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Lipophorin acts as a shuttle of lipids to the milk gland during tsetse fly pregnancy

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ABSTRACT

During pregnancy in the viviparous tsetse fly, lipid mobilization is essential for the production of milk to feed the developing intrauterine larva. Lipophorin (Lp) functions as the major lipid transport protein in insects and closely-related arthropods. In this study, we assessed the role of Lp and the lipophorin receptor (LpR) in the lipid mobilization process during tsetse reproduction. We identified single gene sequences for GmmLp and GmmLpR from the genome of *Glossina morsitans morsitans*, and measured spatial and temporal expression of *gmmlp* and *gmmlpr* during the female reproductive cycle. Our results show that expression of *gmmlp* is specific to the adult fat body and larvae. In the adult female, *gmmlp* expression is constitutive. However transcript levels increase in the larva as it matures within the mother's uterus, reaching peak expression just prior to parturition. GmmLp was detected in the hemolymph of pregnant females and larvae, but not in the uterine fluid or larval gut contents ruling out the possibility of direct transfer of GmmLp from mother to offspring. Transcripts for *gmmlpr* were detected in the head, ovaries, midgut, milk gland/fat body, ovaries and developing larva. Levels of *gmmlpr* remain stable throughout the first and second gonotrophic cycles with a slight dip observed during the first gonotrophic cycle. GmmLpR was detected in multiple tissues, including the midgut, fat body, milk gland, spermatheca and head. Knockdown of *gmmlp* by RNA interference resulted in reduced hemolymph lipid levels, delayed oocyte development and extended larval gestation. Similar suppresion of *gmmlpr* did not significantly reduce hemolymph lipid levels or oogenesis duration, but did extend the duration of larval development. Thus, GmmLp function as the primary shuttle for lipids originating from the midgut and fat body to the ovaries and milk gland to supply resources for developing oocytes and larval nourishment, respectively. Once in the milk gland however, lipids are apparently transferred into the developing larva not by lipophorin but by another carrier lipoprotein.

1. Introduction

The reproductive process in the tsetse fly represents a drastic shift in physiology from oviparous reproduction (egg deposition) to obligate viviparity (intrauterine development and nourishment of a progeny over the duration of larval development) (Meier et al., 1999; Tobe and Langley, 1978). To accommodate this mode of reproduction, tsetse reproductive morphology has undergone drastic alterations (Tobe and Langley, 1978). The birth canal is adapted into a uterus to accommodate the developing larvae and the accessory gland (milk gland) is modified and expanded to generate nourishment for the intrauterine progeny (Tobe and Langley, 1978). The primary nutrients within tsetse milk are lipids and proteins with amino acids and sugars as minor components (Cmelik et al., 1969; Denlinger and Ma, 1974). To date, four proteins have been identified as components of the milk, and are generated within the milk gland (Attardo et al., 2006; Guz et al., 2007; Yang et al., 2010). Up to 10 mg of lipids, consisting mostly of triacylglycerol (TAG) and phospholipids are transferred from mother to larva each gonotrophic cycle (Cmelik et al., 1969; Denlinger and Ma, 1974). However, little is known about this process. Lipids for milk production are not generated in the milk gland, rather they are produced and stored in the fat body or are acquired directly from blood feeding (Langley et al., 1981; Tobe and Langley, 1978). The lipids from both sources are moved to the milk gland for incorporation into the milk secretion (Langley et al., 1981; Tobe and Langley, 1978). Currently, the factors responsible for lipid transport from the sites of nutrient uptake (digestive tract) or storage (fat body) through the hemolymph and to the milk gland have not been examined.

Lipophorins (Lp) are critical in insects for lipid transport between tissues (Ryan and van der Horst, 2000; Soulages and Wells, 1994; Shapiro et al., 1988; Van der Horst and Rodenburg, 2010; Van der Horst et al., 2002). The Lp gene is expressed as a single

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transcript that is cleaved into two separate proteins after translation (Sundermeyer et al., 1996; Atella et al., 2006). Lipid loading and unloading occurs at tissues expressing the lipophorin receptor (LpR) (Canavoso et al., 2001; Van der Horst and Rodenburg, 2010; Van der Horst et al., 2002). Lipophorin transitions from the unloaded high density lipophorin (HDLp) to the lipid-loaded low density lipophorin (LDLp; (Ryan et al., 1986; Arrese et al., 2001; Arrese and Soulages, 2010; Canavoso et al., 2001; Van der Horst et al., 2002). The primary lipid transported by insect lipophorin is diacylglycerol (Chino and Kitazawa, 1981; Chino et al., 1981; Arrese et al., 2001; Arrese and Soulages, 2010; Van der Horst et al., 2002). In some cases, this protein can act as a shuttle for other hydrophobic moieties, such as hydrocarbons, cholesterol, phosophopholipids and fatty acids (Chino and Gilbert, 1971; Katase and Chino, 1984; Fan et al., 2002; Sevala et al., 1999). Upon arrival at target tissues, the protein–lipid complex binds to LpR and lipids are unloaded either with or without endocytosis (Parra-Peralbo and Culi, 2011; Rodenburg and Van der Horst, 2005; Ryan and van der Horst, 2000; Van der Horst and Rodenburg, 2010; Van der Horst et al., 2002; Van Hoof et al., 2005). After unloading, Lp is recycled for subsequent lipid mobilization (Rodenburg and Van der Horst, 2005; Ryan and van der Horst, 2000; Van der Horst and Rodenburg, 2010; Van der Horst et al., 2002; Van Hoof et al., 2005). Lipophorin systems have been thoroughly characterized in blood feeding insects including the yellow fever mosquito, *Aedes aegypti* (Van Heusden et al., 1997; Sun et al., 2000; Cheon et al., 2001, 2006), the malaria mosquito, *Anopheles gambiae* (Atella et al., 2006; Marinotti et al., 2006) and in the kissing bug, *Rhodnius prolixus* (Machado et al., 1996; Grillo et al., 2003; Pontes et al., 2002, 2008), but little is known about lipophorin in tsetse. Tsetse lipophorin (GmmLp) was previously isolated. It contains two subunits, apolipoprotein-I (250 kDa; Apolipo-I) and apolipoprotein-II (80 kDa; Apolipo-II) and has a density of 1.11 g/ml (Ochanda et al., 1991). This lipid protein complex consists of 49% lipids and 51% protein (Ochanda et al., 1991).

The focus of this study is to understand the mechanism of lipid movement during pregnancy and lactation in tsetse. In particular these studies focus on the role of GmmLp as the lipid carrier molecule during the tsetse reproductive cycle. We characterize the molecular biology of lipophorin and its receptor (GmmLpR), and examine expression of *gmmlp* and *gmmlpr* during pregnancy. Localization of GmmLpR was conducted to identify potential target tissues at which lipid loading and unloading occurs. Adult female hemolymph, uterine fluid, larval gut contents and larval hemolymph were examined for the presence of GmmLp to determine if this lipoprotein can facilitate direct transfer of lipids from mother to intrauterine offspring. Lastly, the physiological roles of GmmLp and Gmm LpR during pregnancy were assessed utilizing single stranded RNA based (siRNAi) knockdown. The putative roles of GmmLp and GmmLpR in the oogenesis and larvagenesis processes are discussed.

2. Materials and methods

2.1. Flies

Colonies of *Glossina morsitans morsitans* at Yale University (New Haven, CT, USA) originated from a small population of flies originally collected in Zimbabwe. Flies are maintained at 24 °C and 50-60% RH. Flies receive bovine blood meals via an artificial feeding system every 48 h (Moloo, 1971). Mated female flies were collected for qPCR and western blotting according to developmental markers established in previous studies (Attardo et al., 2006; Yang et al., 2010). In addition, to differentiate between maternal and larval gene expression, progeny were removed from pregnant females and both samples (pregnant females and the larva) were analyzed individually.

2.2. Phylogenetic analysis of Gmmlp Apolipoprotein II and I

BLASTX analysis of tsetse cDNA and genomic read libraries at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/g_morsitans) were utilized to identify tsetse *gmmlp* sequences. Predicted protein sequences were aligned using ClustalX (Mega 4, Thompson et al., 1997) and formatted with BioEdit (Hall, 1999). Pairwise phylogenic tree construction and bootstrap analysis (10000 replicates) were performed using the MEGA3 sequence analysis suite (Kumar et al., 2004).

2.3. Analysis of gmmlp and gmmlpr expression

Levels of *gmmlp* and *gmmlpr* were determined by qPCR utilizing the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules). The data were analyzed with software version 3.1 (Bio-Rad). The primer sequences utilized for *gmmlp* were (F – 5′-TTTGGCTCCG AAATGATGTTCTTGA-3′ and R – 5′-TGTCAATTCCGGACGAAA TTTGTTTGT-3′) and for *gmmlpr*: (F – 5′-GGCCGAACGGCATTA-CATTGGA-3′ and R – 5′-TTGACGACGTTGCGAACCATCA-3′). All treatments were normalized according to tsetse Tubulin (*gmmtub*) expression levels using gene specific primers (F – 5′-CCATTCC CACGTCTTCACTT-3′and R – 5′-GACCATGACGTGGATCACAG-3′) and carried out in triplicate.

For tissue specific expression analysis, cDNA was prepared from 2 μg of total RNA recovered from dissected tissues using Trizol (Invitrogen, Carlsbad CA) and the Superscript 3 cDNA synthesis kit (Invitrogen) according to manufacturers' specifications. Tissues isolated were Malphigian tubules, salivary gland, reproductive tract, fat body, ovary, milk gland, midgut and larva. PCR was conducted with gene-specific primer sets: *gmmlp* (F – 5′-GGAATATGG-TAAAGTGGTGG-3′, R – 5′-TTAGGAGCAAATGCAGGA-3′) and *gmmlpr* (F – 5′-GTATTGGTCGGATTGGGG-3′, R – 5′-TGGCTGACGATACG-GATG-3′). As a control *gmmtub* were used (F – 5′-TCGTTGAC-CATGTCTTGGTGT-3′ and R – 5′-TAGTTCTCTTCAACTTCAGCCTCTT-3′). The PCR amplification conditions were 95 °C for 3 min, thirty cycles of 30 s at 95 °C, 52 or 56 °C for 1 min, and 1 min at 70 °C in a Bio-Rad DNA Engine Peltier Thermocycler (Hercules, CA).

2.4. Lipid quantitation

Adult female hemolymph lipid levels were measured with a modified vanillin reagent assay. Hemolymph (2 μl) was collected utilizing a pulled glass capillary tube using reverse pressure from five female flies. Samples were checked by microscopic analysis to ensure hemolymph was not contaminated with fat body cells. A portion of the combined samples (1 μl) was dried at 50 °C in 5 ml glass test tubes for 2 days. Lipids were dissolved in sulfuric acid at 90 °C for 10 min and allowed to cool to room temperature. The samples were then combined with 4 ml of vanillin reagent (Van Handel, 1985), and the absorbance was measured at 525 and 490 nm. Concentration was determined according to standard lipid levels.

2.5. Western blot and immunohistochemical analyses

Protein was isolated from flash frozen female flies and tissue samples utilizing a Trizol-based protocol modified to dissolve the protein pellets in cracking buffer (8 M urea, 3 M thiourea, 1% dithiothreitol (DTT) and 4% CHAPS). Equal volumes of protein from three flies were combined for each time point, and analyzed by standard western blot protocol (Attardo et al., 2006). Apolipo-II antisera used in this study were generated against *A. aegypti*