Traffic 2007; **8:** 331–338
Blackwell Munksgaard

doi: 10.1111/j.1600-0854.2006.00532.x

Review

Hitch-hiking Between Cells on Lipoprotein Particles

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Cell surface proteins containing covalently linked lipids associate with specialized membrane domains. Morphogens like Hedgehog and Wnt use their lipid anchors to bind to lipoprotein particles and employ lipoproteins to travel through tissues. Removal of their lipid anchors or decreasing lipoprotein levels give rise to adverse Hedgehog and Wnt signaling. Some parasites can also transfer their glycosylphosphatidylinositol-anchored surface proteins to host lipoprotein particles. These antigen-loaded lipoproteins spread throughout the circulation, and probably hamper an adequate immune response by killing neutrophils. Together, these findings imply a widespread role for lipoproteins in intercellular transfer of lipid-anchored surface proteins, and may have various physiological consequences. Here, we discuss how lipid-modified proteins may be transferred to and from lipoproteins at the cellular level.

Key words: glycosylphosphatidylinositol-anchored proteins, hedgehog, intercellular transfer, lipoproteins, lipoprotein receptors, Wnt

Received 2 October 2006, revised and accepted for publication 20 December 2006, published online 30 January 2007

Proteins that are modified with lipids sometimes tightly, and – in case of RABs and ADP ribosylation factors (ARFs) – reversibly associate with cellular membranes (1). Furthermore, the physical properties of lipid anchors are likely to control the localization of their proteins to particular membrane domains, called rafts. Rafts can laterally include or exclude distinct lipids and proteins within a membrane and play pivotal roles in protein sorting and signaling (2,3). Acylated and prenylated proteins occupy different areas on the cytosolic surface of the plasma membrane (4), whereas glycosylphosphatidylinositol (GPI) anchored proteins are enriched in rafts at the cell surface in a controversial and unresolved manner (5–8). Recent findings point to another, inventive role for lipid anchors of surface proteins in trafficking.

Hedgehog, Wnt and epidermal growth factor receptor (EGFR) ligands are families of secretory ligands that travel over more than 30 cell diameters through the aqueous extracellular space, and signal in a concentration-dependent fashion (9,10). Their signal transduction pathways control patterning and proliferation during development and are often involved in the maintenance of the morphology of adult tissues. Some members of these families have been shown to be acylated once or twice (11–13). In addition, Hedgehog proteins contain a cholesterol modification at their C-termini. Hedgehog and Wnt proteins were shown to partition in detergent resistant membrane fractions (DRM), suggesting their presence in rafts (14–16). Their lipid anchors are essential for intercellular trafficking and proper signaling (12,17). Smith–Lemli–Opitz syndrome, desmosterolosis and lathosterolosis are diseases caused by defects in the final stages of cholesterol synthesis. Many of the developmental malformations in these syndromes have been ascribed to defective Hedgehog signaling (18,19).

Like morphogens, GPI-anchored proteins can undergo intercellular transfer without losing their GPI anchor (20–25). Transfer of GPI-anchored proteins to deficient cells has been demonstrated in cell culture, animal models and even in patients. Paroxysmal nocturnal hemoglobinuria (PNH) is a chronic disease with severe hemolytic anemia, and is caused by a defect in the biosynthesis of GPI anchors (26,27). Erythrocytes from patients with PNH are extremely sensitive to complement-mediated lysis, because they lack two GPI-anchored proteins, decay-accelerating factor (CD55) and CD59, which inhibit the formation of the membrane attack complex of complement. Transfer of CD55 and CD59 from microvesicles to PNH erythrocytes reduced their susceptibility to complement-mediated lysis (28). Ectopically expressed CD59 was also transferred from erythrocytes to the vascular endothelial cells, and from seminal fluid to prostasomes (22,29). Furthermore, cell-to-cell transfer of another GPI-anchored protein, Thy-1, occurred in chimeric murine embryoid bodies composed of normal and PNH cells (30). In parasitemic patients, the variant surface glycoprotein (VSG) was transferred from trypanosomal membranes to erythrocytes (31), leading to anemia via destruction of erythrocytes by the immune system. Conversely, some parasites have been shown to acquire host decay-accelerating factor to protect themselves against complement lysis (32).

How proteins with such strong membrane affinity move from producing cells to surrounding cells is puzzling, and more than one mechanism has been proposed to explain how lipid-modified proteins move through tissues (21,33–35). Shedding of GPI-anchored proteins can be mediated by proteases or phospholipases, leaving the lipid anchor in the

donor membrane. GPI-anchored proteins can also be released from cells together with their lipid modification (29,36–38). One of the simplest modes of transport is the formation of micellar multimers (10,29), in which the hydrophobic groups of, for example, Wnt or Hedgehog are arranged in such a way that the protein complex becomes soluble in a polar medium. Alternatively, lipid-anchored proteins could be released on membranous exovesicles containing a complete membrane bilayer, termed exosomes (39,40), surfactant-like particles (41) or nodal vesicular parcels (42). Such particles are generated by vesiculation of plasma membrane protrusions (41) or by an exosome-related mechanism (43,44). Another possibility is that cells pass on Wnt proteins via cell–cell contacts or through long cellular extensions called cytonemes or nanotubules (45–47). Transporters consisting of a membrane bilayer cannot only carry lipid-modified proteins but also transmembrane and cytosolic proteins (48,49).

Lipoprotein-Mediated Transport of Lipid-Modified Proteins

Two recent studies found that lipoprotein particles could act as vehicles for the intercellular movement of lipid-modified proteins (50,51). Lipoprotein particles are large, globular complexes composed of a central core of hydrophobic lipids that are surrounded by a monolayer of membrane lipids. They are held together by one or more members of a family of apolipoproteins, of which some are covalently linked to palmitate as well (52). Lipoprotein particles allow intercellular transport of water-insoluble lipids, fat and signaling metabolites throughout the aqueous circulation of multicellular organisms. In theory, the lipid modification of proteins can anchor the protein in the exoplasmic leaflet of cell membranes as well as in the outer phospholipid layer of lipoproteins (53). In this way, lipid-modified proteins get solubilized and will be transported together with lipoproteins.

In the first study, *Drosophila* Wnt and Hedgehog proteins were found to co-purify with lipoproteins from tissue homogenates, and to co-localize with lipoprotein particles in the developing wing epithelium (50). Furthermore, reduction of lipoprotein levels showed that these particles are required for long-range but not for short-range signaling. Consistent with this idea, multiple *Drosophila* GPI proteins were associated with the lipoprotein fraction in a GPI-specific phospholipase (PLC) dependent way. Despite the many possibilities suggested by this model, direct evidence that lipoproteins function as obligatory, intercellular carriers of lipidated morphogens is lacking. Lipoproteins are indispensable for systemic lipid homeostasis. Perturbation of lipoprotein biosynthesis, intra- and intercellular transport, or degradation could easily affect delicate developmental processes such as morphogen signaling and cell differentiation. It will therefore be very difficult to design an indisputable *in vivo* experiment to demonstrate that lipid-linked morphogens utilize lipoproteins merely as taxi's (34).

Recently, we discovered that the GPI-anchored coat proteins of two parasites, schistosomes and trypanosomes, were physically associated with the host lipoprotein particles in the bloodstream of infected patients (51). As a consequence, cells expressing low-density lipoprotein (LDL) receptors endocytosed parasite GPI-anchored proteins together with LDL particles, which resulted in a lysosomal accumulation of parasite proteins. Upon infection with one of these parasites, the host immune system promptly generates antibodies against the highly antigenic coat proteins, but only inadequately attacks the parasite. In serum from patients suffering from chronic schistosomiasis, we found that antibodies were bound to the patients' own lipoprotein particles, probably via the parasitic antigens. Therefore, LDL particles were co-endocytosed with antibodies by neutrophils via their Fc-receptor. We further demonstrated that the lipoprotein accumulation in neutrophils was associated with enhanced apoptosis. How antibody–antigen–lipoprotein complexes reduce neutrophil viability is unclear, but lipoprotein accumulation induced by anti-apolipoprotein antibodies also efficiently killed neutrophils. These findings are in line with the reduced lipoprotein content and the neutropenia found in patients with chronic schistosomiasis. The intricate relationship between schistosomes and their hosts remains unresolved, but these results provide new clues to how schistosomes may trick the immune system.

The idea that lipid-modified proteins can insert into lipoproteins raises many more questions. It is puzzling how lipid-modified proteins with their lipid anchors inserted in the membrane of producing cells manage to flip into lipoprotein particles. Mammalian plasma lipoproteins have been classified into five major groups based on their size and density, but lipoproteins are also found in cerebrospinal fluid, the interstitial space of the brain and in other body fluids. On what kind of lipoproteins are lipid-modified proteins secreted? Lipoproteins contain different apolipoproteins and some of them are exchangeable (Figure 1). Are there apolipoproteins that specifically bind certain lipid-modified proteins? Contradictory to Wnt and Hedgehog secretion, palmitoylation of the *Drosophila* EGF receptor ligand Spitz (Spi) anchors the protein more tightly to the plasma membrane of producing cells and thereby increases its signaling (12). Thus, can all lipid-modified proteins be secreted via lipoproteins or is specific machinery required to facilitate the release of particular lipidated proteins onto lipoproteins? How does the interaction with receiving cells take place? Can lipidated proteins insert back into membranes? Most importantly, what are the (patho)physiological implications of this resourceful mechanism?

Incorporation into Lipoproteins

Lipidated proteins that hitchhike from cell to cell on lipoproteins might exploit similar strategies and mechanisms as those utilized for intercellular lipid transport (54,55). Their association with and dissociation from

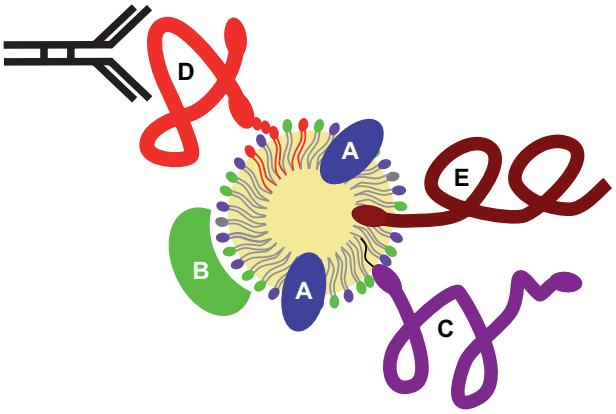

Figure 1: Association of lipid-modified proteins with lipoproteins. The hydrophobic core of a lipoprotein particle is surrounded by a monolayer of membrane lipids. Integral apolipoproteins (A), like apoAI and ApoB100 form belt-like structures around the particle and provide structural stability. Peripheral apolipoproteins (B), such as GPI-PLD, interact with surface lipids via amphipatic helices, and easily transfer between circulating lipoprotein particles. The covalent lipid modification of surface proteins (C) equally well fit in the outer leaflet of the plasma membrane as in the monolayer around lipoprotein particles. Also parasite lipids and lipid-modified proteins (D) can associate lipoproteins. Parasitic GPI anchors are often structurally different from mammalian ones, making them resistant to GPI-specific phospholipases. In theory, membrane proteins that span a membrane only partially or that are cleaved intramembranously, could associate with lipoproteins as well (117). Transport of proteins associated with lipoproteins affect lipoprotein transport and vice versa. For example, LDL receptors bind apoB, and co-endocytose LDL together with parasite GPI-anchored proteins (51). Neutrophils that recognize and endocytose parasite GPI-anchored proteins, also take up LDL via the Fc-receptor (51).

lipoproteins may take place on different sites: in the secretory route where lipoproteins such as LDL and chylomicrons are assembled, at the cell surface or during endocytosis and recycling where for instance high-density lipoprotein (HDL) is (un)loaded with lipids.

Cell surface proteins acquire their lipid modification post-translationally in the endoplasmic reticulum (56–58), the organelle where VLDL, LDL and chylomicrons are assembled as well. It is likely that GPI-anchored proteins and morphogens can be incorporated in these lipoprotein particles during assembly or transport to the cell surface. Several Wnt- and Hedgehog-secreting cells also synthesize their own lipoproteins (59,60). Remarkably, inadequate lipoprotein assembly results in impaired embryonic neurodevelopment (61,62), a phenotype comparable with defective Hedgehog signaling. Furthermore, combining fat metabolism with morphogenic signals allows tissues to modify their organization in response to altered nutrient uptake, and may give novel clues about the role of dietary fat in carcinogenesis (63).

Only a few cell types synthesize lipoproteins themselves. In *Drosophila*, for example, lipoprotein particles are assembled in the fat body and function as a reusable lipid shuttle – they transport lipids from the fat body to tissues and back. Tissues, like the wing imaginal disc, that make Wingless and Hedgehog do not synthesize lipoproteins. These cells can interact with lipoprotein particles present in their external environment either on the cell surface or in the endocytic route. GPI-anchored proteins, and most likely also the lipid-linked morphogens, are enriched at the cell surface. They are endocytosed and recycled back to the cell surface via several mechanisms (64). Association of lipid-modified proteins to lipoproteins could therefore easily happen at the cell surface or during endocytosis and recycling of lipoprotein particles (65,66). In mammals, HDL particles either bind to the cell surface or are endocytosed and recycled back to the cells surface (66). LDL is normally endocytosed and degraded in lysosomes, but some cells may be able to recycle LDL back to the cell surface (67,68). An endosomal pathway exists in trypanosomes that specifically endocytose and recycle surface GPI-anchored proteins together with LDL (69). This route might be required for the release of VSG on host lipoprotein particles into the circulation (51).

Remarkably, secretion of Wnt in *Caenorhabditis elegans* requires the retromer, a multiprotein complex involved in intracellular membrane trafficking (70). Transfer of Wnt to lipoproteins could take place in endosomal organelles, and the retromer could be required for recycling the complex back to the cell surface (71,72).

Lipoprotein Receptors

Each type of lipoprotein has distinct apolipoproteins that mediate binding of the lipoprotein to cell surface receptors. Lipoprotein receptors are indispensable for efficient lipid transfer between cells and lipoproteins, although they are not involved in the actual transfer itself. These receptors bring lipoproteins in close proximity to the membrane after which lipid transfer can take place. Maybe, lipoprotein receptors are involved in transfer of lipid-modified proteins in a similar manner. Two large families of cell surface receptors mediate binding to lipoproteins, the scavenger receptors (73) and the LDL-receptor family (74).

Scavenger receptors are defined as cell surface membrane proteins that bind chemically modified lipoproteins such as acetylated LDL and oxidized LDL (75,76). The prototypic scavenger receptor, Scavenger receptor class B, member 1 (SR-BI) is an HDL receptor that mediates the cellular uptake of cholesterol esters from HDL (77,78). *In vitro* studies have shown that CD55 and CD59 can incorporate into HDL (53), but whether that also occurs *in vivo*, and whether that serves a physiological role remains to be established.

The LDL-receptor family consists of seven structurally related transmembrane proteins. While the LDL receptor plays an essential role in cholesterol homeostasis, the other

LDL receptor related protein family members (LRPs) fulfill a variety of biological functions, many of which are not directly related to lipid metabolism. They bind and endocytose a multitude of extracellular ligands and also directly participate in signal transduction processes. Remarkably, most LRPs have not lost their capability to bind and endocytose lipoproteins or their remnants (74,79). LRP2, for example, serves as a receptor for chylomicron remnants, lipoproteins that shuttle dietary cholesterol from the gut to the liver (74,79). Several members of the LDL-receptor family have been shown to be required during the early stages of embryonic development, in particular Wnt and Hedgehog signaling (74,80). LRP2 has been shown to endocytose Hedgehog (81), and LRP2 knockout mice display defects that are consistent with loss of Hedgehog signaling (82). A recent study however, has suggested an indirect role of LRP2 in Hedgehog signaling (83). LRP5 and LRP6, and maybe also LRP1 are required for Wnt signaling (84,85). Whether these receptors directly interact with Wnts and Hedgehogs alone or whether the interaction is specific for lipoprotein-associated morphogens is unknown (86). Furthermore, it will be interesting to investigate whether these receptors are also involved in the secretion and recycling of morphogens (87).

Heparan Sulfate Proteoglycans

Interestingly, trafficking of both lipid-modified morphogens and lipoproteins also involves another family of cell surface co-receptors. Heparan sulfate proteoglycans (HSPGs) are large molecules composed of repeated sulfated disaccharides covalently attached to core proteins. The HSPGs are abundantly expressed by virtually all mammalian cells and are found on cell surfaces and in the extracellular matrix. The negatively charged sulfate and carboxyl groups of the heparan sulfate in HSPGs interact with positively charged residues on lipoproteins and many other secretory proteins with low affinities. Heparan sulfate proteoglycans of the extracellular matrix play important roles in lipoprotein retention (88), and cell surface HSPGs allow cell adhesion and uptake – even in the absence of lipoprotein receptors – by enhancing the accessibility of lipoproteins to lipoprotein receptors (89,90). Interestingly, heparan sulfate is a basolateral-sorting determinant that may influence the polarized secretion of lipoproteins after synthesis or during transcytosis (91,92).

Heparan sulfate proteoglycans affect trafficking of lipid-modified morphogens via several different mechanisms (33,93,94). Mutations that block the synthesis of heparan sulfates not only impair intracellular accumulation, gradient formation and long-range signaling in *Drosophila* of Wnt and Hedgehog, but also of Decapentaplegic, a non lipid-modified morphogen (95–97). The HSPGs at the cell surface, like *Drosophila* Dlp (33), and in the extracellular matrix [*Drosophila* Trol (98)] might restrict the diffusion of morphogen throughout the epithelium, effectively increasing the local morphogen concentration. Remarkably, diffusion of Hedgehog without a lipid modification is independent of HSPGs (94). Heparan sulfate proteoglycans may restrict morphogen diffusion indirectly by inhibiting the movement of their lipoprotein carrier. Alternatively, HSPGs like *Drosophila* Dally, could affect intracellular trafficking of morphogens. The HSPGs might direct apical/basolateral sorting in morphogen-producing cells, whereas HSPGs in receiving cells might affect lysosomal degradation of morphogens together with their carriers and stimulate their recycling (33,87).

Transporters and Translocators

The mechanisms underlying the biogenesis, maturation and disassembly of lipoprotein particles at the molecular level are complex and not well understood (54,99). The two extremes, monomeric lipid transfer versus fusion with membranes, both require different protein machinery. Transfer of lipidated proteins could occur passively and reversibly, possibly enhanced by the binding of lipoproteins to surface receptors. ATP-binding cassette (ABC) transporters transfer lipids and perhaps also lipid-modified proteins to secretory lipoprotein particles (100). Remarkably, Dispatched – a member of the sterol-sensing receptor family – is essential for the release of Hedgehog from cells. Dispatched is not required for the release of a non–cholesterol modified form, suggesting that Hedgehog requires a transporter-like function of Dispatched (101,102). Another member of this family, Patched binds Hedgehog in receiving cells, and is involved in signaling. However, *C. elegans* has nearly 30 genes related to Patched and Dispatched, but has no Hedgehog (103). A recent study showed that Patched is involved in the secretion of the 3β-hydroxysteroid (pro-)vitamin D3 (104). Confusingly, two other members of this family, Niemann-Pick disease type C1 (NPC1) and Niemann-Pick disease type C1 like 1 (NPC1L1), have been implicated in the transport of cholesterol in the opposite direction: to cytosolic leaflets of cell membranes (105). Ultimately, functional reconstitution of purified members of this family in model membranes is a necessary step to test their ability to move sterol across the membrane bilayer.

Intercellular Movement

Solubilization of anchor-intact lipid-modified proteins by incorporation into lipoproteins enables their transport through aqueous environments. But why are GPI-anchored proteins not abundantly present on lipoproteins in our circulation? GPI anchors can be cleaved by specific phospholipases and the only mammalian member cloned to date is GPI-specific phospholipase D (GPI-PLD). GPI-PLD is relatively abundant in serum (~10 μg/mL) and has a well-characterized biochemistry, but its physiological role is completely unknown (106). GPI-PLD is an HDL-associated protein, and is able to exchange between different lipoprotein classes (107,108).

Although GPI-PLD specifically cleaves GPIs *in vitro*, GPI-PLD appears to cleave GPI-anchored proteins from the cell surface only if the membrane is perturbed with detergents. This raises the possibility that GPI-PLD is catalytically inactive in serum or requires a change in the membrane environment of the substrate to allow cleavage (107,109–111). Possibly, its activity is directed towards GPI proteins released onto lipoproteins. GPI-PLD might prevent systemic spread of GPI proteins released on lipoproteins.

Hedgehog and Wnts are not abundantly present in our bloodstream either and their movement is restricted to specific tissues, where they form concentration gradients. Experimental and mathematical approaches favor the idea that morphogens move along the surface of cells by diffusion, possibly interacting with surface receptors or extracellular matrix components (34,112). Morphogens present on lipoproteins can interact with two independent surface receptors instead of one – one specific for the morphogen and another for the apolipoprotein. This cooperative binding greatly increases the affinity of the morphogen–lipoprotein complex to cells, and therefore contributes to the restricted diffusion of morphogens.

Concluding Remarks

Lipoprotein carriers may determine the intercellular distribution and activity of lipid-anchored proteins. Conversely, the fate and function of lipoproteins might very well be determined by their cargo. Indeed, the importance of HDL-associated proteins in lipid metabolism and atherosclerosis has been increasingly appreciated (113,114). Maybe, many more lipidated proteins utilize this system to communicate to neighboring cells (115). A new and exciting study revealed the presence of GPI-anchored prion on LDL (116). Finally, it seems that parasites utilize this trick as well. Survival of parasites depends on their ability to escape the host innate immune system. Unraveling how lipidated proteins associate with lipoproteins can provide new approaches to tackle morphogen-related cancers, infectious and prion-based diseases, like Creutzfeld–Jacob disease.

Acknowledgment

We are grateful to Maarten R. Egmond and Gerrit van Meer for critical reading of the manuscript.

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