Functional Characterization of the *Bombyx mori* Fatty Acid Transport Protein (BmFATP) within the Silkmoth Pheromone Gland*

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Fatty acid transport protein (FATP) is an evolutionarily conserved membrane-bound protein that facilitates the uptake of extracellular long chain fatty acids. In humans and mice, six FATP isoforms have been identified and their tissue-specific distributions suggest that each plays a discrete role in lipid metabolism in association with fatty acid uptake. While the presence of FATP homologs in insects has been demonstrated, their functional role remains to be characterized. Pheromogenesis is defined as the dynamic period in which all machinery required for sex pheromone biosynthesis is generated and organized within the pheromone gland (PG) cells. By exploiting this unique system in the PG of the silkmoth, *Bombyx mori*, we found that BmFATP is predominantly expressed in the PG and undergoes up-regulation 1 day prior to eclosion. Before eclosion, *B. mori* PG cells accumulate cytoplasmic lipid droplets (LDs), which play a role in storing the pheromone (bombykol) precursor fatty acid in the form of triacylglycerol. RNAi-mediated gene silencing of BmFATP in vivo significantly suppressed LD accumulation by preventing the synthesis of triacylglycerols and resulted in a significant reduction in bombykol production. These results, in conjunction with the findings that BmFATP stimulates the uptake of extracellular long-chain fatty acids and BmFATP knockdown reduces cellular long-chain acyl-CoA synthetase activity, suggest that BmFATP plays an essential role in bombykol biosynthesis by stimulating both LD accumulation and triacylglycerol synthesis via a process called vectorial acylation that couples the uptake of extracellular fatty acids with activation to CoA thioesters during pheromogenesisi.s

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‡The abbreviations used are: PG, pheromone gland; ACP, acyl-CoA-binding protein; ACS, acyl-CoA synthetase; Bmpgdesat1, *B. mori* pheromone gland desaturase 1; DEPC, diethyl pyrocarbonate; ELSD, evaporative light scattering detector; FATP, fatty acid transport protein; LCFA, long-chain fatty acid; LD, lipid droplet; mgACBP, midgut ACBP; MS, mass spectrometry; PBAN, pheromone biosynthesis activating neuropeptide; PBANR, PBAN receptor; pgACBP, PG-specific ACBP; pgFAR, PG-specific fatty acyl reductase; RNAi, RNA interference; TG, triacylglycerol; VLACS, very-long-chain acyl-CoA synthetase; RACE, rapid amplification of cDNA ends; PBS, phospha-buffered saline; UTR, untranslated region.

Lepidopteran sex pheromones are often used by females to lure conspecific males, with most sharing a common progenitor that is de novo synthesized from acetyl-CoA via fatty acid synthesis in the pheromone gland (PG),2 a functionally differenti-
ducing cells can be characterized by the abundance of lipid droplets (LDs) within the cytoplasm; these LDs begin to form one or 2 days prior to eclosion and they rapidly accumulate on the day of eclosion. In contrast, the density of the LDs decreases in response to PBAN stimulation after eclosion (4, 13). We have analyzed the chemical composition of the LDs and confirmed that they are composed of various triacylglycerols (TGs) with the bombykol precursor, Δ10, 12-hexadecadienoate, predominantly sequestered as a major component at the sn-1 and/or sn-3 position of the glycerides, indicating that the LDs play an essential role in storing the bombykol precursor in the form of TGs and releasing it for bombykol production in response to PBAN stimulation (14). In addition, we have recently established a method for targeted disruption of *B. mori* PG-specific genes *in vivo* by the use of RNA interference (RNAi) and have successfully demonstrated that loss of function of the PG-specific acyl-CoA-binding protein (pgACBP) prevents LD accumulation, and consequently, limits the availability of the bombykol precursors (15). However, the precise mechanisms underlying LD accumulation in the PG cells prior to eclosion have yet to be characterized.

Fatty acid transport proteins (FATPs) belong to an evolutionarily conserved family of membrane-bound proteins that facilitate the uptake of extracellular long-chain fatty acids (LCFAs) and/or very long chain fatty acids (VLCFAs) and catalyze the ATP-dependent esterification of these fatty acids to their corresponding acyl-CoA derivatives (16, 17). In humans and rodents, six related members of FATPs (FATP1 to FATP6) have been identified and their tissue expression patterns have been analyzed (18). Although FATP homologs are also present in several insect species (17, 19), their functional role has yet to be characterized. To identify and characterize functional proteins involved in the pheromonogenesis of the silkmoth, *B. mori*, we have generated a PG expressed-sequence tag (EST) data base by constructing a normalized PG cDNA library prepared from newly emerged female moths of the inbred strain (p50) (20). In the course of expression analyses of these EST clones using various tissues as well as PGs isolated during different developmental stages, we found that a gene encoding a FATP homolog in *B. mori* (BmFATP) is predominantly expressed in the PG. In this study, we sought to examine the functional role of BmFATP in *B. mori* pheromonogenesis.

**EXPERIMENTAL PROCEDURES**

*Insects*—Larvae of the inbred p50 strain of *B. mori*, kindly provided by T. Shimada of the University of Tokyo, were reared on mulberry leaves and maintained under a 16L:8D photoperiod at 25 °C. Pupal age was distinguished based on morphological characteristics as described (14).

*Rapid Amplifying cDNA Ends (RACE)*—Messenger RNA was isolated from about 100 μg of PG total RNA by using a MicroFast Track kit (Invitrogen, Carlsbad, CA). RACE was performed using a GeneRacer kit (Invitrogen) according to the manufacturer’s instructions. Computer-assisted sequence analyses were performed using GENETYX-MAC Ver.12.0 (Software Development Co., Tokyo, Japan).

*RT-PCR Analysis*—PGs and other tissues were dissected into insect Ringer’s solution (35 mM NaCl, 36 mM KCl, 12 mM CaCl₂, 16 mM MgCl₂, 274 mM glucose, and 5 mM Tris-HCl, pH 7.5) and mechanically trimmed as described (21). Total RNA was isolated from the trimmed PGs by the method of Chomczynski and Sacchi (22) and first-strand cDNA synthesis was performed using an RNA PCR kit (Takara Bio Inc.) according to the manufacturer’s instructions with 500 ng of total RNA. Fragments of BmFATP were amplified from oligonucleotide primer pairs designed from published sequences (NRPG1860-F1 [sense primer]: 5′-TATACACGTAGGGACACTG-3′ and NRPG1860-R1 [antisense primer]: 5′-TTCAACTCCTAAGGGCACGT-3′). PCR was performed under the conditions of 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. After PCR, 5 μl of the PCR products were electrophoresed on a 1.5% agarose gel in TAE buffer.

*Synthesis and Injection of dsRNA*—The templates for synthesis of dsRNAs corresponding to BmFATP were prepared using gene-specific primers containing T7 polymerase sites (NRPG1860T7-F1 [sense primer]: 5′-CCTAATAACGACTCATACTAGGCGGTATACACGTCAGGGACACTG-3′ and NRPG1860T7-R1 [antisense primer]: 5′-CCTAATAACGACTCATACTAGGCGGTATACACGTCAGGGACACTG-3′; nucleotide sequences corresponding to the T7 promoter region are underlined). PCR was performed under the conditions of 6 cycles of 94 °C for 30 s, 56 °C for 30 s, 68 °C for 90 s followed by 30 cycles of 94 °C for 30 s, 66 °C for 30 s, 68 °C for 90 s using KOD-Plus- (Toyobo, Osaka, Japan) with the resulting products purified (Wizard SV Gel and PCR Clean-Up kit, Promega, Madison, WI) and used as templates to generate dsRNAs using the AmpliScribe™ T7 High Yield Transcription kit (Epicerent Technologies, Madison, WI) according to the manufacturer’s instructions. After synthesis, the dsRNAs were diluted with diethyl pyrocarbonate- (DEPC-) treated H₂O, the RNA concentrations measured (A₂₆₀), and the products were analyzed by gel electrophoresis to confirm annealing. Samples were diluted to the desired concentration (final volume 2 μl) and injected near the abdominal tip of 1-day-old pupae (i.e. pupae 1 day removed from the larval-pupal molt) using a 10-μl microsyringe (Hamilton). Control pupae were injected with 2 μl of DEPC-treated H₂O alone. After injection, pupae were maintained under normal conditions until adult emergence.

*In Vivo Bombykol Analysis*—Adult females were decapitated within 3 h of emergence and maintained at 25 °C for 24 h. They were then injected with either 5 pmol (2 μl) *B. mori* PBAN in PBS or PBS alone. Abdominal tips were dissected 90 min after injection and bombykol production was measured by HPLC as described (23) using a Senshu-Pac NO₂ column (Senshu Scientific Co., Tokyo, Japan).

*Microscopic Examination of Cytoplasmic Lipid Droplets*—Abdominal tips were dissected and mechanically trimmed from normal, decapitated, or RNAi-treated females. The excised glands were fixed with a 4% formalin/PBS solution and stained with Nile Red (a fluorescent probe for intracellular neutral lipids; Molecular Probes Inc. Eugene, OR) as described (13). Fluorescence microscopy was performed with an OLYMPUS BX-60 system equipped with a PM-30 exposure unit and a BH20-RFL-T3 light source (400× magnification). Nile Red imaging was performed with a 330–385-nm band pass excitation filter, a 400-nm dichroic mirror, and a 420-nm long.
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pass barrier filter (OLYMPUS cube WU). Images were processed and merged using Photoshop CS (Adobe Systems Inc., San Jose, CA).

Analysis of Lipid Droplet Components—Five trimmed PGs were prepared from the desired stages of female moths and dipped in 100 μl of aceone for 10 min at room temperature. The dried acetone extracts were dissolved in n-hexane and loaded on either a Senshu-Pak PEGASIL-Silica 120–5 column (Senshu Sci. Co.; 4.6 mm i.d. × 250 mm, pore size: 120 Å) equilibrated with n-hexane/acetone (99/1) or a SSC-C_{22} d docosil column (Senshu Sci. Co.; 4.6 mm i.d. × 250 mm, pore size: 120 Å) equilibrated with acetonitrile/ethanol (6/4). TG components were separated as described previously (14) and detected using an evaporative light scattering detector (ELSD; SEDEX model 75, Sedere, France). Fatty acyl groups in the TGs were identified with Fast Atom Bombardment mass spectrometry (MS) and tandem MS (MS-MS) analyses using a JEOI (Tokyo, Japan) JMS HX/HX-110A tandem mass spectrometer as described (14).

Analysis of Cellular Fatty Acid Uptake using the Fluorescent Fatty Acid BODIPY 500/510 C_{18},C_{12}—Trimmed PGs were prepared from normal or RNAi-treated females 12 h after eclosion and maintained at room temperature for 2 h in insect Ringer’s solution. The PGs were then incubated with 5 μM BODIPY 500/510 C_{18},C_{12} (4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecane) (Molecular Probes, D3823) in 50 μl of insect Ringer’s solution for 10 min in the dark. After incubation, the PGs were washed twice with PBS containing 15 mM fatty acid-free bovine serum albumin and then with PBS alone (24). All steps were performed at room temperature. Incorporation of BODIPY 500/510 C_{18},C_{12} was visualized on an OLYMPUS BX-60 system equipped with a PM-30 exposure unit and a BH20-RFL-T3 light source (400× magnification). Fluorescent imaging was performed with a 330–385-nm band pass excitation filter, a 400-nm dichroic mirror, and a 420-nm long pass barrier filter. Images were processed and merged using Photoshop CS.

Measurement of Radiolabeled Fatty Acid Uptake—Rates of fatty acid transport were determined essentially as described by DiRusso et al. (25). [9,10-3H]Palmitic acid (47.7 Ci/mmol) and [9,10-3H]oleic acid (45.5 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Each trimmed PG, prepared from either a normal or RNAi-treated female moth 12 h after eclosion, was preincubated for 2 h at room temperature in 250 μl of insect Ringer’s solution containing 15 μM fatty acid-free bovine serum albumin, and the assay was initiated by the addition of 80 nM [3H]palmitic acid or [3H]oleic acid. After incubation, each PG was washed three times with PBS containing 0.5% Brj 58, lysed with 200 μl 0.3 M NaOH, and the cell-associated radioactivity was measured by liquid scintillation counting.

Measurement of Fatty Acyl-CoA Synthetase (ACS) Activity—Fatty ACS activity was assayed based on the conversion of [3H]palmitic acid or [3H]oleic acid to their CoA derivatives using a modified method from Hall et al. (16). Each PG prepared from normal or RNAi-treated females 12 h after eclosion was homogenized in 25 μl of 30 mM NaCl, 1% Triton X-100, and 100 mM Tris-HCl, pH 7.5. The homogenate was then incubated at 30 °C for 5 min with 250 μl of 30 mM NaCl, 100 mM Tris-HCl, pH 7.5, 80 mM [3H]-labeled fatty acid, 10 mM ATP, 5 mM MgCl2, 200 μM CoA, and 200 μM dithiothreitol. The reaction was terminated with the addition of 1.25 ml of isopropanole:heptane: H2SO4 (40:10:1, v/v/v), 0.5 ml H2O, and 0.75 ml of heptane to facilitate organic phase separation. The aqueous phase was extracted three times with 0.75 ml of heptane to remove unreacted fatty acids, and the radioactivity was determined by liquid phase scintillation counting.

RESULTS

Cloning of the Bombyx mori FATP Gene (BmFATP) Expressed in the PG—We found a cDNA clone expressed in the PG that encodes the FATP homolog in B. mori, which we have designated as BmFATP. This clone (NRPG1860) is present in the B. mori PG EST data base, which was constructed from our normalized p50 PG cDNA library (20) and then integrated into the public B. mori EST data base (SilkBase). Subsequent RT-PCR amplification and both 3’- and 5’-RACE of NRPG1860 indicated that BmFATP is comprised of a 2,094-nt open reading frame encoding a 698 amino acid protein flanked by a 233-nt 5’-UTR and a 851-nt 3’-UTR. Comparison of the BmFATP sequence with sequences from other organisms revealed high similarities with the Apis mellifera FATP, Anopheles gambiæe FATP, Drosophila melanogaster FATP, Mus musculus FATP4, and Saccharomyces cerevisiae Fat1p (55, 54, 53, 42, and 29% similarity, respectively) (Fig. 1). These FATPs, including BmFATP, can be characterized by two highly conserved sequences, the ATP/AMP signature motif common to the adenylate-forming enzymes and the FATP/VLACS signature motif (24) (Fig. 1). Two studies have described the topology of the FATPs, one for murine FATP1 (26) and the second for yeast Fat1p (27). Analysis of BmFATP with the membrane protein structure prediction program SOSUI revealed that BmFATP contains two possible N-terminal transmembrane domains (amino acid residues 42–64 and 68–90) (data not shown), suggesting that the BmFATP more closely resembles that found in yeast. The yeast Fat1p has two transmembrane-spanning domains, and experimental data have suggested that both N- and C-terminal regions are positioned on the inner face of the plasma membrane, with the ATP/AMP and FATP/VLACS domains of Fat1p facing the cytoplasm (27). A search for FATP gene homologs in the public B. mori genome data base (Silkworm Genome Database) suggested that the BmFATP is a single-copy gene.

Expression Analyses of BmFATP using RT-PCR—To examine the tissue distribution of the BmFATP transcript, we performed RT-PCR analyses using various tissues prepared from p50 female adults and larvae (Fig. 2). In adult tissues prepared 3 h after eclosion, we found that the BmFATP transcript was predominantly expressed in the PG with a less intense signal detectable in other tissues including midgut and fat body (Fig. 2A). Stage-specific expression of BmFATP within the PG indicated that it undergoes significant up-regulation 1 day prior to adult emergence (Fig. 2B). This up-regulation in the PG is reminiscent of PG-specific proteins crucial to B. mori pheromoneogenesis, i.e. pgFAR, Bmpgdesat1, pgACBP, mgACBP, and PBANR (6, 7, 10, 28). Accordingly, these results suggest the
functional involvement of BmFATP in B. mori pheromonogenesis as well. Apart from expression in the PG, we also found the BmFATP transcript prominently expressed in the larval midgut and fat body (Fig. 2C). In addition, a weak but steady signal was detected in the larval brain and silk gland (Fig. 2C).

RNAi-mediated Knockdown of BmFATP Transcripts in Vivo: Effect on Bombykol Production—We have recently established a method for RNAi-mediated knockdown of B. mori PG-specific genes in vivo (15). To elucidate the in vivo function of the BmFATP in the PG during pheromonogenesis, we synthesized double-stranded RNA (dsRNA) corresponding to the open reading frame of BmFATP and examined its inhibitory effect on bombykol production in vivo by using our RNAi protocol (15). When we injected 10 μg of dsRNA for BmFATP into 1-day-old p50 female pupae (i.e. pupae 1 day removed from the larval-pupal molt), BmFATP mRNA levels in the PG after eclosion were prominently reduced compared with the control pupae injected with DEPC-treated H2O (Fig. 3A). In addition, because injection of the BmFATP dsRNA had no effect on pupal development or on adult emergence, we next injected varying concentrations (1, 5, and 10 μg) of the same dsRNA into 1-day-old female pupae. To assess the effects of BmFATP knockdown on bombykol production, the newly emerged female moths were decapitated, injected with 5 pmol of synthetic PBAN, and the amount of bombykol in the PGs measured (see “Experimental Procedures”). As shown in Fig. 3B, a significant dose-dependent reduction in bombykol production was achieved; injections of 1, 5, and 10 μg dsRNA corresponding to BmFATP elicited 11%, 28%, and 44% reduction, respectively. Because no decrease in bombykol production was observed in the control pupae injected with dsRNA corresponding to enhanced GFP (data not shown), these results indicate

![FIGURE 1. Multiple alignment of BmFATP and its related proteins.](image-url) The predicted protein sequence of BmFATP is compared with those of the Apis mellifera, Anopheles gambiae, Drosophila melanogaster, Mus musculus, and Saccharomyces cerevisiae proteins (GenBank accession numbers: XP_624496, XP_321320, NP_725597, AA_H23114, NP_009597). Numbering of each sequence begins at the predicted initiator methionine, and the amino acid residues identical to the B. mori sequence are highlighted in black. The ATP/AMP signature motif consisting of two highly conserved regions is underlined (24). The FATP/VLACS signature motif is underscored with a dotted line.
that disruption of bombykol production was specific to the dsRNA sequence.

RNAi-mediated Knockdown of BmFATP Transcripts in Vivo: Effect on Cytoplasmic LD Dynamics—The most obvious characteristic feature of bombykol-producing cells is the abundance of LDs within the cytoplasm (4, 13). The role of the LDs in bombykol biosynthesis is to store the bombykol precursor fatty acid in the form of TGs and release it in response to the external signal of PBAN (14). To monitor the LD dynamics during pheromogenesis, we have previously stained the LDs with the fluorescent lipid marker, Nile Red. Magnifications, 400×.

FIGURE 2. RT-PCR analyses of BmFATP. A, expression of the BmFATP transcript in adult tissues. Tissues were dissected from newly emerged (day 0) p50 female moths: PG, pheromone gland; BR, brain; FM, flight muscle; EG, egg; MT, Malphigian tubule; FB, fat body; and MG, midgut. B, expression of the BmFATP transcript in the PG at different developmental stages. PGs were dissected from p50 pupae at 3 days (3), 1 day (-1), and 0 days (0) before eclosion, and from p50 adults at 1 day (1) and 2 days (2) after eclosion. C, expression of the BmFATP transcript in larval tissues. Tissues were dissected from 2-day-old fifth instar p50 larvae: BR, brain; SG, silk gland; MT, Malphigian tubule; MG, midgut; and FB, fat body. cDNAs were normalized based on the expression gene, actin.

FIGURE 3. Effects of BmFATP knockdown on in vivo bombykol production. A, RNAi-induced suppression of the BmFATP transcript. Transcript analysis was performed using cDNAs prepared from the total RNA of pupae injected with 0 µg (-) or 10 µg (+) dsRNAs corresponding to BmFATP. B, dose-dependent effects of RNAi-treatment on in vivo bombykol production. One-day-old pupae were injected with 1, 5, and 10 µg of dsRNA corresponding to BmFATP. Results are expressed as percent of bombykol levels compared with non-RNAi-treated females following intra-abdominal injections of 5 pmol of PBAN (*, p < 0.05; **, p < 0.001). Bars represent mean values ± S.D. from independent experimental animals (n = > 9).

FIGURE 4. Effects of BmFATP knockdown on the LD dynamics in bombykol-producing cells. Images are B. mori PG cells of 1-day-old, decapitated females, treated as pupae with DEPC-H2O (Control) or 10 µg BmFATP dsRNA (BmFATP knockdown). Images indicate ± PBAN treatment. Cytoplasmic LDs were stained with the fluorescent lipid marker, Nile Red. Magnifications, 400×.

FIGURE 5. Analysis of TG contents in the PG. One-day-old pupae were injected with DEPC-treated H2O (Control) or 10 ng of BmFATP dsRNA (BmFATP knockdown). After eclosion, females were decapitated within 3 h of emergence, maintained at 25 °C for 24 h, and then injected with PBS alone (-PBAN) or 5 pmol of B. mori PBAN (+PBAN). Five PGs were extracted with acetone 90 min after PBAN injection, and the extracts were subjected to HPLC on a PEGASIL-Silica column (14). Peaks were detected using ELSD and TG fractions are underlined.
expected in the non-RNAi-treated control, Nile Red staining revealed that female moths decapitated immediately after eclosion accumulated numerous LDs in the PG, subsequent PBAN injection caused a striking reduction of the size and number in the LDs (Fig. 4, A and B). In contrast, PGs of female moths treated with BmFATP dsRNA accumulated noticeably smaller LDs (Fig. 4C). This result was further confirmed by measuring the total amounts of TGs in the PGs using normal-phase HPLC on a PEGASIL-Silica column (Fig. 5). In the BmFATP knockdown PG, the overall TG content after decapitation was reduced ~35% compared with the normal PG (Fig. 5, A and C). Taken together, these results indicate that BmFATP plays an essential role in LD accumulation prior to eclosion.

RNAi-mediated Knockdown of BmFATP Transcripts in Vivo—Effect on the incorporation of individual fatty acyl components into the LD TGs; TGs that compose cytoplasmic LDs in the PG cells comprise various TG species in which the bombykol precursor is predominantly sequestered at the sn-1 and/or sn-3 position as a major fatty acyl component (14). Previously, in addition to the bombykol precursor, we analyzed the other fatty acyl components of the TGs and identified them as monoene C16 fatty acyl, Δ11-hexadecenoate, and 3 conventional unsaturated C18 fatty acyls (i.e. oleate, linoleate, linolenate) (14). Consequently, we sought to assess the effects of BmFATP silencing on the incorporation of individual fatty acyl components into the LD TGs. In agreement with our previous results (14) and Fig. 5, overall TG contents in the PGs of both control and RNAi-treated moths were dramatically reduced in response to PBAN (Fig. 6, B and D). In addition, the total amount of TGs in RNAi-treated PGs was again reduced ~35% compared with that of the non-RNAi-treated PGs (Fig. 6, A and C). Furthermore, we found that there was a significant difference in the TG elution profiles between non-RNAi-treated and RNAi-treated PGs before PBAN injection; silencing BmFATP brought about a striking reduction in specific TG peaks (i.e. peaks 2a, 3a, 4a, 5, and 6) (Table 1). Interestingly, MS analysis indicated that the TG species eluted in these peaks are exclusively comprised of two equivalents of unsaturated C18 fatty acyls (i.e. oleate, linoleate, linolenate) (14). Because linoleic and linolenic acids are dietary fatty acids, these results suggest that the role of BmFATP expressed in these peaks is exclusively comprised of two equivalents of unsaturated C18 fatty acyls (i.e. oleate, linoleate, linolenate) (14).

Uptake of an Extracellular Fluorescent Fatty Acid into PG Cells—To confirm the role of BmFATP in the uptake of extracellular LCFAs,

**FIGURE 6. Separation of PG-derived TG components.** One-day-old pupae were injected with DEPC-treated H2O (Control) or 10 mg BmFATP dsRNA (BmFATP knockdown). After eclosion, females were decapitated within 3 h of emergence, maintained at 25 °C for 24 h, and then injected with PBS alone (+PBAN) or 5 pmol of B. mori PBAN (+PBAN). Five PGs were extracted with acetone 90 min after PBAN injection, and the extracts were subjected to HPLC on a C18 docosyl column (14). Peaks were detected by ELSD and the fatty acyl composition of the TG peaks was identified by FAB-MS and MS-MS analyses as described previously (14).

**TABLE 1**

Comparison of peak areas in Fig. 6, A and B

| Peak | Control | BmFATP knockdown | Ratio (%) | Student’s t test | Fatty acyl compositions
|------|---------|-----------------|-----------|-----------------|-------------------|
|      | Avg. peak area (AU) | S.D. | Avg. peak area (AU) | S.D. | B/A×100 | (value) | (value) | (value) | (value) |
| 1 | 121 ± 9 |   | 126 ± 13 |   | 104.1 | NS | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 1a | 79 ± 6 |   | 70 ± 5 |   | 88.6 | NS | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 2 | 139 ± 10 |  128 ± 7 |   | 92.1 | NS | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 2a | 1,071 ± 86 |  431 ± 40 |   | 40.2 | ** | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 3 | 1,879 ± 139 |  1,809 ± 119 |   | 98.3 | NS | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 3a | 1,887 ± 168 |  914 ± 91 |   | 84.3 | ** | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 4 | 2,642 ± 254 |  2,140 ± 254 |   | 81.0 | NS | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 4a | 1,073 ± 106 |  513 ± 34 |   | 47.8 | NS | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 5 | 1,978 ± 236 |  842 ± 96 |   | 42.6 | NS | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 5a | 412 ± 49 |  373 ± 68 |   | 90.5 | NS | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 6 | 350 ± 38 |  186 ± 15 |   | 53.1 | NS | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |
| 6a | 274 ± 27 |  241 ± 28 |   | 88.0 | NS | (C16:2,C16:2,C16:2) | (C16:2,C16:2,C16:2) |

*a* Statistical analysis of avg. peak area control samples vs. BmFATP knockdown samples performed using Student’s *t* test (*n* = 6).

*b* Fatty acyl compositions of the TG(s) in each peak were identified with Fast Atom Bombardment MS and MS-MS analyses (14).

*c* NS, not significant.

*d* **, *p* < 0.01.

*e* *, *p* < 0.05.
plasm in RNAi-treated PG cells was less intense (Fig. 7). In
association with uptake of the fatty acid conjugate within the cyto-

A
DIC

Control

Fluorescent

B

BmFATP knockdown

FIGURE 7. Uptake of extracellular LCFAs into bombykol-producing cells. Uptake of extracellular LCFAs was assessed by the incorporation of the fluorescent fatty acid BODIPY 500/510 C_{1},C_{12}. Trimmed PGs taken from either normal (A) or RNAi-treated (B) female moths on the day of eclosion were incubated with 5 μM BODIPY 500/510 C_{1},C_{12} in 50 μl of insect Ringer’s solution for 10 min in the dark. Images are shown in differential interference contrast (DIC) and fluorescence modes.

BmFATP

FIGURE 8. Effects of BmFATP knockdown on the uptake of extracellular LCFAs into bombykol-producing cells. Uptake of extracellular LCFAs was assessed by the incorporation of [3H]palmitic acid (C_{16}:0) and [3H]oleic acid (C_{18}:1). Trimmed PGs taken from either normal (Control) or RNAi-treated (Knockdown) female moths on the day of eclosion were incubated with 80 nm [3H]-labeled fatty acids in 250 μl of insect Ringer’s solution. Data are given as means ± S.D. from independent experimental animals (n = 6).

TABLE 2

Long-chain fatty ACS activity in PG homogenates prepared from either a normal (Control) or RNAi-treated (BmFATP knockdown) female 12 h after eclosion

Fatty acid uptake (pmol/PG)

| Substrate | ACS activity | Reduction |
|-----------|--------------|-----------|
| Control   | Knockdown    | %         |
| C_{16}:0  | 0.614 ± 0.018| 0.326 ± 0.005* | 53.1   |
| C_{18}:1  | 0.206 ± 0.016| 0.130 ± 0.004* | 63.1   |

*p < 0.01 (vs Control).

trimmed PGs prepared from both normal and RNAi-treated female moths on the day of eclosion were incubated for 10 min in the presence of the fluorescent fatty acid BODIPY 500/510 C_{1},C_{12}, which is known to be an indicator of LCFA incorporation (24). Compared with control cells, the fluorescence associated with uptake of the fatty acid conjugate within the cytoplasm in RNAi-treated PG cells was less intense (Fig. 7). In addition, uptake of extracellular radiolabeled LCFA was prominently reduced in RNAi-treated PGs (e.g., when compared with control PG cells, the uptake of [3H]palmitic and oleic acid was reduced to 59.2 and 51.3%, respectively, after incubation for 30 min) (Fig. 8). These results, taken together, indicate that BmFATP indeed plays a role in the uptake of extracellular LCFA into PG cells.

**Fatty Acyl-CoA Synthetase Activity in PG Cells**—It has been demonstrated that FATP1 has an intrinsic ACS activity with broad specificity for both long and very long chain fatty acids (16). To examine the effect of BmFATP knockdown on the cellular long-chain fatty ACS activity, PG homogenates were prepared from day 0 female moths and fatty acid esterification was measured using radiolabeled LCFA substrates (Table 2). Knockdown of BmFATP decreased the cellular fatty acid esterification activity when [3H]-labeled palmitic and oleic acids were used as substrates (53.1 and 63.1%, respectively).

**DISCUSSION**

FATPs comprise a large evolutionarily conserved family of transmembrane proteins that facilitate extracellular LCFA uptake across the plasma membrane (18, 26, 29). In humans and mice, six different isoforms of FATP (FATP1 to FATP6) have been identified and their distinct tissue-specific distributions of expression suggest that each plays a discrete role in lipid metabolism in association with fatty acid uptake. The molecular mechanisms of LCFA transport across the plasma membrane, however, remain controversial (18, 30).

Genes encoding FATP homologs are present in many organisms including prokaryotes, but not in *Escherichia coli*, and conservation of FATP-mediated LCFA transport has been shown for diverse organisms including nematode (17), yeast (25), and mycobacteria (17). In the present study, during the course of our search for functional molecules involved in *B. mori* pheromonogenesis, we came across a gene that encodes a FATP homolog in *B. mori* (BmFATP) and found that BmFATP is predominantly expressed during the adult stage in the PG and undergoes significant up-regulation 1 day prior to adult emergence (Fig. 2). Furthermore, RNAi treatment for the BmFATP gene in *B. mori* confirmed that BmFATP plays an essential role in the sex pheromone biosynthesis of this moth species (Figs. 3–5).

During pheromonogenesis, female moths of *B. mori* accumulate a large number of LDs within the cytoplasm of PG cells before adult emergence (13). These LDs contain various TGs that play an essential role in providing the bombykol precursor fatty acid in response to the external signal of PBAN after eclosion (4, 13, 14). Consequently, bombykol-producing cells have to store the TGs required for bombykol biosynthesis prior to eclosion. We have previously confirmed that fatty acyls sequen-
tered in these TGs are restricted to 5 long-chain fatty acyls: 2 unsaturated C_{16} fatty acyls (Δ11-hexadecenoate and Δ10, 12-hexadecadienoate) and 3 conventional C_{18} fatty acyls (oleate, linoleate, and linolenate) with Δ10, 12-hexadecadienoate as a major component (14). Because both Δ11-hexadecenoate and Δ10, 12-hexadecadienoate are bombykol biosynthesis precursors, they are synthesized de novo in the PG cells (5). In contrast, because linoleic and linolenic acids are essential fatty acids as precursors, they are synthesized from de novo to store the TGs required for bombykol biosynthesis prior to eclosion. We have previously confirmed that fatty acyls sequen-
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acids even in insects (31), the conventional C_{18} fatty acyls are effectively derived from dietary fatty acids and must be taken up across the plasma membrane. Expression analyses using RT-PCR revealed that BmFATP mRNA is, among the adult p50 tissues assayed, predominantly expressed in the PG and is up-regulated 1 day prior to eclosion (Fig. 2, B and C). Furthermore, RNAi-mediated gene silencing of BmFATP in vivo resulted in a significant reduction of bombykol production, demonstrating the functional relevance of BmFATP in bombykol biosynthesis (Figs. 3–5). In addition, it is obvious that BmFATP plays an essential role in LD accumulation prior to eclosion, because female moths treated with BmFATP dsRNA accumulated noticeably smaller LDs in the PG (Figs. 4 and 5). Interestingly, similar results have been observed when pgACBP gene expression was suppressed, i.e. injection of pgACBP dsRNA significantly reduced overall TG content and resulted in the reduction of bombykol production (15). Consequently, to realize the functional difference between BmFATP and pgACBP on LD accumulation, we further analyzed elution profiles of the LD TGs in RNAi-treated PGs and examined the effects of gene silencing on the incorporation of individual fatty acyl components. We previously reported that pgACBP RNAi-treated samples exhibited a TG elution profile similar to that of the non-RNAi-treated control despite the significant reduction in overall TG content (15). In contrast, silencing BmFATP brought about a striking reduction in specific TG peaks (Table 1) that exclusively contain two equivalents of unsaturated C_{18} fatty acyls (i.e. olate, linoleate, linolenate) (14). Furthermore, our present work using BODIPY 500/510 C_{1},C_{12} as well as 3H-labeled palmitic and oleic acids illustrated the involvement of BmFATP in the uptake of extracellular LCFA into bombykol-producing cells (Figs. 7 and 8). Because the role of FATPs in the uptake of extracellular LCFA has been well documented in diverse organisms (18, 26, 29), these results taken together support the idea that the role of BmFATP in pheromonogenesis is in the uptake of the large amounts of extracellular dietary C_{18} LCFA that constitute the TGs comprising the cytoplasmic LDs in PG cells.

Members of the FATP family exhibit long- and/or very long-chain ACS activity and contain a highly conserved sequence called the FATP/VLACS signature motif (24). FATP1, for instance, exhibits intrinsic ACS activity with broad specificity for both long and very long chain fatty acids and it is hypothesized that the ACS activity of FATP1 is functionally linked to LCFA uptake, i.e. FATP1 facilitates both transbilayer movement of fatty acids and their metabolic activation (esterification) with coenzyme A (16). It is also hypothesized that FATP1 channels exogenous fatty acids into TG biosynthesis because fatty acid incorporation into glycerophospholipids and sphingomyelin was unaffected by FATP1 overexpression (30). Our results reported here are consistent with these views, because knockdown of BmFATP also resulted in a striking reduction in the cellular fatty acid esterification activity as estimated by both radiolabeled palmitic and oleic acids (Table 2). These results, taken together, imply that BmFATP in the PG cells likely facilitates both uptake of extracellular C_{18} fatty acids and subsequent production of C_{18} fatty acyl-CoAs, which are in turn bound to mgACBP (vide infra) and transferred for TG synthesis, the mechanism of which may be similar to the concept of vectorial acylation, which has been proposed as a mechanism driving the trafficking of exogenous fatty acid across the plasma membrane (27).

In B. mori, two ACBPs (pgACBP and mgACBP) are specifically expressed in the PG and undergo up-regulation on the day prior to adult emergence, although mgACBP is also highly expressed in the midgut during larval feeding stages (28). It is known that ACBP specifically binds medium- and long-chain (C_{14}-C_{22}) fatty acyl-CoA esters with high affinity and is structurally highly conserved from yeasts to mammals including insects (32–35). Because disruption of either pgACBP or mgACBP transcription prevented TG accumulation within the cytoplasmic LDs and consequently, the availability of the bombykol precursors, these PG expressed ACBPs possibly function as carriers or cellular deposits for the acyl-CoAs used for the synthesis of the LD TGs (15). Given the abundance of the mgACBP transcript in the larval midgut, it is likely that these ACBPs play distinct roles in TG synthesis, i.e. pgACBP specifically donates de novo synthesized pheromone precursor fatty acyl-CoAs whereas mgACBP donates conventional C_{18} fatty acyl-CoAs derived from dietary lipids. Consequently, our results further imply essential but distinct roles for BmFATP, pgACBP, and mgACBP in the formation of cytoplasmic LDs, the key organelles that store and release the bombykol precursor fatty acid during pheromonogenesis.

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