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Shedding and Uptake of Gangliosides and Glycosylphosphatidylinositol-Anchored Proteins

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Summary:

Gangliosides and glycosylphosphatidylinositol (GPI)-anchored proteins have very different biosynthetic origin, but they have one thing in common: they are both comprised of a relatively large hydrophilic moiety tethered to a membrane by a relatively small lipid tail. Both gangliosides and GPI-anchored proteins can be actively shed from the membrane of one cell and taken up by other cells by insertion of their lipid anchors into the cell membrane. The process of shedding and uptake of gangliosides and GPI-anchored proteins has been independently discovered in several disciplines during the last few decades, but these discoveries were largely ignored by people working in other areas of science. By bringing together results from these, sometimes very distant disciplines, in this review we give an overview of current knowledge about shedding and uptake of gangliosides and GPI-anchored proteins. Tumor cells and some pathogens apparently misuse this process for their own advantage, but its real physiological functions remain to be discovered.

Gangliosides and glycosylphosphatidylinositol (GPI)-anchored proteins come from different biochemical pathways, but they have the same general assembly plan: they both have lipid anchors that tether them to the cell membrane and relatively large hydrophilic domains that protrude in the extracellular space. At the cell membrane gangliosides and GPI-anchored proteins form membrane microdomains called lipid rafts that are involved in the regulation and modulation of numerous cellular processes. Many reviews have been recently published on GPI-anchored proteins [1-4], gangliosides [5-9] and lipid rafts [10-14] and they should be consulted for more detailed consideration of these molecules and their functions. In this review we would like to look at them through a different perspective, not addressing the role of gangliosides and GPI-linked proteins in specific cellular processes, but focusing on their one peculiar characteristic: the ability to be released from the membrane of one cell and incorporated into the membrane of another cell. This process has been independently discovered and rediscovered in several research areas during the past decades, and named with different names, including shedding, release, incorporation, uptake, jumping and even cell-painting. In this manuscript we shall refer to as "shedding and uptake", as originally suggested by Ladisch and colleagues in 1983 [15].

Gangliosides

Gangliosides are a large family of complex glycosphingolipids that contain one or more sialic acid residues (Fig. 1). Based on variations in carbohydrate and ceramide structures, the ganglioside family comprises hundreds of molecular species [16]. Their nomenclature is very complex (IUPAC–IUB Commission on Biochemical Nomenclature 1978), but a simplified system developed by Lars Svennerholm is widely accepted [17]. In this system, the 'ganglio' core is designated by the capital letter G, which is followed by a capital letter designating the total number of sialic acids (M – mono; D – di; T – tri; Q – tetra; P – penta; A – asialo). This is followed by a number designating the length of the neutral 'ganglio' core, with 1 representing the full four-saccharide core and shorter structures having higher numbers. The number of sialic acids linked to the internal Gal residue is designated by a lower case letter (a = 1, b = 2, etc.) and the number of sialic acids linked to the GalNAc residue is designated by a Greek letter (α = 1, β = 2, etc.).

(Fig. 1)

Gangliosides are biosynthesized stepwise as shown in Fig. 2, starting with the addition of galactose in β-linkage to ceramide [18]. A key branch point in brain ganglioside biosynthesis is the addition of sialic acid(s) and/or GalNAc to the growing saccharide chain. Once GalNAc is added, no further sialic acids can be added to the internal galactose residue of the 'ganglio' core. This leads to the generation of ganglioside 'series' bearing no, one, two, or three sialic acids on the internal galactose residue. These have been designated the 0-series, the a-series, the b-series, and the c-series, respectively. Gangliosides exist in all cells, but they are most prominent in the brain whey they represent over 25% of conjugated carbohydrates [16, 19]. A large number of different gangliosides has been isolated from brains of various organisms, but only four structures, GM1a (from traditional reasons, name GM1 will subsequently be used instead of GM1a), GD1a, GD1b, and GT1b (Fig. 1B) constitute the great majority (>90%) of gangliosides in brains from various mammalian species [20]. However, although gangliosides dominate the complex carbohydrate coat (glycocalyx) of nerve cells, their physiological functions are largely undefined.

(Fig. 2)

In principle, gangliosides can function on the cell membrane in three ways: (i) as specific ligands for binding proteins such as MAG [21, 22] and cholera toxin [23]; as glycan arrays that interact with other glycan arrays on adjacent cells [24, 25]; and (iii) as organizers of lipid rafts that modulate activity of various proteins through lateral interactions in the same membrane [26]. In general, cell–cell recognition may occur when glycans on one cell surface bind specifically to complementary binding proteins (lectins) or carbohydrates on an apposing cell surface, whereas cellular regulation may occur through lateral interactions between glycans and signaling molecules on the same membrane [25].

Patterns of ganglioside expression change with cell growth, differentiation, viral transformation, ontogenesis and oncogenesis [24]. In the brain, gangliosides are expressed in cell-type and developmentally specific patterns [27-33]. The same is true in the peripheral nervous system [34], indicating that there is a tight regulation of ganglioside biosynthesis, degradation and intracellular transport [35]. Gangliosides were also reported to be involved in decisions regarding neural growth and myelination (reviewed in [7]), as well as in the development of new axons [36]. Consequently, the expression of specific brain gangliosides was considered to be essential for neuronal differentiation and brain development, but the

unexpectedly mild phenotype of mice deficient for complex gangliosides was a large surprise that significantly amended that hypothesis (reviewed in [9]).

Apparently when ganglioside biosynthetic pathways are blocked by deletion of a specific enzyme, the quantity of total gangliosides is often retained even though the structures are different [37]. Despite major changes in the expression of a particular ganglioside species associated, phenotypic alterations were found to be only subtle [38-40], indicating that none of the specific ganglioside structures is essential, and that different gangliosides (at least those more complex than GM3) can compensate for each other. In that context it is surprising that major brain ganglioside patterns are highly conserved among mammalian species [20, 41] and that major ganglioside polymorphisms have not been reported in the brain, although they have been described in other tissues [42]. Furthermore, recent observation that glycosphingolipid are essential for the development of *Drosophila melanogaster* [43], indicates that the lack of severe phenotype in mice deficient for complex gangliosides might be the consequence of some kind of a backup mechanisms for ganglioside functions that developed later in evolution.

Gangliosides modulate transmembrane signaling

An attractive new line of research of ganglioside function was opened in the nineties when it was found that gangliosides play an important role in the formation and maintenance of lipid rafts, which are supposed to mediate many signaling processes in the cell membrane. Lipid rafts have been extensively reviewed in the last few years [10-13, 44-48], and only some of their aspects will be presented here.

The first indication that gangliosides and other glycosphingolipids can associate and form patches in the cell membrane came from two independent lines of experiments in 1984. Spiegel and colleagues were studying fluorescently labeled gangliosides inserted into cell membranes and observed concerted moving of different gangliosides, that was actually a manifestation of association of gangliosides into lipid rafts [49]. The second line of evidence came from studies of Okada and colleagues who were investigating effects of detergents on membranes and concluded that some gangliosides are located within detergent-insoluble fraction of the membrane [50]. The hypothesis of glycosphingolipid-enriched membrane microdomains (lipid rafts) was formulated by Simons and van Meer in 1988 [51], but more convincing evidence that supported the hypothesis was provided nearly a decade later [26,

52]. Even though the existence and importance of lipid rafts in living cells is still being actively debated [53, 54], several lines of evidence strongly support this hypothesis: fluorescence resonance energy transfer measurements using fluorescent folate showed interactions of folate receptors when they are in proximity in rafts in living cells [55]; biochemical crosslinking demonstrated that GPI-anchored proteins are in proximity in rafts [56]; antibody crosslinking segregated raft proteins from non-raft proteins [57]; photonic force microscopy was used to determine the size of rafts in living cells [58], and electron microscopy was used to visualize clustering of rafts in IgE signaling [58]. However, it was recently shown that crosslinking or proteins inserted into the outer leaflet of the cell membrane through artificially attached lipid anchors can also induce activation of Jurkat T-cell-signaling responses, indicating that at least in some cases, the formation of artificial raft-like patches on the cell membrane might be sufficient to trigger signaling events [59]. In some signaling processes the formation of protein clusters in the membrane was reported to depend on protein-protein, and not protein-lipid interactions [60], thus although lipid rafts apparently play an important role in mediating many signal transduction processes (Table 1), they might be only one of several similar mechanisms.

(Table 1)

Glycosphingolipids in the plasma membrane are able to interact laterally with other membrane molecules modulating their properties (cis-interactions), and the dynamic clustering of sphingolipids and cholesterol in membrane microdomains represent the basis of lipid raft formation [26]. These structures move within the fluid bilayer and function as platforms for the attachment of proteins when membranes are moved around the cell and during signal transduction [61, 62]. The first convincing evidence for the involvement of gangliosides in the modulation of transmembrane signaling through the formation of lipid rafts came from studies of FcεRI, the receptor for IgE on basophils and mast cells. IgE binds constitutively to cell-surface FcεRI. Aggregation of FcεRI after binding of antigen to FcεRI-bound IgE activates the associated Src-family kinase, Lyn, and initiates a signaling cascade that culminates in degranulation. Colocalization experiments showed that the microdomains where tyrosine phosphorylation occurred were enriched in GM1. Fluorescently labeled FcεRI was found to be uniformly distributed in the plasma membrane of unstimulated cells and only transiently translocated to GM1-rich microdomains after antigen addition [63]

The role of gangliosides in the function of receptors for epidermal growth factor (EGF) and platlet-derived growth factor (PDGF) has been extensively studied (reviewed in [5]). GM3 was reported to inhibit dimerization of EGF receptor (EGFR), while GD1a was reported to induce dimerization of the same receptor [64]. Interestingly, the addition of GD1a caused significant EGFR dimerization even in the absence of the growth factor. GD1a apparently creates some kind of a "primed" state of the fibroblast cell membrane and sets the stage for enhanced responsiveness to EGF (Fig. 3). For PDGFR (PDGF receptor), the situation was exactly the opposite; GD1a was found to inhibit dimerization of PDGFR, while GM3 did not have any effect [65]. Because different rafts exist with unique ganglioside composition, specific gangliosides might target the respective receptors through direct interaction to unique rafts. It is also possible that different gangliosides compete to segregate receptors into different rafts resulting in different effects on their activity. Modifying membrane gangliosides through action of a membrane sialidase was reported to be essential for the development of new axons [36], so in addition to shedding, ganglioside composition on the membrane can also be rapidly altered by action of the membrane sialidase.

(Fig. 3)

Uptake of exogenous gangliosides into the cell membrane

Uptake of exogenous gangliosides into cells was first reported by Keenan and colleagues more than 30 years ago [66]. During the subsequent years exogenous gangliosides were administered to fibroblasts [67], astrocytes [68], HeLa cels [69], neuroblastoma cells [70], glioma cells [71], red and white human blood cells [72], as well as normal and leukemic lymphocytes [73]. Exogenously administered gangliosides showed a variety of biological effects depending upon the type of ganglioside and the target cell (for a review see [74]). Initially it was assumed that all exogenous gangliosides that became associated with cells were inserted into outer leaflet of the cell membrane, but subsequent studies demonstrated that exogenously administered gangliosides can be taken up by cells in three different ways: (i) as loosely associated micelles removable by serum proteins; (ii) as a protein-bound serum-resistant, but protease-sensitive ganglioside fraction; and (iii) as gangliosides associated in a protease-resistant manner [75-77].

Gangliosides form aggregates in aqueous media which, depending on their carbohydrate part and ceramide composition, form micelles of different sizes and shapes [78], or in the case of ganglioside GM3 even bilayer structures [79]. Ganglioside micelles appear to be quite stable structures and different micelles do not readily exchange their molecules [80] due to a low off-rate from micelles or membranes at room temperature [81]. When exogenous gangliosides are added to the cells, approximately 20% of micelles that had adhered to the cell surface can be removed by extensive washes with serum-containing media while another 30% of micelles are tightly bound to proteins and can only be released by treatment with proteases like pronase or trypsin [82]. In a relatively slow process most of bound ganglioside molecules eventually escape micelles and, after diffusion through the aqueous phase, insert into the cell membrane, where they behave as endogenous gangliosides [49]. From the analysis of the electron spin resonance spectra it could be shown that over 70% of the incorporated spin-labeled gangliosides are intermixed with other lipids of the host membranes, thus the protease-resistant fraction represent gangliosides incorporated in the cell membrane [77, 83]. The remaining (approx. 20%) could represent either ganglioside molecules clustered in microdomains or ganglioside micelles endocytosed by the cells. The rate of transfer depends on various parameters like ganglioside concentration, temperature, cell type and duration of incubation and can roughly be predicted using a formula developed by Saqr and colleagues [84]. When applied for 24–72 h at 37 ºC ganglioside GM1 incorporates into cultured fibroblasts in a protease-stable fashion in the range of a few nanomoles per mg cellular protein. From this it can be estimated that about 10^9 GM1 molecules can be inserted into the cell membrane of a single cell, corresponding to roughly 3% of total membrane lipid content.

Several lines of evidence suggest that the uptake of gangliosides into cell membrane involves action of some specific proteins. Gangliosides added exogenously to epithelial cell cultures are taken up by the apical membranes, but do not pass the tight junction to the basolateral membranes of the cell [85]. Pretreatment of cells with trypsin reduces ganglioside uptake [86, 87] and prevents adhesion of cells to GM1-coated wells [88]. The recovery of ganglioside-uptake ability requires *de novo* synthesis of proteins [70]. Several proteins were reported to be implicated in binding of gangliosides at the cell surface [89-91], but their identity or exact functions were not determined.

Shedding and uptake of gangliosides at the cell surface

The first indications that gangliosides can be shed from the cell surface and exit into extracellular space came from studies of Kloppel and colleagues who found increased concentrations of gangliosides in sera of humans and mice bearing mammary carcinomas [92]. This was soon followed by the first demonstration of efficient shedding and uptake of gangliosides by Portoukalian and colleagues who reported increased amounts of gangliosides taken up by erythrocytes of melanoma patients [93]. Interestingly, even though plasma concentration of GM3 was increased only by 30%, the concentration of GM3 in erythrocytes increased nearly three times. In the same time, GD3 whose level in serum increased four-fold was undetectable in erythrocytes, clearly indicating that shedding and uptake differently affect different gangliosides.

Many tumor cell lines overexpress gangliosides. For example, malignant melanomas and neuroblastomas overexpress GD3, GD2, and GM2 [94-96], while increased expression of GD1a, GM1, and GM2 was observed in renal cell carcinomas [97]. The process of ganglioside shedding has been intensively studied by S. Ladisch and colleagues in the past 20 years. They found that tumor cells can shed up to 0.5% of their membrane gangliosides per hour [98]. Interestingly, mouse ascites hepatoma cells cultivated at lower cell density were shedding 3 times more gangliosides then cells grown at higher density [99].

Olshevski and Ladisch demonstrated that gangliosides can be effectively transferred from one cell to another in combined cultures separated by a membrane that prevented direct contact between donor and acceptor cells [100]. Inhibition of ganglioside synthesis in donor cells effectively blocked this transfer [101]. Up to 10^7 individual ganglioside molecules were found to insert into a single cell in a co-culture medium with total gangliosides concentration of 7×10^9. The fact that transfer of gangliosides from the lymphoma cells to the fibroblasts occurred at a relatively low concentration of shed gangliosides [100] indicates the potential biological importance of this process. In tumor cells shedding of gangliosides apparently help to suppress the immune response [15, 102], and the inhibition of NF-kappa B in T-cells by shed gangliosides has been proposed as one of the possible mechanisms [103]

Gangliosides are able to spontaneously transfer between membranes at elevated temperatures [104] and the rate of transfer is dependant on both temperature and the physical state of donor and acceptor membranes [105]. Different gangliosides have significantly different

physicochemical properties and it should not be expected that all gangliosides behave in the same way. However, it is likely that under physiological conditions the effective exchange of most gangliosides, or at least monosialogangliosides, requires the intervention of specific exchange proteins [104].

Cells can shed gangliosides both as large membrane vesicles and by preferential release of particular glycolipids [106]. A certain degree of specificity was reported to exist in both shedding and uptake. Young and colleagues reported preferential release of glycosphingolipids with shorter fatty acyl chains, over the corresponding glycosphingolipids with longer fatty acil chains [106, 107]. Shorter forms of ceramide apparently also enables more efficient uptake of gangliosides from the medium as reported by Ladisch and Olson [108]. However, the composition of shed gangliosides was generally found to mirror the composition of gangliosides in donor cells [101, 109], indicating lack of preference for specific carbohydrate structures of gangliosides in the process of shedding.

Kong *et al.* reported that shed gangliosides mostly exist as monomers in the medium [110]. This is very unusual because when exogenous gangliosides were added to the culture medium at same concentrations (10^{-8}-10^{-7} M) they mainly existed in micelles, suggesting that the naturally shed gangliosides are somehow different in their aggregation properties from exogenously added purified gangliosides. This observation is supported by a fact that uptake of shed gangliosides is much more efficient than the uptake of the purified exogenously added gangliosides [100]. Glomerular mesangial cells, neuroblastoma and melanoma cells undergoing apoptosis shed gangliosides in a process that appears to be regulated and occurs in the early stages of the apoptotic process [109]. On the other hand, nearly no shedding was found in cultured Cos7 cells [111]. Taken together, all these results strongly suggest that shedding and uptake of gangliosides is a regulated physiological process that proceeds through action of some specific membrane and/or transfer proteins. Although the identity of these proteins is not known, there are some likely candidates.

Prosaposin is a potential catalyst of ganglioside shedding and uptake

Saposins (also called SAPs – Sphingolipid Activator Proteins) are a group of four highly homologous small heat-stable glycoproteins (called saposins A, B, C, and D) that are required for lysosomal degradation of sphingolipids (for a review see [112]). The first saposin (now called saposin B) was described by Jatzkewitz and his colleagues in 1964 as a heat-stable

factor required for hydrolysis of sulfatides by arylsulfatase A [113]. Cloning of the corresponding cDNA [114] indicated that all four saposins are being synthesized as a single precursor, a 53-kDa protein prosaposin that can be differentially glycosylated into 65-kDa or 70 kDa forms [115]. Prosaposin of 65 kDa is associated with Golgi membranes and targeted to lysosomes where four saposins (A, B, C and D) are generated by its partial proteolysis. Interestingly, the targeting of the 65-kDa protein to lyzosomes is not mediated by the mannose 6-phosphate receptor, but the Golgi apparatus appears to accomplish molecular sorting of the 65-kDa prosaposin by decoding a signal from its amino acid backbone [116]. Each mature saposin contains about 80 amino acid residues and has six equally placed cysteines, two prolines, and a glycosylation site (two in saposin A, one each in saposins B, C, and D). These residues are also completely conserved in saposins from different animal species [117].

In addition to being targeted to lysosomes and cleaved to saposins, prosaposin can be secreted in an uncleaved form and retained at the outer side of the cell membrane [116]. It has been suggested that its association with the cell membrane proceeds through the interaction with membrane gangliosides [118, 119]. Prosaposin can also be found in many biological fluids such as seminal plasma, human milk, and cerebrospinal fluid (reviewed in [120]). Prosaposin is abundant in the brain where it is localized exclusively in certain neurons [121]. Its presence on the neuronal surface was first reported by Fu and colleagues in 1994 [122] and since then many functions have been attributed to the secreted form of prosaposin. Among other effects, it was reported to be neurotrophic [123], to promote myelination after nerve injury [124], to prevent apoptosis of neuronal cells in tissue culture [125], and to act as a neuroprotective and neuroregenerative agent *in vivo* [126].

Prosaposin is the predominant form of saposins in neurons [127] and the majority of effects of prosaposin were observed in neuronal cells. However, recently Misasi and her colleagues reported that prosaposin also prevents TNFα -induced cell death in human histiocytes and demonstrated that this occurs through stimulation of signal cascades in which signal-regulated protein kinases are involved [128]. In a similar way, saposin C itself was shown to prevent apoptosis in prostate cancer cells [128]. These effects are consistent with the observations that prosaposin addition rescues neuroblastoma cells, primary hippocampal neurons [129], Schwann cells [125], and PC12 pheochromocytoma cells [119] from apoptosis induced by various agens. Neurotrophic, neuroregenerative and anti-apoptotic effects of prosaposin are

apparently mediated by modification of signaling pathways and prosaposin was shown to be involved in ERK phosphorylation [130]. Apparently it activates the MAPK pathway by a G-protein-dependent mechanism [131], and through a same or similar mechanism it also stimulates growth, migration, and invasion of prostate cancer cells [132].

A mouse knockout model for prosaposin has been created, but since prosaposin is a precursor of saposins, in addition to affecting membrane functions of prosaposin, the disruption of its gene also abrogates functions of saposins in the endosomal pathway and results in complex phenotype including severe progressive central nervous system disease and early death [133]. In addition to the nervous system, the mostly affected system in mice deficient for prosaposin was the reproductive tract [134, 135]. The prosaposin gene contains 15 exons that can be transcribed into several mRNAs, resulting from alternative splicing of the 9-bp exon 8 [136, 137]. A splicing variant of prosaposin without exon 8 is preferentially expressed in the brain following injury [138], and alternative splicing of the prosaposin gene was assumed to be the mechanism responsible for differential sorting of the different prosaposin forms [139]. However, targeted disruption of this specific splicing variant did not show any specific phenotype, and the levels of secreted prosaposin in serum were similar to those of wild-type mice, indicating that both splicing variants of prosaposin are being secreted to the membrane [140].

Prosaposin and saposins bind different gangliosides differently, with each protein showing preference for specific structures [141]. Different splicing variants of prosaposin were also shown to differentially bind different gangliosides [142]. *In vitro*, prosaposin, as well as saposins, promoted the transfer of gangliosides from donor liposomes to acceptor erythrocyte ghosts [141]. Transfer rates were found to be concentration dependent, and up to 50% of gangliosides were found to be transferred in 60 minutes. On the membrane of neural cells prosaposin was reported to be in complex with gangliosides [119], and neuroblastoma cells incubated in the presence of prosaposin were found to have increased levels of gangliosides on the cell membrane [143]. Saposin is able to extract monomeric lipids from the membrane and functional significance of prosaposin-ganglioside interactions was recently demonstrated in the process of lipid presentation by CD1 proteins during immune recognition [144]. Hiraiwa and colleagues reported that prosaposin purified from milk or medium forms oligomers of varied masses [145] and this was recently confirmed by analysis of recombinant prosaposin expressed in the bakulovirus system [146]. Direct observation by atomic force

microscopy of saposin C effects on membrane bilayers demonstrated ability of saposins to induce membrane reorganization and form raft-like structures [147].

Membrane rafts are places where receptor signaling and processing occurs (for review see [45, 148]). Because different rafts exist with unique ganglioside composition, specific gangliosides might target the respective receptors through direct interaction to unique rafts and it was suggested that the duration and localization of the signal is controlled by the proportion of rafts with unique ganglioside compositions to the number of target receptors [5]. Both gangliosides and prosaposin function through the formation and modulation of lipid rafts, and it is appealing to hypothesize that a possible function of prosaposin on the cell membrane is the regulation of formation and modulation of lipid rafts by insertion or removal of specific gangliosides. Even though there is no direct evidence for functional significance of interactions between saposins and gangliosides, circumstantial evidence seems quite convincing. Prosaposin and gangliosides both exist in rafts at the cell surface [26, 147]. Prosaposin can bind gangliosides, and is able to catalyze their transfer between different vesicles *in vitro* [141]. Both prosaposin and shed gangliosides were reported to be present in milk and cerebrospinal fluid [120, 149, 150]. Gangliosides are being actively shed from the membranes [15], and this process appears to be regulated, indicating that it includes specific protein activity. Another line of evidence comes from the fact that both gangliosides and prosaposin are involved in the same cellular processes. They were both shown to modify signal cascades in which signal-regulated protein kinases are involved [130, 131, 151], they both mediate apoptosis [6, 125, 128, 129], and are involved in decisions regarding neural growth and myelination [7, 123, 124]. Their distribution and expression changes with development [152-154] and in response to brain injury [124, 155]. Both gangliosides and prosaposin are being secreted by tumor cells [15, 156] and were shown to promote tumor development [132, 157]. Mice deficient for prosaposin and mice deficient for complex gangliosides are both infertile [39, 134, 135]. Taken together, all these data suggest that prosaposin has an active role in the regulation of ganglioside shedding and uptake, and consequently functions as modifier of lipid rafts. Although three-dimensional structure of prosaposin is not known, since it has multiple glycolipid binding sites, it is quite possible that at the cell membrane it functions analogously to GM2 activator protein [158, 159] and shuttles gangliosides between neighboring cells, or cells and the extracellular medium.

Glycolipid transfer protein may also be involved in ganglioside shedding and uptake

Glycolipid transfer protein **(**GLTP**)** is a soluble protein that selectively accelerates intermembrane transfer of glycolipids *in vitro*. After the initial discovery in the membrane-free cytosolic extract of bovine spleen [160], proteins with similar activities were found in a wide variety of tissues, including bovine and porcine brain, liver and kidney, as well as in plants [161]. Purified GLTPs from animal spleen and brain consist of single polypeptides of 23-24 kDa and have basic isoelectric points and absolute specificity for glycolipids [162-164]. Even though GLTP transfers glycolipids with shorter sugars more efficiently, it also significantly facilitates exchange of gangliosides between membrane vesicles [165].

Molecular cloning indicated that GLTP is highly conserved among mammals and that bovine and porcine brain cDNAs encode identical 209 amino acid sequences [166]. The structure of GLTP distinctly differ from structures of saposin B [167], saposin C [168], and GM2-activator protein [158]. As recently revealed by x-ray diffraction [169], GLTP is characterized by a novel folding motif among proteins that transfer or bind lipids. The structural data show that complexation of lactosylceramide by GLTP involves a single glycolipid liganding site. The glycolipid liganding site of GLTP is composed of a surface recognition center for the sugar headgroup and a molded-to-fit, hydrophobic tunnel that accommodates the hydrocarbon chains of the ceramide moiety via a cleft-like conformational gating mechanism [169].

Extensive analysis of its transfer properties by Rao and colleagues concluded that GLTP might act as a freely transporting shuttle that carries glycolipids back and forth between the donor and acceptor vesicles [170]. Mutational analysis confirmed that GLTP forms a soluble, stable complex with glycolipids that can be released from the GTLP/complex in the presence of acceptor membranes. Interestingly, the release of glycolipids into artificial membranes was not very efficient, indicating that some acceptor specificity might be involved in the release process [171]. Recent *in vitro* study also concluded that GLTP's ability to both capture glycolipids from the membrane and insert them into the other membrane significantly depends on structure and composition of both membranes, and the authors concluded that this suggests that GLTP might be involved in the assembly of lipid rafts [172].

Even though it is assumed that GLTP is a cytosolic protein, its distribution was never studied in detail, and its physiological functions are mostly unknown. Lin and colleagues suggested that GLTP might function as cytosolic transporter of glycosphingolipids to the membrane

[166], but since glycosphingolipids are generally found on inner leaflet of intracellular vesicles and outer leaflet of the cell membrane, this function does not seem very probable. GLTP orthologs in plants and fungi have been implicated in apoptosis and regulation of vital cellular processes [173, 174], indicating that it might have a similar function in mammalian cells. Even though there is no direct evidence that GLTP is involved in ganglioside shedding and uptake *in vivo*, its ability to perform these functions *in vitro* [164, 165] puts it high on the list of potential candidates.

On the basis of currently published results prosaposin and GLTP appear to be the best candidates for proteins involved in the regulation of ganglioside shedding and uptake, but it is of course possible that some other known or unknown proteins are actually performing this task *in vivo*. Possible alternative candidates might be some of the nonspecific lipid transfer proteins that were reported to be able to transfer different glycosphingolipids [175].

Glycosylphosphatidylinositol-anchored proteins

First indications that proteins might be attached to the cell membrane by lipid anchors appeared in 1963 with the finding that bacterial phospholipase can release alkaline phosphatase from cells [176]. The presence of inositol-containing phospholipid protein anchors was postulated by Ikezawa and colleagues in 1976 [177], but their hypothesis was not widely accepted until 1985, when a body of compositional data about Torpedo electric-organ acetylcholinesterase (AChE) [178], human erythrocyte AChE [179], rat brain and thymocyte Thy-1 [180], and Trypanosoma brucei variant surface glycoprotein (VSG) [181, 182] became available. All glycosylphosphatidylinositols (GPIs) share a common core structure [183]. Phosphatidylinositol is glycosidically linked through carbon 6 of the inositol ring to the reducing end of a nonacetylated glucosamine moiety. Interestingly, GPIs are one of the rare instances in nature where glucosamine is found without either an acetyl group (present in most glycoconjugates) or a sulfate moiety (present in heparin) attached to the amino-group at the 2-position. Three mannosyl residues, linked $\alpha 1\text{–}4$, $\alpha 1\text{–}6$, and $\alpha 1\text{–}2$, respectively, are attached to the glucosamine. The terminal $\alpha 1$ 2 linked mannose is linked to phosphoethanolamine by a phosphodiester linkage. The GPI is attached to the carboxy-terminal carboxyl group of the protein by an amide linkage to the amino group of phosphoethanolamine (Fig. 4). This common core structure can be further modified in a way that depends on both the organism and cell type in which it is synthesized [1].

(Fig. 4)

The whole process of GPI biosynthesis is carried out in the endoplasmic reticulum [184] and nearly 20 enzymes participate in this pathway. Corresponding genes have been cloned from mammals, yeast and protozoa [185]. Once it is completed, the pre-formed anchor is transferred to a specific site upstream of the C-terminal end of the protein in the ER lumen by the action of a transamidase complex, which simultaneously cleaves off the remaining C-terminal peptide [2]. The C-terminal sequence of the protein thus acts as a signal for GPI attachment. It is encoded in the sequences of genomic and cDNA, but does not appear in the final processed protein.

The initial step of GPI synthesis, attachment of N-acetylglucosamine to phosphatidylinositol, depends on the product of a X chromosome gene termed phosphatidylinositol glycan class A (*PIG-A* in humans, Pig-a in mice) [186]. A deficiency in *PIG-A* results in rare human disease named paroxysmal nocturnal hemoglobinuria (PNH) [186-188]. Patients with PNH have abnormal cells of various hematopoietic lineages that are defective in the biosynthesis of GPI-anchored proteins. These include the complement-regulatory proteins, CD55 and CD59, whose absence results in enhanced complement-mediated lysis [189, 190]. Since deficiency of GPI is embryonically lethal [191-193], all PNH patients reported to date acquired a somatic mutation in *PIG-A* [194]. The exact mechanism how one or a few of the large number of pluripotent hematopoietic stem cells that bear mutation in *PIG-A* achieve dominance in the bone marrow and the peripheral blood is not known [195], but it has been recently shown that *PIG-A* deficient cells have lower susceptibility to TNF-α and IFN-γ, what might contribute to their clonal dominance [196].

(Table 2)

Today, hundreds of GPI-anchored proteins are known (see examples in Table 2) and it is estimated that approximately 0.5% of all proteins in lower and higher eukaryotes are being modified in this manner [197]. Although GPI-anchored proteins do not apparently share common features, the presence of the anchor itself appears to confer some important functional and behavioral attributes on proteins to which it is attached. In particular, localization to lipid raft microdomains and cleavage by endogenous and exogenous phospholipases appears to play a major role in transduction of signals across the plasma

membrane (for a recent review see [4]). Recent observation that prion protein and Thy-1 exist in separate lipid rafts, and that the composition of membrane lipids in rafts containing prion protein is different from the composition of lipids in rafts containing Thy-1 [198] suggests that interplay of lipids and GPI-linked proteins in lipid rafts is very specific and carefully regulated.

Release of GPI-anchored proteins by GPI cleavage

The hypothesis that one of the functions of the GPI anchor may be to offer a site for degradation by specific endogenous phospholipases resulting in a release of the protein from the cell surface has been postulated very soon after the existence of GPI-anchors was widely accepted [199]. The removal of the GPI lipid moiety *in vitro* was reported to cause significant alterations in enzymatic activities [200-203] and ligand binding properties [204-206], thus it is quite likely that some GPI-anchored proteins in the membrane are actually reservoirs of inactive proteins that can be activated and rapidly released by GPI cleavage.

Two types of GPI-specific phospholipases, GPI-phospholipase C (GPI-PLC) and GPI-phospholipase D (GPI-PLD) cleave GPI on different sides of the phosphodiester bond between inositol and the lipid part of the anchor (Fig. 4). Very recently, it was demonstrated that angiotensin-converting enzyme (ACE) can also specifically cleave GPI [207]. Several bacterial species secrete PI-specific type C phospholipases, including *Bacillus cereus*, *Bacillus thuringiensis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Clostridium novii*. These enzymes are able to hydrolyze mammalian GPI anchors, and have been extensively used in the study of structure and function of GPI-linked proteins. Several parasitic protozoans, for example, *Tripanosoma brucei* and *Leishmania*, contain endogenous GPI-PLC that converts membrane-bound proteins to hydrophilic soluble forms (reviewed in [4]).

Since the first discovery of bacterial PI-PLC, endogenous mammalian GPI-PLC have been postulated to serve as important regulatory factors, reducing surface expression of GPI-anchored proteins, while simultaneously increasing the levels of soluble protein. Chan and colleagues reported that lipoprotein lipase was released from insulin treated 3T3-L1 adipocytes by cleavage of its GPI anchor and they proposed that activation of an insulin-dependent PI-PLC was responsible [208]. Alkaline phosphatase was also reported to be released in soluble form from myocytes and adipocytes upon insulin stimulation (Romero et

al., 1988), again suggesting the action of a phospholipase C [209, 210]. Park and colleagues reported that endogenous GPI-PLC releases renal dipeptidase from kidney proximal tubules *in vitro* [211] and *in vivo* [212], but mammalian GPI-PLC has yet to be identified.

Mammalian GPI-PLD was discovered in human serum by Davitz and colleagues in 1987 [213]. Despite its high concentration in mammalian serum [214] and relatively well-characterized molecular biology [215] and biochemistry [216] the physiological role of GPI-PLD is not clear. In serum, GPI-PLD is associated with HDL and is apparently not active [217]. Initial reports indicated that GPI-PLD was active against GPI-anchored proteins only in the presence of detergent, and was not able to cleave the anchors of proteins in a native membrane context [218]. Overexpression experiments indicated that it is active in endoplasmic reticulum during GPI synthesis, but also in lipid rafts [219]. Lipid fluidity and packing are the most important modulators of bacterial phospholipase ability to cleave GPI anchors [220] and modulation of membrane lipids were reported to affect GPI-PLD activity *in vitro* [221], so it is quite possible that mammalian GPI-PLD also requires particular membrane composition for activity. The fact that endogenous GPI-PLD was reported to specifically release NCAM from differentiating myoblast cells [222], receptor for urokinase-type plasminogen activator from ovarian cancer cells [223] and carcinoembryonic antigen from human colon cancer cells [224] strongly support this hypothesis.

The angiotensin-converting enzyme (ACE) is a well-characterized zinc peptidase that regulates blood pressure by hydrolyzing bioactive peptides such as angiotensin I and bradykinin [225]. There are two ACE isoforms: a somatic form of around 150-180 kDa, which bears two catalytically active sites, and a smaller isoform (90-110 kDa) found in the testes, which contains a single active site [226]. Kondoh and colleagues recently reported that testicular ACE can specifically release GPI-anchored proteins from the cell membrane [207]. Even when the peptidase activity is abolished by either mutation or inactivation, the enzyme could still cleave GPI-anchored proteins and restore fertility to ACE-deficient sperm. This activity is not protein-specific because it cleaves a variety of GPI-anchored proteins, and its cleavage site is located between the second and the third residue of the conserved mannose core (Fig. 4). GPI-anchor-releasing activity of ACE requires removal of cholesterol from cell membranes, and similarly to GPI-PLD that is also widely present, but mostly inactive, ACE apparently also requires a particular form of substrate presentation on the membrane for activity.

Release of intact GPI-anchored proteins from the cell membrane

In addition to release by enzymatic cleavage, GPI-linked proteins can be released from the cell membrane with their GPI-anchors intact. This release can be in the form of membrane vesicles (exosomes), but also as small aggregates that contain some membrane lipids in addition to GPI-linked proteins [227]. Exosomes are small (50–200 nm) membrane vesicles first described in studies of reticulocyte maturation about 20 years ago [228, 229], that were subsequently demonstrated to be released from various cell types [230-235]. Exosomes were initially thought to correspond to internal vesicles of multivesicular bodies being released in the extracellular space upon their fusion with the cell membrane, but this is apparently only one way how exosomes can be formed since glycolipids and GPI-anchored proteins already embedded in the outer leaflet of the cell membrane can also be efficiently secreted in the form of exosomes [236]. Various GPI-linked proteins, including the prion protein [237] are being actively secreted in exosomes. This process can be quite extensive as demonstrated by reticulocytes that release approximately 50% of acetylcholinesterase in exosomes during differentiation into erythrocytes [238]. Similar vesicles named prostasomes exist in seminal plasma where they assist sperm function [239]. GPI-anchored CD59, CD55 and CD52 were found on prostasomes [240], but also in a form of small aggregates in seminal plasma [227]. While prostasomes bind to target cells and are later internalized, the kinetics of transfer of GPI-anchored molecules from aggregates into cells is consistent with direct incorporation into cell membranes [227].

Shedding and uptake of GPI-anchored proteins

The phenomenon of shedding and uptake of a GPI-linked protein was reported even before GPI-anchors were discovered. While investigating phospholipid exchange between cells and artificial vesicles, Bouma and colleagues showed that acetylcholinesterase and some other erythrocyte proteins were transferred from erythrocytes to the vesicles and that this process was reversible [241]. The rate, direction, and extent of such intermembrane transfers was found to depend on the relative lipid composition and fluidity of the donor and acceptor membranes [242]

Contrary to the release of GPI-anchored proteins by phospholipases C and D that removes GPI and yields soluble protein, shedding releases proteins with intact GPI that are still able to insert into membranes of other cells. Cell-to-cell transfer of GPI-anchored protein has been

reported in a variety of *in vitro* and *in vivo* systems. CD59 was transferred from seminal plasma to erythrocytes and other cells [240], as well as from erythrocytes to endothelial cells in mice made transgenic for this GPI-anchored protein [243]. Thy-1 was transferred between cells in chimeric murine embryoid bodies composed of normal and *PIG-A* "knock-out" cells [244] and trypanosomal variant surface glycoprotein (VSG) was found to be incorporated into erythrocytes of infected patients [245]. High-density lipoproteins (HDL) may act as carriers of CD59 and are capable of transferring this protein to erythrocytes [246]. Transfer between membranes can occur without actual membrane fusion [227] and GPI-anchored proteins are apparently transferred through vesicles or liposomes released from the donor cell [247].

GPI-anchored molecules are clustered in lipid raft membrane microdomains and they actively take part in membrane vesicle formation, resulting in vesicles enriched in GPI-anchored proteins [247]. Storage of erythrocytes results in loss of both CD55 and CD59 from the erythrocyte membrane [248] and creation of erythrocyte microvesicles that are enriched in GPI-linked proteins including CD55 and CD59 [249]. When erythrocytes from PNH patients that were deficient in GPI-anchored proteins were incubated with HDL preparations or erythrocyte microvesicles, there was significant transfer of CD55 and CD59 to the cell surface. Pretreatment of microvesicles and HDL with phosphatidylinositol-specific phospholipase C abrogated protein transfer to deficient cells, indicating that increased cell-associated CD55 and CD59 levels were related to the insertion of an intact GPI moiety, rather than to simple adhesion [250].

In a recent elegant experiment Sloand and colleagues confirmed the ability of GPI-linked proteins to transfer between cells *in vivo* [251]. PNH patients of group A1 blood type were given transfusions of compatible, washed group O blood. Patient's group A1 cells were distinguished from the transfused group O cells by staining with a *Dolichos biflorus* lectin that specifically binds to group A1 erythrocytes. Significant transfer of GPI-linked proteins from donor cells to patient's erythrocytes could be demonstrated as early as 1 day following transfusion and persisted for several days.

GPI-linked proteins transferred from cell to cell appear to be stable and biologically functional [227, 243, 252-254]. For example, transfer of CD55 and CD59 to erythrocytes confers resistance to complement-mediated lysis [250]. For effective transfer to occur, both the GPI anchor and the protein moiety must be intact [255]. Transferred molecules are

inserted into the outer leaflet of the plasma membrane by lipid chains on the GPI moiety and soluble CD59 (that lacks GPI anchor) was found to have only 1/200th the ability of GPI-linked CD59 to inactivate complement [256].

Incubation of rat Thy-1 antigen with murine lymphocytes showed that the rat protein could incorporate into murine cells, and that after the membrane uptake the exogenous protein migrated with the same lateral mobility as endogenous murine Thy-1 protein [257]. Similarly, incorporation of *Trypanosoma brucei* variant surface glycoproteins (VSG) into baby hamster kidney cells showed that the inserted VSG exhibited lateral mobility equivalent to that of endogenous VSG in *T. brucei* [258]. Interestingly neither Thy-1 inserted into lymphocytes [257] nor CD59 incorporated into neutrophils [259] supported transmembrane signaling immediately following transfer. However, CD59 incorporated into U937 monocytic cells and allowed to equilibrate for 2 h at 37°C showed a redistribution into lipid rafts and signaled intracellular Ca^{2+} fluxes [260]. Therefore, exogenously introduced GPI-anchored molecules appear to become functional within the target cell membrane once they have acquired a distribution similar to that of endogenous GPI-anchored proteins, but this process is slow and can take even more than 24 h [255, 261].

GPI-linked proteins were reported not to transfer spontaneously from erythrocytes to liposomes, and it was suggested that *in vivo* GPI-linked membrane proteins do not spontaneously transfer between cell membranes, but that some catalyst is needed [247]. This hypothesis is also supported by the observation that CD4 engineered to have GPI anchor can be efficiently transferred between cell membranes in one type of cells [262], while another cell line expressing CD4-GPI fusion protein failed to release it in any form [263]. However, the identity of a potential protein catalyst of GPI shedding and uptake is not known.

What is a physiological function for shedding and uptake of gangliosides and GPI-anchored proteins?

Tumor cells use shedding and uptake to evade destruction by immune cells [15, 102, 157], and retroviruses exploit shedding for spreading to other cells [264], but these extensively studied mechanisms are actually only examples of a misuse of shedding and uptake, and the real reason why this process developed in the course of evolution still has to be discovered.

One reported function of shedding and uptake is the transfer of GPI-linked proteins and gangliosides from prostasomes and GPI-lipid aggregates released by prostate epithelium to spermatozoa [227]. Since spermatozoa do not synthesize proteins, shedding and uptake here represent an important mechanism by which spermatozoa can acquire new proteins and alter their antigenicity, resistance to immune attack, or other surface properties. Another rather probable function of shedding and uptake is the modulation of lipid rafts and signal transduction. Exogenously added GM1 was reported to inhibit fibroblast growth factor 2 (FGF2)-mediated proliferation in endothelial cells by binding to FGF2 and preventing its interaction with the receptor [265]. In the same time, endogenous GM1 in the cell membrane was found to promote FGF2-mediated fibroblast proliferation [266]. Apparently GM1 in the medium binds to FGF2 in an inhibitory manner, while GM1 in the cell membrane binds FGF2 in a way that promotes its interaction with the receptor [267]. Shedding of GM1 from the cell membrane in the same time decreases promoting activity and increases inhibitory activity of GM1, thus providing a very efficient way of modifying effects of FGF2 on the cell. Shedding of gangliosides from one cell and their uptake by a neighboring cell might also be a way how different cells in a tissue could coordinate reaction to hormonal signals.

Exogenous administration of gangliosides affects membrane distribution of GPI-anchored proteins in lipid rafts [268, 269]. Both GPI-PLD and ACE were reported to require some kind of specific membrane environment to become active, and it is tempting to speculate that modification of lipid rafts by removal or addition of specific gangliosides might create favorable conditions for activity of these enzymes and consequential release of GPI-anchored proteins. In addition to its role in the modulation of lipid rafts, shedding and uptake of gangliosides and GPI-linked proteins might be involved in some other processes. For example it was hypothesized that shed gangliosides might be involved in cell synchronization [270, 271]. The fact that shed ganglioside suppress immune response to cancer cells suggest that this mechanism could actually be used to suppress autoimmune response in some situations. For example, gangliosides are especially enriched in the brain and shed gangliosides in the cerebrospinal fluid might be responsible for suppressing autoimune activity of T-cells that pass blood-brain barrier.

Impact of shedding and uptake of gangliosides and GPI-anchored proteins on the analysis of their distribution by immunohistochemical analysis

The ability of gangliosides and GPI-anchored proteins to move between cells in physiological conditions has profound effects on their behavior in various assay systems *in vitro.* Immunohistochemistry is a very important tool that enables precise localization of various types of biological molecules and structures. However, this method is prone to serious artifacts, and significant care is needed to avoid false interpretation of experimental data [272]. Gangliosides appear to be particularly problematic for immunohistochemical evaluation. This field was for years hampered by the inadequate specificity of antibodies and fixation artifacts [273]. Most of these problems were resolved when adequate fixation techniques were developed and when high-affinity IgG antibodies were raised in mice deficient for complex gangliosides, but recently we reported another serious pitfall of ganglioside immunohistochemistry [274]. Many immunostaining procedures include addition of detergents, either to aid detection of some proteins, or to reduce background staining. However, even when all steps in the procedure are being performed at +4º, the inclusion of even small amount of detergents in the immunostaining buffers causes significant redistribution of gangliosides and GPI-linked proteins from one brain region into another (Fig. 5).

Fig. 5

In addition, tissue sections can not be stored for a long time before immunostaining, nor can they be incubated at 37ºC. Even in detergent-free solutions kept at +4ºC gangliosides are being lost from the tissues during storage. In addition to shedding, interconversion of gangliosides might also be a significant factor in this process. GT1b and GD1b can be easily converted to GD1a by simple removal of one sialic acid (Fig. 2), a process that can occur either through the remaining activity of endogenous membrane sialidases [111] or by spontaneous hydrolysis. Recently we observed that gangliosides can redistribute even in mounted immunostained slides (unpublished results). This phenomenon was observed both for fluorescently labeled antibodies and enzyme-conjugated antibodies. It is somewhat difficult to comprehend that precipitated substrate could move from one place to another, but this apparently happens. One possible explanation for this phenomenon might be the fact that the large proportion of colored product actually precipitates on the complex of primary antibody, secondary antibody, and conjugated enzyme. This complex can be up to a million daltons large and is being anchored to the membrane with a single ganglioside ceramide part. Thus it is easily conceivable that this bulky hydrophilic mass can pull the ceramide out of the

membrane and allow it to move into more hydrophobic environment like myelin rich neuronal fibers in the white matter. The fact that immunostaining of non-fixed cells results in very little or no staining [273], is in the accordance with the hypothesis that attachment of antibodies to a ganglioside can simply pull the ganglioside out of the membrane, in a kind of an *in vitro* shedding process enhanced by addition of antibodies. Fixation apparently creates some kind of mesh on the membrane what makes this more complicated.

A grim consequence of these observations is the fact that tissue sections have to be immunostained for gangliosides and GPI-anchored proteins in detergent-free buffers and that all steps have to be performed at +4ºC. Immunostained sections have to be examined and photographed immediately after mounting onto slides. Unfortunately this was frequently not the case, and a significant amount of previous work on the distribution of these two classes of molecules may need to be re-evaluated.

Fig. 6

Conclusions

The phenomenon of shedding and uptake of gangliosides and GPI-linked proteins have been discovered, forgotten and again discovered several times during the past few decades. In this review we have presented evidence from several nearly completely separated scientific areas that clearly demonstrated the ability of gangliosides and GPI-linked proteins to be actively released from membrane of one cell and inserted in a functional form into membranes of other cells (Fig. 6). This process appears to be regulated, and most probably involves catalytic activity of some proteins that still have to be identified. Functional significance of this phenomenon is not known and it will be very interesting to learn how this complicated process aids in the integration of individual cells into complex organisms.

Figure legends

Fig 1. Gangliosides.

A) GM1 ganglioside consists of neutral core structure Gal β3 GalNAc β4 Gal β4 Glc β1 Cer and one N-acetylneuraminic acid attached to the inner galactose. B) Schematic representation of major gangliosides in vertebrate brain: GM1, GD1a, GD1b and GT1b.

Fig. 2. Schematic representation of the biosynthetic pathway of major gangliosides. Gangliosides are being synthesized by sequential addition of monosaccharides to ceramide. Key enzymes depicted in the pathway are as follows: A: UDP-glucose:ceramide glucosyltransferase; B: UDP-galactose:glucosylceramide β1,4-galactosyltransferase (galactosyltransferase I); C: CMP-NeuAc:lactosylceramide α2,3-sialyltransferase (sialyltransferase I); D: CMP-NeuAc:GM3 α2,8-sialyltransferase (sialyltransferase II); E: UDP-GalNAc:GM3 β1,4-N-acetylgalactosaminyltransferase (GalNAc transferase); F: UDP-Gal:GM2 β1,3-galactosyltransferase (galactosyltransferase II); G: CMP-NeuAc:GM1 α2,3-sialyltransferase (sialyltransferase IV).

Fig. 3. Gangliosides function as modulators of lipid rafts. Gangliosides are specifically enriched in lipid raft domains where they function as modulators of signal transduction through the cell membrane. Effects of gangliosides on the receptor for epidermal growth factor (EGFR) are presented as an example of ganglioside function. Signal transduction through EGFR requires receptor dimerization. The presence of GM3 inhibit dimerization and diminish EGF signaling, while the presence GD1a induces dimerization facilitates EGF signaling [64]. The reaction of cell to EFG can be diminished or enhanced by selective incorporation of EGFR into rafts enriched in GM3, or GD1a, respectively.

Figure 4. Structure of a GPI anchor. All characterized GPI anchors share a common core consisting of ethanolamine-PO_4-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-Ino-1-PO_4-lipid. Heterogeneity in GPI anchors is derived from various substitutions of this core structure that are represented as R groups. Various glycans can be attached to R_1, phosphoetanolamine is frequently found at R_2, and additional fatty acids can be attached at R_3. Cleavage sites of GPI-phospholipase C (GPI-PLC), GPI-phospholipase D (GPI-PLD) and angiotensin-converting enzyme (ACE) are marked by arrows.

Fig 5. Effects of Triton X-100 on the distribution of GD1a ganglioside.

Tissue slices of cerebellum from a wild-type mouse (A) and telencephalon from mice deficient for complex gangliosides (B) were immunostained with antibodies against GD1a. During immunostaining both tissue slices were incubated together in single microtiter wells in the presence of increasing concentrations of Triton X-100 at +4ºC. In the absence of detergents white matter of wild type mouse cerebellum (wm) and telencephalon of mouse deficient for complex gangliosides were completely devoid of GD1a. However, when increasing concentration of Triton X-100 in immunostaining solutions were used, more and more GD1a was transferred from other brain regions of wild type mouse and inserted into corpus callosum (cc) of mouse deficient for complex gangliosides and cerebellar white matter of wild type mouse (for experimental details and more examples of this phenomenon see [274]).

Fig. 6. Shedding and uptake

Gangliosides and GPI-linked proteins can be transferred from cell to cell either directly (A), with help of specific carrier proteins (B), or through small vesicles or micelles (C). The identity of specific proteins that catalyze shedding and uptake on the cell membrane is not known, but experimental data strongly support their existence.

Table 1. Examples of signal transduction processes that involve lipid rafts

Table 2. Examples of GPI-anchored proteins (for a more complete list see a recent review by H. Ikezawa [1])

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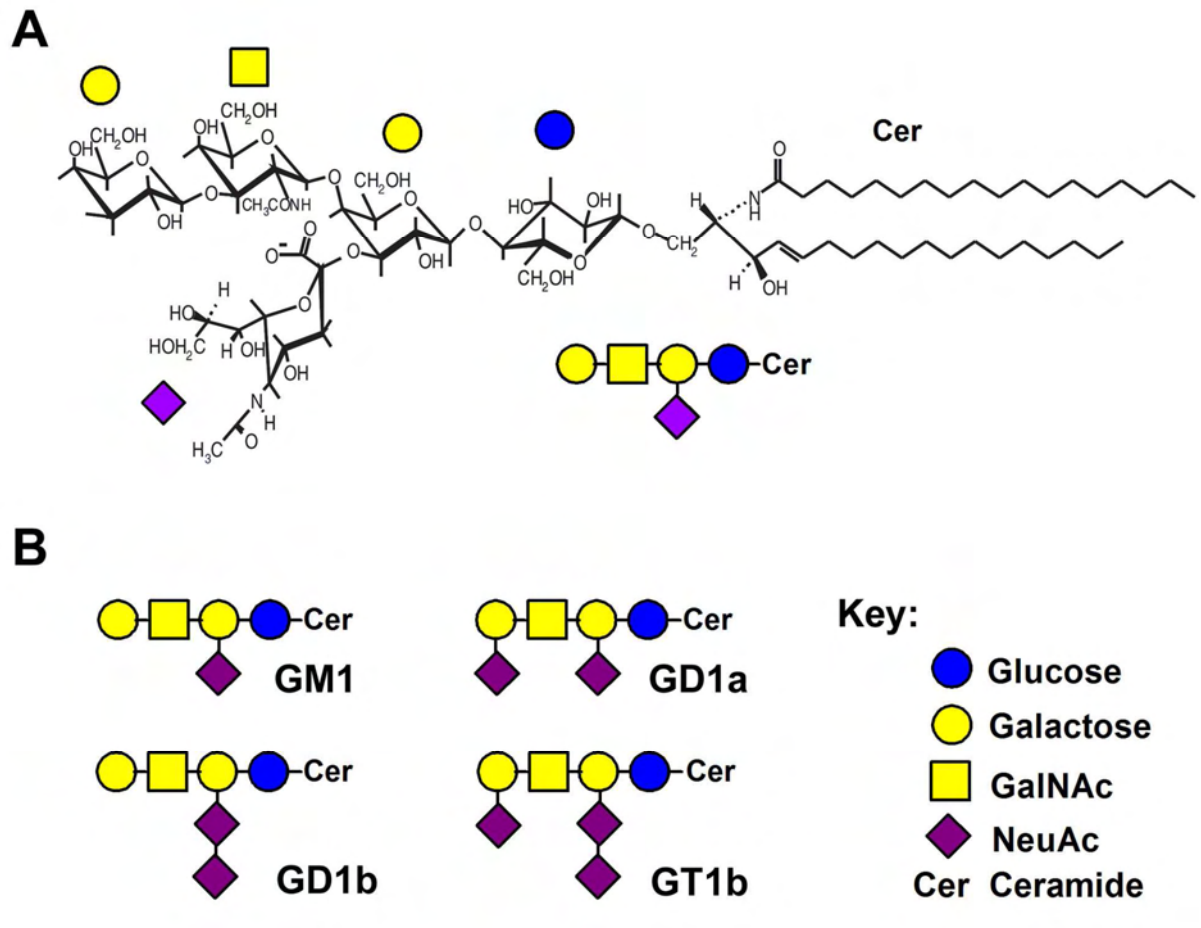
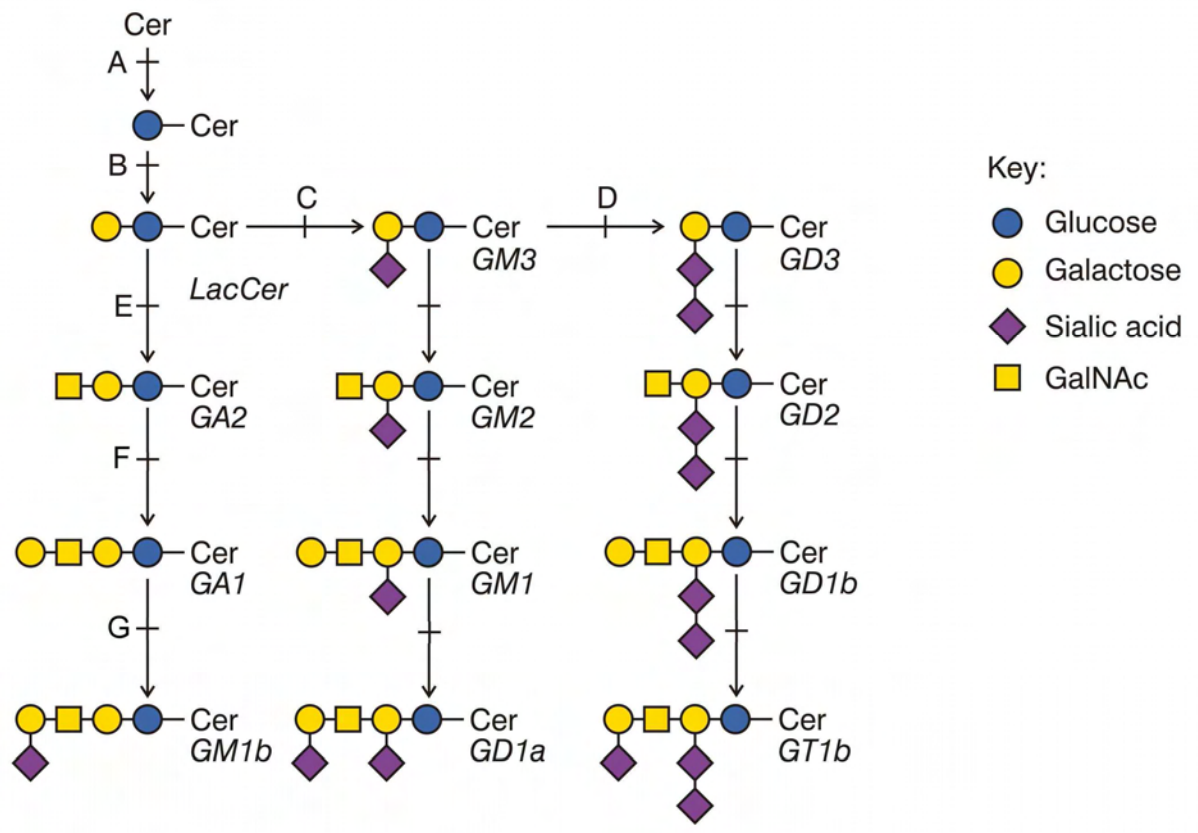
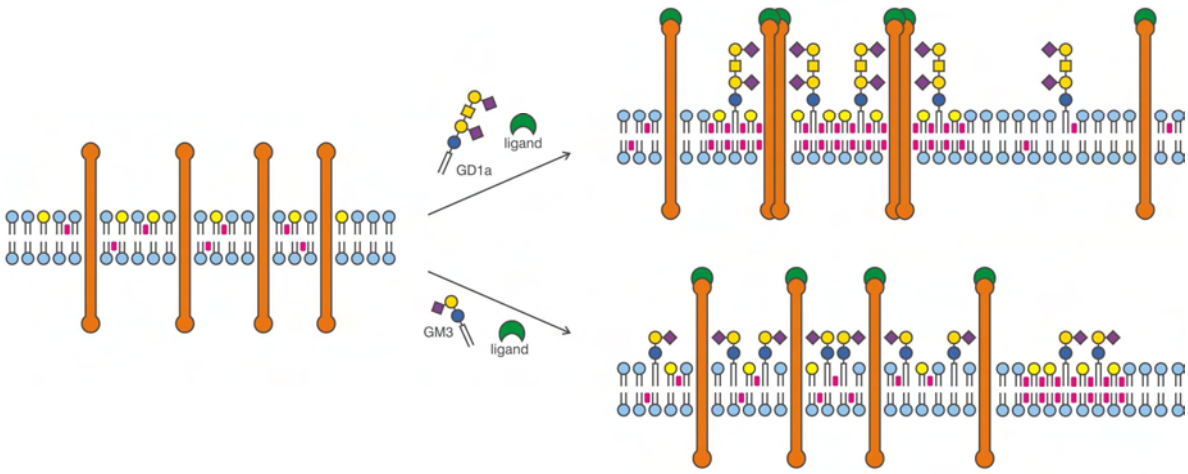


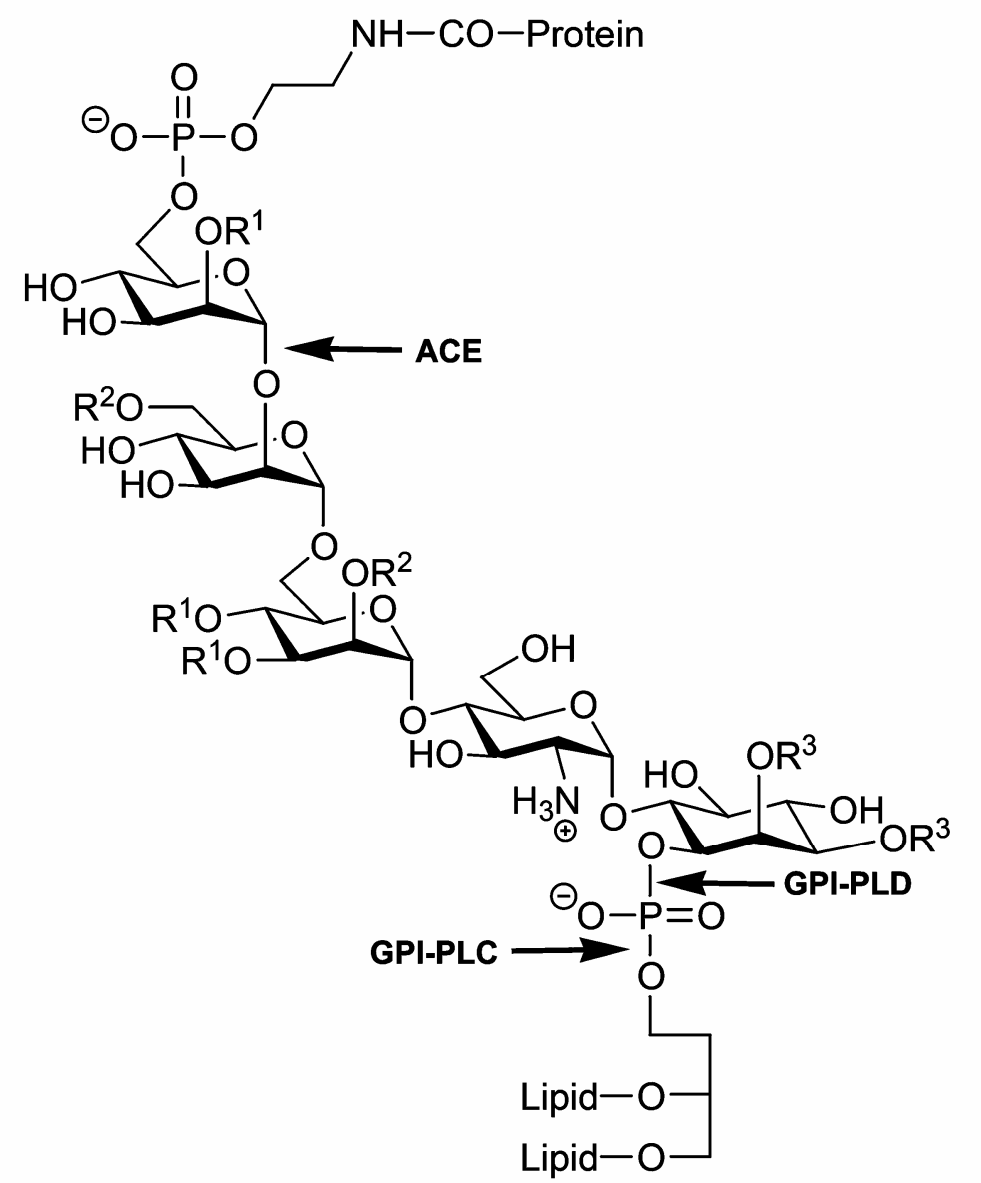
Lauc and Heffer-Lauc, Fig 1.



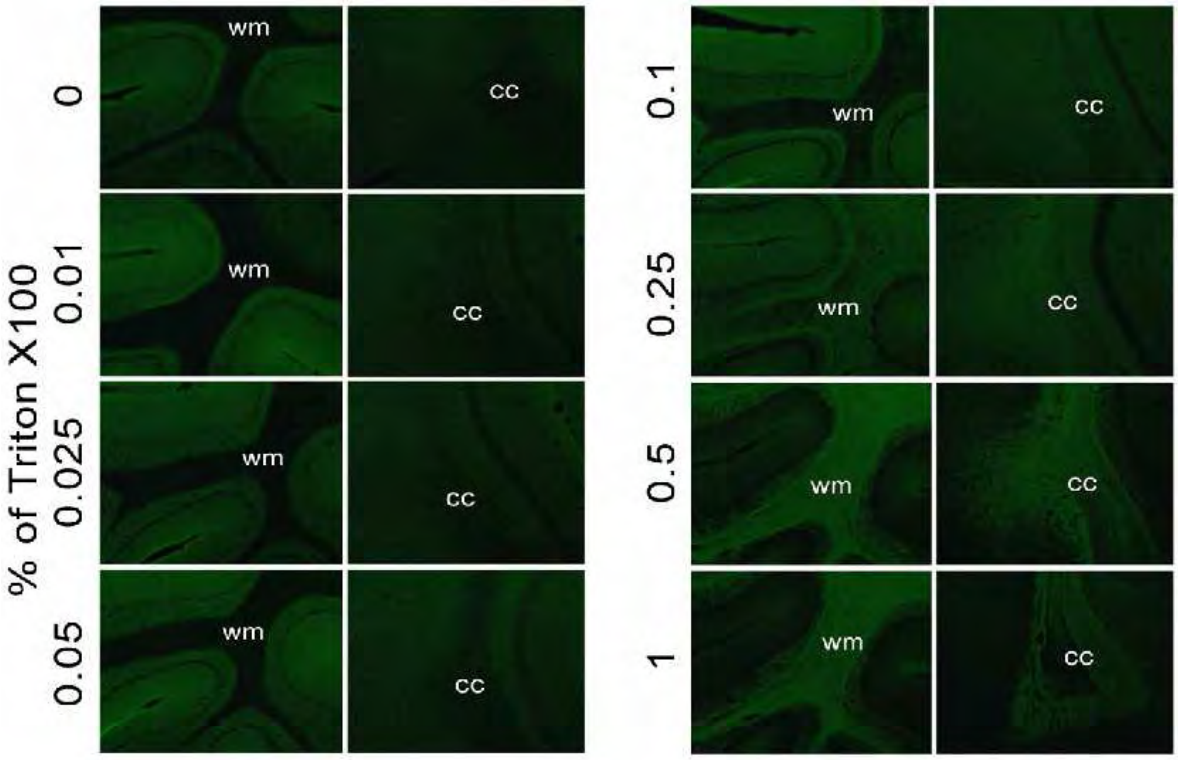
Lauc and Heffer-Lauc, Fig 2



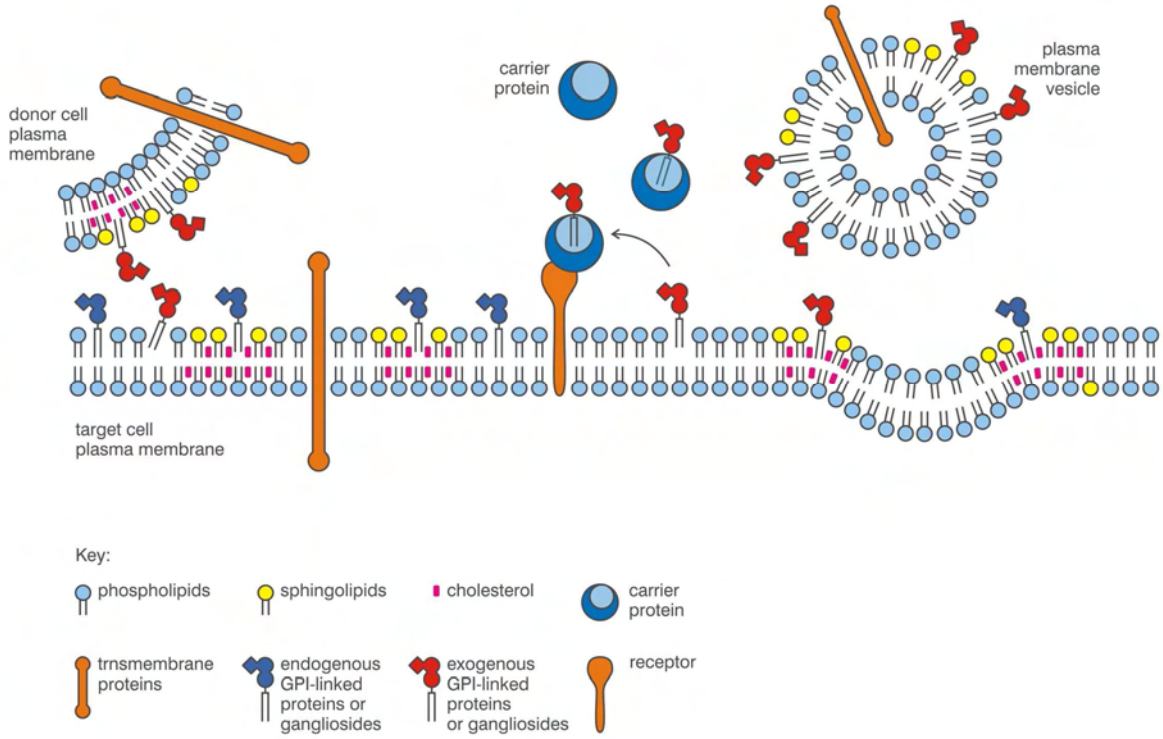
Lauc and Heffer-Lauc, Fig 3



Lauc and Heffer-Lauc, Fig 4.



Lauc and Heffer-Lauc, Fig 5.



Lauc and Heffer-Lauc, Fig 6.

Table 1. Examples of signal transduction processes that involve lipid rafts

- B-cell receptor [275]
- EGF receptor [276]
- Endothelial NOS [277]
- FcεRI receptor [278]
- Insulin receptor [279]
- Integrins [280]
- T-cell receptor [281]

Table 2. Examples of GPI-anchored proteins (for a more complete list see a recent review by H. Ikezawa [1])

Enzymes	**Receptors**	**Other proteins**
Alkaline phosphatase	*Plasmodium* transferrin receptor	Thy-1
Acetylcholinesterase	CD14	CD24
5'-Nucleotidase	CD16	CD55 (DAF)
Alkaline phosphodiesterase I	CD48	CD58
Renal dipeptidase (MDP)	Folate-binding protein	Ly6 family (CD59, Ly6A)
Aminopeptidase P	Urokinase receptor	Carcinoembryonic antigen
NAD^+ glycohydrolase	CNTF receptor	Prions (PrPC, PrPSc)
Carboxypeptidase M	Nogo-66 receptor	NCAM-120
Carbonic anhydrase IV		Tamm-Horsfall glycoprotein
ADP-ribosyltransferase		