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Membranes, detergent resistant membrane fraction, and lipid rafts**

Błony, oporna na detergenty frakcja błon i tratwy lipidowe

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Summary

During the last 85 years our knowledge on the plasma membranes evolved from the lipid bilayer to the current fluid but structured model of dynamic and heterogeneous domains. Detergent resistant membranes (DRMs) are not equivalent to lipid rafts but turned out helpful in our recognition of the complex structure of membranes. Lipid rafts received a definition, got smaller but highly dynamic, and recently were made visible by modern, sophisticated, optical techniques. Some of our published and unpublished results concerning gangliosides, DRMs and lipid raft are briefly discussed.

Key words: detergent resistant membranes, lipid rafts, gangliosides

Streszczenie

W ciągu ostatnich 85 lat znajomość budowy błon komórkowych ewoluowała od dwuwarstwy lipidowej do aktualnego modelu błony będącej mozaiką dynamicznych, heterogennych domen. Nierozpuszczalna w detergentach frakcja błon (DRMs) nie jest jednoznaczna z tratwami lipidowymi, ale okazała się pomocna w poznaniu złożonego charakteru błon komórkowych. Tratwy lipidowe zostały zdefiniowane i okazały się bardzo niewielkimi i dynamicznymi strukturami, widzialnymi przy użyciu skomplikowanych metod mikroskopowych. Niektóre z naszych publikowanych i niepublikowanych wyników dotyczących gangliozydów, DRM i tratw lipidowych zostały krótko omówione.

Słowa kluczowe: oporna na detergenty frakcja błon, tratwy lipidowe, gangliozydy

At the beginning of June 2011, there were over 770 review articles in Medline under the heading *lipid rafts* and about 140 under *lipid rafts in disease.* Thus to write a detailed article on the subject would not be particularly useful. Therefore we take this opportunity to give a prospective Reader a general view on the subject and to discuss in retrospect our published results (1-5) and some unpublished observations (6) on detergent resistant membranes, light membrane fraction, and lipid rafts.

MODEL OF LIPID MEMBRANES

Cell membranes are composed of non-covalently bound lipids and proteins whose weight ratio range from about 7:3 for myelin (7) to 1:4 for inner mitochondrial membrane (8). About 30% of mammalian genome are coding membrane proteins (9). From several lipid classes known to occur in eukaryotic cells (10), most of mammalian membrane lipids belong to sterols, glycerophospholipids, and sphingolipids. The only sterol in animal cell membranes is cholesterol, but glycerophospholipids and sphingolipids are represented by an over a thousand molecular species, differing in the structures of their head groups, fatty acid, and sphingosine residues (11). The progress in studies on lipidomics are likely to expand this list (12, 13). In spite of their structural diversity, all membrane lipids share a common property: they are amphiphatic i.e. have hydrophilic and hydrophobic groups or residues. When studied in Langmuir trough this property orients membrane lipids with hydrophilic groups imbedded in water while the hydrophobic parts protrude into air. At the end of 1924 Gorter and Grendel (14) compared the surface of a monolayer occupied by lipids in Langmuir trough with the surface of erythrocytes used for extraction. Through ingenuity and luck [mutually compensating

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mistakes (15)] they concluded that membrane lipids form a bilayer. In the bilayer model of the membrane hydrophobic residues of lipids in two layers face each other thus avoiding contact with water, while the hydrophilic groups on each side are oriented towards it. Except for archea (archeabacteria) (16) the bilayer turned out to be an universal form of a membrane structure.

THE FLUID MOSAIC MODEL

It took another 10 years to present a model where not only lipids, but also proteins and their spatial relations with the membrane were considered (17). In the Danielli-Dawson model of membrane structure the lipid core was on both sides covered by a continuous layers of proteins. Later on, Robertson refined this model introducing mucoproteins on the exoplasmic and unconjugated proteins on the cytoplasmic side of the lipid bilayer membrane (18). Even as late as 1969 the Danielli-Dawson model was considered valid (19). In 1970 Frye and Eddidin (20) presented results on intermixing of surface antigens after formation of mouse-human heterokaryons. They concluded that membrane allows diffusion of surface protein antigens therefore is fluid. The other important observation on the properties of membrane proteins was discovered by Bretscher (21) that the major erythrocyte glycoprotein is not bound to but, in contradistinction to the previous models, spans the cell membrane. Based on these and their own observation Singer and Nicholson (22) presented the fluid mosaic model of cell membranes. Singer and Nicholson divided proteins into integral (transmembrane) and peripheral. In contradistinction to integral, the peripheral proteins do not span the membrane but are bound to it through electrostatic and hydrogen bonds. It was before the discovery of glycophosphatidylnositol anchored proteins (GPI-AP) of the exoplasmic layer (23) and variously lipidated proteins of the cytoplasmic layer (24, 25) which are anchored to the bilayer through hydrophobic and van der Waals interactions (26). The proteins in this model can diffuse moving freely within the fluid lipid bilayer thus the membrane is a highly dynamic structure. The model turned out to be upgradable accommodating new data (27, 28) however Singer and Nicholson did not really consider the occurrence of domains. Fluidity is not equivalent to chaos and any membrane structure or interaction that would limit it would promote formation of domains, that is the appearance of membrane areas differing from the rest of it.

DYNAMIC, YET STRUCTURED (29) OR MORE MOSAIC THAN FLUID (30)

Plasma membranes are asymmetric structures. Apart from strictly controlled by cells, different distribution of lipids between exoplasmic and cytoplasmic halves of a bilayer (31, 32) membranes show lateral heterogeneity that is, consist of domains. Domains differ widely in properties such as size, half life, and composition affecting their functions. Thus in polarized cells we have apical and basolateral membranes, which in turn, may contain thousands of microdomains (33). One of such microdomains are, or for non- believer, can be lipid rafts. Even though the concept of lipid domains in membranes are much older (34), the roots of the raft theory should be traced to the hypothesis of van Meer and Simons (35) explaining the preferential sorting of GPI-AP (these proteins do not have a transmembrane domain) and glycosphingolipids to the apical membrane of canine kidney cells. As proposed by these authors, GPI-AP in the Golgi apparatus, a subcellular structure where sphingolipids are synthesized (36), form domains with glycosphingolipids. These domains, stabilized by hydrogen bonds, are subsequently exocyticaly transported as a whole, to the plasma membrane. At the moment it is difficult to assess to what extent this hypothesis is universal (33) yet recently it gained support from the observations of Klemm et al. (37) who discovered immunoisolated vesicles enriched in ergosterol and sphingolipids released from trans Golgi network.

Later on two independent observations lain foundation for the raft hypothesis: the recognition of liquid ordered phase in artificial lipid membranes (38-40), and the isolation of detergent resistant membranes (41).

Without bringing in details (42), important but bewildering for a nonprofessional Reader, we should consider that lipids in artificial membranes are in three forms of order or phases (43, 44). At low temperatures the acyl chains of glycerophospholipids and sphingolipids are maximally extended, packed and ordered. The membrane is in the solid ordered *so*, or the gel phase. At high temperature the acyl chains show unrestrained movement around C-C bonds. The membrane is in the liquid disordered *ld* phase. Now the molecules can move around their axis as well as in the “plane” of the membrane (43, 44). These two forms of order are separated by the main transition temperature. This temperature looks sharp and narrow for membranes made of a single phospholipid but becomes broad and poorly defined for membranes prepared from lipid mixtures containing cholesterol, thus indicating the appearance of the third phase, i.e. liquid ordered *lo*. The *lo* phase depends on cholesterol (45). The smooth and rigid structure of cholesterol, which has an “affinity” for long, saturated chains of phospholipids, locates in their vicinity thus preventing their tight packing yet maintaining, to some extent, their extended conformation (43, 44). Phospholipid molecules in *lo* phase have their translational mobility no more than 2-3 fold reduced when compared to *ld* (46). To compare, phospholipids in *so* phase are almost a thousand fold less mobile than in the *ld* phase (44). On the other hand, cholesterol is a condensing agent limiting the fluidity of phospholipids promoting both ways the *lo* phase (47).

DETERGENT RESISTANT MEMBRANES

Detergents are amphiphatic molecules. Due to their reversed cone shape, in water detergents occur as mi-

celles or monomers (48). When added to cell or artificial membrane suspensions in water solutions, detergent monomers incorporate the membrane (48, 49). After a while, the concentration of detergent molecules are so high that the lipids cannot support the membrane structure and instead a mixed micelles are formed (49, 50). Since detergents, such as Triton X-100 are heavier than water, it should be possible to separate the mixed micelles of solubilized membranes from the insoluble fraction. In 1992 Brown and Rose (41) prepared at 4°C the Triton X-100 extract of epithelial cells and subjected it to density gradient centrifugation. As compared with total membrane, the detergent insoluble fraction recovered at a 5%/35% sucrose interface was enriched in GPI-AP, glycosphingolipids, sphingomyelin, and cholesterol. The GPI-AP acquired their insolubility in the detergent after leaving the Golgi apparatus (41).

Since then, DRM fraction has been isolated from all animal cells (51), plant cells containing sterols (52, 53) and recently from sterol synthesizing bacteria (54, 55). The procedure of detergent extraction of cells followed by density gradient centrifugation resulting in the separation of lipid rich detergent insoluble fraction has become widely used for, perhaps, two reasons: It is simple to perform and allows the performer to draw conclusion about basic properties of membranes and their functions.

DRMs were instrumental to the formulation by Simons and Ikonen of the raft hypothesis (56) and later, observations with this membrane fraction were frequently cited in the first review article on lipid rafts and signal transduction (57). Initially, the experiments with model membranes demonstrated that membranes in the *lo* phase are less detergent soluble than in the *ld* phase (58, 59). Thus DRMs isolated from cells could correspond to the area of the membrane in the *lo* phase. This convincing assumption was challenged by the observations of Heerkloz (60). He reported, that addition of Triton X-100 to a uniform membrane preparation caused separation of lipids into patches of *lo* and *ld* phases. However. on the basis of earlier experiments (61) Brown considers effects described by Heerkloz not to have greater effect (51). Likewise, Garner at al. (62) who studied the solubilization of membranes, did not detect domain formation after the addition of a detergent.

After almost 20 years from the publication of Brown and Rose (41) a few observations about DRMs seem (almost) certain. Thus phospholipids (51, 63) and gangliosides (2) with long chain, saturated fatty acid residues prefer DRMs [but compare paper by Pike et al (64)].

Peripheral GPI-AP owe their DRMs association to their lipid anchor (51, 65), a relation to the *lo* phase further strengthened by exchange of GPI-AP unsaturated acyl residues by saturated ones (66). Proteins associated with the cytoplasmic half of the bilayer such as G proteins, Ras proteins, Src-family kinases, depend on palmitoylation and myristoylation (25, 51). So far there is not a single signal in the transmembrane protein structure directing it to DRMs (51, 67). Recently Levental at al. (68) provided data suggesting that palmitoylation regulates raft (and DRM) affinity of transmembrane raft proteins.

Membrane fractions isolated as DRMs have two common properties: relative insolubility in a detergent reflecting primarily the lipids' *lo* phase, and low buoyant density resulting from a higher lipid to protein ratio. Otherwise the fraction is heterogeneous (69). This is an effect of several factors: inherent heterogeneity of the membrane as shown by Brügger et al. (70), use of different detergents, various detergent concentration (71-73), and reconstitution of solubilized membrane components during centrifugation (74). Nevertheless, some proteins, for instance those involved in signaling, the primary function of lipid rafts (56), are enriched in DRMs (75, 76).

Apart from extraction with detergents, homogenization in 0.5M Na_2CO_3 (77) or other buffers (78), were used for density gradient fractionation of membranes (79). The relative value of these procedures should be evaluated by the user (80, 81).

LIPID RAFTS

Almost 10 years after being given a name (56) lipid rafts received a definition, so everybody would know what we are talking about (82). Thus *"membrane rafts are small (10-200 nm), heterogeneous, highly dynamic, sterol and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions".* This definition has important consequences. First, it differentiates DRMs (83) from membrane structures studied in vivo by gentle methods preserving their integrity. Second, it draws our attention to ceramide and sphingomyelin enriched microdomains (84, 85), glycosynapses (86) as well as non-raft domains (87). Third, is demonstrates the growing importance of biophysical and microscopic approaches in experiments on membrane rafts (88).

After about 10 years since the formulation of raft hypothesis (56) and raft engagement in signal transduction (57) our perception of these membrane structures changed considerably. Due to the work of a number of researches, especially Sharma et al. (89), Kusumi et al. (90) and Hancock (91, 92) rafts became smaller and highly dynamic structures. How these small rafts can work was demonstrated by Suzuki et al. (93, 94) by single particle tracing, commented recently by Fedoryszak-Kuśka et al. (67).

How come that the cells' lipid rafts are so small while in artificial membranes the *lo* phase forms a μm domains separated from the *ld* phase? An interesting answer came from an experiment of Yethiraj and Weisshaar, performed *in silico* on the effect of obstacles on formation of large lipid domains (95). These obstacles would be the cytoskeleton-bound transmembrane proteins. Calculations of Yethiraj and Weisshaar agree with

observations of Baumgart et al. (96) who detected plasma membrane separation into large, fluid *lo*/*ld* -like domains in giant plasma membrane vesicles devoid of cytoskeleton. The *lo*/*ld* -like domains were also detected in plasma membrane spheres, again a plasma membrane fragment without a cytoskeleton (97). The importance of cytoskeleton for raft formation is underscored by results reported by Goswami et al. (98). They detected that formation of nanoclusters of GPI-AP depends on cortical actin. Likewise, GM1 and GM3 ganglioside cluster segregation also depended on the status of actin cytoskeleton (99). It is obvious, that tendency of lipids to form *lo* domains is one of many forces ruling the formation and activity of membrane domains.

Seeing is believing. This rather simplistic view is challenged by the size of lipid rafts below 200 nm which evades the resolving power of a conventional microscope. This limitation was overcome by Hell and collaborators (100). They discovered, by stimulated emission depletion far-field fluorescence nanoscopy, that fluorescently labeled sphingolipids, and GPI-AP, but not glycerophospholipids, form microdomains. These microdomains are sensitive to cholesterol depletion. Thus membrane rafts became less illusive yet preserving their elusive charm (101).

GM1, THE GENERAL MARKER FOR DETERGENT RESISTANT MEMBRANES

GM1 ganglioside, or sialosylgangliotetraosyl ceramide, is the most highly cited glycosphingolipid. Its over 5000 citations compare well with 1730 citations for GM3 and outclass its disialo derivative GD1a with 280 citations. GM1 is an important molecule active in the plasma membrane as well as nuclear envelope (102,103) yet its popularity result from being a marker for both DRMs and lipid rafts. The widespread use of GM1 for this purpose results from its highly specific reaction with cholera toxin (104, 105). In membranes GM1 ganglioside can make hydrogen bonds, the basis of the sorting hypothesis of Simons and van Meer (35), take part in electrostatic interactions through its ionized sialic acid residue, and van der Waals bonds with its ceramide moiety. Gangliosides, like the remaining glycosphingolipids are characterized by enormous diversity of their oligosaccharide chains as well as ceramide residues (106).

The objective of our experiments was to find out if, and to what extent, the ceramide residue of GM1 molecululе determines its association with DRMs (2). We have prepared through partial synthesis (107) first fourteen (2) and later another three (6) molecular species of GM1, replacing its fatty acid residue with fatty acids differing in chain length and saturation. We also labeled GM1s with tritium. Following an established procedure, we inserted these GM1s into the membranes of HL-60 cells and studied their distribution between different membrane fractions separated by density gradient centrifugation.

What was new but partly expected: the length and saturation of the ceramide residue had a decisive effect on the occurrence of GM1s in DRMs. Later on we extended these studies to three new GM1s: with 2D hydroxystearic, α-linolenic (three double bonds) and docosahexaenoic acid (six double bonds). When compared, the 2D hydroxystearic did not differ from stearic acid containing GM1. Likewise GM1 with α-linolenic acid had similar distribution as GM1 with monounsaturated fatty acid while the GM1 with docosahexaenoic acid residue was detected in DRMs in the lowest proportion (6).

What was unexpected: cross linking with cholera toxin (CT) placed all GM1s, irrespective of their ceramides, into the DRMs. Also unexpected was an observation, that with saturated, long chain ceramides, depletion of cholesterol did not significantly decrease their recovery in DRMs (2).

A few years later these results are not surprising. As already mentioned, DRMs are a heterogeneous fraction. Not only it was possible to obtain DRMs after cholesterol depletion, but also glycolipid enriched domains withstood, though not in all cells, such treatment (108).

At variance to our observations is a recent publication of Ewers et al. (109). These authors studied the dependence of SV40 virus infection on the ceramide structure of its receptor: GM1. We do not question the elegant experiments with SV40 virus but cannot agree that the ceramide moiety of GM1 by the same mechanism affects the membrane penetration by cholera toxin. We assume, in accordance with earlier work by Fishman et al. (110, 111), that binding of cholera toxin results in translocation of the complex into *lo* phase of the membrane. The next step might be similar as described for the virus.

Apart from detergent extraction we tried to determine the effect of GM1 ceramide on its association with the light membrane fraction prepared through sonication in 0.5M Na_2CO_3 (112). No correlation between the *lo* preferring GM1 ceramide structure and enrichment in this fraction could be detected (3). Moreover, extensive sonication decreased recovery not only of gangliosides but also of GPI-anchored proteins (3).

DO GANGLIOSIDES AND GPI-ANCHORED PROTEINS COMPETE FOR THE SAME MICRODOMAINS?

These experiment were a reflection of an earlier work by Friedrichson and Kurzchalia (113) who discovered an easy dimer formation of GPI-AP when cells were treated with a cross linker. This observation suggested that GPI-AP are very close to each other forming a clusters or a microdomain. Our interest was heightened by observations that gangliosides seem to disperse these domains (114, 115). Since we had available exogeneous gangliosides preferring *lo* or *ld* phases we wanted to know if the competition between GPI-AP and ganglioside reflects the ceramide structure of the latter. We decided to use Jurkat cells and study two GPI-AP: CD55 and CD59. Our attempts at cross linking of these proteins were unsuccessful (6). There are at

least two explanations. First, in experiments described so far (113-115) recombinant, not the native GPI-AP, were used. Second, GPI-AP in Jurkat cells could be present mostly as monomers (89), and not as homo polymers which is a condition for dimer formation after cross linking.

PHOTOREACTIVE AND FLUORESCENT GANGLIOSIDE DERIVATIVES

Photoaffinity labeling (116) and the use fluorescent sphingolipid derivatives (117) are well recognized methods to study membrane structure. However, both procedures are tinted by an original sin: introduction of a photoreactive or fluorescent probe changes the structures of a molecule. We prepared both aryl azide (1, 118) or diazirine (4) substituted ganglioside derivatives. After insertion into the cell membrane the aryl azide substitute ones could not be detected in DRMs (6). The diazirine derivatized GM1 and GM3 gangliosides were in about 40% recovered in DRMs. However, when the latter were used for photoaffinity labeling of Jurkat cells, under conditions where lamellipodium and uropod are formed (119), no difference in the photolabeling pattern of proteins could be detected (6). On the basis of experiments described by Gomez-Mouton such a difference could be expected. Using the same GM1 derivative as described by us, Palestini et al. (120) detected tubulin as the major photolabeled protein while we did not.

As already mentioned, gangliosides exhibit great structural variability. We find it surprising that azidosalicylic acid derivatized GM1 and GM3, not detected in DRMs, differed in their protein photoaffinity pattern (1) while the same gangliosides, diazirine substituted, detected in DRMs in 40% or more, did not (6). Thus it seems possible that non raft proteins can differ in their association with ganglioside derivatives based on the structure of their oligosaccharide chains.

Fluorescent gangliosides were successfully used to detect raft-like domains in kangaroo rat kidney cells (100) and more recently in erythrocyte membranes (121). Our AlexaFluor conjugated ^{3}H labeled GM1s await experimental evaluation (5).

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