A Receptor-type Guanylyl Cyclase Expression Is Regulated under Circadian Clock in Peripheral Tissues of the Silk Moth

LIGHT-INDUCED SHIFTING OF THE EXPRESSION RHYTHM AND CORRELATION WITH ECLOSION*

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The mechanisms by which the circadian clock controls behavior through regulating gene expression in peripheral tissues are largely unknown. Here we demonstrate that the expression of a receptor-type guanylyl cyclase (BmGC-I) from the silk moth Bombyx mori is regulated in the flight muscles in a circadian fashion. BmGC-I mRNA was expressed from the end of the light period through the middle of the dark period. BmGC-I protein expression and cGMP levels were high around the initiation of eclosion events at the beginning of the photoperiod. The rhythm of the BmGC-I and cGMP levels free-ran in constant light and synchronized to the environmental photoperiodic cycle. The circadian regulation of BmGC-I expression was also observed in the legs but not in other tissues examined. BmGC-I therefore represents a circadian output gene that regulates eclosion behavior.

Eclosion behavior is controlled by the circadian clock, which itself is regulated by several genes. The current model holds that the Clock- and cycle-encoded products (CLK and CYC) activate period (per) and timeless (tim) transcription. After the PER and TIM proteins accumulate in the cytoplasm and form heterodimers, the proteins translocate to the nucleus and negatively regulate their own genes by interfering with both CLK and CYC (1, 2). Drosophila strains that are mutant in per, tim, Clock, and cycle genes are defective in the rhythm of eclosion (3–6). However, little is known about circadian clock-regulated output genes, which are thought to be regulated by these clock genes and to control physiological and behavioral rhythms. Recently, the pigment-dispersing factor (PDF) and the Drosophila lark gene, which encodes an RNA-binding protein, have been demonstrated to be circadian clock-regulated output genes, and the strains of Drosophila that are mutant for these genes are defective in the rhythm of eclosion (7–9). These genes are expressed in the brain; in contrast, few circadian clock-regulated output genes have been described in the peripheral tissues.

It has been suggested that the intracellular second messenger cGMP plays a functional role in eclosion behavior. cGMP levels in the ganglion increase at the time of eclosion, and injection of cGMP into pharate pupae prior to natural eclosion induces eclosion behavior in the silk moth, Bombyx mori (10). cGMP formation is catalyzed by guanylyl cyclases (GCs)1. Two types of GC have been described: the soluble type and the receptor type. The soluble-type GC is mainly activated by nitric oxide (NO) (11) and plays an important role in neuronal development (12). The receptor-type GC is generally a peptide hormone receptor similar to the receptor for the mammalian atrial natriuretic peptide hormone (13) and consists of four domains: extracellular, single transmembrane, kinase-like, and guanylyl cyclase. The binding of a ligand to the extracellular domain activates the intracellular guanylyl cyclase domain. Phosphorylation of the kinase-like domain modulates the GC domain (14). In insects, three receptor-type GCs have been identified in the embryo and the nervous system of Drosophila melanogaster (15–17). Another three putative receptor-type GCs are listed in the Drosophila genome sequence data base (flybase.bio.indiana.edu). We identified a receptor-type GC gene, BmGC-I, from the silk moth B. mori, which is present in the flight muscle, antennal lobe, antennal neuron, thoracic ganglion, Malpighian tubules, and midgut (18). However, the physiological roles of BmGC-I have not yet been well elucidated.

In this study, we demonstrate that the expression of BmGC-I is regulated under the circadian clock in the flight muscles and that it is involved in eclosion behavior. BmGC-I protein expression and cGMP levels are high during the initiation of the eclosion event that occurs at the beginning of the photoperiod. The rhythms of BmGC-I protein expression, cGMP levels, and eclosion shift with the environmental photoperiodic cycle and exhibit free-running in constant light. These results suggest that BmGC-I is a putative circadian clock-regulated output gene that controls eclosion behavior in peripheral tissues.

MATERIALS AND METHODS

Animals and Tissues—Pupae of B. mori were obtained from Kyoya Corp., Kyoto, Japan and reared at 25 °C under a 14-h light/10-h dark cycle. Pharate pupae were anesthetized with diethyl ether, and the flight muscles were dissected out and quickly used for measurement of cGMP, extraction and purification of RNA, and Western blotting.

RT-PCR Analysis of BmGC-I mRNA Expression—RNA was isolated from the flight muscles of pharate pupae 0–2 days before eclosion using the guanidine thiocyanate method (19). Aliquots of total RNA were treated with deoxyribonuclease I (Wako Pure Chemical Industries, Ltd.) for 1 h at 37 °C. RT-PCR was performed using a commercial RT-PCR kit (Takara Shuzo Co., Ltd.). 1 μg of total RNA was used for cDNA synthesis. PCR amplifications of the synthesized cDNA were performed according to the following schedule: 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1.34 min, for 30 cycles. The specific primers for BmGC-I were 5′-GGCTGTCACAAAGCAGGACG-3′ and 5′-CCGTT-GCTGTATTACAAACC-3′, corresponding to nucleotides 227–246 and 1241–1261 in the extracellular domain (Fig. 1A). The specific primers

1 The abbreviations used are: GC, guanylyl cyclase; BmGC-I, Bombyx mori GC-I; RT-PCR, reverse-transcriptase polymerase chain reaction; ZT, Zeitgeber time.
for the Actin gene were 5'-ATGTGCAAGCCCGGTTCGG-3' and 5'-CGACACGCGCTTCTAG-3', corresponding to nucleotides 49–68 and 284–351 (20). Products were then analyzed on a 1.0% agarose gel and stained with ethidium bromide. The sizes of the amplified products were 1035 base pairs (BmGC-I) and 303 base pairs (Actin).

Quantitative cGMP Determination in the Flight Muscles—Flight muscles dissected from pharate pupae were quickly frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid. Homogenized samples were washed four times with a 5× volume of diethyl ether and lyophilized. Lyophilized samples were weighed and dissolved in 1 ml of a 0.05 M sodium acetate buffer (pH 5.8) containing 0.02% (w/v) bovine serum albumin. Two 50-μl aliquots were assayed for cGMP using a commercial cGMP enzyme immunoassay kit (Amersham Pharmacia Biotech) after acetylation.

Western Blotting Analysis—Tissues dissected from pharate pupae were homogenized in buffer (50 mM Tris-HCl buffer, pH 7.5, 250 mM sucrose, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 mM dithiothreitol). Homogenized samples were centrifuged at 1000 × g for 3 min, and the supernatants were collected. Proteins in the homogenate were determined using the dye-binding method (23). Fifty microliters of homogenate were separated by SDS-polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 10% milk protein in PBS-T and incubated with a 1:1000 dilution of rabbit anti-BmGC-I polyclonal antibody (Amersham Pharmacia Biotech) for 1 min and exposed to x-ray film (Kodak Co.). Protein expression signals were scanned into a computer using a scanner (Epson Co.) and quantified with NIH image 1.62 software.

RESULTS

BmGC-I Expression and cGMP Levels Are Controlled by Circadian Rhythms and Are Relevant to Elosion Behavior in the Flight Muscles—To examine the temporal pattern of BmGC-I mRNA expression in the flight muscles, we used RT-PCR to amplify 1035 base pairs of mRNA from the extracellular domain (Fig. 1A). Dissected flight muscles were collected from pharate pupae over 24 h at 2–4 h intervals in a 14-h light (Zeitgeber time (ZT) 0-ZT 14/10-h dark (ZT 14-ZT 24) cycle) and then exposed to constant light (circadian time (CT)) for 1 day (C). The open box and closed box represent light and dark periods, respectively. Lanes containing RT-PCR amplifications of BmGC-I mRNAs are indicated by a plus (+). No product was detected in the control samples in which transcripts were amplified in the absence of reverse transcriptase (lanes indicated by a minus (−)). D and E, temporal Western blotting analysis of BmGC-I protein expression in the flight muscles. The upper panel shows the Western blot and the lower panel the BmGC-I signal quantification of the data by densitometry. Silk moth pupae were entrained to a 14-h light (ZT 0-ZT 14/10-h dark (ZT 14-ZT 24) cycle (D) and then exposed to constant light (circadian time (CT)) for 1 day (E). Relative BmGC-I abundance indicates the relative densitometric units, normalized to the value at ZT 6.

We analyzed the expression rhythm of the BmGC-I protein using Western blotting analysis. Western blotting analysis showed that the BmGC-I protein was expressed between about ZT 22 and ZT 24, a 4–5-h delay relative to the mRNA expression (Fig. 1D). The lag of 4–5 h between mRNA expression and protein expression is characteristic of circadian clock genes such as per and tim (22). The BmGC-I protein expression also exhibited a free-running rhythm under constant light (Fig. 1E), and the peak of the BmGC-I mRNA expression under constant light advanced about 2 h from that under the 14-h light/10-h dark cycle (Fig. 1, D and E).

To assess the BmGC-I activity in the flight muscles throughout the 24-h cycle, we measured cGMP levels using an enzyme immunoassay for cGMP. The peak of the cGMP levels was at the subjective beginning of the photoperiod and coincided with that of the BmGC-I protein expression under the 14-h light/10-h dark cycle and under constant light (Fig. 2, A and B). We also found that the peak of cGMP levels coincided with that of elosion events (Figs. 2, C and D). These results indicate that the BmGC-I protein expression is regulated under the circadian clock and is related to elosion behavior in the flight muscles.

The circadian expression of the BmGC-I was slightly affected by constant light; under constant light conditions, the peak of the BmGC-I protein expression, cGMP levels, and elosion advanced about 2 h from those under the entrained condition (Figs. 1, D and E, and 2). In previous studies, the phase of elosion rhythm slightly advances by the first day in constant
BmGC-I Protein Expression Rhythm Synchronizes to the Photoperiodic Cycle—In insects, the circadian clock synchronizes to the photoperiodic cycle. In *Drosophila*, the phase of the locomotor activity rhythm shifts with a light flash in the dark period, and expression rhythms of several circadian clock genes synchronize to the photoperiodic cycle (24). To test whether the rhythm of the BmGC-I protein expression also synchronizes to the photoperiodic cycle, we assessed induction of the BmGC-I protein expression under a condition in which the beginning of the photoperiod was shifted to ZT 20, 4 h prior to the beginning of the entrained photoperiod. The BmGC-I protein expression shifted to ZT 21, 1 h after the beginning of the first photoperiod in the new photoperiodic cycle (Fig. 3A). The peaks of adult eclosion events and of cGMP levels also shifted to ZT 21 (Fig. 3, B and C). These results indicate that the BmGC-I protein expression, cGMP levels, and eclosion rhythm shift with the new photoperiodic cycle.

BmGC-I Protein and Gene Expression in Other Tissues—The products of the circadian clock genes are expressed widely in *Drosophila* tissues: the *per* gene product, PER, is expressed in the antennae, alimentary canal, Malpighian tubule, ovaries, and compound eyes (25, 26). In the silk moth, BmGC-I protein (and mRNA) are expressed widely in the antennae, legs, midgut, and ganglion in addition to the flight muscles (18). We examined the BmGC-I expression rhythm in the legs, midgut, antennae, and ganglion using Western blotting analysis and RT-PCR. In the legs, the BmGC-I protein was expressed at ZT 24 but not at ZT 12 (Fig. 4A), and BmGC-I gene expression was observed at ZT 18 but not at ZT 6 (Fig. 4B). In the midgut, antennae, and thoracic ganglion, the BmGC-I protein was expressed at both ZT 12 and ZT 24 (Fig. 4A), and RT-PCR analysis showed that the BmGC-I gene is expressed at both ZT18 and ZT 6 (Fig. 4B). These results indicate that the BmGC-I expression is regulated under the circadian clock in the muscles and legs but not in the antennae, midgut, or thoracic ganglion.

**DISCUSSION**

BmGC-I is a putative peptide hormone receptor whose ligand has not been identified. The amino acid sequence of BmGC-I is most closely related to the mammalian atrial natriuretic peptide hormone receptors (18), and it is likely that the putative ligand is a member of the atrial natriuretic peptide hormone family.

Here we have demonstrated that BmGC-I protein expression and the peak of cGMP levels in the flight muscles occur near the initiation of eclosion events at the beginning of the photoperiod. These all exhibited free-running under conditions of constant light (Figs. 1 and 2). The BmGC-I protein expression, the peak of cGMP levels, and the peak of eclosion shifted when the beginning of the photoperiod was shifted 4 h prior to that of the entrained condition (Fig. 3). In a previous study, cGMP was suggested to be the second messenger in eclosion events, because injection of cGMP to pharate pupae prior to natural eclosion induces eclosion behavior (10). Furthermore, peptide hormones are thought to be essential for circadian output; for example, the pigment-dispersing factor gene in *Drosophila* and the vasopressin gene in rodents are circadian clock-regulated output genes (27, 28). In light of these reports, we suggest that the putative peptide hormone receptor BmGC-I is a circadian clock-regulated output gene that is involved in eclosion in the flight muscles.

BmGC-I is expressed widely in the silk moth and is found in the antennae, the thoracic ganglion, and the legs in addition to the flight muscles (18). The rhythmic expression of BmGC-I was observed in the flight muscles and the legs but not in the antennae, thoracic ganglion, or the midgut (Fig. 4), suggesting that the functional role of BmGC-I differs from tissue to tissue. In insects, cGMP plays different physiological roles in various tissues, including reception of sex pheromones in male antennae (29, 30), development in neurons (12, 31), and diuresis in the Malpighian tubules (32, 33). However, the physiological role of cGMP in the flight muscles is not well understood. In mammalian skeletal muscles, cGMP mediates oxidation of glucose, inhibition of glycogen synthesis, release of lactate, and transport of glucose (34, 35). Levels of glucose, lipids, and glycogen in skeletal muscle have been demonstrated to follow circadian rhythms in rats (36). It is therefore possible that the rhythm of the BmGC-I expression is relevant to the circadian rhythm of glucose metabolism and that BmGC-I stimulates glucose utilization for muscle contraction. Silk moth pupae frequently contract muscles to cast off pupal skin at eclosion and thus are likely to use glucose at that time. It is important...
Figure 3. The BmGC-I expression levels, cGMP levels, and eclosion rhythm exhibit phase shifts in response to light. Pharate pupae were exposed to a photoperiod whose beginning was shifted to time point ZT 20, 4 h prior to the entrained photocycle (14 h light/10 h dark). A, Western blotting analysis showing induction of BmGC-I expression in response to the beginning of the first photoperiod in the new photoperiodic cycle. B, temporal profile of cGMP levels in the flight muscles. Light induced an increase in cGMP levels at 1 h after the first time point ZT 20, 4 h prior to the entrained photocycle (14 h light/10 h dark cycle). C, temporal profiles of adult eclosion at the first-shifted photoperiodic cycle. Light induced eclosion at 1–2 h after the start of the new photoperiod (indicated by open bars). Adult eclosion in the entrained condition is indicated by closed bars.

Figure 4. Comparison of the BmGC-I expression rhythm in non-flight muscle tissues. A, Western blotting analysis showed a temporal difference in BmGC-I expression between ZT 24 and ZT 12 in the flight muscles and legs. No difference was observed in the antennae, ganglion, and midgut. B, RT-PCR analysis showed a difference in BmGC-I mRNA expression between ZT 6 and ZT 18. Lanes containing RT-PCR amplifications of BmGC-I and Actin are indicated by a plus (+). No product was detected in the control samples in which transcripts were amplified in the absence of reverse transcriptase (lanes indicated by a minus (−)).

REFERENCES
