Biosynthesis and secretion of insect lipoprotein: involvement of furin in cleavage of the apoB homolog, apolipophorin-II/I

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Abstract **The biosynthesis of neutral fat-transporting lipoproteins involves the lipidation of their nonexchangeable apolipoprotein. In contrast to its mammalian homolog apolipoprotein B, however, insect apolipophorin-II/I (apoLp-II/I) is cleaved posttranslationally at a consensus substrate sequence for furin, resulting in the appearance of two apolipoproteins in insect lipoprotein. To characterize the cleavage process, a truncated cDNA encoding the N-terminal 38% of *Locusta migratoria* apoLp-II/I, including the cleavage site, was expressed in insect Sf9 cells. This resulted in the secretion of correctly processed apoLp-II and truncated apoLp-I. The cleavage could be impaired by the furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (decRVKRcmk) as well as by mutagenesis of the consensus substrate sequence for furin, as indicated by the secretion of uncleaved apoLp-II/I-38. Treatment of *L. migratoria* fat body, the physiological site of lipoprotein biosynthesis, with decRVKRcmk similarly resulted in the secretion of uncleaved apoLp-II/I, which was integrated in lipoprotein particles of buoyant density identical to wild-type high density lipophorin (HDLp). These results show that apoLp-II/I is posttranslationally cleaved by an insect furin and that biosynthesis and secretion of HDLp can occur independent of this processing step. Structure modeling indicates that the cleavage of apoLp-II/I represents a molecular adaptation in homologous apolipoprotein structures. We propose that cleavage enables specific features of insect lipoproteins, such as low density lipoprotein formation, endocytic recycling, and involvement in coagulation.**—Smolenaars, M. M. W., M. A. M. Kasperaitis, P. E. Richardson, K. W. Rodenburg, and D. J. Van der Horst. **Biosynthesis and secretion of insect lipoprotein: involvement of furin in cleavage of the apoB homolog, apolipophorin-II/I.** ***J. Lipid Res.*** **2005.** 46: **412–421.**

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Lipoproteins mediate most of the lipid transport in the circulation of animals. In mammals, a single protein component, the nonexchangeable apolipoprotein B (apoB), provides the structural basis for the biosynthesis of neutral fat-transporting lipoproteins (1, 2). Interestingly, the major lipoprotein of insects, lipophorin, contains two structural apolipoproteins, because the insect apoB homolog (3, 4), the precursor apolipophorin-II/I (apoLp-II/I), is cleaved during lipoprotein biosynthesis by the fat body (5, 6).

Cleavage of apoLp-II/I can be related to the activity of furin, a member of the proprotein convertase (PC) family of subtilisin-like serine endoproteases that is mainly active in the *trans*-Golgi network (7). The preferred consensus substrate sequence for furin, R-X-K/R-R, is present in all apoLp-II/I sequences characterized to date (8–11). In agreement with the activity of furin, *Locusta migratoria* apoLp-II/I appears to be cleaved immediately C terminal of its furin substrate sequence, RQKR^{720}, as indicated by the N-terminal sequence of apoLp-I (10).

The predicted furin cleavage site in each insect apoLp-II/I is located in the large lipid transfer (LLT) domain, which constitutes the N-terminal region of apoLp-II/I that has sequence homology to that of apoB, the microsomal triglyceride transfer protein (MTP) large subunit, and vitellogenin (3, 4). In apoB, this domain is essential for lipoprotein biosynthesis. The interaction between the LLT domain of apoB and that of the MTP large subunit enables the assembly of apoB-containing lipoproteins (1, 2). The homology between apoB and apoLp-II/I, as well as the presence of an MTP large subunit in insects (12), suggest that the LLT domain of apoLp-II/I enables lipoprotein

Abbreviations: apoB, apolipoprotein B; apoLp, apolipophorin; decRVKRcmk, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone; HDLp, high density lipophorin; LDLp, low density lipophorin; LDLR, low density lipoprotein receptor; LLT, large lipid transfer; MTP, microsomal triglyceride transfer protein; PC, proprotein convertase.

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biosynthesis in insects as well. Therefore, we hypothesized that the cleavage of apoLp-II/I in the LLT domain functions in the biosynthesis and secretion of insect lipoprotein.

In the present report, we characterize the involvement of insect furin in the cleavage of *L. migratoria* apoLp-II/I and investigate the importance of this posttranslational modification to insect lipoprotein biosynthesis and secretion. To this end, apoLp-II/I cleavage was investigated in a recombinant insect expression system for truncated apoLp-II/I as well as in the locust fat body, the insect tissue that expresses apoLp-II/I and secretes its cleavage products apoLp-I and -II together in the form of a high density lipophorin (HDLp) particle (6, 13). The results indicate that *L. migratoria* apoLp-II/I is cleaved by an insect furin. Uncleaved apoLp-II/I could be secreted and formed a high density lipoprotein. We conclude that cleavage of apoLp-II/I is not required for the biosynthesis and secretion of mature lipophorin. Rather, this posttranslational modification may represent a molecular adaptation enabling specific features of insect lipoproteins. Modeling of apoLp-II/I to the available homologous structures, the lipovitellin crystal structure and an apoB model, indicated the position of the cleavage site in the apoLp-II/I structure, and putative functions for apoLp-II/I cleavage are discussed accordingly.

MATERIALS AND METHODS

Construction of the apoLp-II/I-38 expression plasmid

Standard cloning and sequencing procedures (10, 14) were used to obtain an expression construct for *L. migratoria* apoLp-II/I. The apoLp-II/I-38 truncation variant was constructed by cloning the large *Eco*RI-*Xho*I cDNA fragment from pBK-CMV cDNA clone B20 (10) into pGEM7-Zf+ (Promega). Subsequently, this fragment was transferred to plasmid pALTER-1 (Promega) using *Xba*I and *Sma*I digestion. The *Kpn*I-*Xho*I fragment of this plasmid, encoding apoLp-II/I, was cloned into the insect cell expression vector pIZ/V5-His (Invitrogen) using the same restriction sites, yielding plasmid pIZ/V5-His-mod1. Subsequently, a short *Xho*I-*Sac*II primer fragment, containing a *Bgl*II site, was cloned into pIZ/V5-His-mod1, 3′ of the cDNA, to allow fusion of the open reading frame of the apoLp-II/I cDNA sequence with the V5 epitope and His tag, yielding pIZ/V5-His-mod1* (oligonucleotide sequences of the *Xho*I-*Sac*II primer fragment are 5′-TCGAGGCATGCAGATCTGGCCCGC-3′ and 5′-GGGCCAGATCTGCATGCC-3′). In addition, the *Xho*I-*Cla*I apoLp-II/I cDNA fragment from pBK-CMV cDNA clone C5 (10) was cloned into pGEM7-Zf+, using the *Xho*I and *Cla*I restriction sites, to yield pGEM7-mod2. In a final cloning step, the *Xho*I-*Bam*HI fragment from pGEM7-mod2 was cloned into pIZ/V5-His-mod1* using the unique *Xho*I and *Bgl*II sites of the latter plasmid. DNA sequencing of this plasmid confirmed that the resulting construct in pIZ/V5-His encodes the apoLp-II/I amino acid residues 1–1,287, joined in frame at the C terminus with a vector-encoded V5 epitope as well as a 6×His tag (**Fig. 1A**). Excluding the 21 residue signal peptide (10), this apoLp-II/I truncation variant corresponds to 38% of the total precursor protein and is referred to as apoLp-II/I-38.

Mutagenesis of apoLp-II/I-38

The basic amino acid residues at the P1, P2, and P4 positions (15) within the predicted furin cleavage site RQKR^{720} in apoLp-II/I-38 were changed using the QuickChange Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer's instructions. Mutants (Fig. 1B) were created using the following forward primers, in combination with their complement reverse primers (codons to be modified are underlined; nucleotide positions to be mutated are shown in boldface): QQKR, 5′-ACAGAGAGATTTGAGAAAACATTC**CA**ACAGAAACGATCGGTTTC-3′; DQKR, 5′-CAGAGAGATTTGAGAAAACATTC**GAC**CAGAAACGATCGGTTTCAAAAGATGC-3′; RQAR, 5′-GAGATTTGAGAAAACATTCAGACAG**GC**ACGATCGGTTTCAAAAGATGCTG-3′; RQKQ, 5′-CACAGAGAGATTCGAGAAGACATTCAGACAGAAACAATCGGTTTC-3′; QQAQ, 5′-CACAGAGAGATTCGAGAAGACATTC**CA**ACAG**GC**AC**A**ATCGGTTTC-3′. The forward and reverse primers were used in a PCR procedure of 18 cycles (1 min at 95°C, 45 s at 58°C, and 14 min at 68°C) with 10 ng of the apoLp-II/I-38 construct as the DNA template. After *Dpn*I digestion, the reaction contents were used to transform into XL1-Blue cells (Stratagene). Single zeocin-resistant colonies were cultured and plasmids were isolated. The introduction of mutations in these plasmids was verified by DNA sequencing.

Stable expression of apoLp-II/I-38 constructs in the Sf9 cell line

Spodoptera frugiperda Sf9 cells were maintained in adherent culture in serum-free Insect-Xpress medium (Cambrex) in polystyrene flasks (Greiner) at 27°C in a humidified atmosphere and passed twice each week. Transfections with the wild-type and mutant apoLp-II/I-38 constructs were performed using Cellfectin reagent (Invitrogen) according to the manufacturer's instructions. Stable transformants were selected using 400 μg/ml zeocin (Cayla) and maintained at 100 μg/ml zeocin. Experiments were performed in 25 cm^2 flasks with cells grown to 80% confluence. Incubations with the furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (decRVKRcmk; Bachem) were performed by including either the inhibitor or its solvent methanol in fresh culture medium for 6 h. Culture media were collected and cleared from whole cells and cellular debris by centrifugation (1,000 *g*, 10 min; 22,000 *g*, 10 min, 4°C), and analyzed for apoLp-II/I-38 and cleavage products by immunoblotting.

In vitro incubation of *L. migratoria* fat body tissue

L. migratoria were reared in cages under crowded conditions at 30°C, 40% relative humidity, a light-dark cycle of 12 h/12 h, and a diet of reed grass supplemented with rolled oats. Fat bodies were dissected from day 9 adult males, separated in two halves along the length, and rinsed in saline buffer (10 mM HEPES, 150 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.0). Subsequent incubations were performed in a shaking water bath at 32°C. After washing for 1 h at 32°C in 4 ml of saline buffer, halves of six fat bodies were transferred to 250 μl of fresh saline buffer with 30 μCi of [^{35}S]Met/Cys (Promix; Amersham) supplemented with either 100 μM decRVKRcmk or an equivalent volume of its solvent methanol. Proteins in the incubation medium were analyzed for apoLp-II/I and cleavage products by immunoblotting.

Density gradient analysis

The buoyant density of secreted apoLp-II/I and cleavage products was compared by subjecting the incubation media of decRVKRcmk-treated and control fat body tissue to KBr density gradient ultracentrifugation (13). After gravimetric analysis of density, fractions were assessed for the presence of apoLp-II/I and cleavage products by immunoblotting. Radiolabeled proteins on the blot were visualized by phosphorimaging on a Molecular Imager FX system with Quantity One software (Bio-Rad).

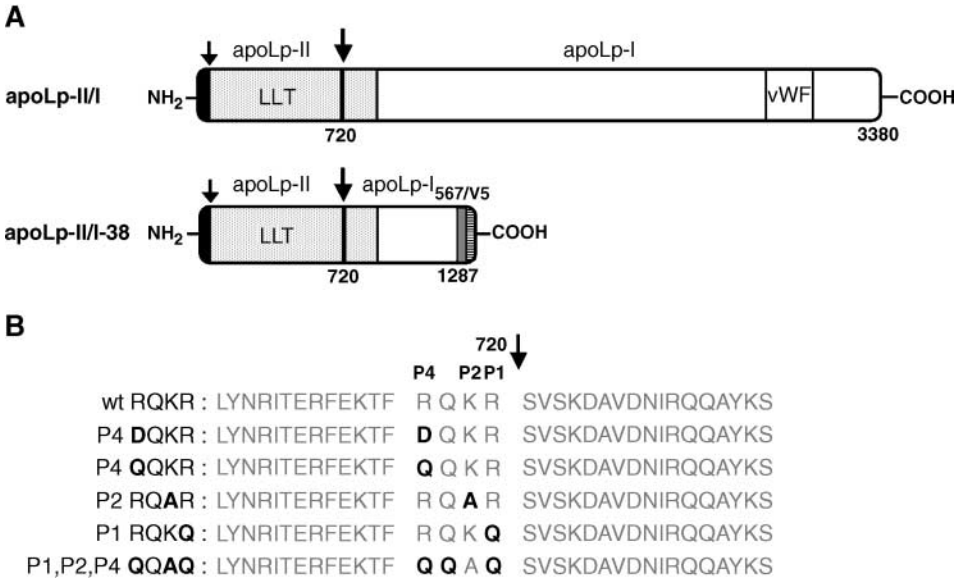

Fig. 1. Expression constructs to characterize apolipophorin-II/I (apoLp-II/I) cleavage. A: The apoLp-II/I-38 cDNA construct encodes the N-terminal 38% of *L. migratoria* apoLp-II/I, including the cleavage site, which is preceded by the consensus substrate sequence for furin at amino acids 717–720, and followed by 567 amino acids of apoLp-I C-terminally fused to the V5 epitope and the 6×His tag (referred to as apoLp-$I_{567/V5}$). LLT, large lipid transfer domain; vWF, von Willebrand factor module D domain. The small arrows indicate the predicted signal peptide cleavage site, and the larger arrows indicate the putative site of cleavage by furin. B: Single and multiple mutations were introduced in the sequence of the apoLp-II/I-38 construct encoding the putative cleavage site at amino acids 717–720. P1 to P4 represent amino acid positions sequentially numbered starting N terminal from the site of apoLp-II/I cleavage (15). For each mutant, the residues 704–737 are shown, with mutated residues in boldface. The putative site of cleavage is indicated by the arrow.

Immunoblot analysis of apoLp-II/I and cleavage products

Proteins were precipitated from incubation media by the addition of TCA to a concentration of 5%. Pellets were resuspended and heated (10 min at 95°C) in Laemmli sample buffer that was modified by the addition of 5% (v/v) 1 M Tris to circumvent acidification by residual TCA. Protein samples were separated using SDS-PAGE (9% slab for transfected cell culture media; 4–10% slab for fat body tissue incubation media). Precision Plus Protein Dual Color Prestained Standards (Bio-Rad) were used as a protein molecular mass marker, and isolated wild-type *L. migratoria* HDLp (6) was used as a positive control. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore), and subsequent immunostaining was performed as described (16). Primary antibodies (1:10,000 dilution) were monoclonal α-V5 (Invitrogen), polyclonal α-II or α-I (17), or polyclonal α-IIC and α-IN (raised in rabbits against the peptides CKSLYNRITERFEKTFRQKR and SVSKDAVDNIRQQAYKSLLC, respectively, which correspond to the C terminus of apoLp-II and the N terminus of apoLp-I, excluding the terminal cysteines). Alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) was used as a secondary antibody (1:10,000 dilution).

Modeling of *L. migratoria* apoLp-II/I

The *L. migratoria* apoLp-II/I sequence was used to identify homologous proteins and conserved domains using the web-based BLASTp program from the National Center for Biotechnology (18). The default settings were used for the BLASTp search. The most significant matches were to sequences from apoLp-II/I, apoB, and vitellogenin, including silver lamprey lipovitellin. Sequence homologies were located within the first 1,000 amino acid residues.

The alignment program CLUSTAL W (19) was used to align the homologous N-terminal regions of silver lamprey lipovitellin, human apoB-100, and *L. migratoria* apoLp-II/I. This alignment was used to generate the structural model of the first 1,009 amino acids (excluding the signal peptide) of locust apoLp-II/I using the program Modeller6 (20), based on the crystal structure of silver lamprey lipovitellin (PDB 1LSH) (21) and an all atom model for apoB (22). To eliminate steric problems and to optimize bond lengths and angles, the apoLp-II/I model was subjected to 250 steps of steepest descent energy minimization using the DISCOVER program package from INSIGHT2 (Accelerys, Inc.). A graphic representation of the model was generated by the Swiss-PdbViewer (23).

RESULTS

Expression and proteolytic processing of apoLp-II/I-38 by Sf9 cells

To study the sequence characteristics of apoLp-II/I that enable posttranslational cleavage, an apoLp-II/I truncation was recombinantly expressed in the insect Sf9 cell line, which expresses a furin homolog (24). The construct used encodes the N-terminal 38% of apoLp-II/I (Fig. 1A). This apoLp-II/I-38 polypeptide includes the signal peptide, the complete sequence for apoLp-II with the consensus substrate sequence for furin, $RQKR^{720}$, followed by 567 N-terminal residues from apoLp-I, fused to the V5 epitope and a 6×His tag (referred to as apoLp-$I_{567/V5}$).

Stable transfection of Sf9 cells with this apoLp-II/I-38

construct resulted in the secretion of apoLp-II and apoLp-$I_{567/V5}$ cleavage products into the incubation medium, as demonstrated by immunoblot analysis (**Fig. 2A**). Secretion of apoLp-$I_{567/V5}$ is indicated by the single reactive band obtained with antibodies directed against apoLp-I (α-IN and α-I; Fig. 2A, lanes 4 and 5) as well as the V5 epitope (α-V5; Fig. 2A, lane 6) at ~66 kDa, similar to the estimated molecular mass of apoLp-$I_{567/V5}$. The weak immunoreactivity of polyclonal α-I (Fig. 2A, lane 5) likely reflects the limited representation of its epitopes in apoLp-$I_{567/V5}$. Secretion of recombinant apoLp-II is indicated by immunoblotting with antibodies directed against apoLp-II (α-II and α-IIC; Fig. 2A, lanes 2 and 3), which results in a single immunoreactive band at ~72 kDa. This recombinant protein appears to migrate identically to the apoLp-II from purified *L. migratoria* HDLp (Fig. 2A, lane 1 vs. lane 2). This similar size indicates that recombinant apoLp-II is similarly glycosylated as wild-type apoLp-II. Indeed, deglycosylation with endoglycosidase H resulted in a single additional apoLp-II immunoreactive band with a decreased molecular mass of ±3 kDa (see supplementary Fig. I), which is similar to that reported for wild-type locust apoLp-II (6). Moreover, the similar migration behavior of recombinant and wild-type apoLp-II indicates that the cleavage of apoLp-II/I-38 proceeds identical to that in fat body (6, 10). Expression of a construct expressing the N-terminal 33% of apoLp-II/I in Sf9 cells, and apoLp-II/I-38 in *Drosophila melanogaster* S2 cells, also resulted in the secretion of the two expected apoLp-II/I cleavage products (data not shown).

Recombinant apoLp-II/I-38 is cleaved at the same site as apoLp-II/I in *L. migratoria*. The α-IIC and α-IN antibodies, raised against synthetic oligopeptides of the sequences that flank the cleavage site N- and C-terminally, specifically recognize recombinant apoLp-II and apoLp-$I_{567/V5}$, respectively (Fig. 2A, lanes 3 and 4). In addition, mutation of the three basic residues in the putative furin cleavage site (yielding the mutant designated $QQAQ^{720}$; Fig. 1B) results in the secretion of uncleaved apoLp-II/I-38 (Fig. 2B), as shown by the single immunoreactive band obtained with α-II, α-IIC, α-I, α-IN, and α-V5 antibodies at ~145 kDa (Fig. 2B, lanes 1–5, respectively), similar to the combined molecular mass of apoLp-II and apoLp-$I_{567/V5}$. The limited immunoreactivity of α-IIC antibody against mutant apoLp-II/I (Fig. 2B, lane 2) likely relates to the introduced mutations. In further experiments, the degree of apoLp-II/I-38 cleavage was quantified using α-V5 antibody. Thus, Sf9 cells cleave recombinant apoLp-II/I-38 into apoLp-II and apoLp-$I_{567/V5}$ at the consensus substrate sequence for furin, similar to apoLp-II/I in locust fat body. These results validate the use of this expression system to characterize apoLp-II/I cleavage.

The secreted recombinant apoLp-II/I-38 cleavage products apoLp-II and apoLp-$I_{567/V5}$ were further characterized for complex formation and lipidation. The coelution of apoLp-II with apoLp-$I_{567/V5}$ after affinity chromatography suggests that both cleavage products can form a complex (see supplementary Fig. II). Upon density gradient ultracentrifugation, the cleavage products apoLp-II and apoLp-$I_{567/V5}$ were found together in the fractions with densities between 1.20 and 1.25 g/ml, as was uncleaved mutant apoLp-II/I-38 ($QQAQ^{720}$) (see supplementary Fig. III). These results indicate that the secreted recombinant apoLp-II/I-38 products are poorly lipidated. Therefore, the hypothesized role for apoLp-II/I cleavage in lipidation was assessed in *L. migratoria* fat body tissue, whereas the present expression system was used to characterize apoLp-II/I cleavage.

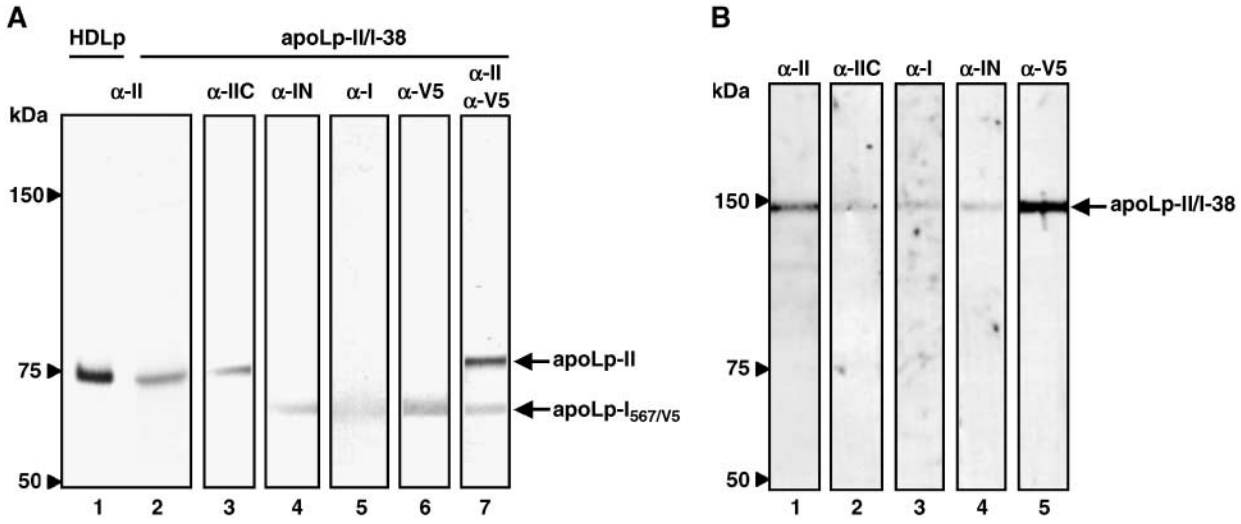

Fig. 2. Recombinant expression and cleavage of apoLp-II/I-38. Incubation medium from stably transfected Sf9 cells was analyzed for the presence of the putative apoLp-II/I-38 cleavage products, apoLp-II and apoLp-$I_{567/V5}$, using antibodies directed against either apoLp-II (α-II and α-IIC) or apoLp-I (α-I, α-IN, and α-V5). A: Immunoblot analysis of media from Sf9 cells stably transfected with the apoLp-II/I-38 construct. B: Immunoblot analysis of medium from Sf9 cells stably transfected with the apoLp-II/I-38 construct in which the cleavage site was mutated from the wild-type sequence $RQKR^{720}$ to $QQAQ^{720}$. Molecular mass standards are indicated at left. Arrows mark the positions of apoLp-II/I-38 and its cleavage products. For reference, lane numbers are indicated at bottom.

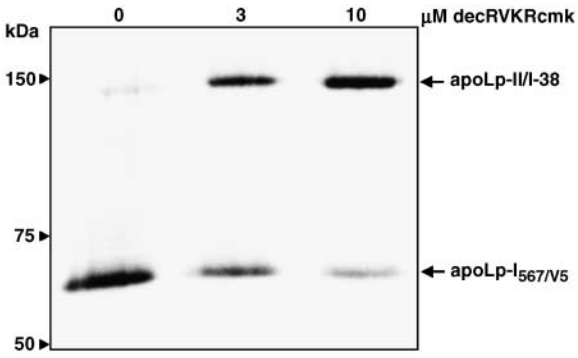

Fig. 3. Effect of the proprotein convertase inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (decRVKRcmk) on the secretion of recombinant apoLp-II/I-38. Sf9 cells stably transfected with apoLp-II/I-38 were incubated for 6 h with decRVKRcmk at concentrations of 0, 3, and 10 μM. Incubation media were analyzed for cleavage of apoLp-II/I-38 by immunoblot analysis with α-V5 antibody. Molecular mass standards are indicated at left, and decRVKRcmk concentrations are shown above the blot. Arrows mark the positions of apoLp-II/I-38 and apoLp-$I_{567/V5}$.

An inhibitor implicates PCs in the cleavage of recombinant apoLp-II/I

DecRVKRcmk is a modified tetrapeptide that irreversibly inhibits a wide range of PCs, including furin (25) and Sfurin, the furin homolog characterized from the insect Sf9 cell line (24). Incubation of Sf9 cells, transfected with the apoLp-II/I-38 construct, with micromolar concentrations of decRVKRcmk resulted in the secretion of uncleaved apoLp-II/I-38, whereas secretion of both apoLp-II and apoLp-$I_{567/V5}$ was concomitantly and dose-dependently decreased, as judged by the immunoreactivity of α-V5 (**Fig. 3**) and α-II (data not shown). Based on the amount of secreted protein, cleavage appeared to be prevented for up to 90% at a concentration of 10 μM decRVKRcmk. The sensitivity to decRVKRcmk indicates the involvement of a PC in the cleavage of apoLp-II/I.

A consensus substrate sequence for furin is required to efficiently process recombinant apoLp-II/I

To provide further evidence for the involvement of PCs in apoLp-II/I-38 cleavage, a negatively charged amino acid was introduced at position P4 (Fig. 1B), which is expected to disturb binding by PCs via charge repulsion at the S4 binding pocket (26–28). As demonstrated by immunoblot analysis of media from stably transfected Sf9 cells using α-V5 antibody, introduction of Asp at the P4 position (R to D; DQKR^{720}) completely prevented cleavage (**Fig. 4A**), implicating the involvement of a PC in the cleavage of apoLp-II/I.

To further identify the sequence that directs the cleavage of apoLp-II/I, the basic residues in the furin consensus substrate sequence of apoLp-II/I-38 (RQKR^{720}) were mutated to neutral (uncharged) amino acids (Fig. 1B). Mutagenesis at the P1 position (R to Q; RQKQ^{720}) abrogated cleavage of apoLp-II/I-38 (Fig. 4B). In contrast, mutation of the cleavage site residues at the P2 (K to A; RQAR^{720}) or P4 (R to Q; QQKR^{720}) position prevented cleavage only partially, reminiscent of the inefficient cleavage at imperfect cleavage sites described for mammalian furins (26, 29). Again, impairment of cleavage did not hamper the secretion of truncated apoLp-II/I by the recombinant expression system (Fig. 4B). These results demonstrate that efficient apoLp-II/I cleavage in Sf9 cells requires the presence of the preferred consensus substrate sequence for furin, R-X-K/R-R, in apoLp-II/I. This indicates the involvement of an insect furin in apoLp-II/I cleavage.

Uncleaved native apoLp-II/I is secreted and forms a stable lipoprotein

To validate the involvement of an insect furin in lipophorin biosynthesis, and to establish the importance of

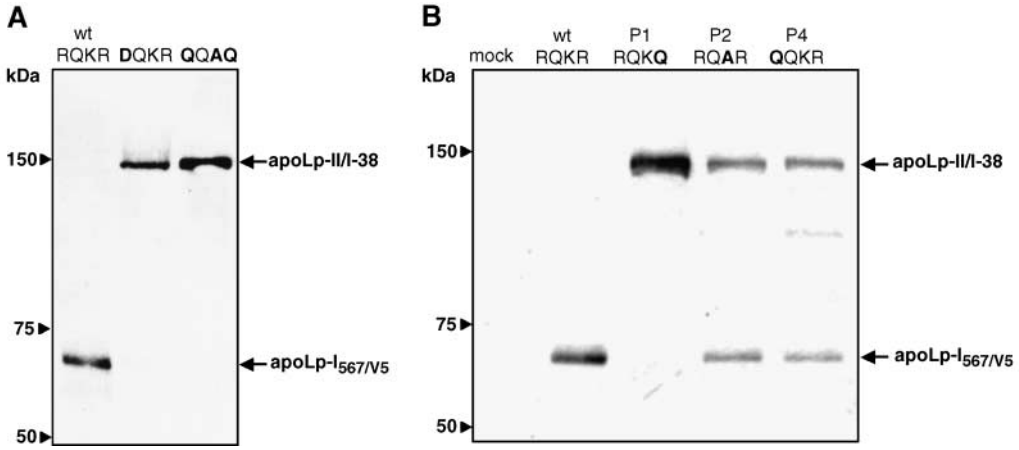

Fig. 4. Cleavage of recombinant apoLp-II/I-38 upon modification of the consensus cleavage site for furin. Sf9 cells were stably transfected with wild-type (wt) and mutant apoLp-II/I-38 constructs (designated as in Fig. 1B), and incubation media were analyzed for cleavage of apoLp-II/I-38 by immunoblotting with α-V5 antibody. A: Effect of a negatively charged amino acid at the P4 position, resulting in the mutant DQKR^{720}. Wild-type and QQAQ^{720} apoLp-II/I-38 transfectants were included to mark uncleaved and cleaved apoLp-II/I-38 products. B: Effect of the mutation of basic residues in the furin consensus substrate sequence to amino acids neutral of charge. Molecular mass standards are indicated at left. Arrows mark the positions of apoLp-II/I-38 and apoLp-$I_{567/V5}$. Mutated residues are shown in boldface.

cleavage for (apo)lipoprotein secretion in a physiological situation, fat body tissue from adult male locusts was treated in vitro with decRVKRcmk. Experiments showed the release of large amounts of HDLp that had adhered to fat body tissue. Therefore, [^{35}S]Met/Cys was included in incubation media to label newly biosynthesized proteins.

To identify secreted apoLp-II/I, incubation media were submitted to density gradient ultracentrifugation, and the resulting fractions were analyzed for radiolabeled proteins by phosphorimaging. Compared with control-treated fat body tissue, incubation with 100 μM decRVKRcmk resulted in the appearance of an additional radiolabeled band of high molecular mass and a concomitant decrease in the putative apoLp-I, in fractions with an average buoyant density of 1.12 ± 0.01 g/ml (**Fig. 5A**), identical to the density previously reported for wild-type HDLp (13). The identity of the radiolabeled proteins in these fractions as apoLp-II/I and apoLp-I was confirmed by immunoblotting with α-I (Fig. 5B). Although apoLp-II could not readily be identified among other labeled proteins, immunoblotting demonstrated its presence in the same fractions as apoLp-II/I and apoLp-I (data not shown). The sensitivity of apoLp-II/I cleavage to decRVKRcmk incubation (~60% with 100 μM inhibitor; Fig. 5A) was reduced in the fat body, compared with the recombinant expression system (~90% with 10 μM inhibitor; Fig. 3). This difference may reflect reduced delivery of decRVKRcmk within the fat body, as the hydrophobic decanoyl group of this inhibitor may cause it to accumulate at the surface of the lipid droplets in this tissue. Thus, fat body tissue can secrete uncleaved apoLp-II/I with a buoyant density identical to wild-type HDLp. Gel filtration chromatography indicates that this secreted apoLp-II/I forms particles with a molecular size identical to wild-type HDLp (data not shown). Together, these results demonstrate that fat body can secrete uncleaved apoLp-II/I that has been integrated in a high density lipoprotein similar to wild-type HDLp. Apparently, the biosynthesis as well as secretion of insect lipoprotein can occur independently of apoLp-II/I cleavage.

Homology modeling of apoLp-II/I indicates the position of the cleavage site

The ability of uncleaved apoLp-II/I to form a lipoprotein highlights the structural homology between insect and mammalian apoB-containing lipoproteins in the region of cleavage, but it also provokes questions regarding its function. To obtain a better understanding of the structural consequences of cleavage, *L. migratoria* apoLp-II/I was modeled to the available homologous structures, the lipovitellin crystal structure (21) and an apoB model (22). The obtained apoLp-II/I model encompasses amino acids 22–1,030 and therefore the complete LLT domain. This apoLp-II/I sequence can form a structure similar to that of the corresponding lipovitellin and apoB regions, as indicated by the recognition of three antiparallel β-sheets

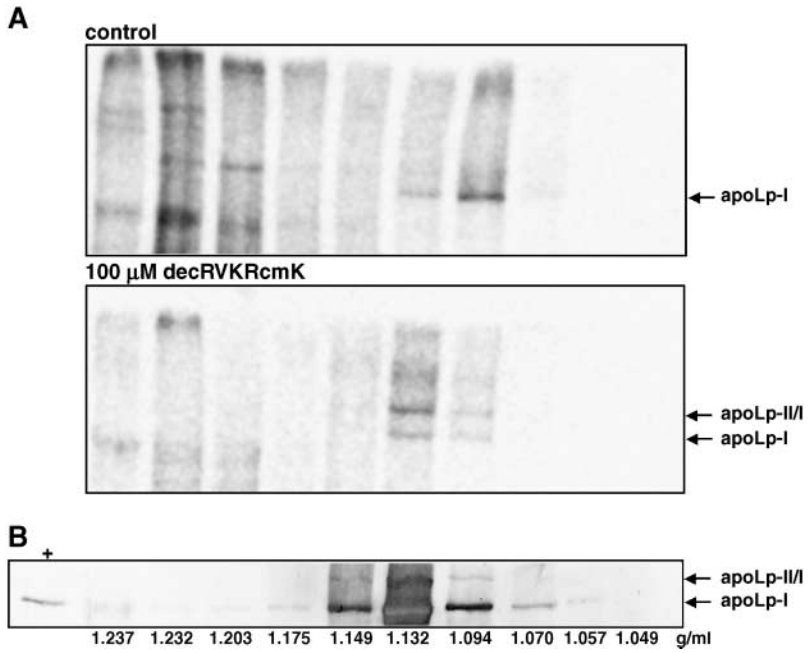

Fig. 5. Buoyant density of apoLp-II/I secreted by fat body. A: Fat body tissue was labeled using [^{35}S]Met/Cys and incubated with 0 (control) or 100 μM decRVKRcmk. Incubation media were subsequently submitted to density gradient ultracentrifugation, fractions were analyzed by SDS-PAGE, and labeled proteins were visualized by PhosphorImager screen autoradiography. The signals of labeled proteins with molecular masses >150 kDa are shown for both the control and 100 μM decRVKRcmk incubations, as indicated. B: Immunoblot detection of apoLp-II/I in ultracentrifugation fractions from decRVKRcmk-treated fat body tissue incubation media, using α-I antibody. Isolated wild-type high density lipophorin was used as a positive control (as indicated by the plus sign). Arrows mark the positions of apoLp-I and apoLp-II/I. The density (g/ml) of each fraction is indicated at bottom.

(βA, βB, and βC) and an extensive α-helical structure (**Fig. 6**). The βA and βB sheets face each other to form a putative lipid pocket and are partly surrounded by the α-helical region. The βC sheet is curved, resulting in a barrel-like structure, which is situated above the putative lipid pocket. The apoLp-II/I cleavage site, located between residues 720 and 721, was situated in an extended loop region (residues 669–748) that connects two β-strands of the βA sheet at the base of the putative lipid pocket. This region could not be modeled confidently. The corresponding sequence was not resolved in the crystal structure of silver lamprey lipovitellin either (21), which is suggestive of structural flexibility in this region of lipovitellin and possibly also in the region of apoLp-II/I cleavage. According to the present model, apoLp-II includes the βC sheet, the α-helical region, and most of the βA sheet, whereas apoLp-I consists of two distal β-strands from the βA sheet, the full βB sheet, and further unmodeled C-terminal sequences.

DISCUSSION

It is well established that insect lipoproteins contain two apolipoproteins, apoLp-I and apoLp-II, that are derived from their common precursor protein apoLp-II/I (6, 8–11). In the present study, we identified and characterized the cleavage site in *L. migratoria* apoLp-II/I using an insect recombinant expression system of Sf9 cells and demonstrated that its cleavage likely involves an insect furin. As locust fat body can secrete uncleaved apoLp-II/I in the form of a lipoprotein particle with a buoyant density and molecular mass identical to wild-type HDLp, we conclude that cleavage of apoLp-II/I by insect furin is not required for the biosynthesis or the secretion of this insect lipoprotein.

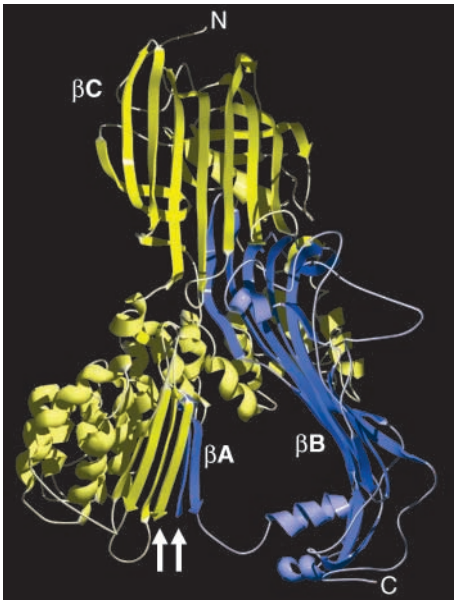

Fig. 6. Model of *L. migratoria* apoLp-II/I. The model of locust apoLp-II/I includes amino acid residues 22–1,030 and was constructed based on sequence homology with silver lamprey lipovitellin and human apolipoprotein B, for which an all atom structure and a model are available, respectively (21, 22). The structures that are part of apoLp-I and apoLp-II after apoLp-II/I cleavage are marked in shades of blue and yellow, respectively. The three β-sheets are indicated by βA, βB, and βC. N and C mark the amino- and carboxyl-terminal sides of the modeled region, respectively. The arrows indicate the β-strands at the base of the putative lipid pocket that are connected by amino acid residues 669–748. ApoLp-II/I is cleaved within this region, between residues 720 and 721.

The involvement of an insect furin in the cleavage of *L. migratoria* apoLp-II/I is indicated by three results. First, apoLp-II/I cleavage in both the Sf9 recombinant expression system and locust fat body can be inhibited by decRVKRcmk, which specifically inhibits PCs (25), including Sfurin, the furin homolog characterized from the insect Sf9 cell line (24). Second, cleavage is completely prevented by mutation of the basic amino acid residue (Arg) at the P4 position to a negatively charged amino acid residue (Asp). Based on the presence of a negatively charged residue at the S4 substrate binding pocket of PCs, this mutation is predicted to impair the substrate interaction via charge repulsion (26–28). Third, complete proteolytic processing requires an intact furin consensus substrate sequence with basic amino acid residues at P1, P2, and P4. Mutation of any of these sites into amino acids neutral of charge results in the secretion of uncleaved apoLp-II/I, yet some cleavage does occur in the P2 (Lys→Ala) and P4 (Arg→Gln) mutants. Mammalian furin is known to cleave, with reduced activity, at sequences that partially match the preferred R-X-K/R-R substrate sequence (26, 29). Likewise, the partial cleavage in these P2 and P4 mutants may result from limited activity of furin at nonideal sites. Together, these results indicate cleavage of apoLp-II/I by an insect furin.

Several furin homologs have been identified in insects. In *D. melanogaster*, cleavage activity and homology have been demonstrated for the dfurin1 and dfurin2 gene products (30–32). Furthermore, a furin was cloned from the insect Sf9 cell line (24). Moreover, a PC with furin-like activity is present in the fat body of *Aedes aegypti* (33). However, no putative furin has yet been identified in *L. migratoria* fat body.

In transfected Sf9 cells as well as locust fat body, uncleaved apoLp-II/I could be secreted in similar amounts as its cleavage products. The recombinant apoLp-II/I-38 cleavage products apoLp-II and apoLp-$I_{567/V5}$ appeared to form a complex, yet were not integrated in a high density lipoprotein, in contrast to apoLp-II/I products secreted by locust fat body, as they were recovered in the very high density range upon density gradient ultracentrifugation (see supplementary Figs. II, III). The poor lipidation of the apoLp-II/I-38 truncation products is in agreement with the decreased lipidation observed for apoB truncations (1, 22) but may also reflect the absence of cofactors for lipidation in the present expression system. Therefore, the possible role of cleavage in the lipidation and secretion of insect lipoprotein was investigated in locust fat body. Here, decRVKRcmk treatment resulted in the secretion of uncleaved apoLp-II/I that formed a stable high

density lipoprotein, as indicated by its density and molecular mass identical to wild-type HDLp. Consequently, the uncleaved precursor apoLp-II/I can function as a single apolipoprotein in the formation of lipoprotein, like its mammalian homolog apoB. Thus, apoLp-II/I can be lipidated to form a high density lipoprotein, irrespective of its cleavage.

The lipidation of apoB starts cotranslationally in the rough endoplasmic reticulum and is completed posttranslationally in the smooth endoplasmic reticulum and/or *cis*-Golgi network, possibly by fusion with an intralumenal lipid droplet (1, 2). The lipidation process starts with the lipidation of the lipid pocket in the apoB LLT domain and requires the lipid transfer activity of MTP (1, 2). Based on the homology between apoB and apoLp-II/I (3, 4) as well as the discovery of an insect MTP (12), insect lipoprotein assembly may also occur early in the secretory pathway. However, cleavage by furin homologs is performed late in the secretory pathway, mainly in the *trans*-Golgi network (34). Therefore, we propose that insect lipoprotein biosynthesis by the fat body involves lipidation of apoLp-II/I to a lipoprotein first, followed by cleavage of apoLp-II/I into apoLp-I and -II. The occurrence of cleavage before any lipidation might result in the parting of apoLp-I and apoLp-II, and hence impairment of lipoprotein biosynthesis. The uncleaved LLT domain in apoLp-II/I may be essential to enable the first steps in lipidation, as in apoB.

Homology modeling shows that the LLT domain of apoLp-II/I can form a putative lipid pocket. Remarkably, the cleavage site is located in an unresolved loop region between two β-strands in the βA sheet at the base of this pocket (Fig. 6). In apoB, the corresponding region is proposed to function in early lipidation events, as it may form a hairpin structure that temporarily closes the basal opening of the lipid pocket by connecting to the βB sheet via salt bridges (22). When the lipid pocket of apoB reaches a certain lipid content during lipoprotein assembly, these salt bridges are proposed to dissociate. This would allow for widening of the V-shaped lipid pocket formed by βA and βB sheets and the progression of apoB lipidation (22, 35). The cleavage of apoLp-II/I might represent an alternative structural solution to unlock the lipid pocket and increase the flexibility of the nonexchangeable apolipoprotein, hence allowing for further lipidation of the lipoprotein particle. Interestingly, insects can lipidate circulating HDLp to a low density lipophorin (LDLp) during conditions that require enhanced lipid transport (e.g., long-term flight and vitellogenesis) (36, 37). Therefore, apoLp-II/I cleavage may function to enable the formation of LDLp from HDLp.

The apparent conservation of apoLp-II/I cleavage in all insects characterized to date reveals the importance of this processing step. Besides LDLp formation, however, apoLp-II/I cleavage may enable other unique insect lipoprotein characteristics, such as the ability of lipophorin to function as a reusable lipid transporter (36) and to be recycled after endocytic uptake by an insect member of the low density lipoprotein receptor (LDLR) family (16). Vitellogenin, another ligand of this receptor family, is homologous to apoB and apoLp-II/I, and is also cleaved at a furin consensus substrate sequence in the LLT domain during biosynthesis in most insect species, but not vertebrates (38). Lipophorin, vitellogenin, and LDLR family members are involved in insect vitellogenesis, the transfer of nutrients to the developing oocyte (38), and perhaps the posttranslational cleavage of apoLp-II/I and vitellogenin facilitates this process (e.g., by enabling receptor binding). In addition, apoLp-II/I cleavage might enable a function for lipophorin in coagulation. Lipophorin has been implicated in this process by its abundant presence in clots (39–42), capacity to aggregate (43, 44), and interactions with other insect hemostasis factors (45). Moreover, the disappearance of apoLp-I but not apoLp-II from plasma during coagulation (46) suggests that apoLp-II/I cleavage enables distinct roles for apoLp-I and apoLp-II in coagulation.

Cleavage of the insect apoB homolog apoLp-II/I appears to be a molecular adaptation within homologous structures and may have a significant impact on insect lipoprotein function. The possibility of obtaining apoLp-II/I lipoprotein from fat body using a furin inhibitor may aid in establishing the physiological role of apoLp-II/I cleavage. Furthermore, the recombinant expression of truncated apoLp-II/I may be used to explore aspects of insect lipoprotein assembly and structure, such as lipid binding regions in apoLp-I and -II, the role of protein-protein and lipid-protein interactions in apoLp-I:apoLp-II complex formation and lipoprotein solubility (47), as well as the cofactors involved in insect lipoprotein biosynthesis. For example, apoB truncations with a length similar to apoLp-II/I-38 can form high density lipoprotein particles upon recombinant expression, but only when cells are supplied with exogenous lipids and (co)express MTP (12, 48). Likewise, the present recombinant expression system may be used to investigate putative roles for lipid availability and insect MTP homologs in the biosynthesis of insect lipoprotein.

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