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Review

Lipoprotein assembly and function in an evolutionary perspective

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Abstract

Circulatory fat transport in animals relies on members of the large lipid transfer protein (LLTP) superfamily, including mammalian apolipoprotein B (apoB) and insect apolipophorin II/I (apoLp-II/I). ApoB and apoLp-II/I, constituting the structural (non-exchangeable) basis for the assembly of various lipoproteins, acquire lipids through microsomal triglyceride-transfer protein, another LLTP family member, and bind them by means of amphipathic α-helical and β-sheet structural motifs. Comparative research reveals that LLTPs evolved from the earliest animals and highlights the structural adaptations in these lipid-binding proteins. Thus, in contrast to apoB, apoLp-II/I is cleaved post-translationally by a furin, resulting in the appearance of two non-exchangeable apolipoproteins in the single circulatory lipoprotein in insects, high-density lipophorin (HDLp). The remarkable structural similarities between mammalian and insect lipoproteins notwithstanding important functional differences relate to the mechanism of lipid delivery. Whereas in mammals, partial delipidation of apoB-containing lipoproteins eventually results in endocytic uptake of their remnants, mediated by members of the low-density lipoprotein receptor (LDLR) family, and degradation in lysosomes, insect HDLp functions as a reusable lipid shuttle capable of alternate unloading and reloading of lipid. Also, during muscular efforts (flight activity), an HDLp-based lipoprotein shuttle provides for the transport of lipid for energy generation. Although a lipophorin receptor – a homolog of LDLR – was identified that mediates endocytic uptake of HDLp during specific developmental periods, the endocytosed lipoprotein appears to be recycled in a transferrin-like manner. These data highlight that the functional adaptations in the lipoprotein lipid carriers in mammals and insects also emerge with regard to the functioning of their cognate receptors.

Keywords: apoB; apolipophorin III; diacylglycerol; insect lipophorin receptor; low-density lipophorin (LDLp); LDL receptor; lipophorin; lipoprotein recycling; large lipid transfer (LLT) domain; microsomal triglyceride-transfer protein (MTP).

Introduction

Lipoproteins are noncovalent assemblies of lipids and proteins, organized as largely spherical particles that allow water-insoluble lipids and other lipophilic components to be transported in the aqueous plasma of the circulatory system of vertebrates as well as invertebrates. The protein components (apolipoproteins) of these particles are amphiphatic in nature and function in both stabilizing the lipid components and mediating particle metabolism.

For the transport of bulk neutral lipids (triacylglycerol, TAG) in the circulation, mammals generally rely on two different classes of TAG-rich lipoprotein particles, each harboring a single copy of integral (non-exchangeable) apolipoprotein B (apoB): liver-derived apoB-100-containing very-low-density lipoproteins (VLDLs) that transport mainly endogenously synthesized lipids, and apoB-48-containing chylomicrons, synthesized by the intestine, that carry diet-derived (exogenous) lipid components. In rodents, however, the apoB-100 mRNA editing mechanism producing apoB-48 in the intestine is also operative in the liver, resulting in an additional production of liver-derived chylomicrons. Selective (non-endocytic) delivery of their lipid cargo, involving hydrolysis of TAG by lipoprotein lipase (LPL) located peripherally on the luminal surface of endothelial cells and interacting with the amino terminal region of apoB (1), results in the progressive conversion of these lipoproteins into smaller remnant particles. Thus, VLDLs are eventually converted into intermediate-density lipoproteins (IDLs) and low-density lipoproteins (LDLs) and chylomicrons into chylomicron remnants. During these conversions, in which high-density lipoprotein (HDL) serves both as a donor and an acceptor for exchangeable apolipoproteins, the remnant particles become enriched in cholesterol esters and are taken up into cells by receptor-mediated endocytosis. In addition to apoB, remnant-associated exchangeable apoE has been recognized as a critical ligand for remnant clearance [for reviews, see refs. (2–11)]. In the latter process of non-selective lipid delivery, the lipoprotein remnants are captured and internalized by members of the LDL receptor (LDLR) family and their apoB and associated lipid components degraded in lysosomes, whereas remnant-derived apoE is recycled (8, 12). In addition, the receptors that release their ligand due to acidification of the endosomal lumen are recycled back to the cell surface for another round of endocytic lipoprotein uptake [(6, 10, 13–17); for reviews, see refs. (18–20)].

The lipoprotein system of insects – which constitute by far the most extensively studied class of invertebrates in this

regard – has revealed another concept for lipid transport. In contrast to mammals, insects use only one single lipoprotein, termed lipophorin, to execute both exogenous and endogenous lipid transport. This multifunctional particle is relatively lipid-poor, displaying a buoyant density similar to that of mammalian HDL (~1.12 g/ml), and is therefore referred to as high-density lipophorin (HDLp). HDLp is produced in the insect fat body, a specialized tissue combining many of the properties and functions of mammalian liver and adipose tissue, including synthesis and storage of lipids. The lipoprotein harbors single copies of its two non-exchangeable apolipoprotein components, apolipophorins I and II (apoLp-I and -II), resulting from cleavage of their precursor apolipophorin II/I (apoLp-II/I) during lipoprotein biosynthesis [for recent reviews, see refs. (21–24)]. Although similar to mammals in that fat body lipid is stored as TAG in intracellular lipid droplets, HDLp transports *sn*-1,2-diacylglycerol (DAG) rather than TAG as its major lipid constituent. Moreover, the conversion of TAG to DAG allows additional DAG to be transferred across the fat body cell membrane and taken up extracellularly by pre-existing HDLp [for review, see ref. (25)]. Virtually all the HDLp-carried DAG is located in the core of the particle, which additionally comprises hydrocarbons and other hydrophobic lipids, including minor amounts of TAG, whereas the core is surrounded by a phospholipid monolayer [mainly phosphatidylcholine and phosphatidylethanolamine; (26)] containing minor amounts of sterols and DAG (27) in which the structural proteins are embedded. The core lipid composition of HDLp contrasts markedly with that of mammalian chylomicrons or VLDLs, in which TAG comprise the bulk component [for reviews, see refs. (9, 22)]. A hallmark feature of HDLp-mediated lipid transport relates to the ability of HDLp particle to circulate in the insect blood (hemolymph) between different sites and to alternately deliver and take up lipids without being internalized or degraded. Thus, HDLp functions as a reusable lipid shuttle, without the requirement of additional synthesis or increased degradation of its apolipoprotein matrix [for reviews, see refs. (21, 22, 24, 28–30)].

Also, during extensive lipid mobilization, such as during prolonged muscular exercise, circulatory lipid transport in mammals and insects use different concepts. In mammals, the abundant serum protein, albumin, transports the free fatty acids (FFAs) resulting from hydrolysis of TAG stores in adipose tissue to the working muscles. By contrast, in insect species engaging in long-term flights – the most energy-demanding process in nature – the robust increase in lipid transport elicited by flight activity relies on circulatory HDLp that once more serves as the basal ingredient for a reusable lipid shuttle. Flight activity triggers the release of peptidergic adipokinetic hormones (AKHs) from a pituitary-like neuroendocrine gland (corpus cardiacum) at the base of the insect brain, that act upon the fat body cells to stimulate the conversion of TAG stores to *sn*-1,2-DAG and its transfer to circulating HDLp [for reviews, see refs. (24, 29–32)]. In this extracellular event, a lipid transfer particle (LTP), bearing functional similarity to the mammalian intracellular and extracellular lipid transfer proteins involved in the redistribution of hydrophobic lipid molecules [reviewed in refs. (21, 22)], has been shown to catalyze the exchange and net transfer of lipids (DAG, but also hydrocarbons, carotenoids and phospholipids although at a lower rate; however, not cholesterol) between lipophorin subspecies (33, 34) and is implicated in facilitating DAG transfer to HDLp [(35); for reviews, see refs. (21, 28, 31, 35, 36)]. By the loading of DAG, the expanding extracellular HDLp almost doubles its diameter and is converted into a less dense form falling into the low-density lipoprotein class [low-density lipophorin (LDLp); buoyant density ~1.04 g/ml]. The increase in lipid content induces several molecules of a low-molecular weight amphipathic exchangeable apolipoprotein, apolipophorin III (apoLp-III, M_r ~ 18 000), to associate with the particle and to unfold to cover its increased surface area. In turn, when the DAG cargo is hydrolyzed by a lipophorin lipase residing at the flight muscle cell surface to provide the FFA used for oxidative energy generation, apoLp-III dissociates, regenerating the original HDLp particle which cycles back to the fat body for another round of lipid uptake and transport (Figure 1) [for reviews, see refs. (21, 24, 28, 37)]. ApoLp-III, which exhibits a dual capacity to exist in both lipid-bound and lipid-free states and plays a crucial role in this unique insect lipoprotein shuttle mechanism, is likewise available for an additional cycle of LDLp-carried DAG transport. ApoLp-III, which bears striking structural similarities to its human counterparts, particularly the 22 kDa N-terminal domain of human apoE, has developed into a valuable model that has provided important insight into structure-function relationships of this class of exchangeable apolipoproteins [for reviews, see refs. (38–41)].

Notwithstanding the functional differences between the lipoprotein systems of mammals and insects, from an evolutionary point of view there are also remarkable structural similarities, as mammalian apoB and insect apoLp-II/I, which constitute the structural basis for the assembly of their respective lipoproteins, were shown to be homologs (42). Additionally, microsomal triglyceride-transfer protein (MTP), an endoplasmic reticulum (ER)-localized dedicated cofactor through which apoB acquires lipids intracellularly, is another member of the protein superfamily comprising apoB and apoLp-II/I [(42, 43); for review, see ref. (23)]. Based on the sequence similarity between apoLp-II/I and apoB, insect HDLp resembles mammalian LDL. Furthermore, the size of the HDLp particle (44, 45) although slightly smaller than that of LDL (46, 47), is of the same order of magnitude and both lipoproteins are practically devoid of exchangeable apolipoproteins. The resemblance of HDLp to LDL was recently extended by the identification of an insect LDLR family member that is capable of endocytic uptake of HDLp into fat body cells, suggesting receptor-mediated endocytosis to constitute an additional mechanism for lipid delivery (48, 49). However, in spite of the high structural similarity of this insect lipophorin receptor (LpR) to mammalian LDLR, endocytosed HDLp appears not to be degraded in lysosomes like mammalian LDL, but is resecreted in a manner similar to transferrin (49). These data indicate that, despite their structural similarities, the functional adaptations in the lipoprotein

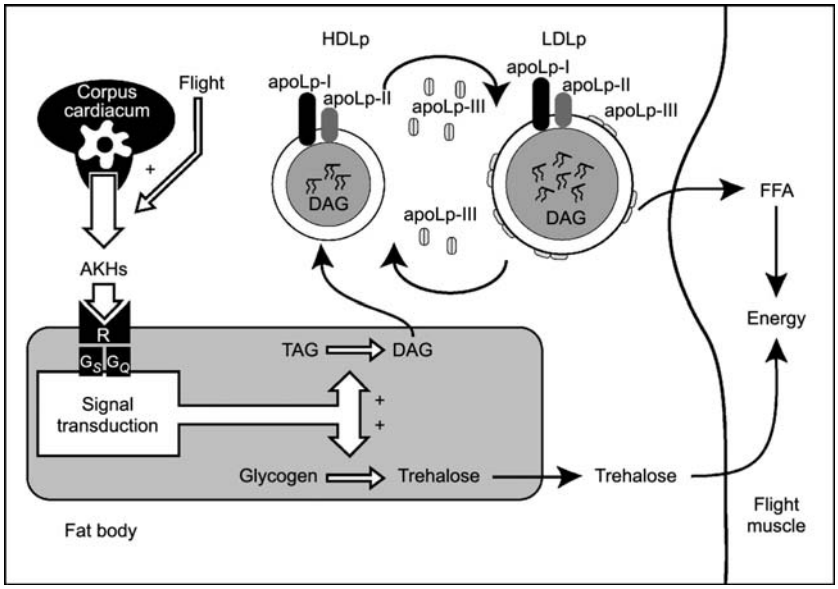

Figure 1 Molecular basis of the lipophorin lipid shuttle.
AKH-controlled DAG mobilization from insect fat body during flight activity results in the reversible conversion of relatively lipid-poor HDLp in lipid-loaded LDLp and apoLp-III from a lipid-free in a lipid bound state. The reversible conformational change in apoLp-III induced by DAG loading of lipophorin is schematically visualized. AKHs: adipokinetic hormones; R: AKH receptor; G: G protein; HDLp: high-density lipophorin; LDLp: low-density lipophorin; apoLp-I, -II and -III: apolipophorin I, II and III; TAG: triacylglycerol; DAG: diacylglycerol; FFA: free fatty acids. Based on data from refs. (21) and (29). All figures (1–8) are (adapted versions of) original artwork created by the authors, and the references for the papers in which the figures were published are provided.

lipid carriers in mammals and insects also emerge with regard to the functioning of their cognate receptors.

This review focuses on recent developments in the molecular and cellular aspects of lipoprotein-mediated circulatory lipid transport and will be confined particularly to the assembly and functioning of mammalian and insect lipoproteins from an evolutionary perspective, even though detailed functioning of both systems appear to have developed differently.

From the viewpoint that insects constitute the largest – and very successful – animal group on earth, understanding of their solutions for circulatory lipid transport has profound biological significance. Such an understanding can additionally provide insight into corresponding processes in mammalian circulatory lipid transport and even into processes that hitherto were not considered to occur in mammals.

Assembly and secretion of lipoproteins in insects and mammals

The non-exchangeable apoLp-I and -II (M_r of approximately 220 000 and 70 000, respectively) of locust HDLp were shown to result from post-translational cleavage of their common precursor apolipoprotein, apoLp-II/I (50), which is arranged with apoLp-II at the N-terminal end and apoLp-I at the C-terminal end (51). The apoLp-II/I cDNA of several insect species has been isolated and characterized (51–55) or identified in genome analysis projects (56–58). Based on sequence similarity and ancestral exon boundaries, these insect apolipoprotein precursors were revealed to belong to the large lipid transfer (LLT) protein (LLTP) superfamily that emerged from an ancestral molecule and includes vertebrate apoB, microsomal triglyceride-transfer protein (MTP) and vitellogenin (Vtg), the egg yolk precursor protein in oviparous species (42). The LLT domain shared by these proteins comprises a large N-terminal domain of approximately 1000 amino acids; the LLT domains of apoB, MTP and Vtg contain a large lipid binding cavity which was proposed to act to store lipids or to transfer lipids to the apolipoprotein in a coordinated manner [(42, 43, 59–64); for reviews, see refs. (22, 23)]. A recent model of locust (*Locusta migratoria*) apoLp-II/I, constructed on homology with the X-ray crystal structure of lamprey lipovitellin, the processed form of Vtg (65), as well as a structural model for a nascent human apoB lipoprotein particle (60), reveals a similar putative lipid pocket in the LLT domain (63) (Figure 2). The cleavage of the insect apoLp-II/I into apoLp-II and apoLp-I occurs between two residues (720 and 721) of the LLT module in an 80-residue long loop connecting two β-strands at the base of this putative lipid pocket.

The assembly of mammalian apoB-containing lipoproteins is widely believed to occur in two steps. The cotranslational lipidation of apoB in the rough endoplasmic reticulum is completed post-translationally in the smooth endoplasmic reticulum and/or *cis*-Golgi network by acquiring the bulk of its neutral lipids (TAG), presumably by fusion with an intralumenal neutral lipid droplet (66–72). The initial cotranslational deposition of lipids in the lipid pocket of the apoB LLT module, which constitutes the first step of lipoprotein particle assembly, requires interaction with MTP (66, 67, 70,

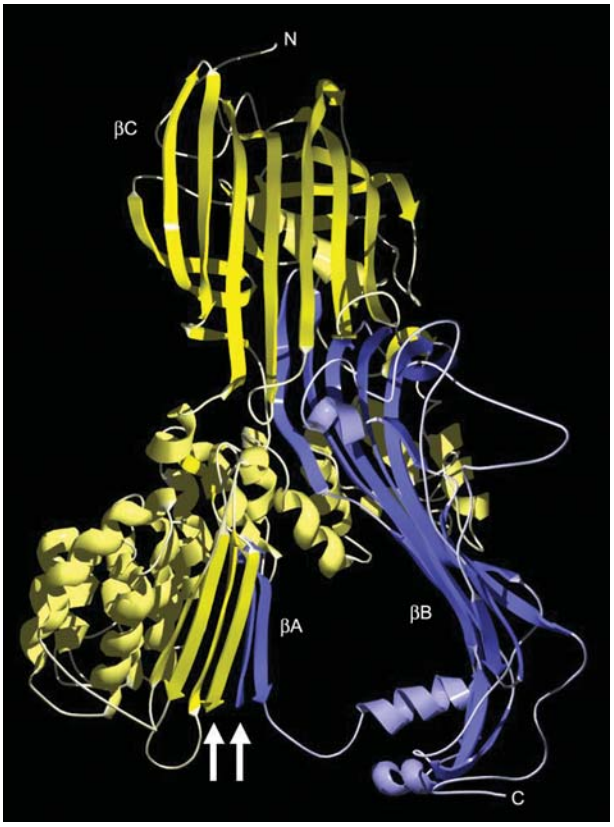

Figure 2 Model of the LLT domain of locust apoLp-II/I.
Model of *L. migratoria* apoLp-II/I (amino acid residues 22–1030), based upon the sequence homology with lamprey lipovitellin (65) and human apoB (60). The structures constituting part of apoLp-I and apoLp-II following apoLp-II/I cleavage are marked in shades of blue and yellow, respectively. The three β-sheets are indicated by βA, βB and βC. The characters N and C mark the amino- and carboxy-terminal sides of the modeled region, respectively. The arrows indicate the β-strands at the base of the putative lipid pocket that are connected by a loop formed by the amino acid residues 669–748 (loop not indicated). apoLp-II/I is cleaved within this loop, between residues 720 and 721 by an insect furin. LLT domain: large lipid transfer domain; apoLp-I, -II and -II/I: apolipophorin I, II and II/I. From ref. (63).

72–76). Based on the homology between apoB and apoLp-II/I, as well as the recovery of an MTP homolog in the fruitfly, *Drosophila melanogaster*, that was able to promote the assembly and secretion of human apoB (77), insect lipoprotein assembly was proposed to proceed similarly to apoB-containing lipoproteins (63). Additional evidence in favor of such a view comes from experimental studies on insect lipoprotein biogenesis that will be elaborated below.

The cleavage of apoLp-II/I was shown to be mediated by an insect furin at a consensus substrate sequence (RQKR) (63). Because protein cleavage by furin homologs is performed late in the secretory pathway, mainly in the *trans*-Golgi network (78), insect lipoprotein biosynthesis was proposed to proceed by initial lipidation of apoLp-II/I to a lipoprotein, whereas cleavage of apoLp-II/I into apoLp-I and -II would occur at a later stage (63). The uncleaved LLT domain in apoLp-II/I, comprising intimately linked regions of apoLp-I and apoLp-II, is likely to be essential to enable the first step in lipidation, as in apoB. Moreover, the occurrence of a cleavage step prior to lipidation might result in the parting of apoLp-I and apoLp-II, and thus in impairment of lipoprotein biosynthesis. In conformity with the above, if cleavage was impaired by a furin inhibitor or mutagenesis of the consensus substrate sequence for furin, uncleaved apoLp-II/I appeared to be lipidated and functioned as a single apolipoprotein in the formation of a lipoprotein particle, similar to mammalian apoB. Because a lipoprotein particle with a buoyant density and molecular mass identical to wild-type HDLp was produced, it was concluded that cleavage of apoLp-II/I by insect furin is neither required for biosynthesis nor for secretion of the insect lipoprotein (63).

The apparent conservation of apoLp-II/I cleavage in all insects characterized to date reveals the importance of this processing step. Vtg is cleaved likewise at a furin consensus substrate sequence in the LLT domain during biosynthesis in most insect species, although not in oviparous vertebrates (79). The rationale for apoLp-II/I cleavage awaits disclosure, but has been suggested to constitute a molecular adaptation relating to the specific functioning of the insect lipoprotein as a reusable lipid shuttle, whereas the increased flexibility of apoLp-I and -II resulting from cleavage of apoLp-II/I can additionally allow for the loading of the particle with an increased lipid cargo and the resultant conversion of HDLp to LDLp during conditions that require enhanced lipid transport, such as flight activity (63).

The structural resemblance between apoLp-II/I and apoB is not limited to the LLT module but also extends to the entire polypeptide chains. Prediction of amphipathic clusters in apoB, based on a computer program (LOCATE) developed by Segrest et al. (80), suggested a pentapartite structure of α-helical domains (α) and amphipathic β-strand domains (β) along the apoB polypeptide, organized as N-$\beta\alpha_1$-β_1-α_2-β_2-α_3-C (62, 80, 81). The α_1 cluster and the N-terminal part of the β_1 cluster constitute the LLT module; based upon homology to lipovitellin (61), the α_1 domain has been expanded to encompass residues 1–1000 and, because of the presence of β-strands in this sequence, renamed to $\beta\alpha_1$ (62) while consequently, the β_1 domain has been shortened. The C-terminal β_1-α_2-β_2-α_3 clusters stabilize the expansion of the initial lipid core in the LLT module and accommodate most of the lipid-binding capacity. Recent data on the amphipathic clusters in apoLp-II/I, likewise obtained by analysis with the program LOCATE, propose that apoLp-II/I contains a similar, although smaller, lipid-associating segment, comprising one C-terminal amphipathic β-sheet and one α-helical domain (α_2) organized along the protein as N-α_1-β-α_2-C, reminiscent of a truncated form of apoB (82) (Figure 3). The α_1 cluster and a small N-terminal part of the β cluster of this tripartite organization constitute the LLT module (and could therefore, in accordance with apoB, be expanded to $\beta\alpha_1$, resulting in the tripartite structure N-$\beta\alpha_1$-β_1-α_2-C); recombinant expression experiments showed the β cluster to accommodate the apoLp-II/I lipid-binding capacity. After cleavage of apoLp-II/I, the β cluster is almost entirely situ-

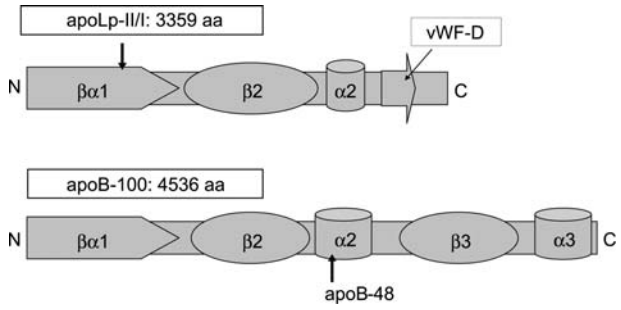

Figure 3 Linear domain structure organization of locust apoLp-II/I and human apoB-100.
The domains are depicted in the N- to C-terminal direction, indicated by N and C, respectively. The LLT domain [or $\beta\alpha_1$ domain; (62)] is shown by a block arrow, the lipid binding domains or β-strand motifs (β_1, β_2) are oval-shaped, the amphipathic α-helical domains (α_1–α_3) are barrel-shaped, and the von Willebrand factor D domain (vWF-D) is displayed by a wide-sized block arrow in the C-terminus of apoLp-II/I. The vertical arrow in bold font in the LLT ($\beta\alpha_1$) domain of apoLp-II/I marks the furin cleavage site. A similar arrow in apoB-100 marks the C-terminal position of apoB-48, the non-exchangeable apolipoprotein of chylomicrons. The domains are not drawn to scale. LLT: large lipid transfer; aa: amino acid. Based on data from ref. (22).

ated in apoLp-I, suggesting apoLp-I, and not apoLp-II, to bind the vast majority of lipids (82). This finding is consistent with HDLp dissociation experiments in which >98% of the total lipid in lipophorin remained associated with apoLp-I (83). Based on the homology between the LLT modules of apoB and apoLp-II/I, the C-terminal sequences of both apolipoproteins can also share a common evolutionary origin. In this regard, it has been speculated that the β_2 and α_3 clusters in apoB would have arisen from duplication of the β_1 and α_2 clusters (82). At its C-terminal end, apoLp-II/I contains a von Willebrand factor D module (51); this module also appears to be present in many different insect Vtgs (84). Although the function of this domain remains enigmatic, it does not appear to be involved in lipid binding (82).

On the basis of the similar structural organization of apoLp-II/I and apoB, the pathway for lipoprotein biogenesis in insects might be assumed to show similarity with that in mammals. Lipoprotein assembly in mammals has disclosed the role of MTP in acquiring the initial binding of lipids to the amphipathic lipid-associating segment of apoB (67, 70, 75) and from the discovery of an MTP homolog in the fruitfly that was able to promote the assembly and secretion of human apoB (77), insect lipoprotein assembly early in the secretory pathway has been proposed to occur similarly (62), as indicated above. The recovery of MTP homologs in all available insect genomes [for review, see ref. (23)] provides significant support for the concept that an MTP-dependent mechanism for initial lipoprotein biosynthesis is also operative in the biogenesis of insect lipoproteins. Moreover, insect MTP was experimentally shown to stimulate insect lipoprotein biogenesis considerably, because coexpression of the *Drosophila* MTP homolog (dMTP) and recombinant full-length locust (*L. migratoria*) apoLp-II/I cDNA in an insect cell (Sf9) expression system resulted in a several-fold increase in the secretion of apoLp-I and -II, as well as uncleaved apoLp-II/I (82). Concomitant with their secretion, dMTP significantly stimulated the lipidation of the apoLp-II/I proteins, because the secreted lipoprotein particles were recovered at a decreased buoyant density compared with control cells lacking the dMTP gene (Figure 4). To determine the amphiphatic region(s) of the apoLp-II/I proteins involved in lipid association, dMTP and a series of C-terminal truncation variants of apoLp-II/I were recombinantly coexpressed in Sf9 cells, revealing that formation of a buoyant HDL particularly requires the amphipathic β cluster (Figure 5) (82). Taken together, these data support a unifying concept for lipoprotein biogenesis and led to the convergence that, regardless of specific modifications, the assembly of lipoproteins both in mammals and insects requires amphipathic structures in the apolipoprotein carriers as well as MTP. Consequently, it has been proposed that lipoprotein biogenesis in animals relies on structural elements that are of early metazoan origin (82).

Molecular diversity and evolution of the LLTP superfamily

The recent discoveries described above highlight the common elements in LLTP structure and lipid binding. Additionally, they have evoked a comparison of the structural uniformity and diversity in this major family of lipid-binding proteins. Analyses of the modular and structural features of the LLTPs known from cloning studies as well as genome sequences have been used to reexamine the evolutionary relationships among LLTPs and the nature of their common ancestor, and to classify the LLTP superfamily into distinct families: (i) apoB-like LLTPs, which include vertebrate apoB and insect apoLp-II/I, but also Vtg from decapod crustaceans

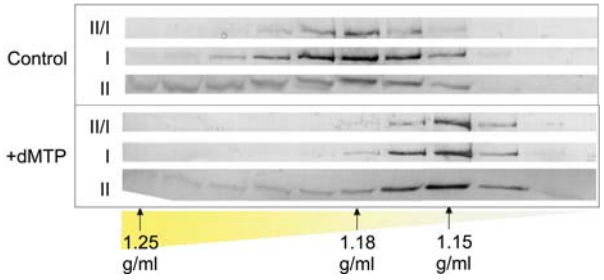

Figure 4 Insect MTP promotes lipidation of recombinant apoLp-II/I proteins.
Density-gradient ultracentrifugation and subsequent analysis of the buoyant density (g/ml) of the secreted proteins resulting from expression of recombinant full-length apoLp-II/I cDNA in insect (Sf9) cells by immunodetection shows that coexpression of dMTP results in the recovery of apoLp-I and -II (I and II, respectively), as well as uncleaved apoLp-II/I (II/I), at a lower density (g/ml), indicating increased lipidation. The decreasing density gradient in the ultracentrifugation analysis is represented by the yellow wedge-shaped diagram at the bottom of the Figure. dMTP: *Drosophila* MTP. Based on data from ref. (82).

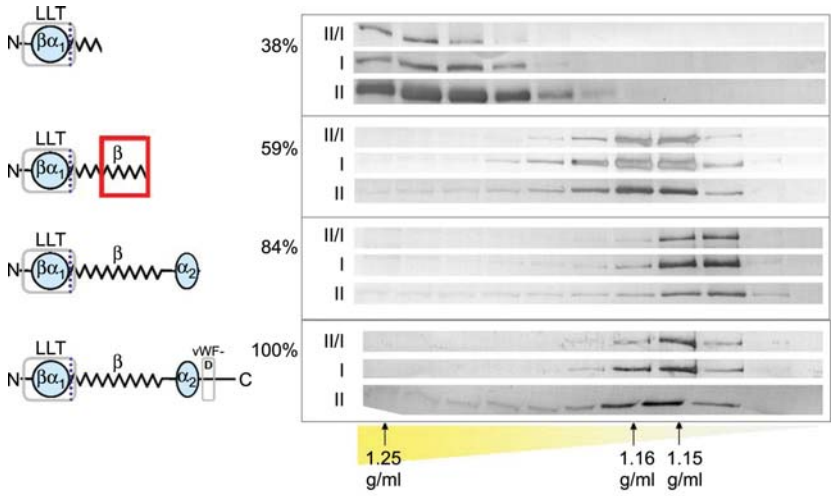

Figure 5 Insect lipoprotein biosynthesis requires the apoLp-II/I amphipathic β-cluster.
To determine which amphipathic regions (β-sheet or α-helical) of the apoLp-II/I products are involved in lipid binding, truncated apoLp-II/I cDNA constructs were generated (38%, 59% and 84%) in addition to the full-length (100%) cDNA. Recombinant coexpression of these constructs with dMTP cDNA in insect (Sf9) cells, followed by density-gradient ultracentrifugation and subsequent analysis of the buoyant density (g/ml) of the secreted proteins [uncleaved apoLp-II/I (II/I), apoLp-I (I) and apoLp-II (II)] by immunodetection, demonstrates that particularly the β-cluster is required for lipid binding, as indicated by the shift in density (g/ml) when a major portion of the β-sheet region (boxed in red) is present in the apolipoprotein (i.e., apoLp-II/I-59%). The dashed vertical line in the LLT domain indicates location of the site of apoLp-II/I cleavage into apoLp-II and -I. The decreasing density-gradient in the ultracentrifugation analysis is represented by the yellow wedge-shaped diagram at the bottom of the Figure. dMTP: *Drosophila* MTP; LLT domain: large lipid transfer domain; vWF-D: von Willebrand factor D domain. Based on data from ref. (82).

(e.g., lobster, crayfish, crab, shrimp); (ii) MTPs of vertebrates and invertebrates, ranging from a nematode and an insect (fruitfly) to zebrafish, human and chicken; and (iii) Vtg-like LLTPs (excluding decapodan Vtg), ranging from molluscs and nematodes via insects to chicken and fish such as lamprey and zebrafish (23).

The clustering of vertebrate apoB, insect apoLp-II/I and decapodan Vtg in the first family (apoB-like LLTPs) is supported by the recognition of a homologous region in these sequences only, the pfam06448 motif (85), located just C-terminal to the LLT module. Moreover, at the N-terminal side, the members of this family are predicted to contain an amphipathic clustering corresponding to N-α-β-α-C, with a relatively long β cluster and additional α cluster. Of the second family (MTPs), the MTP of fruitfly and nematode appeared to have diverged strongly. Nevertheless, the MTPs in nematodes, insects and vertebrates have been documented to stimulate the biosynthesis of other LLTPs (23, 82, 86, 87). The third family (Vtg-like LLTPs) is highly diverse, while the above phylogenetic analysis suggests a closer relation between the Vtgs from nematodes and insects compared with Vtgs from vertebrates. In addition to multiple (related) Vtgs, insects also have another Vtg-like LLTP, melanin-engaging protein. In decapodan crustaceans, however, clotting protein is the only Vtg-like LLTP identified at present. From an evolutionary point of view, the protein termed Vtg in this taxon actually is an apoB-like LLTP and has been proposed to be renamed to apolipocrustacein (23, 88).

The emergence of the LLT module appears to be the hallmark event in the origin of the complete superfamily of LLTPs, as this module provides the basal structure for the binding of multiple lipid molecules (60, 65, 89). The evolution of the LLT module can coincide with the evolution of animal multicellularity, a condition that provoked the need for intercellular lipid transport. The nature of the earliest LLTP has recently been discussed extensively (87). Previously, the evolutionary progenitor to the present LLTPs has been suggested to function in vitellogenesis, as this ancient process is essential to reproduction even in the oldest animal phyla. As such, the sequestration of lipid by Vtg was presumed to be MTP-independent. More recently, however, the predecessor to other LLTPs has been proposed to be an ancient MTP, in view of the currently recognized importance of MTP-mediated lipid transfer in the biosynthesis of both Vtg-like and apoB-like LLTPs (23, 82, 86, 87, 90). Evolution of MTP has resulted in structural adaptations, allowing its differential interactions with multiple substrates and its acquired roles in intracellular lipid mobilization as well as lipoprotein assembly and secretion (87). Whereas human MTP, essential for the assembly and secretion of apoB-containing lipoproteins [for reviews, see refs. (66–68, 70, 72, 74, 75)], is known to transfer neutral lipids (TAG and cholesterol esters) as well as phospholipids, it is recognized that the MTP from *Drosophila* (dMTP) is equipotent to human MTP in the transfer of phospholipids; however, dMTP is defective in neutral lipid transfer activity (90). This led to the conclusion that the phospholipid transfer activity of MTP is sufficient for the assembly and secretion of primordial apoB-containing lipoproteins and additionally suggests this transfer activity, but not the TAG transfer activity, to repre-

sent the earliest function of MTP, evolved for the mobilization of lipid in invertebrates. Accordingly, the ability of fruitfly MTP to stimulate locust apoLp-II/I lipidation and lipoprotein secretion in an insect cell expression system (82), as discussed above, could relate to its capacity to transfer phospholipids rather than TAG or cholesterol esters, which could be supported by the fact that in the resulting HDLp particle, TAG constitutes only a very minor component while cholesterol esters are absent. However, although it was established that the primordial apoB-containing particle is phospholipid-rich (59), studies on the addition of phospholipids to the $\beta\alpha_1$ domain of apoB (i.e., the LLT module, amino acid residues 1–1000), designated apoB:1000, which were examined in an apoB:1000 truncation mutant, demonstrated that the initial addition of phospholipids and initiation of apoB:1000-containing lipoprotein assembly occur independently of MTP lipid transfer activity (91). These data are at variance with the above conclusions reached by Rava et al. (90) and consequently, as yet unknown factor(s) other than MTP were proposed to mediate this early stage of apoB-containing lipoprotein assembly. Comparison of the interfacial properties of two apoB truncation mutants, one of which contains the complete lipoprotein initiating domain (apoB20.1; residues 1–912), and one of which (apoB19; residues 1–862) is incapable of forming nascent lipoproteins (64), established that extension of apoB19 with 50 amino acid residues to form apoB20.1 is associated with an increase in its surface activity and interfacial elasticity. These data are consistent with a model of lipoprotein assembly in which the surface-active initiating domain of apoB interacts with the membrane of the endoplasmic reticulum to recruit neutral and polar lipids (76).

Insect LDLp assembly: structure and function of apolipophorin III

HDLp, the circulatory lipoprotein harboring the product apolipoproteins of cleaved apoB-like insect LLTP (apoLp-II/I), displays the unique features of functioning as a vehicle capable of selective loading and unloading of a variety of lipid components at target sites in the resting situation. However, also during flight activity, HDLp constitutes an essential part of another reusable shuttle (LDLp), used to transport the increased amounts of lipids mobilized from fat body cell stores to the working flight muscles. Vital to this lipid shuttle mechanism during insect flight is the association of the low-molecular weight apoLp-III with the expanding surface of the lipoprotein particle during AKH-induced lipid loading of HDLp and its progressive conversion to LDLp. ApoLp-III belongs to a large family of exchangeable apolipoproteins characterized by a globular amphipathic α-helix bundle conformation [for recent reviews, see refs. (21, 39–41)] and circulates in the hemolymph as a stable, water-soluble protein. However, in response to the loading of HDLp with additional DAG, it binds to the surface area and provides a hydrophilic coating of the expanding particle. Although DAG is accommodated in the core of the resulting LDLp, in view of the relatively polar nature of the lipid it is envisioned that continued DAG accumulation results in partitioning of DAG between the hydrophobic core and the surface phospholipid monolayer of the particle (26, 27, 92–95). By 'sensing' the presence of DAG in the lipophorin surface monolayer, it was proposed that apoLp-III is attracted to the particle surface (96), which is supported by the ability of apoLp-III to bind to a phospholipid bilayer as a function of the concentration of DAG in the bilayer, as demonstrated by experiments using surface plasmon resonance spectroscopy (97). Injection of *L. migratoria* apoLp-III underneath lipid monolayers likewise demonstrated high affinity interaction of the protein with 1,2-DAG, in contrast with a low affinity for phosphatidylcholine (98). Thus, it has been hypothesized that apoLp-III serves to stabilize the DAG-enriched lipoprotein particle, providing an interface between surface-localized hydrophobic DAG molecules and the external aqueous medium [for reviews, see refs. (21, 38)]. This event is fully reversible *in vivo*; as the DAG content of the LDLp particle diminishes, apoLp-III gradually dissociates, eventually leading to regeneration of the HDLp particle, completing a shuttle cycle (cf. Figure 1).

apoLp-III from *L. migratoria* represents the first full-length apolipoprotein in nature for which the three-dimensional architecture has been determined in the lipid-free state by X-ray crystallography (96), offering the possibility of relating the structure of an apolipoprotein to its function. Lipid-free locust apoLp-III, which is 164 amino acid residues long (99, 100) and contains two complex carbohydrate chains (101), is organized as a compact, globular up-and-down bundle of five elongated amphipathic α-helices that orient such that their hydrophobic faces are directed toward the center of the bundle while their hydrophilic side chains are oriented outwards, ensuring solubility in an aqueous environment (96, 102). Importantly, a similar elongated helix bundle structure has been reported for other insect apoLp-IIIs, including the 166-amino acid, non-glycosylated five-helix bundle apoLp-III from another model insect, the sphinx moth, *Manduca sexta* (103–105), but also for the 22-kDa N-terminal domain (four-helix bundle) of human apo E (106–109). Binding of locust apoLp-III to a lipid surface has been postulated to result from a conformational change involving a dramatic lipid-triggered opening of the helix bundle about putative hinge loops, resulting in exposure of its hydrophobic interior to facilitate interaction of the hydrophobic face of the helices with the lipid surface, whereas the polar faces remain in contact with the aqueous environment (38, 96, 110–112). In the nuclear magnetic resonance (NMR) structure of *L. migratoria* apoLp-III, the presence of a 4-residue helix (helix 4′) was observed, which is situated between helix 4 and helix 5 of the five-helix bundle. This helix 4′ was not originally identified in the X-ray structure and has been proposed to act as a recognition motif for initiating apoLp-III interaction with lipoprotein surfaces (113), similar to the helix 3′ connecting helix 3 and helix 4 discovered in the NMR structure of *M. sexta* apoLp-III (105, 110, 114). Even though *L. migratoria* apoLp-III shares only little amino acid sequence identity with the apoLp-III from other

insects including *M. sexta*, locust apoLp-III is able to associate with *M. sexta* lipophorin, suggesting both apoLp-IIIs to be functionally indistinguishable (115). In addition, incubation of recombinant 22-kDa N-terminal domain of human apolipoprotein E with *M. sexta* LDLp resulted in displacement of apoLp-III from the particle surface (116).

apoLp-III expression is developmentally regulated; hemolymph level of the protein is very low in insect larvae and high only in adults (117), thus corresponding with the ability to fly at this stage and the related requirement to transport increased DAG mobilized from fat body TAG stores in LDLp. Interestingly, baculovirus-mediated expression of human apoE in *M. sexta* larvae in which apoLp-III level is low resulted in association of apoE with larval HDLp particles and facilitated the progressive formation of large, buoyant lipoproteins particles with a density even lower than that of normal LDLp, suggesting that in mammals, small apolipoproteins such as apoE are able to affect the amount of lipid packaged into lipoprotein particles and can play a role in buoyant lipoprotein production (118).

The insect apolipoprotein has developed to a unifying model that has provided important insight into structure-function relationships and the molecular details of lipid-binding interaction of the class of exchangeable apolipoproteins, consisting of a relatively small bundle of amphipathic α-helices that reversibly associate with lipoprotein surfaces [for reviews, see refs. (21, 38–41)].

Endocytosis of lipoprotein by the insect LDLR homolog, LpR

Although many structural elements of the lipid transport system of insects are similar to those of mammals, as indicated above, lipoprotein-mediated lipid transport in insects was accepted to deviate significantly from that in mammals in view of the selective mechanism by which the insect lipoprotein transfers its hydrophobic cargo. Indeed, despite the resemblance of insect HDLp to mammalian LDL based on the sequence homology between apoLp-II/I and apoB, circulating HDLp particles alternately function as a lipid donor or acceptor, constituting a reusable lipid shuttle without additional synthesis or increased degradation of their apolipoprotein matrix, as discussed above. However, in apparent contrast to this concept of functioning as a shuttle system, receptor-mediated endocytic uptake of HDLp was demonstrated in fat body tissue of larval and young adult locusts (119). The locust lipophorin receptor (LpR), cloned and sequenced from fat body cDNA, was identified as a novel member of the LDL receptor (LDLR) family (48) that, in addition to the fat body, is particularly expressed in brain, midgut and oocytes. To date, the LpR sequences of several other insect species have been elucidated [(120–127); for review, see ref. (128)]. Locust LpR is expressed only during specific developmental stages of the insect (during a few days after ecdysis, both to the next larval stage and to the adult), suggesting endocytic uptake of HDLp by LpR to occur in these restricted periods. Downregulation of LpR was postponed by experimental starvation of adult locusts immediately after ecdysis, whereas by starving adult locusts after downregulation of LpR, expression of the receptor was re-induced, suggesting LpR expression to be regulated by a deficiency of lipid components in fat body tissue (129). Receptor-mediated endocytosis of HDLp might therefore provide a mechanism for uptake of specific lipid components, independent of the mechanism of selective unloading of the HDLp lipid cargo at the cell surface. Conversely, experiments using HDLp partially delipidated *in vitro*, yielding a particle of buoyant density 1.17 g/ml, indicated that LpR favors the binding of this lipid-unloaded HDLp over HDLp of normal density. Latter data suggest a preferential mechanism for the intracellular loading of specific fat body lipid components onto relatively lipid-poor HDLp, whereas the lipid loading of the particle additionally results in decreased affinity for LpR (130), and thus facilitates the process of HDLp recycling that will be discussed later.

Domain organization of LpR is identical to that of mammalian LDLR (48, 49, 131) (Figure 6), whereas three-dimensional models of the elements representing the ligand binding domain and the epidermal growth factor (EGF) precursor homology domain of locust LpR and mammalian LDLR also bear a striking resemblance (37). Compared with the cluster of seven cysteine-rich repeats in the ligand binding domain of LDLR, however, the ligand binding domain of LpR contains an additional LDLR class A (LA) repeat (Figure 6) similar to the human VLDL receptor (VLDLR) (48, 49), while additionally, the amino acid sequence of the intracellular domain of LpR is extended. The C-terminal amino acid residues of LDLR are different from those of LpR, whereas the C-terminal tail of LpR contains an additional 12 amino acid residues (49, 132). At the functional level, despite their pronounced structural similarity, the specificity of LpR and LDLR for their ligands (HDLp and LDL, respectively) is mutually exclusive (49). Additionally, the functioning of both receptors in lipid transport in insects and mammals appears to be intriguingly different (ligand recycling versus ligand degradation), as discussed below in more detail. Possibly, these specific properties could be attributable to relatively small structural differences governing different properties of ligand binding and/or release.

LpR-mediated recycling of insect lipoprotein

The occurrence of endocytosis of HDLp mediated by the insect LDLR homolog, although restricted to specific developmental periods, highlights once more the existence of remarkable similarities at the structural and molecular levels between the insect and the mammalian lipoprotein systems. At the same time, this data seems to conflict with the process of selective lipid transport between HDLp and fat body cells in which the apolipophorin matrix of the particle is not degraded. However, the pathway followed by the internalized HDLp appears to be different from the classical receptor-mediated lysosomal pathway typical of LDLR-internalized ligands.

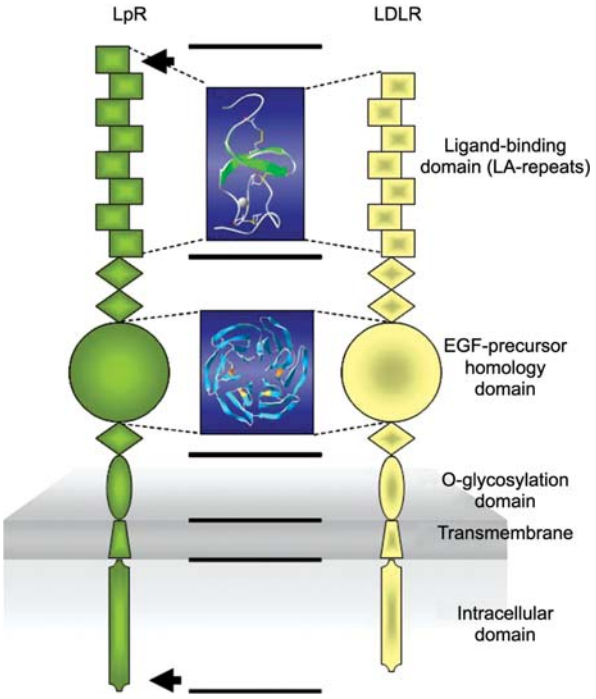

Figure 6 Domain organization of insect LpR and mammalian LDLR.
Schematic representation of the insect lipophorin receptor (LpR) and the mammalian LDL receptor (LDLR), indicating an identical domain organization. Each receptor contains a ligand binding domain composed of LA-repeats (squares), an EGF precursor homology domain composed of two EGF-repeats (diamonds) that are separated from a third one by a propeller containing YWTD-repeats (circle), an O-linked glycosylation domain (oval), a transmembrane domain (trapezium) and an intracellular C-terminal domain (stick). The ligand binding domain of LpR contains an additional repeat and the intracellular domain of LpR is 12 aa longer, as indicted by the arrowheads in LpR. Between the ligand binding domains of LpR and LDLR, a three-dimensional ribbon structure of a single LA-repeat of LpR is presented, showing the repeat-specific three disulfide bonds, and a Ca^{2+} ion. Between the EGF precursor homology domains of the two receptors, the ribbon structure of the β-propeller domain of LpR is indicated, representing the three-dimensional structure of the YWTD repeats containing domain. LA: LDLR class A; EGF: epidermal growth factor. Based on data from refs. (21, 22, 37).

In mammalian cells, endocytosed LDL dissociates from its receptor upon delivery to the low pH milieu of the endosome and is completely degraded in lysosomes [for reviews, see refs. (19, 20)]. By contrast, in the transferrin-mediated pathway of iron uptake, endocytosed transferrin remains attached to its receptor and, following the unloading of its two iron ions, is eventually resecreted from the cells (133, 134). Endocytic uptake of locust HDLp studied simultaneously with human LDL in an LDLR-expressing mammalian cell line (CHO cells) transfected with LpR cDNA revealed both particles to colocalize to the same early endocytic vesicle structures (49). However, whereas LDL was eventually degraded in lysosomes after dissociating from its receptor, HDLp remained colocalized with LpR and was transported to the endocytic recycling compartment (ERC), from which the insect lipoprotein was eventually resecreted ($t_{1/2} \sim 13$ min) in a manner similar to transferrin, thus escaping from lysosomal degradation (49). These data indicate that, in mammalian cells, endocytosed insect HDLp, in contrast to human LDL, follows a recycling pathway mediated by LpR.

Although this behavior of LpR in mammalian cells proposes a novel function of an LDLR family member, recycling of endocytosed HDLp in insect fat body cells remains to be shown. Because a locust fat body cell line is not available, fat body tissue from young adults after ecdysis, endogenously expressing LpR, was used for tracking the intracellular pathway of fluorescently labeled HDLp. The lipoprotein appeared not to be transported to a recognizable ERC-like compartment, but remained in vesicles in the periphery of the cell (135), from which the labeled HDLp disappeared nearly completely with a half-life of approximately 1 h (22, 135). As this time span for the disappearance of labeled HDLp is too short to be interpreted as lysosomal degradation, this data is indicative of resecretion of the ligand and thus supporting the concept of ligand recycling that was demonstrated for LpR-transfected mammalian cells (49).

The above concept, implying that insect lipoprotein, endocytosed by an LDLR family member, is eventually recycled, conflicts with the generally accepted concept of the fate of ligands endocytosed by all the other LDLR family members. Binding assays using flow cytometry demonstrated that, in contrast to the LDL-LDLR complex, HDLp and LpR remain in the complex at endosomal pH (136) (Figure 7). Since in addition to pH lowering, the Ca^{2+} concentration in the early endosome is also lowered to the low micromolar range (137), the HDLp-LpR complex was treated with an EDTA-containing buffer to mimic the effect of the low Ca^{2+} concentration in the endosome. This treatment did not induce complex dissociation either, once more in contrast to the effect of EDTA treatment on the LDL-LDLR complex (136) (Figure 8). These results indicate that endocytic conditions fail to induce dissociation of the complex and imply that HDLp and LpR remain in complex throughout the itinerary from the early endosome to the ERC (136). This remarkable stability of the ligand-receptor complex is likely to provide a crucial key aspect of the recycling mechanism.

Extensive studies have proposed that LDLR releases LDL at endosomal pH by undergoing a conformational change, in which the β-propeller of LDLR interacts with the ligand binding domain, resulting in displacement of LDL (16, 17, 138, 139). Sequence alignment of the amino acid sequence of LDLR with that of LpR revealed that a number of residues crucial for LDL release by LDLR (17, 140, 141), notably Gln540, His562, Glu581 and Lys582, are not conserved in LpR. Changing the complete ligand binding domain of LpR for that of LDLR ($LDLR_{1-292}LpR_{343-850}$) (142) resulted in a hybrid receptor that was able to bind LDL, but unable to release this ligand at endosomal pH, suggesting that the lack of Gln540, His562, Glu581 and Lys582 renders the β-propeller of LpR indeed incapable of inducing LDL release and

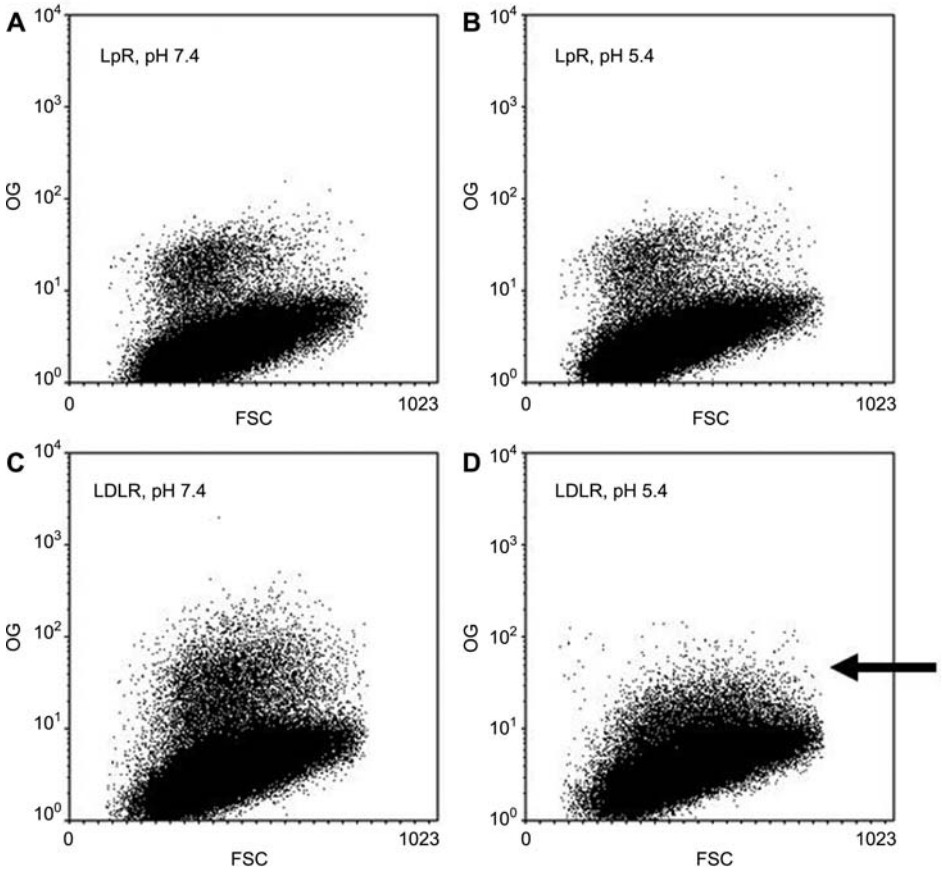

Figure 7 LpR and HDLp remain in complex at endosomal pH.
Mammalian (CHO) cells transfected with LpR (A, B), or LDLR (C, D) as a control, were incubated at pH 7.4 with Oregon green (OG)-labeled ligand, HDLp (A, B) or human LDL (C, D) and washed with the either same buffer of pH 7.4 (A and C) or a buffer of pH 5.4 (B and D) as indicated. The amount of fluorescence (y-mean) per cell is plotted on the y-axis (relative values) and the forward scatter (FSC, relative values) as a marker of cell size on the x-axis. The y-mean of cells in the population of high fluorescence does not decrease upon incubation of the cells with buffer of pH 5.4 for CHO(LpR) cells (B), in contrast to that for CHO(LDLR) cells (D), as denoted by the arrow in (D), indicating dissociation of the LDLR-LDL complex at pH 5.4, whereas the LpR-HDLp complex remains stable at the same pH. Based on data from ref. (136).

causes the lack of HDLp release by LpR. However, the inverse hybrid in which the β-propeller of LDLR was introduced into LpR did not lead to release of HDLp by this hybrid receptor either, implying that other domains produce the remarkable stability of the complex (142). In LDLR, the interface between the most C-terminal LA-repeat (LA-7) and the adjacent cysteine-rich repeat of the EGF domain (EGF-A), the hinge region, has additionally been proposed to play an important role in LDL release by functioning as a rigid scaffold that allows the β-propeller to fold over the ligand binding domain (17, 19, 140). Potentially crucial residues in the hinge region of LDLR (His264, Ser265 and Ile313) are not conserved in LpR and might abolish ligand release by increasing the flexibility of the hinge region. However, a hybrid LpR in which both the hinge region and β-propeller of LDLR were introduced ($LpR_{1-301}LDLR_{252-839}$) failed once more to release HDLp, in spite of the fact that this hybrid contains all the domains that LDLR brings into action for LDL release. Consequently, as these functional LDLR domains appeared unable to evoke HDLp release, the lack of dissociation of the HDLp-LpR complex was proposed to result from the specific binding interaction of the ligand binding domain of LpR with HDLp that can be different from that used by other LDLR family members for the interaction with their ligands (136). In addition, the recently proposed novel mechanism for LDL release in the endosome, in which calcium depletion and decreased stability at acidic pH drive the unfolding of the fifth LA-repeat (LA-5) of LDLR, triggering LDL release from its receptor by the reduced stability of the LDL-LDLR complex (143), would also seem to be ineffective or absent in HDLp-LpR.

Although a molecular mechanism for the stability of the HDLp-LpR complex awaits disclosure, its deciphering might additionally be helpful to explain the ability of LDLR family members to bind a wide range of structurally unrelated ligands.

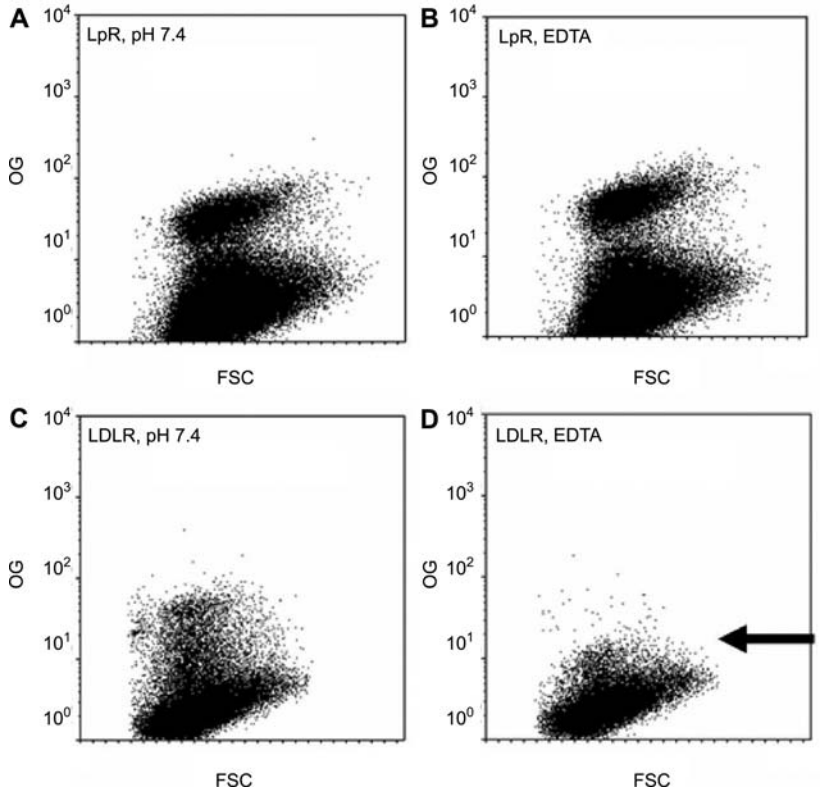

Figure 8 Effect of EDTA treatment on ligand-receptor binding.
Mammalian (CHO) cells transfected with LpR were incubated with Oregon green (OG)-labeled HDLp at pH 7.4 (A). EDTA treatment of these labeled cells was not able to dissociate the complex of HDLp and LpR (B), in contrast to the effect of EDTA treatment on cells transfected with LDLR and preincubated with OG-LDL at pH 7.4 (C and D), as indicated by the arrow in (D). These results imply that the stability of the complex of LpR and HDLp is EDTA resistant, indicating that the low calcium concentration in the early endosome fails to produce dissociation of the complex. Based on data from ref. (136).

Expert opinion

From the notion that circulatory lipid transport relies on members of the LLTP superfamily including mammalian apoB, insect apoLp-II/I, Vtg and the ubiquitous MTP, this review compares lipoprotein assembly and function of mammals and insects in an evolutionary perspective. This choice is based on the recent advances in our knowledge of the lipoprotein systems of both animal classes that are proposed to constitute extremities of divergent branches of the phylogenetic tree (144). However, comparative research reveals that LLTPs evolved from the earliest animals and have been identified in most animal phyla, vertebrate as well as invertebrate (23), underscoring the structural adaptations in these lipid-binding proteins.

In view of the currently recognized importance of MTP-mediated lipid transfer in the biosynthesis of both Vtg-like and apoB-like LLTPs, the predecessor to other LLTPs has been proposed to be an ancient MTP (23, 82, 86, 87, 90). It cannot be excluded, however, that the LLT module evolved first as part of a larger multidomain protein that could have displayed functions unrelated to any of the functions presently ascribed to LLTPs (23). Evolution of MTP has resulted in structural adaptations, allowing its differential interactions with multiple substrates and its acquired roles in intracellular lipid mobilization as well as lipoprotein assembly and secretion (87). In this regard, it should be noted that in the X-ray crystal structure of lamprey lipovitellin a homodimer was observed (89), which could suggest that dimerization of individual ancient MTP molecules might have constituted a mechanism by which MTP lipid transfer function has evolved.

The data obtained for mammalian and insect lipoprotein biogenesis support a unifying concept, proposing that the assembly of lipoproteins both in mammals and insects requires amphipathic structures in their apolipoprotein carriers as well as MTP (82). However, considering the relatively poor lipid loading of insect apoLp-II/I compared with mammalian apoB, resulting in the secretion of a high-density particle in insects rather than a VLDL as in mammals, it is conceivable that of the two-step lipidation mechanism operative in the assembly of mammalian apoB-containing lipoproteins, the second lipidation step is absent in insect lipoprotein biosynthesis. Such a difference in intracellular

lipid mobilization and transfer in the lipoprotein-producing cells of both animal groups could also be supported by the difference in the major neutral lipid components of the newly synthesized lipoproteins in insects and mammals (DAG versus TAG, respectively). Moreover, although human MTP is known to transfer TAG as well as phospholipids, it is recognized that *Drosophila* MTP is equipotent to human MTP in the transfer of phospholipids; however, it is defective in TAG transfer activity (90), suggesting the initial step of lipidation of apoLp-II/I, resulting in synthesis of primordial insect lipoprotein particles, to consist of transfer of phospholipids.

Although it remains enigmatic why mammals and insects use extremely different functional concepts in their mechanisms of circulatory lipid delivery (lipoprotein degradation versus lipoprotein recycling), the reusability of the insect lipoprotein could constitute a more widespread phenomenon. For instance, in mammalian reverse cholesterol transport, HDL particles remove cholesterol from peripheral tissues and deliver cholesteryl esters to the liver by interaction with the receptor SR-BI without apparent uptake or degradation of the HDL particle [for review, see ref. (22)]. Moreover, it has recently been shown that HDLp-mediated selective delivery also applies to the specific transport and delivery of the lipid-anchored morphogens Wingless and Hedgehog in *Drosophila* [(145, 146); for reviews, see refs. (147, 148)]. Although both the *Drosophila* lipophorin receptor 2 (LpR2) and the Hedgehog receptor (Patched), which is also a lipophorin receptor interacting with HDLp, are able to stimulate lipophorin internalization, the latter process was shown not to play a major role in Hedgehog protein signal transduction but rather to be important for Hedgehog gradient formation (146).

The involvement of insect lipoproteins in functions beyond circulatory lipid transport puts forward the concept that a similar function and mechanism could also be present in the mammalian system, in which the Wingless homolog, Wnt (149), and Hedgehog (150) equally have important functions. Indeed, it was recently hypothesized that LDL might transport Hedgehog through the mammalian bloodstream (147, 151). If so, the subsequent incorporation of Hedgehog-carrying LDL into atherosclerotic plaques could result in beneficial effects such as revascularization or other regenerative processes of surrounding tissue, which would imply an unexpected role for LDL in cardiovascular disease (151).

The recent identification of LDLR family receptors in insects that are able to endocytose HDLp has revealed another similarity between the lipoprotein systems of insects and mammals, particularly relating to the processing of HDLp and LDL. Although endocytic uptake of HDLp would seem to conflict with the concept of HDLp acting as a reusable lipid shuttle, the lipoprotein endocytosed by the insect LDLR homolog, LpR, appeared to be recycled in a manner analogous to the resecretion of receptor-bound transferrin. Such a pathway, in which the lipoprotein is ultimately resecreted, possibly after reloading with lipid components (130), clearly is of physiological relevance in insects although the precise function of the process awaits disclosure. This concept of LDLR family member-mediated ligand recycling has not been proposed to apply to mammals and could generate new ideas to identify hitherto undiscovered functions of other family members. Particularly the functioning of the VLDL receptor, which has been proposed to act in a ternary complex with VLDL and LPL in the delivery of fatty acids derived from TAG to lipid storage depots, could resemble that of LpR [for reviews, see refs. (22, 152, 153)]. Based on the amino acid sequence of their C-terminal domains, insect LpRs resemble the VLDL receptors, although they segregate into a specific group distinct from the groups encompassing other LDLR family members, including the group of the VLDL receptors (132). Recent mammalian studies have shown that in addition to LDLR family members, other glycosylated membrane-associated proteins (including glycerophosphatidylinositol-anchored HDL-binding protein 1, GPIHBP1) can constitute an extracellular platform for lipoprotein docking and TAG hydrolysis, allowing fatty acid delivery to cells, possibly mediated by the fatty acid translocase CD36 (11). For LpR-mediated lipid delivery, involvement of insect forms of these glycosylated membrane associated proteins might not be relevant; however, it cannot be excluded that these proteins could be involved in the LDLp-mediated fatty acid delivery to the flight muscle cells during long-term flight activity of insects such as the locust, a process at which extracellular lipophorin lipase activity has been indicated [for reviews, see refs. (21, 28, 37)]. The presence of an insect form of CD36 or other fatty acid translocases in the membrane of flight muscle cells has not been established yet.

Recycling of endocytosed HDLp is likely to depend on a particular interaction between receptor and ligand. Binding studies using the flow cytometry assay indicate that the lipoprotein-LpR complex, in contrast to the LDL-LDLR complex, is resistant to dissociation at endosomal conditions, namely a low pH as well as a decreased concentration of calcium mimicked by treatment with EDTA. These features of the HDLp-LpR complex are proposed to provide a major key to the recycling mechanism (136). Sequence alignment of the amino acid sequence of LDLR with that of LpR revealed that a number of residues crucial for LDL release by LDLR are not conserved in LpR, both in the β-propeller as well as in the hinge region that, in LDLR, functions as a rigid scaffold allowing the β-propeller to fold over the ligand binding domain and to displace LDL. Based on the binding and dissociation capacities of several mutant and hybrid receptors, including one in which both the entire β-propeller and hinge region of LDLR were introduced into LpR, the lack of dissociation of the HDLp-LpR complex was proposed to result from the specific binding interaction of the ligand binding domain of LpR with HDLp (136). Uncovering the molecular mechanism underlying this remarkable stability of the HDLp-LpR complex might as well be important in the face of the ability of LDLR family members to bind a wide range of structurally unrelated ligands.

In general, the different functioning of lipoproteins and their receptors in the insect system renders the latter system

a useful and important alternative model for studying the molecular mechanisms underlying processes of lipid transport and utilization in the mammalian system, also relating to human disorders and disease. The utility of comparative studies for targeting the role of LLTPs in human disease is illustrated by the recent comparison of lipid transfer activity of MTP from fruitfly and man, revealing that the transfer of phospholipids and neutral lipids can selectively be inhibited in human MTP (90). Such a selective inhibition can open new perspectives as a therapy for lowering blood lipid levels and to specifically control the production of atherogenic apoB lipoproteins (154).

Moreover, also in aspects of lipid metabolism outside the realm of lipoprotein assembly, particularly in the mechanism of fat storage and mobilization, mammals and insects recently appeared to be very similar, underscoring the value of a non-mammalian model organism in elucidating the molecular aspects of the regulation of energy homeostasis and dysfunction of this balance resulting in obesity. For example, packaging fat in intracellular lipid droplets and the mechanisms guiding mobilization of stored fat are conserved between mammals and insects (155–159). Lipid droplets, that are progressively recognized to represent ubiquitous dynamic organelles regulating intracellular TAG metabolism, are surrounded by a phospholipid monolayer coated with specific proteins, belonging to the evolutionary ancient PAT (perilipin/ADRP/TIP47) family of proteins, that participate in the regulation of TAG storage and lipolysis (156, 157, 160–165). For the lipid droplet-associated proteins in mammals PERILIPIN was recently adopted as a unifying nomenclature (165). The crystal structure of the C-terminal part of the representative PAT protein TIP47 indicated a four-helix bundle resembling apoE. The structure suggests an analogy between PAT proteins and apolipoproteins in which the amphipathic helices interact with lipid while other parts are involved in protein-protein interactions (166).

Similar to mammalian adipocytes, the TAG accumulated in cytosolic lipid storage droplets of insect fat body cells provide the major long-term energy reserves of the organism, for which *Drosophila* recently emerged as a powerful system, partly owing to its well-developed genetics (162, 167, 168). Generation of loss-of-function mutants evidenced that simultaneous loss of the receptor for AKH – and thus the signaling pathway for lipid mobilization related to β-adrenergic signaling in mammals – and the lipid droplet-associated TAG lipase brummer (bmm), a homolog of human adipose TAG lipase [ATGL, for recent reviews, see refs. (169, 170)], caused extreme obesity and blocks acute storage fat mobilization in flies (168). It is interesting to note that ATGL is the predominant TAG lipase in mammalian adipocytes, whereas hormone sensitive lipase and other lipases are responsible for DAG and MAG hydrolysis. The efflux of DAG from insect fat body cells following bmm action would suggest a lack of the other 'downstream' lipases found in adipocytes. To further demonstrate the functional similarity between mammalian and *Drosophila* TAG lipases, bmm was shown to localize at the lipid droplet surface and to antagonize a perilipin-related lipid droplet surface protein (LSD-2) (167) that functions as an evolutionary conserved modulator of lipolysis (162). Moreover, *Drosophila* key candidate genes for lipid droplet regulation were identified, the functions of which are conserved in the mouse. These include regulation of lipolysis by the vesicle-mediated Coat Protein Complex I (COPI) transport complex required for limiting lipid storage by regulating the PAT protein composition and promoting the association of TAG lipase at the lipid droplet surface and composition (171, 172). Recently, a new regulator of lipid homeostasis in *Drosophila* was identified, the *schlank* gene, encoding a member of the Lass/ceramide synthase family required for *de novo* synthesis of ceramide, while schlank appeared to be also involved in regulating the balance between lipogenesis and lipolysis and reduction of fat body TAG stores in the fruitfly (173), suggesting a novel role for ceramide synthases in the regulation of body fat metabolism. Taken together, these studies using an insect model could be of direct relevance to cellular lipid storage in general and hold the promise of identifying molecular aspects of the regulation of energy homeostasis central to human diseases, including mechanisms involved in dysfunction of this balance resulting in excessive lipid storage.

Outlook

Because insects constitute the largest and most abundant – as well as a very successful – animal class on earth, which has evolved to use all types of available nutrients, understanding of their solutions for circulatory lipid transport will provide new and fundamental insights that have a high biological significance, but can additionally provide insight into corresponding processes in mammalian circulatory lipid transport, and even into processes that hitherto were not considered to occur in mammals.

The comparison of lipid transfer activity of MTP from fruitfly and man revealed that *Drosophila* MTP is defective in neutral lipid transfer activity, suggesting that the transfer of phospholipids and neutral lipids can selectively be inhibited in human MTP (90), provides novel possibilities for targeting the role of MTP and other LLTPs in human disease. In the next years to come, it could be expected that this will have opened new perspectives on inhibiting MTP activity to specifically control the production of apoB-containing lipoproteins (154).

Indeed, although mammalian apoB-containing lipoprotein assembly is critical for lipid absorption and TAG homeostasis, accumulation of apoB-containing lipoproteins and their remnants in plasma induces atherosclerosis and plays an important role in the pathobiology of type 2 diabetes and obesity. Thus, intervention in apoB-containing lipoprotein production and modulation of their lipid composition during biosynthesis represent potent therapeutic targets (87). In this regard, insight into evolutionary, structural and cell biology of the mechanisms of lipoprotein assembly, secretion and functioning is a premier issue that will have greatly advanced in the next few years.

In contrast to mammalian apoB, apoLp-II/I is cleaved post-translationally between two residues of the LLT module in a loop connecting two β-strands at the base of the putative lipid pocket. The rationale for this cleavage step remains enigmatic, but has been suggested to constitute a molecular adaptation relating to specific functions of the insect lipoprotein, including its functioning as a reusable lipid shuttle in the resting situation that could additionally allow for the loading of the particle with an increased lipid cargo and conversion of HDLp to LDLp during conditions that require enhanced lipid transport. The apparent conservation of apoLp-II/I cleavage in all insects characterized to date underscores the importance of this processing step. Moreover, Vtg, another LLTP homologous to apoB and apoLp-II/I, is also cleaved at a furin consensus substrate sequence in the LLT domain during biosynthesis in most insect species. It is expected that in the next few years more insight will be obtained in this cleavage step; it remains to be shown whether this step could provide a crucial key to the functioning of insect HDLp as a reusable lipid shuttle capable of alternate unloading and reloading of lipid, and perhaps as well to the unexpected recycling mechanism of the insect lipoprotein after being endocytosed by the insect homolog of LDLR. For some notorious insect species this mechanism, and perhaps also that of lipophorin assembly, can offer possibilities for application in pest control.

Highlights

- Animal circulatory fat transport relies on members of the large lipid transfer protein (LLTP) superfamily, including mammalian apolipoprotein B (apoB), insect apolipophorin II/I (apoLp-II/I), microsomal triglyceride-transfer protein (MTP) and vitellogenin (Vtg).
- The LLTP superfamily can be classified into three distinct families: (i) apoB-like LLTPs including apoLp-II/I; (ii) MTPs of vertebrates and invertebrates; and (iii) Vtg-like LLTPs.
- In vertebrates and insects, the LLT domain shared by these proteins constitutes a lipid pocket, proposed to act to store or transfer lipids. ApoLp-II/I is cleaved by a furin into apoLp-II and -I within the LLT module.
- Prediction of amphipathic α-helical and β-sheet clusters in apoB proposed a pentapartite structure in an N-α_1-β_1-α_2-β_2-α_3-C configuration, whereas apoLp-II/I is proposed to be a tripartite structure, organized as N-α_1-β-α_2-C, reminiscent of a truncated form of apoB.
- In an insect cell expression system, *Drosophila* MTP stimulated both the lipidation of insect apoLp-II/I and the secretion of lipoprotein particles several-fold, suggesting that the initial step in insect lipoprotein assembly is MTP-dependent similar to mammals.
- The remarkable structural similarities between mammalian and insect lipoproteins notwithstanding important functional differences relate to the mechanism of lipid delivery.
- In mammals, partial delipidation of apoB-containing lipoproteins eventually results in endocytic uptake of their remnants that are degraded in lysosomes, whereas in insects the cleaved apoLp-II/I-containing HDLp functions as a reusable lipid shuttle capable of alternate unloading and reloading of lipid.
- In contrast to mammals, also during muscular efforts (flight activity) insects use a lipoprotein (HDLp)-based reusable shuttle mechanism (LDLp) in which apoLp-III, a small bundle of amphipathic α-helices that reversibly associate with lipoprotein surfaces, allows for the enhanced transport capacity of lipid for energy generation.

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