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Structure and expression of the lipophorin-encoding gene of the malaria vector, *Anopheles gambiae*

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Abstract

Lipophorin is the major hemolymph protein responsible for lipid transport among tissues of insects. This protein may be a lipid source for the development and reproduction of human malaria parasites in mosquitoes, and therefore could be a target to disrupt malaria parasite development in the vector. The lipophorin of *Anopheles gambiae* was purified by KBr gradient ultracentrifugation and showed variation in density from 1.111 to 1.143 g/ml during development. The amount and density of lipophorin increase in blood-fed females, indicating an adaptation of vitellogenic mosquitoes to an elevated rate of lipid transport to the developing eggs. The *A. gambiae* lipophorin gene is composed of eight exons and transcribes an mRNA that is 10,516 nucleotides in length. The predicted initial translation product is a preproapolipophorin consisting of 3332 amino acids, which is processed by proteolysis to generate two mature apolipophorins: apolipophorin-I (Mr=280,000) and apolipophorin-II (Mr=81,000). The gene is expressed in the fat body tissues throughout development. An elevated transcriptional activity of the lipophorin gene during vitellogenesis is consistent with the presence of putative *cis*-regulatory elements (GATA and ecdysone responsive elements) in its 3′-end flanking DNA sequence.
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1. Introduction

Lipophorins are the major lipoproteins found in the hemolymph of insects. The molecular mass of the native molecule is ~700 kDa, and comprises approximately 40% lipid and 60% protein (Chino and Downer, 1982). Lipophorins are composed of two different types of apoproteins, Apolipophorin-I (ApoLp-I) (~250 kDa), and Apolipophorin-II (ApoLp-II) (~80 kDa) (Kanost et al., 1990; Arrese et al., 2001; Canavoso et al., 2001). The two apoproteins are products of a single gene that is expressed in the fat body of the insects, and are derived from the cleavage of a common precursor, the proapolipophorin (Weers et al., 1993; Bogerd et al., 2000; Kutty et al., 1996). A third smaller lipophorin subunit of ~18 kDa, encoded by a different gene, designated Apolipophorin-III, has been detected in some insect species (Van Hoof et al., 2002).

The main proposed function of lipophorins is to transport lipids throughout the insect body by loading dietary lipids in the midgut and delivering them to other tissues (Canavoso et al., 2001). They also can carry lipids from sites of synthesis and storage to sites of utilization such as muscles and oocytes. Other functions suggested for lipophorins include the transport of hydrocarbons to the cuticle and cholesterol to the prothoracic glands, binding of xenobiotics, involvement with plasma coagulation and transport of hormones (Katase and Chino, 1984; Chino et al., 1974; Haunerland and Bowers, 1986; Shapiro, 1988, deKort and Koopmanschap, 1989; Fan et al.,

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2002). Panakova et al. (2005) proposed recently a novel developmental regulatory function for the lipophorin in which this protein acts as a vehicle for the movement of lipid-linked morphogens and glycophosphatidylinositol-linked proteins. They demonstrated that lipophorins mediate intercellular transfer of Hedgehog, a developmental signaling protein, by being endocytosed along with the morphogen.

Despite the importance of lipoproteins in the development and reproduction of insects and their apparent direct interactions with some parasites (Nakahara et al., 1999; Folly et al., 2003; Vlachou et al., 2005), little is known about the lipophorins of anophelines, vectors of human malaria parasites and other pathogens. Here we describe the purification and characterization of the *Anopheles gambiae* lipophorin, analyze its gene structure and lipid transport and expression profile.

2. Materials and methods

2.1. Insects

The *A. gambiae* pink-eye strain (Githeko et al., 1992) was reared at 25 °C, 70–80% relative humidity and 18/6 light/dark cycles. Larvae were fed on finely ground fish food (Tetramin) mixed 1 : 1 with yeast powder. Adults were maintained in cages with access ad libitum to water and raisins. Females were fed when necessary on anesthetized mice.

2.2. Lipophorin purification

Larvae, pupae and adults (~300 animals in each sample) were homogenized separately in 3 ml of buffer A (TBS pH 7.5, 5 mM EDTA, 1 mM benzamidine, 0.5 mM PTC and 1 mM TLCK) containing 3.7 M KBr, using a Potter Elvehjem homogenizer. The homogenates were centrifuged at 14,000 *g* for 10 min at 4 °C, the soluble portion transferred to ultracentrifuge tubes and overlaid by 3 ml of buffer A. These preparations were centrifuged for 18 h at 32,000 rpm, 4 °C, in a Beckman Sw55.Ti rotor. Fractions of 0.2 ml were removed from the top of the formed gradients and stored at 4 °C for further analysis.

2.3. Polyacrylamide gel electrophoresis and molecular weight determination

SDS-PAGE was carried out according to Laemmli (1970) using 7% or 4–20% gels. Gels were stained for protein with Coomassie blue R. Pre-stained protein molecular weight standards were purchased from Bio Rad and New England BioLabs.

2.4. Antibody production and immunoblot analyses

Approximately 100 μg of purified lipophorin was resolved by SDS-PAGE and the protein species corresponding to ApoLp-I and ApoLp-II were excised from the gel. The gel fragments containing the apolipoproteins were forced through an 18 gauge needle and the resulting material was emulsified with Freund's complete adjuvant prior to injection into a rabbit. Similar injections were performed 14 and 28 days after the first immunization and the rabbit was bled seven days after the last injection. The specificity of the serum was tested by immunoblotting using peroxidase-labeled goat anti-rabbit IgG as a secondary antibody. The immunoblots were developed using ECL kit (Amersham) substrate and X-ray films for signal detection.

2.5. ELISA and lipophorin quantification

ELISA assays used for quantification were initiated by coating the wells of microtiter plates with lipophorin in an overnight incubation at 4 °C. The plates were washed three times with TBS (50 mM Tris, pH 8.0; 150 mM NaCl) containing 1% Tween 20, three times with TBS, and then incubated for 2 h with TBS containing 2% bovine serum albumin (BSA). After this incubation, the PBS/BSA was aspirated from the wells and a 1 : 5000 dilution of the anti-lipophorin serum in PBS containing 0.1% Tween 20 was added and the wells incubated for another 2 h. After washing as described above, anti-IgG conjugated to horseradish peroxidase diluted 1 : 1000 with PBS containing 0.1% Tween 20 was added to the wells and incubated for 2 h. After further washing a developing solution containing *o*-phenylenediamine (3 mg/ml) in 0.15 M acetic acid, 0.15 M NaH_2PO_4, 0.02% H_2O_2 and 0.01% thimerosal was added to the wells and incubated in the dark for 5–10 min. Reactions were stopped by addition of 50 μl 7 N H_2SO_4 and absorbances at 492 nm were determined. Standard curves were obtained using purified lipophorin as the reference.

2.6. Lipophorin density determination

KBr density gradient ultracentrifugation was carried out as described above. Each sample was analyzed for density by measuring the refraction indices and lipophorin content by ELISA. Three samples, each representing a different pool of ten animals, were analyzed for each developmental stage and the values were determined as the mean and respective standard deviation.

2.7. Lys-C digestion and peptide sequencing

Purified lipophorin was submitted to SDS-PAGE and the apolipophorins stained with Coomassie blue. Stained bands were excised, digested with Endoproteinase Lys-C and the resulting peptides resolved in a reverse-phase HPLC column and sequenced. Proteolytic digestion, peptide purification and sequencing were performed at the Macromolecular Structure Analysis Facility (University of Kentucky, Lexington, USA).

2.8. RT-PCR, RACE and cDNA cloning

Oligonucleotide primers, LpF: 5′-AAGAAGGA(A/G)GA(T/C)CT(C/G)CT(C/G)AC-3′ and LpR: 5′-C(A/G)AT(C/G)AC(A/G)TC(T/C)TCCTG(C/G)GC-3′, were synthesized based on the lipophorin amino acid sequences for use with RT-PCR. Total RNA from fourth-instar larvae was extracted with Trizol (Invitrogen), treated with DNAse (RQ1, Promega) and used as template for reactions performed with a one-step RT-PCR kit

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