review

Molecular diversity and evolution of the large lipid transfer protein superfamily[S]

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Abstract **Circulatory lipid transport in animals is mediated to a substantial extent by members of the large lipid transfer (LLT) protein (LLTP) superfamily. These proteins, including apolipoprotein B (apoB), bind lipids and constitute the structural basis for the assembly of lipoproteins. The current analyses of sequence data indicate that LLTPs are unique to animals and that these lipid binding proteins evolved in the earliest multicellular animals. In addition, two novel LLTPs were recognized in insects. Structural and phylogenetic analyses reveal three major families of LLTPs: the apoB-like LLTPs, the vitellogenin-like LLTPs, and the microsomal triglyceride transfer protein (MTP)-like LLTPs, or MTPs. The latter are ubiquitous, whereas the two other families are distributed differentially between animal groups. Besides similarities, remarkable variations are also found among LLTPs in their major lipid-binding sites (i.e., the LLT module as well as the predicted clusters of amphipathic secondary structure): variations such as protein modification and number, size, or occurrence of the clusters. Strikingly, comparative research has also highlighted a multitude of functions for LLTPs in addition to circulatory lipid transport. The integration of LLTP structure, function, and evolution reveals multiple adaptations, which have come about in part upon neofunctionalization of duplicated genes. Moreover, the change, exchange, and expansion of functions illustrate the opportune application of lipid-binding proteins in nature. Accordingly, comparative research exposes the structural and functional adaptations in animal lipid carriers and brings up novel possibilities for the manipulation of lipid transport.**—Smolenaars, M. M. W., O. Madsen, K. W. Rodenburg, and D. J. Van der Horst. **Molecular diversity and evolution of the large lipid transfer protein superfamily.** ***J. Lipid Res.*** **2007.** 48: **489–502.**

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Lipid transport potentially poses a problem to multicellular organisms, as lipids are hydrophobic and aggregate in an aqueous environment. In animals, the homologous members of the large lipid transfer (LLT) protein (LLTP) superfamily enable circulatory lipid transport, as these proteins can bind up to hundreds of lipid molecules (for reviews, see Refs. 1, 2). In this way, LLTPs constitute lipoproteins that endow the uptake and transport of a multitude of lipid species. In addition to lipid transport, LLTPs have also been implicated in animal development (3), reproduction (for reviews, see Refs. 4–6), and immunity (7–11) as well as aging and lifespan regulation (12, 13). Moreover, an LLTP, apolipoprotein B (apoB), constitutes lipoproteins that are a major causative agent of atherosclerosis in human and are involved in other metabolic disorders, such as obesity and type 2 diabetes mellitus (for reviews, see Refs. 14–16).

LLTPs have been identified in most animal phyla, in vertebrates as well as invertebrates. In addition to apoB, this protein family includes insect apolipoprotein [apolipophorin-II/I (apoLp-II/I)], vitellogenin (Vtg), and microsomal triglyceride transfer protein (MTP), based on sequence similarity and ancestral exon boundaries (17). Each of these family members binds lipids, although their functions are different. ApoB is present in vertebrates and mediates postprandial lipid uptake as well as lipid redistribution in the body (for review, see Ref. 18). ApoLp-II/I functions as a general-purpose lipid transporter in insects. However, in contrast to its vertebrate homolog apoB, apoLp-II/I additionally mediates the transport of stored lipid for energy generation during muscular activity (for reviews, see Refs. 19–21). Vtg is the major yolk protein found in females of most egg-laying animals, nonmammalian vertebrates as well as invertebrates, supplying the

Abbreviations: aa, amino acids; apoB, apolipoprotein B; apoLp, apolipophorin; ARP, apolipophorin-II/I-related protein; CP, clotting protein; LLT, large lipid transfer; LLTP, large lipid transfer protein; MEP, melanin-engaging protein; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; Vtg, vitellogenin; vWF, von Willebrand factor.

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developing oocyte with nutrients, including lipids (for reviews, see Refs. 4, 6). Often, multiple Vtg genes are present and expressed in a species (4, 22–25). MTP is present in vertebrates and invertebrates and has been shown to enhance the biosynthesis of other LLTPs, as demonstrated for apolipoproteins and Vtg (26–28).

LLTPs clearly differ in the amount and types of lipid that they bind. Whereas MTP and Vtg bind mostly phospholipids, apoB and apoLp-II/I can additionally bind up to several hundred neutral lipid molecules: for apoB, mostly triacylglycerol, and for apoLp-II/I, mostly diacylglycerol. In addition, the insect apoLp-II/I and several invertebrate Vtgs are proteolytically cleaved during their biogenesis, unlike other LLTPs. The resulting polypeptides remain in complex. Accordingly, insect lipoprotein contains two protein moieties, the apoLp-II/I cleavage products apoLp-I and apoLp-II (29, 30). Unlike apoB, apoLp-II/I, and MTP, vertebrate Vtgs form stable homodimers (31, 32). Mammalian MTP does constitute a stable complex with the protein disulfide isomerase (PDI) (33).

Despite the differences described above, recent research has also highlighted the similarity among LLTPs with respect to their structure, lipid binding, and biosynthesis. All LLTPs share an N-terminal LLT module (17) that constitutes the basal lipid binding structure (31, 34–39). In apoB, it primes additional lipid binding by more C-terminal clusters of amphipathic α-helices and β-strands. Such clusters have been demonstrated to increase the lipid binding in apoB as well as apoLp-II/I (40, 41; for reviews, see Refs. 1, 36, 42; M. M. W. Smolenaars, A. De Morrée, J. Kerver, D. J. Van der Horst, and K. W. Rodenburg, unpublished data). Moreover, MTP facilitates the biosynthesis of diverse LLTPs in distinct lineages of the animal kingdom (26, 28, 43).

Inspired by recent discoveries that highlight the common elements in LLTP structure and lipid binding, this review discusses the structural uniformity as well as diversity in this major family of lipid binding proteins. First, analysis of the modular and structural features of the LLTPs known from cloning studies as well as genome sequences is used to classify the LLTP superfamily into distinct families. Next, we discuss the origin of LLTP features in relationship to their function, to arrive at novel insights and perspectives on the functional and structural dynamics of these lipid-binding proteins.

LLTP FAMILY MEMBERS

LLTPs (**Fig. 1**) are defined by the presence of a lipid binding domain, the LLT module (17). This module comprises the conserved N-terminal region of ~900 amino acid (aa) residues (17, 31, 34, 35, 37, 44, 45). Based on its presence, apoLp-II/I, apoB, Vtg, and MTP were previously recognized as members of the LLTP superfamily (17). Our multiple and iterative searches (see supplementary Methods) for LLTPs have now also identified several differently named proteins from invertebrates as LLTPs. These include the clotting protein (CP) from the shrimp *Metapenaeus ensis* and the crayfish *Cherax quadricarinatus* (7, 8), M-177 from the dust mite *Euroglyphus maynei* (46), and melanin-engaging protein (MEP) from the beetle *Tenebrio molitor* (47) (Fig. 1, **Table 1**). The sequences of these proteins were not available at the time of the first description of the LLTP superfamily (17).

Analysis of complete genome annotations can reveal all of the LLTPs encoded in a single species. According to our survey of the human, chicken, and zebra fish genome annotations, the subset of LLTPs encoded by vertebrates includes MTP, apoB, and, except in mammals, also one or more Vtgs. Apparently, human has only two LLTPs, apoB and MTP. Similarly, apoB and MTP were the only LLTPs identified in the current genome annotations of mammals (including those of *Bos taurus*, *Canis familiaris*, *Mus musculus*, and *Rattus norvegicus*), supporting the view that apoB and MTP are the only LLTPs present in mammals. Interestingly, two apoB protein sequences of 3,730 and 4,418 aa were predicted to be encoded by two distinct genes in the zebra fish *Danio rerio* (see supplementary Table I). A literature survey indicates that it remains to be established whether both apoB variants constitute lipoproteins in zebra fish.

Insects have previously been reported to encode and express the LLTP types MTP, apoLp-II/I, and, in general, multiple Vtgs (4, 17, 36). However, no Vtg is identified in the genome annotations of the fruit fly *Drosophila melanogaster* (48), in accordance with the finding that fruit fly and other higher (cyclorrhaphan) Diptera species rely on another unrelated protein, a lipase, as a major yolk constituent (for reviews, see Refs. 49, 50). Importantly, our database survey recognized two additional LLTP family members in insects. The first one is MEP, which was previously identified in the beetle as a factor that regulates melanogenesis, an immune response of insects to invading pathogens (47). MEP appears to be present in most insects, as it was also identified in the genome annotations of the honey bee (*Apis mellifera*) and the fruit fly (see supplementary Table I). The second novel insect LLTP was recently noted in the fruit fly genome annotations (48) (see supplementary Table I) and has not been described before in the literature. We have named it apolipophorin-II/I-related protein (ARP), in view of its relatively high similarity to apoLp-II/I. An as yet prematurely annotated ARP homolog was also predicted in the genome of the honey bee (see supplementary Table I), indicating that ARP may be present in most insects. Thus, the fruit fly has at least four distinct LLTP genes: MTP, apoLp-II/I, MEP, and ARP. Unlike this higher Dipteran, other insects additionally have one to three relatively closely related Vtgs (51).

In another class of arthropods, the decapodan Crustacea (e.g., lobster, crayfish, crab, shrimp), two distinct LLTPs have been identified, a CP (7, 8) and a yolk protein named Vtg (52–60). Although both LLTPs have been identified only in distinct species, the relatively close relationship among these species as well as the divergence of the identified proteins strongly suggest that the subset of LLTPs present in decapodan Crustacea con-

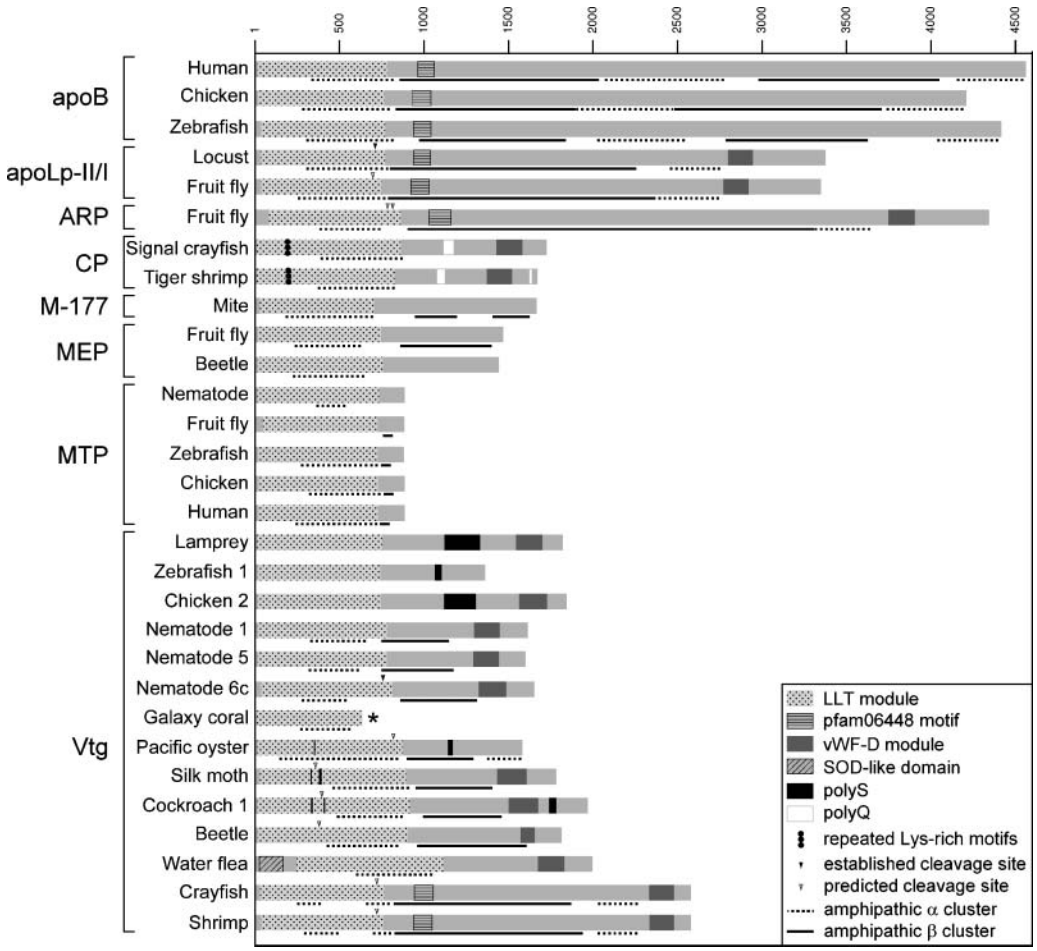

Fig. 1. Large lipid transfer (LLT) proteins (LLTPs) and their modular architecture. Selected LLTPs (see supplementary Table I) are shown with size proportional to length, with the N and C termini at the left and right sides, respectively. Recognized domains are indicated by boxes at their respective positions within the LLTPs. The LLT module delimited by the currently recognized conserved segments (see supplementary Fig. II) is indicated by a dotted box, the pfam06448 motif by a box with parallel horizontal lines, the von Willebrand factor (vWF)-D module by a dark gray box, and the superoxide dismutase-SOD domain in the vitellogenin (Vtg) of water flea by a diagonally lined rectangle. Regions rich in polyserine tracts are indicated by black boxes, the polyglutamine regions in clotting protein (CP) are indicated by white boxes, and the repeated lysine-rich motifs are indicated by stacked black circles. Clusters of amphipathic α-helices or amphipathic β-strands, as predicted using LOCATE (see supplementary Methods and Fig. I), are indicated by dotted and solid black lines, respectively, below each LLTP. Found (evidenced) and predicted sites of posttranslational cleavage during biosynthesis are indicated by closed and open arrowheads, respectively. An asterisk indicates that the analyzed sequence is not complete. For additional details, see supplementary Table I. apoB, apolipoprotein B; apoLp, apolipophorin; ARP, apolipophorin-II/I-related protein; MEP, melanin-engaging protein; MTP, microsomal triglyceride transfer protein; SOD, superoxide dismutase.

sists at least of one CP and one Vtg. In view of the absence of a genome sequence for this taxon, however, the complete subset of LLTPs in this animal group remains to be established.

In contrast to animal groups and species described above, the nematode *Caenorhabditis elegans* encodes an MTP as well as six Vtgs (61) but no apolipoprotein. Thus, the subset of LLTPs can differ strongly between distinct animal groups. Strikingly, all animals (for which a complete genome is available) appear to encode an MTP, in agreement with its proposed ubiquitous importance in LLTP assembly. In addition, most animals encode multiple Vtgs, whereas most often only a single gene is present for other LLTPs (e.g., apoB, apoLp-II/I, and MTP).

LLTPs are found throughout the animal kingdom. Unfortunately, LLTPs remain to be identified in most of the oldest invertebrate phyla, such as the Porifera (sponges). However, the occurrence of LLTPs appears to be limited to animals. Although several hits on bacteria, plants, and unicellular eukaryotes were obtained, none of these met the identification criteria (see supplementary Methods). This suggests that the LLTP superfamily of lipid binding proteins arose early in animal evolution, possibly upon the development of animal multicellular-

TABLE 1. The distribution and functions of identified LLTPs

Family	LLTP	Species Range	Function	Reference
MTPs	MTP	Metazoa	Facilitate biosynthesis of other LLTPs	26–28, M. M. W. Smolenaars, A. De Morrée, J. Kerver, D. J. Van der Horst, and K. W. Rodenburg, unpublished data
			Lipid antigen presentation (mammals)	9, 10
ApoB-like LLTPs	ApoB	Deuterostomes (vertebrates, echinoderms)	Lipid transport to maintain homeostasis	18
			Vitellogenesis/placenta	5, 106, 107
			Vision: place lipids in Bruch's membrane?	108
	Apolipophorin-II/I	Insects	Lipid transport to maintain homeostasis	20, 21, 109
			Vitellogenesis	82–84
			Coagulogen	110
			Immunity: β-1,3-glucan recognition	110
			Immunity: lipopolysaccharide detoxification	11, 111
			Morphogen transport during embryogenesis	3
	Apolipophorin-II/I-related protein	Insects	Currently unknown	This study
	M-177	Arachnids	Currently unknown	46
	Decapodan Vtg	Crustaceans	Vitellogenesis	52–54
Vtg-like LLTPs	Vtg	Metazoa	Vitellogenesis	4, 6, 62, 112
	Melanin-engaging protein	Insects	Immune system: regulation of melanogenesis	47
	Clotting protein	Crustaceans	Coagulogen	7, 8

apoB, apolipoprotein B; LLTP, large lipid transfer protein; MTP, microsomal triglyceride transfer protein; Vtg, vitellogenin.

ity, a condition that prompted the need for intercellular lipid transport.

Modular organization of LLTPs

The features of exemplary LLTPs (see supplementary Table I), including size, recognized modules, amphipathic clusters, established and putative posttranslational cleavage sites, polyserine tracts, and repeated lysine-rich and glutamine-rich regions, are summarized in Fig. 1.

The defining domain of LLTPs, the LLT module, is situated at the N terminus in virtually all LLTPs. The Vtg from the water flea *Daphnia magna* is the only exception known at present, as it has a 145 aa superoxide dismutase-like domain N terminal to the LLT module (62) (Fig. 1). Apart from their common N-terminal LLT module, however, LLTPs differ to a great extent in size and organization. MTPs are the smallest of the LLTPs, with ~890 aa, and contain little more sequence than the LLT module. Other family members span from ~1,350 to 4,500 aa and constitute additional domains (Fig. 1).

In addition to an LLT module, many LLTPs also contain a single von Willebrand factor (vWF)-D module that is situated near the C terminus (Fig. 1). ApoB, MTPs, M-177, Vtgs from several animals (e.g., the oyster *Crassostrea gigas*, zebra fish Vtg1), and the MEP from beetle lack this module. A search for additional domains in LLTPs revealed a single additional conserved motif, pfam06448, located from aa ~950 to aa ~1,070, uniquely present in apoLp-II/I, ARP, decapodan Vtg, and apoB only. The presence of this pattern suggests that these vertebrate and arthropodan LLTPs are relatively closely related.

Apart from the LLT module, vWF-D module, and the pfam06448 motif, hardly any primary sequence similarity can be observed among LLTPs from vertebrate and invertebrate animals. Structural predictions on LLTPs using the dedicated program LOCATE, however, have indicated the presence of large clusters enriched in either amphipathic α-helices or amphipathic β-strands (i.e., α and β clusters), also in segments of unidentified or poor primary sequence similarity (35, 63, 64). In apoB lipoproteins, these clusters constitute scaffolds for interaction with lipid molecules (for reviews, see Refs. 1, 2). For apoB, apoLp-II/I, ARP, and some Vtgs (from decapodan Crustacea and oyster), an evident alternating clustering of α and β clusters is predicted by LOCATE (Fig. 1; see supplementary Fig. I) (63, 64). The amphipathic clusters are distributed along the full length of these proteins as N-α-β-α-C, with the exception of apoB, for which an extended clustering conforming to N-α-β-α-β-α-C is predicted. M-177, insect Vtgs, and nematode Vtgs are predicted to contain smaller and less evident clusters of amphipathic structures according to N-α-β-C, whereas such clusters are less evident for CP, MEP, and vertebrate Vtgs. In particular, the predicted amphipathic β-strand clusters in apoB, apoLp-II/I, ARP, decapodan Vtg, and M-177 are relatively large (~700 to 2,400 aa) compared with other LLTPs (Figs. 1; see supplementary Fig. I). Thus, although there may be little sequence identity among LLTPs in substantial parts, similarities in the organization of amphipathic regions are observed among subgroups.

Several relatively unique features have also been observed in some LLTPs. C-terminal from the LLT module, vertebrate Vtgs most often contain multiple polyserine tracts, occupying a region of ~40 to ~210 aa (Fig. 1). As noted below, several invertebrate Vtgs contain shorter polyserine tracts (<15 aa) in the LLT module. Polyserine tracts are present in Vtgs only, not in other LLTPs. These

tracts are believed to act as substrates for kinases; the negatively charged phosphoserines may affect the solubility of the proteins or chelate essential metal ions needed in vitellogenesis (4). In addition, both characterized decapodan CPs contain an ~50 aa glutamine-rich and an ~20 aa lysine-rich region, with the shrimp CP additionally containing a polyglutamine tract near its C terminus. These regions may enable the intermolecular cross-linking of CPs by transglutamination during hemolymph coagulation (7, 8).

An additional noteworthy feature is the absence of conserved potential N-linked glycosylation sites (N-X-S/T) in the LLT or vWF-D modules (see supplementary Fig. II) or of the pfam motif of LLTPs (data not shown). Because of the lack of sequence similarity in the amphipathic α and β clusters throughout the superfamily, conservation of such glycosylation sites could not be established in these clusters. Glycosylation is known to be important for the folding, processing, transport, or function of proteins in general. Several LLTP members are known to be N-linked glycosylated, of which the functionality has been described; in Vtgs, glycosylation has also been proposed to provide oocytes with a source of carbohydrate during embryogenesis (4), and the glycosylation of the N-terminal half of apoB affects the assembly and secretion of apoB-containing lipoprotein (65). The apparent absence of conservation of such a site in the analyzed LLTP sequences could indicate that N-linked glycosylation does not support a general or evolutionarily conserved aspect of LLTP functioning.

Thus, LLTPs differ in size, amphipathic elements, and the presence of a vWF-D module, pfam06448 motif, small sequence elements, and posttranslational modifications. In the next section, architectural features of their common element, the LLT module, are discussed.

Architecture of the LLT module

The LLT module is the only region conserved among all LLTPs. Babin and coauthors (17) previously recognized the LLT module as constituted by 21 conserved sequence motifs, neighbored and interspaced by nonconserved regions. In our novel alignment of the LLT module from selected LLTPs (see supplementary Table I), we observe 13 sequence blocks of nearly contiguous conserved sequence (see supplementary Fig. II). These conserved segments largely correspond to the conserved N1–N5 and N7–N20 sequence motifs, previously recognized by Babin et al. (17). The other conserved regions observed in that study (N6, N21, and N22) were also recognized in all compared LLTPs, except in MTP from fruit fly and nematode. In the latter sequences, these regions could not be recognized unambiguously as a result of the relatively high sequence divergence.

At the structural level, the LLT module is constituted by four major elements, as revealed by the X-ray crystal structure (**Fig. 2**) of lipovitellin, an N-terminal cleavage product of lamprey Vtg. From the N to the C terminus, these are: *1*) a barrel-like β-sheet C (βC); *2*) a coiled horseshoe-shaped α-helical bundle with α-helices arranged in two layers; *3*) the amphipathic β-sheet βA; and *4*) the amphipathic β-sheet βB (Fig. 2) (31). Homology modeling to the lamprey lipovitellin structure supports a similar structural organization for the LLT module in mammalian MTP (34), mammalian apoB (39), and insect apoLp-II/I (30). The four structural elements are assumed to perform similar functions within LLTPs. The α-helical bundle plays an important role in dimeric interactions, such as homodimerization of Vtg (31), heterodimerization of MTP with PDI (34), and transient heterodimerization of MTP and apoB (66, 67); the two amphipathic β-sheets βA and βB constitute the larger part of a cavity in which lipids are bound (31, 34, 35, 37, 39); the βC-sheet in apoB (and possibly also in other LLTPs) interacts with the same sheet in MTP (34) and may assist in the transfer of lipids to the lipid cavity (39).

Mapping of the noted conserved sequence segments onto the lamprey lipovitellin X-ray crystal structure (Fig. 2) reveals that they are situated in three structural elements of the LLT module. Segments 1–5 are located in the barrel-like βC-sheet, segments 6–10 in the α-helical bundle, and segments 11–13 in the βA-sheet. Considering the βB-sheet, sequence conservation is restricted to the N21 and N22 motifs (17) that are present in all LLTPs except for the investigated invertebrate MTPs, nematode and fruit fly MTP. Interestingly, the barrel-like βC-sheet has a single α-helix inside that appears to be constituted by a highly conserved heptameric motif (N-L/I/V/M-K/R-K/R-G-L/I/V), situated in the conserved segment 3. This motif is only absent from nematode MTP. In the lipovitellin structure, it appears to interact with a nearby highly conserved hexameric aa motif (P-S/T-X-G-L/V/I/M-P) located in a loop between two β-strands from the βB-sheet that extend into the barrel-like βC-sheet. Thus, all four basic structural elements of the LLT module have been retained throughout evolution in all LLTPs, except in invertebrate MTPs. Here, the regions that encode the βB-sheet and its periphery have not been conserved.

Several other remarkable features have been observed in the LLT module of some LLTPs. These include posttranslational cleavage sites (apoLp-II/I, ARP, and several Vtgs), repeated lysine-rich motifs in CPs (i.e., five S-K-T-S motifs in shrimp CP and three T-K-T-T-G motifs in the crayfish CP) (7, 8), and an additional stretch of ~60 aa in Vtg of water flea. Strikingly, these variations are consistently located in loops between secondary structure elements and outside of the conserved segments of the LLT module (Fig. 2). Such localizations are likely to enable possible novel functionalities for LLTPs, whereas the basal structure of the LLT module that accommodates lipid binding is retained. A further discussion of the integration of the structure, function, and evolution of LLTPs is presented below.

Three families of LLTPs

The analysis of structural features enables a reexamination of the evolutionary relationships among LLTPs and the nature of their common ancestor. These could not be established previously as a result of a lack of sequence information (17). Using novel sequences, our extensive

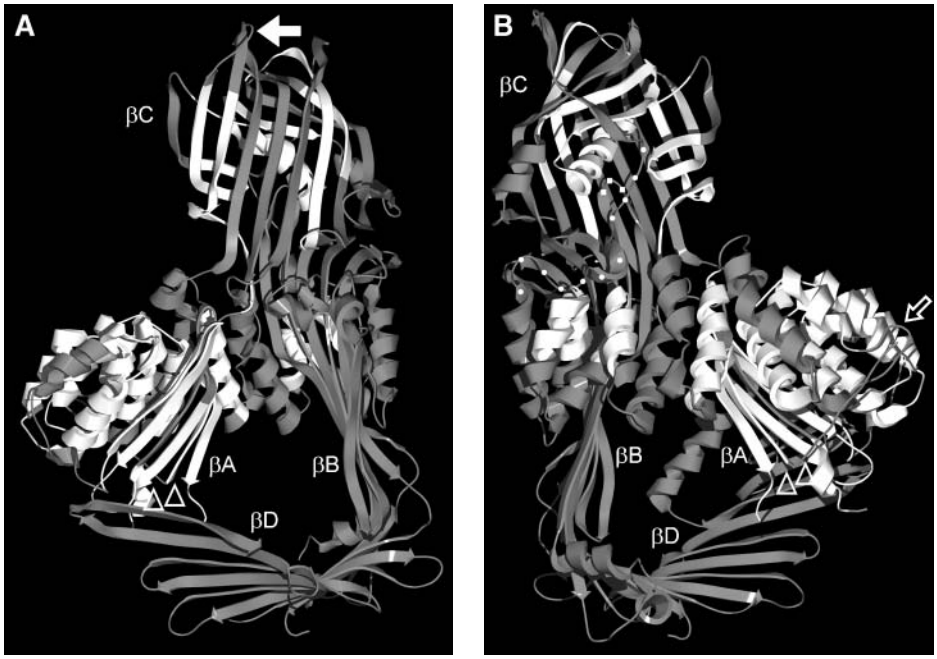

Fig. 2. Mapping of LLT module sequence variation onto the lamprey lipovitellin structure. Ribbon representations of the lamprey lipovitellin X-ray crystal structure (31) that includes the complete LLT module. Regions encoded by sequence that has been conserved among all LLTPs are shown in white, and the rest are shown in gray. A: Front view. Several structures outside of the LLT module obstructing the view of the lipid binding cavity [C terminal from amino acid (aa) 1,456] have been left out. B: Back view. The three β-sheets present in the LLT module are indicated by βA, βB, and βC. Sequences C terminal to the LLT module encode part of the βB-sheet and the whole βD-sheet. The closed arrow in the front view (A) indicates the loop in which decapodan CPs have an insert of repeated lysine-rich motifs. In the back view (B), the open arrow indicates the loop in which the Vtg of water flea (*Daphnia magna*) contains an additional stretch of ~60 aa, relatively enriched in lysine residues; white circles indicate the region that contains the two polyserine tracts with intermittent cleavage sites in a Vtg of cockroach (*Periplaneta americana*) and silk moth (*Bombyx mori*); and white squares indicate the location of the conserved motif in a loop extending from the βB-sheet into the barrel-like βC-sheet. Open arrowheads point to the β-strands of the βA-sheet that are connected by a loop of unknown structure that contains a consensus substrate sequence for cleavage by furin in apoLp-II/I, ARP, decapodan Vtg, and several other invertebrate Vtgs. The βA-, βB- and βD-, and βC-sheets in this figure refer to the C-sheet, A-sheet, and N-sheet, respectively, described by Anderson, Levitt, and Banaszak (31). In the process of apolipoprotein homology model building, they were renamed as indicated (30, 38, 39).

phylogenetic analyses using Bayesian as well as maximum likelihood methods (see supplementary data) on the aligned conserved segments in the LLT module now yield a phylogenetic tree that reveals three major families within the superfamily of LLTPs (**Fig. 3**): *1*) vertebrate and invertebrate MTPs; *2*) vertebrate apoB, insect apoLp-II/I, insect ARP, mite M-177 allergen, and decapodan Vtg; and *3*) Vtg (excluding decapodan Vtg), CP, and MEP. From here on, we refer to these three LLTP families as MTPs, apoB-like LLTPs, and Vtg-like LLTPs, respectively.

The first family is constituted by vertebrate and invertebrate MTPs. The MTPs of the fruit fly and particularly of the nematode appear to have diverged strongly, as evident from their relatively long branch lengths in the phylogenetic tree (Fig. 3). Nonetheless, in nematodes, insects and vertebrates alike, MTP has been reported to stimulate the biosynthesis of other LLTPs (26–28; M. M. W. Smolenaars, A. De Morrée, J. Kerver, D. J. Van der Horst, and K. W. Rodenburg, unpublished data). Because the only putative LLTP substrates for the nematode MTP are the relatively closely related Vtgs, its divergence may reflect the absence of the selective pressure induced by multiple distinct substrates, as is the case in insects as well as vertebrates that have multiple distinct LLTP substrates (i.e., Vtg-like and apoB-like LLTPs).

The second family, referred to as apoB-like LLTPs, includes vertebrate apoB, insect apoLp-II/I, fruit fly ARP, mite M-177, and Vtg from decapodan Crustacea (Fig. 3). In addition, the grouping of vertebrate apoB, insect apoLp-II/I, insect ARP, and decapodan Vtg in this family is supported by the recognition of a homologous region in these sequences only, the pfam06448 motif (68), located just C terminal to the LLT module (Fig. 1). Moreover, at the N-terminal side, 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 an additional α cluster. Although the fruit fly ARP is clearly related to apoLp-II/I, phylogenetic support for its grouping with apoLp-II/I rather than with other apoB-like LLTPs is relatively poor, highlighting the molecular diversification of ARP as opposed to apoLp-II/I. Phylogenetic analysis suggests that mite M-177 is also a member of this apoB-like

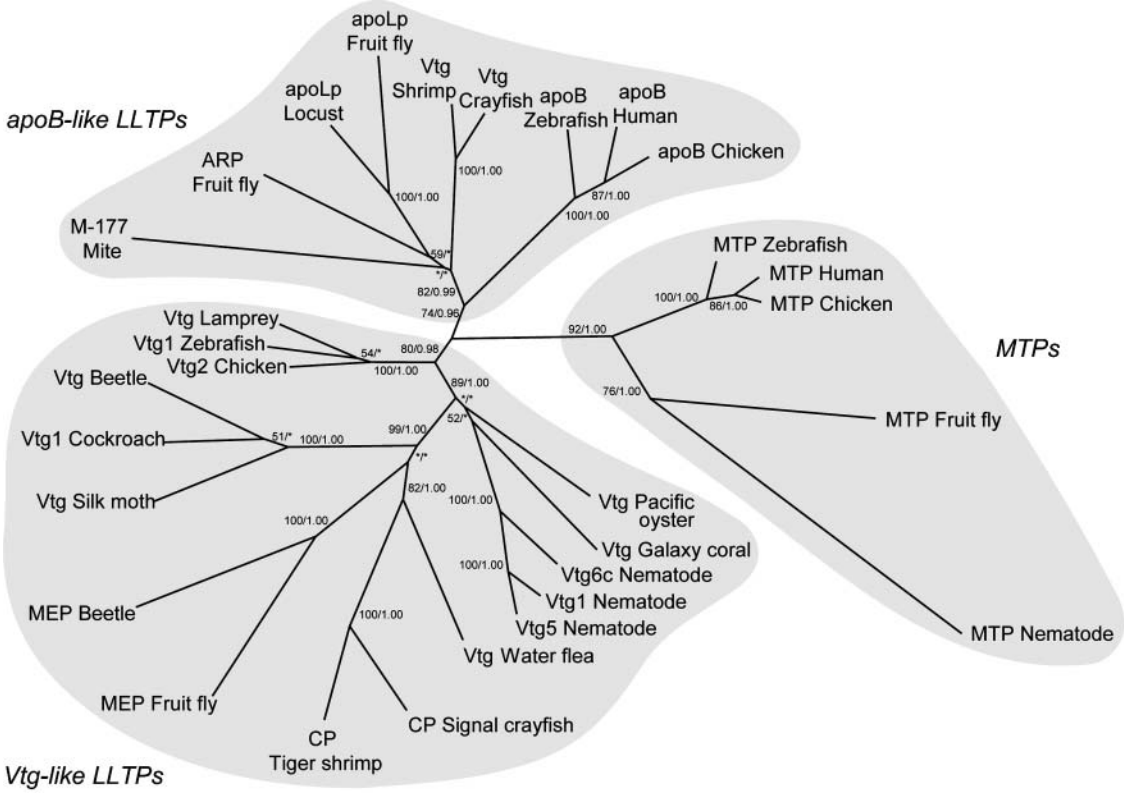

Fig. 3. Phylogenetic tree of the LLTP superfamily. This unrooted phylogenetic tree was constructed using the aligned segments of the LLT module (see supplementary Fig. II) by maximum likelihood analyses (see supplementary Methods). Numbers indicate maximum likelihood bootstrap and Bayesian posterior probability values (left and right of slash, respectively) for each node. Only support values ≥50% for maximum likelihood and ≥0.95 for Bayesian analyses are shown, whereas nodes with lower support are indicated by asterisks. The members of the three recognized LLTP families, the MTPs, the Vtg-like LLTPs, and the apoB-like LLTPs, are denoted by separate gray backgrounds.

LLTP family, even though it lacks the pfam06448 motif and an extended β cluster and additional α cluster that are present in other apoB-like LLTPs. Additional sequences of LLTPs from arachnids may help define the context of the divergence of M-177. Importantly, the Vtg of decapodan Crustacea is not directly related to the Vtg genes of other taxons, as assumed by others (52–60), but is an apoB-like LLTP. This observation explains previous phylogenetic analyses on Vtgs only (57, 62, 69, 70), in which decapodan Vtg failed to group with other invertebrate Vtgs. Phylogenetic analysis of the aligned vWF-D modules (in total only ~100 aa alignable sequence) also supports the grouping of decapodan Vtg with insect apoLp-II/I and ARP, apart from an unresolved group of other LLTPs with a vWF-D module (data not shown), further supporting the notion that decapodan Vtg is an apoB-like LLTP.

The third family, referred to as Vtg-like LLTPs, includes vertebrate as well as invertebrate Vtgs (except for Vtg from decapodan Crustacea), decapodan CP, and insect MEP (Fig. 3). The family of Vtg-like LLTPs is highly diverse. Previous phylogenetic analyses of Vtgs differed regarding the relationship among nematode, insect, and vertebrate Vtgs. Several analyses based on the conserved N-terminal regions of Vtgs revealed a closer relationship between the Vtgs from nematodes and insects compared with Vtgs from vertebrates (61, 68). In contrast, other analyses suggest a closer relationship between the Vtgs from nematodes and vertebrates compared with Vtgs from insects (4, 71–73). The difference between the two groups of analyses may be explained by the fact that the Vtg trees were unrooted (73), rooted using an insect Vtg rather than a non-Vtg LLTP (72), or interpreted without taking the included apoB outgroup into account (4, 73). The present phylogenetic analysis suggests a closer relationship between the Vtgs from nematodes and insects compared with Vtgs from vertebrates, in agreement with the present view on animal phylogeny (74). In addition to multiple (related) Vtgs, insects also have another Vtg-like LLTP, MEP. In decapodan Crustacea, however, CP is the only Vtg-like LLTP identified at present, as the protein that is named Vtg in this taxon actually is, from an evolutionary point of view, an apoB-like LLTP (Fig. 3).

INTEGRATING THE STRUCTURE, FUNCTION, AND EVOLUTION OF LLTPs

Structurally variant LLTPs that spread across the population will have some beneficial functional feature, for example, because the variant has a modified catalytic activity

or has additional interaction partners. Likewise, the superfamily of LLTPs has arisen and expanded as its (new) members proved beneficial to fitness. To understand the observed structural uniformity and diversity in the superfamily of LLTPs, we investigated the relationship between the structure, function, and evolution of its members.

Origin and early evolution of LLTP domains and families

The emergence of the LLT module appears to be the hallmark event in the origin of the complete superfamily of LLTPs, as it provides the basal structure for the binding of multiple lipid molecules (31, 37, 39). The evolution of the LLT module may 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 come to discussion (28). Previously, the common ancestor of the present LLTPs was suggested to function in vitellogenesis, as this ancient process is essential to reproduction in even the oldest animal phyla. More recently, however, an ancient MTP has been proposed to be the predecessor to other LLTPs, in view of the current importance of MTP in the biosynthesis of both Vtg-like and apoB-like LLTPs (26–28, 75). We note that the first LLTP could as well have been quite different from any current LLTP, in form as well as function. It cannot be excluded that the defining domain in LLTPs, the LLT module, evolved first as part of a larger multidomain protein that had a function unrelated to any of the functions ascribed to LLTPs at present.

The currently recognized LLTP families (i.e., MTP, apoB-like, and Vtg-like) appear to have arisen early after the conception of the first superfamily members, possibly before the evolution of Bilateria, in view of the presence of distinct LLTP families in all investigated (Bilaterian) phyla. A recent preliminary apoB sequence (accession number XP_785809) from the genome of a sea urchin (*Strongylocentrotus purpuratus*) suggests a closer relationship between Vtg-like and apoB-like LLTPs, as opposed to MTPs. The sea urchin apoB contains an N-terminal LLT module and a pentapartite amphipathic structure similar to that of vertebrate apoB (LOCATE predictions). Strikingly, this apoB also has a C-terminal vWF-D module. In view of the shared ancestry of echinoderms and vertebrates, this poses the possibility that the ancestral vertebrate apoB lost its vWF-D module. Accordingly, the presence of a vWF-D module appears to be the ancestral state for apoB-like LLTPs. Therefore, the apoB-like and Vtg-like LLTP families may stem from a common ancestor that consisted of an LLT module as well as a vWF-D module. This appears to be a more parsimonious possibility than the independent gain of the vWF-D module in the apoB-like and Vtg-like LLTP families.

The organization of amphipathic clusters in Vtg-like and apoB-like LLTPs has been subject to changes. Among apoB-like LLTPs, the number, size, and organization of these clusters clearly vary. The amphipathic clusters in vertebrate apoB may have expanded from an ancestral N-α-β-α-C organization to the pentapartite N-α-β-α-β-α-C clustering by the duplication of a β and α cluster in the ancestral vertebrate apoB gene. Compared with apoLp-II/I, the amphipathic β cluster region of ARP appears to have expanded, resulting in the larger size of ARP. Unfortunately, reconstruction of these modifications is prevented by the scarce sequence similarity within and among the involved regions of different LLTPs.

Thus, several scenarios for the origin of the three LLTP families and their structural features can be suggested. For example, the three families could originate from an ancestral LLTP constituted of an LLT module only. After gene duplications, one of the MTP-like LLTPs gained a vWF-D module and became the ancestor to both the apoB-like and Vtg-like LLTPs. After its duplication, the common ancestor to the apoB-like LLTP gained extensive amphipathic α and β clusters, whereas the common ancestor to the Vtg-like LLTP specialized in other ways. Alternatively, the ancestral LLTP was constituted by an N-terminal LLT module as well as a C-terminal vWF-D module. While the MTP family arose by the loss of the sequences C terminal to the LLT module, the Vtg-like and apoB-like LLTPs diverged as the ancestor to apoB-like LLTP developed extensive amphipathic stretches. Likely, any structural changes at the origin of LLTP families were accompanied by functional diversification (i.e., neofunctionalization) to warrant selective advantages for the new families. Likewise, the ancestral MTP specialized in stimulating the secretion of other LLTPs via heterodimerization, the ancestral Vtg-like LLTP in nutrient transfer to oocytes, and the ancestral apoB-like LLTP in general-purpose lipid transport.

A role for functional dynamics in the expansion of LLTP families

Functional diversification of gene duplicates likely has driven the subsequent expansion of LLTP families as well. The frequent occurrence of LLTP gene duplication (and loss) is clearly illustrated by the large variation in the number of Vtg genes between taxa and even within closely related species, such as the 7 Vtg genes present in the zebra fish (25), 2–30 within different species of salmonids (24), 4 in the amphibian *Xenopus laevis* (22), and 6 in the nematode *C. elegans* (61). These copies of Vtg have been found to originate from whole genome duplication as well as local gene amplification (24, 25). They appear to be maintained as a result of subfunctionalization, because egg production and fertility can depend on the number of yolk protein gene copies (76). Conversely, Vtg genes can also be lost entirely when they become redundant, as exemplified by the higher Diptera (including the fruit fly) that lack a Vtg gene because their common ancestor reverted to a lipoprotein lipase-related protein as the major yolk protein (50). Likewise, Vtg-like LLTPs were lost in placental mammals when massive yolk accumulation in the oocyte was abandoned, as indicated by the continued presence of this family in the platypus (data not shown), an egg-laying mammal basal to placental mammals.

The genes of apoB-like LLTPs and MTPs would appear to be as prone to duplication as those of Vtg-like LLTPs. However, relatively few additional apoB-like LLTPs have

been found in a single species, such as ARP in insects, an additional apoB gene in zebra fish (see supplementary Table I), and a second apoB-like Vtg in the shrimp *M. ensis* (57, 58). The duplicate apoBs in zebra fish likely arose in an ancestor upon whole genome duplication (77). In mammals, RNA editing of apoB mRNA results in the secretion of a truncated apoB, apoB-48 (for reviews, see Refs. 78–80), effecting the expression of an additional type of apoB. From this point of view, the evolutionary arrival of this process has resulted in a de facto gene duplication of apoB. In contrast to the apoB-like LLTPs, no duplicate MTPs have been observed at all. The relatively limited expansion of the apoB-like and MTP families indicates that their duplicates are retained less often than Vtg-like LLTPs. Thus, novel copies of apoB-like and MTP family members do not often attain a selective advantage. Because the neofunctionalization of a gene is often preceded by its subfunctionalization (81), this may primarily reflect the absence of initial subfunctionalization of duplicates of apoB-like LLTPs and MTPs.

An overview of the functions ascribed to LLTPs (Table 1) suggests several instances of neofunctionalization within LLTP families. For example, MEP is an insect Vtg-like LLTP that functions in regulating melanogenesis reactions in insect immunity (47), unlike any other LLTP. In view of the presence of other Vtg-like LLTPs in insects (e.g., Vtgs), MEP appears to originate from a duplicated Vtg gene that has been retained as a result of the acquisition of a novel function. CP, the single Vtg-like LLTP identified in decapodan Crustacea to date, may represent another example of neofunctionalization, as this protein functions as the major hemolymph coagulogen rather than as a yolk protein (7, 8, 50, 52, 53). In the same taxon, an apoB-like LLTP, named Vtg, constitutes the major yolk protein, rather than a Vtg-like LLTP as in most animals. This likely reflects a shift in the relative importance of yolk proteins, as other apoB-like LLTPs have also been reported to engage in nutrient supply, especially of lipids, to the developing oocyte in insects as well as nonmammalian vertebrates (5, 82–84). Therefore, rather than a duplicate, decapodan CP may also be the Vtg ortholog that has been retained by neofunctionalization in the face of redundancy, when an apoB-like LLTP became the major yolk protein. Entirely opposite to decapodan Crustacea, however, most insects depend on an apoB-like LLTP (apoLp-II/I) for coagulation and on a Vtg-like LLTP (Vtg) as the major yolk protein (30; for review, see Ref. 4). Thus, members from phylogenetically distinct LLTP families have attained similar functions in vitellogenesis or coagulation in distinct taxa. This could reflect the taxon-specific division of multiple functions that used to be performed by the common ancestor of current apoB- and Vtg-like LLTPs, although it is also possible that these functional similarities arose independently and are the result of parallel functional adaptations.

Correlating diversity in structure and function

For LLTPs, the structure-function relationship has particularly been studied with respect to lipid binding, especially focused on apoB. ApoB lipoproteins, constituted of a single apoB that envelopes a core of hundreds of lipid molecules (for reviews, see Refs. 1, 2), are completely assembled intracellularly by the lipidation of apoB (for reviews, see Refs. 14, 84–86). This process starts already during its translation, upon translocation of the nascent polypeptide chain into the endoplasmic reticulum (87). The LLT module of apoB is the first part to be translated and translocated, and it gathers lipids soon after its folding, a process that is facilitated by its interaction with the MTP/PDI heterodimer (34, 88, 89). At this cotranslational stage, the nascent apoB polypeptide binds relatively few lipids, for a large part phospholipids (36, 38), in a cavity constituted principally by the βA- and βB-sheets of the LLT module (38, 39, 89). Additional amphipathic structure has been predicted in the linker region between the βA- and βB-sheets that may close the cavity temporarily (39). The bound lipids constitute a nucleus for further lipid binding. In apoB, expansion of this lipid core is enabled by the transfer of neutral lipids by MTP and the concomitant association of amphipathic β-strands that have just been translocated into the endoplasmic reticulum (40; for reviews, see Refs. 1, 2, 36). In particular, sequence located in the pfam06448 motif has been suggested to be critical for the formation of a stable, neutral lipid-containing nascent lipoprotein particle (38). In a second stage, after translation, apoB can acquire most of its neutral lipids (triacylglycerol and cholesteryl esters), presumably by fusion with a neutral lipid droplet in the smooth endoplasmic reticulum and/or *cis*-Golgi apparatus (84). This intralumenal lipid is accreted in the secretory pathway via MTP (90–92). Thus, in apoB the N-terminal LLT module is the basal lipid-binding structure that sets the stage for the proper folding and lipid association of further C-terminal amphipathic clusters. Consequently, apoB appears to be organized to enable the binding of a large quantity of lipids upon its biosynthesis. The functionality of this organization may apply to all apoB-like LLTPs.

MTP plays an essential role in the lipidation and secretion of apoB in mammals (for reviews, see Refs. 14, 85, 93). Current evidence suggests a similar role for MTP in insects in apoLp-II/I lipoprotein biosynthesis (M. M. W. Smolenaars, A. De Morrée, J. Kerver, D. J. Van der Horst, and K. W. Rodenburg, unpublished data) and in nematodes as well as nonmammalian vertebrates in Vtg production (26, 27). Unlike vertebrate MTPs, however, the fruit fly and nematode MTPs display no sequence similarity to other LLTPs in the βB-sheet. In its periphery, they also lack the highly conserved motif, located in a loop extending from the βB-sheet into the barrel-like βC-sheet, which appears to interact with another highly conserved α-helix within the barrel-like βC-sheet (Fig. 2B). Previously, fruit fly and bee MTP were noted to diverge from vertebrate MTPs by the absence of amphipathy in helix A, at the boundary of the βB-sheet (36, 45). Thus, a large part of the βB-sheet and peripheral structure may not be present in insect and nematode MTPs. Given the roles of these structures in constituting the lipid binding cavity and facilitat-

ing lipid transfer (45), their absence may have profound functional implications. Strikingly, the fruit fly MTP was recently found to lack triacylglycerol and cholesteryl ester transfer activity, but it did transfer phospholipids in the in vitro assay used (75). Possibly, neutral lipid transfer depends on the βB-sheet and peripheral structures. Accordingly, the ability of fruit fly MTP to stimulate apoLp-II/I lipoprotein biogenesis in vitro (M. M. W. Smolenaars, A. De Morrée, J. Kerver, D. J. Van der Horst, and K. W. Rodenburg, unpublished data) could relate to its capacity to transfer phospholipids rather than triacylglycerol or cholesteryl ester.

Although the LLT module is the first to associate with lipids during biosynthesis, the amphipathic clusters of apoB and apoLp-II/I stabilize the majority of bound lipids (for reviews, see Refs. 1, 2). ApoB-like LLTPs are predicted to contain one or two extended clusters enriched in amphipathic β-strands. The (partial) presence of such a β cluster dramatically increases the binding of (neutral) lipids by truncated apoB and apoLp-II/I (40) (for reviews, see Refs. 1, 2; M. M. W. Smolenaars, A. De Morrée, J. Kerver, D. J. Van der Horst, and K. W. Rodenburg, unpublished data). In apoB, the two β clusters have been predicted to form nearly continuous amphipathic β-sheets of sufficiently high lipid affinity to nearly irreversibly associate with lipids (2, 94, 95). Thus, the β clusters in apoB-like LLTPs may function to enhance their lipid binding capacity, in accordance with the role of these LLTPs in lipid distribution.

In this respect, the observation that Vtg-like LLTPs bind far less (neutral) lipids than apoB-like LLTPs such as apoB and apoLp-II/I (18, 96–98) may reflect the presence of less amphipathic structures, resulting in a decreased lipid binding capacity. Vtg-like LLTPs are predicted to contain amphipathic β-strands, yet often in smaller or less clearly defined clusters, as in apoB (Fig. 1) (35). In contrast to apoB-like and Vtg-like LLTPs, MTPs lack any sequence C terminal to the LLT module. Thus, the suspected role of MTPs in the transfer of lipids to other LLTPs may result from their limited number of amphipathic β-strands of high lipid affinity.

In addition to one or two β clusters, apoB-like LLTPs are also predicted to contain one or two C-terminal α clusters of high calculated lipid affinity (Fig. 1) (63, 64). Amphipathic α-helices from the α clusters of mammalian apoB have been reported to reversibly associate and dissociate from the lipid core upon changes in lipid content (95, 99), such as also occur in vivo in lipid transport by lipoproteins constituted of apoB-like LLTPs, such as the insect lipoprotein, lipophorin, and the vertebrate lipoproteins containing apoB (18, 20). From this point of view, the absence of a predicted C-terminal α cluster in nearly all Vtg-like LLTPs is in agreement with their limited dynamics in lipid content, as expected from their functions in yolk supply (Vtg), coagulation (CP), and immunity (MEP) (Table 1). Compared with vertebrate apoB, insect apoLp-II/I contains only one α cluster that also is of relatively small size. Accordingly, the exchangeable apolipoprotein apoLp-III, which is constituted of amphipathic α-helices and enables additional lipid binding by insect lipoprotein, has been proposed to compensate for the relatively small number of amphipathic α-helices present in apoLp-II/I, so insect lipoprotein remains stable upon changes in lipid content (M. M. W. Smolenaars, A. De Morrée, J. Kerver, D. J. Van der Horst, and K. W. Rodenburg, unpublished data).

Several LLTPs also contain specific structural features that may facilitate functions in addition to lipid transport. For example, the repeated lysine-rich motifs in CP are located on top of the barrel-like βC-sheet (Fig. 2), a location that may best allow for the suggested role of these motifs in the cross-linking of CP by transglutaminase upon hemolymph coagulation (7, 8). In addition, the LLT module of the Vtg of water flea is preceded N terminally by a superoxide dismutase-like domain, unlike any other currently reported LLTP. The fusion of this domain to Vtg has been suggested to play a role in the transport of Cu(II) or in the immediate detoxification of superoxides resulting from Vtg metabolism (62).

The vWF-D module, present in many apoB-like LLTPs and most Vtg-like LLTPs, is always located near the C terminus (Fig. 1). By recombinant expression of apoLp-II/I truncation variants, it was demonstrated not to be essential for insect lipoprotein formation (M. M. W. Smolenaars, A. De Morrée, J. Kerver, D. J. Van der Horst, and K. W. Rodenburg, unpublished data). Interestingly, mammalian secretory mucin and vWF contain multiple vWF-D modules that enable their multimerization (100, 101). Accordingly, the single vWF-D module might facilitate the sequestration of Vtg in the oocyte and the clotting of apoLp-II/I lipoproteins during coagulation in insects. However, the functional significance of the single vWF-D module in LLTPs at its specific C-terminal location remains unknown.

Several Vtg-like LLTPs also contain polyserine tracts, unlike any of the MTPs or apoB-like LLTPs. These serine residues are heavily phosphorylated, likely resulting in the binding of calcium (4, 102). The physiological role of this process is not clear yet. Strikingly, the region enriched in polyserine tracts is particularly large in most vertebrate Vtgs, suggesting a taxon-specific role. Accordingly, we speculate that the extensive polyserine region in vertebrate Vtgs represents a molecular adaptation to enhance calcium deposition to the developing oocyte to facilitate skeletal formation in the future embryo.

Some LLTPs are cleaved by a furin-like protease (103) during biosynthesis (e.g., apoLp-II/I). This insect LLTP is cleaved within the LLT module in a loop between two β-strands of the βA-sheet. The cleavage appears not essential for initial lipidation, although a putative role in enabling secondary extracellular lipidation, as occurs in some insects during sustained flight, cannot be excluded. Alternatively, apoLp-II/I cleavage (29) may be essential to allow for distinct roles of apoLp-I and -II during coagulation or insect lipoprotein receptor interaction (30; for review, see Ref. 104). Putative cleavage sites are also present in the apoB-like Vtg of decapodan Crustacea at the same location (52–60), and also in some insect Vtgs at vary-

ing locations in the LLT module (for review, see Ref. 4). With respect to the occurrence of the cleavage sites in the LLT module of the latter LLTPs, we propose, in analogy to the explanation for the insect apolipoprotein, that cleavage of LLTPs occurs subsequent to initial biogenesis. The role of this cleavage in the LLT module may, in general, well serve a function succeeding biogenesis and may possibly also involve extracellular functions of the apoB-like Vtg and insect Vtgs (30).

In conclusion, the noted variation in the molecular organization of LLTPs can be related to functional aspects and thus appears to reflect molecular adaptations. However, many of the proposed structure-function relationships remain to be established experimentally, also with regard to lipid binding. For example, how do the specific domains in the LLT module cooperate in the initial assembly of lipids, especially in view of the heterodimerization of MTP with apoB and perhaps other LLTPs? Does the lipid transfer activity of MTP result from specific features of its LLT module and/or the absence of C-terminal lipid-retaining amphipathic sequences? Does the pfam06448 motif in apoB-like LLTPs mediate a specific function in neutral lipid binding during cotranslational lipoprotein assembly? Moreover, recent data suggest a role for specific sequences in apoB in the posttranslational acquisition of particularly neutral lipids (105; for review, see Ref. 14). Several remarkable features noted in some LLTPs may also enable functions beyond lipid binding. However, these do not seem to interfere with lipid binding, a function that has been conserved and appears to rely on similar mechanisms and principles in LLTPs across the animal kingdom.

CONCLUSIONS AND FUTURE DIRECTIONS

This examination of the structure, function, and evolution of LLTPs reveals the plasticity of this superfamily of lipid binding proteins. The three major LLTP families recognized in this review (i.e., MTPs, Vtg-like LLTPs, and apoB-like LLTPs) arose in the earliest animals, suggesting that the LLT module came about coincident with animal multicellularity. Ever since, novel LLTPs have arisen by duplication events. Although most duplicates are lost, some are retained because they share a function (subfunctionalization) or gain a different functionality (neofunctionalization). The continuous expansion, degradation, and modification of LLTPs is illustrated by the presence of different sets of LLTP families in current animal groups (MTP and apoB-like, MTP and Vtg-like, or MTP, apoB-like, and Vtg-like) as well as by the observation that some evolutionarily closely related proteins (e.g., MEP and Vtg in insects) have acquired distinct functions, whereas some evolutionarily distant proteins (e.g., Vtg and the decapodan apoB-like Vtg) have acquired similar functions. However, the early origin and evolution of this superfamily and its specific structural elements (i.e., the LLT module, vWF-D module, and amphipathic clusters) remain elusive at present. Current efforts on additional animal genomes, particularly from the ancient metazoan phyla Cnidaria and Porifera, may provide further insight into the early origin and diversification of LLTPs.

The integration of LLTP structure, function, and evolution reveals common structural elements as well as multiple putative adaptations. For example, the structural organization of particularly apoB-like LLTPs appears to be adapted to facilitate lipid transport. For apoB, the N-terminal location of the LLT module allows it to cotranslationally acquire lipids first. This results in a lipid core on which its further C-terminal amphipathic structures can properly fold as they concomitantly stabilize additional lipids. Although all LLTPs investigated to date bind lipids, comparative research has also highlighted a multitude of functions for LLTPs in addition to circulatory lipid transport. A wealth of LLTP functionality may even await discovery, as illustrated by the recent discoveries that apoLp-II/I lipoproteins transport morphogen proteins during early embryonic development of the fruit fly (3) and that mammalian MTP facilitates lipid antigen presentation (9, 10). Moreover, a function for the newly established insect LLTP, ARP, remains to be discovered. It may also be of interest to further investigate whether the adaptive advantage of multiple Vtg genes relates to increased yolk accumulation only (76) or also to new functions. Moreover, the functional implications of the observed structural variations remain to be established in most cases. Accordingly, the LLTP superfamily provides a rich substratum for future integrative and comparative studies on the evolution and adaptation of lipid binding proteins throughout the animal kingdom.

The recent study by Rava et al. (75) also illustrates the utility of comparative studies in understanding and targeting the role of LLTPs in human disease. The comparison of the lipid transfer activity of MTP from fruit fly and human revealed that the transfer of phospholipids and neutral lipids can selectively be inhibited in human MTP. This opens a new perspective on inhibiting MTP activity to specifically control the production of atherogenic apoB lipoproteins, an approach that was severely hampered by aspecific effects. As comparative research on molecular, biochemical, and cellular aspects of apoB-like LLTPs has come of age only during the last couple of years (30, 36, 40, 75), the time is now to experiment with the structural and functional diversity of diverse LLTPs to arrive at novel opportunities for modulating lipid transport in health and disease.

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