolipids, 33% glycerophospholipids and 33% cholesterol, and with the sphingolipids situated in the noncytoplasmic leaflet the apical surface is practically covered by sphingolipids and cholesterol (see Simons and van Meer, 1988). The >4-fold enrichment of (glyco)sphingolipids on the apical over the basolateral surface (yellow) and the opposite situation for PC (red) is maintained by the tight junction (TJ). SM and complex glycosphingolipids are synthesized in the Golgi lumen. They do not cross membranes spontaneously, which is also true for PC. The 10-fold enrichment of cholesterol at (both domains of) the plasma membrane as compared to ER then suggests the following sorting events in the Golgi lumen: sphingolipids + cholesterol into apical carriers, PC + cholesterol into basolateral transport vesicles and PC into retrograde transport vesicles (pink). Possibly, a similar sorting event enriches the inner leaflet of the plasma membrane in PS, disaturated phospholipids and cholesterol (blue) (van Meer, 1989). The term 'raft' is routinely used for the less fluid phase, but may be problematic when multiple phases coexist.

them to diffuse past the tight junction. Similarly, SM was unable to translocate from the luminal towards the cytosolic surface of the Golgi (Jeckel *et al.*, 1992) and endosomes (Koval and Pagano, 1991). The sphingolipid gradient in the Golgi must therefore reside on its luminal aspect. Cholesterol rapidly moves between and across membranes. Its gradient must be determined by its high affinity for sphingolipids.

Local synthesis and specificity in transport

The glycerophospholipids PC, PE, PS and PI are synthesized on the cytosolic surface of ER and Golgi (Henneberry *et al.*, 2002; Vance and Vance, 2004), while PE is also generated by PS decarboxylation in mitochondria. The ceramide transfer protein CERT, bound to the Golgi via the lipid PI-4P, was required for delivering ceramide substrate for new SM synthesis (Hanada *et al.*, 2003) by the newly identified SMS1 in the Golgi (Huitema *et al.*, 2004; Yamaoka *et al.*, 2004), with SMS2 interconverting the signaling lipids ceramide and diacylglycerol at the cell surface (Huitema *et al.*, 2004). The glycosphingolipid glucosylceramide (GlcCer) is produced on the cytosolic side of the Golgi (Ichikawa *et al.*, 1996), followed by translocation towards the Golgi lumen and (partial) conversion to lactosylceramide and complex glycosphingolipids.

Lipid rafts

From (i) the tight junction barrier in the exoplasmic leaflet of the plasma membrane, (ii) the assignment of the sphingo-

lipids to this leaflet (Figure 3) and (iii) sphingolipid synthesis occurring inside the Golgi, the compositional differences between the apical and basolateral plasma membrane had to be caused by specificity in transport. Indeed, newly synthesized fluorescent GlcCer and SM were sorted from each other before reaching the cell surface. Their apical/basolateral ratios were sufficiently different to explain the differences in steady-state lipid composition (van Meer *et al.*, 1987). The simplest interpretation was that glycosphingolipids laterally segregated from SM and were concentrated in transport carriers destined for the apical surface. Extended to the sphingolipid gradient in the Golgi, the hypothesis predicts that both SM and glycosphingolipids are sorted towards the exit of the Golgi, where epithelial sorting would be superimposed on this general principle (van Meer, 1989). Extensive data on the ability of sphingolipids and not glycerolipids to act as hydrogen bond donors provided the physical basis for this hypothesis.

Cholesterol rapidly flips across membranes and readily transfers as a monomer between membranes (Baumann *et al.*, 2005). As cholesterol preferentially interacts with sphingolipids, especially SM, its intracellular distribution will be essentially determined by the sphingolipids. This explains its sorting with the sphingolipids to the plasma membrane. At the same time, it should be realized that sphingolipids at 37°C form a solid gel phase, which is fluidized by cholesterol. Importantly, mixtures of SM, PC and cholesterol can spontaneously segregate into a liquid-ordered phase enriched in SM and cholesterol and a disordered phase (e.g. de Almeida *et al.*, 2003), behavior originally observed in PC-cholesterol mixtures (see Ipsen *et al.*, 1987). This refines the physical basis for the lipid sorting model (Figure 3). On the luminal surface of the Golgi, PC domains are sorted into the retrograde pathway to the ER, PC/cholesterol domains follow the basolateral route and sphingolipid/cholesterol domains end up in a pathway towards the apical surface. Apical and basolateral pathways may also exist in nonepithelial cells and may serve as independently regulated transport pathways for different cargo proteins. Sphingolipid sorting via lipid 'rafts' (the more ordered environment including a subset of proteins) has been shown convincingly at the plasma membrane and endosomes (Sharma *et al.*, 2003). It is disturbed in a number of lysosomal storage diseases (Pagano, 2003; Futerman and van Meer, 2004).

As proteins target, dock and fuse transport carriers, the different lipid environments must recruit a specific set of transport proteins including SNAREs and Rabs. The first proteins tentatively assigned to sphingolipid/cholesterol rafts were the GPI-anchored proteins and their apical sorting information was assigned to the glycolipid moiety by which they are anchored on the luminal leaflet (Lisanti *et al.*, 1988). A significant advance was the finding that GPI proteins and glycosphingolipids were enriched in membranes found after detergent extraction in the cold (Brown and Rose, 1992). Although it still lacks a solid physical basis, this simple method has been broadly applied to predict whether a protein is raft-like. Importantly, it uncovered the involvement of lipid rafts in signaling (Lisanti *et al.*, 1994). Many of the relevant kinases are anchored to the cytosolic side of the plasma membrane by acyl chains, suggesting that this membrane leaflet displays lipid heterogeneity as well. Inner leaflet rafts, which seem also required for sorting PS to the plasma

membrane (Figure 3), may consist of disaturated phospholipids, maybe some SM, and cholesterol. They could be stabilized by the presence of overlying ordered lipid rafts in the outer leaflet, whereby the opposed rafts may be connected via transmembrane proteins. Some of these proteins may recognize rafts by having long hydrophobic domains to fit the thicker membrane of the raft (Bretscher and Munro, 1993). Furthermore, oligomerization can move molecules into rafts (Dietrich *et al*, 2001). How multispinning proteins partition between phases remains to be elucidated.

According to the dogma, rafts are small and transient unless stabilized by some ordering component (Kusumi *et al*, 2004). This could be proteins, like caveolin on the inside of the membrane (Lisanti *et al*, 1994; Parton, 1994) or activated receptors, a lipid, like ceramide generated by signaling sphingomyelinases (Gulbins *et al*, 2004; London and London, 2004), or lipid-anchored proteins (Brügger *et al*, 2004). In lipid transport, the forming lipid domain must be stable on the time scale of budding. It must collect the cognate transport proteins, and situate itself in the transport carrier. On a macroscale of 10 μm , a lipid domain can bud spontaneously, driven by the tendency of the system to lower the line tension between the phases by shortening the phase boundary (Baumgart *et al*, 2003). Owing to their high curvature, the budding of 60–90 nm diameter vesicles from cellular organelles must involve additional energy-dependent mechanisms (see below). However, once generated, the curvature itself may drive and stabilize lipid segregation. When 50–100 nm diameter tubes were drawn from homogeneous SM/PC/cholesterol (1:1:1) liposomes, a liquid-disordered phase formed and partitioned preferentially into the tube, whereas a raft marker remained in the liposome (Roux *et al*, 2005). This predicts that budding vesicles will be enriched in the most fluid lipids (Figure 3) and that vacuole remnants may transport the remaining ordered lipids. A membrane may harbor different types of lipid rafts containing specific GPI proteins (Brügger *et al*, 2004) or glycosphingolipids (Gomez-Mouton *et al*, 2001), which may underlie the generation of storage organelles with specific glycolipid compositions (Walkley, 2004).

Lipid translocators

The simple glycosphingolipid GlcCer is produced on the cytosolic surface and the higher glycolipids on the luminal side of the Golgi (see above). Indeed, GlcCer crosses the Golgi membrane via an energy-independent mechanism (Lannert *et al*, 1994). Still, GlcCer might bypass this event and be translocated across the plasma membrane (Figure 4). Indeed, a translocator was found to be responsible for the apical enrichment of fluorescent GlcCer (van Helvoort *et al*, 1996). It was identified as the ATP-binding cassette (ABC) transporter ABCB1, the multidrug transporter MDR1. ABCB1 (MRP1) translocated fluorescent sphingolipids across the basolateral surface. Whether ABCB1 and -C1 translocate natural long chain lipids and whether this is physiologically relevant remains unclear. ABCB4 (MDR2/3), the first ABC transporter connected to lipid translocation in 1993, transports PC into the bile (Borst and Oude Elferink, 2002). Also in erythrocytes, PC is subject to outward translocation, and translocation is correlated with the expression of ABCB1 and -B4 (Kälin *et al*, 2004). Other ABC transporters have since been found involved in outward transport of sterols. Mutations in

Figure 4 Lipid translocation across membranes. Lipids can freely flip bidirectionally across the ER membrane which is probably protein-mediated and nonspecific (Vishwakarma *et al*, 2005). This property is lost in the Golgi, where active translocation has been reported towards the cytosol (purple arrow) by one or more members of the 'aminophospholipid translocator' subfamily of P-type ATPases (Natarajan *et al*, 2004). Transport towards the lumen (orange arrow) may occur by ABC transporters. Specific ABC transporters have been found in apical and basolateral membranes of epithelial cells (yellow and red arrow) (Borst and Oude Elferink, 2002) and the same probably applies to the inward transporting P-type ATPases (blue and green arrow).

ABCA1 cause Tangier disease and mutations in ABCG5/G8 cause sitosterolemia. It is presently not clear how many of the 48 human ABC transporters are involved in lipid translocation, what are their substrates and how they are regulated.

The enrichment of SM and PC in the exoplasmic and of the aminophospholipids PE and PS in the inner leaflet of the erythrocyte membrane depends on an active translocator (Seigneuret and Devaux, 1984), identified as a P-type ATPase (Tang *et al*, 1996). In yeast, the subfamily has five members, distributed over the various membranes of the vacuolar transport system. Knocking out the two plasma membrane members abolished aminophospholipid translocation to the inner leaflet (Pomorski *et al*, 2003). Unexpectedly, fluorescent PC was also translocated similar to the situation in some mammalian epithelial cells. Moreover, knocking out a Golgi member inhibited secretory vesicle budding from the Golgi (Natarajan *et al*, 2004), and an additional knockout of the plasma membrane ones reduced endocytosis (Pomorski *et al*, 2003). The lipid translocators may convert ATP to a mass imbalance between the two membrane leaflets, and the resulting pressure increase in the cytosolic leaflet may induce curvature. In mammals, the 15 members of this lipid-translocating P-type ATPase family may have unique locations and specificities and may drive vesicle budding from the various organelles.

Cholesterol, having a small headgroup, flips across membranes in seconds but also exchanges between membranes in minutes. Apparently, this is not sufficiently fast for cellular processes as a number of membrane proteins are involved in its transport. These include the endosomal membrane proteins MLN64 and Niemann-Pick type C disease NPC1, and the luminal NPC2, involved in moving endocytosed cholesterol out into the cytosol by an unknown mechanism. One other family member is involved in intestinal cholesterol

uptake. Other proteins involved in cholesterol transport are STAR, at the mitochondria, SCP-2 in the cytosol and peroxisomes and the oxysterol binding protein OSBP at the Golgi (Soccio and Breslow, 2004). While all of these proteins are most likely facilitators of some form of cholesterol transport, a variety of ABC transporters in the plasma membrane use ATP to extrude cholesterol (Borst and Oude Elferink, 2002). The actual substrates and the molecular mechanism of action of all of these proteins remain to be determined.

Lipid function and homeostasis

Cells use lipids for structural and specialized functions. The bulk lipids enable them to form closed membrane compartments while at the same time allowing the budding, fission and fusion of transport carriers. In addition, cells use the phase properties of the lipids to relocate membrane proteins during protein sorting and signal transduction. Small amounts of specialized lipids play essential roles in these processes, like LPA and lysobisphosphatidic acid in budding and ceramide and diacylglycerol in plasma membrane signaling. The signaling functions of phosphoinositides and their compartmentation have been studied in great detail (Wenk and de Camilli, 2004), as have those of lipids with known receptors like LPA, sphingosine-1-phosphate and prostaglandins, including steroid hormones and other lipids with nuclear receptors (Edwards *et al*, 2002; Rawson, 2003). Although we understand the basic requirements that lipids must fulfill for the housekeeping functions, the function of various major lipids, for example plasmalogens (ether lipids), remains unclear (Brites *et al*, 2004). Similarly, we have only a rudimentary insight in the function of the enormous variety in glycosphingolipids. Finally, specific lipids are being identified as part of functional protein complexes (Palsdottir and Hunte, 2004). The metabolic enzymes for 1000 cellular lipids have survived evolution. Thus, each lipid must in one or more ways be of functional use to the organism.

It is becoming increasingly clear that cells have intricate mechanisms to maintain a balanced lipid composition. This is illustrated by the beautiful system that regulates lipid levels through sterol sensors in the membrane and effectors at the transcriptional level and the similar PE-regulatory system in flies (Rawson, 2003). No doubt, these are part of a wide network of control systems regulating the lipid composition (the lipidome) of the various organelles (Vance and Vance, 2004) and their size (Rudge *et al*, 2004). The balance of the bulk lipids regulates the flux in vesicular pathways (Bankaitis

and Morris, 2003; Levine and Holthuis, 2005) and, no doubt, a host of other basic physiological parameters in the cell.

Challenges and contributions to be expected from new methodology

First, we need a more detailed determination of the local concentration of lipids in time. New mass spectrometric techniques, by which lipids can be measured in a highly sensitive, accurate and reproducible (and even high-throughput) manner (Brügger *et al*, 2000, 2004; Wenk, 2005), are now getting to the single-cell level. Still at the subcellular level, we probably still need improved methods of cell fractionation. Alternatively, the subcellular localization can now be addressed by highly sensitive imaging by fluorescence and electron microscopy, using for example phosphoinositide-specific binding domains (Rudge *et al*, 2004). A major challenge remains to use tools that do not change the local organization: The lipids must be instantly frozen and labeled quantitatively without being allowed to move around (Parton, 1994). Great progress has been made in the development of photoactivatable and fluorescent lipids that closely mimic natural lipids (Kuerschner *et al*, 2005), but cellular metabolism recognizes even the best mimics as unique. This sends us back to the drawing board and careful biophysical studies to assess to what extent the exciting behavior of model lipids reflects that of natural lipids (Sharma *et al*, 2003), and of which ones. The new mass spectrometry and NMR techniques also hold great promise for the studies of lipid-protein interactions that are so badly needed for understanding the dynamics and function of membrane proteins beyond detergent resistance. Finally, major developments are taking place in computational lipidomics (Forrester *et al*, 2004). Methods are being developed for dealing with large databases and interpreting the data in the context of the cell as a system (Alvarez-Vasquez *et al*, 2005), and it will be exciting to see what predictions and new insights these approaches will come up with. Cellular lipidomics, or how cells use lipids for their vital functions: a lifting fog and thrilling vista.

Acknowledgements

I am grateful to the EC for its specific support action The European Lipidomics Initiative (www.lipidomics.net; LSSG-CT-2004-013032), to the EMBO members for recognition and to my fellow scientists for inspiration. I thank Maarten Egmond for preparing the space-filling structures of Figure 1.

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