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Formation of functional cell membrane domains: the interplay of lipid- and protein-mediated interactions

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Numerous cell membrane associated processes, including signal transduction, membrane sorting, protein processing and virus trafficking take place in membrane subdomains. Protein–protein interactions provide the frameworks necessary to generate biologically functional membrane domains. For example, coat proteins define membrane areas destined for sorting processes, viral proteins self-assemble to generate a budding virus, and adapter molecules organize multimolecular signalling assemblies, which catalyse downstream reactions. The concept of raft lipid-based membrane domains provides a different principle for compartmentalization and segregation of membrane constituents. Accordingly, rafts are defined by the physical properties of the lipid bilayer and function by selective partitioning of membrane lipids and proteins into membrane domains of specific phase behaviour and lipid packing. Here, I will discuss the interplay of these independent principles of protein scaffolds and raft lipid microdomains leading to the generation of biologically functional membrane domains.

Keywords: raft membrane domains; membrane phases; protein complexes; T-cell antigen receptor

1. BASICS OF THE RAFT CONCEPT

Compartmentalization into a raft/non-raft structure is believed to reflect complex phase behaviour of lipid membranes. Accordingly, raft membrane domains are in a tightly packed L_o membrane phase while non-raft regions are L_d, a phase of loose lipid packing (Brown & London 1998). In the L_o phase cholesterol aligns with aliphatic lipid hydrocarbon chains, which therefore straighten out and become ordered. In spite of their dense packing, lipids in L_o phases retain lateral and rotational mobility (Almeida *et al.* 1992).

A L_o/L_d-phase separation has been thoroughly characterized in model membranes composed of cholesterol, pure phospholipids and/or sphingolipids (for a comprehensive review of this issue see London (2002)). In these model membranes segregation of L_o and L_d phases has been thoroughly characterized using electron spin resonance, nuclear magnetic resonance and fluorescence quenching measurements. A L_o/L_d phase separation was observed when 'natural conditions' were mimicked in artificial lipid bilayers containing phosphoglycerides, SM and cholesterol at a temperature of 37 °C (Ahmed *et al.* 1997). Cholesterol was required for the formation of L_o phases in membranes containing SM while cerebroside glycolipids formed ordered membrane domains by themselves (Sankaram & Thompson 1991; Xu & London 2000).

Recent studies showed important features required for cholesterol's capability to induce L_o phases in SM containing membranes: a small polar headgroup, the planarity of the steroid ring system, and an optimal length and structure of the aliphatic site chain at the D7 ring of cholesterol. Moreover, these analyses identified sterols that antagonize L_o phase formation and in this regard function as 'anti cholesterols' (Xu & London 2000).

In contrast to the mostly saturated aliphatic hydrocarbon chains of the ceramide sphingolipid core, natural phosphoglycerolipids frequently harbour a polyunsaturated fatty acid at their SN-2 position (White 1973). The *cis*-configured C=C bonds of these fatty acids induce kinks in the hydrocarbon chain, impeding tight packing and formation of L_o phases by these lipids (Simons & Ikonen 1997). Therefore raft/non-raft structure of lipid membranes not only reflects distinct physical properties in a lipid bilayer but a difference in chemical composition, i.e. a segregation of cholesterol–sphingolipid rich L_d-phase membrane domains (rafts) from phosphoglyceride rich L_o membranes (Brown & London 1997; Simons & Ikonen 1997).

A well-characterized hallmark of artificial bilayers in the L_o phase is their relative resistance to solubilization by detergent Triton X100 at low temperatures (Schroeder *et al.* 1998). Therefore, Triton X100 resistant membrane fractions have been used to define cellular raft domains and to identify raft-resident proteins. Prominent constituents of DRMs are GPI-anchored proteins, which are believed to insert into outer lipid bilayer leaflets of the raft domains via saturated alkyl/acyl chains of the GPI moiety (Brown & Rose 1992). DRMs also concentrate proteins that are anchored in the inner leaflet of plasma membrane rafts by fatty acylation (palmitoylation and myristoylation). These are, for example, members of the Src kinase

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family of tyrosine kinases (for example Lck, Fyn and Lyn) or some Gα subunits of heterotrimeric G proteins (Resh 1999). By contrast, a prenyl plasma membrane anchor like that of many small GTPases (for example N-ras) confers little affinity for DRMs possibly because the branched and bulky structure of the prenyl group interferes with tight lipid packing (Melkonian *et al.* 1999). A number of transmembrane proteins are associated with DRMs, for example the influenza virus haemagglutinin (Scheiffele *et al.* 1997; Melkonian *et al.* 1999) and the TCR signalling adaptor LAT, which both require palmitoylation sites for their association into DRMs (Zhang *et al.* 1998).

2. STRUCTURE OF RAFT LIPID DOMAINS IN ARTIFICIAL MEMBRANES

Using fluorescence microscopy and AFM it was possible to visualize L_o and L_d phases in free or glass-supported membrane bi- or monolayers. In mixtures of purified glycerolipids, cholesterol and sphingolipids, shape and connectivity of the lipid domains, bilayer thickness and the partition coefficients of specific constituents have been precisely measured. The raft domains in these membranes have diameters in the range 200 nm to several micrometres and are therefore readily detectable by fluorescence microscopy (Dietrich *et al.* 2001). These experiments allowed monitoring and quantification of the selective partitioning of GM1 glycosphingolipid, GPI anchored protein Thy-1 and lipid analogue protein into raft domains. Consistently, fluorescence tagged DPPE (two saturated palmitoyl C16 chains) translocated into ordered domains if crosslinked with antibodies while DOPE (two monounsaturated C18 oleyl chains) partitioned in the disordered phase before and after antibody-mediated crosslinking (Dietrich *et al.* 2001). A recent analysis of a supported bilayer by AFM showed partitioning of placental alkaline phosphatase GPI anchored protein into ordered membranes (Saslowsky *et al.* 2002). In addition, AFM analysis of supported bilayers showed the important feature of bilayer thickening upon formation of ordered domains due to increased gauche *trans*-transitions and hence straightening of the aliphatic hydrocarbon chains of the lipid constituents (see figure 1). Interestingly, these studies suggested some bilayer coupling, i.e. that opposing lipids in both bilayer leaflets adopted the L_o phase (Rinia *et al.* 2001). Altogether these experiments in artificial membranes recapitulated membrane properties which were predicted by the raft hypothesis: a separation of L_o and L_d phases and a preferential segregation of GPI anchored proteins and glycosphingolipid into ordered membrane domains.

3. STRUCTURE OF RAFT DOMAINS IN CELLS

Raft domains in living cells appear to be very small and are probably heterogeneous. This may explain why they have escaped direct visualization by any microscopical technique (Mayor *et al.* 1994). Indirect evidence for small rafts was obtained using single particle tracking of the thermal position fluctuation, showing that raft-associated membrane proteins are stably associated to a small cholesterol-dependent lipid assembly of *ca.* 50 nm diameter (Pralle *et al.* 2000, p. 283). Such domains can harbour around 3000 lipids and few protein molecules. Energy transfer and chemical crosslinking were used to measure the distance between GPI-anchored proteins in the plasma membranes of cells. These studies indicated that the distance between GPI-anchored model proteins was relatively unaffected by their density in the plasma membrane, suggesting that they are concentrated in small membrane domains. Energy transfer and crosslinking between GPI-linked proteins was sensitive to cholesterol extraction and did not occur for transmembrane versions of the respective model proteins (Mayor *et al.* 1994; Friedrichson & Kurzchalia 1998). Similarly, energy transfer measurements showed that GFP variants carrying a myristoyl/palmitoyl membrane anchor are concentrated in cholesterol-dependent lipid domains, showing that the inner leaflet of the plasma membrane bilayer also exhibits a raft/non-raft structure. The energy transfer patterns of GFP variants, anchored in the plasma membrane by prenyl groups, indicated a random distribution (Zacharias *et al.* 2002). However, other energy transfer measurements using fluorescence labelled antibodies against GPI anchored proteins or glycosphingolipid GM1 binding choleratoxin B-subunit failed to detect concentration within microdomains (Kenworthy *et al.* 2000). These discrepancies may be explained by the distribution of the raft markers in multiple small microdomains. Depending on the experimental systems used, these domains may therefore contain only raft molecules of a kind.

Particle tracking experiments have shown confinement zones for membrane proteins. These zones have been attributed in part to protein-mediated obstruction of free diffusion. However, recent data have shown that Thy-1 and GM1 confinement was sensitive to cholesterol extraction and that the bilayer viscosity of these domains was increased, indicating that these domains adopt an ordered membrane phase (Dietrich *et al.* 2002). The relationship between these confinement zones and rafts remains unknown.

Clearly, the lipid domain structure of the plasma membrane is more complex then in simple L_o/L_d biphasic model systems. The plasma membrane contains hundreds of different lipid species. It seems likely that this diversity allows multiple different types of lipid domains and different degrees of lipid packing and order in the plasma membrane. A comparison of membranes insoluble in different detergents suggested that membrane domains of different solubility contain subsets of membrane raft domains which differ in their molecular compositions. For example, Brij 98 DRMs contain Thy-1 GPI anchored protein membranes and a subfraction of these DRMs which strongly enrich in T-cell receptor complexes. By contrast, Triton X100 insoluble membranes contain Thy-1 but largely exclude TCR (Drevot *et al.* 2002).

A concept of different subsets of raft membrane domains is strongly supported by the observation that different ganglioside species segregated in migrating lymphocytes, leading to concentration of GM3 in the leading edge of the cell and a GM1 concentration at the trailing uropod of the cell (Gomez-Mouton *et al.* 2001). In migrating neutrophils DRMs concentrate in the uropod (Seveau *et al.* 2001). It was proposed that this segregation is mediated by the actin cytoskeleton; however, the precise molecular mechanism remains to be defined.

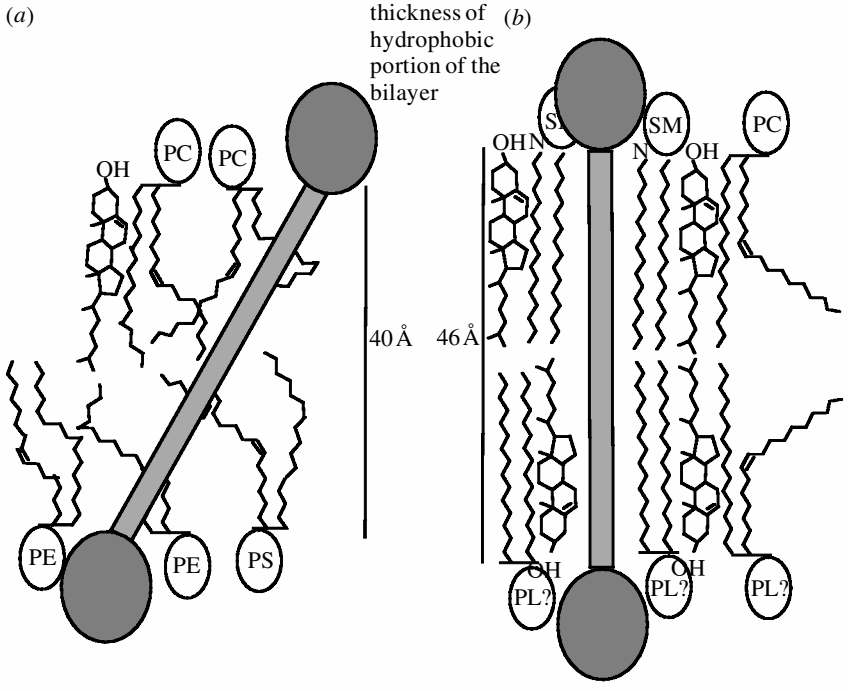

Figure 1. Conformational changes of proteins can induce a change in the lipid order of their environment. (*a*) A tilted transmembrane region of a protein within a disordered lipid environment. (*b*) Erecting the TM regions causes thickening of the lipid bilayer and increase of the acyl chain order in order to fit the hydrophobic TM regions. PC: phosphatidylcholine; PE: phosphatidylethanolamine; PL: glycerophospholipid. The structure and composition of the inner leaflet of rafts remain to be defined.

The plasma membrane contains most of the cell's cholesterol and sphingolipids and therefore most probably exhibits in general a high degree of lipid packing and order. Indeed, detergent extraction with TX100 showed that a large fraction of the cells (more than 50%) of the PM of cells consists of detergent resistant membranes (Hao *et al.* 2001). Therefore rafts can be maximally enriched in a specific PM region compared with the whole PM by a factor of two. This may explain why GM1 is at most twofold enriched at the immunological synapse even though T-cell signal transduction is believed to occur in raft membrane domains (Burack *et al.* 2002). Taken together these considerations suggest that rafts by themselves are unlikely to function as platforms for any biological process but that protein-mediated interactions generate specific assemblies of proteins and lipids that define substructures in the plasma membrane.

4. ELASTICITY OF THE RAFT/NON-RAFT EQUILIBRIUM

Exogeneously administered GPI-anchored protein CD59 was shown to rapidly integrate into the plasma membrane of U937 cells but to partition into DRMs with a delay of 20 min (Van den Berg *et al.* 1995). Likewise, newly synthesized Fyn Src-related tyrosine kinase, overexpressed in fibroblasts, is anchored into cell membranes (most probably the plasma membrane) within 2–5 min via its myristoyl and palmitoyl fatty acylations, but acquires detergent resistance within 10–20 min after synthesis (Van't Hof & Resh 1997). These surprisingly long delays may be explained as the times Fyn's and CD59's acyl chains require to adopt an extended conformation, which would be accompanied by partitioning into ordered lipid domains. Within the ordered domain the all-*trans* conformation of the acyl chains could be stabilized keeping the protein raft-associated. This underlines the potential elasticity of a dynamic L_o/L_d equilibrium in the plasma membrane in which acyl chain conformation may determine whether raft phases form or disintegrate in membranes and around proteins (figure 1).

In support of such an elasticity, Dietrich *et al.* could demonstrate in a L_o/L_d-biphasic supported membrane system that upon crosslinking with an antibody a DPPE–FITC translocates from a disordered into an ordered phase domain (Dietrich *et al.* 2001).

The current data indicate that raft domains in the plasma membrane are small and highly dynamic. However, large and stabilized raft-like membrane domains in a highly ordered lipid phase can be formed by lateral crosslinking of plasma membrane proteins and raft-associated lipids on the cell surface (Mayor *et al.* 1994; Thomas *et al.* 1994). Independently crosslinked DRM-associated molecules coalesce into common patches and this is inhibited by disruption of raft domains using cholesterol-depletion. By contrast, patched raft components and patches of transferrin receptor as a non-raft marker were sharply separated (Harder *et al.* 1998). Patches induced by crosslinking of immunoglobulin E receptors concentrate lipid dye DIC16, a lipid dye which is highly selective for ordered lipid domains. Interestingly, the lipid dye is immobile in these patches, suggesting that within these clusters lipids adopt a solid gel-like phase (Thomas *et al.* 1994).

An elastic system which allows changes in lipid ordering may have important implications for raft functions. The lipid environment surrounding a receptor may change following engagement of ligands. This may be caused by

receptor oligomerization or by conformational changes of TM regions. For example, a straightening or erection of a tilted TM domain may cause a thickening of the bilayer in adaptation to the hydrophobic TM domains. Consequently packing and order of lipid domains in the environment of the TM protein increases.

5. INTERPLAY OF PROTEIN AND RAFT LIPID MEDIATED INTERACTIONS: MISCIBILITY AND STABILIZATION

As discussed, elementary lipid raft domains are not likely to sufficiently concentrate molecules to form functional platforms for biological processes. Therefore an interplay of lipid-based elementary raft units and protein-mediated assembly of specific protein complexes generates functional domains in cell membranes.

A large number of reports have shown that virus trafficking i.e. entry, intracellular transport and budding, is tightly associated to raft membrane domains (Manes *et al.* 2000; Alfsen *et al.* 2001; Pelkmans *et al.* 2001). Assembly of HI virions occurs in raft membrane domains of the plasma membrane. This process is mediated by the myristoylated protein Gag, which by itself is relatively soluble in Triton X100 detergent. Gag self-associates at the plasma membrane to form lipid–protein assemblies termed 'barges' which are detergent insoluble, but are biochemically distinguishable from DRMs (Lindwasser & Resh 2001). Achieving membrane anchorage of Gag by a more potent raft anchor derived from the Fyn kinase increases its incorporation into 'barges'. However, if the self-assembly is disrupted by deletion of important Gag binding protein segments the Fyn anchored Gag completely partitions into DRM, presumably as monomer (Lindwasser & Resh 2001). Taken together these results show the distinct contribution of raft association and protein-mediated stabilization required for the formation of 'barges' and the subsequent budding of active HI virus particles. A similar cooperation of raft and protein mediated interactions may be responsible for budding of the influenza virus (Zhang *et al.* 2000).

Several large subdomains in the plasma membrane are organized by multi membrane spanning proteins. The most prominent raft stabilizing proteins are the caveolins, a family of palmitoylated hairpinlike membrane proteins which organize flask-shaped caveolar membrane invaginations (Kurzchalia & Parton 1999). More recently the CD86 member of the tetraspanin family was shown to assemble MHC class II oligomers in intracellular compartments, possibly generating MHC clusters important for T-cell activation (Kropshofer *et al.* 2002). A membrane domain containing pentaspan membrane protein prominin was isolated from microvilli and may be responsible for the formation of specific cholesterol-rich domains at the brush border of epithelial cells (Roper *et al.* 2000).

Following activation of TCR multimolecular signalling, complexes form in raft microdomains of the T lymphocyte's plasma membrane. These complexes assemble around LAT, a transmembrane adaptor protein, which nucleates downstream signalling enzymes and adaptor molecules. LAT is targeted to DRMs via dual acylations, which are required for LAT's ability to transduce TCR evoked signals (Zhang *et al.* 1998). Using biochemical and morphological approaches it was shown that T-cell signalling complexes form by a selective signalling dependent and protein mediated anchoring of LAT in the vicinity of activated TCR (Harder & Kuhn 2000; Bunnell *et al.* 2002). The formation of these signalling assemblies required palmitoylation and hence DRM targeting of LAT showing the involvement of raft domains in the assembly. However, general raft-associated proteins did not specifically accumulate in the signalling assemblies (Harder & Kuhn 2000). These observations can be explained by relatively transient raft-dependent interactions between LAT and TCR. The complexes are stabilized by the formation of a signalling protein scaffold, which is held together by multiple SH2- and SH3-domain mediated protein–protein interactions.

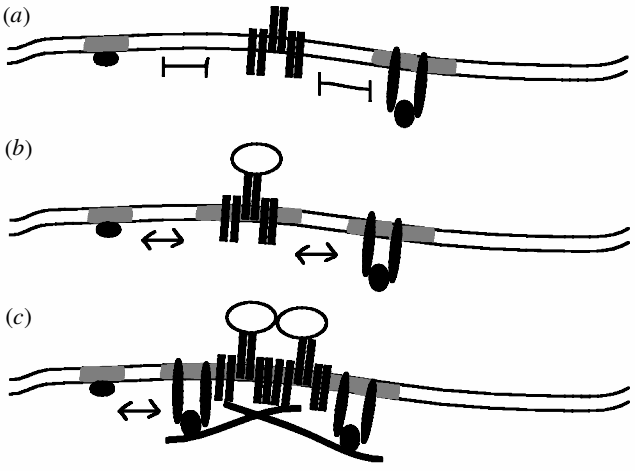

Figure 2. Interplay of raft lipid and protein mediated interactions in the early TCR activation and signalling complex formation. (*a*) Induced phase miscibility: engagement of TCR triggers transition of its membrane environment to form an L_o membrane-phase (*b*) miscible with the membrane environment of DRM-associated Src-kinases. Facilitated access of raft-associated Src-kinases to engaged TCR leads to increased phosphorylation and triggers (*c*) Formation of multimolecular signalling complexes by protein–protein interactions such as SH2/SH3 mediated interactions.

In addition to facilitating interactions, rafts may function by impeding random encounters between membrane proteins which reside in different membrane phases. One may speculate that such segregation is important for early events that lead to TCR (or other immunoreceptors) triggering following engagement by a ligand. Receptor engagement could shift the TCR complex from a less ordered to a high order membrane phase. Consequently the interactions with raft resident proteins such as the Src-related tyrosine kinase would become facilitated leading to the critical tyrosine phosphorylations of the TCR cytoplasmic portions, which set off the protein mediated assembly of signalling complexes (figure 2).

6. SUMMARY

The current data suggest a role of rafts in the regulation of dynamic interactions between membrane proteins. Rafts can facilitate encounters between membrane proteins, or they can impede interactions between proteins in

different membrane phases. Any functional assembly in raft domains has to be stabilized by protein scaffolds. The future challenge will be to develop methods to assess the structure of functional membrane domains and to obtain an integrated picture of protein scaffolds and lipid in these entities.

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GLOSSARY

AFM: atomic force microscopy
DOPE: dioleyl phosphatidylethanolamine
DPPE: dipalmitoyl phosphatidylethanolamine
DRM: detergent resistant membrane
GFP: green fluorescent protein
GPI: glycosyl phosphatidylinositol
HI: human immunodeficiency
L_d: liquid-disordered
L_o: liquid-ordered
MHC: major histocompatibility complex
PM: plasma membrane
SM: sphingomyelin
TCR: T-cell antigen receptor
TM: transmembrane