

9 Lipid Transport

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9.1. Historical Perspective	317
9.1.1. Lipophorin Structure, Assembly, and Morphology	317
9.1.2. Lipophorin Subspecies	319
9.2. Flight-Related Processes	319
9.2.1. Adipokinetic Hormone	319
9.2.2. Strategy of the Adipokinetic Cells	320
9.2.3. Effect of Adipokinetic Hormones on Lipid Mobilization	322
9.3. Apolipophorin III	325
9.3.1. Lipid-Free Helix Bundle Structure	325
9.3.2. Lipid-Induced Conformation Change	326
9.3.3. Initiation of ApoLp-III Lipid Binding	328
9.3.4. ApoLp-III Alternate Functions	329
9.4. Lipophorin Receptor Interactions	330
9.4.1. Receptor-Mediated Endocytosis of Lipophorin	330
9.4.2. Lipophorin Receptor-Mediated Ligand Recycling	332
9.5. Other Lipid-Binding Proteins	333
9.5.1. Lipid Transfer Particle	333
9.5.2. Carotenoid-Binding Proteins	334
9.5.3. Fatty Acid-Binding Proteins	335
9.5.4. Vitellogenin	336

9.1. Historical Perspective

9.1.1. Lipophorin Structure, Assembly, and Morphology

Lipophorin was discovered nearly 50 years ago as a major hemolymph component and key transport vehicle for water-insoluble metabolites (for reviews, see [Beenackers et al., 1985](#); [Chino, 1985](#)). Lipophorin is generally regarded as a multifunctional carrier because it displays a broad ability to accommodate hydrophobic biomolecules. In essence, lipophorin can be described as a non-covalent assembly of lipids and proteins, organized as a largely spherical particle. The core of the particle is made up of hydrophobic lipid molecules, such as diacylglycerol (DAG), hydrocarbons, and carotenoids. DAG, which serves as the transport form of neutral glycerolipid in hemolymph, provides an energy source for various tissues through oxidative metabolism of its fatty acid constituents. Hydrocarbons, in the form of long-chain aliphatic alkanes and alkenes, are extremely hydrophobic lipid molecules that are deposited on the cuticle, where they serve to prevent desiccation and may function as semiochemicals. Carotenoids are plant-derived pigments used for coloration, and as a precursor to visual pigments ([Canavoso et al., 2001](#)). Another important lipid component of lipophorin is

phospholipid. In general, the major glycerophospholipids present are phosphatidylcholine and phosphatidylethanolamine ([Wang et al., 1992](#)). These amphiphilic lipids exist as a monolayer at the lipophorin particle surface, positioned in such a way that their fatty acyl chains interact with the hydrophobic core of the particle while their polar head groups are presented to the aqueous milieu. In this manner, the phospholipid moieties of lipophorin serve a key structural role. The other major structural component of lipophorin is protein. All lipophorin particles possess two apolipoproteins, termed apolipophorin I (apoLp-I) and apolipophorin II (apoLp-II). ApoLp-I and apoLp-II are integral components of the lipophorin particle, and cannot be removed without destruction of lipophorin particle integrity. It is recognized that apoLp-I and apoLp-II are the product of the same gene, and that the two proteins arise through post-translational cleavage of their common precursor protein ([Weers et al., 1993](#)). This finding is consistent with the fact that apoLp-I and apoLp-II are found in a 1:1 molar ratio in lipophorin particles. Their common precursor protein is arranged with apoLp-II at the N-terminal end and apoLp-I at the C-terminal end ([Bogerd et al., 2000](#)), and therefore is termed apoLp-II/I. The apoLp-II/I cDNA of several insect species has been isolated and characterized or identified in genome analysis

projects (for review, see [Van der Horst and Rodenburg, 2010a](#)); 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 apolipoprotein B (apoB), microsomal triglyceride-transfer protein (MTP), and vitellogenin (Vg) ([Babin et al., 1999](#)). The LLT domain shared by these proteins comprises a large N-terminal domain of about 1000 amino acids; the LLT domains of apoB, MTP, and Vg contain a large lipid-binding cavity which was proposed to act to store and transfer lipids to the apolipoprotein in a coordinated manner (for reviews, see [Rodenburg and Van der Horst, 2005](#); [Smolenaars et al., 2007a](#)). 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 Vg ([Thompson and Banaszak, 2002](#)), as well as a structural model for nascent human apoB lipoprotein ([Richardson et al., 2005](#)), reveals a similar putative lipid pocket in the insect LLT domain ([Smolenaars et al., 2005](#)). The cleavage of insect apoLp-II/I into apoLp-II and apoLp-I is mediated by an insect furin, acting at a consensus substrate sequence (RQKR) between two residues (720 and 721) of the LLT module ([Smolenaars et al., 2005](#)). Since protein cleavage by furin homologs is performed late in the secretory pathway, mainly in the *trans*-Golgi network, insect lipoprotein biosynthesis was proposed to proceed by initial lipidation of apoLp-II/I to a lipoprotein, while cleavage of apoLp-II/I into apoLp-I and -II would occur at a later stage ([Smolenaars et al., 2005](#)). 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. Indeed, it was shown that 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 with a buoyant density and molecular mass identical to wild type lipophorin ([Smolenaars et al., 2005](#); [Van der Horst and Rodenburg, 2010a](#)); it was therefore proposed that cleavage of apoLp-II/I by insect furin is required neither for biosynthesis nor for secretion of the insect lipoprotein. Although the apparent conservation of apoLp-II/I cleavage in all insects characterized to date reveals the importance of this processing step, at present it is not known if one or the other apoLp possesses additional functions aside from its primary role in stabilizing lipophorin particle integrity, although a role in receptor interactions is implied (see below). The structural role is fulfilled by the capacity of apoLp-I and apoLp-II to interact with lipid and create an interface between the

non-polar core of the particle and the external environment. In this capacity, apoLp-I and apoLp-II function in a manner similar to that proposed for apoB in vertebrate plasma. This is consistent with the finding that the genes encoding these proteins are derived from a common ancestor ([Babin et al., 1999](#)).

The structural resemblance between apoLp-II/I and apoB is not limited to their LLT modules, but extends to the entire polypeptide chains. Prediction of amphipathic clusters in apoB proposed a pentapartite structure of α -helical domains (α) and amphipathic β -strand domains (β) along the apoB polypeptide, organized as N- α_1 - β_1 - α_2 - β_2 - α_3 -C ([Segrest et al., 2001](#)); 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 propose the polypeptide to contain a similar, albeit smaller, (tripartite) structure, organized as N- α_1 - β - α_2 -C; recombinant expression experiments demonstrated the β cluster to accommodate the apoLp-II/I lipid-binding capacity ([Smolenaars et al., 2007b](#)). After cleavage of apoLp-II/I, the β cluster is almost entirely situated in apoLp-I, suggesting that apoLp-I, and not apoLp-II, binds the vast majority of lipids ([Smolenaars et al., 2007b](#)). This finding is consistent with lipophorin dissociation experiments in which >98% of the total lipid in lipophorin remained associated with apoLp-I ([Kawooya et al., 1989](#)). 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 ([Hussain et al., 2001](#); [Shelness and Sellers, 2001](#); [Ledford et al., 2006](#)). From the discovery of an MTP homolog in the fruit fly, *Drosophila melanogaster*, which was able to promote the assembly and secretion of human apoB ([Sellers et al., 2003](#)), insect lipoprotein assembly early in the secretory pathway has been proposed to occur similarly ([Smolenaars et al., 2005](#)). The recovery of MTP homologs in all available insect genomes ([Smolenaars et al., 2007a](#)) 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, since co-expression of the *Drosophila* MTP homolog (dMTP) and recombinant full-length *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 ([Smolenaars et al., 2007b](#); for reviews, see [Van der Horst et al., 2009](#); [Van der Horst and Rodenburg, 2010a](#)). Concomitant with their secretion, dMTP significantly stimulated the lipidation of the apoLp-II/I proteins, since the secreted lipoprotein particles were recovered at a decreased

buoyant density compared to control cells lacking the dMTP gene. Recombinant co-expression of dMTP and a series of C-terminal truncation variants of apoLp-II/I in Sf9 cells revealed that formation of a buoyant lipoprotein particularly requires the amphipathic β cluster (Smolenaars *et al.*, 2007b). Taken together, these data support a unifying concept for lipoprotein biogenesis, and led to the conclusion 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 (Smolenaars *et al.*, 2007b; for reviews, see Van der Horst *et al.*, 2009; Van der Horst and Rodenburg, 2010a).

9.1.2. Lipophorin Subspecies

One of the hallmark features of lipophorin-mediated lipid transport relates to the dynamic nature of the particle. Lipophorin isolated from various life stages is generally of a unique density and lipid composition. For example, lipophorin from *Manduca sexta* fifth instar larvae displays a density of 1.15 g/ml, with a particle diameter in the range of 16 nm. By contrast, lipophorin isolated from adult hemolymph is of lower density and larger diameter. Indeed, in *M. sexta* a broad array of unique lipophorin subspecies has been identified, each with characteristic properties (Prasad *et al.*, 1986). On the basis of this diversity, a nomenclature system has been adopted that distinguishes various lipophorin subspecies based on their density. Since most particles fall within the density limits of 1.21 and 1.07 g/ml, the term “high-density lipophorin” (HDLp) is commonly used. Because many lipophorin subspecies are present at well-defined developmental stages, a suffix may be added to denote this. Hence, HDLp-P and HDLp-A may be used to distinguish HDLp from pupal and adult hemolymphs, respectively.

One of the features of HDLp-A is its ability to associate with a third apolipophorin, apoLp-III. In insect species that use lipid as a fuel for flight (such as *L. migratoria* and *M. sexta*), apoLp-III is present in abundance in adult hemolymph as a lipid-free protein. Whereas a small amount of apoLp-III may be associated with HDLp under resting conditions, flight activity induces association of large amounts of apoLp-III with the lipophorin particle surface (Van der Horst *et al.*, 1979). This process, which is dependent upon the uptake of DAG by the lipophorin particle, leads to the conversion of HDLp into low-density lipophorin (LDLp). LDLp has a larger diameter, a significantly increased DAG content, and a lower density. In studies of this conversion, it has been shown that apoLp-III associates with the surface of the expanding lipophorin particle as a function of DAG enrichment (for reviews, see Soulages and Wells, 1994a; Ryan and Van der Horst, 2000; Van der Horst and Ryan, 2005; Van der Horst and Rodenburg, 2010a, 2010b). Thus, it has

been hypothesized that apoLp-III serves to stabilize the DAG-enriched particle, providing an interface between surface-localized hydrophobic DAG molecules and the external aqueous medium. It is envisioned that continued DAG accumulation by HDLp results in partitioning of DAG between the hydrophobic core of the particle and the surface monolayer (Wang *et al.*, 1995). The presence of DAG in the surface monolayer exerts a destabilizing effect on the particle structure, and, if allowed to persist, would result in deleterious particle fusion and aggregation. By “sensing” the presence of DAG in the lipophorin surface monolayer, apoLp-III is attracted to the particle surface and forms a stable binding interaction. This event is fully reversible, and, upon removal of DAG from the particle, apoLp-III dissociates, leading to regeneration of HDLp. Importantly, it is recognized that lipophorin particles can then bind additional DAG, forming a cycle of transport. It is noteworthy that these concepts about apoLp-III association/dissociation from lipophorin emerged from physiological studies of flight activity in *L. migratoria* conducted in the late 1970s and early 1980s in The Netherlands and England (Mwangi and Goldsworthy, 1977, 1981; Van der Horst *et al.*, 1979, 1981). A cartoon depicting metabolic and biochemical processes related to the induction of flight-related lipophorin conversions and the accompanying increase in neutral lipid transport capacity is presented in **Figure 1**. Elaboration of various aspects of this central scheme will occur in subsequent sections. At this point, however, it should be noted that this generalized mechanism differs fundamentally from metabolic processes in vertebrates, where lipoproteins do not have a function in the transport of energy substrates during exercise (Van der Horst *et al.*, 2002; Van der Horst and Rodenburg, 2010a). That said, it is evident that novel insight into structural and functional aspects of vertebrate lipid transport processes can be gained from the study of insect lipid transport (for reviews, see Rodenburg and Van der Horst, 2005; Van der Horst *et al.*, 2009; Van der Horst and Rodenburg, 2010a).

9.2. Flight-Related Processes

9.2.1. Adipokinetic Hormone

Insect flight involves the mobilization, transport, and utilization of endogenous energy reserves at extremely high rates. In insects that engage in long-distance flight, the demand for fuel, particularly lipids, by the flight muscles can remain elevated for extended periods of time. Adipokinetic hormones (AKHs), synthesized and stored in neuroendocrine cells, play a crucial role in this process, as they integrate flight energy metabolism. Insect AKHs comprise a family of short peptides consisting of 8–11 amino acid residues. Over 40 bioanalogs of this family have been identified in representative species of

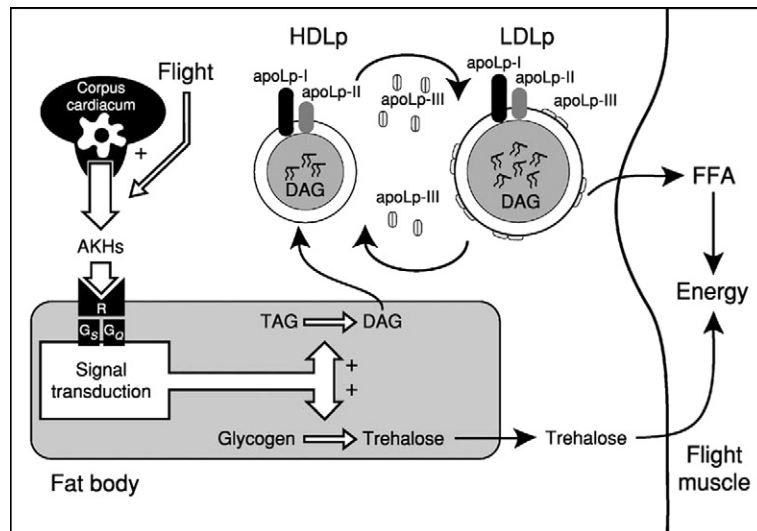


Figure 1 Molecular basis of the lipophorin lipid shuttle. AKH-controlled DAG mobilization from insect fat body during flight activity results in the reversible alternation of lipophorin from a relatively lipid-poor (HDLp) to a lipid-rich (LDLp) state, and apoLp-III from a lipid-free to a lipid-bound state. The reversible conformational change in apoLp-III induced by DAG loading of lipophorin is schematically visualized. AKHs, adipokinetic hormones; R, receptor; G, G protein; HDLp, high-density lipophorin; LDLp, low-density lipophorin; apoLp-I, -II, -III, apolipophorin I, II, III; TAG, triacylglycerol; DAG, diacylglycerol; FFA, free fatty acids.

Based on data from several insect species, particularly *Locusta migratoria* and *Manduca sexta*, reviewed in Ryan and Van der Horst (2000) and Van der Horst *et al.* (2001).

Reprinted with permission from Van der Horst D.J., Ryan R.O., 2005. Lipid transport. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (eds), *Comprehensive Molecular Insect Science*, Vol. 4. Elsevier, Amsterdam, pp. 225–246.

most insect orders; in spite of considerable variation in their structures, they are clearly related (for reviews, see Gäde, 1997, 2009; Van der Horst *et al.*, 2001; Oudejans and Van der Horst, 2003). All AKHs are N-terminally blocked by a pyroglutamate (pGlu) residue, and all but one (Köllisch *et al.*, 2000) are C-terminally amidated. Initiation of flight activity induces the release of AKHs from the intrinsic AKH-producing cells (adipokinetic cells) in the glandular lobes of the corpus cardiacum, a neuroendocrine gland located caudal to the insect brain and physiologically equivalent to the pituitary of mammals. The fat body plays a fundamental role in lipid storage, as well as in the process of lipolysis controlled by the AKHs. Binding of these hormones to their G protein-coupled receptors at the fat body target cells triggers a number of coordinated signal transduction processes that ultimately result in the mobilization of carbohydrate and lipid reserves as fuels for flight activity (see **Figure 1**). Energy-yielding metabolites are transported via the hemolymph to the contracting flight muscles. Carbohydrate (trehalose) in the circulation provides energy for the initial period of flight, and is replenished from glycogen reserves. However, similar to sustained activity in many other animal species, flight activity of insects covering vast distances non-stop is powered principally by mobilization of endogenous reserves of triacylglycerol (TAG), the most concentrated form of energy available to biological tissues. As a result of TAG mobilization, the concentration of *sn*-1,2-DAG in the

hemolymph increases progressively, and gradually constitutes the principal fuel for flight. The mechanism for hormonal activation of glycogen phosphorylase, the enzyme determining the rate of glycogen breakdown and trehalose biosynthesis, has been well established. In contrast, the mechanism by which the pivotal enzyme TAG lipase catalyzes AKH-controlled production of the DAG on which long-distance flight is dependent is less well understood.

For a considerable part, the success of insects in long-distance flights is attributable to their system of neuropeptide AKHs integrating flight energy metabolism, involving the transfer of energy substrates, particularly lipids, to the flight muscles, as discussed above. Therefore, in the following sections, recent advances in the strategy of adipokinetic cells in hormone storage and release will be discussed, along with the effects of the AKHs on lipid mobilization.

9.2.2. Strategy of the Adipokinetic Cells

In view of their involvement in the regulation and integration of extremely intense metabolic processes, the AKH-producing cells (adipokinetic cells) of the corpus cardiacum constitute an appropriate model system for studying neuropeptide biosynthesis and processing, as well as the coherence between biosynthesis, storage, and release of these neurohormones (for reviews, see Ryan and Van der Horst, 2000; Van der Horst *et al.*, 2001; Diederer *et al.*,

the corpus cardiacum (Bogerd *et al.*, 1995). Following their synthesis in the rough endoplasmic reticulum in the cell bodies, the AKH prohormones are transported to the Golgi complex and packaged into secretory granules at the trans-Golgi network, whereas proteolytic processing of the prohormones to bioactive AKHs is presumed to take place in the secretory granules (for reviews, see Van der Horst *et al.*, 2001; Diederer *et al.*, 2002; Oudejans and Van der Horst, 2003). All three AKHs were shown to be co-localized in the same secretory granules, and are released simultaneously during flight (Harthoorn *et al.*, 1999). Since the membranes of exocytosed secretory granules fuse with the plasma membrane, the total content of the granules is released into the hemolymph. Consequently, in addition to bioactive AKHs the APRPs, and possibly other products encoded by the AKH genes, are also released during flight activity and might execute specific functions. Intriguingly, aligning of all known AKH pre-prohormone genes showed the APRP region to be better conserved in evolution (nematodes, insects, crustaceans) than that of AKH, suggesting an important biological role (De Loof *et al.*, 2009). However, although APRPs have been tested extensively in a large variety of bioassays, APRP function has not yet been uncovered (Hatle and Spring, 1999; De Loof *et al.*, 2009). A recent peptidomic survey of the locust neuroendocrine system confirmed the corpora cardiaca of both *L. migratoria* and *S. gregaria* to contain the two processing products of the APRPs, AKH-JP I and II (Clynen and Schoofs, 2009). However, whether the AKH-JPs are released is not yet clear (Baggerman *et al.*, 2002; Huybrechts *et al.*, 2002); in bioassays, AKH-JP I and II appeared neither to stimulate lipid release from the fat body nor to activate fat body glycogen phosphorylase – both key functions of the AKHs (Baggerman *et al.*, 2002).

The secretory activity of the adipokinetic cells, which has been investigated *in vitro* primarily for AKH-I, is subject to many regulatory substances, including neurogenic locustatachykinins and humoral crustacean cardioactive peptide (CCAP) as initiating factors, trehalose as an inhibitor, and several positive and negative modulators (for reviews, see Van der Horst *et al.*, 1999; Vullings *et al.*, 1999; Van der Horst, 2003; Van der Horst and Ryan, 2005). Data on the release of AKH from the corpora cardiaca *in vitro* show that regulatory substances (including CCAP) affect the release of all three AKHs in proportion to their concentration in the corpus cardiacum (Harthoorn *et al.*, 2001). However, the only natural stimulus for the release of the AKHs is flight activity, and the relative contributions of all known substances effective in the process of release of these neurohormones remain to be established *in vivo*.

The amount of AKHs released during flight represents only a few percent of the huge stores harbored in the adipokinetic cells. On the other hand, only a limited part

of these AKH stores appear to be actually releasable. In studies in which young secretory granules were specifically labeled, these newly formed secretory granules were preferentially released (last in, first out) (for reviews, see Van der Horst *et al.*, 2001; Diederer *et al.*, 2002; Oudejans and Van der Horst, 2003). Following the biosynthesis of new AKH prohormones, their packaging into secretory granules and their processing to bioactive AKHs, which takes less than 1 hour, granules containing newly synthesized AKHs appeared to be available for release during a restricted period of approximately 8 hours before they are supposed to enter a pool of older secretory granules that appear to be unable to release their content upon secretory stimulation. This indicates that only a relatively small readily releasable pool of new secretory granules exists. Therefore, an important question is whether the secretory output of AKHs during flight would induce a stimulation of the rate of AKH biosynthesis. The mRNA levels of all three AKH pre-prohormones, however, did not appear to be affected by flight activity, while the rate of synthesis of AKH prohormones and AKHs was not affected either (Harthoorn *et al.*, 2001). Apparently, a coupling between release and biosynthesis of AKHs is absent. Inhibition of AKH biosynthesis *in vitro* by Brefeldin A, a specific blocker of the transport of newly synthesized secretory proteins from the endoplasmic reticulum to the Golgi complex, resulted in a considerable decrease in the release of AKHs induced by CCAP, highlighting once more that the regulated secretion of AKHs is completely dependent on the existence of a readily releasable pool of newly formed secretion granules (Harthoorn *et al.*, 2002). Therefore, we conclude that the strategy of the adipokinetic cells to cope with variations in secretory output of AKHs apparently is to rely on the continuous biosynthesis of AKHs, which produces a readily releasable pool that is sufficiently large and constantly replenished.

An important question remaining unanswered, however, is: what might be the rationale for the storage of such large quantities of hormones that are not accessible for secretory release?

9.2.3. Effect of Adipokinetic Hormones on Lipid Mobilization

9.2.3.1. Adipokinetic hormone receptors Binding of the AKHs to their plasma membrane receptor(s) at the fat body cells is the primary step to the induction of signal transduction events that ultimately lead to the activation of target key enzymes and the mobilization of lipids as a fuel for flight. Although the AKHs constitute extensively studied neurohormones, and their actions have been shown to occur via G protein-coupled receptors (GPCRs) (for reviews, see Van Marrewijk and Van der Horst, 1998; Vroemen *et al.*, 1998), the general properties of which are remarkably well conserved during evolution (for review,

see Vanden Broeck, 2001), insect AKH receptors have been identified only recently. However, in *L. migratoria*, which produces three different AKHs and may be envisaged to have (three) different AKH receptors, the receptor(s) are as yet unidentified. The first insect AKH receptors characterized at the molecular level, namely those of *D. melanogaster* and the silkworm *Bombyx mori* (Staubli *et al.*, 2002), were shown to be GPCRs structurally related to mammalian gonadotropin-releasing hormone (GnRH) receptors. No other AKH receptors were isolated until 2006, when an AKH receptor from the American cockroach *Periplaneta americana* was identified (Hansen *et al.*, 2006); the production of two intrinsic AKHs (*Periplaneta* AKH-I and -II) may suggest the presence of a second AKH receptor. A similar cockroach AKH receptor was also identified by Wicher *et al.* (2006); there are, however, differences in one amino acid residue, as well as in the response towards the two *Periplaneta* AKHs (cf. Hansen *et al.*, 2006). In the malaria mosquito *Anopheles gambiae* an AKH receptor has been characterized in addition to an orphan receptor, the close relationship of which to the insect AKH receptors identified thus far suggesting that this receptor is an AKH receptor as well (Belmont *et al.*, 2006). For the yellow fever mosquito *Aedes aegypti*, two splice variants of the AKH receptor gene, differing at their C-terminal ends, were reported (Kaufmann *et al.*, 2009); it was postulated that both receptor variants could selectively bind the two AKH peptides found in *Ae. aegypti*. The signaling of the AKH receptor of *B. mori* and its peptide ligands (*Bombyx* AKH1, -2 and -3) have been recently characterized at the molecular and functional levels (Zhu *et al.*, 2009). Recent cloning studies demonstrating that the GnRH receptor in the nematode *Caenorhabditis elegans* is stimulated by both a *C. elegans* AKH-GnRH-like peptide and *Drosophila* AKH suggest that the AKH-GnRH signaling system arose very early in metazoan evolution (Lindemans *et al.*, 2009).

9.2.3.2. Signal transduction of adipokinetic hormones The signal transduction mechanism of the three locust AKHs has been studied extensively, and involves stimulation of cAMP production, which is dependent on the presence of extracellular Ca^{2+} . Additionally, the AKHs enhance the production of inositol phosphates, including inositol 1,4,5-trisphosphate (IP_3), which may mediate the mobilization of Ca^{2+} from intracellular stores. This depletion of Ca^{2+} from intracellular stores stimulates the influx of extracellular Ca^{2+} , indicative of the operation of a capacitative (store-operated) calcium entry mechanism. The interactions between the AKH signaling pathways ultimately result in mobilization of stored reserves as fuel for flight (for reviews, see Van Marrewijk and Van der Horst, 1998; Vroemen *et al.*, 1998; Ryan and Van der Horst, 2000; Van der Horst *et al.*, 2001; Van Marrewijk, 2003;

Van der Horst and Ryan, 2005). The concentration of DAG in the hemolymph increases progressively at the expense of stored TAG reserves in the fat body, which implies hormonal activation of the key enzyme, fat body TAG lipase. In a bioassay, all three AKHs are able to stimulate lipid mobilization, although their relative potencies are different. This recalls the concept of a hormonally redundant system involving multiple regulatory molecules with overlapping actions (for reviews, see Goldsworthy *et al.*, 1997; Vroemen *et al.*, 1998). Results obtained with combinations of two or three AKHs, which are likely to occur together in locust hemolymph under physiological conditions *in vivo*, revealed that the maximal responses for the lipid-mobilizing effects were much lower than the theoretically calculated responses based on dose-response curves for the individual hormones. In the lower (probably physiological) range, however, combinations of the AKHs were more effective than the theoretical values calculated from the responses elicited by the individual hormones (for review, see Van Marrewijk and Van der Horst, 1998).

The mechanism by which TAG lipase catalyzes AKH-controlled production of the DAG on which long-distance flight depends is only partly understood, mainly due to technical problems in isolating or activating the lipase. In vertebrates, hormone-sensitive lipase (HSL) and adipose TAG lipase (ATGL) are the key enzymes in the control of lipid mobilization from TAG stores in adipose tissue, and although contrary to insects, free fatty acids (FFA) are released into the blood for uptake and oxidation in muscle, there is a clear functional similarity between vertebrate adipose tissue HSL and ATGL on one hand, and insect fat body TAG lipase on the other (for reviews, see Ryan and Van der Horst, 2000; Van der Horst *et al.*, 2001; Van der Horst and Oudejans, 2003; Van der Horst and Ryan, 2005; Van der Horst and Rodenburg, 2011).

9.2.3.3. Activation of lipolysis in insect fat body

Regarding the process of lipid mobilization, recent data reveal insects to be very similar to mammals (for review, see Van der Horst and Rodenburg, 2011). For example, packaging lipid in intracellular lipid droplets and the mechanisms guiding mobilization of stored lipids are conserved between insects and mammals (Kulkarni and Perrimon, 2005; Martin and Parton, 2006; Brasaemle, 2007; Murphy *et al.*, 2009; Walther and Farese, 2009). Lipid droplets, which 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 (Martin and Parton, 2006; Brasaemle, 2007; Londos *et al.*, 1999; Miura *et al.*, 2002; Grönke *et al.*, 2003; Gross *et al.*, 2006; Arrese

et al., 2008a; Bickel *et al.*, 2009). Similar to mammalian adipocytes, the TAG accumulated in cytosolic lipid storage droplets of insect fat body cells provides the major long-term energy reserve of the organism, for which *Drosophila* recently emerged as a powerful system, to a large extent due to its well-developed genetics (Grönke *et al.*, 2003, 2005, 2007). Generation of loss-of-function mutants evidenced that simultaneous loss of the AKH receptor – and thus the signaling pathway for lipid mobilization, which is 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 Zechner *et al.*, 2009; Zimmermann *et al.*, 2009), caused extreme obesity and blocked acute storage fat mobilization in flies (Grönke *et al.*, 2005). Intriguingly, excessive fat storage in flies lacking *bmm* function reduced the median lifespan by only 10%, and acute TAG mobilization is impaired but not abolished in *bmm* mutants (Grönke *et al.*, 2005), suggesting that, as in mammals, mobilization of TAG in *Drosophila* is controlled by more than one TAG lipase (Grönke *et al.*, 2005; Kulkarni and Perrimon, 2005). In addition, *Akhr*^{null} mutant flies appeared to be markedly starvation resistant, suggesting that their higher TAG content confers a survival benefit. Consequently, lipolytic mechanisms independent of the AKH pathway must exist in *Drosophila*, enabling *Akhr* mutants to mobilize TAG reserves, although they retain considerable energy stores as well when challenged with starvation (Bharucha *et al.*, 2008).

In addition to a similar TAG lipase, two lipid storage droplet (Lsd) proteins (Lsd1 and -2) belonging to the PAT protein family were identified in insects (Miura *et al.*, 2002; Grönke *et al.*, 2003; Teixeira *et al.*, 2003; Arrese *et al.*, 2008a, 2008b, 2008c; Bickel *et al.*, 2009), suggesting that the overall processes of lipid storage and mobilization in insects may function similar to those in vertebrates. To further demonstrate the functional similarity between mammalian and *Drosophila* TAG lipases, the lipid droplet surface-localized *bmm* was shown to antagonize a perilipin-related lipid droplet surface protein (Lsd2) (Grönke *et al.*, 2005) that functions as an evolutionarily conserved modulator of lipolysis (Grönke *et al.*, 2003). 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 (Beller *et al.*, 2008; Guo *et al.*, 2008).

In contrast to the mechanism of lipid mobilization in *Drosophila*, however, the main TAG lipase in the fat body of *M. sexta* was identified as the homolog of *D. melanogaster* GC8552. This protein, which was named triglyceride

lipase (TGL), is conserved among insects and also displays significant phospholipase A₁ activity (Arrese *et al.*, 2006; for review, see Arrese and Soulages, 2010). TGL shares significant sequence similarity with vertebrate phospholipases, but shows no homology to the main vertebrate adipose TAG lipase, ATGL.

In vertebrates, mobilization of TAG stores in adipose tissue is facilitated by the phosphorylation of several key proteins, including HSL and lipid droplet PAT proteins such as perilipin. The principal substrate for HSL is DAG, which is provided by the upstream ATGL (for reviews, see Watt and Steinberg, 2008; Zechner *et al.*, 2009). In insect fat body, AKH induces increased cAMP levels, which in turn may lead to increased PKA activity (reviewed in Van der Horst *et al.*, 2001; Gäde and Auerswald, 2003). Although the resulting PKA-mediated protein phosphorylation is considered a major factor in the activation of lipolysis (Arrese and Wells, 1994; for reviews, see Van der Horst *et al.*, 2001; Van der Horst and Ryan, 2005), *in vitro* studies showed the phosphorylation level of TGL in *M. sexta* fat body to be unchanged by AKH (Patel *et al.*, 2006). Instead, activation of lipid droplets by phosphorylation of Lsd1 was identified to mediate AKH-induced lipolysis (Arrese *et al.*, 2008b; for review, see Arrese and Soulages, 2010). Also in mammalian adipocytes, the PKA-mediated phosphorylation of perilipin at the surface of the lipid droplets is directly involved in the activation of lipolysis (Londos *et al.*, 2005) as mentioned above, and the phosphorylation of perilipin mediates the translocation of the likewise phosphorylated HSL to the surface of perilipin-coated lipid droplets (Szalryd *et al.*, 2003; Wang *et al.*, 2009; for reviews, see Martin and Parton, 2006; Brasaemle, 2007; Walther and Farese, 2009; Bickel *et al.*, 2009).

In spite of the similarities in overall processes of lipid storage and mobilization in insects and mammals, however, both the transport form and the transport vehicle of the lipid substrate mobilized from the TAG stored in lipid droplets are different. During prolonged exercise of mammals, long-chain FFAs are mobilized from adipose tissue TAG stores and transported in the circulation bound to the abundant serum protein, albumin, for uptake and oxidation in the working muscles. However, in the locust and other insect species recruiting fat body TAG depots to power their flight muscles during migratory flight, the TAG-derived lipid is released as DAG into the hemolymph, as indicated above, and transported to the flight muscles in LDLp particles as discussed earlier (see section 9.1.2. and Figure 1).

It is interesting to note that in mammalian adipocytes ATGL is the predominant TAG lipase, whereas HSL and monoacylglycerol (MAG) lipase are the major lipases responsible for the hydrolysis of DAG and MAG, respectively. The net result of the consecutive actions of these three enzymes is the hydrolysis of a fatty acyl side chain

from TAG, DAG, and MAG, and the release of the liberated FFAs and glycerol from the cells. The efflux of DAG from insect fat body cells following bmm action would suggest a lack of (the net activity of) the other downstream lipases found in adipocytes (for review, see Van der Horst and Rodenburg, 2010a).

L. migratoria DAG were shown to be stereospecific, revealing the *sn*-1,2 configuration, thus demonstrating stereospecific conversions to be involved in their production from TAG (for reviews, see Beenackers *et al.*, 1985; Van der Horst, 1990). Data on the (nonapeptide) AKH-stimulated synthesis of *sn*-1,2-DAG in the fat body of *M. sexta* support the hypothesis of stereospecific hydrolysis of fat body TAG stores (Arrese and Wells, 1997; for reviews, see Gibbons *et al.*, 2000; Arrese *et al.*, 2001).

9.3. Apolipophorin III

9.3.1. Lipid-Free Helix Bundle Structure

ApoLp-III was discovered in the late 1970s and early 1980s by research groups in Europe and North America (for reviews, see Blacklock and Ryan, 1994; Ryan and Van der Horst, 2000; Weers and Ryan, 2006). ApoLp-III was first isolated from hemolymph of *L. migratoria* (Van der Horst *et al.*, 1984) and the tobacco hawkmoth, *M. sexta* (Kawooya *et al.*, 1984). *M. sexta* apoLp-III is a 166-aa protein that lacks tryptophan and cysteine (Cole *et al.*, 1987). However, the well-characterized apoLp-III from *L. migratoria* is 164 residues long, and lacks cysteine, methionine, and tyrosine (Kanost *et al.*, 1988; Smith *et al.*, 1994). *M. sexta* apoLp-III is non-glycosylated, while *L. migratoria* apoLp-III contains two complex carbohydrate chains (Hård *et al.*, 1993). The sequences of numerous apoLp-III have been reported, and important aspects are summarized in Table 1. Sequence analysis predicts that all apoLp-IIIs are composed of a predominantly amphipathic α -helix secondary structure, consistent with far ultraviolet circular dichroism (CD) studies (Ryan *et al.*, 1993; Weers *et al.*, 1998). An important breakthrough in our understanding of the structure of apoLp-III occurred with the determination of the X-ray crystal structure of *L. migratoria* apoLp-III (Breiter *et al.*, 1991). These authors showed that apoLp-III exists as a globular, up-and-down amphipathic α -helix bundle in the absence of lipid. The molecule is composed of five discrete α -helix segments that orient their hydrophobic faces toward the center of the bundle. Using a convenient method for bacterial overexpression, recombinant *M. sexta* apoLp-III was enriched with stable isotopes (Ryan *et al.*, 1995; Wang *et al.*, 1997a). Application of heteronuclear multidimensional nuclear magnetic resonance (NMR) techniques to isotopically enriched *M. sexta* apoLp-III yielded a complete assignment of this protein (Wang *et al.*, 1997b). Structure calculations revealed a five-helix bundle molecular architecture,

Table 1 Insect Species from which ApoLp-III has been Identified and Characterized*

ApoLp-III	Number of Residues, Mass	Glycosylation
Orthoptera		
<i>Locusta migratoria</i>	162–164, 20 kDa	14%
<i>Gastrimargus africanus</i>	20 kDa	5.3%
<i>Acheta domesticus</i>	161, 17.2 kDa	–
<i>Barytettix psolus</i>	20 kDa	5%
<i>Melanoplus differentialis</i>	20 kDa	5%
Lepidoptera		
<i>Manduca sexta</i>	166, 18.4 kDa	–
<i>Bombyx mori</i>	164, 18.3 kDa	–
<i>Bombyx mandarina</i>	164	–
<i>Galleria mellonella</i>	18.1 kDa	–
<i>Spodoptera litura</i>	166, 18.3 kDa	–
<i>Acherontia atropos</i>	20 kDa	–
<i>Diatraea grandiosella</i>	17 kDa	–
<i>Heliothis virescens</i>	18.0 kDa	–
<i>Hyphantria cunea</i>	165, 18.3 kDa	–
<i>Hyalophora cecropia</i>	18 kDa	–
Coleoptera		
<i>Derobrachus geminatus</i>	18 kDa	+ (% NA)
Hemiptera		
<i>Lethocerus medius</i>	19 kDa	–
<i>Thasus acutangulus</i>	20 kDa	–

*See Weers and Ryan (2006) for individual references.

representing the first full-length apolipoprotein whose high resolution solution structure has been determined in the absence of detergent (Wang *et al.*, 2002) (Figure 3). In keeping with the X-ray structure of *L. migratoria* apoLp-III, this structure also reveals an up-and-down bundle of five amphipathic α -helices. Interestingly, however, Wang and coworkers identified a distinct short segment of α -helix that connects helices 3 and 4 in the bundle (termed helix 3'). This sequence segment (P95DVEKE100) aligns perpendicular to the long axis of the bundle, and, as discussed below, has been shown to play a role in the initiation of apoLp-III lipid interaction. More recently, Fan *et al.* (2001, 2003) employed multidimensional NMR techniques to obtain a complete assignment and solution structure determination for *L. migratoria* apoLp-III. This work is significant in that it permits direct comparison between the X-ray crystal structure and the NMR structure. Interestingly, Fan and colleagues provide previously unreported structural evidence for a solvent exposed short helix that is positioned perpendicular to the long axis of the helix bundle. These authors propose that this short helix can serve as a recognition helix for initiation of apoLp-III lipid interaction, leading to conformational opening of the helix bundle in a manner that is different from the original proposal on the basis of the X-ray crystal structure of this protein. Another important aspect of this

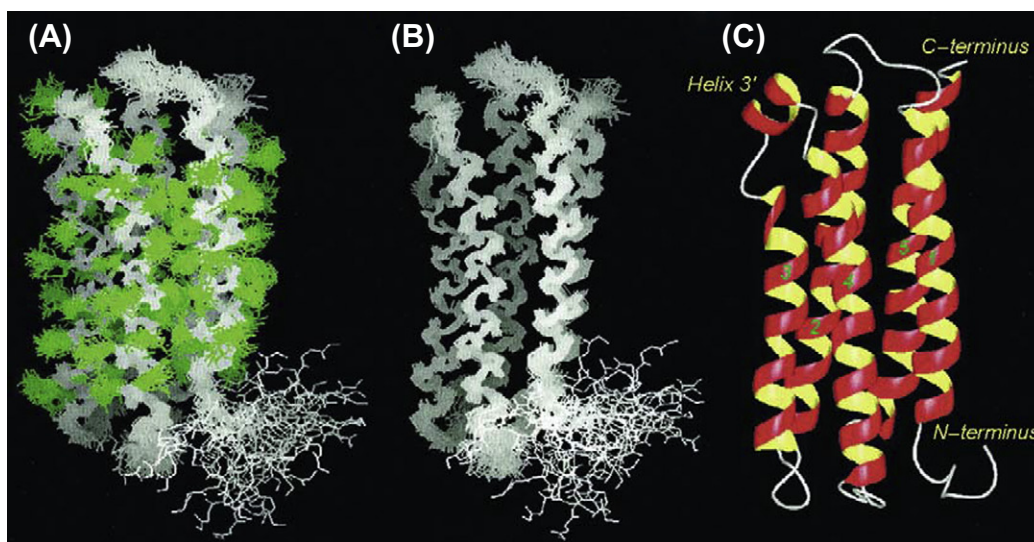


Figure 3 Nuclear magnetic resonance (NMR) visualizations of structure of lipid-free *Manduca sexta* apoLp-III. (A, B) Superposition of 40 NMR-derived structures of apoLp-III, with backbone atoms displayed in white and side chain heavy atoms displayed in green. (C) Ribbon representation of an energy-minimized, average structure of apoLp-III (PDB code 1EQ1). Reproduced with permission from Wang, J., Sykes, B.D., Ryan, R.O., 2002. Structural basis for the conformational adaptability of apolipoprotein III, a helix bundle exchangeable apolipoprotein. *Proc. Natl. Acad. Sci. USA* 99, 1188–1193; ©National Academy of Sciences of the United States of America.

work is that buried interhelical H-bonds provide a driving force for the helix bundle recovery of apoLp-III from the lipid-bound open conformation.

9.3.2. Lipid-Induced Conformation Change

The up-and-down antiparallel organization of helical segments in apoLp-III allows for a simple opening of the bundle about putative “hinge” loops that connect the helices as originally proposed by Breiter *et al.* (1991). The model suggests that apoLp-III initiates contact with lipid surfaces via one end of the helix bundle. Conformational opening could then occur with retention of helix boundaries present in the bundle configuration. Such an event would result in substitution of helix–helix interactions in the bundle conformation for helix–lipid interactions. Current evidence suggests that this conformational transition is triggered by availability of a suitable lipid surface, and is reversible (Singh *et al.*, 1992; Liu *et al.*, 1993; Soulages and Wells, 1994b; Soulages *et al.*, 1995, 1996). Thus, it is conceivable that helices 3 and 4 move away from helices 1, 2, and 5 in concert as the bundle opens about the loop segments connecting helices 2 and 3, and helices 4 and 5 (Breiter *et al.*, 1991; Narayanaswami *et al.*, 1996a).

A well-known property of amphipathic exchangeable apolipoproteins in general is an ability to disrupt phospholipid bilayer vesicles and transform them into apolipoprotein–phospholipid disc complexes (Pownall *et al.*, 1978). This property provides a useful method to investigate aspects of the proposed lipid-induced helix bundle molecular switch process. The disc-shaped complexes

formed between apoLp-III and dimyristoylphosphatidylcholine (DMPC) are of uniform size and composition, permitting detailed analysis of their structural organization (Wientzek *et al.*, 1994). Attenuated total reflectance Fourier transformed infrared spectroscopy has been employed to characterize helix orientation in apoLp-III–DMPC disc complexes (Raussens *et al.*, 1995, 1996). This analysis, and more recent studies (Soulages and Arrese, 2001), reveal that apoLp-III helical segments interact with phospholipid fatty acyl chains around the perimeter of the disc complex.

Several independent studies have provided convincing evidence that apoLp-III undergoes a significant conformational change upon association with lipid. Kawooya *et al.* (1986) used a monolayer balance to investigate apoLp-III behavior at the air–water interface, while Narayanaswami *et al.* (1996b) studied the unique fluorescence properties of the lone tyrosine in *M. sexta* apoLp-III. Near-ultraviolet CD analysis of *L. migratoria* apoLp-III indicates that helix realignment and reorientation occurs upon interaction with phospholipid vesicles (Weers *et al.*, 1994). Sahoo *et al.* (2000) used pyrene excimer fluorescence spectroscopy to investigate lipid-binding induced realignment of helix 2 and helix 3 in *M. sexta* apoLp-III. In this study, cysteine residues were introduced into the protein by site-directed mutagenesis (N40C and L90C). These sites were selected for introduction of cysteine residues based on the fact that they reside in close proximity in the helix bundle conformation. Covalent modification of the cysteine thiol groups with pyrene maleimide yielded a double pyrene labeled apoLp-III. In the absence of lipid, pyrene labeled

apoLp-III adopts a helix bundle conformation. Fluorescence spectroscopy experiments revealed normal pyrene emission at 375 and 395 nm (excitation 345 nm) as well as excimer (excited state dimer) fluorescence at longer wavelengths (460 nm). Control experiments verified that the excimer peak arose from intramolecular pyrene–pyrene interactions in the labeled protein, and was not due to intermolecular interactions. Because it is known that excimer fluorescence is manifest only when pyrene moieties are within 10 Å of one another, this property was used to assess the effect of lipid binding. The observation that excimer fluorescence was greatly reduced when apoLp-III was complexed with DMPC was taken as evidence for a conformational change in the protein upon lipid binding that results in relocation of helix 2 away from helix 3.

In fluorescence studies of apoLp-III, carried out by Soulages and Arrese (2000a, 2000b), site-directed mutagenesis was used to create various mutant apoLp-IIIs with a single tryptophen residue in each of the five helical segments of the protein. Data obtained in this study suggest that apoLp-III undergoes a conformational change that brings helices 1, 4, and 5 into contact with the lipid surface, while others (helices 2 and 3) appear to behave differently. In other studies, Soulages *et al.* (2001) used disulfide bond engineering to show that conformational flexibility of helices 1 and 5 of *L. migratoria* apoLp-III plays an important role in the lipid-binding process. Dettloff *et al.* (2001a) reported that a C-terminal truncated apoLp-III from the wax moth *Galleria mellonella*, comprising the first three helical segments of the protein, retains structural integrity and an ability to interact with lipid surfaces. More recently, Dettloff *et al.* (2002)

expanded this work to encompass two additional three-helix mutants derived from *G. mellonella* apoLp-III, a C-terminal fragment comprising helices 3–5, and a core fragment comprising helices 2–4. All three truncation mutants retained their ability to solubilize bilayer vesicles of DMPC – an event that led to large increases in their α -helix content. The N-terminal and core fragment, but not the C-terminal fragment, were able to interact with phospholipase C modified human low-density lipoprotein, thereby preventing its aggregation. This result suggests that impairment of the lipid interaction properties of the C-terminal fragment has occurred as a result of removal of N-terminal helix segments. Taken together, it appears that the minimal essential elements required for apoLp-III lipid-binding function are less than the intact five-helix bundle. Recent experiments have provided evidence that opening of the helix bundle is even more dramatic than originally postulated. It is now proposed that the protein adopts a fully extended belt-like conformation (Garda *et al.*, 2002; Sahoo *et al.*, 2002) (Figure 4). Garda *et al.* (2002) employed fluorescence resonance energy transfer methods, while Sahoo *et al.* (2002) used pyrene excimer fluorescence to probe aspects of helix repositioning upon interaction with DMPC. In both of these studies, knowledge of the three-dimensional structure of the apoLp-III in the absence of lipid (i.e., the helix bundle conformation) allowed for structure-guided site-directed mutagenesis to introduce strategically placed cysteine residues to which fluorescent reporter groups could be covalently attached. Subsequent characterization studies yielded a unifying model of apoLp-III conformation on disc complexes wherein the resulting structure resembles

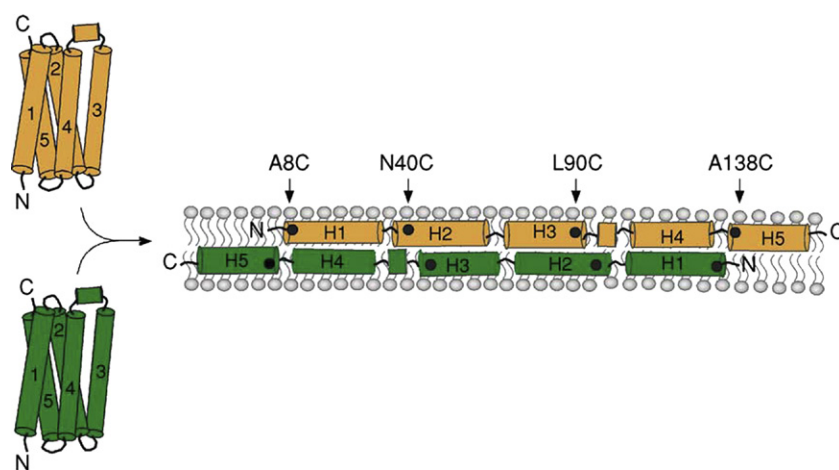


Figure 4 Model of apoLp-III bound to phospholipid discoidal complexes. ApoLp-III complexes with phospholipids on a discoidal particle adopting an extended α -helical conformation. Lipid-triggered association involves extension of H1 away from H5, helix bundle opening and repositioning of H2 and H3. The positions of cysteine substitution mutations employed in this and previous analyses are indicated: A8C, N40C, L90C, and A138C; H1, H2, H3, and H5. Apolp-III adopts an extended helical conformation around the periphery of discoidal phospholipid bilayer complexes, with neighboring molecules aligned antiparallel with respect to each other, and shifted by one helix.

Reprinted with permission from Sahoo, D., Weers, P.M.M., Ryan, R.O., Narayanaswami, V., 2002. Lipid-triggered conformational switch of apolipoprotein III helix bundle to an extended helix organization. *J. Mol. Biol.* 321, 201–214; ©Elsevier.)

concepts and models that describe the organization of human apolipoprotein A-I on nascent high-density lipoproteins (Klon *et al.*, 2002).

Recently, Vasquez *et al.* (2009) employed *L. migratoria* apoLp-III as a model to study apolipoprotein lipid-binding interactions. To investigate the role of positive charges on lipid binding, lysine residues in apoLp-III were acetylated. Modified apoLp-III possessed a reduced amount of α -helix, and a slight increase in protein stability. While the ability to solubilize DMPC vesicles was unchanged, the rate of anionic dimyristoylphosphatidylglycerol (DMPG) vesicle solubilization was reduced two-fold. These results indicate that the eight lysine residues in *L. migratoria* apoLp-III are not required for the protein's ability to bind zwitterionic phospholipids, but are required for optimal binding to anionic lipid surfaces, likely through an electrostatic effect, and this may provide a means of modulating apoLp-III interaction with complex membrane environments *in vivo*. Chiu *et al.* (2009) employed differential scanning calorimetry to measure the binding interaction of *L. migratoria* apoLp-III with liposomes composed of mixtures of lipids. Association of apoLp-III with multilamellar liposomes occurred over a temperature range around the liquid crystalline phase transition of the lipid in question. Thus, surface defects arising from non-ideal packing at the lipid phase transition temperature influence apoLp-III binding properties.

9.3.3. Initiation of ApoLp-III Lipid Binding

Analysis of the structure of *L. migratoria* apoLp-III indicates the presence of solvent-exposed leucine residues at one end of the protein (Breiter *et al.*, 1991). These authors proposed that this region of the molecule functions as a "hydrophobic sensor" that recognizes potential lipid surface binding sites. Surface plasmon resonance spectroscopy studies revealed that small amounts of DAG induce binding of apoLp-III to a phospholipid bilayer with its long molecular axis normal to the lipid surface (Soulages *et al.*, 1995). This interaction is proposed to be the first step in formation of a stable binding interaction. Site-directed mutagenesis was performed to determine whether alteration in the hydrophobicity of the putative sensor region of *L. migratoria* apoLp-III affects its ability to initiate contact with lipid surfaces (Weers *et al.*, 1999). In this study three partially exposed leucine residues, located at the end of the protein containing the loop segments that connect helices 1 and 2 and helices 3 and 4, were mutated to arginine. Three single arginine-to-leucine substitution mutants and a triple mutant were expressed in *Escherichia coli*, and characterized in terms of their structural and stability properties. The effect of these mutations on phospholipid bilayer vesicle transformation into disc complexes versus lipoprotein binding suggests that the former binding interaction has an electrostatic

component. Taken together, the data support the view that the end of the molecule bearing Leu 32, 34, and 95 is responsible for initiating contact with potential lipid surface binding sites.

The solution structure of *M. sexta* apoLp-III revealed the presence of helix 3' at one end of the protein globule (Wang *et al.*, 1997b, 2002). One possibility is that helix 3' reorientation facilitates contact with a lipid surface by exposing the hydrophobic interior of the helix bundle. The lipid surface could then trigger a molecular switch to induce conformational opening of the helix bundle and formation of a stable binding interaction. To investigate this, protein engineering was employed to remove helix 3' and replace it with a sequence that has a high probability of forming a β -turn (Narayanaswami *et al.*, 1999). Characterization of the lipid-binding properties of this "helix-to-turn" mutant apoLp-III revealed defective lipid-binding properties. In more refined site-directed mutagenesis studies, it was determined that Val 97, located in the center of helix 3', is a critical residue for initiation of apoLp-III lipid binding. As described above, a similar short helix was identified in *L. migratoria* apoLp-III based on its solution structure (Fan *et al.*, 2003). This helix, however, is present at the opposite end of the apoLp-III helix bundle, suggesting that, if it is a recognition helix, bundle opening is different from that proposed for *M. sexta* apoLp-III by Narayanaswami *et al.* (1999) and Wang *et al.* (2002).

The role of the conformational flexibility of helices and loops in *L. migratoria* apoLp-III in lipid-binding activity was investigated by disulfide bond engineering experiments (Chetty *et al.*, 2003). The ability of helix-tethered apoLp-III mutants to interact with phospholipid vesicles, mixed micelles, and spherical lipoprotein particles was studied. The authors determined that: (1) opening of the helix bundle does not require the separation of loops 2 and 4; (2) α -helices 3 and/or 4 are involved in the insertion of apoLp-III in both phospholipid bilayers and monolayers; and (3) interaction of helices 1 and/or 5 with the lipid surface is required for the formation of stable lipoprotein complexes. In *L. migratoria* apoLp-III, most hydrophobic residues are buried in the protein interior. However, it was postulated that the presence of polar residues in the hydrophobic protein interior may contribute to protein instability and lipid-binding induced conformational opening (Weers *et al.*, 2005). To test this, Thr-31 was changed to alanine by site-directed mutagenesis. Lipid-binding studies using phospholipid vesicles showed that Thr31Ala apoLp-III was able to transform phospholipid vesicles into discoidal particles, but at a three-fold reduced rate compared to wild type apoLp-III. In contrast, less stable apoLp-III mutants displayed an increased ability to transform phospholipid vesicles. Thus, an inverse correlation exists between protein stability and phospholipid vesicle transformation activity. Furthermore, these data

suggest that Thr-31 is a key determinant of apoLp-III lipid-binding activity.

Studies of the effect of the glycosyl moieties of *L. migratoria* apoLp-III on its lipid-binding properties have also been investigated. [Soulages et al. \(1998\)](#) showed that recombinant apoLp-III, which lacks covalently bound carbohydrate, displayed a much stronger interaction with phospholipid vesicles than natural insect-derived apoLp-III. From the X-ray structure of *L. migratoria* apoLp-III in the absence of lipid, it is known that both glycosylation sites (at residues 18 and 85) are localized in the central region of the long axis of the bundle. Further study of this phenomenon revealed that apoLp-III sugar moieties interfere with helix bundle penetration into the bilayer surface during disruption and transformation into disc complexes ([Weers et al., 2000](#)). Thus, it is apparent that structural aspects of the helix bundle as well as the composition of the lipid surface influence the ability of apoLp-III to initiate and form a stable lipid-binding interaction.

9.3.4. ApoLp-III Alternate Functions

Based on the developmentally timed upregulation of its mRNA, apoLp-III has been implicated in muscle and neuron programmed cell death ([Sun et al., 1995](#)). When considered in light of its known lipid interaction properties, it is conceivable that apoLp-III functions in membrane dissolution and/or lipid reabsorption during metamorphosis. ApoLp-III has also been identified as a hemagglutinating agent in larval hemolymph of *G. mellonella* ([Ishikawa et al., 1998](#)). It interacts with lipoteichoic acids and with surface components of Gram-positive bacteria ([Halwani et al., 2000](#)), binds to fungal conidia and β -1,3-glucan ([Whitten et al., 2004](#)), and has been implicated in the detoxification of lipopolysaccharide (LPS) ([Dunphy and Halwani, 1997](#)). Given its recognition and binding properties, apoLp-III may serve a surveillance role as a pattern recognition receptor ([Kanost et al., 2004](#)). Since its function as a lipid transport protein is required only when large amounts of lipids are transported, a large pool of apoLp-III may be available for immediate protection against foreign invaders. Indeed, such a role could explain the presence of apoLp-III in hemolymph during life stages wherein its association with lipophorin is not required or does not occur.

It has been reported that apoLp-III functions in insect immunity ([Wiesner et al., 1997](#)). Indeed, results suggest that lipid-associated apoLp-III manifests this biological activity ([Dettloff et al., 2001b, 2001c](#)). These authors hypothesized that LDLp serves as an endogenous signal for immune activation, specifically mediated by apoLp-III interaction with hemocyte membrane receptors. From a structural standpoint, truncated variants of *G. mellonella* apoLp-III (see above) that retain function represent useful tools to probe the structural and physiological roles of

apoLp-III in innate immunity. Support for this general concept has emerged from studies of *G. mellonella* apoLp-III variants wherein point mutations were introduced at residues 66 and 68 ([Niere et al., 2001](#)). The observation that mutation-induced decreases in apoLp-III lipid interaction properties correlate with decreased immune-inducing activity is consistent with the hypothesis that apoLp-III immune activation is related to the conformational change that accompanies lipid interaction of this protein.

Fluorescence studies, exploiting the unique tyrosine residue in *G. mellonella* apoLp-III (Tyr-142), provided additional evidence for LPS interaction ([Pratt and Weers, 2004](#)). In the absence of lipid, Tyr-142 fluorescence is quenched. However, upon incubation with LPS or detoxified LPS, Tyr-142 fluorescence intensity is enhanced, indicating relocation of this residue to a new environment. Dissociation constants (K_d) measured by apoLp-III titration were estimated at approximately 1 μ M ([Leon et al., 2006a](#)). Increasing the ionic strength had no effect on the K_d , and nor did removal of LPS phosphate moieties. Interestingly, a truncated apoLp-III variant missing two complete helices retained LPS-binding activity. To further investigate the structural requirements for LPS binding, [Leon et al. \(2006b\)](#) used a protein engineering approach. These authors introduced two cysteine residues in close spatial proximity, resulting in disulfide bond formation between the residues. Tethering the helix bundle in this manner abolished the ability of apoLp-III to undergo conformational opening. Furthermore, tethered apoLp-III was unable to bind LPS. Disulfide bond reduction, however, restored helix bundle opening and LPS-binding capability. It may be concluded that helix bundle opening is required for LPS binding, and, since the interior of the bundle is hydrophobic, it appears that apoLp-III–LPS interactions are governed by hydrophobic interactions.

Although they lack an adaptive immune system, insects possess an innate immune system that recognizes and destroys intruding microorganisms. Its operation under natural conditions has not been well studied, as most studies have introduced microbes to laboratory-reared insects via artificial mechanical wounding. One of the most common routes of natural exposure and infection, however, is via food. [Freitak et al. \(2007\)](#) examined the immune system response to non-infectious microorganisms via simple oral consumption. Eight proteins were highly expressed in the hemolymph of the larvae fed bacteria including apoLp-III. Studies in the fall webworm, *Hyphantria cunea*, also demonstrated an immune activation role for apoLp-III, including induction of antimicrobial peptide expression and *E. coli* membrane binding ([Kim et al., 2004](#)). Changes in apoLp-III concentration and lipophorin interconversions after immune challenge are known ([Dettloff et al., 2001a; Mullen and Goldsworthy, 2003](#)), and it has been suggested that lipid-bound

apoLp-III acts as the immune activator, while lipid-free apoLp-III does not display this activity (Dettloff *et al.*, 2001b; Niere *et al.*, 2001; Whitten *et al.*, 2004). The reason for this is unclear, but a ligand-induced conformational change may create new structural features on the surface of apoLp-III that may form a signal for secondary immune responses.

Giannoulis and colleagues (2007) studied the effects of LPS and lipoteichoic acid on the immune response of *Malacosoma disstria*. LPS induced an increase in the number of damaged hemocytes, and limited removal of the entomopathogenic bacterium *Xenorhabdus nematophila* from hemolymph. Similar effects were observed with the lipid A moiety of LPS. At the same time, the effects of LPS and lipid A on hemocyte were abrogated by polymyxin B, an antibiotic that binds to lipid A, confirming that LPS is the hemocytotoxin. Lipoteichoic acid elicited nodulation and enhanced phenoloxidase activation. Importantly, apoLp-III interfered with the effects of LPS, lipid A, and lipoteichoic acid on hemocytes and prophenoloxidase until a critical threshold was exceeded.

The potential for two roles for apoLp-III raises several issues. For example, Adamo *et al.* (2008) tested the hypothesis that competition between apoLp-III immune function and lipid transport causes transient immunosuppression in crickets. Both flying and an immune challenge reduced the amount of free apoLp-III in hemolymph. The authors showed that immune function is sensitive to the amount of free apoLp-III in hemolymph. Reducing the amount of free apoLp-III in hemolymph, using AKH, produced immunosuppression. Likewise, increasing apoLp-III levels after flight by pre-loading insects with trehalose, or by injecting isolated apoLp-III, reduced immunosuppression. Thus, it appears that competition between lipid transport and immune function can lead to transient immunosuppression.

Seo *et al.* (2008) studied the antioxidant properties of apoLp-III and compared them to its mammalian counterpart, apolipoprotein A-I (apoA-I). In order to compare the antioxidant abilities of apoLp-III and apoA-I in the lipid-free and lipid-bound state, both proteins were purified and synthesized individually as a palmitoylcholine (POPC)-reconstituted high-density lipoprotein (rHDL) using the same molar ratio. In the lipid-bound state, apoLp-III and apoA-I showed good antioxidant activities against copper-mediated low density lipoprotein (LDL) oxidation. However, while lipid-free apoA-I treatment prevented cellular uptake of oxLDL in macrophages, lipid-free apoLp-III did not. These results indicate that the putative conformational change of apoLp-III during lipid association is critical for the maintenance of antioxidant activity, and that the physiologic role of apoLp-III may differ when it is in the lipid-free state and the lipid-bound state.

On a broader scale, it is important to understand the molecular details of this family of proteins (Narayanawami

and Ryan, 2000) because their property of reversible inter-conversion between water-soluble and lipid-bound states could have application beyond their natural biological settings. Indeed, as work on this system continues, it is evident that apoLp-III and analogous helix bundle apolipoproteins represent novel biosurfactants with potentially useful properties, including biodegradability. An example of such an application is the use of apoLp-III to generate functional nanolipoprotein particles (NLPs; Chromy *et al.*, 2007). These authors made NLPs from DMPC in combination with apoLp-III or mammalian apolipoproteins. Predominantly discoidal in shape, these particles have diameters of 10–20 nm, and a uniform height of 4.5–5 nm. The apolipoprotein employed, the lipid-to-lipoprotein ratio, and the assembly parameters affect the size and homogeneity of NLPs generated. These particles have myriad potential uses, including membrane protein solubilization (Bayburt and Sligar, 2010), drug delivery (Ryan, 2008), and diagnostic imaging (Cormode *et al.*, 2009).

9.4. Lipophorin Receptor Interactions

9.4.1. Receptor-Mediated Endocytosis of Lipophorin

In the concept of lipid transport during intense lipid utilization in insects, a major difference between the functioning of lipoproteins of mammals and insects is the selective mechanism by which insect lipoproteins transfer their hydrophobic cargo. Circulating HDLp particles may serve as a DAG donor or acceptor, dependent on the physiological situation, and function as a reusable lipid shuttle without additional synthesis or increased degradation of the apolipoprotein matrix, as discussed above. In apparent contrast to this concept, in fat body tissue of larval and young adult locusts, receptor-mediated uptake of HDLp was demonstrated (Dantuma *et al.*, 1997). A receptor has been cloned and sequenced from locust fat body cDNA, and identified as a novel member of the LDL receptor (LDLR) family, which is particularly expressed in fat body, oocytes, midgut, and the brain (Dantuma *et al.*, 1999). When stably transfected in an LDLR-deficient Chinese hamster ovary (CHO) cell line, the locust lipophorin receptor (LpR) mediated endocytic uptake of fluorescently labeled HDLp that was absent in mock-transfected cells, suggesting that the receptor may function *in vivo* as an endocytic receptor for HDLp (Dantuma *et al.*, 1999). To date, the LpR sequences of several other insect species have been elucidated (Cheon *et al.*, 2001; Lee *et al.*, 2003a, 2003b; Seo *et al.*, 2003; Gopalapillai *et al.*, 2006; Ciudad *et al.*, 2007; Guidugli-Lazzarini *et al.*, 2008; Tufail *et al.*, 2009; for review, see Tufail and Takeda, 2009). Domain organization of LpR is identical to that of mammalian LDLR (Dantuma *et al.*, 1999). However, the ligand-binding domain of LpR contains one additional

cysteine-rich repeat compared to the seven repeats in LDLR, and is therefore identical to that of the human very low-density lipoprotein (VLDL) receptor (VLDLR), which also contains eight consecutive cysteine-rich repeats in this domain (schematically depicted in **Figure 5**). The amino acid sequence of the longer cytoplasmic tail of LpR is unique for insect lipophorin receptors: the 12 C-terminal amino acid residues of LDLR are completely different from those of LpR, whereas the C-terminal tail of LpR contains an additional 10 amino acid residues (Van Hoof *et al.*, 2002; Rodenburg *et al.*, 2006). Three-dimensional models of the elements representing both the ligand-binding domain and the epidermal growth factor precursor homology domain of locust LpR bear a striking resemblance to those of mammalian LDLR (Van der Horst *et al.*, 2002). Despite their pronounced structural similarity, however, the ligand specificity of LpR and LDLR for lipophorin and LDL, respectively, is mutually exclusive (Van Hoof *et al.*, 2002). Additionally, the functioning of both receptors in lipid transport in insects and mammals

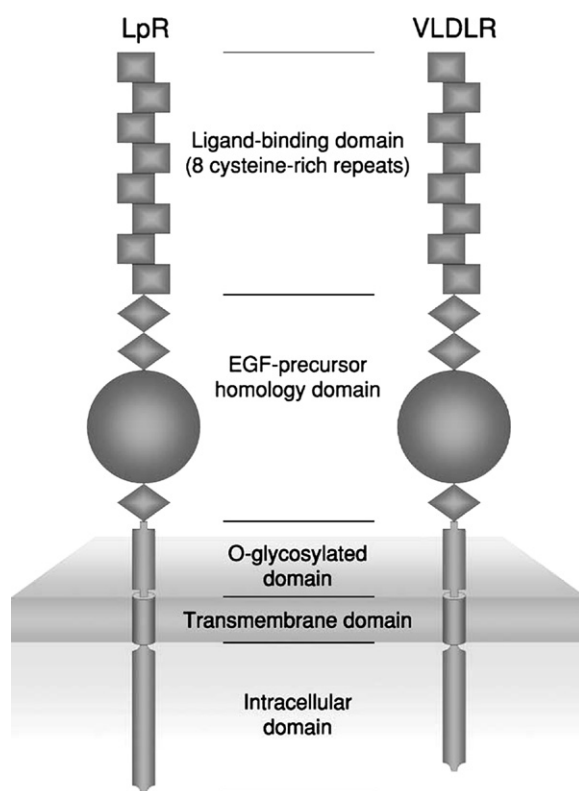


Figure 5 Schematic representation of the insect lipophorin receptor (LpR) and the mammalian VLDL receptor (VLDLR), indicating the identical domain organization. The mammalian LDL receptor has the same organization, but one less ligand-binding repeat. EGF, epidermal growth factor. Based on data from Dantuma *et al.*, 1999. Reprinted with permission from Van der Horst D.J., Ryan R.O., 2005. Lipid transport. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (eds), *Comprehensive Molecular Insect Science*, Vol. 4. Elsevier, Amsterdam, pp. 225–246.

appears to be intriguingly different (ligand recycling *versus* ligand degradation), as discussed below in more detail. Possibly, these specific properties may be attributable to relatively small structural differences governing different properties of ligand binding and/or release.

Interaction of HDLp with a specific high-affinity binding site or receptor in the cell membrane of the fat body and other tissues of several insect species has been well documented (for reviews, see Ryan and Van der Horst, 2000; Van der Horst and Ryan, 2005). In the binding of human LDL to its receptor, the most C-terminal 1000 amino acids in apoB are involved (Borén *et al.*, 1998). Remarkably, although both the sequence and domain structure of insect apoLp-II/I resemble that of apoB100 (Babin *et al.*, 1999; Smolenaars *et al.*, 2007b; for reviews, see Van der Horst *et al.*, 2002, 2009; Van der Horst and Rodenburg, 2010a), apoLp-II/I does not show homology to this C-terminal part of apoB100, leaving the receptor-binding domain of apoLp-II/I to be disclosed.

Immunocytochemical localization of HDLp has demonstrated the presence of the lipoprotein in endosomes of fat body of the larval dragonfly *Aeshna cyanea* (Bauerfeind and Komnick, 1992) and in developing mosquito oocytes (Sun *et al.*, 2000), suggesting endocytosis of circulating HDLp. In addition, the uptake of HDLp in the fat body of young adult locusts was shown to be receptor mediated (Dantuma *et al.*, 1997). Van Hoof *et al.* (2003) presented evidence for the involvement of LpR in the endocytic uptake mechanism for HDLp in the locust that is temporally present during specific periods of development. Shortly after ecdysis, when lipid reserves are depleted, LpR is expressed in fat body tissue of young adult locusts as well as larvae, and fat body cells are able to endocytose the complete HDLp particle. On the fourth day after (larval or imaginal) ecdysis, however, expression of LpR is downregulated and drops below detectable levels; consequently, HDLp is no longer internalized. Downregulation of LpR was postponed by experimental starvation of adult locusts immediately after ecdysis. Moreover, by starving adult locusts after downregulation of LpR, expression of the receptor was re-induced. These data suggest that LpR expression is regulated by a deficiency of lipid components in fat body tissue (Van Hoof *et al.*, 2003). 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 lipid cargo of circulatory HDLp particles at the cell surface.

On the other hand, in contrast to specific uptake of lipid components by receptor-mediated endocytosis of HDLp, experiments using HDLp that was 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. The latter data would suggest a preferential mechanism for the

intracellular loading of specific fat body lipid components onto relatively lipid-poor HDLp, while the lipid loading of the particle additionally results in decreased affinity for LpR (Roosendaal *et al.*, 2009), and thus facilitates the process of HDLp recycling (discussed in the following section).

9.4.2. Lipophorin Receptor-Mediated Ligand Recycling

Receptor-mediated uptake of HDLp in newly ecdysed adult and larval locusts may provide a mechanism for the uptake of specific lipid components, in addition to the mechanism of selective unloading of HDLp lipid cargo at the cell surface. However, the downregulation of LpR expression in fat body cells after these developmental periods suggests that this receptor is not involved in the lipophorin shuttle mechanism operative in the flying insect. Nevertheless, an endocytic uptake of HDLp seems to conflict with the selective process of lipid transport between HDLp and fat body cells without degradation of the lipophorin matrix. However, the pathway followed by the internalized HDLp appears to be different from the classical receptor-mediated lysosomal pathway typical of LDLR-internalized ligands.

In mammalian cells, LDL and diferric transferrin have been used extensively to study intracellular transport of ligands that are internalized by receptor-mediated endocytosis (Goldstein *et al.*, 1985; Brown and Goldstein, 1986; Mellman, 1996; Mukherjee *et al.*, 1997). Whereas LDL dissociates from its receptor upon delivery to the low pH milieu of the endosome and is completely degraded in lysosomes (for reviews, see Jeon and Blacklow, 2005; Beglova and Blacklow, 2005), transferrin remains attached to its receptor and, following the unloading of its two

iron ions, is eventually re-secreted from the cells (Ghosh *et al.*, 1994; Maxfield and McGraw, 2004). The endocytic uptake and intracellular trafficking of fluorescently labeled locust HDLp were studied simultaneously with fluorescently labeled human LDL or transferrin in LDLR-expressing CHO cells transfected with LpR cDNA, by multicolor confocal laser scanning microscopy, and provided evidence for different intracellular routes followed by the mammalian and insect lipoproteins (Van Hoof *et al.*, 2002) (Figure 6). Both HDLp and LDL appeared to co-localize to the same early endocytic vesicle structures. However, whereas LDL was eventually degraded in lysosomes after dissociating from its receptor, HDLp remained coupled to LpR and was transported to a non-lysosomal juxtanuclear compartment. Co-localization of HDLp with transferrin (Van Hoof *et al.*, 2002) (Figure 6) identified this organelle as the endocytic recycling compartment (ERC), from which internalized HDLp was eventually resecreted (half-life ~13 minutes) in a manner similar to that operative in the transferrin recycling pathway, thus escaping from lysosomal degradation.

The above 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 remained to be shown. Since 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 from which, during a chase, the labeled HDLp disappeared almost completely (Van Hoof *et al.*, 2005a),

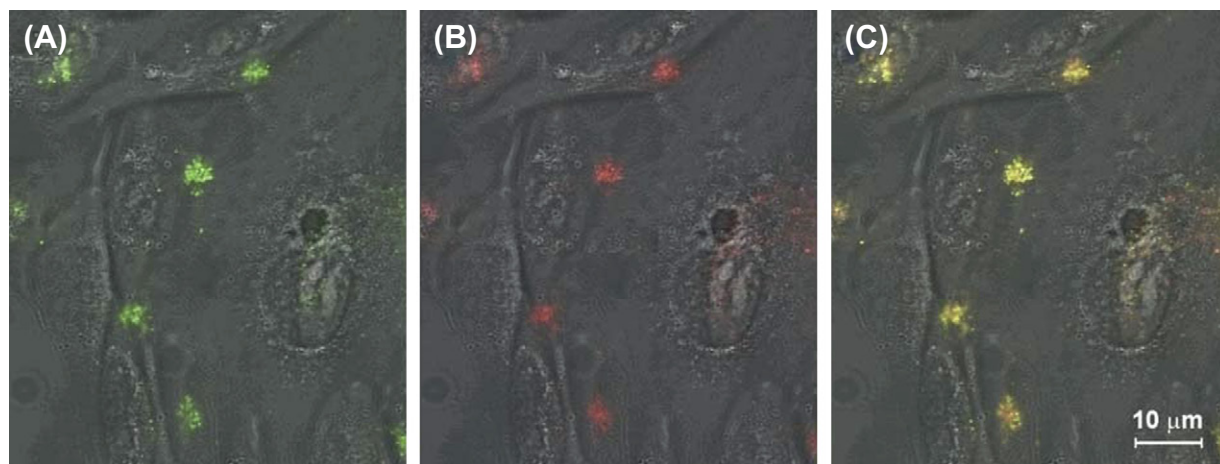


Figure 6 Confocal laser microscopic digital image of Chinese hamster ovary cells incubated with fluorescently labeled HDLp (A) and transferrin (B) after a chase period of 20 min. Co-localization of both ligands is visualized in yellow when images (A) and (B) are merged (C).

Reproduced with permission from Van der Horst, D.J., Van Hoof, D., Van Marrewijk, W.J.A., Rodenburg, K.W., 2002. Alternative lipid mobilization: the insect shuttle system. *Mol. Cell. Biochem.* 239, 113–119; ©Kluwer Academic Publishers.

indicative of re-secretion of the ligand and thus supporting the concept of ligand recycling that was demonstrated for LpR-transfected mammalian cells (Van Hoof *et al.*, 2002).

The above concept, which implies that lipophorin is recycled following endocytosis by an LDLR family member, conflicts with the generally accepted fate of ligands endocytosed by all 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 (Roosendaal *et al.*, 2008). Since, in addition to pH lowering, the Ca^{2+} concentration in the early endosome is also lowered to the low micromolar range (Gerasimenko *et al.*, 1998), 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 (Roosendaal *et al.*, 2008). 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 (Roosendaal *et al.*, 2008). This remarkable stability of the ligand-receptor complex is likely to provide a crucial key to 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 (Herz, 2001; Rudenko *et al.*, 2002; Innerarity, 2002; Beglova *et al.*, 2004a). 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 (Beglova *et al.*, 2004a, 2004b; Boswell *et al.*, 2004), notably Gln540, His 562, Glu581, and Lys582, are not conserved in LpR. Changing the complete ligand-binding domain of LpR for that of LDLR (LDLR₁₋₂₉₂LpR₃₄₃₋₈₅₀) (Van Hoof *et al.*, 2005b) 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, His 562, Glu581, and Lys582 renders the β -propeller of LpR indeed incapable of inducing LDL release and 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 (Van Hoof *et al.*, 2005b). 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 (Beglova *et al.*, 2004a, 2004b; Jeon and Blacklow, 2005). 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 (LpR₁₋₃₀₁LDLR₂₅₂₋₈₃₉) 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, since 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, which may be different from that used by other LDLR family members for the interaction with their ligands (Roosendaal *et al.*, 2008). However, the molecular mechanism for the stability of the HDLp-LpR complex awaits disclosure.

Additionally, even though the acidic endosomal environment of endocytosed HDLp has been postulated to facilitate the transfer of lipid components other than DAG or cholesterol (Dantuma *et al.*, 1997), both the precise function of the process of receptor-mediated endocytosis and the rationale for its occurrence during specific stages of insect development remain to be elucidated.

9.5. Other Lipid-Binding Proteins

9.5.1. Lipid Transfer Particle

In mammals, specialized proteins function in redistribution of hydrophobic lipid molecules. A wide variety of distinct lipid transfer proteins have been characterized, and their metabolic roles investigated. In 1986, a lipid transfer particle (LTP) was isolated from *M. sexta* larvae and shown to facilitate vectorial redistribution of lipids among plasma lipophorin subspecies (Ryan *et al.*, 1986a, 1986b). In subsequent studies, LTP was implicated in the formation of LDLp from HDLp in response to AKH (Van Heusden and Law, 1989). The concept that LTP functions in flight-related lipophorin conversions correlates well with observed increases in LTP concentration in adult hemolymph compared with other developmental stages (Van Heusden *et al.*, 1996; Tsuchida *et al.*, 1998). When compared to other lipid transfer proteins, however, LTP displays unique structural characteristics. For example, it exists as a high molecular weight complex of three apoproteins (apoLTP-I, 320,000 kDa; apoLTP-II, 85,000 kDa; and apoLTP-III, 55,000 kDa) and 14% non-covalently associated lipid (Ryan *et al.*, 1988). LTPs exhibiting similar structural properties have been isolated from *L. migratoria*, *P. americana*, *B. mori*, and *Rhodnius prolixus* hemolymph (Hirayama and Chino, 1990; Takeuchi and Chino, 1993; Tsuchida *et al.*, 1997; Golodne *et al.*, 2001). The large size of LTP permitted examination of its structural properties by negative stain electron microscopy (Ryan *et al.*, 1990a; Takeuchi and Chino, 1993). LTP from distinct species

displays a highly asymmetric morphology with two major structural features: a quasispherical head region, and an elongated cylindrical tail that appears to possess a central hinge. The lipid component resembles that of lipophorin in that it contains predominantly phospholipid and DAG (Ryan, 1990). An important question arising from these physical characteristics relates to the requirement of the lipid component as a structural entity and/or its involvement in catalysis of lipid transfer. Studies employing lipoproteins containing radiolabeled lipids in incubations with LTP have revealed that the lipid component of the particle is in dynamic equilibrium with that of lipoprotein substrates (Ryan *et al.*, 1988). Thus, it is evident that the lipid moiety is not merely a static structural component of LTP; rather, it can be considered as a functional element in the mechanism of lipid transfer.

9.5.1.1. Lipid substrate specificity Experiments have been conducted to examine the ability of LTP to utilize various substrate lipids. As reviewed earlier (Ryan and Van der Horst, 2000; Arrese *et al.*, 2001), LTP catalyzes the exchange and net transfer of DAG, in keeping with its proposed role in lipophorin interconversions *in vivo*. Extending this concept, Canavoso and Wells (2001) incubated radiolabeled midgut sacs with lipophorin-containing medium. These authors found that transfer of DAG from the midgut sacs to lipophorin was blocked by preincubation with antibody against LTP, supporting the view that LTP functions in DAG export from the midgut to lipophorin. In a similar manner, LTP was shown by Jouni *et al.* (2003) to be required for DAG transfer from lipophorin to *B. mori* ovarioles.

In studies of other potential lipid substrates, Singh and Ryan (1991) used [¹⁴C]acetate to label the DAG and hydrocarbon moiety of lipophorin *in vivo*. Subsequent lipid transfer experiments revealed that LTP is capable of facilitating transfer of hydrocarbon among lipoprotein substrates, suggesting that LTP plays a role in the movement of these extremely hydrophobic, specialized lipids from their site of synthesis to their site of deposition at the cuticle (Takeuchi and Chino, 1993). Interestingly, the rate of LTP-mediated hydrocarbon transfer was slower than DAG transfer. In other work, *B. mori* LTP was employed in studies of LTP-mediated carotene transfer among lipophorin particles (Tsuchida *et al.*, 1998). Again, compared to DAG transfer, the rate of LTP-mediated carotene redistribution was much slower. Taken together, these results suggest that LTP may have a preference for DAG versus hydrocarbon or carotenes. Alternatively, the observed preference for DAG may be a function of the relative accessibility of the substrates within the donor lipoprotein. The ability of LTP to facilitate phospholipid transfer was studied by Golodne *et al.* (2001). These authors observed that LTP-mediated phospholipid transfer is non-selective. In contrast to the requirement for LTP to mediate transfer of

DAG, hydrocarbon, carotenoids, and phospholipids, Yun *et al.* (2002) provided evidence that LTP does not function in cholesterol transfer or redistribution in *M. sexta*. Rather, cholesterol is proposed to diffuse among tissues via mass action, freely transferring between lipophorin and tissues, depending on the physiological need. In keeping with this interpretation, Jouni *et al.* (2003) found that cholesterol transfer from lipophorin to *B. mori* ovarioles was unaffected by antibodies directed against LTP, whereas DAG transfer was inhibited.

9.5.1.2. Mechanism of facilitated lipid transfer In general, lipid transfer catalysts may act as carriers of lipid between donor and acceptor lipoproteins, or transfer may require formation of a ternary complex between donor, acceptor, and LTP. Based on the observed LTP-mediated net transfer of DAG from HDLp to human LDL (Ryan *et al.*, 1990b), a strategy was developed to address this question experimentally (Blacklock *et al.*, 1992). [³H]-DAG-HDLp and unlabeled LDL were covalently bound to Sepharose matrices and packed into separate columns connected in series, followed by circulation of LTP or buffer. Circulation of LTP, but not buffer, resulted in a concentration-dependent increase in the amount of radiolabeled DAG recovered in the LDL fraction, revealing that LTP facilitates net lipid transfer via a carrier-mediated mechanism.

Blacklock and Ryan (1995) employed LTP apolipoprotein-specific antibodies to probe the structure and catalytic properties of *M. sexta* LTP, obtaining evidence that apoLTP-II is a catalytically important apoprotein. In a similar manner, Van Heusden *et al.* (1996) employed LTP antibody inhibition experiments to demonstrate that all three LTP apoproteins are important for lipid transfer activity. These authors found that, unlike apoLp-III, apoLTP-III is not found as a free protein in hemolymph (Van Heusden *et al.*, 1996), despite the fact that it dissociates from the complex following exposure to non-ionic detergent (Blacklock and Ryan, 1995).

9.5.2. Carotenoid-Binding Proteins

In insects, the involvement of lipophorin in the hemolymph transport of dietary carotenoids is well documented (Tsuchida *et al.*, 1998). In keeping with its general function to accept and deliver hydrophobic lipid cargo, lipophorin may be anticipated to selectively deposit these isoprenoids at specific tissues. The mechanism involved is not fully understood, but likely involves LTP, the lipophorin receptor, and other proteins. Carotenoids fulfill several important roles in insects. Certain carotenoids are provitamins for vitamin A, which is required as the visual pigment chromophore (Giovannucci and Stephenson, 1999). Studies in *Drosophila* have shown that mutations in the *ninaD* gene result in blindness due to a defect in cellular

uptake of carotenoids (Kiefer *et al.*, 2002). The *ninaD* gene encodes a protein that possesses significant sequence identity with mammalian class B scavenger receptors (i.e., SR-BI and CD36). In mammals, SR-BI is involved in cholesterol homeostasis, and mediates cholesterol flux between target cells and lipoproteins (Jian *et al.*, 1998; Yancey *et al.*, 2000). Insofar as lipophorin is structurally related to mammalian lipoproteins, it is conceivable that *ninaD* mediates transfer of carotenoids from lipophorin in a mechanistically similar manner (Kiefer *et al.*, 2002). Consistent with this concept, *ninaD* mRNA levels are particularly high in pupae, suggesting a role in the transport/delivery of zeaxanthin from fat body to the developing eye during pupation (Giovannucci and Stephenson, 1999). In a screen for mutations that affect the biosynthesis of rhodopsin in *Drosophila*, Wang *et al.* (2007) identified a second class-B scavenger receptor, named Santa Maria. Subsequent studies revealed that Santa Maria functions upstream of vitamin A formation in neurons and glia. The protein is co-expressed, and functionally coupled, with the β , β -carotene-15, 15'-monooxygenase (*NinaB*), which converts β -carotene to all-trans-retinal.

Another vivid example of carotenoid transport is the production of a yellow cocoon in the silkworm, *B. mori* (Tabunoki *et al.*, 2002). A carotenoid-binding protein (CBP) from silk glands of *B. mori* larvae was identified (Tabunoki *et al.*, 2002). The function of this 33-kDa protein was investigated using *B. mori* mutants. Interestingly, only in larvae carrying the dominant *Y* (yellow hemolymph) gene, was CBP present in the villi of the midgut epithelium, suggesting that CBP may be involved in absorption of carotenoids. Tsuchida *et al.* (2004a) fed radiolabeled triolein to *B. mori* mutants, and found no defects in fatty acid uptake from midgut or delivery to fat body and silk glands. However, analysis revealed that yellow coloration of hemolymph was attributable to its carotenoid content. Lipophorin from the *Y+I* mutant exhibited the highest concentration of total carotenoids (55.8 $\mu\text{g}/\text{mg}$ lipophorin) compared to 3.1 $\mu\text{g}/\text{mg}$ in the *+Y+I* mutant, 1.2 $\mu\text{g}/\text{mg}$ in the *YI* mutant, and 0.5 $\mu\text{g}/\text{mg}$ in the *+YI* mutant. Thus, although lipid metabolism in the mutants is normal, defective lutein uptake was associated with the recessive *Y*-gene. Tsuchida *et al.* (2004b) studied the expression of CBP in the larval midgut and silk glands from wild type and four naturally occurring carotenoid transport mutants. CBP was expressed throughout the fifth stadium, with the highest expression on days 4–5 in the silk gland and days 3–5 in the midgut. Microscopic immunocytochemistry demonstrated uniform expression along the brush border of columnar cells in the midgut epithelium, consistent with a role in absorption of dietary carotenoids. CBP was also highly expressed along the distal membrane of the middle part of the silk gland, indicating a function in the uptake of carotenoids from lipophorin. When the middle silk glands and midguts of

the four mutant strains were incubated with rabbit anti-CBP antibody, only proteins of the *Y*-gene dominant mutants cross reacted with the antibody, supporting the hypothesis that CBP is a *Y*-gene dependent protein.

Tabunoki *et al.* (2004) reported that CBP expression in the silk glands of larvae matched the period of carotenoid uptake into the silk gland. When these authors provided double-stranded CBP RNA to *B. mori* larvae, reduced expression of CBP in the silk gland was observed, along with a decrease in yellow pigmentation in the cocoon. Thus, CBP modulates cocoon pigmentation via its role in the uptake of carotenoids by silk gland tissue. The deduced amino acid sequence of CBP indicates it is a member of the steroidogenic acute regulatory (StAR) protein family, despite the fact that CBP binds carotenoids rather than cholesterol. In 2005, Sakudoh and colleagues identified a novel isoform of CBP, Start1, in *B. mori* that is comprised of a membrane-spanning N-terminal “MENTAL” domain together with a C-terminal lipid-binding “START” domain. This molecular architecture is identical to mammalian MLN64 and *Drosophila* Start1 proteins that are implicated in cholesterol transport and regulation of steroidogenesis. Interestingly, *B. mori* Start1 is expressed in both white and yellow cocoon strains, while CBP is only detected in the yellow cocoon strain. *B. mori* Start1 mRNA abundance in the prothoracic gland, the main ecdysteroidogenic tissue, positively correlates with changes in the hemolymph ecdysteroid level. Genomic analysis revealed that *B. mori* Start1 and CBP are generated from the same gene locus by alternative splicing. Thus, alternative splicing of the Start1/CBP gene generates unique protein isoforms whose endogenous ligands, sterol or carotenoid, are structurally different. Sakudoh and coworkers (2007) showed that, in the *Y*-recessive strain, the absence of a specific exon generates a non-functional CBP mRNA. Germ-line transformation with a wild type CBP gene, however, induced carotenoid uptake and normal cocoon coloration. More recently, Sakudoh *et al.* (2010) used positional cloning and transgenic rescue experiments to identify Cameo2, a transmembrane protein gene belonging to the CD36 family. In mutant larvae, Cameo2 expression was strongly repressed in the silk gland, resulting in colorless silk glands and white cocoons. The developmental profile of Cameo2 expression, CBP expression, and lutein pigmentation in the silk gland of the yellow cocoon strain are correlated. Thus, it may be considered that selective delivery of lutein requires the combination of two components: CBP as a carotenoid transporter in cytosol; and Cameo2 as the cell surface receptor.

9.5.3. Fatty Acid-Binding Proteins

Hydrolysis of LDLp-carried DAG by a lipophorin lipase at the flight muscles (for review, see Van der Horst *et al.*, 2001, 2010b) results in the extracellular production of FFAs. After uptake by the flight muscle cells, these FFAs

are oxidized for energy generation. The mechanism by which the extracellularly liberated FFAs are translocated across the plasma membrane is as yet unknown, but may involve membrane fatty acid transporter proteins similar to those identified in mammals, i.e., fatty acid translocase (FAT)/CD36, a family of fatty acid transport proteins (FATP1-6), and plasma membrane-associated fatty acid-binding protein (FABP_{pm}) that not only facilitate but also regulate cellular fatty acid uptake, for instance through their inducible rapid (and reversible) translocation from intracellular storage pools to the cell membrane (for reviews, see Bonen *et al.*, 2007; Glatz *et al.*, 2010; Schwenk *et al.*, 2010). The intracellular transport of FFAs in insect flight muscle cells closely resembles that in mammalian skeletal red muscle cells, and is mediated by a fatty acid-binding protein (FABP) (for review, see Haunerland, 1997). This insect FABP belongs to the cytoplasmic FABPs that comprise a family of 14- to 15-kDa proteins that bind fatty acid ligands with high affinity and are involved in shuttling fatty acids to cellular compartments, modulating intracellular lipid metabolism, and regulating gene expression (for reviews, see Boord *et al.*, 2002; Storch and McDermott, 2009). Intriguingly, in contrast to FABP in mammals, locust FABP is an adult-specific protein, the expression of which is directly linked to metamorphosis; the concentration of FABP in locust flight muscle cytosol is over three times that in the mammalian heart (for review, see Haunerland, 1997), suggesting an adaptation to the extremely high metabolic rate of fatty acid oxidation for energy generation during migratory flight (Haunerland *et al.*, 1992; Van der Horst *et al.*, 1993; Qu *et al.*, 2007). The flight muscle tissue of migratory birds such as the Western sandpiper also contains unusually high amounts of FABP (Guglielmo *et al.*, 2002), supporting a role of FABP in migratory flight as suggested above.

The high amino acid sequence similarity (82%) between the FABP of *L. migratoria* flight muscle and that of human skeletal muscles (Maatman *et al.*, 1994) is reflected in a strong similarity in their three-dimensional structures (for review, see Van der Horst *et al.*, 2002). The structure of *L. migratoria* FABP complexed with a fatty acid, however, although resembling the closely related mammalian heart- and adipocyte-type FABPs, is characterized by binding features that differ significantly from the typical hairpin-turn ligand shapes of the latter forms. As a result of these evolutionary variations, insect FABPs may display a much greater diversity in intracellular lipid binding than that observed for the mammalian transport proteins (Lücke *et al.*, 2006).

9.5.4. Vitellogenin

Oocyte development in adult females involves the accumulation of large amounts of lipid, most of which is extra-ovarian in origin and is delivered by lipophorin. Another

lipid-binding protein that serves a role in lipid transport to the oocyte is Vg; although its overall contribution to the oocyte lipid accumulation is relatively modest (about 5%) (Sun *et al.*, 2000), it is by far the most abundant yolk protein precursor accumulated in all insect species (for review, see Tufail and Takeda, 2009). While the structural properties of insect Vgs are diverse (for review see Tufail and Takeda, 2008), they generally possess 10% lipid, primarily phospholipid and glycerolipid. Vg is synthesized and assembled in the fat body, secreted into hemolymph, and taken up by oocytes. Vg uptake is facilitated by members of a subfamily of the LDLR family that have been characterized so far in *Drosophila* (Schonbaum *et al.*, 1995), *Ae. aegypti* (Sappington *et al.*, 1996), *Solenopsis invicta* (Chen *et al.*, 2004), *P. americana* (Tufail and Takeda, 2005), *Blattella germanica* (Ciudad *et al.*, 2006), and *Leucophaea maderae* (Tufail and Takeda, 2007; for review, see Tufail and Takeda, 2009). Cheon *et al.* (2001) demonstrated that the ovarian vitellogenin receptor (VgR) is only distantly related to LpR, another ovarian LDLR homolog with a different ligand; a recent phylogenetic analysis places insect VgRs and LpRs in separate groups, and reveals that insect LpRs are more closely related to vertebrate VLDLRs/VgRs and LDLRs than to insect VgRs (Tufail and Takeda, 2009). These data imply that the receptor-mediated mechanisms involved in the uptake of lipid and the accumulation of yolk protein precursors (which provide a key nutrient source for the developing oocyte) utilize two separate receptors (VgR and LpR), which are specific for their respective ligands, Vg and HDLp. Considerable early work was performed to characterize the lipid transport properties of Vg (for review, see Kunkel and Nordin, 1985), while more recent work has focused on molecular and evolutionary aspects of vitellogenin proteins and receptor-mediated endocytosis. These aspects, which are beyond the scope of the present chapter, have been comprehensively reviewed elsewhere (Sappington and Raikhel, 1998; Raikhel *et al.*, 2002; Tufail and Takeda, 2008, 2009).

With respect to HDLp internalization, it is important to note that immunocytochemical data in the mosquito (Sun *et al.*, 2000) revealed that only a small amount of HDLp accumulates in developing oocytes as yolk protein, comprising 3% of total ovarian proteins upon completion of protein internalization. Since lipid accounts for 35–40% of the insect egg dry weight (Kawooya and Law, 1988), Sun *et al.* (2000) proposed that internalization of HDLp is unlikely to be the major route of lipid delivery to the developing oocyte (for review, see Ziegler and Van Antwerpen, 2006). A dual mechanism for lipophorin-mediated lipid delivery to oocytes (a lipophorin shuttle mechanism involving LDLp and internalization of HDLp, with stripping of most of its lipid) had been demonstrated earlier (Kawooya and Law, 1988; Kawooya *et al.*, 1988; Liu and Ryan, 1991; Van Antwerpen *et al.*, 2005). In addition, recently LDLp

formation in the eggs of *B. mori* was reported, in which the apoLp-III associated with LDLp was apparently synthesized in the eggs (Tsuchida *et al.*, 2010). However, considering that the LpR involved in uptake of HDLp by mosquito oocytes bears a high structural similarity to the LpR discovered in locust fat body cell membranes (Dantuma *et al.*, 1999; Cheon *et al.*, 2001), the precise mechanism of LpR-mediated endocytosis in the oocyte, and the fate of HDLp, remain open questions. In light of the ligand recycling mechanism discussed earlier for lipid delivery to the fat body, the possibility exists that LpR recycles its ligand after intracellular trafficking, providing another mechanism for the uptake of specific lipid components by the oocyte.

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