Molecular identification and lipid mobilization role of adipokinetic hormone receptor in Spodoptera litura (F.)

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Abstract

Energy homeostasis is essential for organisms to maintain fluctuation in energy accumulation, mobilization. Lipids as the main energy reserve in insects, their metabolism is under the control of many physiological program. This study aimed to determine whether the adipokinetic hormone receptor (AKHR) was involved in the lipid mobilization in the Spodoptera litura. A full-length cDNA encoding AKHR was isolated from S. litura. The SlAKHR protein has a conserved seven-transmembrane domain which is the character of a putative G protein receptor. Expression profile investigation revealed that SlAKHR mRNA was highly expressed in immature stage and abundant in fat body in newly emerged female adults. Knockdown of SlAKHR expression was achieved through RNAi by injecting double-stranded RNA (dsRNA) into the 6th instar larvae. The content of triacylglycerol (TAG) in the fat body increased significantly after the SlAKHR gene was knocked down. And decrease of TAG releasing to hemolymph with increase of free fatty acid (FFA) in hemolymph were observed when the SlAKHR gene was knocked-down. In addition, lipid droplets increased in fat body was also found. These results suggested that SlAKHR is critical for insects to regulate lipids metabolism.

Introduction

The insect fat body is a dynamic tissue involved in energy storage and metabolism, and regulation. Insect adipocytes store a great amount of lipid reserves as cytoplasmic lipid droplets (Arrese and Soulages, 2010). The intermediary metabolism of the fat body is regulated by many hormonal signals. Lipids metabolism is tightly coupled with a large array of physiological processes such as feeding preference and frequency change (Konuma et al., 2012; Fukumura et al., 2018), digestion regulation (Bil et al., 2014), egg-laying (Lindemans et al., 2009) etc. Adipokinetic hormone (AKH), a peptide hormone, is the first factor that stimulates lipid mobilization (Stone et al., 1976; Gäde et al., 1997). Reports had proven that the major function of the AKH is the regulation of triacylglycerol lipase to break down the stored glycogen and triacylglycerol (TAG) in the fat body. When the AKH is released into the hemolymph and binded by a G protein-coupled receptor, adipokinetic hormone receptor (AKHR), various cellular signaling pathways come into play (Cae Rs et al., 2012; Alves-Bezerra et al., 2016). The detailed signaling cascades for many insect species is not well known yet.

Lipid storage and mobilization are important for energy homeostasis in insect development, reproduction etc. And the lipid droplets for the main energy store are primarily TAG (Canavoso et al., 2001; Zhou et al., 2018b). Signaling molecule can affect gene transcription (Xu et al., 2012). When energy needed, AKH signaling pathways are activated and the ester bonds in TAG are hydrolyzed to form diacylglycerols which are released into the hemolymph (Ryan and van der Horst, 2000). Then the alanine/acyt-CoA system is open. And the free fatty acids that are released from triacylglycerols undergo β-oxidation, and the ATPs are generated for vital movement (Auerswald et al., 2005). But which enzyme(s) are being activated by the AKHs is still not known.

In the present study, we identified the adipokinetic hormone receptor (SlAKHR) in the the tobacco cutworm (Spodoptera litura) which was not reported in genome database using molecular biological approaches. The function of the SlAKHR gene in the mobilization of TAG was confirmed by dsRNA injection knockdown. The obtained data will confirm whether SlAKHR gene is involved in lipid mobilization related to the tobacco cutworm.
Materials and methods

Insect rearing

The Spodoptera litura population was gotten from School of Life Sciences, Sun Yat-sen University. And they were reared on an artificial diet (Chen et al., 2000) at 25 ± 1°C in a 14L:10D photoperiod and 70–80% relative humidity in intelligent light incubator from 2013. The newly emerged adults were transferred to a one-end open cylindrical plastic cage (Φ = 12 cm, H = 25 cm) covered with filter paper in inner around and a cotton cloth at the open end, honey added as a diet supplement.

Gene cloning

Total RNA was isolated from about 100 mg fat body from 6th larvae using the Trizol reagent according to the manufacturer’s specifications (Invitrogen, USA). First-strand complementary DNA was synthesized from 2 μg total RNA using a first strand synthesis kit (PrimeScript™ II 1st Strand cDNA Synthesis Kit, TaKaRa, Japan). The intermediate fragment of AKHR was amplified by a pair of degenerate primers which were designed based on the conserved amino acid sequences of the reported insect AKHRs in NCBI database (table 1). The PCR was carried out with the following conditions: initial preheating for 5 min at 94 °C, 35 cycles at 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min, and with a final extension at 72 °C for 10 min using the primer pair SlAKHR-F1 and SlAKHR-R1.

For the full-length cDNA cloning, A Rapid Amplification of cDNA Ends (RACE) Kit (Clontech, Japan) was used. Specific primers for the 5’- and 3’-Rapid RACE were designed based on partial SlAKHR cDNA sequence amplified by the degenerate primers SlAKHR-F1 and SlAKHR-R1. The specific primers GSP1 and NGSP1 were used for 5’-RACE, while GSP2 and NGSP2 used for 3’-RACE (table 1).

Expression profiles of SlAKHR

The AKHR transcript levels were detected by quantitative real-time PCR (qRT-PCR) using an iCycler iQ (BIO-RAD, Hercules, CA) and SYBR Premix Ex Taq (Takara, Japan). Briefly, each qRT-PCR mixture contained 10 μl total volume: 5 μl TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara), 0.4 μl forward and 0.4 μl reverse primers (10 μM), 3.2 μl MilliQ water and 1 μl cDNA. The target gene primer pair SlAKHR-qF1 and SlAKHR-qR1 was used with a housekeeping gene (Ribosomal protein L10, RPL10, GenBank Acc. No. KC866373) primers SlRPL10-qF1 and SlRPL10-qR1. The qRT-PCR was performed in a CFX Connect Real-Time System (BIO-RAD) under the following conditions: one cycle for 30 s at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at 60°C and 30 s at 72°C. The data were normalized to the reference gene (RPL10) expression. The relative expression levels were calculated by 2−ΔΔCT method (Livak and Schmittgen, 2001).

To investigate the expression pattern of different developmental phases and tissues, we got the 1st to 6th larvae, pupae, newly emerged adults. And the head, ovaries, midgut, fat body of newly female adults were carefully separated and collected. The specimens or tissues were rinsed in PBS buffer (0.01 M, pH = 7.4)

| Table 1. Primers used in this study |
|-------------------------------------|
| **Primer name** | **Primer sequence (5’-3’)** |
|-----------------|--------------------------|
| For gene cloning |                          |
| SlAKHR-F1       | GCATTTRGCWGTNGCYGAYYT    |
| SlAKHR-R1       | CGTGAAACRATGGTCTGGCC     |
| GSP1            | GATTACGCCAAGCTTACGTCGTC  |
| NGSP1           | GATTACGCCAAGCTTACGTCGTC  |
| GSP2            | GATTACGCCAAGCTTACGTCGTC  |
| NGSP2           | GATTACGCCAAGCTTACGTCGTC  |
| For qTR-PCR     |                          |
| SlAKHR-qF1      | TGTACGTCTCCTCCTGCTTC     |
| SlAKHR-qR1      | TGTACGTCTCCTCCTGCTTC     |
| Sl RPL10-qF1    | GATGACATGGATGGATG        |
| Sl RPL10-qR1    | GACTTGGAGTAAGAGAAG       |
| For SlAKHR dsRNA synthesis |                |
| SlAKHR ds-F1    | CTTCCCAAGTCTTGTCTTATC    |
| SlAKHR ds-R1    | ACATGAAACCAACACATGGC    |
| T7 SlAKHR ds-F  | GGACTTCAATAGCAGTCTATAGGGCCCTCCAGCTCTGCTGCTTAC |
| T7 SlAKHR ds-R  | GGACTTCAATAGCAGTCTATAGGGCCCTCCAGCTCTGCTGCTTAC |
| EGFP dsRNA-F1   | GCTGACCCTGAGATTCATC      |
| EGFP dsRNA-R1   | GAACCTCAGCGAGACACATG    |
| T7 EGFP ds-F    | GGATCCTAATAGCATCTATAGGGCGTGAACCTGATTCATC      |
| T7 EGFP ds-R    | GGATCCTAATAGCATCTATAGGGCGTGAACCTGATTCATC      |

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several times, and each tissue was pooled from 8–10 individuals. Total RNA was isolated from all samples and obtained the first-strand cDNA as above.

**Gene characterization and phylogenetic analysis**

The signal peptide of the AKHR was predicted with the SignalP server (http://www.cbs.dtu.dk/services/SignalP). And the transmembrane domain predicting was executed under the the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM). The alignment of all sequences were generated using the MultiAlin website server (http://multalin.toulouse.inra.fr/multalin/multalin.html). The neighbor-joining (NJ) tree was produced in MEGA 6.0 software (Tamura et al., 2013) with Maximum-likelihood analysis using the Jones-Taylor-Thornton model (1000 bootstrap replicates). AKHR amino acid sequences used for phylogenetic analysis derived from other reported sequences.

**RNA interference**

According to the manufacturer recommendations of T7 RiboMAX™ Express RNAi System (Promega), two pairs of primers (SlAKHR ds-F1,SlAKHR ds-R1, T7 SlAKHR ds-F and T7 SlAKHR ds-R) (table 1) were designed to synthesize the 544-bp (734–1278 bp) region of the SlAKHR gene that included a T7 promoter region in both the sense and antisense strands. The SlAKHR cDNAs from fat body was used as a template. The amplification reactions protocol comprised 36 cycles of 95°C for 40 s, 55°C for 45 s and 72°C for 60 s, with a final extension step of 72°C for 10 min. The sequence was verified by sequencing (Huada gene company, Shenzhen, China). The EGF gene (DQ768212.1) was used as a control dsRNA. The PCR primers EGFP dsRNA-F1 and EGFP dsRNA-R1 were used to amplify the EGF fragment (547 bp) (126–672 bp) (table 1), and the dsRNA was synthesized by T7 RiboMAX™ Express RNAi System as above. The final dsRNA production corresponding to SlAKHR and EGF genes were eluted into sterilized DEPC dH2O, The quality and concentration of dsRNAs were determined by Nano-Drop Spectrophotometer (Implen, München, Germany) and their integrity were confirmed by a 1% agarose gel electrophoresis. Then they were stored at −80°C and used up within 1 week.

For RNAi bioassays, newly 6th larvae were anesthetized on ice for 1 min before injection. dsRNAs were prepared and injected into the body cavity between the 3rd and 4th abdominal segment using a 10 μl micro-syringe syringe (Hamilton) and the injection point was sealed immediately with wax (Dong et al., 2013). The injected larvae were then returned to the artificial diet under the conditions described above. The efficiency of RNAi was assayed by comparing the brightness of the EGFP gene that included a T7 promoter region in both the sense and antisense strands, and EGFP genes were eluted into sterilized DEPC dH2O, The dsRNA production corresponding to SlAKHR and EGF genes were eluted into sterilized DEPC dH2O, The quality and concentration of dsRNAs were determined by Nano-Drop Spectrophotometer (Implen, München, Germany) and their integrity were confirmed by a 1% agarose gel electrophoresis. Then they were stored at −80°C and used up within 1 week.

**Lipid droplet staining and microscopy**

The dissected fat bodies were fixed with 4% paraformaldehyde on a glass slide for 2 h at room temperature and then washed with chilled PBS (0.01 M, pH = 7.4) three times (5 min × 3). Then these fat bodies were submerged in Nile red solution (5 μl Nile red (1 mg/ml−1) in 495 μl PBS) for overnight at 4°C (Wang et al., 2017). After washing for 5 min with PBS for three times, the fat bodies were re-stained in DAPI (2 ng/μl−1) for 10 min, next 5 min washing with PBS for three times (Zhao and Huang, 2019). The fat body were mounted on glass slides in 80% glycerol and visualized under a fluorescence microscope (Nikon, Japan) at 510–560 nm excitation wavelength combined with a 580 nm emission filter.

**Statistical analysis**

Results were presented as means ± SD (standard deviation) based on three independent biological replications. Differences between two groups were analyzed by Student’s t-test (*P < 0.05; **P < 0.01). One-way ANOVA followed by a Fisher’s protected LSD multiple comparison was used for the comparison among more than two different conditions. Statistical analyses were performed using SPSS 20.0 (Chicago, USA).

**Results**

**Gene character and phylogenetic analysis**

The full-length SlAKHR cDNA was obtained after sequencing and splicing. It indicated that the SlAKHR gene was 2539 bp, including an ORF of 1197 bp that encoded a 398 amino acids protein (accession No. MZ424871). No signal peptide cleavage was found under website server. The multi-sequence alignment showed that AKHR protein shared a high homology with other insects AKHR. Seven transmembrane domains (TM) were
Figure 1. Alignment of the SlAKHR protein with other species. The transmembrane domains (determined by TMHMM 2.0) are indicated with numbers from I to VII. *Bombyx mori* (GenBank Acc. No. NP_001037049.1), *Chilo suppressalis* (GenBank Acc. No. ALM88332.1), *Glossina morsitans* (GenBank Acc. No. AEH25943.1), *Grapholita molesta* (GenBank Acc. No. QPZ46758.1), *Locusta migratoria* (GenBank Acc. No. ANW09575.1), *Manduca sexta* (GenBank Acc. No. ACE00761.1), *Periplaneta americana* (GenBank Acc. No. ABB20590.1), *Pyrrhocoris apterus* (GenBank Acc. No. ARV86499.1), *Drosophila melanogaster* (GenBank Acc. No. NP_477387.1).
predicted in the protein, which indicates that this protein is a member of the GPCR superfamily (fig. 1).

For the phylogenetic analysis, it was chosen to compare the known and predicted insect AKHRs. Our tree showed that SlAKHR belongs to the same clade as the other AKHRs and was most related to the Manduca sexta AKHR (fig. 2).

**Expression profiles of SlAKHR**

To get more insights into the expression character of the SlAKHR gene in the S. litura, the transcription levels of SlAKHR gene in different developmental stages and a set of tissues in newly female adults were calculated by qRT-PCR. The result showed that SlAKHR gene was relatively low expression level during the early larval stage, and highly expressed when growing up (fig. 3a). In females, the mRNA was abundant in fat body and moderately expressed in midgut (fig. 3b).

**Effects of SlAKHR knockdown on lipid metabolism**

To confirm the role of SlAKHR in lipid metabolism, dsRNA-injection knockdown of SlAKHR was performed. qRT-PCR analysis were carried out after 12, 24, 36, 48 h and it showed a high silencing efficiency after 36 h (fig. 4a). To detect the function of SlAKHR on lipid metabolism, TAG contents in fat body and hemolymph were investigated. It showed that lipids content in fat body was increased but it was decreased in hemolymph after the SlAKHR gene knockdown (fig. 4b, c).

To explore the further changes of lipid decomposition products, the content of FFA in hemolymph was also analyzed. The graph revealed that the content of FFA was increased in SlAKHR RNAi-treated group compared to control treated group (fig. 4d).

**Effects of SlAKHR knockdown on TAG accumulation**

Five μg SlAKHR dsRNA was injected to each 6th star larva. Twenty-four hour later, fat body was isolated and Nile-red & DAPI staining was performed to visualize the lipid droplets. The results showed that after SlAKHR knockdown, lipid droplets in the fat body was accumulated compared with the control (fig. 5).

**Discussion**

In the present study, we cloned and sequenced the full cDNA sequence encoding AKHR from the S. litura (SlAKHR) successfully. But it was not found in the reported genome database. Several evidences confirm that the SlAKHR gene isolated here is indeed an AKHR of S. litura. Determination through TMHMM showed that there were seven transmembrane domains (fig. 1). AKHR is a glycoprotein hormone receptor containing seven transmembrane helices characteristic for the family of GPCRs (Wang et al., 2017). Secondly, AKHRs and the vertebrate gonadotropin-releasing hormone receptors are structurally and evolutionarily related (Hauser et al., 1998; Lindemans et al., 2011). Furthermore, SlAKHR was highly homologous to other receptors that had been reported (fig. 2). And the results of multiple amino acids sequence alignment showed that there were multiple conserved sites.

Our results indicate that SlAKHR was relatively low in earlier juvenile individuals and accumulated with their growing (fig. 3a).

The chronic accumulation and acute lipid mobilizing is mainly regulated by AKH signaling pathway (Grönke et al., 2007; Lu et al., 2018). Insect fat body is an important organ of biosynthetic and metabolic location (Law and Wells, 1989). And its cells control the synthesis and utilization of energy stores (Arrese and Soulages, 2010). The highest expression of SlAKHR in the fat body of females was observed in this study as other insects, D. melanogaster (Staubli et al., 2002), M. sexta (Ziegler et al., 2011), G. morstians (Caers et al., 2016), R. prolixus (Alves-Bezerra et al., 2016) and B. dorsalis (Hou et al., 2017). These findings most probably support the main lipids mobilization organ is fat body (Alves-Bezerra et al., 2016).
Insect mid-gut is closely associated with food digestion and immerse in fat body cells. qRT-PCR indicated that SlAKHR was moderately expressed in the midgut (fig. 3b) next to the fat body. This result might hint this tissue also be involved in lipid metabolism functionally, as reported in D. melanogaster (Hauser et al., 1998), P. regina (Stoffolano et al., 2014), P. americana (Bodláková et al., 2017), and B. dorsalis (Wang et al., 2017).

Focusing on the intended function, SlAKHR mRNA was specifically knocked-down via dsRNA injection (fig. 4a). The intermediary metabolism of the fat body is regulated by many hormonal signals. The amount of TAG present in the cells act in insect energy homeostasis of lipid synthesis and breakdown. Both the amount and size of lipid droplets in fat body cells can be altered by lipogenesis and lipolysis (Grönke et al., 2005). In this study, Knockdown of SlAKHR resulted in the accumulation of TAG in fat bodies and prevent it releasing to hemolymph (figs 4b, c and 5). This result indicated that SlAKHR controlled the metabolism of TAG in fat body. Similar results were obtained in G.
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