MOBILIZATION OF LIPID STORES IN *MANDUCA SEXTA*: ROLE OF ADIPOSE TRIGLYCERIDE LIPASE

By

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MOBILIZATION OF LIPID STORES IN *MANDUCA SEXTA*: ROLE OF ADIPOSE TRIGLYCERIDE LIPASE

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Abstract: Triglycerides (TG) stored in lipid droplets (LDs) are the main energy reserve in all animals. The mechanism by which animals mobilize TG is complex and not fully understood. Several proteins surrounding the LDs have been implicated in TG homeostasis such as mammalian Perilipin A and insect lipid storage proteins (Lsd). Lipid storage protein 1 (Lsd1) is a conserved insect protein and plays significant roles in the regulation of TG metabolism. Most of the knowledge on LD-associated proteins comes from studies using cells or LDs leaving biochemical properties of these proteins uncharacterized. Here we describe the purification of recombinant *Ms*Lsd1, its reconstitution in lipoprotein particles and the mutagenesis studies in putative phosphoryation sites and conserved region of Lsd1. Moreover, mobilization of TG depends on the action of lipases. The fat body triglyceride lipase from *Manduca sexta*, *Ms*TGL, is the only insect lipase that has been purified and characterized, so far. This study also describes another enzyme, adipose triglyceride lipase (ATGL), and investigates a possible link between ATGL expression and nutrition levels in *M.sexta*. The ATGL cDNAs from *M. sexta* fat body encoding a 64KDa protein were cloned. Northern blot analysis detected two bands corresponding to the 3.9 and 2.4 kb transcripts, respectively. The protein sequence has the consensus lipase catalytic motif (GxSxG) and conserved “patatin-like”domain which is a key signature of ATGL enzymes isolated from other organisms. ATGL is mostly associated to the lipid droplet. Sf9 cells over-expressing the *Ms*ATGL showed lower content of cellular TG and a higher TG hydrolase activity of purified protein indicating that it is a lipase. *Ms*ATGL is up-regulated during the physiological non-feeding periods but lower than TGL with the exception of 3rd-day pre-pupal. Both levels of transcripts and expression of ATGL were dramatically up-regulated by starvation in a time-dependent manner indicating that ATGL is highly sensitive to the nutritional status of animals and may play a key role under starvation conditions. Thus, ATGL and TGL coordinately catabolize stored TGs in *M.sexta*. These studies provide the starting point for future studies on the mechanism and function of *Ms*Lsd1 and ATGL.

TABLE OF CONTENTS

Chapter Page

LIST OF FIGURES

Figure Page

Figure Page

ABBREVIATIONS

A-Kinase	cAMP dependent protein kinase A
ADRP	Adipose Differentiation-Related Protein
ATGL	Adipose triglyceride lipase
AKH	Adipokinetic Hormone
ATP	Adenosine triphosphate
cDNA	Complementary DNA
cAMP	cyclic 3', 5'-adenosine monophosphate
DG	Diacylglycerol
DMPG	1,2-DiMyristoyl-sn-glycero-3-PhosphoGlycerol
DTT	Dithiothretiol
DIG	Digoxigenin
DEPC	Diethyl pyrocarbonate
EDTA	Ethylenediamietetraacetic acid
FFA	Free Fatty Acid
HDLp	High density lipophorin
HSL	Hormone sensitive lipase
LD	Lipid droplet

LDLp	Low density lipophorin
Lsdp-1	Lipid storage droplet protein-1
LTP	Lipid transfer protein
mM	milli Molar
MOPS	3-(*N*-morpholino) propane-sulfonic acid
MG	Monoglyceride
MS	Mass Spectrometry
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PKA	cAMP dependent protein kinase A
PL	Phospholipids
rpm	revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SSC	Saline sodium citrate
TG	Triacylglycerol
TIP47	Tail interacting 47-kDa protein
TLC	Thin layer chromatography
^{3}H	Tritium

CHAPTER I

INTRODUCTION

Energy metabolism is a fundamental property of animal life. Neutral lipids in the form of triglycerides (TG) are the predominant form of storage of fatty acids and comprise the main energy reserve in all known organism (Wolins, Brasaemle et al. 2006). The storage and release of this energy involves a carefully regulated balance between TG synthesis and hydrolysis. Insects accumulate TG as lipid droplets (LDs) within the cytoplasm of fat body cells during feeding periods, and rely on these reserves to support the energy requirements associated with physiological non-feeding periods, sustained flight and embryo development (Beenakkers, Van der Horst et al. 1985, Ziegler and Van Antwerpen 2006). The insect fat body, which combines many of the properties and functions of vertebrate liver and adipose tissue, is the principal organ for the storage of lipids. LDs are considered as organelles. They are macromolecular assemblies of lipids surrounded by phospholipids and proteins.

The tobacco hornworm, *Manduca sexta*, is widely used as a model insect to study insect physiology and biochemistry. Fig 1 illustrates the life cycle of *M. sexta*. It has three different stages: larvae, pupa and adult. During larval stage (~20 days) insects feed constantly and the amount of TG in the fat body increases from a few micrograms to ~80 mg at the end of larval development (Fernando-Warnakulasuriya et al., 1988). During subsequent development, the lipid

reserves are mobilized to sustain the life of the adult insects (moth), which feed occasionaly (Ziegler 1991, Arrese, Canavoso et al. 2001, Canavoso, Jouni et al. 2001, Arrese and Soulages 2010). Due to these metabolic features, *M. sexta* represents an excellent model for studying the basic mechanisms involved in either the synthesis/deposition of TG in larvae or the mobilization of TG in adult insects (moth).

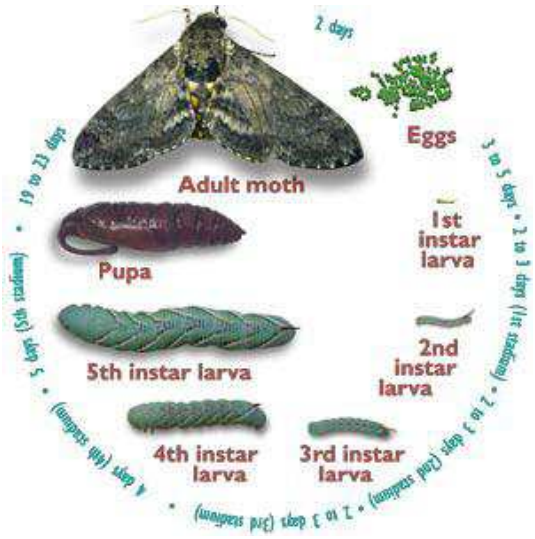

Figure 1. Life Cycle of *Manduca sexta.* The three different life stages in *M. sexta* are larva, pupa, and adult. On average, the complete metamorphosis from an egg to an adult takes about 1.5 month. Each instar is marked by a headcap, representing the developmental stages. The stadium is the period of time between larval molts (Source: http://insected.arizona.edu/manduca/Mand_cycle.html).

The mobilization of TG stored from LDs requires "lipolysis", which is the process that breaks ester bonds between long chain fatty acids and the glycerol backbone in TG. This process is catalyzed by lipases (Holm, Osterlund et al. 2000, Lass, Zimmermann et al. 2006).

In vertebrates, lipolysis involves at least three lipases: adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoglyceride lipase (MGL). ATGL is the rate-limiting enzyme for the initiation of TG catabolism, generating DG and FA. HSL efficiently hydrolyzes

DG to generate MG and FA (Haemmerle, Zimmermann et al. 2002). The final step is performed by MGL to result glycerol and FA (Fig 2).

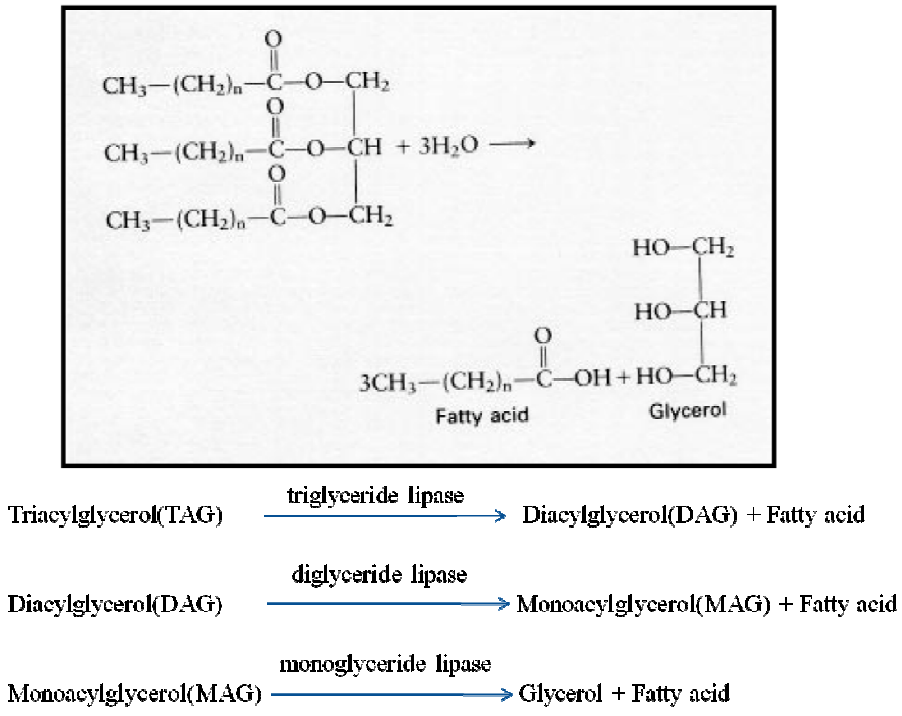

Figure 2. Lipolysis of Triacylglycerols. The three ester bonds of TG are hydrolyzed by their respective lipases to generate DG, MG and finally Glycerol with released free fatty acids for oxidation.

Unlike vertebrates, where stored fatty acids are mobilized as free fatty acids, in insects, most fatty acids are released from the fat body to hemolymph as *sn*-1,2-diacylglycerol (DG) (Beenakkers, Van der Horst et al. 1985, Arrese, Rojas-Rivas et al. 1996, Gade, Hoffmann et al. 1997). In circulation DG is carried by lipophorin, the insect lipoprotein, for delivery to tissues, *e.g.* the flight muscle, and ovaries, where it is hydrolyzed to free fatty acids (Arrese and Wells 1997).

Two fat body lipases have been identified so far: ATGL, and triglyceride lipase (TGL). TGL is a major lipase in *Manduca sexta* (Arrese, Howard et al. 2010) and is the only insect lipase that has been purified and characterized in *vitro* (Arrese and Wells 1994). TGL is a polypeptide with a

relative mass of 74-76 KD that has been identified as the homolog of *Drosophila melanogaster* CG8552, whose function was studied by expression of the Drosophila protein in insect cell lines (Arrese, Patel et al. 2006). TGL shares significant sequence similarity with vertebrate phospholipases from the phosphatidic acid phospholipase A1 (PAPLA1) family (Arrese, Patel et al. 2006), but it shows no homology to the main triglyceride hydrolases of vertebrate adipocytes, HSL and ATGL. TGL is well conserved among insects (Arrese, Patel et al. 2006). The enzyme can catalyze the hydrolysis of tri-, di-, and mono-oleoylglycerols, but shows highest affinity for tri- or di- oleoylglycerol. The fat body lipase exhibits a preference for hydrolyzing the primary ester bonds of acylglycerols, and does not show stereoselectivity toward either the *sn*-1 or *sn*-3 position of trioleoylglycerol. Its activity has an optimum pH (7.9) and can be inhibited by diisopropylfluorophosphate, ATP, ADP, Mg^{2+}, and NaF (Arrese and Wells 1994). In addition to its main triglyceride and diglyceride hydrolase activities, TGL has a significant phospholipase A1 activity (Arrese, Patel et al. 2006) with the ability to hydrolyze the phospholipids of the outer layer of the LDs. This activity is expected to facilitate the access of TGL to the core of the LDs where TG molecules localize. TG hydrolysis necessarily involves the interaction of the lipase with the lipid droplet. However, TGL does not achieve a tight association with the LDs and experimentally is only found in the cytosol regardless of the lipolytic conditions (Patel, Soulages et al. 2005). TGL is constitutively phosphorylated *in vivo*, and its phosphorylation level is unchanged by AKH, the main lipolytic hormone of insects (Patel, Soulages et al. 2006). But TGL activity was 2.4-fold higher when assayed against lipid droplets isolated from AKH-stimulated fat bodies, suggesting an effect of AKH on the lipid droplets (Patel, Soulages et al. 2005). Subsequent studies to investigate the AKH-induced changes in the phosphorylation level of lipid droplet proteins identified Lsd1 as the major LD associated phosphoprotein (Arrese, Rivera et al. 2008). More importantly *in vitro* studies showed that the phosphorylation level of Lsd1 correlated with TGL activity (Patel, Soulages et al. 2005). The activity of TGL can be directly modulated by

PKA-mediated phosphorylation of Lsd1 (Patel, Soulages et al. 2004). However, the mechanism by which Lsd1 phosphorylaiton activates TGL is unknown.

As mentioned above, LDs play an active role in the release of stored fatty acids. The lipid droplet surface, composed by phospholipids and proteins, represents a barrier for the hydrolysis of TG, which resides in the core of the particle ((Patel, Soulages et al. 2005). However, in the presence of the proper stimuli changes on the surface of the lipid droplet ensure a rapid hydrolysis of TG.

Only few proteins have shown a strong preference to associate with LDs in animal cells. This small set of proteins were grouped under the PAT family (Pfam 03036) (Lu, Gruia-Gray et al. 2001, Miura, Gan et al. 2002) and comprises proteins such as Perilipin, TIP47 and ADRP, in vertebrates, and lipid storage droplet protein-1 and -2, Lsd1 and Lsd2, in insects. These proteins share sequence similarity in the N-terminal region, a region called the PAT domain. These proteins do not have a known enzymatic activity, but as suggested by studies in vertebrates (Brasaemle 2007, Ducharme and Bickel 2008) and in insects ((Patel, Soulages et al. 2005, Bickel, Tansey et al. 2009, Beller, Thomas et al. 2010), they play a major role in the degradation of TG and its regulation. Since the PAT domain is not required for targeting Perilipin and ADRP to the LDs its role remains uncertain (Garcia, Sekowski et al. 2003, Nakamura and Fujimoto 2003). Perilipin is the best characterized lipid droplet protein so far and is a critical regulator of lipolysis in vertebrate adipocytes. Depending on its phosphorylation level, Perilipin can prevent or stimulate triglyceride hydrolysis (Brasaemle 2007). The insect genomes encode two proteins of the PAT family (Bickel, Tansey et al. 2009), Lsd1 and Lsd2. Insect PAT proteins also localize in LDs (Miura, Gan et al. 2002, Teixeira, Rabouille et al. 2003), but the overall sequence similarity with the vertebrate family members is very low.

In *Manduca* Lsd proteins are particularly abundant in LDs from the fat body of adult insects. Although both proteins are found in LDs from adult fat body, Lsd1 is the predominant Lsd

protein (Arrese, Mirza et al. 2008). Previous studies have linked the expression levels (Arrese, Mirza et al. 2008) and the phosphorylation (Patel, Soulages et al. 2005, Arrese, Rivera et al. 2008) of Lsd1 with the ability of *M.sexta* fat body to hydrolyze TG and mobilize FA. The relative abundance of Lsd1 in LDs from adult insects, as compared to LDs of larval fat body and ovaries, is consistent with the physiological state of adult insects, which are mobilizing fatty acids from the fat body to the ovaries. Moreover, adult insects are also mobilizing and oxidizing FA to support basal metabolism since these insects are kept without food. Similarly, the low levels of Lsd1 in LDs from larval fat body or from ovaries are also consistent with the notion that these tissues are accumulating rather than mobilizing fatty acids.

Lsd1 is the main target of the phosphorylation cascade triggered by AKH in the *Manduca sexta* fat body of adult insects. AKH is produced by the corpora cardiac (Gade, Hoffmann et al. 1997, Gade and Auerswald 2003). During energy demanding processes like flight and reproduction, AKH is secreted into hemolymph, which elicits a glucagon-like action mediated by a G-protein-coupled receptor that activates both inositol phosphate and cAMP signaling responses (Gade, Hoffmann et al. 1997, Van der Horst, Van Marrewijk et al. 2001, Staubli, Jorgensen et al. 2002). Studies in the locust fat body showed that cAMP and/or Ca^{2+} are involved in mediating the action of AKH mobilizing lipids (Lum and Chino 1990). In adult *M. Sexta* the lipolytic response induced by AKH is associated with a rapid activation of cAMP-dependent protein kinase A (PKA) and sustained increase in calcium influx (Arrese, Flowers et al. 1999). The lipolytic response in insects seems to be mainly controlled through reversible phosphorylation /dephosphorylation reactions. The brief signal transduction of AKH on lipid mobilization is illustrated in Fig 3. AKH binding triggers activation of the adenylate cyclase (AC) and concomitant increase in the cAMP concentration followed by PKA activation. PKA phosphorylates Lsdp-1 (Step 1). Phosphorylation of Lsd1 enhances binding of the lipase to the surface of the lipid droplet and/or its catalytic activity (Step 2). Lipid droplet bound lipase

catalyzes the hydrolysis of phospholipid (Step 3) allowing the access of TG to the lipid surface (Step 4) and its subsequent hydrolysis also catalyzed by the lipase. The lipolytic process ends by release of the lipase from the lipid surface. TGL does not bind tightly to the lipid droplets, even under conditions of high lipolysis. Additional binding of the lipase to the lipid droplet would be prevented by dephosphorylation of Lsd1 mediated by a protein phosphatase (PP) (Step 5). Thus, PKA phosphorylaiton of Lsd1, activates the lipase and lipids are mobilized from the fat body as *sn*-1,2-diacylglyceride (DG) (Beenakkers, Van der Horst et al. 1985, Arrese, Rojas-Rivas et al. 1996, Gade, Hoffmann et al. 1997). In circulation, DG is carried by lipophorin, the insect lipoprotein, for delivery to tissues (e.g., the flight muscle) and ovaries, where it is hydrolyzed to fatty acids by a membrane-bound lipophorin-lipase (Law and Wells 1989, Van Heusden and Law 1989).

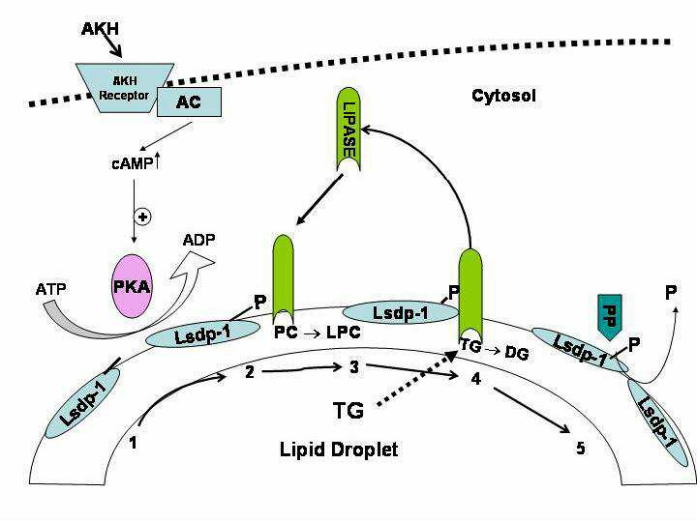

Fig. 3. Current model for the mechanism of AKH induced lipolysis. AKH binding triggers activation of the adenylate cyclase (AC) and concomitant increase in the cAMP concentration followed by PKA activation. PKA phosphorylates Lsdp-1 (Step 1). Phosphorylation of Lsdp-1 enhances binding of the lipase to the surface of the lipid droplet and/or its catalytic activity (Step 2). Lipid droplet bound lipase catalyzes the hydrolysis of phospholipid (Step 3) allowing the access of TG to the lipid surface (step 4) and its subsequent hydrolysis also catalyzed by the lipase. The lipolytic process ends by release of the lipase form the lipid surface. The insect TG-lipase does not bind tightly to the lipid droplets, even under conditions of high lipolysis. Additional binding of the lipase to the lipid droplet would be prevented by dephosphorylation of Lsdp-1 mediated by a protein phosphatase (PP) (step 5). PC: Phosphatidylcholine; LPC: lysophosphatidylcholine. Source: (Arrese, Patel et al. 2006).

However, the complete sequence of reactions underlying the AKH signaling mechanism has not been elucidated in any insect system yet. The mechanism by which Lsd1 phosphorylation, alone or in conjunction with other lipid droplet proteins, activates lipolysis in insects is unknown. The study of the function of the lipid droplet proteins will provide new insights into the mechanisms of lipid deposition and mobilization. In this project, we show the purification of recombinant Lsd1 and its assembly in lipoproteins complexes, as well as investigation the effect of *Ms*Lsd1 on the TGL activity by mutagenesis studies.

As mentioned above, the AKH-induced activation of lipolysis is a complex process that involves not only the activation of the substrate-Lsd1 phosphorylation-but also changes in the cytosol. Most of the AKH lipolytic (~70%) response can be accounted for by changes induced in the lipid droplets whereas changes in the cytosol are responsible for 30% of the lipolytic response (Patel, Soulages et al. 2006). The nature of changes in the cytosol that includes the activation of TGL remains to be elucidated. Studies on the lipolytic activity of cytosolic fractions of fat bodies have shown an AKH-dependent activation of TG hydrolase activity in moth (Arrese and Wells 1997), beetle (Auerswald, Siegert et al. 2005) and locust (Auerswald and Gade 2006). However, the lipolytic activation was modest and the mechanism of such activation that seems to be independent of TGL phosphorylation is unknown (Patel, Soulages et al. 2004).

A single AKH peptide is responsible for the mobilization of glycogen and lipids in *M. sexta* (Ziegler, Eckart et al. 1990). However, the effect of AKH on the mobilization of energy reserves is dependent on the developmental stages. In larval stage, AKH activates glycogenolysis whereas stimulates lipolysis in adult insects (Ziegler 1991). Therefore differences in TGL expression could also be part of the regulation of lipolysis, especially during developmental stages in *M. sexta*. Both the fat body TG hydrolase activity and expression of TGL during *M. sexta* development have been studied recently (Arrese, Howard et al. 2010). Lipase activity increases as larva grow to the last instar and, then, decreases to minimal levels during pupa stage. Lipase

activity is progressively restored in adult insects reaching maximum values at this stage as showed in Fig 4 (Arrese, Howard et al. 2010). TGL expression amount detected by Western Blot showed a good correlation with the lipase activity measured in the same samples (r^2=0.95) which reaffirmed that *M. sexta* TGL is the main fat body lipase.

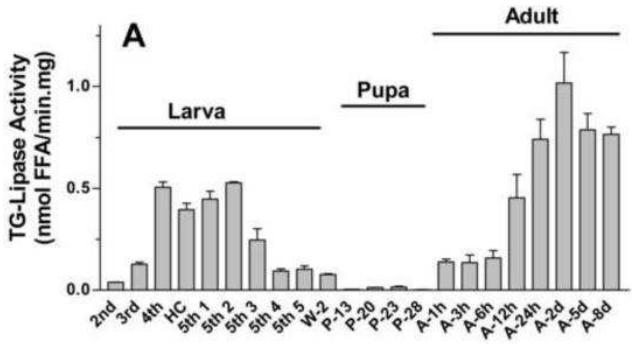

Fig 4. Fat body lipase activity during development Abbreviations: HC, head capsules; W, wanderer; P, pupa; and A, adult (males). Source: (Arrese, Howard et al. 2010)

However, it is known that during physiological non-feeding periods, such as HC (the molt from 4th to 5th instar) and wander stage, the insects mobilize the TG reserves to support the energy requirements as judged by the moderate increase of lipid concentration in the hemolymph (Ziegler, Willingham et al. 1995). During the feeding phase of the 4th and 5th instar larvae the lipid level remained largely unchanged (<2 mg/ml), whereas it was nearly doubled in the molt from 4th to 5th. And there was a pronounced increase (from about 2 to nearly 10 mg/ml) in wandering larvae (Fig 5) (Ziegler, Willingham et al. 1995). Interestingly, the non-feeding larval periods, i.e., head capsule slippage of the last larval molt (4th to 5th), late fifth, and wanderers, were not accompanied by an increase in fat body lipase activity. This observation is not consistent with the increase of the lipid level in the hemolymph suggesting that an additional lipase could be present in the fat body of *M.sexta*.

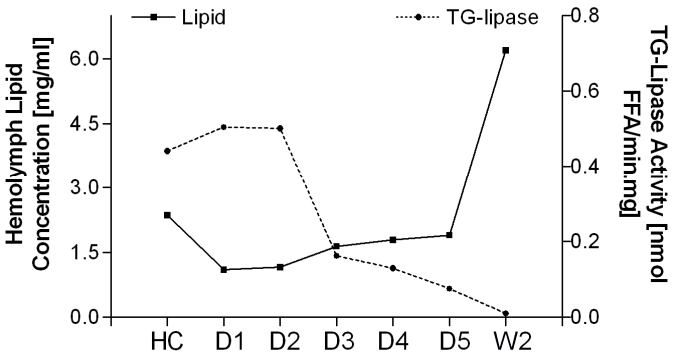

Fig 5. TG-lipase activity and hemolymph lipid concentration during development of *Manduca sexta*. Source: (Ziegler, Willingham et al. 1995, Arrese, Howard et al. 2010)

Additional enzymes may act as TG hydrolase when TGL activity is low during non-feeding periods. In 2006, Brummer lipase, a homolog of human ATGL was identified in Drosophila. Brummer over-expression renders lean flies whereas its deletion caused accumulation of triglyceride and therefore obesity flies (Gronke, Mildner et al. 2005). We are interested in the functions of ATGL in *M. Sexta* which is little known compared to TGL.

This project has two parts. Part one is to further investigate the role of Lsd1 in the activation of lipolysis. For this purpose we proposed two specific aims: 1) To express, purify and characterize recombinant *Ms*Lsd1; 2) To make Lsd1 mutants targeting conserved phosphorylation sites for *in vitro* studies to investigate the role of each phosphorylation site on the activity of TGL.

The second part of this project was designed to investigate the role of ATGL in *Manduca sexta* lipolysis. The specific aims were: 1) Clone the full length of ATGL cDNA from the fat body of *M. sexta*; 2) Characterize *Ms*ATGL including the study of the developmental expression at mRNA and protein levels.

CHAPTER II

EXPERIMENTAL PROCEDURE

Experimental Insects

Manduca sexta eggs were purchased from Carolina Biological supplies (NC) and larvae were reared at 25°C on artificial diet (Bell and Joachim 1976). At the end of the fourth larval instar the first sign of the molt was identified by the appearance of head capsule slippage. Typically slipped head capsule (HC) lasts about 29h and insects in the middle of this period were used as HC stage. The initiation of wandering behavior was detected by the exposure of the dorsal aorta (Arrese, Howard et al. 2010). For the experiments using starved larvae, 5^{th} instar day 1 larvae (weight ~2.0g) were subjected to starvation for different periods of time (6h, 12h, 24h, and 30h). For re-feeding experiments, after 18h or 24h starvation, the insects were re-fed for 3h and 6h, respectively. Adult insects were maintained at room temperature without food. In some cases adult insects were injected with 13mg of trehalose 2h before experiments (Arrese, Rojas-Rivas et al. 1996). For each time point, 3-5 fat bodies were pooled to prepare total RNA and lipid droplet fractions.

Materials

pIEx-1 Ek/LIC vector, pET-32 Ek/LIC vector, pET-30 Ek/LIC vector, JM-109 competent cells, NovaBlue GigaSingles competent cells and E. coli Rosetta 2 cells were purchased from Novagen.

pGEM T- Easy cloning system was from Promega. Insect Gene Juice and anti S-tag monoclonal antibodies were purchased from Novagen. Miniprep kit was from Qiagen (Qiagen Inc., Valencia, CA). Triton X-100 was from Aldrich. Sf9 cells, Sf-900 II SFM medium, gel electrophoresis molecular weight markers and electrophoresis items were from Invitrogen (Carlsbad, CA). InfinityTM Triglycerides Reagent was from Thermo. [Tri-9,10-^{3}H(N)]oleoylglycerol was purchased from Perkin Elmer Life Sciences (Boston, MA). Fatty acid free bovine serum albumin (BSA), Kanamycin, and Penicillin were purchased from Sigma Chemicals Co. (St. Louis, MO). Thin Layer Chromatography (TLC) plates were from Whatman. DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and DMPG [1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol (Na+ salt)] were purchased from Sigma. All other chemicals were of analytical grade. DNA sequencing was performed by the Core Facility of our department using an ABI Model 3700 DNA Analyzer.

Cloning the full length cDNA of *Ms*ATGL

Total RNA was isolated from the fat bodies of 5th day 1 of *M. sexta* using Trizol reagent (Invitrogen). From total RNA, mRNA was subsequently isolated using a NucleoTrap® mRNA Kit (BD Biosciences). mRNA was reversed transcribed using the SMARTerTM RACE cDNA Amplification Kit (BD Biosciences) according to the manufacturer's instructions. The resulting RACE-Ready cDNA was used as PCR template. 3'-RACE was performed using forward specific primer 1139F, 5'-CGACTCCGCCAACAGTGGAATAGTCAACTG-3' with reverse primer provided within the kit. The PCR run began with an initial denaturation step at 94°C for 2 minutes; 30 cycles at 94°C for 30 seconds, 68°C for 3minutes, and a final extension at 68°C for 4 minutes. Two major bands (2800bp and 1300bp) were obtained. After gel-purification from each band, these products were used as templates respectively for second PCR by 1212F, 5'-CTGCCCTACCGAGTGCCCATCGAC -3' with reverse primer provided within the kit using the same PCR program. Each reaction yielded one product (1300bp) that was subsequently cloned and sequenced. For 5'-RACE the reverse specific primer 1352, 5'-

CTGGCGGCCACCTCGGACCTGTCG-3' and nested primer 609R, 5'-CTGCAACAAATCTTCCCTGGT-3' were used with the forward primers supplied within the kit. A 700bp product was cloned and sequenced. The full length cDNA of *M. sexta* ATGL was amplified from cDNA by PCR using the forward and reverse primers ATGL-1F, 5'-AAGCAGTGGTATCAACGCAGAGTACGCGGGGGCA-3 and ATGL-2274R, 5'-CAAATATTCAGTAAGACACAGGTACTCAGTCAGTGGTGTAGCCA-3', respectively. The 2400bp PCR product was cloned into the pGEM-T Easy Vector and sequenced in both directions using our departmental Core Facility ABI Model 3730 DNA Analyzer. The cDNA sequence of *M. sexta* ATGL has been deposited into GenBank (Accession number AEJ33048.1).

Using sequence information from the *Manduca* genome a new primer was designed to verify the existence of a larger transcript. To further investigate this, we did 3'-RACE PCR using 2266F, 5'-CGATAGTTTGGCTACACCACTGACTGAG -3' with reverse primer provided within the kit followed by a nested PCR using 2266F and nested reverse primer 3830R, 5'-CTTTCACAACATAAGCCTGTCATAACAATTTAACATCG -3'. A 1600bp product was obtained and sequenced. The full length sequence of *M. sexta* ATGL was amplified from cDNA by PCR using ATGL1', 5'- GGGGCAGTTTCTTCGGTTTCTCTGTTGTGGA -3 with ATGL3779R, 5'- CCAAACTTCAACAGAGACAGACTCATTGCTG -3', respectively and we isolated a 3900bp product, which was cloned into the pGEM-T Easy Vector and the sequence was confirmed in both directions.

Northern blot analysis

Plasmid with 2.4kb full length *Ms*ATGL insert cloned into pGEM vector was used as template to amplify a 700 bp region by PCR using forward primer 5'-GTTGGTGTCGCTGTGTGCTTCAAGAAATACGCGCCA-3 and reverse primer ATGL-716R-T7, 5'-TAATACGACTCACTATAGGGAGGAACCGCAGCGCGT-3'. The resulting T7

promoter sequence (underlined) contained product was used to produce digoxigenin (DIG) labeled antisense RNA probe by in *vitro* transcription using the DIG RNA labeling kit (SP6/T7) (Roche). The kit was used according to manufacturer's instructions. One or two micrograms of total RNA was loaded in a 1 % agarose / 1.2% formaldehyde gel, and transferred to a nylon membrane by capillary blotting. The membrane was baked at 65°C for 20 min, and then pre-hybridized in church buffer (0.5M Na_2HPO_4, 7% SDS, 1mM EDTA, PH 7.0) with 0.5% (w/v) blocking reagent (Roche) at 68°C for 1h followed by hybridization with a final concentration of 100ng/ml ATGL probe in DIG Easy Hyb solution (Roche) at 68°C overnight. After washing with 2× SSC (0.3M NaCl, 30mM Sodium citrate), 0.1% SDS (w/v) at RT for 10 min twice followed by 0.1× SSC, 0.1% SDS at 68°C for 15 min twice, the membrane was blocked at RT in blocking solution [1% blocking reagent in Maleic acid buffer (0.1M Maleic acid, 0.15M NaCl, PH 7.5)] and then incubated in anti-DIG-AP 1:10000 solution. The membrane was continuously washed by Maleic acid buffer containing 0.3% Tween-20 before equilibration in detection buffer (0.1M Tris, 0.1M NaCl, PH 9.5). Alkaline phosphatase activity was detected using CDP-Star chemiluminescent reagent (Roche) and exposed to X-ray films. The films were scanned using a ScanMaker i900 (Microtek).

Expression and purification of *Ms*ATGL

Plasmid with 2.4kb a full length *Ms*ATGL insert cloned into pGEM vector was used to amplify the coding region of ATGL from the position corresponding to the first methionine to the stop codon by PCR. The left and right primers were ATGL lic-F 5′-GACGACGACAAGATGAACTTGTCGTTCGCCGGTTG-3’ and ATGL lic-R 5’-GAGGAGAAGCCCGGTTATTCGGCGTAAGTGACGTAGCTAG-3’. The product was ligated into the vector pIEx-1 Ek/LIC that contains an N-terminal His-Tag and S-Tag coding sequences and is designed for transient transfection and protein expression in Sf9 cells. Sf9 insect cell line is a clonal isolate derived from the parental *Spodoptera frugiperda* cell line. E. coli strain NovaBlue

GigaSingles cells were transformed with the generated plasmid for amplification. Plasmid DNA was extracted from isolated colonies using the Qiagen Miniprep kit and sequenced. Sf9 cells were transfected with the plasmid for *Ms*ATGL protein expression. Positive transfected cells were confirmed by western bloting.

Sf9 cells (50 ml of culture volume in flask with a density of 1-1.3× 10^6 cells/ml) were transfected with the pIEx1-ATGL plasmid (2.4µg plasmid/ml culture) dissolved in Insect Gene Juice according to the manufacturer's instructions. Suspension cultures were grown at 28°C with shaking at 150rpm. Cells contained in 50ml culture were harvested 24h after transfection and sedimented by centrifugation. Cells were washed once in cold PBS and re-suspended in 3ml of lysis buffer (25 mM sodium phosphate, pH 7.8, containing 20% glycerol and 1mM EDTA, 1mM DTT) followed by sonication (30 times with 1-s bursts at 40% power) (Jenkins, Mancuso et al. 2004). After centrifugation at 16,000g for 30min at 4°C the procedure was repeated with the pellet. Both supernatants were combined and used for protein purification. 50ml without transfected Sf9 cells with the same process were used for control. Both experimental and control supernatants were adjusted to 20mM imidazole and 500mM NaCl, and combined with 1ml of Ni^+ resin pre-equilibrated with the buffer (25mM sodium phosphate, 0.5M NaCl, pH 7.8, containing 20% glycerol). The slurry was incubated for 1h at 4°C and centrifuged at 300g for 5min. The resin was washed with ten bed volumes of same buffer containing 20mM imidazole, 40mM imidazole, 60mM imidazole, respectively. The fusion protein was eluted with 500µl buffer (200mM imidazole in buffer-25mM sodium phosphate, 0.5M NaCl, pH 7.8, containing 20% glycerol). Fractions were kept on ice in refrigerator and assayed for lipase activity.

Triglyceride (TG) content in Sf9 cells

Sf9 cells were grown in suspension in serum-free Sf-900 II SFM at 28 °C ± 0.5 °C in non-humidified, ambient-air incubator with shaking at 150 rpm. The cells with a density of 6-8×

10^5 cells/ml were seeded into 12-well plates (Corning® Costar®) for 2ml/well. When the confluency was >95%, the cell medium was supplemented with oleic acid (0.86 mM) for 24h followed by transfection (4µg plasmid/well) using Insect GeneJuice™ (Novagen/EMD Biosciences). After 24h cells were collected, rinsed with 500µl cold PBS and lysed with 75µl buffer (20mM Tris, 50mM NaCl, PH 7.4). Cellular TG content was determined in the lysates by a colorimetric assay using the Infinity triglyceride reagent kit (ThermoTrace Ltd, Mlebourne, Australia) according to the manufacturer's protocol. Triolein was used as standard. Protein concentration was determined by absorbance spectroscopy. TG content was expressed as nmol TG / mg protein. Data were statistically analyzed using one-way ANOVA.

Assay for Lipase Activity of purified ATGL

The final assay volume of 0.1 ml contained 85 mM K_3PO_4, pH 7.0, 2 mM EDTA, 7.5 mM DTT, 0.22mM [9,10-^{3}H] Triolein (4mCi/mmol), 2 mM Triton X-100, and ~30 µg of purified *Ms*ATGL protein. The mixture was incubated at 37°C in a water bath with 200rpm shaking. After 30 min, the reaction was terminated by the addition of 500 µl of a mixture of chloroform-methanol-benzene (2:2.4:1, v/v/v) and 40 µl of 1 M HCl. Blank reactions did not contain enzyme. Radiolabeled lipids from the organic phase were separated by TLC using hexanes:ethyl ether:formic acid (70:30:3, v/v/v) as developing solvent. Regions of the plate corresponding to TG, DG, monoacylglycerol (MG), and FFA were scraped and quantified by liquid scintillation counting. Enzyme activity was expressed as as nmol TG hydrolyzed/min-mg protein or nmol DG produced/min-mg protein (Arrese, Gazard et al. 2001).

***Ms*ATGL antibody**

A partial clone coding from the first methionine to the amino acid 449 was used to generate recombinant protein using the ligation-independent cloning (LIC) system. The amplified product was ligated into the vector pET-32 Ek/LIC after being treated with LIC-qualified T4 DNA

polymerase. The plasmid (pET-32-ATGL) was then transformed into E. coli strain NovaBlue GigaSingles. The positive clones were confirmed by DNA sequencing. Recombinant plasmid was used to transform E. coli Rosetta 2 (DE3). Fusion recombinant protein was purified by Ni affinity chromatography. Tag sequences were removed by thrombin and the resulting protein in conjunction with the peptide TSHHDALLAYYYLDGENKV conjugated with Keyhole limpet hemocyanin (KLH) was used to raise antibodies in rabbit at Cocalico Biologicals (Reamstown, PA).

Subcellular fractionation

Fat body tissue was collected after washing away the hemolymph using insect saline containing sodium bicarbonate 10 mM, HEPES 10 mM, sucrose 100 mM, potassium chloride 40 mM, sodium chloride 10 mM, calcium chloride 8 mM and magnesium chloride 30 mM at pH 6.5. Fat bodies from 3-4 insects were pooled and homogenized with a Potter-Elvehjem glass homogenizer fitted with Teflon pestle, using 3ml of homogenization buffer (HB) consisting of 50 mM Tris, pH 7.4, 0.25 M sucrose, 2 mM EDTA, 0.2 mM benzamidine, 10 mg/l leupeptin, 1 mg/l aprotinin, 2 mM dithiothreitol) (Arrese, Howard et al. 2010). All steps were carried out on ice. The homogenate was overlaid with 2ml of HB buffer without sucrose, and subjected to ultracentrifugation at 100,000 x g for 1 hr in a Beckman Ti 18 rotor. Three fractions were collected: fat cake, cytosol and pellet. For lipid droplets purification, the fat cake was re-suspended in HB and sucrose concentration was adjusted to 15% (w/v). A layer of 2 ml buffer without sucrose was laid on top and samples were centrifuged in SW40 rotor at 100,000 x g for 1hr. Purified lipid droplets were collected from the top and re-suspended in HB. Typically lipid droplets of two insect fat bodies were re-suspended in 0.5 ml of HB buffer. To isolate the membrane from the pellet, the pellet sample obtained from the first ultracentrifugation was re-suspended in 15 ml of HB and re-centrifuged at 100,000 x g for 1hr. The resulting pellet was

dissolved in 1ml of HB and centrifuged at 500 x g for 15min in order to remove the cell debris and nuclei. The resulting supernatant was used as the membrane fraction.

Western blotting

For western blotting, 30-40µg proteins were separated by SDS-PAGE (4-15%) and transferred to nitrocellulose membranes. Immuno-detection was performed using anti-ATGL antibody (1:20,000 in 7% milk). After incubation of membrane with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000 in 7% milk), peroxidase activity was detected using ECL chemi-luminescence reagents (Amersham Biosciences, Piscataway, NJ) and exposed to X-ray films. The films were scanned using ScanMaker i900 (Microtek).

Real-time quantitative PCR expression analysis

For expression analysis of ATGL transcripts during development, total RNA was extracted from a pool of dissected fat bodies (n = 3) from various stages using the Trizol reagent (Invitrogen). 1µg total RNA was reverse transcribed at 25°C for 5min, 42°C for 30min, followed by 85°C for 5min in a 20µl final volume containing 4µl of qScript cDNA supermix (Quanta). Real-time quantitative PCR (qPCR) analysis was performed in triplicate using the 7500 Real-Time PCR System from Applied Biosystems. Each reaction mixture contained 0.01µg cDNA template and 0.3 µM primers with a 20µl final volume using PerfeCTa SYBR Green FastMix, low ROX (2×) (Quanta) according to manufacturer's instructions. ATGL specific primers (forward, 5′-GGTCCCTCGGCCCGTTCA-3′ and reverse, 5′-TCTTCCCACCATACACCCTCGTGA-3′), and TGL specific primers (forward, 5′-ATGAACGATAGTACGGAAAGGAAAAGAGATAGCGA-3′ and reverse, 5′-CCCGCCATATTGATTTATCTTCGACATCCA-3′) were used in corresponding reactions. The simultaneous detection of the transcript of ribosomal protein S3 (*Ms*rpS3) was performed using primers (forward, 5′-TACAAACTCATTGGAGGTCTGGCCGT-3′, and reverse 5′-ACGAACTTCATGGACTTGGCTCTC-3′). The primer concentrations and

cycling conditions were optimized in preliminary experiments to avoid saturation. Samples were incubated in the light-cycler apparatus for an initial denaturation at 95°C for 1 min, followed by 40-45 cycles alternating 95 °C for 2s and 60°C for 45s. Quantification of relative gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Expression of each transcript was normalized with the transcript of *Ms*rpS3. At least two independent sets of total RNA were independently analyzed in triplicate.

Expression and purification of recombinant *Ms*Lsd1

Total mRNA was reverse transcribed using oligo-d(T)18-primer and the cDNA was used to amplify the coding region of Lsd1 (ACF24761.1) by PCR. The left and right primers were NLIC-msLSD1, 5′-GACGACGACAAGGTGACTCGAAGCCAAAAACCGAACATG-3' and Clsd1-lic, 5′-GAGGAGAAGCCCGGTCTAGTTCAGCCCGTTGATAGCCGCTA-3'. The product was ligated into the vector pET-32 Ek/LIC that contains an N-terminal coding sequence for thioredoxin followed by His-Tag and S-Tag coding sequences. E. coli strain NovaBlue GigaSingles cells were transformed with the recombinant plasmid. Positive clones were confirmed by DNA sequencing. E. coli Rosetta 2 cells were transformed for protein expression. Expression of the recombinant protein was induced with 1mM IPTG in 1 liter of suspension culture. After 4 h, bacteria were collected and rinsed in 20ml equilibration buffer (50mM Na_2HPO_4, 150mM NaCl, pH 7.6). The pellet was resuspended in 20ml Lysis Buffer (50mM Tris, pH 8.0, 100mM NaCl, 1mM EDTA, 1mM PMSF) containing 0.125mg/ml lysozyme and incubated at 4°C for 20min followed by sonication (3 times with 30-s bursts at 20% power). The incubation was repeated after the preparation was adjusted to 0.1% (v/v) Triton X-100. After adding 180ml Lysis Buffer, the preparation was centrifuged at 10,000g for 20min. The fusion protein was found in the pellet, which was resuspended in 50mM Na_2HPO_4, 150mM NaCl, 10mM $MgCl_2$, pH 7.4 and incubated with DNAse I at 28 °C for 2h. Sample

was centrifuged (10,000g, 20min) and the pellet was resuspended in equilibration buffer containing 10mM DTT. After centrifugation (10,000g, 15 min) the procedure was repeated for four times. Then the pellet was resuspended in 20mM Tris, pH 7.6,150 mM NaCl, 2 M Urea and 10mM DTT. After centrifugation (5000g, 25 min) the procedure was repeated for four times. The final pellet was resuspended in 20mM Tris, pH 7.6, 150mM NaCl, 3 M Urea and 10mM DTT. The preparation was centrifuged (5000g, 25 min) and the supernatant was collected. The stock solution was kept in the freezer.

Reconstitution of Trx–Lsd1 in lipoprotein particles and thrombin cleavage

DMPC/DMPG liposomes were prepared by adding 146ul of 10% (m/v) octylglucoside into a glass vial containing a thin film of DMPC/DMPG (0.84mg). The vial was vortexed vigorously for 30s at RT. Then DMPC/DMPG liposomes were mixed with 0.75 mg of fusion protein (Lsd1) or mutant proteins in 20mM Tris, pH 7.6, 150mM NaCl, 3 M Urea and 10mM DTT containing 10mM β-mercaptoethanol followed by exhaustive dialysis for 12 h against phosphate buffer (50mM Na_2HPO_4 pH7.4, 150 mM NaCl, 0.01% β-mercaptoethanol), and another 24 h against phosphate buffer (5mM Na_2HPO_4 pH7.4, 15 mM NaCl) at 4°C. Cleavage of Trx–Lsd1 was carried out by incubating the entire preparation with 1.3 U of thrombin for 3.5 h at RT followed by dialysis against the second phosphate buffer using dialysis membrane with 12–14 kDa cutoff.

Circular dichroism (CD)

CD spectroscopy was performed with a Jasco-715 (Jasco Corporation, Tokyo, Japan) spectropolarimeter using a 0.1 cm path length cell over the 195–260 nm range. The spectra were acquired every 1 nm with a 2 s averaging time per point and a 1 nm bandpass. Quadruplicates of the spectra were averaged, corrected for background, and smoothed. Protein concentrations were

determined by UV at 280 nm using extinction coefficients of 52,955 $M^{-1}cm^{-1}$ and 37,360 $M^{-1}cm^{-1}$ for Trx–Lsd1 and Lsd1, respectively. The mean residue ellipticity (deg cm^2 $dmol^{-1}$) was calculated from the corresponding number of residues of Trx–Lsd1 and S-tagged-Lsd1. The secondary structure of Lsd1 was estimated with the program Selcon3 using a 29-protein dataset of basic spectra (Sreerama, Venyaminov et al. 2000).

Mutagenesis of *Ms*Lsd1

The wild type *Ms*Lsd1-42 coding sequence in the pET30 plasmid cloned before was used as template to do the mutagenesis reactions (~30ng plasmid was used for each reaction). pET30 Ek/LIC vector contains N-terminal cleavable His-Tag and S-Tag coding sequences for detection and purification. All the mutants were prepared using the Quickchange (Stratagene) kit for site-directed mutagenesis according to manufacturer's instructions. Mutagenesis primers were used to introduce the desired replacements. Successful mutations were confirmed by sequencing of mutated plasmids in both directions using our departmental Core Facility ABI Model 3730 DNA Analyzer.

Statistics

Statistical comparisons were made by the student's t test. $p < 0.05$ was considered to be significant.

Other methods

Protein concentrations were determined by the Bradford dye-binding assay using bovine serum albumin as standard (Bradford 1976). SDS-PAGE was performed according to Laemmli (Laemmli 1970) and the proteins were visualized by Coomassie Brilliant Blue R staining. The deduced amino acid sequence was obtained using the translate tool at ExPASy (http://ca.expasy.org/tools/dna.html) (Arrese, Howard et al. 2010) and the multiple amino acid

sequence alignment was done at ExPASy (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phosphorylation site prediction was done at ExPASy (http://www.cbs.dtu.dk/cgi-bin/nphwebface?jobid=netphos,5057910902D06974&opt=none).

CHAPTER III

RESULTS AND DISCUSSION

Expression and purification of recombinant *Ms*Lsd1 in *E.coli*

In *M. sexta*, the mobilization of energy reserves is regulated by AKH. The activity of the main lipase TGL correlates with the phosphorylation level of the lipid droplet-associated protein, Lsd1. AKH-induced lipolysis provokes a rapid phosphorylation of Lsd1, and this event accounts for the majority of the lipolytic response induced by AKH.

Lsd1 is tightly bound to the lipid droplets. It can be only partially dissociated with urea and detergents. Expression of recombinant Lsd1 (rLsd1) in *E. coli* was attempted as a mean to generate sufficient protein for functional studies. *Manduca sexta* Lsd1 was expressed as a fusion protein with thioredoxin (Trx–Lsd1) using the pET-32 Ek/LIC vector that also inserts a His-tag and an S-tag coding sequence between Trx and Lsd1(Fig.6). A thrombin cleavage site separates the Trx/His tag from S-tag/Lsd1.

Fig. 6. Scheme of Trx-lsd1 protein construct including the protease cleavage sites for thrombin and enterokinase.

Expression of Trx–Lsd1 was attained in *E. coli* strain Rosetta 2. Maximal expression of rTrx–Lsd1 (60kDa) occurred 4 h after IPTG induction when Trx–Lsd1 was the major protein of the

lysate. Trx–Lsd1 was found in the insoluble fraction of the bacteria lysate. Solubilization was achieved by sonication in the presence of lysozyme and detergent as described in methods. Exploiting the insolubility of Trx–Lsd1 we removed contaminant proteins by washing the pellet several times with PBS buffer containing 2 M urea and 10mM DTT. Then Trx–Lsd1 was solubilized in PBS buffer containing 3 M urea and 10mM DTT. This procedure yielded a pure preparation of Trx–Lsd1 (Fig.7A, lane 1). Thrombin cleavage of Trx–Lsd1 yielded a single product of 45 kDa and the 14 kDa N-terminal domain (Trx–His) (Fig.7A, lane 2). Fig 7B shows the purified *Ms*Lsd1.

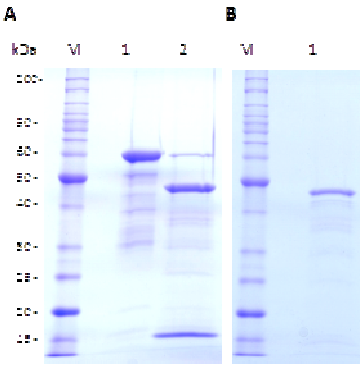

Fig. 7. Expression and purification of recombinant *Ms*Lsd1. SDS-PAGE of Trx-*Ms*Lsd1 before and after thrombin cleavage (Lane 1A and 2A) and purified *Ms*Lsd1 (Lane 1B).

*Ms*Lsd1 reconstituted with phospholipids is stable in aqueous solution

In order to be able to characterize Lsd1, first of all it is needed to have the protein soluble in an aqueous medium in the absence of denaturant or detergent. The protein was stabilized in aqueous buffer by binding to lipids. We prepared lipoprotein particles of Trx–Lsd1 by using the octylglucoside dialysis method, which is amply used to prepare discoidal lipoproteins with apolipoproteins (Jonas, Kezdy et al. 1989, Chetty, Arrese et al. 2003). Trx–Lsd1 in buffer containing urea and octylglucoside was incubated with two types of lipids DMPG and DMPC,

and subjected to exhaustive dialysis in phosphate buffer. A clear solution of Lsd1 was obtained after dialysis. The absence of aggregates was an indication that rLsd1bound to lipids and because of this the protein was stable in solution . The removal of the Trx–His N-terminal region from the fusion protein was obtained by cleavage with thrombin and this step did not affect stability of the lipoprotein complexes. The lipoprotein particles of Lsd1 and DMPG/DMPC formed a clear and stable solution in aqueous buffer. Analysis of the particle size by nondenaturing gel electrophoresis indicated that Lsd1/DMPG particles had an apparent diameter of 7.8 nm and Lsd1/DMPC particles had an apparent diameter of 12 nm (Fig.8A). The far-UV CD spectrum of both Lsd1/DMPG and Lsd1/DMPC showed the typical features of the spectra of α-helical proteins confirming that the protein is folded. Lsd1/DMPC seems to be less structured than Lsd1/DMPG (Fig.8B).

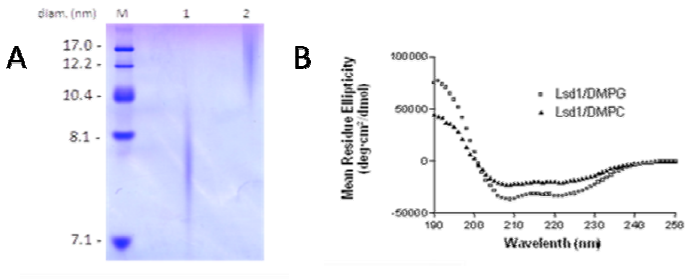

Fig. 8. Reconstitution of *Ms*Lsd1 with phospholipids ("artificial LDs"). A) Native gels showing the formation of complexes Lsd1-DMPG (Lane 1) and Lsd1-DPMC (Lane 2); B) Far UV CD spectrum of Lsd1 in lipid complex, CD spectrum in phosphate buffer.

Alignment of Lsd1 proteins

*Ms*LSD1 protein is a regulator of lipolysis whose activity is modulated by PKA-mediated phosphorylation as mentioned in the introduction. A previous study showed there was a direct effect of PKA phosphorylation of Lsd1 on the activity of TGL (Lsd1phosphorylation enhances the activity of TGL) (Arrese, Rivera et al. 2008). However, the mechanism is not known. As a first step to elucidate the mechanism by which this protein activates TGL we wanted to identify

the conserved elements in Lsd1. To investigate this issue, both the alignment of the deduced Lsd1 protein sequences from multiple organisms and phosphorylation site prediction of *Ms*LSD1were performed. As seen in Fig.9, several conserved Ser and Thr which were predicted phosphorylation sites, in addition a 100% conserved "EPENQARP" region was also identified. The sketch of *Ms*Lsd1structure showing key conserved residues and structure regions are illustrated in Fig 10.This analysis provided us with the preliminary molecular insight for a mutagenesis study.

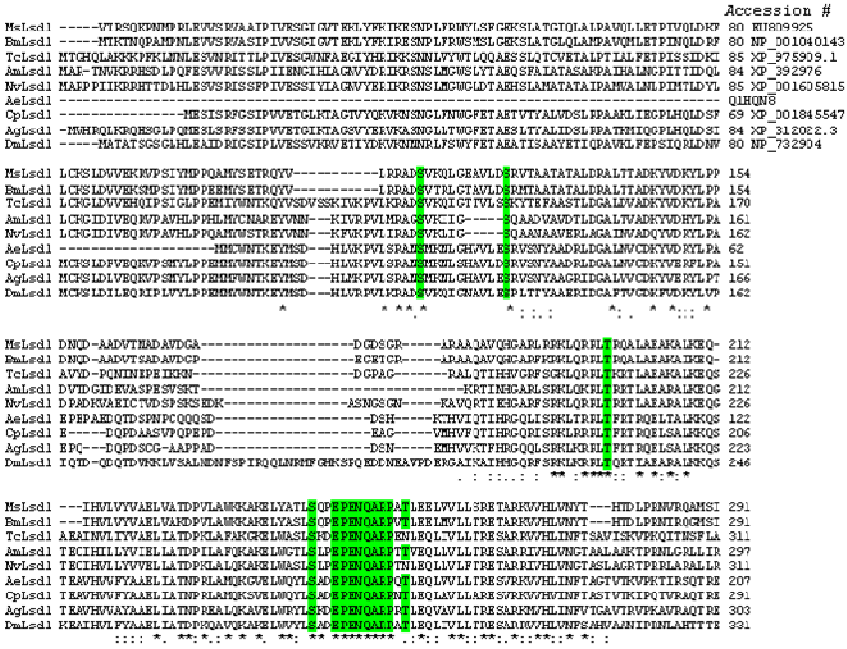

Fig.9. ClustalW Alignment of Lsd1 deduced amino acid sequences. *M. sexta, Ms; B.mori, Bm; Tribolium c., Tc; Apis mel, Am; Nasonia v., Nv; Aedes aegypti, Ae; Culex pipiens, Cp;Anopheles gambiae, Ag; Drosophila mel, Dm.* The conserved amino acids are colored with green.

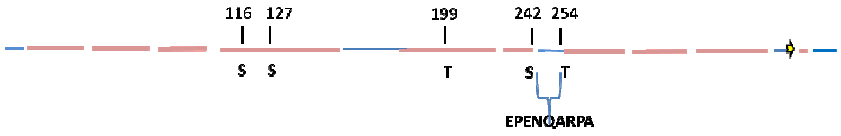

Fig.10. Sketch of *Ms*Lsd1-42 structure showing key conserved residues and structure regions. The figure shows the locations of conserved phosphorylation sites presented in *Ms*Lsd1. They are within the α-helix. It also shows the key conserved sequence (EPENQARP) located in the random coil. —— α-helix, —➔ β-sheets, —— random coil

The following mutants: triple mutant S116D/S127D/T199E; double mutant S242D/T254E; and "EPENQARP" deletion mutant were prepared by site directed mutagenesis as part of this project. Site directed mutagenesis was performed as indicated in Materials and Methods. The mutations were confirmed by DNA sequencing. Wild-type and mutated Lsd1s were expressed, purified and reconstituted in lipoprotein particles using DMPC. The effect of these mutations on the lipase activity will be tested *in vitro*. At this time, some of these experiments are in progress and preliminary results obtained by Zengying Wu indicate that the conserved "EPENQARP" region may have inhibitory activity to TGL. Moreover, the triple mutant mimicking the phosphorylated Lsd1 showed an increase in the lipase activity.

The second part of this project is to investigate the role of Adipose triglyceride lipase (ATGL) in the hydrolysis of TG stores in *M.sexta*. ATGL is an evolutionary conserved lipase that governs the mobilization of fatty acids in human adipose tissue in conjunction with HSL. The importance of ATGL in insects was shown in *Drosophila* where the loss of Brummer lipase (ATGL homolog) causes accumulation of TG, whereas its over-expression renders lean flies. To begin the study, we attempted to clone, express and obtain antibodies against *Ms*ATGL.

Cloning and analysis of *Ms*ATGL cDNA sequence

A previous study conducted by Jorge Zamora in the lab of Dr. Michael Wells' lab at University of Arizona led to the identification of a partial clone of *Manduca Sexta* ATGL. Our lab had access to such material and that clone was used to generate antibodies as explained below. Moreover that sequence was also used to design gene specific primers to perform RACE experiments to clone the full length of *Ms*ATGL cDNA using cDNA synthesized from fat body mRNA. The 3'- RACE reactions produced two major products, ~2800 and 1300bp that were purified and used for a second round of PCR performed with nested primers as indicated in methods. These PCR reactions resulted in same length products ~1300bp that were cloned into the pGEM vector and sequenced. On the other hand, 5'- RACE amplified a product of ~700bp, which was sequenced. These studies provided an *Ms*ATGL cDNA sequence of 2.4 kb which was cloned. The full-length coding sequence encodes for a 550-amino acid protein containing a patatin-like domain (Fig. 11) which is a key signature of ATGL enzymes isolated from other organisms. The sequence has an open reading frame (ORF) of 1646 bp (positions 246-1892) coding a 550 amino acid protein (ATGL) with a theoretical molecular weight of 64 kDa (Fig. 11). The 3' non-coding region of *Ms*ATGL consists of ~470 nucleotides including a poly(A) tail of 25 residues.

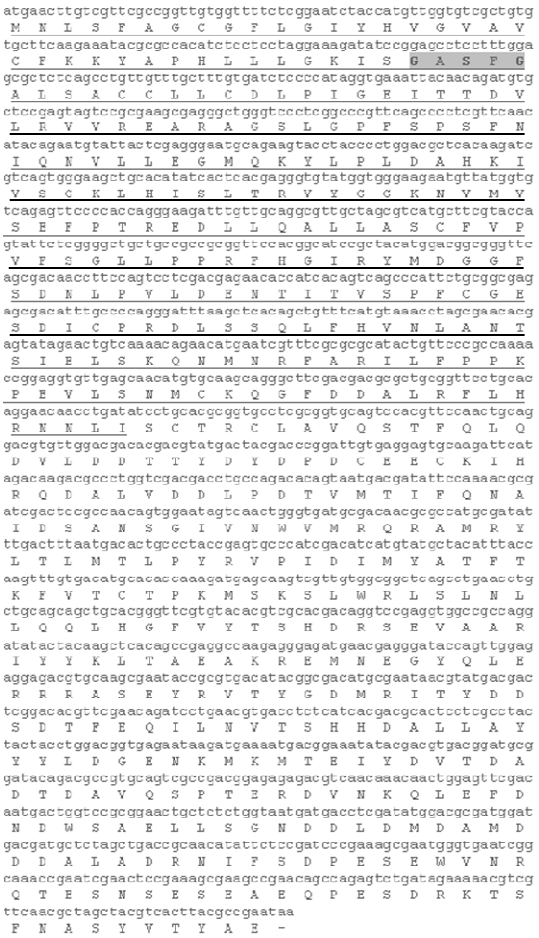

Fig. 11. ***M.sexta.*** **cDNA coding region nucleotide (246–1892) is shown above the deduced amino acid sequences (1–550).** The amino acid sequences underlined represent Patatin-like phospholipase domain. The consensus lipase catalytic motif is highlighted in gray.

A comparison of the deduced *Ms*ATGL protein sequence with the sequences of other putative insect ATGLs shows significant conservation (~50% amino acid identity) and 95% identity to *B.mori* (Fig. 12). The conservation is particularly high in N-terminal region where a potential functional domain is found: the N-terminal region that contains the Patatin-like phospholipase domain in which the lipase consensus sequence (GXSXG) containing the active site serine is located. We also did the amino acid sequence alignment between *Ms*ATGL and vertebrate ATGLs (TTS-2.2, GS2, adiponutrin in human and desnutrin in mice) (data not shown). The result showed only ~30% amino acid identity. However, the lipase catalytic motif is still conserved.

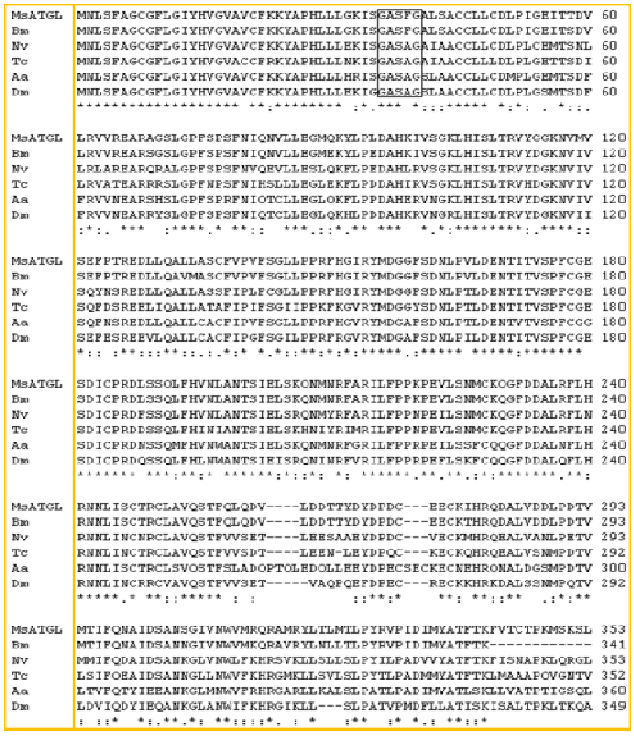

Fig.12. Multiple alignment of five insect ATGL deduced amino acid sequences. MS (*M. sexta*, AEJ33048.1), Bm (*B. mori*, NP 001165929.1), Nv (*Nasonia vitripennis* , XP 001602845.1), Tc (*Tribolium castaneous*, XP 970721.2), Aa (*Aedes aegypti*, ABL75463.1)and Dm (*Drosophila melanogaster*, NP 001163445.1). The lipase catalytic motif is framed.

Northern Blot analysis: *Ms*ATGL has two transcripts

Northern blot analysis of *Ms*ATGL using poly (A) - RNA extracted from fat body of D_2 adult male detected a major band corresponding to a transcript of 4.0 kb and a weak band corresponding to transcripts of 2.4 kb, respectively (Fig. 13A).

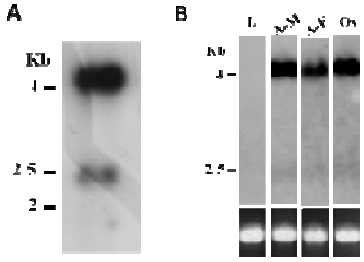

Fig. 13. Northern blot of *Manduca sexta* RNA. A) PolyA-RNA extracted from the fat body of 2-day adult male insects; B) Total RNA from the fat body of larva (L), adult male (A-M), adult female (A-F) and ovaries (Ov). RNA was probed with a 0.67 kb DIG labeled anti-sense RNA probe.

More recently we had information from the *Manduca* genome project and we were able to identify in the lab the gene corresponding to ATGL (Fig.14). This gene has 9 exons and 8 introns. After transcription and splicing, the mature mRNAs have two different lengths (2.4 and 3.9 Kb) due to the alternative polyadenylation. Both transcripts are translated into 64KDa ATGL Protein. The average length of intron is ~3000 bp. The *drosophila ATGL* gene (Gene ID: 39611) has two transcripts: variant A (8 exons) and variant B (7 exons, exon #7 is missing compared to variant A). The length of first intron is ~6900 bp while the left introns are ~100 bp. The *Mouse ATGL* gene (Gene ID: 66853) also has two transcripts: variant 1 (9 exons) and variant 2 (8 exons, exon #6 is missing compared to variant 1). The first intron is ~1800 bp while the left introns are ~150 bp. The *human ATGL* gene (Gene ID: 57104) has only one transcript (10 exons) and the average length of intron is ~500 bp.

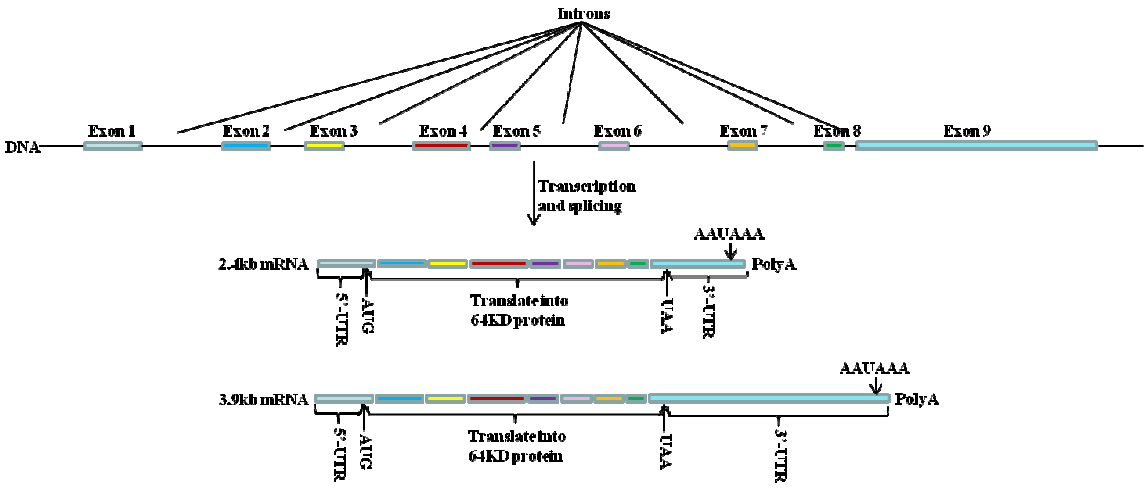

Fig.14. Sketch of *MsATGL* gene. There are 9 exons and 8 introns. After transcription and splicing, the mature mRNAs have two different lengths due to the alternative polyadenylation which is later translated into 64KDa ATGL Protein.

To further investigate this, the terminal regions of 2.4 kb sequence together with the *Manduca Sexta* genome sequence were used to design gene-specific primers as described in methods for RACE PCR. The 3'-RACE amplified a single product of ~1600bp, which was sequenced. On the other hand, for 5'-end the same sequence as 2.4kb cDNA was obtained. These studies provided

an *Ms*ATGL cDNA sequence of 3.9 kb. The 2.4 kb cDNA is totally included in the 3.9 kb cDNA, which is 1620 bp longer in the 3'-non coding region including a poly(A) tail of 30 residues. Therefore, they have the same coding region which translates to a 550 amino acid protein with a theoretical molecular weight of 64 kDa (Fig. 11). The presence of two different transcripts encoding the same protein anticipates complex mechanisms of the functions and regulations of ATGL. We were able to clone and sequence cDNA corresponding to these two transcripts.

Characterization of *Ms*ATGL: ATGL is a lipase

Recombinat *Ms*ATGL was expressed in Sf9 insect cells that were previously incubated with optimal amount of oleic acid to induce an accumulation of cellular triglycerides. For this purpose, cell medium was supplemented oleic acid for 24h prior to the transfection. The success of transfection was confirmed by western blot (Fig. 15A). The over-expressed Sf9 cells showed a significantly lower content of cellular TG than in control suggesting that recombinant ATGL exhibits lipase activity against triglycerides (Fig. 15B).

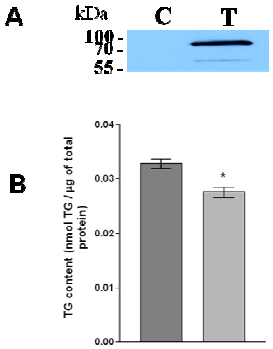

Fig.15. Expression and partial Characterization of *Ms*ATGL in Sf9 cells. *Ms*ATGL was ligated into pIEx-1 Ek/LIC vector that contains N-term His•tag and S•tag coding sequences and used to transfect Sf9 cells. **A)** Homogenates of transfected (T) Sf9 cells and control (C) cells (30µg/lane) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-ATGL antibody. Immuno-reactive bands were visualized by probing the blot with an anti-rabbit Ig G horseradish peroxidase conjugate followed by reaction with ECL reagents; **B)** Effect of *Ms*ATGL expression on the TG content of Sf9 cells. Data are expressed as means ± SE (n = 4–5). Statistical comparisons were made by t-Student test. Data that are expressed as means ± SEM (n = 4).

Recombinant ATGL expressed in Sf9 cells was purified by Ni-affinity (Fig. 16). In *vitro* analysis of the lipase activity of the purified protein was studied using artificial substrates. Fig. 16 shows the changes in lipase activity normalized by protein content (nmol product/min-mg of protein). The DG-producing activity was significantly higher ($P < 0.05$) than MG. This suggests that ATGL has higher TG- than DG- hydrolase activity. In other words, the main product of TG hydrolysis catalyzed by ATGL is diacylglycerol (DG).

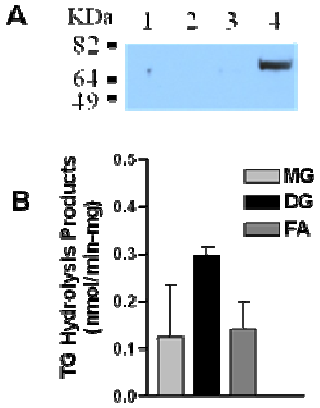

Fig.16. Expression, Purification and partial Characterization of *Ms*ATGL in Sf9 cells
*Ms*ATGL was ligated into pIEx-1 Ek/LIC vector that contains N-term His•tag and S•tag coding sequences and used to transfect Sf9 cells. **A)** Purification of recombinant ATGL in Sf9 cells by Ni-affinity chromatography. The soluble fraction from ATGL over-expressed Sf9 cells was bound to Ni^{+} resin. Recombinant ATGL His-tagged protein was eluted by a gradient of imidazole in lysis buffer (lane1-20mM imidazole; lane 2-40mM imidazole; lane 3-60mM imidazole and lane 4--200mM imidazole). The fractions (15µl /lane) were loaded for western blot analysis using anti-ATGL polyclonal antibody; **B)** Affinity-purified recombinant ATGL catalyzes TG hydrolysis to form Diolein (DG). 50ul sample fractions (~30ug protein) were examined for lipase activity against an emulsion of [^{3}H-triolein] and Triton X-100. Products of TG hydrolysis were separated by TLC. Data that are expressed as means ± SEM (n = 4).

Subcellular localization of *Ms*ATGL

A polypeptide from the first methionine to the amino acid 449 of *Ms*ATGL was generated in *E.coli* and used to generate antibodies in rabbits as indicated in materials and Methods. Fig17 panel A shows the SDS-PAGE of the purified fusion ATGL (lane 1, 65KD) and the resulting partial cleavage of fusion ATGL with thrombin (lane 2, 53KD). Panel B shows the Western Blot analysis using the anti-ATGL antibody generated in rabbit.

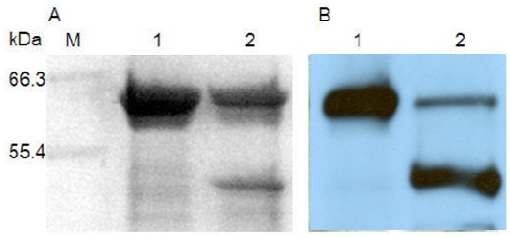

Fig.17. Purification of recombinant *M. sexta* ATGL and antibody production. rATGL was expressed in *E.coli* as a fusion protein and purified from the bacteria lysate by standard procedures. Fusion ATGL was separated by SDS-PAGE, transferred to nitrocellulose, and probed with antiserum. **Lane 1**: Fusion ATGL**; Lane 2**: partial cleavage of fusion ATGL. **A)** SDS-PAGE; **B)** Western Blot

The subcellular distribution of *Ms*ATGL among cytosolic, lipid droplet, and membrane fractions of fat body cells was investigated by immunoblotting using an anti-*M. sexta* ATGL antibody. Fig. 18 shows ATGL is mostly associated to the lipid droplet fraction of the cells.

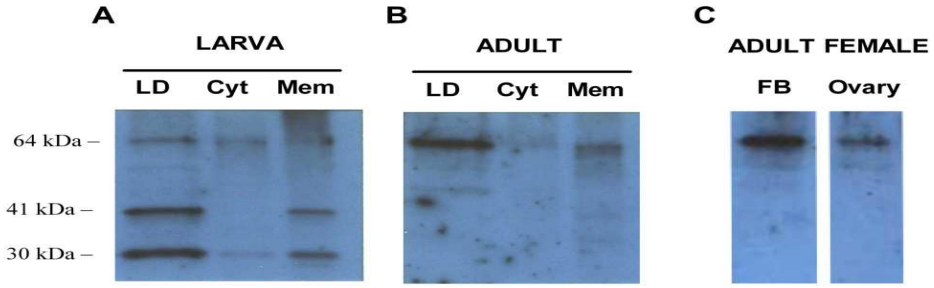

Fig. 18. Western analysis of the expression and sub-cellular distribution of ATGL in *M.sexta* fat body. Lipid droplets (LD), cytosol (Cyt) and membrane (Mem) fractions were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-ATGL antibody. A) and B) Samples from larva and adult fat bodies; C) LD isolated from fat body and ovaries from female insects.

Expression of ATGL during development

To gain insights into the role of ATGL on TG mobilization, developmental changes in the levels of its transcript to that of rpS3 (control) were quantified by RT-PCR which was carried out as described in methods. rpS3 is a highly conserved protein component of the small ribosomal subunit (Jiang, Wang et al. 1996, Lyamouri, Enerly et al. 2002). *Manduca* rpS3 has been previously used as control in studies dealing with fat body expression of proteins (Jiang, Wang et

al. 1999, Yu and Kanost 1999, Arrese, Mirza et al. 2008). RT-PCR analysis throughout development revealed that the transcript is present at all developmental stages, larvae, wander, and adults (Fig 19A). *Ms*ATGL transcription is up-regulated during the physiological non-feeding periods of the larval stages (HC and W). High levels of expression are also observed in the adult male and female insects which are also non-feeding periods (M and F). The levels decreased significantly in feeding larvae (D1, D2, and D5). *Ms*ATGL transcript was also detected female ovary, where the level is in the same range as wander stages.

The expression of ATGL protein in different developmental stages of *M. sexta* was investigated by immunodetection using anti-ATGL antibody. The lipid droplets isolated from fat body were directly separated by SDS-PAGE, transferred to the membrane and analyzed by western blotting. As seen in Fig. 19B, *Ms*ATGL (64 KD) is up-regulated during the non-feeding periods (HC, W3 M and F) together with female ovary. More bands (such as 41 KD and 30 KD) were detected in larvae stages from HC to W1. Interesting, the transcription of ATGL mRNA correlates with the appearance of the 64 KDa ATGL protein for the most part (HC, D5, W1, W3, M and F). The smaller bands identified by anti ATGL antibody (41 KDa and 30 KDa) could originate from cleavage of the 64KDa protein. This observation is in agreement with the fact that no transcript was detected by Northern blot. Moreover the cleavage of 64 KD ATGL seems to be increased in the feeding period of the 5th instar larva.

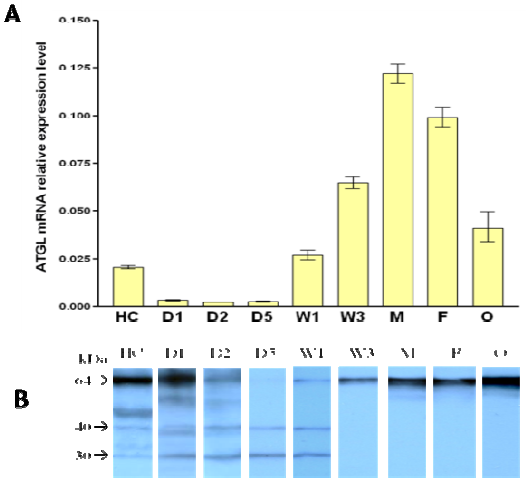

Fig. 19. Changes of *Ms*ATGL during development: A) mRNA levels. Total RNA was extracted from fat bodies and subjected to real-time PCR. Results show the means of independent triplicate samples± SD, normalized against ribosomal protein S3; B) Protein levels by Western Blot of lipid droplets and fat bodies and ovary. Abbreviations: HC, head capsules; D1,D2,D5, larva day 1, day 2 and day5, respectively; W, wanderer day 1 and 3: M and F , adult male and female day 2-3; O, ovary.

Major behavioral and metabolic differences distinguish the larval and adult stages of *M. sexta*. Among those differences is the fact that larvae eat constantly and accumulate lipid reserves in the fat body, whereas adult insects consume the lipid stores to support the energy demand s imposed by reproduction and flight. Since HC, wander and adult stages need to mobilize TG stored in the fat bodies, it makes sense that ATGL transcript and expression are up-regulated while in feeding-larvae stages it is degraded. Because ATGL is the important lipid droplets associated lipase to hydrolyze TG for energy supplying. However, the biological significance of this specific degradation is unknown. Perhaps these products could play an important role during larvae stages. In fact, ATGL degradations were also observed during the transfection studies in Sf9 cells (Fig. 20: when the Sf9 cells were transfected for 48h, ATGL was significantly degraded compared with the transfection of 24h). More work remains to be done in order to solve the issues.

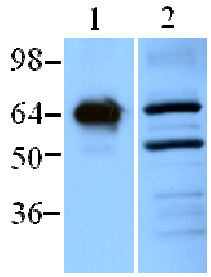

Fig.20. Western blot analysis of expression of *Ms*ATGL in Sf9 cells. *Ms*ATGL was ligated into pIEx-1 Ek/LIC vector that contains N-term His•tag and S•tag coding sequences and used to transfect Sf9 cells. Homogenate of transfected Sf9 cells (30µg/lane) was separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-ATGL antibody. Lane 1: transfection for 24h; Lane 2: transfection for 48h.

As introduction chapter mentioned, the concentration of lipid in the hemolymph was nearly doubled in the molt from 4th to 5th instar and there was a pronounced increase (Fig.5) in wandering larvae and prepupae, which were not consistent with the main lipase TGL activity pattern (TGL activity is lower during the molt from 4th to 5th instar, wandering larvae and prepupae stages). Here we investigate the transcript level of ATGL during development which shows the similar pattern to the hemolymph lipid concentration (Fig.21). This suggests that ATGL does play a critical role during the physiological non-feeding periods of the larval stages (HC and W).

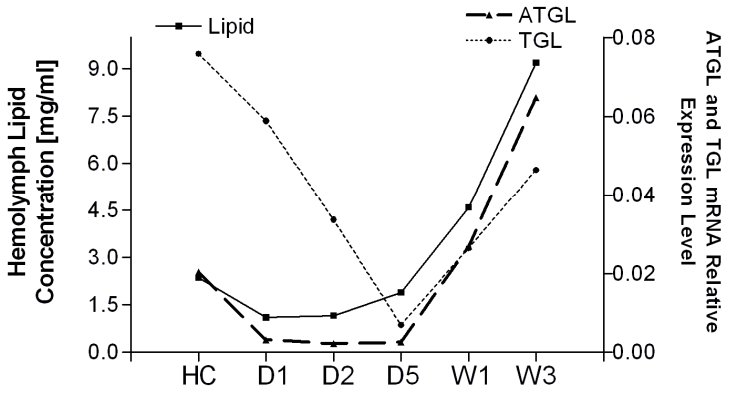

Fig. 21. Changes of hemolymph lipid concentration with *Ms*ATGL and TGL expression during development of *Manduca sexta*. Results for mRNA levels of ATGL and TGL show the means of independent triplicate samples, normalized against ribosomal protein S3. Data assembled from: (Ziegler, Willingham et al. 1995, Tobler and Nijhout 2010)

To infer the biological function of ATGL from its changes during development, its level of mRNA was compared with the expression of TGL. The expression of TGL mRNA was higher than that of ATGL at all times during development (Fig.22), but for the wander stages especially at W3.

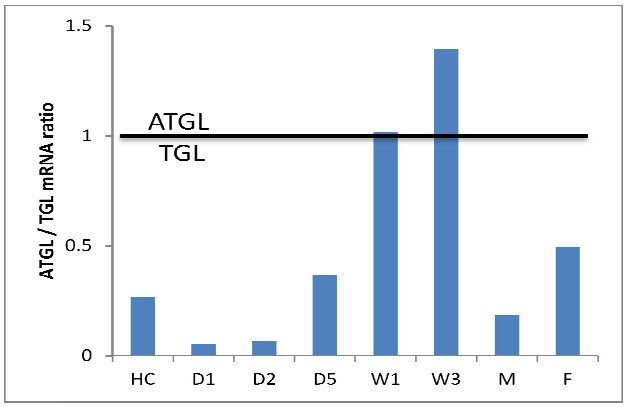

Fig.22. Ratio between mRNA levels of ATGL and TGL during development. Values are the ratio of average values for the expression of ATGL and TGL in the fat body.

Effect of starvation and re-feeding on ATGL expression in the fat body of insects at the larval stages

Given the very low abundance of ATGL under feeding conditions and increased levels during the physiological non-feeding periods we investigated into more detail the effect of starvation in the feeding larvae insects. 5th D1 larvae were subjected to starvation for different periods of time (6h, 12h, 24h, and 30h) and levels of transcripts and proteins associated with the lipid droplets were determined. Both levels of ATGL transcripts and proteins were dramatically up-regulated by starvation in a time-dependent manner. For 6h starvation the transcripts increased to ~7 fold compared to D1 and D2 control while it became ~30 fold when starved for 30h (Fig.23A). Quantification analysis of protein level in Fig. 23C shows that the amount of large form increased to ~8 fold compared to D1 after 30h starvation. These observations indicate that the expression of ATGL is highly sensitive to the nutritional status of insects and may play a key role under starvation conditions during larval stages. This conclusion was confirmed by re-feeding experiments. As we can see in Fig. 23D, 18h starved larvae were re-fed for 3h and 6h the level of ATGL mRNA dropped significantly after re-feeding for 3h. The degradation of ATGL was observed at 3h re-feeding using 24h starved larvae, and the levels of ATGL protein dropped

dramatically after re-feeding for 6h (Fig.23F). The levels of ATGL mRNA from both male and female adult were also investigated. We mimicked the adult re-feeding by injecting trehalose, because they were maintained at room temperature without food which was in a starvation status. Fig 23E shows that levels of ATGL were dramatically down-regulated (~1 fold) after injection of trahelose for 24h regardless of the sex of the insects.

Moreover, the expression of ATGL protein correlates with the appearance of the 4.0 kb transcript rather than 2.4 kb transcript by Northern blot analysis (Fig. 23B). The biological significance of this observation is unknown.

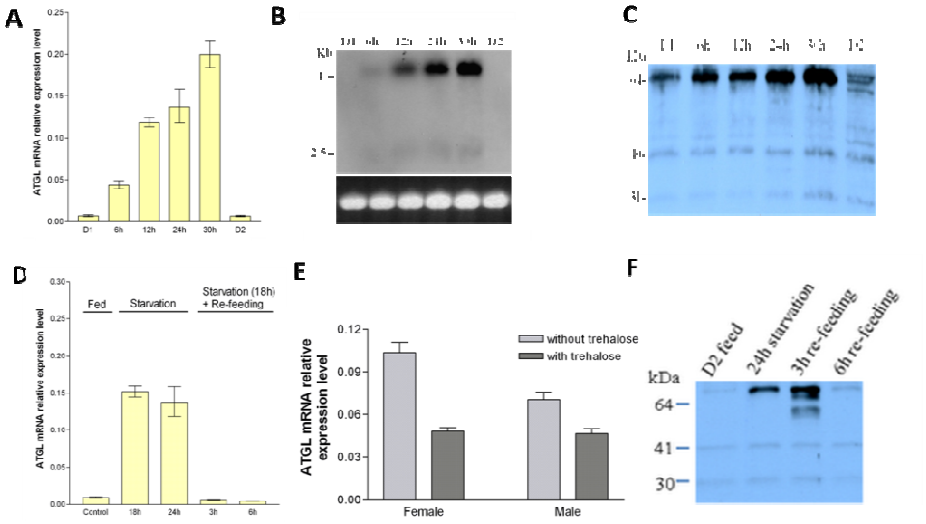

Fig.23. Expression of ATGL during starvation and re-feeding. A) mRNA measured by RT-PCR; B) Northern analysis of total RNA; C) and D) Western blot analysis of LD fractions. Larvae from 5th instar day 1 were subjected to starvation for different periods of times. Each condition had 3-5 insects pooled together for total RNA extraction and fat body homogenates. 0.01µg cDNA was used for each RT-PCR reaction. 2µg total RNA was loaded for northern blotting. 40µg protein from LD fraction was loaded for western blot.

Likewise the effect of starvation of 5th-instar larva on the levels of TGL mRNA was investigated (Fig.24). The expression of TGL was significantly increased after 12, 24 and 30h of starvation. During prolonged starvation (>6h) TGL levels are higher than ATGL. However, during short-term starvation (6h) the transcription of ATGL was dramatically induced, whereas the expression of TGL was decreased, such that ATGL and TGL mRNA levels become equally abundant. The

differences in ATGL levels observed between feeding and non-feeding periods of larval stage were not observed for TGL suggesting a specific role of ATGL in lipid mobilization under starvation conditions and the two lipases seem to be controlled by independent mechanisms.

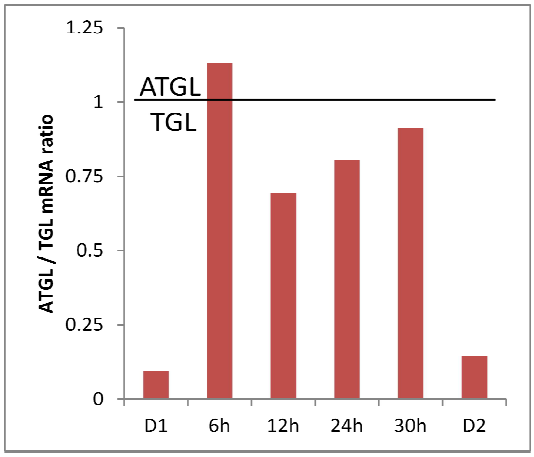

Fig.24. Ratio between mRNA levels of ATGL and TGL during starvation. Values are the ratio of average values for the expression of ATGL and TGL in the fat body.

According to Tobler and Nijhout (Tobler and Nijhout 2010), nutrient deprivation affected glucose concentration in hemolymph and the effect also depended on the timing of the starvation. Glucose levels declined precipitously soon after the animal had been deprived of food and glucose concentration had become almost undetectable after 24h starvation (Fig.25). This decrease in hemolymph glucose appeared to trigger the secretion of AKH from the corpora cadiaca which activated fat body glycogen phosphorylase. Probably because of the activited glycogen phosphorylase, the hemolymph trehalose level stayed high (A Gies, T Fromm, R Ziegler 1988). But during prolonged starvation (>6h), both the transcript level of ATGL and TGL was dramatically increased which suggestes that the up-regulated expression of lipases plays roles in hydrolyzing TG in stored to release lipid into hemolyph for energy supplying. However, more work need to be done to figure out the exact signal pathways.

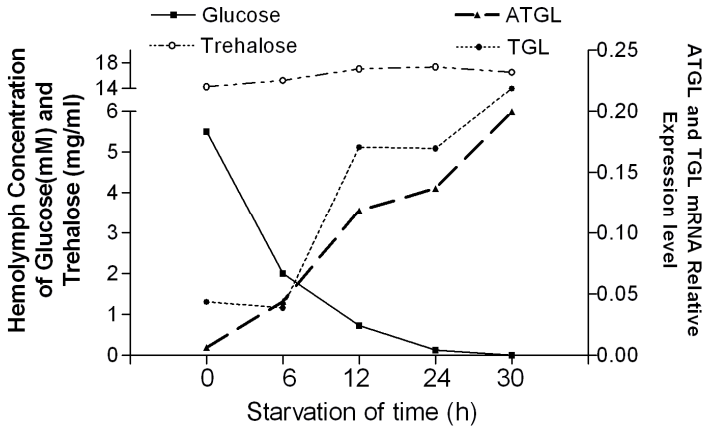

Fig. 25. Changes in hemolymph sugar levels and the expression of ATGL and TGL in starving larvae of the 5th instar. Results for mRNA levels of ATGL and TGL show the means of independent triplicate samples, normalized against ribosomal protein S3. Data assembled from: (Tobler and Nijhout 2010) (A Gies, T Fromm, R Ziegler 1988)

Effect of ATGL on TGL mediated lipolysis

In order to assess the contribution of ATGL activity to the overall TG hydrolysis, the endogenous lipase activity associated to lipid droplets was measured. Unlike TGL which is a cytosolic lipase, ATGL is found associated with LDs. Lipid droplets were isolated from larval fat bodies under fed (low ATGL content) and starved conditions (high ATGL content) (Fig. 23) and tested for lipase activity using [^{3}H]-triolein that was incorporated into the lipid droplets by vortexing the lipid droplet aliquot containing ~650 nmol TG into a thin film of [^{3}H]-triolein (0.1 µCi). Lipid droplets showed a very low lipase activity and no significant difference were observed between fed and starved conditions (Fig 25). By contrast, when lipid droplets were incubated in the presence of cytosol (TGL) a very robust lipase activity was observed (Fig 26). This result points out the difficulty of measuring lipase activity of ATGL. This was also the case with recombinant ATGL. Different condition (pH, detergents, ion strength) were tested without being able to improve the lipase activity of ATGL. More research is needed to solve this issue.

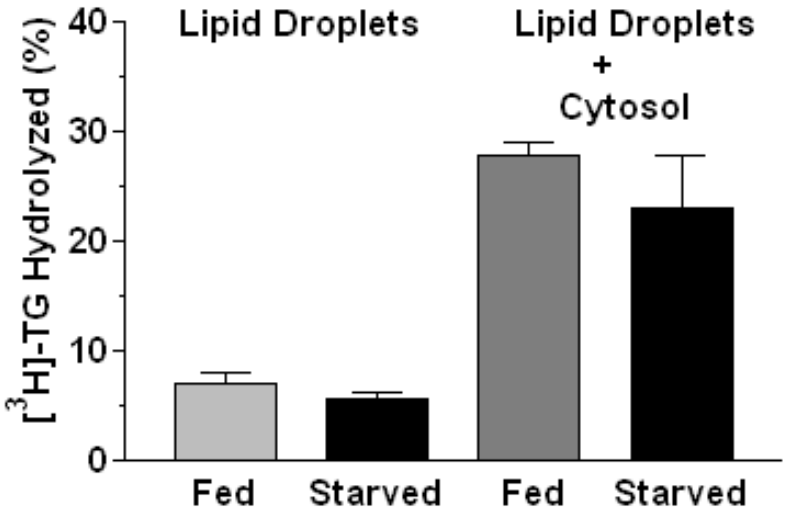

Fig. 26. Lipase activity associated to lipid droplets (LD) and lipase activity of lipid droplets plus cytosol (LD+cytosol). Larvae from 5th instar day 1 were subjected to starvation for 12h. Each condition had 3-5 insects pooled together for LD and cytosol extraction. 25ul sample fractions (~650 nmol TG) were examined for lipase activity against an emulsion of [^{3}H-triolein] and Triton X-100.

Concluding remarks:

Trigycerides (TG) stored in lipid droplets (LDs) are the main energy reserves in all animals. Mobilization of fatty acids from TG depends on the action of lipases, and is stimulated by adipokinetic hormones (AKH) in several insects. In Manduca sexta, the effect of AKH on the mobilization of energy reserves is dependent on the developmental stages. In larval stage, AKH activates glycogenolysis whereas it stimulates lipolysis in adult insects. In adult *Manduca sexta* AKH promotes a rapid phosphorylation of the lipid droplet-associated protein Lsd1, and a concomitant activation of the rate of hydrolysis of TG by the main lipase (TGL). Since LDs are complex organelles that contain a large number of proteins, the study of the mechanism of lipolysis would be facilitated if one would have an *in vitro* system that uses purified proteins. Here we describe the purification of recombinant *Ms*Lsd1 and its reconstitution with lipids to form lipoprotein complexes suitable for functional and structural studies. We also describe mutagenesis studies of *Ms*Lsd1 targeting conserved phosphorylation sites and a short region localized towards the C-term of the protein that is a feature of all insect Lsd1. These studies suggest that the mutated sites are relevant elements of Lsd1 judging by the effect of the mutations on TG-lipase activities.

The expression of the fat body TGL and Lsd1 during *M. sexta* development have been studied. Both TGL and its regulator -Lsd1- are more abundant in adult insects when insects rely solely in lipid stores for all the energy demand including flight and reproduction. Interestingly, during physiological non-feeding periods of larva *M. sexta*, TG-lipase activity is lower than in the feeding periods. However, the lipid concentrations in hemolymph are somewhat higher than the feeding larva. This contradiction suggests that additional enzymes may act upon TG stores mobilizing lipids to the hemolymph. A major lipase in Drosophila is the Brummer lipase, a homolog of human ATGL. This enzyme which is a highly conserved lipase could be also present in Manduca and we wondered if this lipase could be playing a role in the non feeding state. In the

present study we identified Adipose triglyceride lipase (ATGL) in *Manduca sexta*. Two *Ms*ATGL cDNAs (2.4kb and 3.9kb) encoding a 550 amino acid protein with a theoretical molecular weight of 64KDa were cloned. This enzyme was mostly associated to the lipid droplets and catalyzed the hydrolysis of TG to diacylglycerides (DG), while TGL (74-76KDa) was a cytosolic lipase and the main product was monoacylglycerides (MG) in *vitro*. *Ms*ATGL is up-regulated during the physiological non-feeding periods but lower than TGL with the exception of 3rd-day pre-pupal. However, this expression pattern of ATGL coincides well with the hemolymph lipid concentrations. Our data demonstrate that ATGL plays a critical role during the physiological non-feeding time when TGL activity is low, which allows the insects survive during these periods. Unlike TGL whose activation is very rapid -within minutes- and is modulated by the PKA-dependent phosphorylation of Lsd1triggerd by AKH, the activation of lipolysis by ATGL is a slower process. For example, the enzyme was up regulated in 6h after starvation. The decreasing of glucose levels in hemolymph during starvation coincides with the onset of ATGL up regulation suggesting that the nutritional status of the animal may play a key role for the regulation of ATGL expression.

Clearly, an understanding of these metabolic pathways is of fundamental importance in insect biochemistry. This study shows for the first time that ATGL is important besides TGL. The differences in ATGL levels observed between feeding and non-feeding periods of larval stage were not observed for TGL suggesting a specific role of ATGL in lipid mobilization under starvation conditions and the two lipases seem to be controlled by independent mechanisms.

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