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Review

The GPI-anchor and protein sorting

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Abstract. Glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) are a diverse class of proteins that are anchored to the membrane solely via means of a post-translational lipid modification, the GPI-moiety. Since their discovery in the late 1970s, years of research have provided significant insight into the functions of this ubiquitous modification. In addition to the structure and biosynthesis of the GPI-moiety, perhaps the best-studied feature of this glycolipid is its ability to impart characteristic membrane-trafficking properties to the proteins that it anchors. Study of the mechanism of sorting of GPI-APs has brought to light the importance of lateral heterogeneities in cell membranes, termed *rafts*, in biological sorting processes. The focus of this review is to examine the emerging role of the GPI-anchor and mechanisms involved in GPI-AP sorting in the context of intracellular trafficking pathways.

Key words. GPI-anchor; membrane trafficking; sorting; endocytic pathway; biosynthetic pathway; rafts; signaling; sphingolipid; cholesterol.

The structure and biosynthesis of the glycosylphosphatidylinositol-moiety

The observation that certain proteins are likely to be anchored to membranes via phosphatidylinositol (PI)-specific phospholipase C (PI-PLC)-releasable lipidmodifications was originally made in the late 1970s when highly purified PI-PLCs from bacteria were shown to specifically release alkaline phosphatase (AP), acetylcholinesterase (AChE) and 5′-nucleotidase from cell membranes of a variety of mammalian tissues [1–4]; phospholipases with other specificities were unable to duplicate the effect of PI-PLC. That proteins are anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI)-moiety became evident in 1985 when the structure of the glycolipid anchor was characterized by analyses of the membrane anchors of *Torpedo* electric ray organ AChE [5], human erythrocyte AChE [6], rat brain and thymocyte Thy-1 [7], and the variant surface glycoprotein (VSG) protein of *Trypanosoma brucei* [8, 9]. These studies led to the characterization of the structure of the GPI-anchor (fig. 1). Below we summarize salient features of the structure and biosynthesis of the GPI-anchor. In addition, we refer readers to recent reviews which contain similar comprehensive accounts [10–16].

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Structural diversity of GPI-moieties

The discovery of the phenomenon of GPI-anchoring resulted in an explosion in research into the distribution, structural analysis, biosynthesis, and function of this ubiquitous protein modification in many animal and cell types. Several studies have now established that GPI-APs represent a large class of functionally diverse proteins (table 1). The only common feature among these proteins is their attachment to the exoplasmic leaflet of the membrane via a post-translational lipid modification, the GPI-anchor (fig. 1) [10, 13, 15].

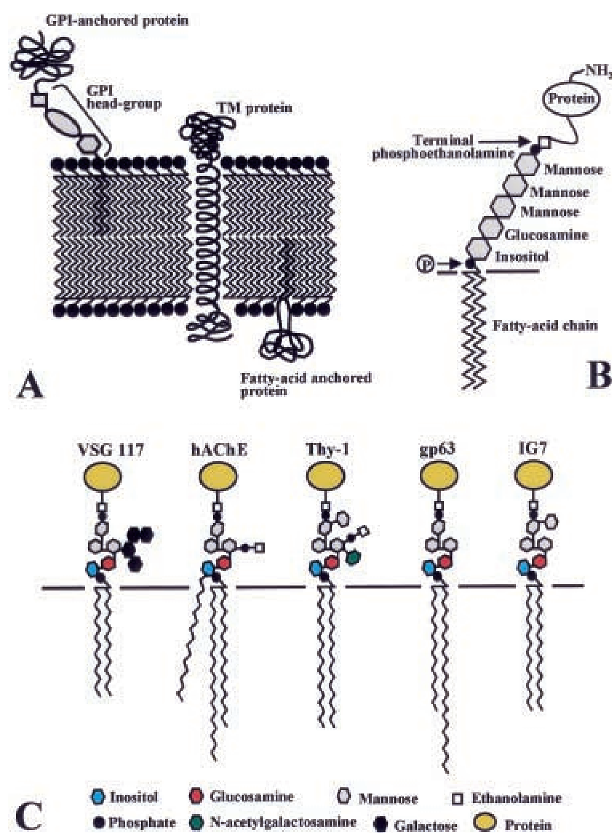

Figure 1. (*A*) Membrane topology of GPI-anchored proteins in comparison to transmembrane (TM) proteins and fatty-acid linked proteins. (*B*) Core residues of the GPI-anchor. (*C*) Comparison of representative GPI-anchored proteins from *Trypanosoma brucei* (VSG 117), human erythrocytes (hAChE), rat brain (Thy-1), *Leishmania major* surface protease (gp63), and *T. cruzi* antigen (IG7).

Table 1. Functional diversity of GPI-anchored proteins.

Enzymes Acetylcholinesterase alkalinephosphatase, 5′-nucleotidase, dipeptidase, lipoprotein lipase, ART1 ART2 (RT6)	*Surface antigens* Thy-1, Ly-6 (TAP), Qa-2, Sca-2, CD24, CD48 (sgp-60), CD52 (CAMPATH-1), CD-5 (DAF), CD59, CD73, cerebroglycan, ceruloplasmin, prion proteins
Adhesion molecules NCAM, ApCAM, OBCAM F3/F11/contain), TAG-1 (axonin-1), fasciclin II, BIG-1, BIG-2, neurotrimin, LFA-3	*Other (lower eukaryotes)* Variant surface glycoprotein (VSG), SSp-4, sialic acid acceptor, 160-kDa flagellar antigen, CS protein, MSP-1 protease, MSP-2, MSP-4, gp63 metalloprotease, PSA-2, PARP/procyclin, GP-2
Receptors Folate receptor, CNTFR-α GDNFR-α, CD87 (uPAR) NTNR-α, CD14, glypicans FcγRIIIb,	

On the basis of the glycan structure, GPI-moieties may be classified into three classes [13]: type-1 which have a Manα1-6Manα1-4GlcNα1-6PI motif, type-2 which have a Manα1-3Manα1-4GlcNα1-6PI motif, and type-3 containing a hybrid GPI with a Manα1-6(Manα1-3)Manα1-4GlcNα1-6PI motif. The GPI-moiety present in GPI-APs belongs to the type-1 class. There are close to 100 distinct GPI-APs identified so far [14], only a few of which are listed in table 1. The core structure of the GPI-anchor consists of a single phospholipid spanning the exoplasmic membrane leaflet and a complex head-group consisting of a phosphodiester-linked inositol, a glucosamine linked to the inositol, a linear chain of three mannose sugars linked to the glucosamine, and a phosphoethanolamine (P-EtN) linked to the terminal mannose residue (fig. 1B). The protein is attached to the GPI via an amide bond between the carboxy-terminal residue of the protein and the amino group of the P-EtN (fig. 1B). The core structure of the GPI-anchor is conserved across all species studied so far [10, 14].

Variation in the structure of the GPI-anchor arises both due to compositional differences in the lipid portion and to numerous (protein- cell-, or species-specific) side-chain substitutions on the tetrasaccharide backbone of the conserved head-group (fig. 1C) [10, 15]. The lipidic part of most GPI-anchors is made of diacyl chains (e.g., *T. brucei* VSG [8], *Torpedo* AChE [17]). In a number of cases, however, the lipidic portion of the GPI-anchor consists of alkyl-acyl chains (e.g., decay-accelerating factor, DAF [18], erythrocyte AChE [19], placental alkaline phosphatase [20], and folate-binding protein [21]), although other lipid structures are also present [15]. Further diversity in the core structure of the GPI-anchor arises due to an additional fatty acid ester linked to the inositol ring of many GPI-APs, rendering them resistant to cleavage by PI-PLC (human AChE in erythrocytes, fig. 1C [19]; PARP in *T. brucei* [22]; human alkaline phosphatase [23]). Diversity also arises due to side-chain substitutions of an additional one or two P-EtN residues on conserved mannoses seen in many GPI-anchors from different species (e.g., hAChE, fig. 1C [24]; Thy-1, fig. 1C [25]; gpi10 [26]). One of the most prominent reasons for GPI-anchor diversity is glycan substitution on the conserved mannose residues in several different species (see fig. 1C) [10, 15].

GPI-moieties in a cell are not always found covalently linked to proteins; many cells have a large pool of GPIs that are non-protein-linked or 'free' [10, 15]. These include free GPI-anchors which could represent excess material that is not consumed in protein or glycoconjugate anchoring, or type II GPIs that could be precursor end-products of the GPI biosynthetic pathway [15]. Some prominent examples of free GPIs in lower eukaryotes (e.g., *Leishmania* and trypanosomes) include glycoinositolphospholipids (GIPLs; containing type I, type II, or

the hybrid type III, glycans) and lipophosphoglycan (LPG; containing type II glycan) [11, 13, 27].

GPI-APs are widely distributed in eukaryotic organisms [11, 14]. In mammals and other vertebrates, GPI-APs have been found in almost all tissues and cells examined. They have been found in representatives from many other groups of eukaryotes, including protozoa, yeast, slime molds, nematodes, molluscs, and insects [14]. Prokaryotes such as *Mycobacterium lepre* and *M. tuberculosis*, on the other hand, have abundant GPI-like molecules that contain only mannoses in the glycan backbone and are often linked to mannan and arabinan polymers instead of a protein [28, 29]. Recently, a prokaryotic protein, ice-nucleation protein, has been reported to be present in GPI-linked form in the bacterium *Pseudomonas syringae* [30].

Biosynthesis of the GPI-moiety and GPI-APs

Biosynthesis of the GPI-moiety takes place primarily in the endoplasmic reticulum (ER) membrane system [12, 15]. The complete GPI-anchor structure is fully assembled prior to attachment to the protein via a series of enzymatic steps that sequentially add the various GPI components. In mammals many of the enzymes involved in each step have been identified as *PIG* gene products [15, 16]. Topological and enzyme location studies have revealed that the first two steps in GPI biosynthesis commencing with the addition of N-acetyl-glucosamine to PI and its subsequent deacetylation occur on the cytosolic side of the ER membrane [31–33]. In *T. brucei*, the remaining steps in GPI biosynthesis also occur on the cytoplasmic face of the ER [34], but in mammals, the topology of steps subsequent to deacetylation is not clear [15]. Attachment of the GPI to the protein involves cleavage of the lumenally located transmembrane-anchored pre-protein at a loosely defined signal (hydrophobic stretch) close to its transmembrane domain followed by attachment of the cleaved ectodomain to the fully assembled GPI via a transamidation reaction [12]. The components of the transamidase enzyme have been recently identified in yeast and their homologues have been found in mouse and humans [35–37]. The protein-anchoring step resulting in the addition of the GPI-moiety to the protein must occur in the lumen of the ER, as the protein including its GPI-attachment site is lumenally disposed [34, 38]. Consistent with this, the protein components of the transamidase complex (Gpi8p and Gaa1p) have a membrane topology in which the bulk of the protein is lumenally oriented [35, 36]. The lumenal location of the GPI-addition step suggests that cytoplasmically located GPI precursors have to be flipped across the bilayer into the lumen before attachment can proceed. A flippase/translocase might be necessary for the process but it has not yet been identified.

Membrane trafficking and sorting pathways inside cells

After attachment of the GPI-anchor, GPI-APs are transported to the cell surface via the 'canonical' secretory pathway [39]. These proteins are then subject to the biosynthetic and endocytic sorting processes available to molecules of the endomembrane system of cells. The sorting events and sites in the context of both biosynthetic and endocytic pathways are shown in figure 2; each membrane discontinuity in a cell offers a potential site for the sorting of proteins and lipids which is key to the main-

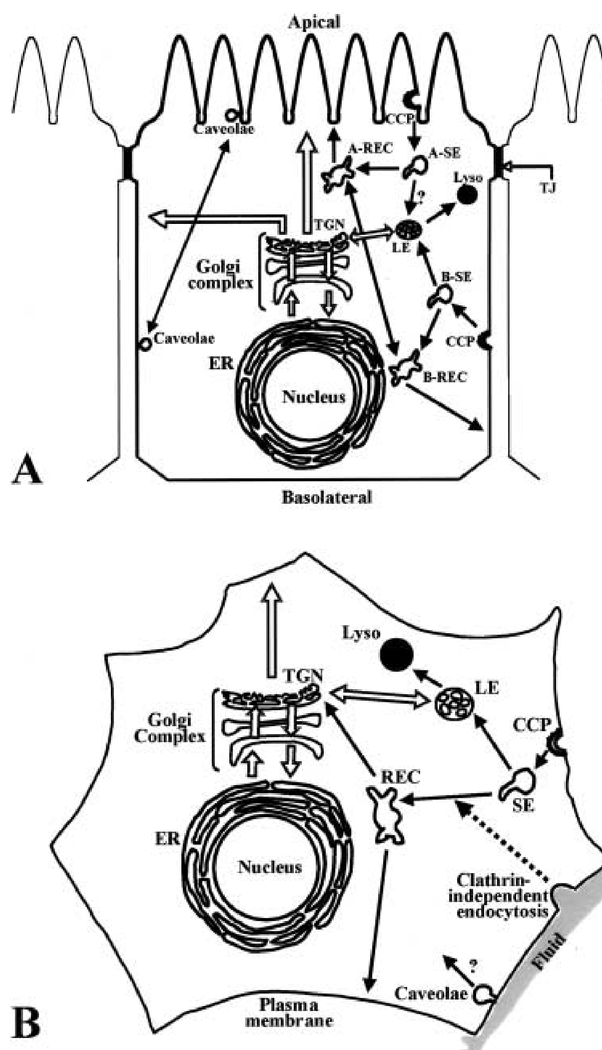

Figure 2. Sorting steps in the biosynthetic (thick open arrows) and endocytic (thin solid arrows) pathways in polarized MDCK epithelia (*A*) and non-polarized CHO fibroblasts (*B*). The transcytotic route (in *A*) is shown as a double-headed solid arrow. CCP, clathrin-coated pit; A-SE, apical sorting endosome; B-SE, Basolateral sorting endosome; A-REC, apical recycling endosomal compartment; B-REC, basolateral recycling endosomal compartment; LE, late endosome; Lyso, lysosome; TGN, trans-Golgi network; ER, Endoplasmic reticulum; TJ, Tight junction.

tenance of their structural and functional identity. In both polarized and non-polarized cells, the distinct composition of subcellular compartments is maintained by dynamic sorting processes.

In the biosynthetic pathway, sorting is a key feature of trafficking from the ER. Anterograde fully folded secretory or membrane proteins are sorted from their misfolded counterparts or from the ER resident proteins prior to their packaging into Golgi-targeted transport intermediates [40, 41]. Soluble ER resident proteins that escape the ER along with the forward-moving cargo are specifically retrieved back from the post-ER compartments via the coatomer protein complex I-mediated retrograde pathway [40, 42]; a process that aids in the maintenance of ER composition.

Traffic from the Golgi to the cell surface is not a default process: the trans-Golgi network (TGN) is a major site for the segregation of molecules destined to different locations inside the cell. This is particularly evident in polarized cells, in which the plasma membrane is demarcated into two functionally distinct domains, the apical and basolateral domains in the case of epithelial cells, and axonal and dendritic membrane domains in neurons. These domains differ in their protein and lipid compositions [43–45]. Development and maintenance of the polarized distribution of proteins and lipids in these cells is now understood to be due to sorting of protein and lipid cargo via several means including distinct trafficking routes from the TGN and, in some cases, by transcytosis (interdomain exchange of molecules via the endocytic pathway) [46–48].

Sorting of proteins is also a characteristic feature of endocytosis; many transmembrane receptors [e.g., transferrin receptors (TfRs) and low-density lipoprotein (LDL) receptors (LDLRs)] are segregated into clathrin-coated pits prior to internalization [49–51]. Subsequently, the majority of these receptors (e.g., TfRs and LDLRs, lacking a lysosome-specific targeting signal) are further sorted in the sorting endosomes (SEs) from the lysosomally directed cargo and either delivered to the recycling endocytic compartment (REC) [49, 50] or recycled to the plasma membrane [52]. The REC, a highly tubular endocytic compartment, often located in a pericentriolar location in non-polarized cells, is the site from which receptors are recycled to the plasma membrane. Certain proteins that recycle between the TGN and plasma membrane are delivered to the TGN via the endocytic pathway [53, 54], involving the REC, as in the case of TGN 38 [53], or involving the mannose-6-phosphate (M6P) receptor (MGPR) pathway from the late endosomes, as in the case of furin [54]. At the conjunction of the biosynthetic and endocytic pathways, lysosomes are maintained primarily by sorting and separate packaging of lysosomal cargo in the Golgi [e.g., sorting of M6P-linked proteins via M6PRs], and sometimes by sorting and delivery of the lysosomal proteins via the endocytic route from the plasma membrane (e.g., Lamp1) [55].

The mechanisms that bring about sorting of most transmembrane proteins in the context of various trafficking pathways have been characterized in some detail. The common theme in these sorting processes is the recognition of specific sequence motifs in the cytoplasmic domain of transmembrane proteins by specific cytosolic sorter proteins. For example, sorting of receptors during clathrin-mediated endocytosis is mediated by direct protein-protein interaction between the tyrosine-based signal motif (of the type YXXϕ) in the cytoplasmic tail of endocytosed receptors and the specific cytosolic multimeric adaptor protein complex, AP-2 [56, 57]. In the Golgi, sorting of lysosomally targeted M6PRs into clathrin-coated pits appears to occur via recognition of signal sequences in the cytoplasmic domain of these receptors by the multimeric adaptor protein complex, AP-1 [58, 59]. However, a recent report contesting this has instead suggested a retrograde sorting role for AP-1 that aids in recycling M6PRs from the late endosomes to the TGN [60]. Another example is the sorting of lysosomal membrane glycoproteins via the adaptor protein complex, AP-3 [61, 62]. Sorting of basolaterally destined proteins in polarized epithelia is directed by cytoplasmic tail signal sequences which include tyrosine or dileucine-based motifs followed by a stretch of acidic residues [63]; a novel type of adaptor protein complex has been shown to be necessary for this sorting step [64]. Regardless of the exact step in sorting, an essential feature of adaptor protein complex-mediated sorting is that it involves direct protein-protein interactions.

Sorting is not solely based on protein sequences: N- and O-linked glycosylations can act as sorting determinants for trafficking of proteins to specific cellular destinations in the biosynthetic pathway [65–71]. In some cases the mode of lipid attachment also serves as a targeting signal for the protein. For example, palmitoylation acts as a signal for targeting of apolipoprotein B (apoB) to large vesicular structures corresponding to a subcompartment of the ER [72]. Many cytoplasmically located soluble proteins are also translocated to specific membrane-bound compartments following lipid modifications. Targeting of Ras-GTPases (Hras and Nras) to specific cellular membranes is dependent on lipid modifications; isoprenylation of a CAXX motif and its subsequent carboxymethylation is necessary for targeting of the protein to the endomembrane system (e.g., ER, Golgi) while its subsequent palmitoylation is necessary for targeting to the plasma membrane [73]. Fatty-acyl modifications found in the non-receptor protein tyrosine kinase family (NRPTKs) are sufficient to target green fluorescent protein (GFP) to specific cellular locations [74].

Intracellular distribution of GPI-APs

The intracellular distribution of GPI-APs and free GPIs has been studied in some detail. A large fraction of GPI-APs are found on the cell surface anchored to the extracellular leaflet in both lower and higher eukaryotes [10, 14]. Recent detailed studies in a protozoan parasite, *Leishmania,* using biotinylation, fluorescence-activated cell sorting (FACS) analyses, and ultrastructural analysis have shown that GPI-APs are located in various cellular compartments including the cell surface, the ER, the Golgi, and in exo- and endocytic structures [75]. In mammalian cells, following biosynthesis, GPI-APs traverse the Golgi system, prior to delivery to the plasma membrane, as revealed by the presence of Golgi-enzyme-modified forms of a GPI-AP, placental alkaline phosphatase [76]. GPI-APs are also found in vesicular transport intermediates during their biosynthetic trafficking [77, 78]. GPI-APs have also been shown to be extensively associated with the endocytic system in mammalian cells [79–81]. Some studies had suggested that GPI-APs are present in flask-shaped plasma membrane invaginations, called caveolae, but these results were later shown to be artifactual (see below). Free GPIs are widely distributed among cellular compartments [15]. Unlike their protein-anchored counterparts, free GPIs are likely to be present on both lumenal (or extracytoplasmic) and cytoplasmic leaflets of the membrane [15]. They have been found to be evenly distributed in the apical and basolateral domains in epithelial cells [82]. Trafficking of free GPIs to various cellular locations could occur via any of the traffic options available to lipids [83, 84] including lipid transfer carrier proteins, contact-site-mediated transfer, and vesicular transport [15].

Functions of the GPI-anchor: a survey

The striking conservation of the core structure of the GPI-anchor from yeast to mammals suggests that this modification is of prime functional significance [14]. However, any functional argument is compounded by the fact that many GPI-APs can function equally well when substituted by a transmembrane proteinaceous anchor. For example, GPI-anchored and transmembrane versions of DAF show equal efficiency in protection from complement-mediated cytotoxicity [85]. Furthermore, T-lymphocyte activation via CD73 (5′-nucleotidase) occurs equally well when the GPI-anchor is replaced with a transmembrane domain [86]. Some GPI-anchored proteins (e.g., CD58) occur naturally in both GPI-anchored and transmembrane isoforms, which do not show functional dissimilarities [87]. In the case of several other GPI-APs, GPI-anchoring appears to be an absolute requirement for their function. For example, efficient and regulated uptake of folate by GPI-anchored folate receptors is abolished when the GPI-anchor is replaced by a transmembrane protein domain [88]. The GPI-anchor is critical for Ly-6A/E-mediated T cell activation [89]. Binding of T-cadherin to lipoproteins is dependent on the presence of the GPI-anchor [90]. Recently, the GPI-anchor was shown to be necessary for functioning of the tectal neuron protein, CPG15; axonal arborization and synapse maturation are inhibited in a truncated form of CPG15 that lacks the GPI-anchor [91].

The sensitivity of the GPI-anchor to cleavage by the enzyme PI-PLC was key to identifying the lipid-based anchoring of GPI-APs. Thus, at the very least, the GPI-moiety undoubtedly provides a more stable anchorage to proteins than other lipid-based anchors like myristoyl, palmitoyl, and farnesyl groups. The latter modifications found in a variety of cytosolic proteins allow only weak transient membrane anchorage [92, 93]. Does GPI only serve as a membrane anchor? If so, why do certain proteins have a GPI-anchor instead of a more stable transmembrane protein domain? The GPI-anchor does appear to be responsible for slower turnover rates of GPI-APs compared to transmembrane proteins, especially those that are internalized by clathrin-coated pits, suggesting that the presence of the GPI-anchor may endow proteins with a longer plasma membrane residence time [94–96]. Whether this property of the GPI-APs is important for cellular physiology is not clear.

The plasma membrane of many parasitic protozoans (e.g., trypanosomes and *Leishmania*) are abundant in GPI-APs. In these organisms, the GPI-moiety, beside providing membrane anchorage, serves to greatly increase the packing density of GPI-APs (e.g., VSG in trypanosomes) without compromising the membrane bilayer required for functioning of other membrane proteins [10]. The high packing density of GPI-APs mediated by the GPI-anchor and the space-filling effected by numerous side-chain glycan modifications on the GPI lead to formation of a ‘glycocalyx’ that presumably aids in the formation of a protective coat against harsh conditions of the host gut or blood [10]. Moreover, the GPI-anchor may allow rapid switching between 1000 or more antigenic variants of VSG in trypanosomes which may be a crucial step in evasion of the host immune response [97]. GPI-APs are not sufficiently abundant in the plasma membrane of higher eukaryotes to allow such a high packing density. Therefore, their role as a protective coat is unlikely to be manifest in higher eukaryotic cells.

In mammalian cells, the GPI-moiety, besides acting as a membrane anchor, has been shown to provide GPI-APs with the capacity to transduce signals across the bilayer resulting in several intracellular responses: oxidative burst, Ca^{2+} influx, protein tyrosine phosphorylation, cytokine secretion, and proliferation or inhibition of growth

are only some examples [98–100]. A prerequisite for signaling via GPI-APs is the cross-linking of these proteins [98–100]. Most signaling studies of GPI-APs have been carried out using antibody-mediated cross-linking of the proteins. However, how GPI-APs may be cross-linked under physiological conditions has not been clarified. A likely scenario for cross-linking of GPI-APs at the cell surface is homotypic interactions across cells between dimeric cell adhesion molecules such as Tag-1 [101]. Co-immunoprecipitation studies have revealed that GPI-APs are associated with cytosolic NRPTKs like $p56^{lck}$, $p59^{fyn}$, Hck, and Fgr, suggesting that signaling via GPI-APs involves tyrosine phosphorylation [100].

In some instances, GPI-APs interact specifically with transmembrane proteins capable of intracellular signaling. This appears to modulate ligand-receptor interactions and the intracellular signaling capacity of these transmembrane proteins. GPI-anchored cell surface receptors like glial-cell derived neurotrophic factor (GDNFR-α) and neurturin (NTNR-α) have been found to interact with Ret, a transmembrane receptor tyrosine kinase [102, 103]. In myeloid cells, three GPI-linked receptors, CD14 [lipopolysaccharide (LPS) receptor], FcγRIIIb (CD16b), and CD87 (urokinase-type plasminogen activator receptor) are involved in lateral interactions with the β2 integrin CR3 [104]. Furthermore, the efficiency of signaling via FcγRIIa (a transmembrane protein of the FcγR family of IgG receptors) in neutrophils and transfected T cells depends on co-clustering with GPI-anchored FcγRIIIb [105]. However, whether the signal transduction due to these interactions is similar to the signaling via GPI-APs mediated by antibody cross-linking is not known. As discussed above, direct protein-protein interaction of the GPI-AP protein ectodomain and a transmembrane protein that serves to link the GPI-APs to the cytosolic signaling components is only one of the several possibilities by which GPI-APs can transduce a signal across the membrane bilayer. Other mechanisms for signaling via GPI-APs may include lectin-like interaction of signaling-competent transmembrane proteins with the GPI-moiety. Alternatively, GPI-APs may indirectly modulate the activity of the cytosolic signaling molecules via their association with membrane rafts, maintained by purely lipidic interactions (see below).

Several GPI-APs are known to be involved in cell-cell interactions (table 1) and the anchor is thought to provide a means to rapidly regulate the formation and breakage of adhesion contacts [106]. The nervous system has a repertoire of GPI-anchored cell adhesion molecules [107] that have been postulated to play a role in neurite outgrowth by regulated release of these proteins [108, 109]. The released adhesion molecules themselves have been shown to posses different biological functions including outgrowth and fasciculation of neurites [110]. Some adhesion molecules such as NCAM, ApCAM, and fasciclins exist in both GPI-linked and transmembrane isoforms, and the differential expression, degradation, or localization of these forms may have functional significance. For example, the transmembrane form of ApCAM has been hypothesized to promote fasciculation of axons, while the GPI-linked form stabilizes synaptic contacts [111]. Recently, the GPI-anchored and transmembrane isoforms of *Manduca sexta* adhesion molecule fasciclin II (MFasII), expressed in a cell-type specific manner, were shown to perform different functions; the GPI isoform, expressed in glial cells and newly generated neurons, functions strictly in cell-adhesion whereas the transmembrane isoform, expressed in differentiating neurons, aids in cell motility and fasciculation [112]. The GPI-anchored isoforms of the human carcinoembryonic antigen (CEA) class of intercellular adhesion molecules are overexpressed in cancer cells while the transmembrane isoform is down-regulated [113]. Ectopic expression of GPI-anchored isoforms of CEA leads to inhibition of myogenic differentiation while the transmembrane isoform promotes differentiation [114], suggesting that the transmembrane isoform acts as a tumor suppressor. Additionally, cross-linking of the adhesion molecules can lead to signal transduction events similar to the situation in lymphocytes. This may have several regulatory roles, including axonal chemoattraction and repulsion, growth cone collapse, and target-induced differentiation [115]. Cross-linking of F3 on neurons and transfected cells indeed results in increased association with Fyn, and enhanced tyrosine phosphorylation [116, 117].

Being lipid-anchored, the GPI-APs may be released in an intact form from the membrane and reincorporated into them with relative ease compared to transmembrane proteins [118]. Transfer of GPI-APs either between cells or between liposomes and cells, called hopping or painting, is quite often observed in vitro [119–121]. Such a mechanism of transfer in vivo could be a potential means to acquire new GPI-APs, and hence new properties, by cells that normally lack these proteins [122]. One study attributed the presence of GPI-anchored DAF and CD59 on endothelial cells, normally lacking these proteins and hence potentially susceptible to complement-mediated immune attack, to intercellular transfer of these proteins from circulating erythrocytes [123]. However, the possibility that the presence of GPI-APs on endothelial cells is due to adherence of fragments of erythrocytic membrane was not ruled out in these studies. Post-testicular acquisition of certain GPI-APs by spermatozoa (that normally do not synthesize these proteins) has been speculated to occur via intercellular transfer from cells of the male genital tract [124].

GPI-APs can also be released from the plasma membrane by the action of endogenous hydrolases including PI-PLC, PI-specific phospholipase D (PI-PLD), and GPI-specific PLC [125] in both lower and higher eukaryotes

[126, 127]. In *T. cruzi,* PI-PLC-mediated release of GPI-anchored proteins, SSp-4, and the polymorphic family of trans-sialidase/sialidases has been observed [128–130]. Endogenous PI-PLC releases the GPI-anchored 76-kDa serine protease in *Plasmodium falciparum* and *Plasmodium chaboudi* which is thought to be necessary for the activation of the enzyme [131, 132]. GPI-PLC-mediated release of VSG in trypanosomes has also been reported [133]. Such a release may be vital to rapidly switch the surface expression of VSG (several variants of which are expressed by trypanosomes [97]) to escape immune attack [125]. Studies in some higher eukaryotes have shown that the soluble form of GPI-anchored 5′-nucleotidase present in membrane extracts of the electric organ of *Torpedo marmorata* (electric ray) and bovine cerebral cortex is due to the release of the protein by a PI-specific phospholipase C (PI-PLC) [127]. Release of the cancer marker CEA into the extracellular medium of colonic epithelial cells has been attributed to endogenous PI-PLC-mediated release of the GPI-anchored form of CEA [134]. However, the functional importance of release via these phospholipases remains unclear.

The GPI-anchor and sorting in the biosynthetic pathway

Perhaps, the clearest function for the GPI-anchor is in providing GPI-APs with specific membrane-trafficking properties distinct from those of proteins that are transmembrane anchored, despite the fact that GPI-APs lack a proteinaceous transmembrane anchor and thus cytoplasmic extensions. One of the earliest clues to the significance of the GPI-anchor in imparting specific trafficking properties came from studies of GPI-APs in the biosynthetic pathway of polarized epithelial cells. All detectable endogenous GPI-APs (110-, 85-, 70-, 55-, 38-, and 35-kDa proteins) in polarized MDCK cells were specifically targeted to the apical domain [135]. Since then, several studies have confirmed that the GPI-anchor acts as a targeting signal for the apical transport of GPI-APs in many different polarized epithelial cell types [135–137]. These studies also showed that proteins that are normally targeted to the basolateral domain when attached to a GPI-anchoring sequence not only acquire the GPI-anchor but are also targeted to the apical domain [135, 136]. In neurons, many GPI-APs are specifically targeted to the axonal membrane [45, 138]. However, the localization of GPI-APs in neurons appears to be more complex, depending on cell type and developmental stage [139].

Targeting of GPI-APs to the apical membrane domains is not without exceptions. In Fischer rat thyroid (FRT) cells, six out of nine endogenously expressed GPI-APs are directly delivered to the basolateral domain, the other three being apically disposed [140]. In these studies, 50% of a transfected GPI-AP, DAF, was localized to the basolateral domain while another protein, herpes simplex gD-1, fused to the GPI-anchoring signal sequence of DAF (gD-1-DAF) was exclusively localized to the basolateral domain. The apical targeting role of the GPI-anchor has been recently challenged by the fact that the ectodomain of many GPI-APs in the absence of a GPI-anchor is apically secreted, suggesting that the protein ectodomain of these GPI-APs may contain apical targeting signals [141]. An exception to the apical-targeting rule of the GPI-anchor is also seen in a mutant MDCK cell line (MDCK II, concanavalin A resistant), defective in an unknown glycosylation step. In these cells, two of the five endogenous GPI-APs show equal distribution to the apical and basolateral surfaces [137]. Recent studies have also questioned the view that GPI-anchors act as apical-targeting signals in MDCK cells. Benting and co-workers [68] showned that N-glycans are sufficient to target any protein (transmembrane or GPI-anchored) from the Golgi to the apical domain in normal MDCK cells. These observations, however, do not rule out the apical-targeting function of the GPI-anchor; instead, they suggest that other targeting signals, as yet not fully characterized, might come into play, possibly in a complex hierarchical manner or, in certain cases, in a cell-specific manner, during apical transport of proteins [70, 141].

Besides acting as an apical-targeting signal or, more precisely, as a component of an apical-targeting signal, the GPI-anchor also acts as a sorting determinant at an early step in biosynthetic trafficking, i.e., exit from the ER. In yeast cells, a GPI-AP, Gas1p, appears to be sorted and separately packaged from other cargo molecules in the ER membrane during their exit to the Golgi [78, 142]. The ER transport protein Emp24p interacts directly with Gas1p and when mutated (Emp24pΔ) causes a defect in Gas1p packaging in the ER but not of other cargo proteins like Gap1p and gpαF [142]. Recently, Muniz et al. [78] demonstrated conclusively that Gas1p is indeed present in ER-to-Golgi trafficking intermediates that are devoid of other cargo markers.

The GPI-anchor and sorting in the endocytic pathway

For the endocytic pathway of mammalian cells there is considerable controversy regarding the precise pathway followed by GPI-APs. However, there is a general consensus that these proteins are trafficked differently from other membrane components. In 1988, like most other plasma membrane components, GPI-anchored folate receptors were found to be internalized and, subsequently, recycled to the plasma membrane [143]. This study also found that during its intracellular passage, FR-GPI passed through an acidic compartment where the folate ligand bound to the receptor was presumably released. In-

vestigating the mechanism of FR-GPI internalization, Rothberg and co-workers [144, 145] found that these proteins were present in caveolae. Based on these findings, the authors proposed a mechanism for folate uptake, termed potocytosis [146]. According to this model, FR-GPIs (and possibly other GPI-APs) bound to their ligand are enriched in caveolae and are sequestered from the external environment by transient closure of the caveolae from the plasma membrane. The caveolae subsequently undergo acidification causing release of the ligand from the receptor. This mechanism, however, had to be significantly revised when it was shown that GPI-APs are not constitutively enriched in caveolae [147–149]; they may be induced into a caveolar localization due to cross-linking of the GPI-APs that occurred during immunolocalization studies. Fluorescence microscopy and ultrastructural analysis of FR-GPI distribution using fluorescently-labeled and gold-conjugated primary antibody to FR-GPI, respectively, revealed that GPI-APs were not enriched in caveolae [147], but addition of secondary antibody caused redistribution of GPI-APs into caveolae. Schnitzer and co-workers have purified caveolae from the rest of the plasma membrane using a (detergent-free) cationic colloidal silica coat method, and have shown the lack of GPI-AP enrichment in caveolae [150]. These findings reopened the question as to how GPI-APs are internalized from the cell surface.

Investigations into the mechanism of GPI-AP internalization by a number of groups have led to the conclusion that these proteins are not internalized via a clathrin-dependent mechanism. Drugs that effectively blocked internalization of transferrin and H2 class I molecules known to be internalized via clathrin-mediated endocytosis were unable to block internalization of GPI-APs, Thy-1, and Ly6-A.2 in lymphocytes [151]. Keller and co-workers [79] using both fluorescence and electron microscopy have found that in CHO cells, GPI-APs are internalized via non-clathrin coated vesicles. Recently, GPI-AP-specific toxins have also been used to study the endocytic fate of GPI-APs. Skretting and co-workers [152] have shown that internalization of diphtheria toxin, that binds to a genetically engineered GPI-AP form of the diphtheria toxin (DT) receptor, occurs independently of clathrin-coated pits. Overexpression of the dominant-negative form of dynamin that blocks clathrin-mediated endocytosis was unable to block GPI-receptor-mediated DT internalization, and depletion of cholesterol (in ways known to inhibit caveolae formation) also did not affect DT internalization. However, the authors also found that binding of the toxin could not be completely inhibited using PI-PLC, an enzyme which completely hydrolyzes GPI-anchored proteins in PI-PLC-sensitive lines; DT binding is relatively insensitive to PI-PLC treatment even when the GPI-anchored form of the receptor is used. Therefore, if potentially non-GPI-anchored forms of the receptor are also involved in the pathways studied by the authors is not clear. Based on studies with *Helicobacter pylori* vacuolating toxin (VacA) which requires GPI-APs for internalization and subsequent vacuolation, blocking clathrin-mediated internalization does not affect the internalization of VacA [153]. However, the stoichiometry and specificity of binding of the toxins to endogenous receptor molecules is unclear; VacA forms higher-order oligomers [154] and may induce clustering of potential GPI-anchored receptors with attendant consequences [147]. The binding of the toxin is reported to be 'non-specific,' and the intoxication process alters endosomal morphology [153]. These characteristics of the two studies preclude the authors from making specific claims about the route and mechanism of GPI-AP internalization.

Recent studies in our laboratory have revealed that the GPI acts as a sorting signal for targeting GPI-APs into a novel internalization route distinct from the caveolae and clathrin-mediated pathways. At very early times of internalization, GPI-APs are found enriched in compartments termed GPI-AP-enriched endosomal compartments (GEECs) [155; S. M. Sabharanjak and S. Mayor, unpublished data]. These compartments also contain the bulk of the endocytosed fluid phase but are devoid of markers of the clathrin-mediated pathway [155; Sabharanjak and Mayor, unpublished data]. GPI-APs are delivered to the REC from peripheral GEECs via tubular intermediates [Sabharanjak and Mayor, unpublished data]. There has been considerable confusion in the literature [including recent overviews; ref. 51] regarding the very existence of such a 'non-coated' pathway, precisely because of the lack of specific markers to confirm its existence and to study its function [50, 156]. The discovery of the GPI-anchor as a specific sorting signal for this pathway will aid in the understanding and detailed characterization of this constitutive endocytic process, which also accounts for the major portion of the fluid-phase uptake.

Characterization of the endocytic trafficking pathways of GPI-APs has shown that regardless of their mode of internalization, these proteins, during their intracellular passage, accumulate inside cells in compartments that are also enriched for the recycling markers of the clathrin-mediated pathway [81] and the fluid phase [80]. Kinetic studies of the endocytic trafficking of GPI-APs have brought to light interesting differences in the properties of GPI-APs compared to other endocytic markers. GPI-APs are internalized at a rate similar to that of those membrane components that lack any specific internalization signals. Measurement of GPI-AP recycling rates have revealed that they are recycled to the plasma membrane at a three- to fourfold slower rate than most recycling membrane components including TfRs. The slower recycling manifests as a longer retention of GPI-APs in endosomes compared to other recycling markers [81].

The observation that endocytosed GPI-APs are present in two distinct compartments, namely the GEECs and the REC, raises the question as to which of the two sites is responsible for retention of GPI-APs. The location of endocytic retention has different implications for the mechanism of GPI-AP sorting. A retention of GPI-APs due to slower exit from GEECs would suggest that slower recycling of GPI-APs is a characteristic property of these compartments. On the other hand, if the REC is the rate-limiting step in GPI-AP recycling, for retention of GPI-APs to occur, they would have to be sorted/segregated from other recycling components in this compartment, as suggested by previous studies [81]. In recent studies from our laboratory, using FR-GPI as a model GPI-AP, the rate-limiting step has been identified as the exit from the REC [157]: GPI-APs accumulate in the REC at a rate which is comparable to the overall recycling rate of GPI-APs, whereas in the GEECs, they accumulate at a two-fold faster rate [157]. Presence of the GPI-anchor is necessary for the successful retention of GPI-APs, as their transmembrane isoforms exit the REC at a rapid rate, similar to that of the transmembrane recycling marker, TfR [157]. These observations show that GPI-APs are sorted/segregated from other recycling components in the REC.

Mechanism of GPI-AP sorting

As discussed above, the lack of a cytoplasmic tail for interactions with cytoplasmic adaptor protein complexes poses a singular problem in understanding the mechanism of GPI-AP sorting from other membrane molecules. Several studies have now shown that cholesterol and sphingolipids are required, often specifically, for the sorting of GPI-APs. In polarized epithelia, targeting of GPI-AP to the apical domain of the plasma membrane is affected by cholesterol and sphingolipid depletions. In MDCK cells, depletion of sphingolipids leads to the missorting of GPI-anchored GP-2, whereupon it is equally distributed to the apical and basolateral surfaces; sorting of a basolateral marker, E-cadherin, remains unaffected under these conditions [158]. Moreover, the effect of sphingolipid depletion on GPI-AP apical sorting is fully reversed by addition of a short-chain ceramide, *N*-6 [7-nitro-2,1,3benzoxadiazol-4-yl] aminocaproyl sphingosine galactoside (C_6-NBD-ceramide) [158]. However, unlike sphingolipid depletion, depletion of cholesterol has a more complex effect. In MDCK cells, depletion of cholesterol to ~25% does not affect apical localization of gD1-DAF, but there is a 40–50% reduced surface expression and a concomitant increase in the intracellular pool of this protein that does not co-localize with markers of the ER, Golgi, or the endocytic pathway [159]. Studying the effect of cholesterol depletion on another apical membrane marker, HA (hemagglutinin antigen protein of influenza viruses), hypothesized to utilize a mechanism similar to that of GPI-AP for apical delivery, Keller and Simons [160] found that upon cholesterol depletion (to ~60–70%), there was a 60% reduction in the apical delivery of HA in both polarized MDCK cells and non-polarized BHK cells. However, they also found that, unlike gD1-DAF [159], both HA and gp80 (an apically secreted glycoprotein) were significantly missorted to the basolateral domain upon cholesterol depletion. Under these conditions there was no defect either in the localization or the extent of delivery of a basolateral marker (VSV-G; vesicular stomatitis virus glycoprotein). The effect of cholesterol-depletion on GPI-AP trafficking was not determined in the latter study. The differential effect on the trafficking of apical markers seen upon cholesterol depletion could simply reflect a different extent of cholesterol depletion in the two studies.

The effect of cholesterol and sphingolipid depletions on GPI-AP biosynthetic trafficking has also been studied in cell types other than MDCK cells. In human monocytes, cholesterol depletion leads to a reduction in the surface localization of the GPI-AP, CD14, an effect similar to that observed for gD1-DAF in cholesterol-depleted MDCK cells [161]. In neurons, the GPI-AP, Thy-1, normally targeted to the axonal membrane, is missorted upon sphingolipid depletion, while delivery of a dendritic membrane marker under these conditions is not affected [162].

In the endocytic pathway, cholesterol depletion has a profound effect on retention of GPI-APs in CHO fibroblasts; upon cholesterol depletion, GPI-APs recycle at a faster rate, comparable to that of the other recycling components [81]. In recent studies, we have shown that sphingolipid depletion also leads to specific acceleration in the endocytic recycling of GPI-APs in CHO fibroblasts [157]. Depletion of sphingolipids either via drug treatments such as fungal toxins or D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol treatments [fumonisin B_1 and (PDMP)] or by growing mutant CHO cells [defective in sphingolipid biosynthesis due to a mutation in the serine-palmitoyltransferase (SPT) enzyme] in a sphingolipid-depleting medium causes the specific loss of the endocytic retention of GPI-APs; they recycle at a rapid rate, comparable to that of other recycling markers [157]. The effect of sphingolipid depletion on GPI-AP recycling is reversed upon exogenous addition of sphingolipid analogues [157]. These studies also suggest that the role of sphingolipids in regulating endocytic retention of GPI-APs is structural rather than due to signaling. First, sphingolipid analogues that are incapable of any known signaling activity are able to rescue the defect in endocytic retention of GPI-APs in FB_1-treated cells [157]. Second, inhibition of sphingolipid biosynthesis via means that do not result in the accumulation of any signaling-competent precursors as in SPT mutant CHO cells

or the accumulation of different precursor species upon treatment with the drugs FB_1 or PDMP have the same effect on GPI-AP retention [157]. The dependence of intracellular sorting of GPI-APs on sphingolipid and cholesterol levels suggests a mechanism distinct from those that rely primarily on protein-protein interactions (e.g., clathrin-mediated endocytosis) and indicates a role for lipid-based interactions.

Lateral heterogeneities, lipid trafficking, and the 'raft' hypothesis

The requirement for cholesterol and sphingolipids in the sorting of the lipid-anchored GPI-APs suggest that lipid heterogeneities may play a role in these sorting processes. The fluid-mosaic model, proposed by Singer and Nicolson [163], suggests that biological membranes are likely to be a homogenous two-dimensional fluid allowing free diffusion of its transmembrane protein and lipid constituents. This model proposed a short-range order which is probably mediated by specific protein (and perhaps protein-lipid) interactions leading to the formation of stoichiometrically defined aggregates within the membrane, whereas, organization of lipids or lipid-protein entities was proposed to be random over long ranges. Since then, numerous studies, particularly in artificial membranes, have led to the conclusion that homogeneity of artificial membrane bilayers at long ranges may be an exception rather than a rule. Theoretical models and a number of experimental techniques like differential scanning calorimetry (DSC), intervesicular transfer rates of various lipids, scattered spectroscopy, fluorescence quenching, fluorescence recovery after photobleaching (FRAP), and ultrastructural analysis have validated the existence of long-range heterogeneities or phase-separated domains in artificial membranes [164–166].

Broadly, the lipid bilayer can have three possible forms whose co-existence is temperature dependent: gel, liquid-disordered (l_d) and liquid-ordered (l_o) states. While the first is observed at very low temperatures (often below the T_m of constituent lipids of a membrane), the other two states co-exist at temperatures above the T_m of the constituent lipids [166]. It is the co-existence of the latter two states that manifests as phase-separation in membranes at physiological temperatures. Several parameters play a role in generating such phase differences [for a recent review see ref. 167]. Acyl-chain mismatch between constituent lipids in a liposome results in phase-separation of species with matched acyl chain lengths. One of the most commonly observed phase-separations is that of sphingomyelin and glycosphingolipids from other phospholipids in mixed bilayers [165, 168]. Compared to sphingomyelin, glycosphingolipids have a stronger tendency to phase-separate in liposomes. The higher degree of interaction of glycosphingolipids could be due to the hydrogen-bonding capacity of the sugar residues in addition to the packing offered by matched saturated acyl chains of these lipids. Cholesterol (and possibly other sterols) play a major role in modulating the size and extent of these phase-separated domains due to their planar 'rigid' structure that can potentiate the organization of liquid-ordered domains in membranes [164]. Indeed, the packing density of sphingolipids is higher in the presence of cholesterol than when phosphatidylcholine (PC) alone is present [169]. Using fluorescence-quenching and intervesicle-partitioning methodologies, Wang and Silvius [170] concluded that N-(diphenylhexatrienyl)propionyl (DPH 3:0)-labeled gluco- and galactocerebroside partition into sphingolipid-enriched domains in sphingolipid/phosphatidylcholine/cholesterol bilayers with substantially higher affinity than analogous phospholipids, like phosphatidylcholine, or ceramides, or sphingolipids with other polar head-groups (e.g., sphingomyelin). The authors suggest that the DPH3:0-labeled sphingolipids whose N-acyl chain length approximates the long saturated N-acyl chains of natural membrane glycosphingolipids are better suited for studying the 'raft' affinity of different sphingolipids because, unlike these, the short acyl chain analogues like C_6-NBD- and C_5-bodipy-labeled sphingolipids exhibit generally very weak affinities for sphingolipid/ cholesterol-rich domains. Although studies in artificial systems do not reveal the architecture of domains in living cell membranes, they provide guidelines for investigating this phenomenon in cells.

The strongest evidence for the existence of phase-separated domains in biological membranes has come from studies involving the role of sphingolipids and cholesterol in various cellular functions, including the membrane-trafficking studies of GPI-APs discussed earlier. However, prior to the studies of GPI-AP-trafficking, studies on the distribution and trafficking of lipids in polarized epithelial cells had provided clues to the existence of specialized domains in biological membranes [43]. Analysis of the lipid composition of a large number of polarized epithelial cell types revealed that the apical domain in these cells contains unusually high levels of glycosphingolipids compared to either basolateral membrane or plasma membrane of non-polarized cells: there is a two- to fourfold enrichment of glycosphingolipids and a two- to fourfold depletion of phosphatidylcholine in the apical compared to the basolateral membrane [43, 171]. Basolateral membrane, on the other hand, has a lipid composition similar to membranes of non-polarized cells [43, 172]. Investigating the mechanism by which the polarized distribution of glycosphingolipids is achieved, van Meer and co-workers [173] showed that the preferential delivery (two- to threefold over the basolateral domain) of a fluorescent lipid marker, C_6-NBD-glucosylceramide, in MDCK cells was due to intracellular sorting of this lipid class to the apical domain via the Golgi.

C_6-NBD-sphingomyelin was found equally distributed to both domains in these studies. The enrichment of C_6-NBD-glucosylceramide in the apical domain and C6-NBD-SM in the basolateral domain has also been found in other model cell systems, CaCo-2 and HepG2, and such differential localization is due to either preferential apical delivery via the Golgi (CaCo-2 cells [174]) or via transcytosis (HepG2 cells [175]).

However, not all glycosphingolipids appear to be preferentially delivered to the apical domain. Studying the biosynthetic delivery of radiolabeled C_6-galactosylceramide, van der Bijl and co-workers [176] came to the conclusion that while this class of glycosphingolipid was preferentially delivered to the basolateral domain of MDCK cells, it was delivered preferentially to the apical domain in CaCo-2 cells. The authors concluded that the sorting of lipids in polarized epithelia was a cell- and sphingolipid-type-specific phenomenon. Based on lipid- and GPI-AP-polarity studies, Simons and Ikonen [177] have postulated the existence of short- and long-range lateral organization that results from dynamic clustering of sphingolipids and cholesterol. These entities act as moving platforms or 'rafts' within the fluid bilayer with which specific proteins can associate (see fig. 3).

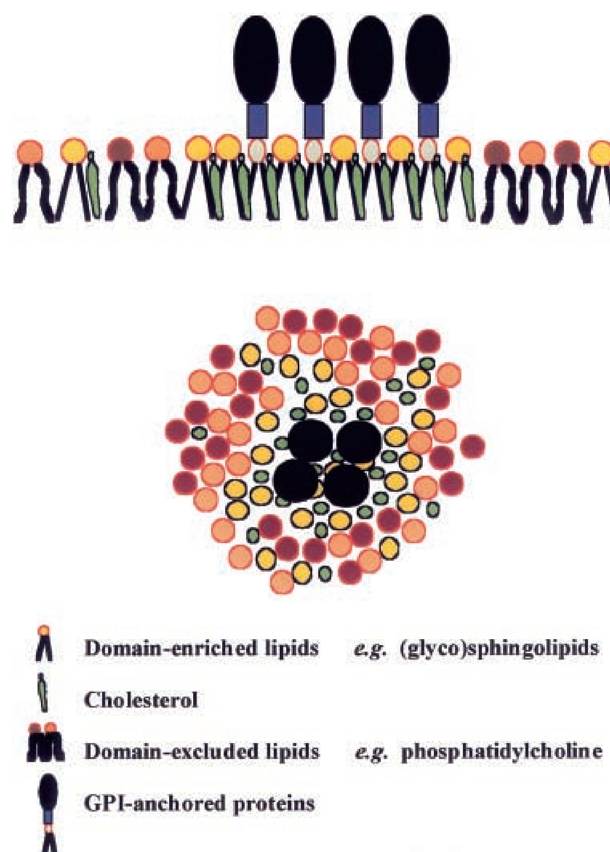

Figure 3. Domain organization of GPI-anchored proteins. GPI-anchored proteins are depicted in small, submicron-sized domains or rafts in association with other raft components, cholesterol and (glyco)sphingolipids, consistent with available information on the organization of these proteins. These domains or rafts are likely to be self-organizing and enriched in specific proteins and lipids, while excluding others, as proposed by the raft hypothesis (reproduced from Mayor and Kurzchalia [215]).

Evidence for rafts in living systems

The existence of rafts in biological systems has primarily been deduced from biochemical analysis of membrane lipids and proteins in detergent-resistant membrane fractions of various cell types. In 1992, Brown and Rose [76] demonstrated that in MDCK cells, GPI-APs en route to the apical domain become resistant to cold Triton X-100 (TX-100) solubilization in the TGN; the TX-100-insoluble complexes are low-density complexes since they float as buoyant membranes in the sucrose density gradient [76, 178]. Apart from GPI-APs, the authors also showed that certain sphingolipids (e.g., cerebrosides, sphingomyelin, lactosyl-ceramide, Forssman antigen, sulfatides, and the ganglioside GM3) were enriched in the detergent-insoluble complexes. Since then, several studies have identified a large number of components associated with such detergent-resistant complexes (also called detergent-resistant membranes or DRMs) including GPI-APs, a variety of sphingolipids, cholesterol, VIP21-caveolin, lipid-linked NRPTKs, and HA [179, 180]. These observations have led to the proposal that DRMs are biochemical equivalents of rafts in vivo [76, 177, 178]. In support of this hypothesis, depletion of either cholesterol or sphingolipids was shown to lead to increased solubilization of raft components including GPI-APs and HA which are otherwise detergent resistant [161, 181, 182].

Caveolae have been equated to rafts based on analysis of DRMs. First, the protein component of caveolae, caveolin, is also found enriched in DRMs [183]. Furthermore, caveolin itself is present as a stoichiometric complex with cholesterol [184] and has been shown to photo-cross-link to the glycosphingolipid, GM1 (monosialoganglioside 1) [185]. Since both cholesterol and sphingolipids are present in DRMs, these observations have led several investigators to propose that caveolae and caveolin are responsible for raft assembly [179, 180].

The mechanism by which proteins and lipids partition into DRMs has now been studied in some detail [reviewed in refs. 166, 186]. The requirement for saturated acyl chains in lipids appears to be important for their partitioning into DRMs [187]. However, co-purification with detergent-insoluble material does not itself alone constitute proof of pre-existing segregated domains in cells. Furthermore, the detergent itself may cause a coalescence or removal of components and hence may not reveal the true nature of rafts [188].

The contention that caveolae are responsible for raft assembly has been challenged based on studies on lympho-

cytes and neuroblastoma cells that lack detectable caveolin and caveolae. In these cells, GPI-APs, NRPTKs, cholesterol, and sphingolipids are still found in the TX-100-insoluble fraction [189, 190]. In endothelial cells, Schnitzer and co-workers [150] found that TX-100-insoluble isolates consist of a mixture of linear membrane sheets, large non-caveolar vesicles (>150 nm), free caveolar vesicles (<80 nm) and, occasionally, caveolae attached to the large vesicles. These studies showed that caveolae are not the only component of detergent-resistant membranes in cells that express high levels of caveolin. Therefore, to exclusively equate caveolae with 'detergent-resistant rafts' is incorrect. Moreover, electron microscope studies of the detergent-resistant membranes using gold-conjugated secondary antibodies to detect GPI-APs revealed that GPI-APs are mainly present in an annular ring at the neck of caveolae [150]. However, in these studies, redistribution of GPI-APs in unfixed agarose sections due to cross-linking by the application of gold-conjugated secondary antibodies was not ruled out.

The detergent-insolubility criterion of rafts itself requires critical examination. Sphingomyelin, a major component of the detergent-resistant complexes (and hence expected to be a raft component), unlike other proposed components of rafts, is enriched in the basolateral domain in MDCK cells [43]. Missorting of GPI-APs that occurs upon sphingolipid depletion in MDCK cells can be rescued by replenishment with short-chain sphingolipid analogues like C_6-NBD-ceramide [158] which are incapable of being incorporated into detergent-resistant complexes in cell membranes [E. R. Smith and V. L. Stevens, personal communication; L. J. Leung and E. Rodriguez-Boulan, personal communication; ref. 191]. A short-chain ceramide, N-hexanoyl-D-sphingosine, is also capable of rescuing the defect in endocytic sorting of GPI-APs in sphingolipid-depleted cells [157]. In addition, a moderate depletion of cholesterol results in the loss of the association of GPI-APs with detergent-resistant membranes, without affecting their sorting to the apical domain [159]. The inconsistent nature of the detergent-resistant criterion is further highlighted by the observations of Lipardi et al. [141]. They found that in FRT cells, the GPI-APs, PLAP (placental alkaline phosphatase) and the neurotrophin receptor-PLAP chimera (NTR-PLAP) are targeted to the apical domain in a sphingolipid-dependent manner. However, the insolubility of these proteins in cold TX-100 (>60%) is not significantly altered upon sphingolipid depletion compared to untreated controls [141]. Analysis of apical sorting and detergent insolubility of a variety of HA mutants (defective in apical delivery) revealed that while some HA mutants were incapable of being incorporated in DRMs other mutants (also defective in apical sorting) were still present in DRMs [192]. The authors concluded that association of HA with detergent-insoluble complexes is a necessary but not sufficient criterion for their apical targeting. The choice of detergent also has a bearing on the components detected in the insoluble fraction. Insoluble complexes of CHAPS-extracted cells show the presence of cholesterol and sphingolipids but not GPI-APs [193]. Together, these observations suggest that detergent-insoluble complexes are not true correlates of functional rafts. These observations also raise doubts about the validity of these biochemical methods for detection of rafts.

Detergent-free techniques capable of detecting rafts in living cell membranes have begun to gain considerable importance. Conventional fluorescence microscopy reveals that GPI-APs are uniformly distributed on the cell surface [81, 147]. Lack of detection of segregated GPI-APs in the plasma membrane is likely due to their arrangement in structures below the detection limit of fluorescence microscopy (>300 nm). Electron microscopy using gold-conjugated primary antibody to GPI-APs has also shown that these proteins are uniformly distributed at the cell surface [147–149]. However, this technique is insensitive and may be limited by fixation artifacts.

Several biophysical methods have also been used to address the issue of rafts in vivo [reviewed in ref. 194]. Using a single-particle tracking (SPT) method, one report showed that a fraction of glycosphingolipid, GM1, as well as Thy-1, a GPI-AP, are transiently confined to zones (TCZs) of 200–300 nm diameter in the plasma membrane [195]. These TCZs are diminished when glucosylceramide synthesis is inhibited [195]. Using SPT at higher temporal resolution, Pralle and co-workers [196] detected ~26 (±13)-nm-sized cholesterol-stabilized complexes of GPI-APs; cholesterol depletion causes increased diffusion of the GPI-APs. Two-dimensional surface scanning resistance measurements using large beads in a laser trap revealed non-elastic barriers of sizes frequently between 50–100 nm and less frequently between 200–300 nm [197]. The authors state that these barriers were not connected to form domains or regions of confinement. However, the inherent cross-linking of GPI-APs via antibody-coated beads and the absence of comparative measurements on a non-GPI-linked (transmembrane) protein make it difficult to correlate these results to rafts.

Studies from the laboratory of Kurzchalia [198], using classical chemical cross-linking techniques, have demonstrated that at least a fraction of GPI-APs are present in clusters of at most ten molecules at the cell surface; two to three molecules per cluster is the most common species. Using FRET-based methodology that measures the change in emission polarization upon homo-transfer of energy, Varma and Mayor [199] demonstrated the existence of cholesterol-dependent subresolution clusters of GPI-APs at the surface of living unfixed cells. A

FRET-based study carried out in MDCK cells revealed that the GPI-APs are randomly distributed in the plasma membrane [200, 201], arguing against the organization of GPI-APs at large length scales at the cell surface [194]. Recent evidence from our laboratory suggests that GPI-APs are likely to exist as small clusters at FRET-length scales in the plasma membrane of cells [R. Varma, S. Chatterjee, S. RoyChoudhury, M. Rao and S. Mayor, unpublished data]. These observations reconcile the apparently contradictory results from the two different FRET methodologies [M. Edidin; see http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2001/67/pe1].

Although many of these studies have provided clues to the existence of rafts at the level of the plasma membrane, virtually nothing is known about their existence in other cellular locations. On the other hand, there is functional evidence for the role of rafts in sorting processes at these sites, i.e., the TGN and endosomes. In this regard, biophysical techniques based on FRET offer a great deal of promise not only for detection of rafts but also in dissecting their architecture at these locations. However, it is still the functional properties of GPI-APs and other lipid-anchored proteins that provide evidence consistent with the existence of rafts or functional segregation of lipid-anchored components in living cells.

The GPI-anchor, rafts, and cellular physiology

GPI-anchor-dependent endocytic sorting is important for the physiology of some GPI-APs; replacement of the GPI-anchor with a transmembrane proteinaceous anchor leads to the loss of endocytic retention of FR-GPI [157] and a concomitant impairment in folate uptake [88]. The pathophysiology of GPI-anchored prion proteins is also likely to be critically dependent on endocytic sorting mediated by the GPI-anchor. Replacement of the GPI-anchor on the cellular prion protein prevents efficient conversion to the scrapie form in acidic endosomes [202]. Signaling via GPI-APs in lymphocytes and neurons is well documented, and abrogation in signaling activity is observed for many GPI-APs (but not all) when the GPI-anchor is replaced by a transmembrane domain [105, 203].

In many cases, along with the GPI-anchor, cholesterol and sphingolipid-dependent segregation of GPI-APs is essential for the biology of GPI-APs, suggesting that rafts are critical for the observed physiology. For example, folate uptake via FR-GPI is severely impaired in cholesterol-depleted cells [204], in which FR-GPI is no longer retained in the endosomes [81]. Folate uptake via FR-GPI is also severely impaired upon depletion of sphingolipids [205] possibly due to disruption of the sphingolipid-dependent endocytic-retention machinery [157]. These studies have a direct bearing on the observation that developmental abnormalities like cleft palate, which occurs due to folate deficiency, is also manifested in newborn mice as a result of fumonisin intoxication [206]; other effects of lowering of sphingolipids may also contribute to the observed pathology. Template-driven conversion of prion proteins into the infectious scrapie form is affected by both cholesterol and sphingolipid depletion [202, 207]. Signaling via GPI-APs in lymphocytes is also abrogated by cholesterol depletion [208]. These observations strongly implicate sphingolipid- and cholesterol-dependent rafts as critical determinants of GPI-AP function in sorting and signaling processes.

Rafts are likely to be involved in cellular functions other than GPI-dependent sorting and signaling; alteration of raft components also affects cell migration. Stimulation of cell migration by insulin-like growth factor 1 (IGF-1) results in redistribution of raft-associated components like ganglioside GM1, GFP-GPI, and ephrinB1 to the leading edge of the migrating cell, and the IGF-1-stimulated movement is severely affected upon cholesterol depletion [209].

Manifestation of pathological conditions also occurs as a result of defects in GPI-AP biosynthesis. For example, the pathology of the disease paroxysmal nocturnal hemoglobinuria is due to defects in GPI-AP synthesis in blood cells [210, 211]. Disease conditions due to genetic alterations (e.g., Niemann-Pick type C disease [212]), and environmental toxins (fumonisin B_1 [213]), and sphingolipid-storage diseases [214] that lead to changes in cholesterol and sphingolipid levels, respectively, may manifest their effects due to disruption of rafts involved in GPI-AP trafficking and signaling.

In conclusion, the mechanisms underlying GPI-AP protein trafficking and sorting are likely to have implications for understanding raft structure and function, and their role in cellular physiology.

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