Annu. Rev. Cell Dev. Biol. 1998. 14:111–36

FUNCTIONS OF LIPID RAFTS IN BIOLOGICAL MEMBRANES

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KEY WORDS: membrane domain, glycosphingolipid, GPI-anchored protein, signal transduction, membrane trafficking

ABSTRACT

Recent studies showing that detergent-resistant membrane fragments can be isolated from cells suggest that biological membranes are not always in a liquid-crystalline phase. Instead, sphingolipid and cholesterol-rich membranes such as plasma membranes appear to exist, at least partially, in the liquid-ordered phase or a phase with similar properties. Sphingolipid and cholesterol-rich domains may exist as phase-separated "rafts" in the membrane. We discuss the relationship between detergent-resistant membranes, rafts, caveolae, and low-density plasma membrane fragments. We also discuss possible functions of lipid rafts in membranes. Signal transduction through the high-affinity receptor for IgE on basophils, and possibly through related receptors on other hematopoietic cells, appears to be enhanced by association with rafts. Raft association may also aid in signaling through proteins anchored by glycosylphosphatidylinositol, particularly in hematopoietic cells and neurons. Rafts may also function in sorting and trafficking through the secretory and endocytic pathways.

CONTENTS

1081-0706/98/1115-0111$08.00

INTRODUCTION

Because most biological phospholipids have low acyl chain melting temperatures (T_m), cellular membranes are generally thought to exist in a fluid, liquid-crystalline (l_c) phase. However, the plasma membrane and some organelles of the secretory and endocytic pathways in eukaryotic cells are rich in sphingolipids, which have elevated T_m, and sterols, which can have profound effects on membrane phase (Silvius et al 1996). This mixture of lipids raises the possibility of complex phase behavior in these membranes.

In fact, a number of recent studies suggest that eukaryotic cell plasma membranes are not entirely in the conventional l_c phase (Brown & London 1998). Instead, they may be, at least partially, in the cholesterol-rich liquid-ordered (l_o) phase. The l_o phase is characterized by a high degree of acyl chain order and is favored by high-T_m lipids with saturated acyl chains such as sphingolipids, when they are mixed with cholesterol. The strongest experimental support for this idea has come from a seemingly unrelated finding; i.e. in the cold, plasma membranes are partially resistant to solubilization by non-ionic detergents such as Triton X-100 (Brown & London 1998). After Triton extraction, insoluble lipids remain in the form of detergent-resistant membranes (DRMs; also called detergent-insoluble glycolipid-enriched membranes or DIGs) (Simons & Ikonen 1997).

We recently reviewed the evidence equating DRMs and lipid bilayers in the l_o phase and the possible domain structure that could result from formation of a separate l_o phase in biological membranes (Brown & London 1998). Readers are referred to this and three other recent reviews of sphingolipid and cholesterol-rich membranes (Simons & Ikonen 1997, Brown 1998, Rietveld & Simons 1998). Due to space limitations, these topics are summarized only briefly in this review. Here, we first discuss the relationship between DRMs, lipid rafts, caveolae, and low-density plasma membrane fragments. We then focus on how phase separation may function in biological membranes.

DRMS, RAFTS, CAVEOLAE, AND LOW-DENSITY MEMBRANES

DRMs

Most of the sphingolipids and some of the cholesterol in mammalian cell membranes are detergent-resistant in the cold, and can be isolated as DRMs (Brown & Rose 1992). In contrast, most of the cellular phospholipid is detergent soluble. Detergent-insolubility was found to correlate with lipid T_m, providing a clue that insolubility of cellular lipids might reflect their phase behavior (Schroeder et al 1994). Further support for this link came from findings that genuine l_o phase model membranes are detergent resistant and that DRMs isolated from cells have physical properties similar to those of l_o phase membranes (Schroeder et al 1994, Ahmed et al 1997, Schroeder et al 1998).

DRMs can be isolated from almost all mammalian cell types. Immature oligodendrocytes (Krämer et al 1997) and immature hippocampal neurons (C Dotti, unpublished data) are exceptions in that they are very poor sources of DRMs. However, as hippocampal neurons mature, their sphingomyelin content and their ability to produce DRMs both increase (C Dotti, unpublished data), suggesting that a high sphingolipid content is required for DRM formation. DRMs have not been well studied in other eukaryotes, although they have been isolated from *Saccharomyces cerevisiae* (Kübler et al 1996) and possibly from *Tetrahymena* (Zhang & Thompson 1997).

DRMs isolated from mammalian cell lysates have the appearance of vesicles, sometimes mixed with membrane sheets. Most probably originate from the plasma membrane, although some are also derived from intracellular membranes. However, DRMs examined in situ look very different, as first shown by Mayor & Maxfield (1995a). Detergent-resistant plasma membrane is a continuous sheet, interrupted by holes similar to those in Swiss cheese (Figure 1). Thus plasma membrane–derived DRMs do not exist as vesicles in cells. In fact, they may not be a collection of discrete structures, as the term DRMs suggests. This will be important later, when we consider the possible domain organization of the plasma membrane, and the relationship between DRMs and other structures such as caveolae.

HOW PROTEINS AND LIPIDS ASSOCIATE WITH DRMS A specific group of membrane proteins is present in DRMs when they are isolated from mammalian cell lysates. Because DRMs are in an l_o-like state when they are isolated, they should be the molecules with the greatest tendency to partition into an ordered environment that is enriched in lipids with saturated acyl chains. In agreement with this idea, sphingolipids are enriched in DRMs, whereas phospholipids are

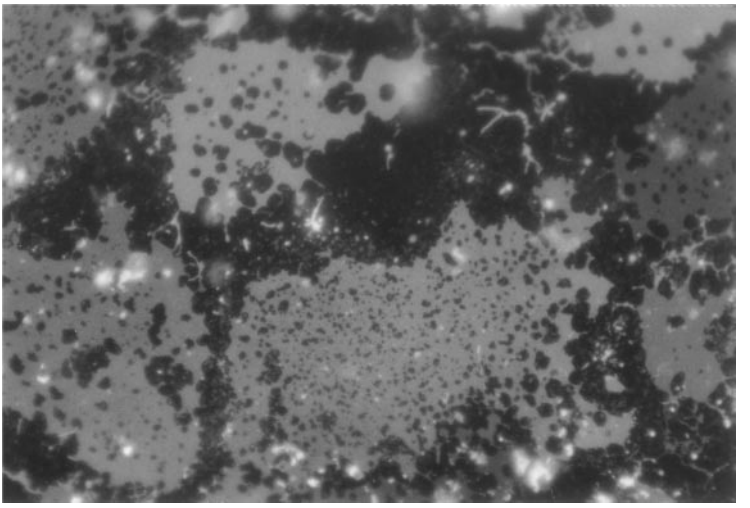

Figure 1 Detergent-resistant plasma membrane (DRM). COS cells were removed from glass coverslips by sonication, leaving a plasma membrane sheet behind as described by Huang et al (1997). After fixation, the membrane was incubated with 1% Triton X-100 for 30 min at 4°C and washed. DiI, a fluorescent lipophilic probe that should partition into any membrane under these conditions, was then added to visualize DRMs.

relatively depleted (Brown & Rose 1992). As expected from their high T_m, glycosphingolipids are enriched in DRMs. However, as sphingomyelin and cholesterol-rich DRMs can be isolated from mammalian cells that do not contain glycosphingolipids (K Ivarson, K Grove & DA Brown, unpublished data), these lipids are not absolutely required for DRM formation.

Surprisingly, phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) is enriched in DRMs, although its acyl chains are highly unsaturated (Pike & Casey 1996). The DRM-associated pool of PtdIns 4,5-P_2 is preferentially turned over in response to bradykinin and EGF, suggesting that this localization is important in signaling. It will be interesting to determine how PtdIns 4,5-P_2 is localized to these membranes.

Many DRM proteins are linked to saturated acyl chains, which is likely to make them prefer an ordered environment. Proteins can be linked to saturated acyl chains in two ways; either in the form of a glycosylphosphatidylinositol (GPI) anchor (McConville & Ferguson 1993) or through acylation with myristate or palmitate. Except in the DRM-poor cells noted above, GPI-anchored proteins in mammalian cells generally associate with DRMs, in a manner that requires the GPI anchor (Rodgers et al 1994, Arreaza & Brown 1995). The

importance of acylation in DRM targeting has been shown most clearly for Src family nonreceptor protein tyrosine kinases (Resh 1994, Milligan et al 1995, Robbins et al 1995). With a few exceptions (Lisanti et al 1994, Dorahy et al 1996), most groups have found that association of these kinases with DRMs requires dual modification by both myristate and palmitate (Shenoy-Scaria et al 1994, Robbins et al 1995). Several other palmitoylated DRM proteins have also been identified (Brown & London 1997), and acylation may be a widely used DRM targeting signal. In fact, most of the proteins in DRMs isolated from the MDCK cultured epithelial cell line are either GPI anchored or acylated (KA Melkonian & DA Brown, unpublished data.) Mutation of either palmitoylation site in the dually palmitoylated neuronal protein GAP-43 (Maekawa et al 1997) blocks association with DRM (S Arni, SA Keilbaugh & DA Brown, unpublished data). Similarly, mutation of any of the three palmitoylation sites of the transmembrane influenza hemagglutinin protein blocks DRM association (MG Roth, unpublished data). Caveolin, a marker of caveolae (see below), may be unusual, as elimination of its three palmitoylation sites does not affect association with DRMs (Dietzen et al 1995). GPI-anchorage and acylation are the only known signals for DRM targeting. Other signals must exist, however, as some DRM proteins contain neither modification.

Rafts

Several early studies suggested that glycosphingolipids might cluster in membranes (Thompson & Tillack 1985). Simons & van Meer (1988) proposed that these glycolipid rafts might be involved in sorting (see below). Lipids in the rafts were suggested to be clustered by a network of hydrogen bonds. Glycosphingolipid-rich DRMs, discovered later, were presumed to be isolated rafts (Brown & Rose 1992). Further work showed that lipids associate with DRMs based largely on their degree of acyl chain order (Schroeder et al 1994). This appears to be a more important determinant of DRM association than hydrogen bonding capability (Brown & London 1998). Thus the focus of raft research has shifted to cholesterol and sphingolipid-rich ordered domains that may have characteristics of the l_o phase. The term rafts has recently been adapted to refer to such domains (Rietveld & Simons 1998), and we use the word in this sense here.

DO RAFTS EXIST IN CELL MEMBRANES? Although detergent-insolubility studies strongly suggest that plasma membranes are not in the l_c state, a key unanswered question is whether membranes contain discrete domains in different phases. It is possible that DRMs exist in intact cell membranes as rafts floating in a detergent-soluble l_c phase sea. [Similarly, in very sphingolipid-rich membranes, discrete l_c phase domains could exist in a continuous l_o-like

sea (Rietveld & Simons 1998).] Model membrane studies show that these are viable models, because liposomes with sphingolipid and cholesterol levels similar to those in the plasma membrane are partially detergent insoluble (mimicking the behavior of cell membranes); they also show phase separation (Ahmed et al 1997, Schroeder et al 1998). However, it is important to note that partial detergent insolubility by itself does not prove that membranes contain rafts. As we have discussed, membranes present in a single uniform phase with properties intermediate between the l_o and l_c phases could also exhibit partial insolubility (Brown & London 1998). In this case, although rafts would not exist constitutively, they might form in a regulated manner.

Morphological studies have provided further insight into the question of whether rafts exist. Proteins (such as GPI-anchored proteins) and lipids (such as glycosphingolipids) that have an affinity for an ordered environment should partition into rafts if they are present and should act as useful morphological markers. The conclusion of several studies using this approach is that if rafts exist, they are very difficult to see. GPI-anchored proteins generally appear uniformly distributed in the plasma membrane (Maxfield & Mayor 1997), and it has been difficult to obtain evidence of glycosphingolipid clusters larger than a few molecules (Rock et al 1990).

However, there is much better evidence that rafts exist after certain proteins and lipids are clustered in the membrane. One of the best examples is the IgE receptor in basophils (see below). Additional evidence comes from the fact that proteins that associate with DRMs and thus are expected to prefer an ordered environment can cocluster when both are clustered independently. This has been shown for two different GPI-anchored proteins (Mayor et al 1994) and recently also for a GPI-anchored protein and a transmembrane DRM protein (T Harder, P Scheiffele, P Verkade & K Simons, unpublished data). The affinity of these independently clustered proteins for each other suggests that both are present in rafts.

We have incorporated these observations into three possible models for raft structure (Brown & London 1998). Briefly, stable rafts might exist, but individual proteins and lipids might have a relatively low affinity for them. The affinity might be increased by clustering. Alternatively, rafts might be very small, and might coalesce when the components are clustered. Finally, it is even possible that clustering of components might induce phase separation and cause raft formation. In any case, these studies highlight the importance of clustering of proteins and lipids that have an affinity for an ordered environment in triggering the stable association of these molecules with large rafts. This property must be kept in mind when considering how rafts may function.

A major outstanding question is how (or whether) rafts form in the cytoplasmic leaflet of the bilayer (discussed further by Rietveld & Simons 1998). The

fact that Src-family kinases are present in DRMs, although their acyl chains have access only to the inner leaflet, suggests that rafts exist there, as does the fact that DRMs have a bilayer appearance (Brown & Rose 1992). However, sphingolipids are largely concentrated in the extracellular leaflet. Some glycerophospholipids may also participate in raft formation, a property that could be critical in the inner leaflet (Brown & London 1997). How rafts in opposite leaflets might communicate remains a mystery. Monolayer coupling in sphingomyelin-containing bilayers has been observed in model systems (Schmidt et al 1978), and it is conceivable that the long sphingolipid acyl chains affect phospholipid organization in the opposite leaflet.

Caveolae

Caveolae (recently reviewed by Parton 1996, Anderson 1998, Okamoto et al 1998) are 50–70-nm plasma membrane pits implicated in endocytosis, lipid traffic, and signal transduction. A 22-kDa protein, caveolin (VIP21), is closely associated with caveolae and may play an important structural role in their formation (Rothberg et al 1992, Fra et al 1995). Caveolin is also present in the Golgi/*trans*-Golgi network (TGN) and in post-Golgi transport vesicles (Dupree et al 1993), although caveolae have not been detected in intracellular membranes. The function of Golgi caveolin and the relationship between the Golgi and cell-surface pools of the protein are not known, although the presence of caveolin in transport vesicles suggests a role in vesicle formation (Simons & Ikonen 1997, Anderson 1998). In addition, several observations suggest that caveolae and/or caveolin are important in cholesterol traffic (Anderson 1998, Rietveld & Simons 1998).

It was recently proposed that the term caveolae be broadened beyond its original definition (Anderson 1998). The rationale for this suggestion is twofold. First, caveolae are dynamic structures, and their degree of invagination can be regulated. Under some experimental conditions, caveolae flatten and are not detectable as invaginations. Thus the original definition may seem too limited. Second, as described below, fragmented plasma membrane can be fractionated, and a low-density subfraction that is enriched in caveolae can be isolated. Low-density membranes with similar properties can be isolated from cells that do not contain caveolae. Thus it was proposed that the word caveolae be broadened to include these membranes.

However, the degree of similarity between these membranes and caveolae is not clear, and the membrane of invaginated caveolae may have unusual properties that are not shared by other membranes. For these reasons, we use the word caveolae here to refer only to caveolin-associated plasma membrane invaginations. It should be kept in mind that their morphology may be altered under some conditions.

Several observations initially suggested that DRMs exist in cells as rafts within caveolae. First, DRM markers such as GPI-anchored proteins and glycosphingolipids can concentrate in caveolae (although see below). Caveolin, the best marker of caveolae, is also concentrated in DRMs. In addition, isolated DRMs sometimes have the size and shape of caveolae (Chang et al 1994).

However, further results show that DRMs are not the same as caveolae. First, it now appears that most GPI-anchored proteins are not constitutively concentrated in caveolae but rather show a uniform distribution in the plasma membrane (Maxfield & Mayor 1997, Brown & London 1998). GPI-anchored proteins move into, or close to (Schnitzer et al 1995), caveolae only after being clustered in the membrane by antibody-mediated cross-linking. As a further indication that DRMs and caveolae are not the same, DRMs can be isolated from cells that do not have caveolae. Finally, in situ visualization (Figure 1) clearly shows that DRMs are not restricted to caveolae.

LIPID COMPOSITION Caveolae are widely believed to be enriched in cholesterol and sphingolipids. However, this belief leans heavily on the assumptions that all DRMs are caveolae and that DRMs exist in membranes as distinct sphingolipid/cholesterol-rich rafts. As discussed above, the origin of DRMs is not as clear as it initially appeared. For this reason, data on the lipid composition of caveolae must be carefully re-examined.

The strongest evidence that sphingolipids are enriched in caveolae is that the ganglioside GM1 is concentrated in caveolae when detected with cholera toxin (Tran et al 1987, Parton 1994). However, cholera toxin is pentavalent. Thus GM1, like GPI-anchored proteins, may be concentrated in caveolae only after it is aggregated. Consistent with this possibility, cholera toxin-bound GM1 is more detergent insoluble than is free GM1 (Hagmann & Fishman 1982). This suggests that toxin binding increases the affinity of the lipid for an ordered environment. The distribution of GM1 has not been examined with a monovalent probe and remains uncertain.

The plasma membrane distribution of sphingomyelin and several neutral glycosphingolipids has also been examined (Fujimoto 1996). They were found to be randomly distributed in the membrane but concentrated in caveolae after being clustered. Another study has been cited as evidence that sphingomyelin is concentrated in caveolae (Liu & Anderson 1995). Fifty to seventy percent of the total plasma membrane sphingomyelin was found in low-density caveolin-rich membranes thought to be purified caveolae. However, as the fraction of total plasma membrane lipid in these fractions was not determined, it is not known if sphingomyelin was enriched there over its concentration in bulk membrane. In addition, it is now clear that these low-density membranes are not homogeneous caveolae (see below).

It is also not clear if cholesterol is enriched in caveolae. In an early study, the cholesterol-binding compound filipin preferentially labeled rings around the necks of caveolae when added to cells for a short time (Simionescu et al 1983). After longer exposure to the drug, heavy labeling of the entire plasma membrane was generally observed. As the authors pointed out, rings of filipin around caveolae could reflect either a concentration of cholesterol or a difference in the accessibility of cholesterol to the probe. Two later studies suggested that cholesterol is enriched in caveolae. First, nystatin, which complexes with cholesterol, caused caveolae to flatten and prevented GPI-anchored proteins from concentrating in them (Rothberg et al 1992). In a second study, caveolin was expressed in lymphocytes, which do not normally contain caveolae. Expression of caveolin in these cells (a procedure that induces formation of caveolae) (Fra et al 1995) selectively increased the cholesterol:protein ratio in a low-density caveolae-containing plasma membrane fraction, suggesting that the presence of caveolin caused an enrichment of cholesterol (Smart et al 1996). In another study, however, the distribution of cholesterol in the plasma membrane was examined morphologically using a modified biotinylated perfringolysin O toxin (Fujimoto et al 1997). Cholesterol detected with this reagent appeared evenly distributed in the membrane unless it was first clustered by cross-linking with streptavidin. Only then was it concentrated in caveolae-rich regions of the plasma membrane.

Thus the question of whether sphingolipids and cholesterol are concentrated in caveolae has not been resolved. Nevertheless, it is clear that GPI-anchored proteins and glycosphingolipids, which should prefer an ordered environment, concentrate in or near caveolae when they are clustered. This is the best indication that the lipid environment in caveolae may be more ordered than the surrounding membrane. It is not clear how this is accomplished, although caveolar proteins (possibly caveolin itself) are likely to play an important role.

PURIFICATION Several recently described methods for purifying caveolae have provided a critical advance in the biochemical characterization of these structures (Anderson 1998). However, the purity of the isolated caveolae remains somewhat controversial. For instance, caveolae isolated from endothelial cells by two groups using similar techniques contain different proteins (Schnitzer et al 1995, Stan et al 1997). In one of the most widely used methods, low-density caveolae are separated from high-density bulk plasma membrane on Optiprep gradients (Smart et al 1995). However, a recent study of heterotrimeric G proteins showed that caveolae are not the only membranes in this fraction (Huang et al 1997). This was demonstrated by examining the distribution of $G_{i\alpha}$ between light and heavy membranes and also its plasma membrane localization, as determined by electron microscopy. Although almost all the $G_{i\alpha}$ was present in

the gradient fractions proposed to contain purified caveolae (Smart et al 1995), only a small fraction of the protein was present in caveolae as detected morphologically (Huang et al 1997). It is thus difficult to determine what fraction of these low-density membranes are actually caveolae.

Low-Density Plasma Membrane Fragments

Fragmented plasma membrane from a variety of cells including lymphocytes (Hoessli & Rungger-Brändle 1983, Arni et al 1996, Smart et al 1996), fibroblasts (Smart et al 1995), epithelial cells (Huang et al 1997), and neurons (Wu et al 1997) can be separated into high- and low-density fractions on density gradients. The low-density membranes are enriched in GPI-anchored proteins and caveolin, suggesting that they are rafts. However, where the lipid composition has been examined, these membranes, compared with high-density membranes, do not appear to be enriched in cholesterol or sphingolipid (Hoessli & Rungger-Brändle 1983, Arni et al 1996). In another study, light membranes from neurons were reported to be enriched in cholesterol, sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine compared with heavy membranes (Wu et al 1997). However, lipids isolated from light and heavy membranes containing equal amounts of protein were compared. Because light membranes have a higher lipid:protein ratio, even if the lipid composition of the two fractions were identical, every lipid would appear to be enriched in a light membrane sampled for equal amounts of protein. There was no indication of a difference in lipid composition between the two membranes in this study.

In summary, lipid analysis has not proven that low-density plasma membrane fragments correspond to rafts. Nonetheless, the fact that a number of key signaling molecules are enriched in the low-density membranes underscores their significance (Anderson 1998). Further characterization of these membranes will be very important.

RAFTS AND SIGNAL TRANSDUCTION

Rafts may be important in transmembrane signal transduction at the cell surface. Most of the evidence for this role comes from studies of signaling through GPI-anchored proteins, as is discussed in the following section. First, however, we describe how transmembraneous receptor proteins may also associate with rafts during signaling in some hematopoietic cells.

Transmembrane Signaling Proteins in Hematopoietic Cells

FCϵRI The best evidence for the involvement of rafts in signaling comes from studies of FcϵRI, the receptor for IgE on basophils and mast cells. IgE binds constitutively to cell-surface FcϵRI. Aggregation of FcϵRI by binding of antigen to FcϵRI-bound IgE activates the associated Src-family kinase, Lyn, initiating

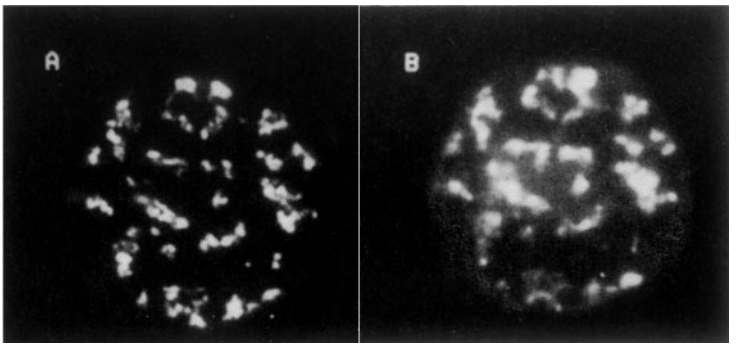

Figure 2 DiI colocalizes with clustered IgE receptor. DiI was incorporated into the plasma membrane of RBL cultured basophilic leukemia cells, where it appeared uniformly distributed (Thomas et al 1994). Cell-surface FcϵRI was then clustered with biotinylated IgE and streptavidin before visualization of FcϵRI; visualized with (*A*) FITC-IgE and (*B*) diI, in the same cell. Reproduced with permission from Thomas et al (1994).

a signaling cascade that culminates in degranulation (Field et al 1997). In an elegant series of studies, FcϵRI-bound biotinylated IgE was clustered with streptavidin, generating large, easily detectable cell-surface clusters. Strikingly, as shown in Figure 2, a saturated-chain lipid probe, diI, colocalized with clustered receptors (Thomas et al 1994). DiI is known to partition preferentially into more-ordered gel phase domains in two-phase model membranes (Spink et al 1990). Of several other fluorescent probes examined, regardless of charge, only those predicted to prefer an ordered environment coclustered with the receptor (Thomas et al 1994). Similar results were obtained from another group when FcϵRI was clustered by addition of antigen. In this study, receptor clusters colocalized with clustered GM1 (Stauffer & Meyer 1997).

Field et al showed that a fraction of FcϵRI becomes detergent insoluble by associating with DRMs when it is clustered (Field et al 1997). Two additional observations showed that association of the receptor with DRMs correlates with signaling. First, the receptor is recruited into DRMs and phosphorylated by Lyn with the same kinetics. More strikingly, only the receptor that is in DRMs serves as a substrate for Lyn (Field et al 1997). These studies provide the clearest indication to date that rafts can form under physiological conditions in cell membranes. They show that clustering of FcϵRI leads to formation, stabilization, or reorganization of rafts in the membrane and that the rafts can be visualized with diI. These studies also suggest a role for rafts in signaling.

OTHER RECEPTORS Signaling in a variety of hematopoietic cells (including basophils) involves tyrosine phosphorylation of conserved sequences in the cytoplasmic domains of cell-surface receptors by Src-family kinases (Isakov

1997). This mechanistic similarity suggests that rafts are important in signaling in other cells as well as in basophils. For example, antibody-mediated cross-linking of the cell-surface transmembrane protein CD20 triggers signaling in B cells and tumor cells and recruits the protein into DRMs (Deans et al 1998).

Cell-surface receptors do not always associate with DRMs, possibly implying that they do not associate with rafts. However, in vitro studies have shown that detergent insolubility is not always a perfect measure of the l_o phase (Brown & London 1998). Receptors could have a moderate affinity for rafts in vivo and still not associate stably with DRMs. In fact, although the studies described above show that FcϵRI associates with rafts, detergent insolubility of the receptor is somewhat difficult to detect (Field et al 1997).

Additional indirect evidence suggests that transmembrane receptors associate with rafts during signaling. For instance, activation of T cells through the T cell receptor (TCR) is impaired in cells that are defective for GPI anchor synthesis (Romagnoli & Bron 1997). Cross-linking of GPI-anchored proteins in these cells can stimulate signaling, in a manner that may involve rafts (see below). Thus recruitment of the TCR to rafts via association with GPI-anchored proteins may enhance its signaling. It is also interesting to note that depletion of cellular cholesterol can inhibit signaling in mast cells (Shakarjian et al 1993) and T cells (Stulnig et al 1997), possibly by affecting the structure of rafts.

How might association of receptors with rafts be important in signaling? One possibility is suggested by the puzzling nature of signaling in T cells (Germain 1997). The TCR complex and peptide-bound MHC molecules are both monomeric and interact with only low affinity. However, productive signaling appears to require the formation of oligomeric complexes of these molecules. It is not clear how these oligomeric complexes form. It has been proposed that low-affinity homophilic interactions between molecules of TCR, MHC, and the co-receptor CD4 may cooperate to form a stable complex (Germain 1997). Recruitment of these proteins into rafts could concentrate them enough to facilitate protein-protein interactions.

In addition, the activity of Src-family kinases may be inhibited (Rodgers & Rose 1996) or stimulated (Arni et al 1996, Kabouridis et al 1997) by association with rafts. For instance, in T cells, DRM-associated Lck is less active than detergent-soluble Lck, probably because CD45, the tyrosine phosphatase required for activation of Lck, is excluded from rafts (Rodgers & Rose 1996).

Signaling Through GPI-Anchored Proteins

Clustering or ligation of cell-surface GPI-anchored proteins can trigger transmembrane signal transduction. In some cases this occurs through activation of Src-family kinases (Brown 1993, Zisch et al 1995). Because neither protein penetrates the bilayer, how they communicate—and how GPI-anchored

proteins send any information across the membrane—present a puzzle. A reasonable scenario is that GPI-anchored proteins bind in *cis* (in the plane of the membrane) to transmembrane proteins. These proteins could transmit signals across the membrane, for instance, by binding directly or indirectly to Src-family kinases. In the following, we consider GPI-anchored proteins known to be involved in signaling and the progress made in identifying some of their transmembrane signaling partners. We also discuss how rafts may be involved in these signaling pathways.

HEMATOPOIETIC CELLS Antibody-mediated cross-linking of GPI-anchored proteins on the surface of most hematopoietic cells can stimulate Src-family kinases, leading to signaling events that include calcium flux as well as some downstream events (Robinson 1991, Brown 1993, Dráberová & Dráber 1993, Morgan et al 1993, van den Berg et al 1995).

A GPI-anchored protein for which *cis* signaling partners have been identified is FcγRIIIb (CD16), a member of the FcγR family of IgG receptors. Binding of circulating immune complexes to Fcγ receptors clusters the receptors and stimulates signaling (Unkeless et al 1995, Green et al 1997). FcγRIIIb, the only GPI-anchored FcγR, is restricted to primate neutrophils, where it coexists with the more widely distributed transmembrane form of the protein, FcγRIIa. Although the role of FcγRIIIb in signaling was controversial, it is now clear that clustering of this protein by itself can mediate calcium flux. Further signaling events require FcγRIIa, including its cytoplasmic domain. However, signaling through FcγRIIa is enhanced by coclustering with FcγRIIIb, suggesting a unique role for the GPI-anchored form of the protein. In fact, in neutrophils and transfected T cells, coclustering of FcγRIIa with either FcγRIIIb or unrelated GPI-anchored proteins enhances calcium flux, in a manner that depends on the GPI anchor (Green et al 1997). This finding highlights the role of the GPI anchor in this process.

GPI-anchored proteins can affect integrin signaling in hematopoietic cells. FcγRIIIb, as well as two other GPI-anchored proteins, the urokinase-type plasminogen activator receptor (uPAR, CD87) and CD14, the receptor for lipopolysaccharide, can bind in *cis* to β_2 integrins, which are found only on hematopoietic cells (Stockinger 1997, Todd & Petty 1997). These interactions, which are sometimes reversible and physiologically regulated, appear to have important and complex effects on integrin signaling and function (Todd & Petty 1997).

NEURONS Binding of ligands to several GPI-anchored proteins in neurons can transmit signals across the membrane. One of the best characterized is the GPI-anchored protein contactin (F3, F11), which appears to mediate interactions of neurons with the local environment during development (Peles et al 1997).

Proteins that bind contactin both in *cis* and in *trans* (i.e. on adjacent cells) have been identified. Binding of the receptor-type protein tyrosine phosphatase β (RPTPβ) to contactin in *trans* can stimulate signaling by contactin. A transmembrane protein, Caspr (contactin-associated protein), binds contactin in *cis* (Peles et al 1997). Contactin was shown to provide a link between RPTPβ and Caspr in adjacent cells. Caspr is a good candidate for a *cis* signaling partner for contactin because it contains a mosaic of domains implicated in protein-protein interactions (Peles et al 1997). In particular, a Pro-rich region in the cytoplasmic domain of Caspr was shown to bind in vitro to SH3 domains of a subset of proteins, including those of Src and Fyn. (However, interaction between Caspr and Src in vivo could not be demonstrated.) Caspr associates with a Triton-insoluble fraction that might be either DRMs or cytoskeleton (Einheber et al 1997).

Contactin also binds the transmembrane neuronal immunoglobulin-superfamily members Ng-CAM (Brümmendorf et al 1993) and Nr-CAM (Morales et al 1993), although the role of this interaction in signaling through contactin is not clear. Interactions between contactin, L1 (the mouse homologue of Ng-CAM), and Fyn were demonstrated by coimmunoprecipitation from mouse brain (Olive et al 1995).

Several other neuronal GPI-anchored receptors can mediate signaling upon ligand binding; one example is Thy-1 (Doherty et al 1993). In other cases, such as the receptors for ciliary neurotrophic factor (CNTF) (Economides et al 1995), glial cell neurotrophic factor (GDNF) (Massagué 1996), and the closely related neurturin (Buj-Bello et al 1997), multi-subunit receptor complexes contain a GPI-anchored protein component. GPI-anchored proteins can also act as ligands for EPH-family receptor tyrosine kinases in adjacent cells (Davis et al 1994).

OTHER CELLS Signaling through GPI-anchored proteins appears to be uncommon in cells other than hematopoietic cells or neurons. However, uPAR can mediate signaling in a variety of mammalian cells. uPAR is the receptor for the urokinase-type plasminogen activator (uPA), which cleaves plasminogen to plasmin. Signaling through the uPA/uPAR complex has been implicated in cell adhesion, migration, and differentiation independently of the enzymatic activity of uPA (Yebra et al 1996, Planus et al 1997). Its *cis* interactions with integrins appear to be important in this signaling, as was mentioned above for interactions between β_2 integrins and uPAR in neutrophils. For example, uPAR cooperates with the integrin αvβ5 in mediating migration of carcinoma cells on vitronectin (Yebra et al 1996). In another study, embryonic kidney cells were shown to bind fibronectin via a β1 integrin (Wei et al 1996). Expression of uPAR (which binds vitronectin in vitro) in these cells prevented them from binding to fibronectin. However, the transfected cells now bound vitronectin, in a manner that required

both uPAR and the integrin cytoplasmic domain. This study showed that uPAR and the integrin could cooperate to mediate binding of cells to vitronectin.

ROLE OF RAFTS IN SIGNALING EVENTS It is not known whether association of GPI-anchored proteins with rafts is important in any of these signaling pathways. The role of rafts may differ between proteins and between cell types. At one extreme, the fact that soluble forms of the GPI-anchored subunits of CNTFR and GDNFR can substitute for the GPI-anchored forms (Economides et al 1995, Massagué 1996) suggests that these proteins do not need to associate with rafts in order to signal.

In other cases, the presence of GPI-anchored and associated signaling proteins in DRMs and/or caveolae suggests that localization of the proteins in rafts may be important. For instance, DRMs containing both GPI-anchored proteins and Src-family kinases can be isolated from hematopoietic cells (Brown 1993) and neurons (Olive et al 1995, Zisch et al 1995). In addition, uPAR, β_2 integrins, and Src-family kinases have been isolated from monocytes in the same DRMs (Bohuslav et al 1995), and uPAR, integrins, and caveolin formed complexes in transfected fibroblasts, suggesting that the protein complex might localize to caveolae in these cells (Wei et al 1996). Another study has also shown that certain integrins can be coimmunoprecipitated with caveolin in fibroblasts (Wary et al 1996). It should be cautioned, however, that the presence of two proteins in the same DRM does not demonstrate that they interact (Mayor & Maxfield 1995a). For example, even if they did not interact directly, two proteins in DRMs might be coimmunoprecipitated if the entire DRM were isolated in the process. In fact, proteins could be linked via association with DRMs even if, by some criteria, they appear to be fully solubilized. As an example, although a GPI-anchored protein could no longer be pelleted by centrifugation after detergent extraction at 37°C, it still had a low density, thus indicating that it associated with DRM lipid (Naslavsky et al 1997).

There may be two distinct paradigms for signaling through GPI-anchored proteins. The first and best characterized model is specific binding of GPI-anchored proteins with signaling capability to one or more transmembrane proteins in *cis*. As discussed above, in some cases, such as CNTFR and GDNFR, association of these complexes with rafts does not appear to be crucial for signaling. In other cases, such as the binding of FcγRIIIb or uPAR to β_2 integrins (described above), association of the complexes with rafts may facilitate signaling.

A second paradigm may be found in some hematopoietic cells (for example, T cells) that can be stimulated by clustering of virtually any GPI-anchored protein (Robinson 1991). This lack of specificity could result from binding of an unidentified linker protein to the GPI anchor itself. However, it is interesting that no GPI-anchored proteins in T cells have been shown to bind β_2

integrins (Stockinger 1997) nor have any transmembrane signaling partners for GPI-anchored proteins in T cells been identified. This may mean that signaling uses a different mechanism; GPI-anchored proteins may not bind directly to transmembrane signaling partners. Instead, rafts that are formed or stabilized when GPI-anchored proteins are clustered may play an important role in signaling through GPI-anchored proteins in T cells. For instance, transmembrane proteins that are downstream of GPI-anchored proteins in signaling might be concentrated and activated simply by partitioning into these rafts, without binding the GPI-anchored protein directly. It is even conceivable that in some cases no transmembrane linker protein is involved and that rafts in the outer bilayer leaflet are somehow coupled to rafts in the inner leaflet, possibly through monolayer coupling, as mentioned above (Schmidt et al 1978).

In either of these models, how would clustering of GPI-anchored proteins enhance signaling? This would depend on the structure of rafts in the membranes (Brown & London 1998). If rafts normally contain only a few molecules, then clustering of GPI-anchored proteins would cause small rafts to coalesce, bringing raft-associated transmembrane proteins close together. Alternatively, clustering of GPI-anchored proteins might induce formation of rafts in a previously uniform membrane, allowing recruitment of transmembrane proteins with an affinity for rafts.

Signaling in Caveolae

Several groups have found that a variety of cell-surface signaling pathways are concentrated in caveolae or in low-density plasma membrane domains (Anderson 1998). [However, in contrast, one group failed to detect an enrichment of signaling molecules in purified caveolae (Stan et al 1997).] Several signaling proteins, including heterotrimeric G proteins, Ras, Src, endothelial nitric oxide synthase (eNOS), and protein kinase C have been reported to bind caveolin directly and to be inactivated when bound (Oka et al 1997, Okamoto et al 1998). It should be noted that another group failed to detect binding of G proteins to caveolin or an effect of caveolin on the activity of $G_{o\alpha}$ (Huang et al 1997).) Exciting recent studies have shown that eNOS can be up- or down-regulated by alternative binding to either caveolin or calmodulin (Feron et al 1998). Thus with the divergent reports as caveats, most studies point to an important role for caveolae as signaling centers, as reviewed in detail elsewhere (Anderson 1998, Okamoto et al 1998). The role of the caveolar lipid environment in signaling remains to be explored.

CELL-SURFACE PROTEOLYSIS

Two cell-surface proteases may be regulated by association with rafts or caveolae. The first is uPA. uPAR (the receptor for uPA) appears to be constitutively

localized to caveolae in melanoma cells, as detected morphologically (Stahl & Mueller 1995). As most GPI-anchored proteins localize to caveolae only after cross-linking (Maxfield & Mayor 1997), uPAR may be concentrated there either by binding to other caveolar proteins, or through an unusually high affinity for an ordered lipid environment. Treatment of cells with nystatin or filipin to disrupt caveolae inhibits cell-surface plasmin generation by uPA, suggesting that caveolar localization may regulate uPA enzymatic activity (Stahl & Mueller 1995).

A second example is the coagulation cascade on endothelial cells. This involves tissue factor (TF), a transmembraneous protease receptor. Briefly, a proteolytically active complex formed by TF and the serine protease factor VIIa can be inhibited by binding of other components (Sevinsky et al 1996). During this down-regulation process, the complex was shown to move into caveolae and gain an affinity for DRMs by associating with an unidentified GPI-anchored protein (Sevinsky et al 1996). The complex in DRMs was inhibited more than would be expected from binding of inhibitory proteins, suggesting that the membrane environment itself has an additional inhibitory effect. Thus regulating the activity of cell-surface proteases such as uPA and the TF complex may be an important function of rafts and/or caveolae.

SECRETORY AND ENDOCYTIC PATHWAYS

Membranes of the Golgi, TGN, and endocytic pathway can contain significant amounts of cholesterol and sphingolipid (Steer et al 1984, Coxey et al 1993, Cluett & Machamer 1996) and may have some l_o-like character. In agreement with this idea, a GPI-anchored protein first becomes Triton-insoluble in the medial Golgi during biosynthetic transport (Brown & Rose 1992). However, there is no direct evidence that rafts form in intracellular membranes. Because these membranes are difficult to access with externally added probes, the morphological approaches that have provided evidence for raft formation in the plasma membrane have not been applicable.

Regardless, it has been suggested that rafts function in sorting of lipids and proteins in the secretory and endocytic pathways. This could explain how the distinct lipid compositions of the plasma membrane and organelles of the secretory pathway are maintained in the face of membrane traffic in both directions through the pathway. Lipids could be sorted by preferential inclusion of rafts in nascent transport vesicles. Alternatively, rafts might be selectively excluded from the vesicles. Furthermore, sorting of cargo proteins could be coupled to lipid sorting if the proteins partitioned preferentially into rafts (Bretscher & Munro 1993, Simons & Ikonen 1997). As described in the next sections, such sorting mechanisms have been proposed to operate at several steps in the secretory and endocytic pathways.

Sorting in Epithelial Cells and Neurons

Coupled sorting of lipid microdomains and proteins was first proposed by Simons and van Meer in a model for sorting of apical and basolateral proteins and lipids in polarized epithelial cells (Simons & van Meer 1988). Glycosphingolipids are more abundant in apical than basolateral membranes. To explain this distribution, glycosphingolipid-rich rafts containing apically directed proteins were proposed to form in the TGN and to be packaged into apical transport vesicles. A similar mechanism has been proposed to mediate sorting of axonal and dendritic membrane proteins in neurons; axonal proteins may associate with rafts (Dotti et al 1991).

The behavior of GPI-anchored proteins initially appeared to provide experimental support for the raft model (Brown & Rose 1992). These proteins, which partition into DRMs during biosynthetic transport, are targeted apically in epithelial cells. This correlation suggested that DRM association might lead to apical targeting. This suggestion was strengthened by other evidence (independent of DRM association) that GPI anchors were apical sorting signals (see below). Thus the idea that the anchors mediated sorting by partitioning into rafts was very appealing. However, as discussed next, further findings forced a re-evaluation, and showed that—apart from DRM association—there is no evidence that GPI anchors play a role in apical sorting. Furthermore, although several apical proteins in intestinal cells associate with DRMs (Danielsen 1995), DRM association does not correlate well with apical targeting of transmembrane proteins in MDCK cells (Sargiacomo et al 1993, Melkonian et al 1995, Arreaza & Brown 1995).

The evidence implicating GPI anchors in sorting came from studies of hybrid proteins expressed in MDCK cells (Brown et al 1989, Lisanti et al 1989). For example, PLAP-G, a fusion protein containing the extracellular domain of the GPI-anchored placental alkaline phosphatase (PLAP) linked to the transmembrane and cytoplasmic domains of the vesicular stomatitis virus glycoprotein (VSV G), is targeted to the basolateral membrane (Figure 3). One interpretation of this result is that GPI anchors are apical sorting signals. However, the data are equally consistent with the opposite conclusion: that signals in the cytoplasmic domains of the basolateral proteins contain positive signals. In fact, cytoplasmic domains of basolateral proteins are now generally believed to contain such positive signals. Furthermore, the apical localization of two additional hybrid proteins, PLAP654t (Casanova et al 1991) and PLAP-HA (Arreaza & Brown 1995), showed that the GPI anchor is not required for apical targeting of PLAP (see Figure 3 for details). In addition, PLAP-HA is detergent soluble, indicating that DRM association is not required for apical targeting.

Apical/basolateral sorting now appears to be very complex and may involve a hierarchy of sorting signals (Mays et al 1995, Yeaman et al 1997, Simons &

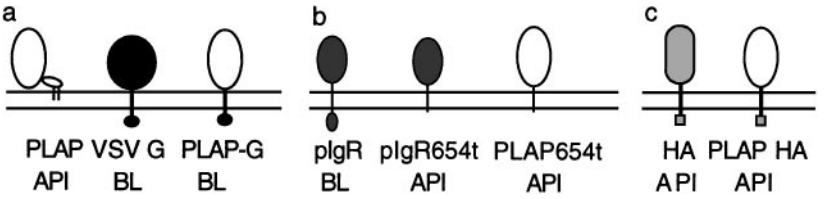

Figure 3 The GPI anchor is not required for apical sorting of PLAP. (*a*) Placental alkaline phosphatase (PLAP), a GPI-anchored protein, is apical, wheras PLAP-G, a hybrid protein containing the extracellular domain of PLAP and the transmembrane and cytoplasmic domains of the basolateral vesicular stomatitis glycoprotein (VSV G), is basolateral (Brown et al 1989). One explanation of this result is that the GPI anchor of PLAP is an apical signal. Alternatively, a dominant signal contributed by VSV G might specify basolateral targeting of PLAP-G. (*b*) The polymeric immunoglobulin receptor (pIgR) is targeted basolaterally by a signal in the cytoplasmic domain (Casanova et al 1991). pIgR654t (a truncated pIgR with a two–amino acid cytoplasmic domain) is apical (Casanova et al 1991). PLAP654t (PLAP fused to the transmembrane and cytoplasmic domains of pIgR654t) is also apical, indicating that GPI anchorage is not required for apical targeting of PLAP. (*c*) Influenza hemagglutinin and the PLAP HA hybrid are both apical (Arreaza & Brown 1995). As the short cytoplasmic domains of PLAP HA and especially PLAP654t are unlikely to contain apical signals, apical sorting of PLAP, PLAP654t, and PLAP HA may require a signal in the PLAP extracellular domain. Such a signal would be recessive to a basolateral signal in the cytoplasmic domain of VSV G, explaining basolateral targeting of PLAP-G.

Ikonen 1997, Weimbs et al 1997). It is generally believed that basolateral sorting signals are located in the cytoplasmic domains of proteins to be sorted, whereas apical signals are in extracellular domains. If two conflicting signals are present in the same protein, one may be dominant and specify targeting. Thus apical transport of PLAP could be mediated by a signal anywhere in the protein, if this signal is recessive to a basolateral signal in the cytoplasmic domain of PLAP-G.

On the other hand, studies showing that reducing cellular cholesterol or sphingolipid levels can lead to selective mis-sorting of apical and axonal proteins support the raft model. For instance, inhibition of sphingolipid synthesis caused mis-sorting of a GPI-anchored protein in MDCK cells (Mays et al 1995). In addition, inhibition of sphingolipid synthesis with fumonisin caused mis-sorting of a GPI-anchored protein that is normally targeted to axons in primary hippocampal neurons, and increased its Triton solubility (Ledesma et al 1998). Depletion of cellular cholesterol by 60 to 70% in MDCK cells caused mis-sorting of the apical influenza hemagglutinin (HA) protein, which associates with DRMs (Keller & Simons 1998). However, in another study, a more modest reduction in cellular cholesterol in these cells did not affect sorting of a GPI-anchored protein, although its detergent solubility was increased (Hannan & Edidin 1996).

Although the raft model for sorting is appealing, several questions about it have been raised (Weimbs et al 1997). For example, it is surprising that DRMs

are rich in sphingomyelin (Brown & Rose 1992), which is not apically targeted (Simons & van Meer 1988), and that several basolateral proteins associate with DRMs (Sargiacomo et al 1993, Melkonian et al 1995). Further study will be required to conclusively demonstrate the involvement of rafts in apical sorting.

Cholesterol and Protein Sorting in the Golgi

There is a gradient of cholesterol across the Golgi cisternae, with higher levels on the *trans* side (Coxey et al 1993). To explain this gradient, it was proposed that cholesterol-rich membrane domains are selectively transported forward through the Golgi toward the plasma membrane (Bretscher & Munro 1993). Cholesterol-poor domains may be left behind or actively transported in the opposite direction by retrograde transport. As plasma membrane proteins often have longer transmembrane spans than do Golgi proteins, they might partition preferentially into the thicker cholesterol-rich domains, leaving Golgi proteins in cholesterol-poor domains in the Golgi. In support of this model, mutagenesis studies showed that the same protein can be directed to either the Golgi or plasma membrane by altering the length of the transmembrane span (Munro 1995). In addition, model membrane studies have shown that insertion of a hydrophobic peptide into a bilayer requires the proper relationship between membrane width (which is affected by cholesterol content) and peptide length (Ren et al 1997, Webb et al 1998).

ER to Golgi Transport of GPI-Anchored Proteins in Yeast

ER to Golgi transport of GPI-anchored proteins in yeast is selectively retarded when sphingolipid synthesis is inhibited (Skrzypek et al 1997, Sütterlin et al 1997). This suggests that rafts form in the ER and that GPI-anchored proteins must partition into them for efficient transport (Sütterlin et al 1997). However, the concentration of sphingolipids in the yeast ER is very low (Patton & Lester 1991), arguing against spontaneous formation of rafts. Additional information came from an in vitro budding assay, measuring packaging of the GPI-anchored protein Gas1p into transport vesicles that bud from ER membranes. The assay was performed using ER membranes from either wild-type yeast or from a mutant strain that is defective for sphingolipid synthesis and for ER to Golgi transport of Gas1p in vivo (Sütterlin et al 1997). Gas1p packaging was equally efficient in both cases. Thus slow transport of GPI-anchored proteins in the mutant does not result from an inability to associate with any sphinoglipid-rich rafts in the ER, but from defects in a step after vesicle budding.

Rafts in Endosomes

A provocative observation suggests that rafts exist in endocytic compartments in fibroblasts. A GPI-anchored protein delivered to early endosomes after internalization was found to recycle to the cell surface more slowly than bulk membrane

(Mayor & Maxfield 1995b). However, after depletion of cholesterol (Mayor & Maxfield 1995b, Maxfield & Mayor 1997) or sphingolipids (Chatterjee et al 1997), GPI-anchored proteins recycle as fast as bulk membrane. Association of GPI-anchored proteins with rafts in the endocytic pathway may slow their recycling.

RAFTS AND DISEASE

Some pathogens may take advantage of the ordered state of mammalian cell membranes for infection. For example, fusion of simian forest virus with endosomal membranes during infection requires both sphingolipid (Nieva et al 1994) and cholesterol (Phalen & Kielian 1991). Another virus, SV40, can be internalized into mammalian cells via caveolae and then delivered to the lumen of the ER (Stang et al 1997). Internalization of pathogenic *Escherichia coli* via caveolae may promote pathogen survival by preventing fusion of phagosomes with lysosomes (Baorto et al 1997). Some evidence suggests that aerolysin toxin (Abrami et al 1998), cholera toxin (Tran et al 1987), and Shiga toxin (Sandvig et al 1996) enter mammalian cells via rafts. As detailed elsewhere, rafts may also be involved in prion diseases, Alzheimer's disease, and cancer (Anderson 1998). Further work will show whether the properties of rafts can be used in treatment or prevention of any of these diseases.

ACKNOWLEDGMENTS

We thank C Dotti, MG Roth, and K Simons for sharing unpublished data. Work on this article was supported by National Institutes of Health Grants GM 47897 (to DAB) and GM 48596 (to EL).

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