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Apolipophorin-III expression and low density lipophorin formation during embryonic development of the silkworm, *Bombyx mori*

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ABSTRACT

We examined the expression of apolipophorin-III (apoLp-III) during embryonic development of the silkworm *Bombyx mori*. ApoLp-III mRNA was first expressed 24 h after oviposition, which corresponds to the time of germ band formation. The amount of apoLp-III in the eggs increased from day 2, peaked on day 4, and then gradually decreased until hatching (on day 9.5). ApoLp-III was apparently synthesized during early embryogenesis, as radioactive amino acids were incorporated into newly synthesized apoLp-III in three-day-old eggs. Moreover, radioactive apoLp-III was found only in the embryo and not in the extraembryonic tissue. KBr density gradient ultracentrifugation of egg homogenates showed that apoLp-III was associated with low-density lipophorin (LDLp). These results suggest that LDLp is required for the delivery of lipids for organogenesis during embryogenesis.

1. Introduction

Lipophorin, a major hemolymph component and key transport vehicle for lipids, is composed of three proteins: apolipophorin-I (apoLp-I), apolipophorin-II (apoLp-II), and apolipophorin-III (apoLp-III) and lipids. ApoLp-I and -II are necessary components for lipophorin, while apoLp-III is an exchangeable apolipoprotein. The functions of apoLp-III associated with lipophorin have been intensively investigated in the hawkmoth *Manduca sexta* [Lepidoptera: Sphingidae] and the migratory locust *Locusta migratoria* [Orthoptera: Acrididae] (Soulages and Wells, 1994; Narayanaswami and Ryan, 2000; Weers and Ryan, 2006). ApoLp-III can exist free in the hemolymph or in association with lipophorin (Shapiro et al., 1988). When large amounts of lipids are transported during flight, apoLp-III becomes associated with high-density lipophorin (HDLp), forming a diacylglycerol-rich, low-density lipophorin (LDLp) (Wheeler and Goldsworthy, 1983). ApoLp-III is released from LDLp into the hemolymph after the delivery of diacylglycerol to the flight muscles, and LDLp is converted back to HDLp (Shapiro and Law, 1983).

During vitellogenesis, LDLp is the major lipid shuttle for yolk deposition in *M. sexta*. Lipid is accumulated as triacylglycerol and formes lipid droplets in oocyte (Kawooya and Law, 1988; Kawooya et al., 1988; Van Antwerpen et al., 1993, 2005). The fundamental aspects of lipid utilization during embryonic development in insects are not understood. This includes the transport of lipids from lipid droplets or yolk granules to the embryo and their digestion, as well as lipid mobilization during early embryogenesis prior to organogenesis. The previous observations show that lipid droplets are distributed overall in the cytoplasm in *Bombyx mori* (Miya et al., 1972) and some droplets are found in the embryonic tissues before blastokinesis of *B. mori* (Yamahama et al., 2008). In Lepidoptera, the lipid droplets in the embryo can be taken up from the extraembryonic yolk at the time of the dorsal closure (Truman and Riddiford, 1999; Lamer and Dorn, 2001). And during late embryogenesis, the developing embryo ingests yolk granules (Yamashita and Yaginuma, 1991). The amount of triacyglyceride in the egg decreased beginning day 5 after oviposition (Miura and Shimizu, 1987). However, nothing is known how lipid is used by the embryo during the very early stage of embryogenesis prior to organogenesis. In this report, we examined the developmental expression profile of apoLp-III and show that apoLp-III is expressed in the eggs. ApoLp-III is synthesized *de novo* during early embryogenesis and it associates with HDLp, forming LDLp in eggs before the end of organogenesis. The possible function of apoLp-III in early embryogenesis is discussed.

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2. Materials and methods

2.1. Insects

Insects (*Bombyx mori*, N4 strain) were reared on an artificial diet (Nosan Kogyo Co., Japan) at 25 °C with a 12:12 light/dark photoperiod. The eggs were kept under the same conditions.

2.2. ApoLp-III purification and production of antibody against apoLp-III

One hundred moths, one day after emergence, were injected with 50 nmol of adipokinetic hormone/10 μL water per moth. After 2 h, the moths were injected with 100 μL of 20 mM phosphate buffer (pH 6.7), 150 mM NaCl (phosphate-buffered saline, PBS) containing 5 mM EDTA, 1 mM glutathione, and 1 mM AEBSF (Calbiochem, CA, USA), and hemolymph was collected by the flushing-out method (Chino et al., 1987). The hemolymph was centrifuged at 800 *g* for 5 min to remove hemocytes. Lipophorin was isolated by KBr density gradient ultracentrifugation as described by Tsuchida et al. (1997). After centrifugation, LDLp was collected from the top of the centrifuge tubes and pooled. The LDLp fraction (4 mL) was desalted by Biospin Column P-30 (BioRad, CA, USA) which was equilibrated with PBS. LDLp was precipitated by dialyzing the sample overnight against ice-cold distilled water and then centrifuged at 20,000 *g* for 10 min. The precipitated LDLp was dried in a lyophilizer and 10 mL of methanol were added to remove the lipids. The methanol was then discarded and the precipitate lyophilized again. Distilled water was added to the sample to extract apoLp-III. ApoLp-I and apoLp-II are insoluble in water and were removed by centrifugation. The supernatant was subjected to reverse-phase HPLC (Cole et al., 1987). Antibodies to apoLp-III were raised in Japanese white rabbits by subcutaneous injection of purified apoLp-III with adjuvant (Corixa Corp., WA, USA).

2.3. SDS-PAGE

Hemolymph was collected daily from newly hatched larvae through the adult stage by puncturing a leg with a needle. The collected hemolymph was placed on a small piece of Parafilm on ice, diluted five-fold with PBS and centrifuged to remove the hemocytes. The supernatant was then boiled for 3 min with an equal volume of 2× SDS-PAGE sample buffer (Laemmli, 1970). Eggs were collected daily and homogenized in 100 μL of PBS/mg of eggs with a polytron homogenizer. The homogenate was centrifuged at 20,000 *g* for 20 min. An equal volume of 2× SDS-PAGE sample buffer was added to the supernatant and the mixture was boiled for 3 min. Electrophoresis was performed in 4–15% linear gradient or 8% single percentage polyacrylamide gels containing SDS. A 10 μL sample was applied to each lane. The gels were run at 40 mA for 4 h then stained with Coomassie Brilliant Blue R-250(CBB).

2.4. Western blotting

Proteins were separated for immunoblotting by SDS-PAGE and transferred to a PVDF membrane (BioRad) using the method of Towbin et al. (1979). The membranes were then incubated at room temperature for 1 h with blocking buffer (20 mM Tris–HCl, pH 7.8, 500 mM NaCl, 0.05% Tween 20 (TTBS), 1% bovine serum albumin (BSA), and 1% gelatin). The membranes were then incubated for 1 h with anti-apoLp-III rabbit serum in TTBS containing 0.1% BSA, and washed three times for 10 min each with the same buffer. After washing, the membranes were incubated for 1 h with anti-rabbit IgG antibodies conjugated to alkaline phosphatase (Jackson Immuno Res. Lab, PA, USA) in TTBS containing 0.1% BSA, and then washed three times for 10 min each with TTBS. Finally, the membranes were incubated with a developing kit (BioRad).

2.5. In vivo synthesis of apoLp-III

A total of 100 eggs were injected on day 3 with a [^{3}H(U)] amino acid mixture (Perkin-Elmer, MA) at 3.7 kBq/egg using a microsyringe with a glass needle and incubated for 6 h at 25 °C. The eggs were dissected to separate embryonic and extraembryonic regions, and then each region was homogenized with a Polytron homogenizer in 5 mL of 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% Tween 20, 0.02% NaN_3, 0.2% BSA, 1 mM AEBSF, 1 mM aprotinin, 1 mM chymostatin, 1 mM leupeptin, and 1 mM pepstatin A (sample buffer). The supernatant was recovered after centrifugation at 20,000 *g* for 20 min. Part of the sample was boiled for 3 min with SDS-PAGE sample buffer. For immunoprecipitation, the rest of the sample was lyophilized and the lipids were extracted according to Bligh and Dyer (1959). After delipidation, PBS was added to the sample, and the mixture was vortexed and centrifuged to remove the precipitated proteins. 20 μL of anti-apoLp-III rabbit serum or control rabbit serum were added to 1 mL of the supernatant, and incubated it overnight at 4 °C. We next added 50 μL of a protein A-Sepharose suspension (GE Healthcare, PA, USA) to the sample and centrifuged it. The precipitate was washed twice with 500 μL (PBS) and then added 50 μL 1× SDS-PAGE sample buffer, and boiled for 3 min. The samples were subjected to SDS-PAGE. After electrophoresis, the gel was stained with CBB, soaked in EN3HANCE (Perkin-Elmer, MA, USA) and processed for fluorography.

2.6. Observation of embryo

Eggs laid during 1 h period were incubated at 25 °C until larval hatching. For observation of embryonic morphology, eggs were fixed in Carnoy's solution and then treated according to the method of Yaginuma et al. (1990).

2.7. Northern hybridization

Eggs were collected 0, 4, 8, 12, and 24 h after oviposition and daily from day 2 (48 h after oviposition) to day 8 (1.5 days before hatching) and frozen until analyzed. Total RNA was isolated with Trizol® (Invitrogen, CA, USA) from the eggs at various time points according to the manufacturer's instructions. A ^{32}P-labeled riboprobe was synthesized and labeled with [γ-^{32}P] CTP using *B. mori* apoLp-III cDNA inserted into pGEM (Yamauchi et al., 2000) with the Riboprobe® system (Promega, PA, USA). For Northern blotting, 10 μg of total RNA were electrophoresed in a 1% agarose gel containing formaldehyde, stained with ethidium bromide, and visualized under UV light before being transferred to a Hybond N+membrane (GE Healthcare) (Alwine et al., 1977). The blotted RNA was hybridized with the radiolabeled riboprobe at 68 °C overnight in Ultrahyb (Applied Biosystems, TX, USA). The membranes were washed twice in 2× SSC containing 0.1% SDS at 68 °C, and twice in 0.1× SSC containing 0.1% SDS at 68 °C. The hybridized radioactivity was visualized by BAS-2500 (Fuji Film Co., Japan).

2.8. Ultracentrifugation

We homogenized four-day-old eggs in ice-cold PBS (10 μL/mg egg) containing 5 mM EDTA, 1 mM glutathione, and 1 mM AEBSF (Calbiochem, CA, USA) using a polytron homogenizer. The sample was then immediately centrifuged at 20,000 *g* for 20 min. Next we added 8.9 g of KBr to the supernatant and brought it to a total volume of 20 mL with PBS. The preparation was overlaid with 0.9% NaCl in 36.2-mL Opti Seal tubes (Beckman Coulter, CA, USA). The tubes were then centrifuged at 4 °C for 4 h at 50,000 rpm using a VTi50 rotor. After centrifugation, 1 mL fractions were collected from the top of the tubes and the fractions were monitored by SDS-PAGE after dialysis against PBS. For further purification, the LDLp and HDLp fractions were