

Insect lipoprotein biogenesis depends on an amphipathic β cluster in apolipoprotein II/I and is stimulated by microsomal triglyceride transfer protein

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Abstract Lipoproteins transport lipids in the circulation of an evolutionally wide diversity of animals. The pathway for lipoprotein biogenesis has been revealed to a large extent in mammals only, in which apolipoprotein B (apoB) acquires lipids via the assistance of microsomal triglyceride transfer protein (MTP) and binds them by means of amphipathic protein structures. To investigate whether this is a common mechanism for lipoprotein biogenesis in animals, we studied the structural elements involved in the assembly of the insect lipoprotein, lipophorin. LOCATE sequence analysis predicted that the insect lipoprotein precursor, apolipoprotein II/I (apoLp-II/I), contains clusters of amphipathic α -helices and β -strands, organized along the protein as N- α_1 - β - α_2 -C, reminiscent of a truncated form of apoB. Recombinant expression of a series of C-terminal truncation variants of *Locusta migratoria* apoLp-II/I in an insect cell (Sf9) expression system revealed that the formation of a buoyant high density lipoprotein requires the amphipathic β cluster. Coexpression of apoLp-II/I with the MTP homolog of *Drosophila melanogaster* affected insect lipoprotein biogenesis quantitatively as well as qualitatively, as the secretion of apoLp-II/I proteins was increased several-fold and the buoyant density of the secreted lipoprotein decreased concomitantly, indicative of augmented lipidation. **■** Based on these findings, we propose that, despite specific modifications, the assembly of lipoproteins involves MTP as well as amphipathic structures in the apolipoprotein carrier, both in mammals and insects. Thus, lipoprotein biogenesis in animals appears to rely on structural elements that are of early metazoan origin.—Smolenaars, M. M., A. de Morrée, J. Kerver, D. J. Van der Horst, and K. W. Rodenburg. **Insect lipoprotein biogenesis depends on an amphipathic β cluster in apolipoprotein II/I and is stimulated by microsomal triglyceride transfer protein.** *J. Lipid Res.* 2007. 48: 1955–1965.

Supplementary key words evolution • metabolism • *Locusta migratoria* • *Drosophila melanogaster* • Sf9 cells • lipid droplet • large lipid transfer protein • apolipoprotein • lipophorin • apolipoprotein B

Lipoproteins function in the transport of lipids in the circulation of vertebrates as well as invertebrates. In mammals, the biosynthesis of these macromolecular protein-lipid complexes occurring in liver and intestine involves the intracellular transfer and subsequent stabilization of lipids onto a single protein component, the nonexchangeable apolipoprotein B (apoB) (for reviews, see Refs. 1–7).

The major lipoprotein of insects, lipophorin, is stabilized by apolipoproteins derived from apolipoprotein II/I (apoLp-II/I), a homolog of apoB (8). In contrast to apoB, apoLp-II/I is cleaved during lipoprotein biosynthesis, resulting in the presence of two apolipoproteins in the secreted insect lipoprotein, apoLp-I and apoLp-II (8–12; for reviews, see Refs. 13–17). Cleavage of apoLp-II/I is not essential for lipophorin biogenesis, as uncleaved apoLp-II/I is also able to form a lipoprotein (11). Lipophorin is produced in cells of the fat body, an organ that combines the functions of liver and adipose tissues in vertebrates. Lipophorin is secreted with a buoyant density in the high density or very high density lipoprotein range, dependent on the species (18–22). Accordingly, lipophorin contains far fewer associated lipids than apoB lipoproteins that are secreted as very low density lipoproteins or chylomicrons. Moreover, the major neutral lipid in the lipophorin of most insects is diacylglycerol (13–17, 23, 24), rather than the triacylglycerol that dominates the lipid content of newly biosynthesized apoB lipoproteins. In the context of these differences between the mammalian and insect lipoprotein (i.e. neutral lipid load and content), it remains to be established whether the biosynthetic properties of the two lipoprotein species differ.

Abbreviations: aa, amino acids; apoB, apolipoprotein B; apoLp-II/I, apolipoprotein II/I; dMTP, *Drosophila melanogaster* microsomal triglyceride transfer protein; HDLp, high density lipophorin; LLT, large lipid transfer; MTP, microsomal triglyceride transfer protein; vWF, von Willebrand Factor.

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Manuscript received 29 September 2006 and in revised form 9 March 2007 and in re-revised form 25 April 2007 and in re-re-revised form 29 May 2007.

Published, JLR Papers in Press, June 13, 2007.
DOI 10.1194/jlr.M600434-JLR200

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Unlike the biosynthesis of lipophorin, the molecular mechanism of apoB lipoprotein biogenesis has been studied extensively (1–7). ApoB lipoprotein biogenesis starts upon the translation of apoB mRNA and the translocation of the nascent polypeptide into the endoplasmic reticulum. Subsequently, the N-terminal ~900 amino acids (aa) of apoB fold into the large lipid transfer (LLT) module that interacts with microsomal triglyceride transfer protein (MTP). This interaction is required for the deposition of lipids in the lipid binding cavity of apoB's LLT module. Accordingly, MTP prevents misfolding of the more C-terminal amphipathic apoB sequences and the resulting intracellular retention and degradation of apoB. After apoB translocation, the lipoprotein particle continues along the secretory pathway and acquires the bulk of its neutral lipids (triacylglycerol), putatively by fusion with an intraluminal neutral lipid droplet.

ApoB can bind thousands of lipid molecules by amphipathic structures that ultimately envelope and stabilize a core of lipids, thus shielding hydrophobic lipids from the hydrophilic environment (for reviews, see Refs. 25, 26). Prediction of amphipathic α -helices and amphipathic β -strands by the program LOCATE suggested the presence of five amphipathic clusters in apoB, enriched in either of these secondary structure elements, organized along the apoB polypeptide as N- α_1 - β_1 - α_2 - β_2 - α_3 -C (27, 28). The α_1 cluster and the N-terminal part of the β_1 cluster constitute the LLT module. 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 actually possess most of the lipid binding capacity (26). The β_1 cluster appears to be especially important in buoyant apoB-containing lipoprotein formation, because the mammalian intestine in fact produces lipoproteins of the lowest buoyant density (i.e., chylomicrons) from C-terminally truncated apoB (apoB-48) that contains the α_1 and β_1 clusters, and a small part of the α_2 cluster only, as a result of the editing of apoB RNA (for reviews, see Refs. 29–31). Thus, mammalian lipoprotein assembly is enabled by the molecular architecture of apoB as well as MTP function.

Despite apparent differences in the biogenesis of insect apoLp-II/I and mammalian apoB lipoproteins, the presence of an LLT module in apoLp-II/I (8, 11) and MTP homologs in insect genomes (32, 33) suggests that lipoprotein biosynthesis in insects may be dictated by similar structural elements as in mammals. Therefore, we studied the involvement of two major elements of mammalian lipoprotein biogenesis, amphipathic clusters in the structural apolipoprotein as well as MTP, in the assembly of *Locusta migratoria* apoLp-II/I into a high density lipophorin (HDLp), the mature form of lipophorin that enables the circulatory transport of lipids. Secondary structure prediction as well as recombinant expression of apoLp-II/I and MTP from *Drosophila melanogaster* (dMTP) in insect Sf9 cells provide direct evidence for a major role of apoLp-II/I amphipathic clusters and MTP in the biosynthesis of insect lipoprotein. Consequently, the mechanisms of lipoprotein assembly in vertebrates and invertebrates appear to have a common evolutionary origin.

MATERIALS AND METHODS

LOCATE analysis

LOCATE (26–28, 34) was used to predict amphipathic α -helices and β -strands with high lipid affinity in apoLp-II/I and apoB sequences. The following sequences were downloaded from GenBank in FASTA format and analyzed: apoLp-II/I from *L. migratoria* (accession number AJ130944), *D. melanogaster* (AAC47284.1), and *Manduca sexta* (AAB53254.1), and apoB from *Homo sapiens* (P04114), *Gallus gallus* (XP_419979.1), and *Danio rerio* (XP_694827.1). Amphipathic α -helices and β -strands in these sequences were predicted by LOCATE. In addition to the use of default parameter settings, the exhaustive search option was activated, as well as the snorkel option for amphipathic α -helix prediction. Lipid affinities were calculated using the Λ_3 algorithm (29).

Construction of the dMTP expression plasmid

A cDNA clone (SD01502) encoding dMTP was obtained from the Berkeley *Drosophila* Genome Project. To express dMTP in insect Sf9 cells, its complete cDNA was cloned into the pIB/V5-His vector (Invitrogen). To this end, the dMTP coding sequence was amplified by PCR (forward primer, 5'-CGCGCCGATATCATG-GAGAACAAAAATAAGAAGTGCCTG-3'; reverse primer, 5'-GCA-GGGCTCGAGTACAAGTCCCTAAAAATGAGGTTGCAC-3'). The PCR product was purified and ligated into the pGEM-T vector (Promega) and subsequently transferred into the pIB/V5-His vector using *EcoRV*/*XhoI* double digestion, in-frame with the vector-encoded V5/His epitope tag. To prevent the inclusion of possible PCR-introduced mutations, an *EcoRI*/*AfeI* dMTP fragment was transferred from the original cDNA clone into the dMTP:pIB/V5-His construct. Subsequent DNA sequencing verified that the dMTP cDNA sequence in the obtained expression construct was correct.

Construction of truncated and full-length apoLp-II/I expression plasmids

Subclones of the *L. migratoria* apoLp-II/I cDNA (10) were used to step-wise extend a previously established apoLp-II/I-38 construct, consisting of the pIZ/V5-His expression vector encoding 38% (1,287 aa) of the complete apoLp-II/I polypeptide (11). A fragment corresponding to apoLp-II/I mRNA nucleotides 3,387 to 6,021 was amplified from one of the apoLp-II/I cDNA subclones by PCR (forward primer, 5'-CAGAAGGAATCACTGTT-GATGG-3'; reverse primer, 5'-TCTAGATGGCCATTTCCACTG-TATG-3') using *PfuTurbo* DNA polymerase (Stratagene). After SuperTaq DNA polymerase (HT Biotechnologies, Ltd.)-mediated tailing with a terminal adenosine nucleotide, this fragment was cloned into pGEM-T. The transfer of a *PacI*/*SacII* fragment from the selected clone into the apoLp-II/I-38 clone (in the pIZ/V5-His vector) resulted in the apoLp-II/I-59 expression vector, which encodes the N-terminal 2,004 aa of apoLp-II/I, in-frame with the V5 epitope and the 6xHis tag. The apoLp-II/I fragment in pGEM-T was subsequently extended to apoLp-II/I mRNA nucleotide 8,505 by the transfer of a *BaeI*/*XbaI*-digested fragment from an apoLp-II/I cDNA subclone. The subsequent transfer of a *PacI*/*SacII* fragment from the resulting pGEM-T construct into the apoLp-II/I-38 clone resulted in the expression vector apoLp-II/I-84, which encodes the N-terminal 2,832 aa of apoLp-II/I, in-frame with the V5 epitope and 6xHis tag. The subsequent cloning of a *BstEII*-digested PCR-amplified fragment (forward primer, 5'-CTGGTGGTGACCATACAAAGG-3'; reverse primer, 5'-TCTAGACCTCCTTTAACACCCTTAG-3') into the *BstEII*/*XbaI*-digested apoLp-II/I-84 construct resulted in the apoLp-II/I-100 construct that encodes the full-length 3,380 aa *L. migratoria* apoLp-II/I with a C-terminal V5 epitope and a 6xHis tag. The

sequences of the obtained constructs were confirmed to be correct by DNA sequencing of both sense and antisense strands of the apoLp-II/I cDNA of two independently isolated plasmid clones that were obtained standardly from separate PCRs. Several nonsynonymous and synonymous nucleotide differences, as well as three nearly adjacent nucleotide deletions and a 16 nucleotide frame-shifted segment, were observed compared with the published sequence (10). These differences likely reflect inaccuracies in the published sequence and have been deposited in the European Molecular Biology Laboratory nucleotide sequence database at European Bioinformatics Institute, Hinxton, as an update to the *L. migratoria* apoLp-II/I nucleotide and protein sequences (accession number AJ130944, version 2).

Transient transfection and incubation of Sf9 cells

Spodoptera frugiperda Sf9 cells were maintained in adherent culture in serum-free Insect-Xpress medium (Cambrex) in polystyrene flasks (Greiner) at 27°C and passed twice a week. Transfections were performed on six-well plates with cells grown to 80% confluence. Transfection mixture was prepared by adding DNA and 6 µg of polyethylenimine (Polysciences Europe) to 50 µl of culture medium. After 15 min of incubation at room temperature, the transfection mix was added to the wells (on 600 µl of culture medium). Cells were incubated with DNA for 5 h and subsequently washed with culture medium. The amount of DNA transfected was increased according to construct size, using 0.75, 0.75, 1, 1.2, and 1.5 µg/well for the dMTP, apoLp-II/I-38, -59, -84, and -100 constructs, respectively. For coexpression experiments, transfection mixtures were supplemented with either 0.75 µg of dMTP construct or 0.3 µg of control (pIB/V5-His) vector. For determinations of buoyant density and antibody reactivity, transfections were performed in 75 cm² tissue culture flasks, with reaction volumes for transfection scaled up according to surface increase. Incubations were performed at 40 h after transfection. Cells were rinsed twice with culture medium and incubated for 7 h in PBS (pH 7.4) supplemented with 0.75 mM CaCl₂, 0.75 mM MgCl₂, and 1 g/l β-D-glucose. Incubation media were subsequently sampled as described previously (11).

Density gradient analysis

The buoyant density of recombinant apoLp-II/I proteins as well as dMTP was analyzed by subjecting incubation media from transfected cells to KBr density gradient ultracentrifugation (21). Resulting gradients were divided into aliquots in 11 fractions, collected from bottom to top. After gravimetric analysis of density, fractions were precipitated with TCA and assessed for the presence of apoLp-II/I proteins and dMTP by immunoblotting. The mean density and standard deviation were calculated for the peak buoyant density fractions from three independent experiments.

Immunoblot analysis of apoLp-II/I and cleavage products

TCA-precipitated proteins from incubation media were analyzed for the presence of recombinant apoLp-I, apoLp-II, uncleaved precursor apoLp-II/I, and dMTP by SDS-PAGE (5–10% slab) and subsequent immunoblot analysis (11). The molecular weight of the analyzed proteins was calculated from a logarithmic plotting of the position of molecular weight marker proteins on the gel after electrophoresis and staining. Primary antibodies (dilution, 1:10,000) were either monoclonal α-V5 (Invitrogen) or the polyclonals α-II and α-I, directed against apoLp-II and apoLp-I, respectively (35). The relative amount of incubation medium used for immunoblot analysis was varied according to the apoLp-II/I variant used, to enable consistent detection

of apoLp-II/I proteins. Secreted proteins were quantified by densitometry of unadjusted immunoblots using Image/J (developed at the U. S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). The detection of dMTP as well as apoLp-I and apoLp-II/I with the α-V5 antibody enables the direct comparison of expression levels upon the concomitant analysis of the three proteins. Data from single blot experiments only were used for quantification and comparison. ApoLp-II is detected independently using the polyclonal antibody described above (35). Therefore, direct quantitative comparison of apoLp-II with dMTP, apoLp-II/I, and apoLp-I is not possible. For several figures, the immunoblots were digitally clarified by modification of the γ factor, as indicated in the figure legends.

RESULTS

Clusters of amphipathic secondary structure in apoLp-II/I

The computer program LOCATE (26–28, 34) was used to predict amphipathic secondary structure elements (i.e., α-helices and β-strands) of high lipid affinity in available insect apoLp-II/I sequences. The prediction indicated the presence of two clusters of amphipathic α-helices of relatively high calculated lipid affinity in the *L. migratoria* apoLp-II/I. The clusters are located at aa 275 to 750 and 2,400 to 2,750 (Fig. 1A) and were named α₁ and α₂, respectively. These regions correspond to part of the LLT module and a less conserved region just N-terminal to the von Willebrand Factor (vWF) D-module (vWF-D; aa 2,815 to 3,004; 8), respectively. Compared with the α₁ cluster, the α₂ cluster contains a higher density of predicted amphipathic α-helices that also have a higher calculated lipid affinity than the helices in α₁. Most amphipathic β-strands are located between apoLp-II/I aa 750 and 2,250, with a concentration of strands of particularly high calculated lipid affinity between aa 800 and 950. The C-terminal part of apoLp-II/I (aa > 2,750) is not clearly enriched in either amphipathic α-helices or amphipathic β-strands (Fig. 1A).

A similar clustering of amphipathic α-helices and β-strands is predicted for other characterized insect apoLp-II/Is, which also appear to contain a region enriched in amphipathic β-strands between two clusters of amphipathic α-helices and a C-terminal part that is not particularly enriched in either of these amphipathic structures (Fig. 1C). This organization of amphipathic clusters in insect apoLp-II/I may be summarized as N-α₁-β-α₂-C and termed tripartite. The LOCATE analysis of vertebrate apoB (Fig. 1B, C) confirmed the pentapartite amphipathic cluster organization of apoB as N-α₁-β₁-α₂-β₂-α₃-C (27, 28). The three insect clusters may correspond to the three N-terminal clusters of apoB (i.e., α₁, β₁, and α₂); however, three important differences were recognized. First, in all analyzed apoLp-II/I sequences, the β cluster constitutes at least 200 residues more than either of the β clusters of analyzed vertebrate apoB (Fig. 1C). Second, the β cluster of apoLp-II/I is predicted to contain amphipathic β-strands of particularly high calculated lipid affinity from aa 800 to 950, at its N-terminal boundary, unlike the β₁ and β₂ clusters

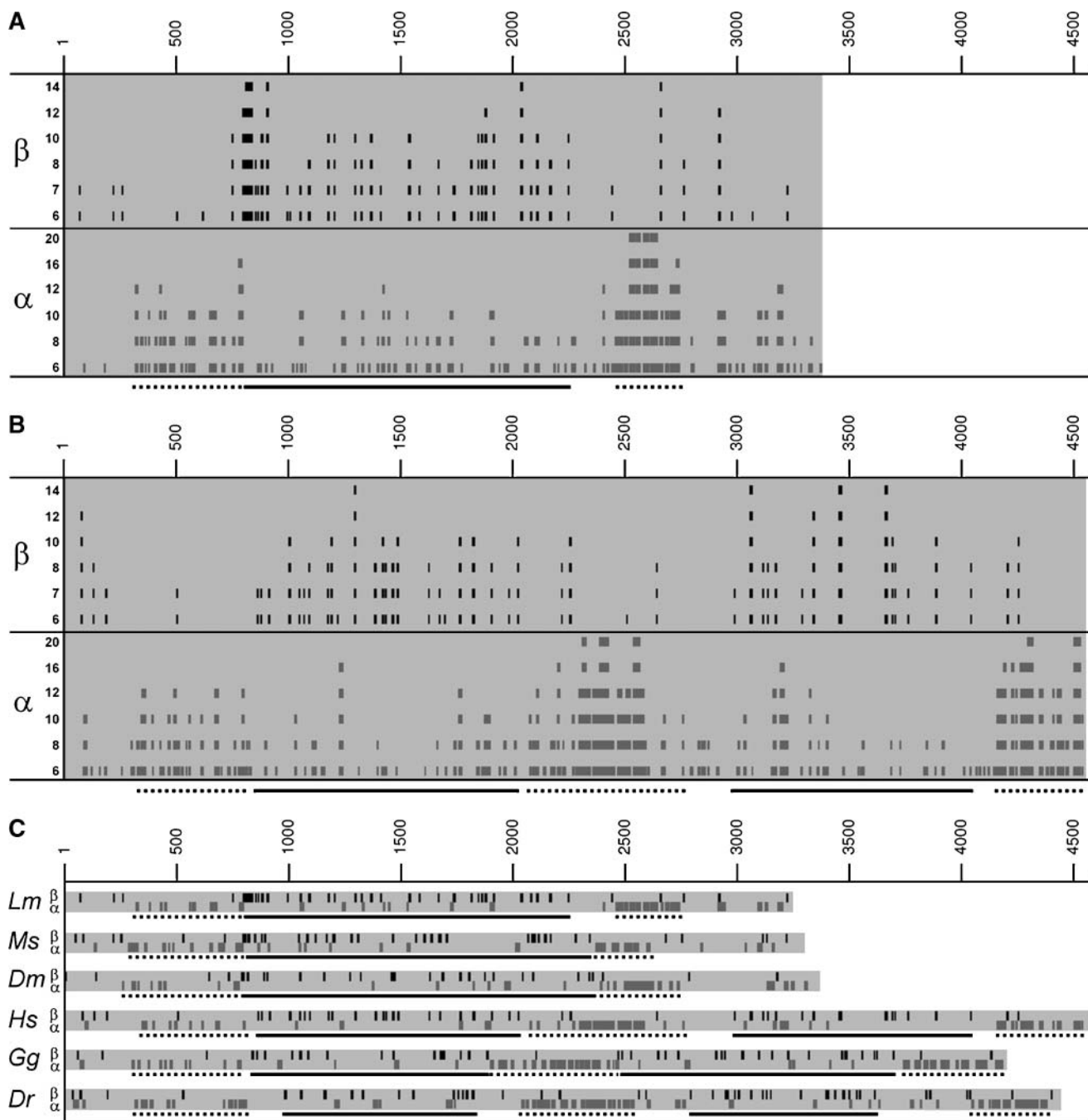


Fig. 1. Clusters of amphipathic secondary structure in apolipoprotein II/I (apoLp-II/I). Amphipathic α -helices and β -strands in apoLp-II/I and apolipoprotein B (apoB) were predicted using LOCATE. Predicted α -helical or β -strand amphipathic stretches are indicated by boxes at their predicted amino acid positions (horizontal axis), provided that their calculated lipid affinity is above the chosen threshold for calculated lipid affinity (vertical axis). Upper panels show predicted amphipathic β -strands, and lower panels show predicted amphipathic α -helices. Solid or dotted black lines below the panel of each large lipid transfer protein indicate the designated clusters enriched in either amphipathic α -helices or amphipathic β -strands, respectively. **A:** LOCATE analysis of *L. migratoria* apoLp-II/I, with the threshold for calculated lipid affinity increasing from 6 to 20 and from 6 to 14 kcal/mol for amphipathic α -helices and β -strands, respectively. **B:** LOCATE analysis of human apoB, with the threshold for calculated lipid affinity increasing from 6 to 20 and from 6 to 14 kcal/mol for amphipathic α -helices and β -strands, respectively. **C:** Amphipathic α -helices and β -strands predicted within apoLp-II/I from *L. migratoria* (*Lm*), *D. melanogaster* (*Dm*), and *M. sexta* (*Ms*) as well as apoB from *H. sapiens* (human; *Hs*), *G. gallus* (chicken; *Gg*), and *D. rerio* (zebrafish; *Dr*) at a calculated lipid affinity threshold of 10 and 7 kcal/mol, respectively.

in apoB (Fig. 1B). These sequences correspond to the β B sheet that constitutes one side of the lipid binding cavity of apoLp-II/I's LLT module, which is opposite to the side that becomes cleaved in apoLp-II/I (11). Third, the α_2 cluster of apoLp-II/I is smaller than the α_2 and α_3 clusters in vertebrate apoB, comprising ~ 300 rather than ~ 500 residues.

Expression of apoLp-II/I variants

To assess the role of the identified apoLp-II/I amphipathic clusters in lipoprotein formation, constructs encoding a series of C-terminal apoLp-II/I truncations in addition to wild-type apoLp-II/I were created (Fig. 2A). C-terminal truncations of apoB were previously used to demonstrate the role of its β_1 cluster in lipid binding and lipoprotein formation (36–42). The created apoLp-II/I constructs encode the N-terminal 1,287, 2,004, and 2,832 aa and the complete 3,380 aa of *L. migratoria* apoLp-II/I, fused at the C terminus to a V5/His epitope tag. Excluding the signal sequence, these C-terminal variants correspond to 38, 59, 84, and 100% of apoLp-II/I, respectively. All constructs contain the complete LLT module, including the full apoLp-II sequence (Fig. 2A).

ApoLp-II/I-38 encodes the complete LLT module and one-third of the β cluster, apoLp-II/I-59 encodes nearly the complete β cluster, and apoLp-II/I-84 encompasses all three recognized amphipathic clusters. ApoLp-II/I-100 encodes the complete apoLp-II/I precursor, with all recognized clusters as well as the C-terminal region, including the vWF-D module. ApoLp-II/I-38 expression products have been characterized previously (11).

Recombinant expression of the truncation variants apoLp-II/I-59 and apoLp-II/I-84 and the wild-type variant apoLp-II/I-100 in Sf9 cells resulted in the secretion of the expected apoLp-II/I cleavage protein products, as judged from immunoblotting of incubation medium from transfected Sf9 cells (Fig. 2B). ApoLp-II protein was identified by the appearance of immunoreactivity to α -II at a molecular mass of ~ 72 kDa in SDS-PAGE for all variants expressed. ApoLp-I protein was identified by immunoreactivity to α -V5 and α -I, with molecular mass increasing proportional to the apoLp-II/I truncation length (i.e., ~ 145 , ~ 220 , and ~ 250 kDa for apoLp-II/I-59, apoLp-II/I-84, and apoLp-II/I-100, respectively). Because the α -II antiserum harbors a mild cross-reactivity to apoLp-I (J. M. Van Doorn, personal communication), a minor immuno-

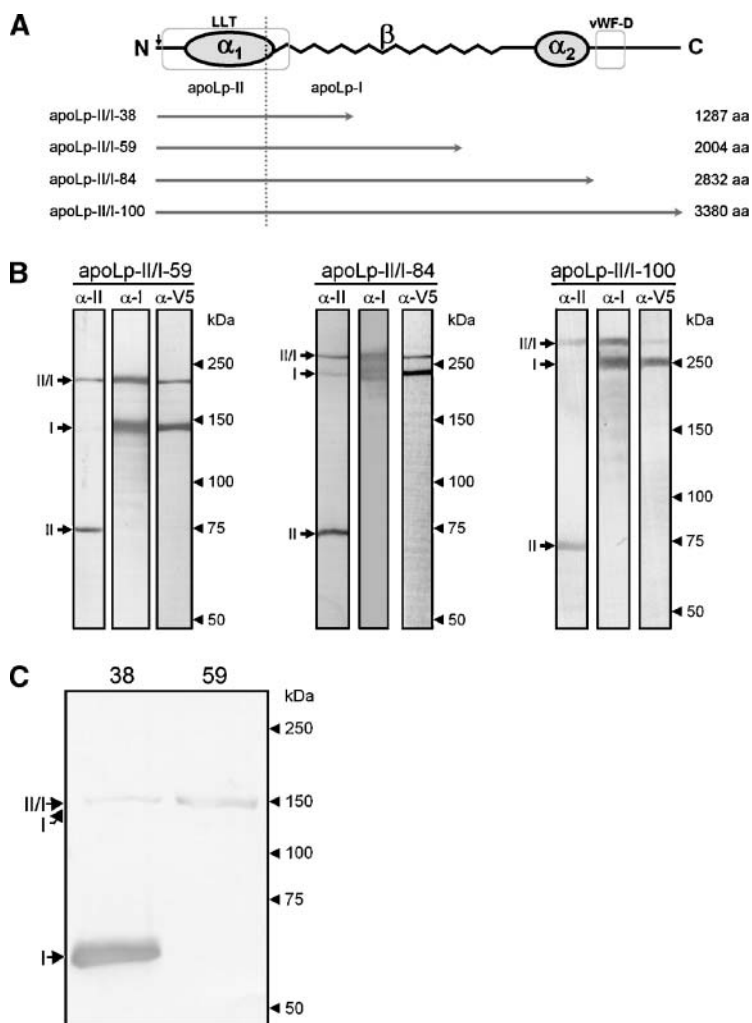


Fig. 2. Expression of apoLp-II/I variants. Distinct expression constructs encoding C-terminal variants of *L. migratoria* apoLp-II/I were created and subsequently expressed in Sf9 cells. A: Comparison of the proteins encoded by the different constructs, indicated by gray horizontal arrows, to the complete *L. migratoria* apoLp-II/I. The names of the constructs at left of the arrows correspond to the encoded percentage of the complete apoLp-II/I, excluding the signal peptide. The total number of encoded apoLp-II/I amino acids is shown at right. The recognized structural elements in apoLp-II/I are indicated schematically. Boxes denote the large lipid transfer (LLT) and von Willebrand Factor D (vWF-D) modules, whereas regions enriched in amphipathic α -helices, designated α_1 and α_2 , are indicated by ovals, and the region enriched in amphipathic β -strands, named β , is indicated by the zigzag line. The small vertical arrow indicates the predicted signal peptide cleavage site, and the dashed vertical line indicates the location of the site of apoLp-II/I cleavage into apoLp-II and apoLp-I. B: Recombinant expression of the novel apoLp-II/I constructs apoLp-II/I-59, -84, and -100 in Sf9 cells. Immunoblot analysis of incubation medium, using antibodies directed against apoLp-II (α -II) and apoLp-I (α -I and α -V5), demonstrates the secretion of (truncated) uncleaved apoLp-II/I as well as its cleavage products apoLp-II and (truncated) apoLp-I (marked by arrows and I, II, or II/I). The positions of molecular mass standards are indicated at right by arrowheads. The relative amount of incubation medium applied to the immunoblot was varied according to the apoLp-II/I variant used to enable the detection of variants with relatively low secretion levels. For enhanced clarity, immunoblots are shown with γ settings modified. C: Recombinant expression of apoLp-II/I-59 (lane 59) compared with the expression of apoLp-II/I-38 (lane 38) by Sf9 cells. Cells were transiently transfected, and equal volumes of the incubation media were assessed for the presence of recombinant apoLp-I (I; for apoLp-II/I-38 and apoLp-II/I-59) and apoLp-II/I (II/I; for apoLp-II/I-38) by immunoblotting with α -V5 antibody. In lane 38, the apoLp-I part as well as the uncleaved precursor protein (~ 150 kDa) are visible.

reactivity with apoLp-I was observed in some cases. The immunoreactivity of α -I, α -II, and α -V5 also identified significant amounts of uncleaved apoLp-II/I in the incubation media for all variants, with molecular masses of \sim 230, \sim 290, and \sim 325 kDa, respectively, for these variants. Notably, secretion of precursor for the apoLp-II/I-84 variant was detected in a few experiments only. In general, the secretion of uncleaved apoLp-II/I was likely related to the relatively high expression level achieved with the present transfection protocol, as transfection according to a previous protocol did not result in the secretion of uncleaved apoLp-II/I-38 protein (11). From this point on, we refer to the three secreted insect proteins together (i.e., apoLp-I, apoLp-II, and their precursor apoLp-II/I) as apoLp-II/I proteins.

The relative amount of apoLp-II/I proteins secreted by transfected cells differed between the apoLp-II/I variants. Compared with apoLp-II/I-38 proteins, immunoblot densitometry revealed that >10 times fewer apoLp-II/I-59

proteins were secreted (Fig. 2C) and at least 20 times fewer apoLp-II/I-84 and apoLp-II/I-100 proteins (data not shown). These differences may reflect different expression levels, as a result of decreased transfection efficiency with larger expression plasmids, or a decreased secretion rate attributable to difficulties in the proper folding of apoLp-II/I proteins.

Lipoprotein formation of apoLp-II/I variants

To assess the role of the identified apoLp-II/I amphipathic clusters in lipid binding, we determined the buoyant density of the recombinant secreted apoLp-II/I variants using density gradient ultracentrifugation. The apoLp-II/I-100 proteins were all recovered at a buoyant density of 1.18 ± 0.02 g/ml (Fig. 3G), suggesting the formation of high density lipoprotein particles. The apoLp-II/I-84 proteins were recovered at a similar buoyant density (1.17 ± 0.02 g/ml; Fig. 3E). In contrast, apoLp-II/I-38 proteins were found in the very high density lipo-

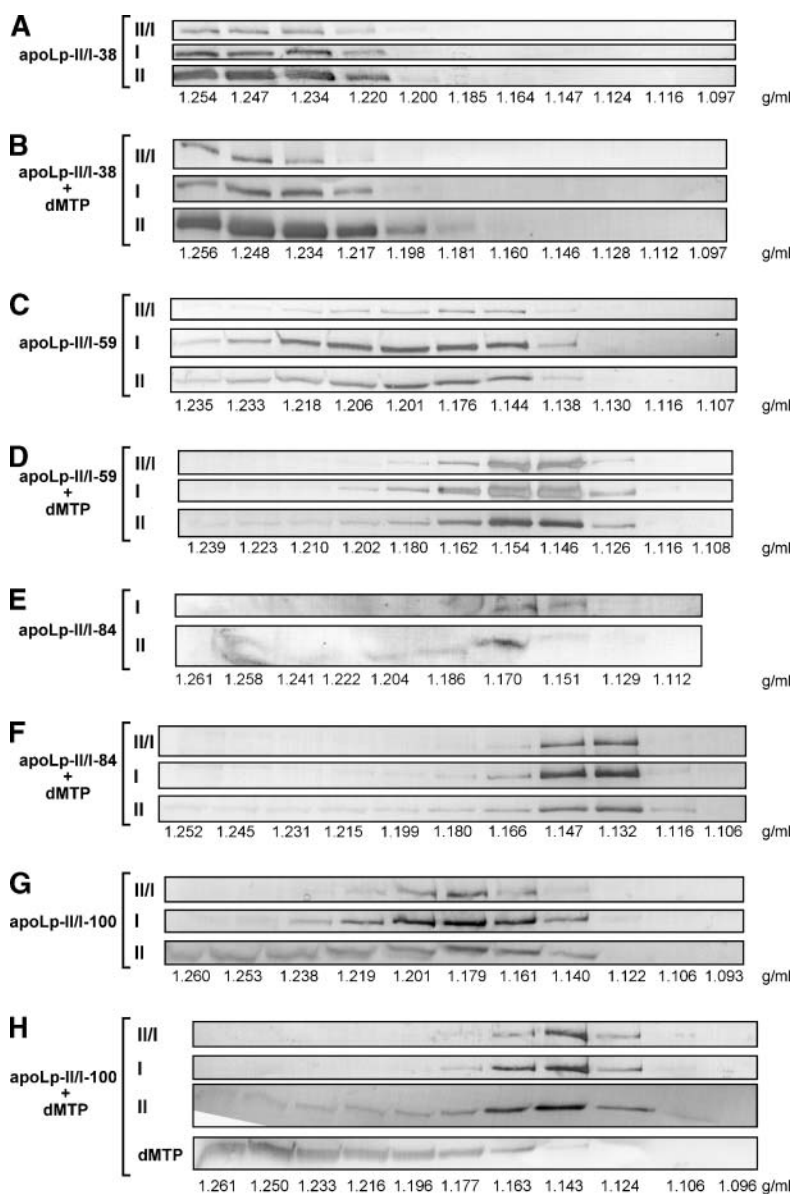


Fig. 3. Coexpression of *Drosophila melanogaster* microsomal triglyceride transfer protein (dMTP) selectively decreases the buoyant density of recombinant apoLp-II/I proteins. Sf9 cells were transiently transfected with one of the different apoLp-II/I constructs, along with the dMTP construct or control vector, and incubation medium was submitted to density gradient ultracentrifugation. The resulting gradients were divided into fractions that were analyzed for apoLp-II/I proteins by immunoblotting with α -V5 and α -II antibodies. The relative amount of fractions used for immunoblotting was varied between the gradients according to secretion levels, to enable consistent detection of apoLp-II/I proteins. Panels show immunoblot results for the distribution of apoLp-I, apoLp-II, and apoLp-II/I among the fractions of each gradient and are accordingly marked I, II, and II/I, respectively. For all gradients, the buoyant density of each fraction is indicated under the lower panel. Parts A–H show the density distribution of apoLp-II/I proteins for different sets of transfected constructs, representative of at least three independent transfections. A: ApoLp-II/I-38 and control. B: ApoLp-II/I-38 and dMTP. C: ApoLp-II/I-59 and control. D: ApoLp-II/I-59 and dMTP. E: ApoLp-II/I-84 and control. F: ApoLp-II/I-84 and dMTP. G: ApoLp-II/I-100 and control. H: ApoLp-II/I-100 and dMTP. In H, a panel has been included that shows the distribution of dMTP in the density gradient, which is representative of its coexpression with any of the apoLp-II/I variants.

protein range only, at 1.24 ± 0.02 g/ml, indicating a very poor lipidation, if any (Fig. 3A). ApoLp-II/I-59 proteins floated at an intermediate position of 1.19 ± 0.03 g/ml (Fig. 3C). Gel filtration analysis (data not shown) confirmed that apoLp-I and apoLp-II from the apoLp-II/I-59, -84, and -100 variants form lipoprotein complexes, as both cleavage products were recovered at the same elution volume, displaying estimated molecular masses of 230, 500, and 550 kDa, respectively. ApoLp-II/I-38-derived apoLp-I and apoLp-II, however, were recovered at an estimated molecular mass of 100 kDa, below their combined protein molecular mass of ~ 145 kDa, indicating that they did not form a complex.

In a previous study, we found that apoLp-II and truncated apoLp-I of apoLp-II/I-38 formed a complex (11). In that study, the complex stability of apoLp-II/I-38 was not assessed with regard to that of other apoLp-II/I variants and therefore was assumed to be stable. In this study, compared with the stability of the more extended apoLp-II/I variants, we conclude that apoLp-II and apoLp-I derived from apoLp-II/I-38 may form an unstable protein-protein or protein-lipid complex, whereas apoLp-II/I-59, -84, and -100 variants form stable high density lipoproteins. The differences between the apoLp-II/I variants in expression level, as apoLp-II/I-38 proteins also failed to produce a high density lipoprotein at lower expression levels (11). Compared with apoLp-II/I-38, apoLp-II/I-59 contains a much larger part of the β cluster, amounting to nearly the complete β cluster, whereas apoLp-II/I-84 contains the entire β cluster and the α_2 cluster. Therefore, sequences in the β cluster, and possibly also the α_2 cluster, are proposed to enable apoLp-II/I to acquire the lipids required to constitute a HDLp.

Effect of dMTP on apoLp-II/I lipoprotein formation

The recombinant lipoprotein expression system was subsequently used to investigate a putative role for MTP in apoLp-II/I lipoprotein assembly and secretion. A previously reported insect MTP from *D. melanogaster* (dMTP) (32, 43) was cloned for coexpression with the apoLp-II/I constructs. As antibodies to dMTP are currently not available, dMTP cDNA was cloned in-frame with a V5/His epitope tag to enable detection of the encoded protein. Transfection of this dMTP construct in Sf9 cells resulted in the secretion of dMTP, as shown by an α -V5 immunoreactive band at ~ 100 kDa (Fig. 4A). This molecular mass is similar to that reported for dMTP upon expression in the mammalian COS-1 cell line (32). The comparison of secretion levels by immunoblot densitometry revealed that significant amounts of dMTP are secreted: amounting to half of the secreted apoLp-II/I-38 proteins (as measured by the combined immunodensities of apoLp-I and apoLp-II/I, which are comparable because of concomitant detection, unlike apoLp-II) and more than five times the secretion level of apoLp-II/I-59 proteins (Fig. 4A).

The effect of dMTP coexpression on the secretion of apoLp-II/I proteins by recombinant Sf9 cells was

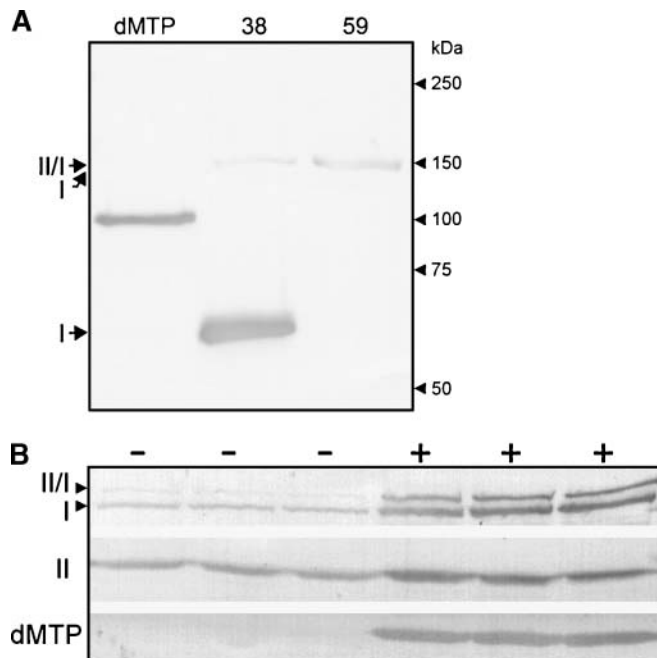


Fig. 4. Coexpression of dMTP enhances the secretion of recombinant apoLp-II/I proteins. A: Recombinant expression of dMTP by Sf9 cells (lane dMTP) compared with the expression of apoLp-II/I-38 (lane 38) and apoLp-II/I-59 (lane 59) alone. Cells were transiently transfected, and equal volumes of the incubation media were assessed for the presence of recombinant dMTP as well as apoLp-I (I; for apoLp-II/I-38 and apoLp-II/I-59) and apoLp-II/I (II/I; for apoLp-II/I-38) by immunoblotting with α -V5 antibody. In lane 38, the apoLp-I part as well as the uncleaved precursor protein (~ 150 kDa) are visible. This panel is from the same immunoblot shown in Fig. 2C. B: Effect of dMTP coexpression on apoLp-II/I-100 proteins. Sf9 cells were cotransfected with the apoLp-II/I-100 construct as well as the dMTP construct (+) or control vector (-). The amount of apoLp-I, apoLp-II, and uncleaved apoLp-II/I secreted into the incubation medium was assessed by immunoblotting using α -II antibody to detect apoLp-II and α -V5 antibody to detect apoLp-I, apoLp-II/I, and dMTP. Relevant sections of the blots are shown, with contrast enhanced for visibility.

assessed by densitometry of incubation media immunoblots. Compared with the control (pIB/V5-His vector-cotransfected cells), coexpression of dMTP with apoLp-II/I-100 in Sf9 cells resulted in a 4-fold increase of secreted apoLp-II/I proteins (Fig. 4B, Table 1). Coexpression of dMTP was verified by α -V5 immunoreactivity at 100 kDa. dMTP affected the secretion of apoLp-II/I-59 proteins to a similar extent as for apoLp-II/I-100, whereas the secretion of apoLp-II/I-84 proteins was stimulated by >10 -fold. In contrast, the increase in secretion of apoLp-II/I-38 proteins was stimulated by <2 -fold (Table 1). For all variants, the stimulation factor of secretion by coexpression of dMTP was larger for uncleaved apoLp-II/I than for apoLp-I or apoLp-II. However, the absolute amount of the secreted precursor protein was only about half of that of the apoLp-I protein, at most. The larger increase in secretion of the precursor protein upon dMTP coexpression likely reflects the limited furin capacity of Sf9 cells to cleave apoLp-II/I in the secretory

TABLE 1. Stimulation of the secretion of apoLp-II/I-38, apoLp-II/I-59, apoLp-II/I-84, and apoLp-II/I-100 proteins upon dMTP coexpression

Apolipoprotein	Uncleaved	ApoLp-I	ApoLp-II	Total Apolipoprotein
ApoLp-II/I-38	9.2 ± 3.3	1.3 ± 0.2	1.7 ± 0.7	1.6 ± 0.2
ApoLp-II/I-59	10.4 ± 3.2	4.0 ± 1.2	3.9 ± 1.6	4.7 ± 1.9
ApoLp-II/I-84	n.d.	18.3 ± 6.0	2.9 ± 0.9	16.4 ± 1.5
ApoLp-II/I-100	12.5 ± 5.4	3.3 ± 0.9	2.0 ± 0.6	4.3 ± 1.0

ApoLp-II/I, apolipoprotein II/I; dMTP, *Drosophila melanogaster* microsomal triglyceride transfer protein. Sf9 cells were cotransfected with one of the apoLp-II/I constructs as well as the dMTP construct. The amount of apoLp-II/I, apoLp-I, and apoLp-II secreted into the incubation medium was assessed by densitometric analysis of immunoblots. As a measure of the overall dMTP-induced stimulation of apoLp-II/I proteins, in uncleaved as well as cleaved form, the stimulation of the combined amounts of uncleaved apoLp-II/I and its cleavage product apoLp-I (concomitantly detected) is shown in the total apolipoprotein column (calculation: [apoLp-II/I_{dMTP} + apoLp-I_{dMTP}]/[apoLp-II/I_{control} + apoLp-I_{control}]), where "control" indicates the expression of apolipoprotein in the absence of dMTP). The stimulation factors are expressed as means of at least three independent transfections ± SD. In the absence of MTP coexpression, the secretion of precursor was detected in only a few experiments for apoLp-II/I-84; therefore, its stimulation could not be determined accurately (n.d.).

pathway (11). Accordingly, the stimulation of apoLp-II/I secretion results in the secretion of relatively more uncleaved apoLp-II/I. For apoLp-II/I-100 and, in particular, apoLp-II/I-84, the stimulation of secretion was consistently stronger for apoLp-I than for apoLp-II. Possibly, this is the result of the instability of apoLp-I after secretion in the absence of dMTP coexpression. Expression levels of dMTP were identical among expressed variants, as determined by immunoblot densitometry (data not shown). Thus, insect MTP enhances the secretion of recombinant apoLp-II/I proteins by Sf9 cells.

To assess the effect of MTP on the lipidation of insect lipoproteins, we determined the effect of dMTP coexpression on the buoyant density of the secreted apoLp-II/I proteins. Coexpression of dMTP was confirmed by α-V5 immunoreactivity at 100 kDa (Fig. 3H). dMTP coexpression clearly affected the peak buoyant density of the apoLp-II/I proteins for all variants, except apoLp-II/I-38 (Fig. 3A, B), because the peak buoyant density decreased from 1.19 ± 0.03 g/ml to 1.15 ± 0.01 g/ml for the apoLp-II/I-59 proteins (Fig. 3C, D), from 1.17 ± 0.02 g/ml to 1.14 ± 0.01 g/ml for the apoLp-II/I-84 proteins (Fig. 3E, F), and from 1.18 ± 0.02 g/ml to 1.14 ± 0.02 g/ml for the apoLp-II/I-100 proteins (Fig. 3G, H). For all variants, the uncleaved apoLp-II/I displayed the same buoyant density distribution as its cleavage products apoLp-I and apoLp-II, confirming that apoLp-II/I cleavage is a dispensable step in HDLp biogenesis (11).

dMTP was recovered in the higher density range of the gradient, mostly at a density of >1.20 g/ml (Fig. 3H), which is similar to that of the apoLp-II/I-38 proteins (Fig. 3A), demonstrating that it was not or was only poorly lipidated and that it was not secreted, by virtue of its association with apoLp-II/I lipoproteins, as already indicated by its secretion by Sf9 cells in the absence of apoLp-

II/I cotransfection (Fig. 4A). In summary, these findings show that, in a recombinant expression system, an insect MTP promotes the secretion as well as the lipidation of apoLp-II/I proteins.

DISCUSSION

It is well established that the two apolipoproteins of insect lipoprotein, apoLp-I and apoLp-II, are derived from cleavage of their precursor protein apoLp-II/I (9, 11–17). Yet, the mechanism that allows apoLp-II/I to acquire lipids and accordingly form a lipoprotein remains to be characterized. In this study, we identified clusters enriched in either amphipathic α-helices or amphipathic β-strands in apoLp-II/I and demonstrated the minimal requirement of the β cluster for biogenesis of a buoyant lipoprotein using a novel, insect cell-based recombinant expression system for insect lipoprotein (i.e., Sf9 cells transfected with dMTP and apoLp-II/I cDNA). ApoLp-II/I may acquire at least part of its bound lipids from MTP, as we found dMTP to promote the secretion and lipidation of lipoprotein. These effects of dMTP depend on the presence in apoLp-II/I of the larger part of the predicted amphipathic β cluster (i.e., in apoLp-II/I-59). Thus, insect lipoprotein biogenesis appears to involve the same major structural elements as mammalian apoB lipoprotein biogenesis (26). Moreover, in accordance with our earlier study (11), it appears that the cleavage of apoLp-II/I is not a prerequisite for lipoprotein biogenesis, because uncleaved apoLp-II/I was also lipidated and displayed a buoyant density similar to that of native HDLp.

LOCATE analysis predicts insect apoLp-II/I to contain three regions enriched in either amphipathic α-helices or amphipathic β-strands that are organized along the polypeptide as N-α₁-β-α₂-C. This tripartite organization is reminiscent of a truncated form of its vertebrate homolog apoB, which was predicted to contain a pentapartite organization, N-α₁-β₁-α₂-β₂-α₃-C (27, 28). A tripartite organization is actually revealed in apoB-48, the truncated form of apoB that is present in chylomicrons (2, 3). The β cluster of apoLp-II/I, however, is significantly longer than either of the two β clusters in apoB, whereas apoLp-II/I's α₂ cluster is only about two-thirds the size of the α₂ or α₃ clusters in apoB. Based on their sequence similarity in the N-terminal LLT module, which includes the α₁ cluster and a small part of the β₁ cluster, apoB and apoLp-II/I have been recognized as homologs (8, 33, 44). Therefore, the amphipathic clusters C-terminal from the LLT module of apoB and apoLp-II/I may also share a common evolutionary origin. From this perspective, one speculative scenario to explain these differences between apoB and apoLp-II/I amphipathic clusters would be that the β₂ and α₃ clusters in apoB have arisen from a duplication of the β₁ and α₂ clusters.

Recombinant expression of apoLp-II/I proteins demonstrates the importance of the β cluster region in the biogenesis of insect lipoprotein, as only C-terminal variants of apoLp-II/I with at least the larger part of the β cluster were secreted as buoyant lipoproteins. Therefore, the binding

of the bulk of lipids appears to be organized by the β cluster as a whole. In addition, the α_2 cluster may also enhance lipid acquisition, as suggested by the slightly lower density of apoLp-II/I-84 lipoprotein, compared with apoLp-II/I-59 lipoprotein, in the presence and the absence of dMTP coexpression. On the other hand, apoLp-II/I-84 also contains an additional ~ 250 aa of the β cluster region that might mediate the additional lipid association. Inclusion of the region C-terminal to the α_2 cluster hardly affects lipoprotein density. This region includes the vWF-D module (8), which apparently does not function in lipid binding.

After the cleavage of apoLp-II/I into apoLp-I and apoLp-II (11), the β cluster is almost entirely situated in apoLp-I. From this perspective, the finding that inclusion of the β cluster enables HDLp biogenesis suggests that apoLp-I, and not apoLp-II, binds the vast majority of lipids. This finding is in accordance with HDLp dissociation experiments in which $>98\%$ of the total lipid in lipophorin remained associated with apoLp-I (45). Nonetheless, apoLp-I and apoLp-II most probably cannot be regarded as independent entities during insect lipoprotein biogenesis, as lipidation likely occurs before the cleavage of apoLp-II/I and the apoLp-I and apoLp-II regions are intimately linked within the structure of the LLT module (11).

Whereas insect lipoprotein appears to bind lipids via the amphipathic β cluster in apoLp-II/I, our results also suggest that insect MTP enhances the lipid transfer to apoLp-II/I. Coexpression of dMTP clearly stimulated the lipidation of apoLp-II/I-59, apoLp-II/I-84, and apoLp-II/I-100, as judged from their shift to a lower buoyant density. This stimulation is dependent on the presence of the larger part of the β cluster, as apoLp-II/I-38 failed to form a buoyant lipoprotein regardless of dMTP coexpression. In addition, dMTP coexpression resulted in a several-fold increase in the secretion of recombinant apoLp-II/I proteins for all of the variants studied (Table 1). Similarly, MTP coexpression enhances the secretion of apoB truncations longer than the N-terminal 1,000 aa of apoB (46, 47). Our finding for apoLp-II/I secretion in the absence of dMTP coexpression is noteworthy in the light of the inability of mammalian cells to secrete apoB in the absence of MTP (32, 46–48). The secretion and limited lipidation of apoLp-II/I variants by Sf9 cells in the absence of dMTP coexpression may indicate that MTP is not an absolute requirement in insect HDLp biogenesis. However, it cannot be excluded that Sf9 cells express an endogenous MTP, especially given that Sf9-derived cells have been reported to possess apoB lipoprotein assembly capacity (47). In any case, dMTP coexpression clearly enhanced the cellular capacity of Sf9 cells to produce HDLp, suggesting that also in vivo, MTP functions in insect lipoprotein biogenesis.

Given the specific features of insect lipoproteins and lipid transport, it appears unlikely that the mechanism of apoLp-II/I lipoprotein biogenesis is identical to that of apoB lipoprotein biogenesis. For one, upon secretion, insect lipoprotein contains far fewer lipid molecules than

apoB lipoproteins (i.e., several hundred vs. $>1,000$, respectively). ApoB acquires the majority of these lipids after its cotranslational lipidation by fusion of the primordial lipoprotein with an intraluminal neutral lipid droplet, resulting in a very low density lipoprotein (1–7). Therefore, the relatively modest lipidation of apoLp-II/I to a high density lipoprotein could result from the absence of this second step in insect lipoprotein biosynthesis. In addition, the major lipid components in newly biosynthesized insect HDLp and mammalian apoB lipoprotein are diacylglycerol and triacylglycerol, respectively (15, 21), suggesting that the lipoprotein-producing cells of insects and mammals differ in their intracellular lipid mobilization. The lipid transfer activity of MTP may have been optimized accordingly, as suggested by the absence of in vitro triacylglycerol transfer activity by dMTP, in contrast to human MTP (32, 43, 47). The presence of several structural divergences in dMTP compared with human MTP does suggest its specific adaptation (33).

In conclusion, we propose that lipoprotein biogenesis in insects depends on the same major structural elements as in mammals (i.e., MTP as well as amphipathic structures in the carrier apolipoprotein). This implies an early metazoan origin for the current mechanisms of lipoprotein assembly. Specific features of insect lipoprotein (e.g., binding fewer and different lipids) appear to result from the divergence and adaptation of the function of proteins containing an LLT module (i.e., a large lipid transfer protein) over time (33, 44). Thus, further research on the mechanism of insect lipoprotein biosynthesis may provide novel venues to manipulate the biosynthesis of apoB lipoproteins and accordingly contribute to the treatment of atherosclerosis and other lipid disorders. ■

The authors thank Jan M. Van Doorn and Marcelle A. M. Kasperaitis for technical assistance and Jere P. Segrest and Martin K. Jones (Department of Medicine, University of Alabama at Birmingham Medical Center) for providing the program LOCATE. The authors thank Gregory S. Shelness (Department of Pathology, Wake Forest University School of Medicine) for valuable discussions and Carol C. Shoulders (Medical Research Council Clinical Sciences Centre, Imperial College London) for critical reading of the manuscript. This work was supported by the Department of Biology of Utrecht University.

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