LIPID RAFTS AND SIGNAL TRANSDUCTION

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Signal transduction is initiated by complex protein–protein interactions between ligands, receptors and kinases, to name only a few. It is now becoming clear that lipid micro-environments on the cell surface — known as lipid rafts — also take part in this process. Lipid rafts containing a given set of proteins can change their size and composition in response to intra- or extracellular stimuli. This favours specific protein–protein interactions, resulting in the activation of signalling cascades.

EXOPLASMIC LEAFLET
Lipid layer facing the extracellular space.

The Singer–Nicholson fluid mosiac concept is still the textbook model of how the cell membrane is organized. It proposes that the lipid bilayer functions as a neutral two-dimensional solvent, having little influence on membrane protein function. But biophysicists find that lipids exist in several phases in model lipid bilayers, including gel, liquid-ordered and liquid-disordered states, in order of increasing fluidity[1]. In the gel state lipids are semi-frozen, whereas at the other extreme, the liquid-disordered state, the whole lipid bilayer is fluid, as proposed by the Singer–Nicholson model. In the liquid-ordered phase, phospholipids with saturated hydrocarbon chains pack tightly with cholesterol (BOX 1) but nevertheless remain mobile in the plane of the membrane[2]. Despite a detailed biophysical characterization of model membranes, it has been difficult to show that lipids exist in these different phases in the complex environment of the cell.

Lipid rafts

A turning point came when the lipid raft hypothesis was formulated more than ten years ago[1,3,4]. It originated from studies on epithelial cell polarity, and its central postulate was the existence of lipid rafts, consisting of dynamic assemblies of cholesterol and sphingolipids (BOX 1), in the EXOPLASMIC LEAFLET of the bilayer. The preponderance of saturated hydrocarbon chains in cell sphingolipids allows for cholesterol to be tightly intercalated, similar to the organization of the liquid-ordered state in model membranes. The inner leaflet is probably rich in phospholipids with saturated fatty acids and cholesterol[5], but its characterization is still incomplete. It is also not clear how the inner leaflet is coupled to the outer leaflet. One possibility is that long fatty acids of sphingolipids in the outer leaflet couple the exoplasmic and cytoplasmic leaflets by interdigitation. Transmembrane proteins could also stabilize this coupling. The membrane surrounding lipid rafts is more fluid, as it consists mostly of phospholipids with unsaturated, and therefore kinked, fatty acyl chains and cholesterol. In other words, lipid rafts form distinct liquid-ordered phases in the lipid bilayer, dispersed in a liquid-disordered matrix of unsaturated glycerolipids[1,6]. The raft concept has long been controversial, largely because it has been difficult to prove definitively that rafts exist in living cells. But recent studies with improved methodology have dispelled most of these doubts (BOX 2).

One of the most important properties of lipid rafts is that they can include or exclude proteins to variable extents. Proteins with raft affinity include glycosylphosphatidylinositol (GPI)-anchored proteins[1,7], doubly acylated proteins, such as Src-family kinases or the α-subunits of heterotrimeric G proteins[8], cholesterol-linked and palmitoylated proteins such as Hedgehog[9], and transmembrane proteins, particularly palmitoylated ones[1]. GPI-anchored proteins or proteins that carry hydrophobic modifications probably partition into rafts owing to preferential packing of their saturated membrane anchors. It is not yet clear why some transmembrane proteins are included into rafts, but mutational analysis has shown that amino acids in the transmembrane domains near the exoplasmic leaflet are critical[10].

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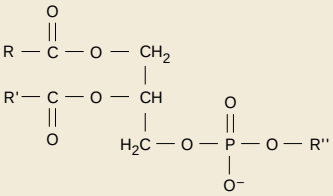
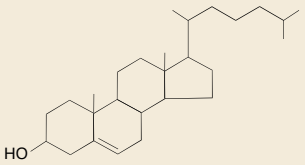
Box 1 | Glossary of lipid structures

Phospholipids

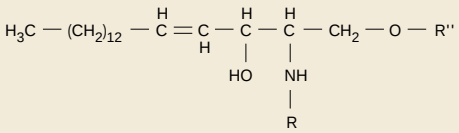


R, R', Hydrocarbon chains of fatty acids
R'', Head-group

Cholesterol



Sphingolipids



TRANSCYTOSIS
Transport of macromolecules across a cell, consisting of endocytosis of a macromolecule at one side of a monolayer and exocytosis at the other side.

APICAL PLASMA MEMBRANE
The surface of an epithelial cell that faces the lumen.

BASOLATERAL PLASMA MEMBRANE
The surface of an epithelial cell that adjoins underlying tissue.

SOMATODENDRITIC MEMBRANE
The surface of a neuron that surrounds the cell body and dendrites.

BIOSYNTHETIC PATHWAY
Secretory or membrane proteins are inserted into the endoplasmic reticulum. They are then transported through the Golgi to the *trans*-Golgi network, where they are sorted to their final destination.

ENDOCYTIC PATHWAY
Macromolecules are endocytosed at the plasma membrane. They first arrive in early endosomes, then late endosomes, and finally lysosomes where they are degraded by hydrolases. Recycling back to the plasma membrane from early endosomes also occurs.

Palmitoylation can increase a protein's affinity for rafts, but it is not sufficient for raft association[11]. It is likely that a given protein can associate with rafts with different kinetics or partition coefficients. For instance, a monomeric transmembrane protein may have a short residency time in rafts, spending most of its time outside rafts. But when the same protein is crosslinked or otherwise oligomerized, its affinity for rafts increases[12]. As we will discuss below, a common theme is that the clustering of separate rafts exposes proteins to a new membrane environment, enriched in specific enzymes, such as kinases, phosphatases and perhaps palmitoylases and depalmitoylases. Even a small change of partitioning into a lipid raft can, through amplification, initiate signalling cascades. These dynamic features of initial raft association have so far received little attention, but we predict that they are crucial for the activation of many signal transduction pathways.

Caveolae

One subset of lipid rafts is found in cell surface invaginations called caveolae (BOX 3). These flask-shaped plasma membrane invaginations were first identified on the basis of their morphology by Palade[13] and Yamada[14] in the 1950s. Caveolae are formed from lipid rafts by polymerization of caveolins — hairpin-like palmitoylated integral membrane proteins that tightly bind cholesterol[15,16]. The general function of caveolae is not clear. They have been implicated in endocytosis[15] and TRANSCYTOSIS of albumin and other proteins across the endothelial monolayer[17]. In developing myocytes, ribbons of many caveolae organize into T-tubules[18], which are required for the calcium regulation of muscle contraction. Caveolae also function during signal transduction[19], but they are not absolutely required as several cell types that lack caveolin, such as lymphocytes and neurons, can nevertheless signal through rafts.

Raft distribution and trafficking

The distribution of lipid rafts over the cell surface depends on the cell type. In polarized epithelial cells and neurons, lipid rafts accumulate in the APICAL and axonal plasma membrane, respectively. BASOLATERAL and SOMATODENDRITIC MEMBRANES also contain rafts, but in smaller amounts[4]. Interestingly, caveolae are present mainly on the basolateral side of epithelial cells[20], which faces the blood supply and is more active during signal transduction. In lymphocytes and fibroblasts, rafts are distributed over the cell surface without obvious polarity. We can roughly estimate the fraction of the cell surface covered by rafts by comparing the ratio of the main raft and non-raft exoplasmic leaflet lipids, sphingolipids and phosphatidylcholine, respectively. Typically, sphingolipids make up about 45% of the cell surface in fibroblasts[21] and roughly 30% in lymphocytes[22], but these values are upper limits and may also be cell-type dependent.

Raft lipids are most abundant at the plasma membrane, but can also be found in the BIOSYNTHETIC and ENDOCYTIC PATHWAYS. Whereas cholesterol is synthesized in the endoplasmic reticulum (ER), sphingolipid synthesis and head-group modification are completed largely in the Golgi[23]. As these data predict, cholesterol–sphingolipid rafts first assemble in the Golgi[1]. Movement of lipid rafts out of the Golgi seems to be mainly towards the plasma membrane, as vesicles going back to the ER contain little sphingomyelin and cholesterol[24]. The inclusion of proteins into rafts is important for polarized delivery to the cell surface in many cell types[4,25,26]. Lipid raft trafficking does not end with surface delivery — rafts are continuously endocytosed[27] from the plasma membrane. From early endosomes, rafts either recycle directly back to the cell surface or return indirectly through recycling endosomes, which could also deliver rafts to the Golgi[28].

Raft size

One reason why it has been so difficult to prove that rafts exist in cells is that they are too small to be resolved by standard light microscopy. But if raft components are crosslinked with antibodies or lectins in living cells, then raft protein and lipid components cluster together, and

Box 2 | Key papers on the existence of rafts in cell membranes

- Fluorescence resonance energy transfer measurements using fluorescent folate to show interactions of folate receptors when they are in proximity in rafts in living cells[31].
- Biochemical crosslinking of GPI-anchored proteins when they are in proximity in rafts[32].
- Antibody crosslinking of raft proteins into patches segregating from non-raft proteins[12].
- Photonic force microscopy measurements of the size of rafts in living cells[30].
- Visualization of rafts and clustered rafts in IgE signalling by electron microscopy[81].

Box 3 | Confusion about the relationship between caveolae and rafts

The problem has arisen primarily because of the difficulty of isolating pure caveolae. Several methods have been used — the first was simply Triton X-100 extraction at 4 °C (REF. 82). When it became clear that this fraction contains not only plasma membrane caveolae but rafts (detergent-resistant membranes or DRMs) from all cellular membranes[83], new methods were devised. The most frequently used of these is based on density gradient centrifugation[84] but it is also not capable of isolating pure caveolae[35]. Immuno-isolation has been used with conflicting results[85–87], so that the only safe method now used to identify caveolar proteins is double-label immunoelectron microscopy[80,88].

Confusion persists because definitions of caveolae remain vague. In a recent review, Anderson defined caveolae as "meant to encompass a membrane system with specific functions essential for normal cell behavior"[19]. Lisanti *et al.* describe rafts, in the absence of caveolae, as "caveola-related domains"[16]. To resolve these issues, we propose to distinguish between rafts, DRMs and caveolae. We reserve the use of the term "caveolae" for morphologically defined cell surface invaginations (containing caveolin), as originally proposed half a century ago[13,14].

raft and non-raft components separate into micron-sized quilt-like patches[12,29]. In fibroblasts, raft proteins rapidly diffuse in assemblies of roughly 50 nm diameter[30], corresponding to about 3,500 sphingolipid molecules. The number of proteins in each raft depends on the packing density, but is probably not more than 10–30 proteins. We do not yet know whether individual raft proteins are randomly distributed between different rafts, or whether they are grouped in specialized rafts. Clusters of up to 15 molecules of the same protein have been observed within the same raft[31,32], supporting the view that some proteins are distributed non-randomly. However, other studies indicate that such clusters may only represent a small population[33]. Either way, given its small size, a raft can statistically contain only a subset of all available raft proteins. This conclusion may have profound consequences on how signalling through rafts can be dynamically activated by raft clustering, as will be discussed later.

Methods to study rafts

The formulation of the raft hypothesis was influenced by the discovery that, on entering the Golgi, some proteins form large complexes with lipids, which resist solubilization by non-ionic detergents[34]. Detergent-resistant membrane (DRM) complexes float to low density during SUCROSE GRADIENT CENTRIFUGATION and are enriched in raft proteins and lipids, providing a simple means of identifying possible raft components. Despite the ease and usefulness of non-ionic detergent extraction, this method is not without pitfalls[7]. A raft protein can be connected to the cytoskeleton, so it will not float after detergent extraction. Or its association with rafts can be so weak that it is solubilized by the detergent. Moreover, changes in detergents and extraction conditions can produce strikingly different results[7,29,35].

One useful approach in raft research has been the manipulation of raft lipid constituents (BOX 4). This treatment leads to the dissociation of proteins from rafts, which can be readily detected by common methods used to analyse raft association (TABLE 1). This type of methodology has greatly contributed to our understanding of raft function *in vivo*. Such experiments have, for example, reinforced the conclusion that the fatty acids that mediate protein binding to rafts are usually saturated. Indeed, feeding cells with polyunsaturated fatty acids leads to the replacement of saturated fatty acids with unsaturated ones in acylated proteins, causing these proteins to dissociate from rafts[36]. Similarly, the addition of exogenous GANGLIOSIDES to cells can lead to their incorporation into rafts and, as a result, also cause proteins to dissociate from rafts[37].

The lack of standardized methodology has led to confusion in the current nomenclature between rafts, detergent-resistant membranes and caveolae (TABLE 2). In the hope of dissipating some of this confusion, we summarize the advantages and disadvantages of some methods used to study rafts in TABLE 1, and propose a more standardized nomenclature in TABLE 2.

Rafts in signal transduction

The most important role of rafts at the cell surface may be their function in signal transduction (TABLE 3). It is well established that, in the case of tyrosine kinase signalling, adaptors, scaffolds and enzymes are recruited to the cytoplasmic side of the plasma membrane as a result of ligand activation[38]. One way to consider rafts is that they form concentrating platforms for individual receptors, activated by ligand binding. If receptor activation takes place in a lipid raft, the signalling complex is protected from non-raft enzymes such as membrane phosphatases that otherwise could affect the signalling process. In general, raft binding recruits proteins to a new micro-environment, where the phosphorylation state can be modified by local kinases and phosphatases, resulting in downstream signalling. To highlight these principles, examples of signalling pathways that involve lipid rafts are described below.

Immunoglobulin E signalling. The first signalling process convincingly shown to involve lipid rafts was immunoglobulin E (IgE) signalling during the allergic immune response[39–41] (FIG. 1a). This signalling pathway is activated when IgE binds through its Fc segment to receptors (FcεRI) residing in the plasma membrane of MAST CELLS and BASOPHILS. FcεRI is monomeric and binds one IgE molecule. The receptor is activated by the bind-

Box 4 | Common tools to disrupt rafts

Cholesterol sequestration
- Antibiotics:

  Filipin | Nystatin | Amphotericin
- Pore-forming agents:

  Saponin | Digitonin | Streptolysin O

Cholesterol depletion
- Methyl-β-cyclodextrin

Inhibition of cholesterol biosynthesis
- Lovastatin

Perturbation of raft stability
- Exogenous cholesterol
- Exogenous gangliosides
- Exogenous polyunsaturated fatty acids

SUCROSE GRADIENT CENTRIFUGATION
Allows separation of cellular membranes according to their size and/or density by centrifugation.

GANGLIOSIDES
Anionic glycosphingolipids that carry, in addition to other sugar residues, one or more sialic acid residues.

MAST CELL
A type of leukocyte, of the granulocyte subclass.

BASOPHIL
Polymorphonuclear phagocytic leukocyte of the myeloid series.

Table 1 | Techniques to identify rafts

Approach*	Information available	Live cells	Comments	References
Flotation of detergent–resistant membranes (DRMs)	Identifies putative raft association Identifies possible raft proteins	No	• Easy to do • Most common approach for identifying putative proteins involved in signalling • Artefacts possible • Weak associations with rafts are difficult to detect	1, 4, 7
Antibody patching and immunofluorescence microscopy	Identifies putative raft association	No	• Easy to do • Common approach • Better than flotation for detecting weak raft associations • Cell–cell variability makes quantification difficult	12, 29
Immunoelectron microscopy	Determines location of raft components	No	• Promising results • Requires technical expertise	80, 81, 88
Chemical crosslinking	Identifies native raft protein complexes	Yes	• Straightforward • Choice of appropriate conditions and reagents is semi-empirical	32
Single fluorophore tracking microscopy	Monitors the diffusion and dynamics of individual raft proteins or lipids	Yes	• Requires highly specialized equipment and expertise	89
Photonic force microscopy	Determines the diffusion constant, size and dynamics of individual rafts	Yes	• Very informative technique • Requires highly specialized equipment and technical expertise • Time-consuming acquisition and analysis	30
Fluorescence resonance energy transfer (FRET)	Detects whether two raft components are spatially close (for example, <10 nm)	Yes	• Powerful approach • Choice of appropriate donor and acceptor probes is important	31, 33

*The disruption of rafts by cholesterol depletion or sequestration is especially useful as a control for each of these approaches.

ing of oligomeric antigens to receptor-bound IgE. Crosslinking of FcεRI by oligomeric antigens activates the transmembrane signalling process, ultimately leading to release of the chemical mediators of allergic reactions.

The Fc receptor is a tetramer composed of one α-, one β- and two γ-chains[41]. The α-chain binds IgE and the β- and the γ-chains contain immune receptor tyrosine-based activation motifs (ITAMs), common to all multi-subunit immune recognition receptors. Crosslinking of two or more of these receptors by antigens recuits the doubly acylated non-receptor Src-like tyrosine kinase Lyn, which is thought to initiate the signalling cascade by phosphorylating ITAMs so that they can bind to Syk/ZAP-70 family tyrosine kinases through their phosphotyrosine residues[39,40]. Syk is activated by phosphorylation and this, in turn, leads to activation of phospholipase Cγ (PLCγ). Finally, downstream signalling results in increased calcium levels in the proximity of the membrane, and this triggers the release of histamine from nearby granules.

IgE signalling was initially thought to be based on protein–protein interactions alone[42]. But several observations indicate that rafts are involved in this process[41]. The first hint came from the finding that FcεRI is soluble in Triton X-100 at steady state, but becomes insoluble in low concentrations of this detergent after crosslinking[39]. Moreover, FcεRI crosslinking causes the redistribution of raft components, including gangliosides and GPI-anchored proteins, to patches that are large enough to be seen by fluorescence microscopy[43,44]. This observation also indicates that raft clustering takes place following receptor activation. A last indication came from the finding that IgE signalling is abolished if surface cholesterol is depleted with METHYL-β-CYCLODEXTRIN[45].

One working model for IgE signalling is that crosslinking increases the raft affinity of FcεRI. In this model, a change in receptor partioning would lead to increased phosphorylation of its ITAMs by raft-associated Lyn kinase, possibly due to the exclusion of inhibitory phosphatases. FcεRI crosslinking could, in addition, bring small individual rafts together. Linker proteins, such as members of the BASH (B cell adaptor containing SH repeats) family and LAT (linker for activation of T cells), are good candidates for this job[46]. As a result of amplification, even small changes in receptor partitioning could produce strong signals. One key issue to be explained is how FcεRI aggregates as small as dimers[42] can initiate the raft clustering process to activate the allergic response.

T-cell antigen receptor signalling. The T-cell antigen receptor (TCR) is another multisubunit immune recognition receptor that engages lipid rafts during signalling[47,48] (FIG. 1b). The TCR is composed of αβ-heterodimers which associate with the CD3 (γδε) complex and the ζ-homodimer. Whereas the α- and β-subunits contain the extracellular binding site for peptides that are presented by the MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) class I and II proteins on the surface of ANTIGEN-PRESENTING CELLS (APCs), the CD3 and ζ-subunits contain cytoplasmic ITAM motifs. The earliest signalling event after TCR engagement is the phosphorylation of ITAM tyrosine residues by the doubly acylated non-receptor Src-like tyrosine kinases, Lyn and Fyn[47,48]. When ZAP-70 binds to phosphorylated ITAMs it is activated and, in

METHYL-β-CYCLODEXTRIN
Carbohydrate molecule with a pocket for binding cholesterol.

MAJOR HISTOCOMPATIBILITY COMPLEX
A complex of genetic loci, occurring in higher vertebrates, encoding a family of cellular antigens that help the immune system to recognize self from non-self.

ANTIGEN-PRESENTING CELL
A cell, most often a macrophage or dendritic cell, that presents an antigen to activate a T cell.

Table 2 | Raft nomenclature

Present raft nomenclature*

Rafts | DRMs | DIGs | DICs | GPI domains | Glycosphingolipid signalling domains | Caveolae-like domains | Microdomains | LDM | Liquid-ordered domains | DIM | GEMs | TIFF

Suggested raft nomenclature

	I. Rafts	II. Clustered rafts	III. DRMs	IV. Caveolae
Components	• Glycosphingolipids • Cholesterol • Lipid-modified proteins containing saturated acyl chains: – GPI-anchored proteins – Doubly acylated Src-type kinases • Transmembrane proteins	• Rafts clustered by: – Antibody – Lectin – Adjacent cell proteins – Physiological crosslinking proteins	• Rafts remaining insoluble after treatment on ice with detergent‡§: Triton X-100 (most popular), Brij-58, CHAPS, NP-40	• Raft proteins and lipids • Caveolins
Properties	• 50 nanometres in diameter • Mobile (~10^{-8} cm^{-2} sec^{-1}) • Liquid-ordered phase	• Large, often hundreds of nanometres to micrometres in size • Often bound to cytoskeleton	• Float to low density in sucrose or Optiprep™ density gradients	• Morphological 'cave-like' invaginations on the cell surface
Comments	• Native rafts are only detected in living cells	• Clustering is used both artificially and physiologically to trigger signalling cascades	• Non-native (aggregated) raft • Variable effects depending on: – Detergent type – Detergent:lipid ratio – Cell type	• Raft subcategory • Highly specialized

* DRM, detergent-resistant membrane; DIG, detergent-insoluble glycolipid-rich domain; DIC, detergent-insoluble complex; LDM, low-density membrane; DIM, detergent-insoluble material; GEM, glycolipid-enriched membrane; TIFF, Triton X-100 insoluble floating fraction.
‡Care should be taken when choosing solubilization conditions for co-immunoprecipitation experiments, as these popular detergents do not solubilize rafts on ice. Co-localization of proteins in rafts or DRMs could be mistaken for direct protein–protein interactions if rafts are not completely solubilized.
§Rafts can be solubilized in octyl glucoside or in the detergents listed above at raised temperatures.

turn, phosphorylates LAT, a transmembrane protein that couples TCR activation to several signalling pathways[49–51]. Several GPI-linked proteins[52,53] and accessory molecules[54–56] help to amplify the T-cell activation events. Phosphatases are also required to switch these pathways on and off [57].

One remarkable feature of T-cell activation is that only 10–100 cognate peptide–MHC complexes, among a total pool of 10^4–10^5 MHC molecules expressed on an APC, have to be recognized by receptors on the T cell to generate an immune response[58]. This is possible only because the same TCR can be activated over and over again. This process can take hours to complete and is facilitated by the assembly of an immunological synapse, several micrometres in diameter, at the contact zone with the APC. A complex series of events involving the actin cytoskeleton[59] leads to the formation of the immunological synapse[60] — a contact zone between APC and T cells, where T-cell activation takes place[47,58,61]. During the formation of the immunological synapse, the T cell polarizes its actin and microtubule networks towards this contact site, and also directs membrane traffic in this direction[59].

Evidence from several laboratories indicates that clustering of rafts is an essential feature in the formation of an immunological synapse[47,48]. As with IgE receptors, monomeric TCR complexes have only weak steady-state raft affinity[29,54]. After receptor crosslinking, their raft residency increases and they become partly insoluble in detergent. Although we still need to understand the precise mechanism of initial TCR engagement *in vivo*, artificial crosslinking of the TCR-associated CD3 subunits[55], or of CD3 and CD28 with antibody-coated beads, can be used experimentally to activate TCR signalling[56]. Under these conditions many proteins, including the hyperphosphorylated TCR multisubunit complex, and cytoplasmic proteins such as ZAP-70, Vav, PLCγ, Grb2 and phosphatidylinositol 3-OH kinase become detergent resistant, indicating possible raft association[55]. Consistent with this interpretation, cholesterol depletion by methyl-β-cyclodextrin dissociates these proteins from rafts and inactivates the signalling cascade[52,55]. The activation of Lck by the TCR could furthermore lead to raft clustering, perhaps

Table 3 | Signal transduction processes involving rafts

Protein	Selected references
FcεRI receptor	40
T-cell receptor	47, 48
B-cell receptor	90
EGF receptor	35, 91
Insulin receptor	92
EphrinB1 receptor	93
Neurotrophin	94
GDNF	63, 65
Hedgehog	68
H-Ras	66
Integrins	95, 96
eNOS	97, 98

through protein adaptors that are constitutively associated with rafts, such as LAT[49,50]. This cascade of interactions, including scaffolding proteins and adaptors, builds up the immunological synapse on the surface of the T cell. Moreover, rafts also function to concentrate MHC class II molecules, loaded with specific peptides, on the APC side of the synapse[62].

GDNF signalling. The glial-cell-derived neurotrophic factor (GDNF) family of ligands is important for the development and maintenance of the nervous system. In addition, they function during differentiation of the kidney and spermatogonia. GDNF binds to a multicomponent receptor complex that is composed of the GPI-linked GDNF receptor-α (GFRα) and the transmembrane tyrosine kinase, RET. The receptor subunits GFRα and RET are not associated with each other in the absence of ligand[63]. But after extracellular GDNF stimulation, RET moves into rafts, where it associates with GFRα. Signal transduction depends on the co-localization of RET and GFRα in lipid rafts, as cholesterol depletion with methyl-β-cyclodextrin decreases GDNF signalling[63].

As with IgE receptor and TCR signalling, the issue of how signalling is activated on GDNF binding is unresolved. GDNF is a dimer and is sufficient to trigger the initial events. Being a dimer, it could crosslink its receptor, but whether this is really the signal-initiating event has not yet been shown. It is also not known whether signalling can occur within a single raft, or whether it requires raft clustering to reach a signalling threshold.

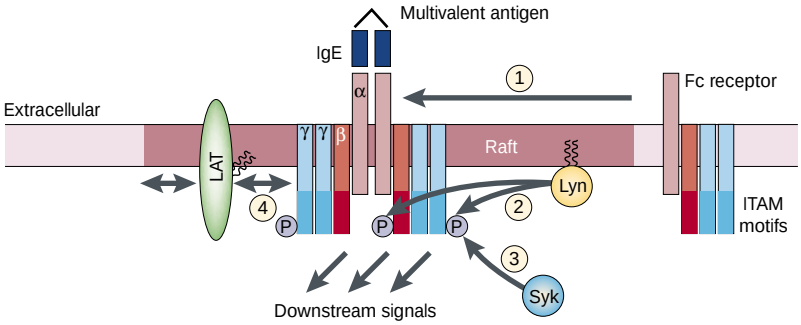
If both GFRα and RET are necessary for GDNF signalling, you would predict that the receptor subunits should localize together *in vivo*. But GFRα is more widely expressed than RET in neural tissue. In fact, GDNF can also signal through GFRα in a RET-independent way[64,65]. Moreover, autophosphorylated RET can trigger different signalling pathways depending on whether it is inside or outside rafts (C. Ibáñez, personal communication).

Ras signalling. The small GTPase Ras is central to many signalling processes. It acts as a switch that, when activated, recruits serine/threonine kinases of the Raf family to the plasma membrane. These, in turn, activate the ERK–MAP kinase pathway and other targets. The two Ras isoforms, K-Ras and H-Ras, are almost identical in sequence but have different signalling properties[66]. Both isoforms have a carboxy-terminal prenylated CAAX sequence, but whereas K-Ras has a polybasic region required for plasma membrane localization, H-Ras is palmitoylated[67] and therefore more likely to partition into lipid rafts. Roy *et al.*[66] showed that expression of a dominant-negative mutant of caveolin strongly inhibited H-Ras-mediated Raf activation, but had no effect on its activation by K-Ras. The expression of this mutant led to a decrease in the number of caveolae on the cell surface, and depleted cell surface cholesterol. The mutant phenotype could be mimicked by depleting cholesterol with methyl-β-cyclodextrin and it could be rescued by addition of exogenous cholesterol. One interpretation of these results is that expression of the caveolin mutant reduces the cholesterol content of the plasma membrane and therefore the number of functional lipid rafts. As H-Ras can signal only through rafts, it can no longer activate Raf. But K-Ras, which does not operate in rafts, is not affected.

Hedgehog signalling. Drosophila melanogaster Hedgehog and its mammalian homologues act as short-range morphogens during tissue patterning. In the absence of Hedgehog signalling, the sterol-sensing membrane protein Patched represses the constitutive signalling activity of a second membrane protein, Smoothened, by forming an inactive Patched–Smoothened complex[68]. Hedgehog binding to Patched releases Smoothened, which activates a signalling cascade that culminates in the upregulation of a specific set of nuclear transcripts.

Hedgehog is an interesting signalling molecule, as it

a **FcεRI signalling**



b **TCR signalling**

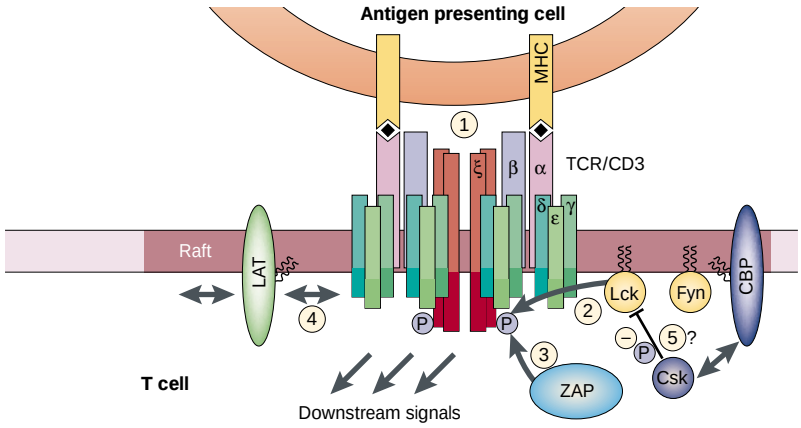


Figure 1 | **Initial signalling events in rafts for a | IgE receptor (FcεRI)- and b | T-cell antigen receptor (TCR)-mediated signalling.** For clarity, only a small subset of involved proteins are shown. A likely sequence of the key initial events is indicated numerically. 1 | Ligand-induced receptor dimerization of the Fc receptor or TCR/CD3 probably increases its raft association, which leads to 2 | phosphorylation of the receptors' immune receptor tyrosine-based activation motifs (ITAMs) by Src-family protein tyrosine kinases (for example, Lyn, Lck and Fyn). 3 | Phosphorylated ITAMs act as a membrane docking site for cytoplasmic Syk/ZAP-70; these are also tyrosine kinases and are activated in the raft by tyrosine phosphorylation. 4 | Syk/ZAP-70 can, in turn, activate other proteins such as LAT, a raft-associated adaptor. Through crosslinking, LAT can recruit other proteins into the raft and further amplify the signal. The complex cascade of later downstream signalling events is not shown. 5 | One possible way of downregulating the signal may occur by binding of the cytosolic kinase Csk to the raft-associated protein CBP. Csk may then inactivate the Src-family kinases through phosphorylation[57].

is post-translationally modified to introduce a cholesterol moiety at the carboxyl terminus[69] and a palmitate moiety at the amino terminus[70]. Cholesterol-modified Hedgehog is membrane bound, and has been shown to associate with lipid rafts in *Drosophila* embryos[9]. The cholesterol modification restricts the signalling range of Hedgehog, making it a short-range morphogen. If Hedgehog is mutated to lose its hydrophobic anchor, it is secreted and can activate cells much further away than normal[71]. So how is membrane-bound Hedgehog released from the cell where it is synthesized, to act as a signal for a target cell several cell layers away? It seems that the association of Hedgehog with rafts is important for its function, but it is not sufficient[68]. If cholesterol is replaced with a GPI-anchor — which should still localize the protein to rafts — Hedgehog is no longer released from the surface of the expressing cells[71]. Another sterol-sensing protein, Dispatched, is also required for the release of Hedgehog[71]. The mechanism of release could involve either displacement of the cholesterol tether or shedding of membrane vesicles from Hedgehog-producing cells. In conclusion, the requirement for lipid rafts during Hedgehog signalling is completely different from that described for other signalling processes. The cell biology of this fascinating signalling process is poorly understood, and awaits a detailed exploration.

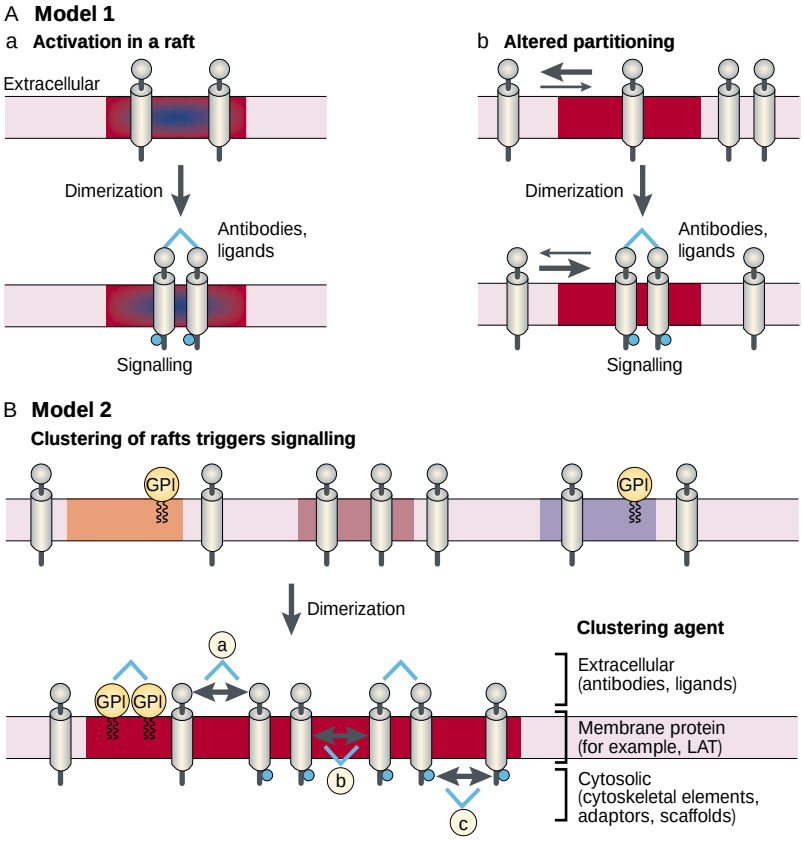


Figure 2 | **Models of how signalling could be initiated through raft(s). A** | In these models, signalling occurs in either single rafts (Model 1) or clustered rafts (Model 2). Following dimerization (or oligomerization) the protein becomes phosphorylated (blue circle) in rafts. In single rafts this can occur by activation **a** | within the raft, or **b** | by altering the partitioning dynamics of the protein. **B** | In the second model we assume that there are several rafts in the membrane, which differ in protein composition (shown in orange, purple or blue). Clustering would coalesce rafts (red), so that they would now contain a new mixture of molecules, such as crosslinkers and enzymes. Clustering could occur either extracellularly, within the membrane, or in the cytosol (a–c, respectively). Raft clustering could also occur through GPI-anchored proteins (yellow), either as a primary or co-stimulatory response. Notably, models 1 and 2 are not mutually exclusive. For instance, extracellular signals could increase a protein's raft affinity (for example, similar to the effect of single versus dual acylation) therefore drawing more of the protein into the raft where it can be activated and recruit other proteins, such as LAT, which would crosslink several rafts.

Models for signal initiation in rafts

Although we are at a stage at which we can build working hypotheses, we still do not know exactly how receptors signal through lipid rafts. As illustrated by the preceding examples (except for Hedgehog signalling) a common theme is that individual rafts cluster together to connect raft proteins and interacting proteins into a signalling complex. For instance, doubly acylated non-receptor tyrosine kinases and G proteins from separate rafts could be brought into contact with a signalling receptor in this way.

Receptors could behave in at least three different ways in rafts (FIG. 2). First, receptors associated at steady state with lipid rafts could be activated through ligand binding (FIG. 2A, a). Second, individual receptors with weak raft affinity could oligomerize on ligand binding, and this would lead to an increased residency time in rafts (FIG. 2A, b). Last, activated receptors could recruit crosslinking proteins that bind to proteins in other rafts, and this would result in raft coalescence (FIG. 2B). These models are not mutually exclusive. Through formation of a raft cluster, a network of interactions between adaptors, scaffolds and anchoring proteins would be built up to organize the signal complex in space and time. This signalling complex would be insulated within the raft clusters from the surrounding liquid-disordered lipid matrix. The formation of clustered rafts would lead to amplification through the concentration of signalling molecules, as well as to exclusion of unwanted modulators.

The interactions that drive raft assembly are dynamic and reversible. Raft clusters can be disassembled by negative modulators and/or by removal of raft components from the cell surface by endocytosis. The coalescence of individual rafts to form raft clusters has been observed repeatedly, for example, when crosslinking raft components with antibodies[12,29]. The movement and behaviour of the raft clusters can also be influenced by interaction with cytoskeletal elements[44,72,73] and second messengers such as the phosphoinositide PtdIns(4,5)P_2, which help organize actin assemblies on the cytoplasmic surface of the rafts[74,75].

Many open issues

Several aspects of raft structure and function still need to be explained. One important area is raft composition and the question of whether more than one kind of raft exists on the cell surface of different cell types[76,77]. Not only do we need to identify raft-associated proteins, but we also have to determine the lipid composition in both the exoplasmic and cytoplasmic leaflets of rafts. As detergent extraction undoubtedly leads to raft aggregation, it is not easy to isolate individual rafts or ligand-activated

raft clusters in such a way that their native state is preserved. Understanding the nature of individual rafts will probably require the development of new methods. The question of how single rafts are crosslinked to form clustered rafts during signal transduction also requires detailed exploration. This will require real-time imaging of the assembly of signalling complexes under normal conditions and during cholesterol depletion.

A pressing issue is to clarify the function of caveolae during signal transduction. We know little about how proteins move into caveolae. Why does crosslinking of GPI-anchored proteins or gangliosides lead to their enrichment in caveolae[78–80]? Could the clustering of rafts induce the formation of caveolae in caveolin-containing cells? What are the protein signals required for caveolar trapping? Caveolae can be internalized, but how is this process regulated? What is the role of actin? These are important questions if we want to understand how raft-associated receptors are downregulated.

This is an exciting time for researchers studying cellular membranes, but the issues at hand can be clarified only by a multidisciplinary approach. After long neglect, the dynamic organization of lipid bilayers is finally back at centre stage.

Links

DATABASE LINKS

Src kinase | caveolin | IgE | FcεRI | Lyn | Syk | ZAP-70 | PLCγ | LAT| TCR | CD3 | Fyn | Vav | Grb2 | Lck | GDNF | RET | Ras | Raf | ERK | Hedgehog | Patched | Smoothened | Dispatched

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ENCYCLOPEDIA OF LIFE SCIENCES

Lipids| Membrane proteins

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- Lipid rafts consist of dynamic assemblies of cholesterol and sphingolipids in the exoplasmic leaflet of the lipid bilayer.
- Lipid rafts can include or exclude proteins selectively, and the raft affinity of a given protein can be modulated by intra- or extracellular stimuli.
- They are too small to be seen by standard microscope techniques. It is also not possible to isolate lipid rafts in their native state. Detergent-resistant membranes, containing clusters of many rafts, can be isolated by extraction with Triton X-100 or other detergents on ice.
- Raft association of proteins can be assayed by manipulating the lipid composition of rafts. If cholesterol or sphingolipids are depleted from membranes, lipid rafts are dissociated, and previously associated proteins are no longer in rafts.
- There is great confusion in the nomenclature for lipid rafts, and Table 2 proposes a new nomenclature.
- Rafts are involved in signal transduction. Crosslinking of signalling receptors increases their affinity for rafts. Partitioning of receptors into rafts results in a new micro-environment, where their phosphorylation state can be modified by local kinases and phosphatases, modulating downstream signalling.
- Raft clustering could also be involved in signal transduction. Several rafts coalesce, resulting in amplification of the signal.
- Some examples for such raft-dependent signalling processes are IgE signalling during the allergic response, T-cell activation and GDNF signalling.
- Rafts are also necessary for Hedgehog signalling during development but the mechanism is very different. Hedgehog is a membrane-bound ligand and needs to be released from its cell of origin so it can signal to cells several layers away. It can be released from the cell when it is anchored in rafts through its cholesterol moiety.

Kai Simons received his M.D. Ph.D. degree in 1964 from the University of Helsinki, Finland. Simons then did postdoctoral research with A.G. Bearn at Rockfeller University in New York. In 1975, he became a Group Leader at the European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany, and he started the Cell Biology Program, which became the focal point for molecular cell biology in Europe. He is the first President of the newly formed European Life Scientist Organization. In addition, he will head the new Max Planck Institute for Molecular Cell Biology and Genetics, which will start its operations in Dresden, Germany, in January 2001. His research interests have been concerned with the life cycle of Semliki Forest virus. This virus had the simplest biological membrane known. From this early work he moved to studies of epithelial polarity and intracellular protein and lipid transport. These studies laid the ground work for the lipid raft concept that now is dominating his research interests.
Derek Toomre studied Glycobiology at the Univeristy of California, San Diego (UCSD), under the supervision of Ajit Varki. He obtained his Ph. D. in Biochemistry in 1996 and was subsequently attracted to the field of cell biology. He is a postdoc in Kai Simons's lab on a Marie Curie Fellowship. His primary focus in the Simons lab has been the application of advanced videomicroscopy techniques including multicolour GFP imaging and total internal reflection fluorescence microscopy (TIR-FM) to study the dynamic processes of membrane sorting, trafficking and fusion in living cells.

Src kinase
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=1445

Hedgehog
http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=hedgehog*&ORG=Hs

caveolin
http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=caveolin*&ORG=Hs

IgE
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=3497

FcεR1
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=2205

Lyn
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=4067

Syk
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=6850

ZAP-70
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=7535

PLCγ
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=5335

LAT
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=27040

TCR
http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=TCR*&ORG=Hs&V=0

CD3
http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=CD3*

Fyn
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=2534

VAV
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=7409

Grb2
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=2885

Lck
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=3932

GDNF
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=2668

RET
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=5979

Ras
http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=ras*

Raf
http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=raf*

ERK
http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=erk

Hedgehog
http://flybase.bio.indiana.edu/.bin/fbidq.html?FBgn0004644

Patched
http://flybase.bio.indiana.edu/.bin/fbidq.html?FBgn0003892

Smoothened
http://flybase.bio.indiana.edu/.bin/fbidq.html?FBgn0003444

Dispatched
http://flybase.bio.indiana.edu/.bin/fbidq.html?FBgn0029088

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Lipids
http://www.els.net/elsonline/fr_loadarticle.jsp?available=1&ref=A0000711&orig=searching&page_number=1&page=search&Sitemap=Lipid&searchtype=freetext&searchlevel=4
membrane proteins
http://www.els.net/elsonline/fr_loadarticle.jsp?available=1&ref=A0000624&orig=searching&page_number=1&page=search&Sitemap=Lipid&searchtype=freetext&searchlevel=4

lab homepage
http://www.embl-heidelberg.de/ExternalInfo/simons/index.html