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Photoperiodic and Circadian Photoreception in the Silkworm, *Bombyx mori*

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We have cloned a cDNA for an opsin (Boceropsin) from the silkworm larval brain which was suggested to contain the photoperiodic receptor. Its deduced amino acid sequence was composed of 381 amino acids and included amino acid residues highly conserved in insect visual pigments. This opsin belonged to the long wavelength photoreceptor group of insect opsins, and are presumed to be photoperiodic receptor. RT-PCR analysis revealed that Boceropsin mRNA is expressed in the larval brain, but not in the subesophageal (Sg) and thoracic ganglion. Immunohistochemical analyses demonstrated that Boceropsin protein is present bilaterally in some defined cells localized in the brain of the *Bombyx* larva. Boceropsin was considered not to be involved in the circadian photoreception, because carotenoids are not indispensable for the photoreception and formation of circadian rhythms in the silkworm.

Key words: potoperiodism, extraocular photoreception, cerebral opsin, circadan rhythm

INTRODUCTION

In the silkworm, which shows the photoperiodic response for the maternal determination of embryonic diapause [1], the photoreceptor involved in the diapause induction has been demonstrated to be cephalic but extraocular [2, 3]. *In vitro* culture experiments using isolated brain-Sg complex demonstrated that the photoreceptor of the photoperiodic clock is localized in the complex [4]. It was demonstrated that dietary carotenoid or vitamin A is essential for the photoperiodic induction of the embryonic diapause in the silkworm [5] and the silkworm brain was found to contain both retinal and 3-hydroxyretinal which are chromophores of insect visual pigments [6]. Here we report cloning of cDNA for an opsin in the silkworm brain and expression of this opsin (Bombyx cerebral opsin:Boceropsin) in some cells of the brain by immunohistochemistry. The photoreceptive function of Boceropsin was discussed.

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MATERIALS AND METHODS

cDNA cloning was performed using a Chinese race of the silkworm, which shows a sensitive photoperiodic response during larval stage. cDNA was made from the total RNA template using a first-strand cDNA synthesis kit (Life Sciences, Inc., St. Petersburg, FL). Reverse transcription of total RNA from the brains was performed using 5 μg of total RNA and 25 units of avian myeloblastosis virus (AMV) reverse transcriptase in a 25 μl cDNA reaction mixture.

Two degenerate primers were designed on the basis of highly conserved regions [(E/D/A)QAKKM and (D/N)P(I/F)VY(G/A)] of invertebrate opsins [8]. The PCR products were electrophoresed on 1% agarose gels, and the amplified DNA band was purified. The products (ca.207bp band) were cloned into pT7 Blue vector (Novagen: Takara Co., Ltd.) for sequencing. The 3'-terminal sequence was cloned by the 3'-RACE (rapid amplification of cDNA ends) method.

The amplified ca. 0.5 kb fragment was purified, cloned into pT7Blue and sequenced as described above. The 5'-terminal portion of the cDNA was cloned by the 5'-RACE method (5'RACE System for Rapid

Amplification of cDNA Ends, Gibco BRL, Gaitherburg, MD). The amplified ca.1.3 kb fragment was subcloned and sequenced (AB064496).

One-step RT-PCR was performed using a kit purchased from QIAGEN (Chatsworth, CA) according to the manufacturer's protocol. One peptide corresponding to the carboxy-terminal 25(CHSTTTDEASSVASGTTV MEEKPTA) of the deduced amino acids sequence was synthesized by Biomedical Center of Takara Shuzo Co., Ltd (Shiga Pref., Japan) and a polyclonal antibody against the peptide conjugated with maleinimide-bovine serum albumin (BSA) was raised in mice. Immunocytochemistry was performed according the method described by Ichikawa *et al.* [9] with slight modifications.

Commercial silkworms were used in circadian hatching rhythm study. The larvae were fed on a carotenoid-deprived artificial diet prepared as described [11]. As a carotenoid-rich diet for a control group, an artificial diet containing mulberry leaf powder (40% dry weight) was used as described before[3]. To obtain lutein-supplemented silkworms for a second control group, we administered 1ml of lutein solution (5 mg/ml in dimethylsulfoxide; Sigma, X-6250) to carotenoid-depleted pupae intraperitoneally at 5 days after pupation..

We compared hatching rhythms of carotenoid-depleted silkworms with those of carotenoid-rich and lutein-supplemented groups under a transfer from continuous light (LL) to continuous darkness (DD). The illuminations in LL was adjusted to 1,000lux. A white fluorescent lamp was used as a source of light. Hatched larvae were manually collected every hour. In darkness, hatched larvae were gathered with a feather without any exposure to light and counted.

RESULTS

To clone cDNA encoding opsin in the silkworm larval brain, PCR was performed using degenerated oligonucleotide primers (SF and SR) corresponding to amino acid sequences highly conserved in invertebrate visual pigments. These primers successfully amplified cDNAs of the visual pigments in the silkworm compound eyes[10]. Amplification of DNA fragments of approximately 200 bases in length was observed by electrophoresis on an agarose gel. The nucleotide sequences of the fragments were determined using subclones, and were found to have a sequence (207 base) of distinct opsin-like protein[7].

Using primers based on the nucleotide sequences of the cloned fragment, we cloned the 5'- and 3'-portions of the cDNA, respectively. To determine whether these RACE products were actually parts of a single transcript, PCR was performed using primers corresponding to the sequences of the 5'- and 3'-untranslated regions obtained by RACE method. We amplified one full-length DNA fragment of 1322bp. The full-length cDNA contained an open reading frame (ORF) encoding a putative protein of 381 amino acids.

This deduced protein showed a high homology with insect visual pigments. Database research revelaed that this protein shows the highest homology of 84% with Manop1 (377 amino acids), one of the visual opsins found in the moth *Manduca sexta*. Similarities with insect visual pigments include a lysine (Lys-327) in the seventh transmembrane domain to serve as a site for shiff base linkage of the chromophore and a pair of cysteines (Cys-131 and Cys-208) in the second and third extracellular loops to stabilize the tertiary structure by disulfide bridge (Fig. 1). Asparagine (Asn-28) residue suspectible to glycosylation is present in the N-terminal region. The C-terminal region of this opsin is rich in serine (S) and threonine (T) residues as potential phosphorylation sites. Like other opsins, this opsin consists of predicted seven transmembrane segments. We have named this opsin Boceropsin because of its isolation from the Bombyx cerebral ganglion.

NJ tree presents the molecular phylogenetic relationship between Boceropisn and other insect opsins. The insect opsins are divided into two group: one is long-wavelength photoreceptor group containing *Drosophila* Rh1 and Rh2 and second is shortwavelength photoreceptor group containing *Drosophila* Rh3 and Rh4. This phylogenetic tree shows that Boceropsin belongs to the short wavelength photoreceptor [9].

To investigate the transcription of the *Boceropsin* gene in the brain, Sg, and Tg, RT-PCR analysis was performed using mRNA isolated from these ganglia of the silkworm larvae. An amplification of 461 bases fragmen of which sequence was just the partial sequence of Boceropsin cDNA was detected in the brain as expected and stemmata, but not in Sg and Tg.

Whole-mount immunohistochemistry was performed to localize the Boceropsin-producing cells in the silkworm brain. Some somata (15-20 μm in

diameters) in the brain showed strong immunoreactivity to the mouse anti-Boceropsin antiserum. In the anterior protocerebrum, there were two groups of bilateral Boceropsin-immunoreactive cells: dorsal anterior protocerebral (DAP) and ventral anterior protocerebral (VAP) cells. DAP cells had large somata and showed intense immunostainning as compared to other immunoreactive cells. Boceropsin immunoreactivity was also found bilaterally in dorsal posterior protocerebral (DPP) cells and lateral posterior tritocerebral (LPT) cells. We found no Boceropsin-immunoreactive cells in Sg. When samples were incubated without the primary antiserum or incubated with the primary antiserum inactivated by preincubation with an excess of the synthetic peptide, no staining was seen in the brain.

To confirm the dispensability of carotenoids in the formation and maintenance of circadian rhythm in the silkworm, an experiment of LL to DD transfer was carried out [11]. The transfer of silkworm eggs from LL to DD produced obvious hatching rhythms, irrespective of the presence or absence of carotenoids. On the first day after the transition, the carotenoid-depleted group showed a robust peak at 13.2 hr. and relatively small numbers of animals hatched in the control, lutein-supplemented and carotenoid-rich groups, and the peaks were obscure. However, the following second and third peak occurred at the same timing in each experimental group.

DISCUSSION

Here we isolated one opsin (Boceropin) cDNA from the silkworm brain, which has been suggested previously to contain a photoperiodic receptor [4]. The molecular phylogenetic tree showed that Boceropsin belongs to the long wavelength receptor group of insect opsins. Boceropsin showed the highest degree of homology to Manop1, one of the *M.. sexta* visual pigments. Chase *et al.* (1997) suggested that Manop1 may be the opsin P520 identified by biochemical methods. These observations suggested that Boceropsin may function as a green-sensitive photopigment in the silkworm brain. Proper action spectrum has not been obtained in the photoperiodism of the silkwom. In a preliminary experiment, Kogure [1] observed that the silkworm was

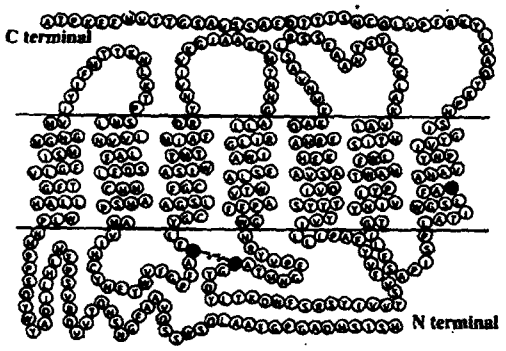

Fig. 1. Structural model for Boceropsin. Seven segments in the amino acid sequence is arranged to membrane topology. Possible chromophore binding lysine residue (327K) and a pair of cysteines (Cys-131 and Cys-208) making a disulfide bridge are shaded.

sensitive to blue-green (350-510 nm) light but not to red light(>600nm). Nakajima (1981) carried out "day interruption" experiments using different wavelengths of light during the photoperiodic sensitive stage and examined the interruption effects on the diapause incidence of the next generation. He found that the 500-550 nm wavelength region was most effective to accomplish a long-day photoperiod. This pronounced green sensitivity in the *Bombyx* photoperiodism suggested that Boceropsin, which has been assumed to be a green receptor on the basis of the molecular phylogenetic tree, is involved in photoperiodic photoreception.

In some insects photoperiodic photoreceptors were reported to reside within the brain. Studies of Williams (1969) using *Antheraea pernyi* suggested that the dorsal region of the protocerebrum containing the lateral neurosecretory cells is essential for the photoperiodic reception. Kono (1973) also suggested the brain-centered photoreception in white butterfly *Pieris rapae*. In this connection we could clone cDNAs encoding same kind of long-wavelength type opsin as Boceropsin from the larval brain of the *A. pernyi* (381 a.a), and *P. rapae* (382a.a)(unpublished data). These pieces of evidence implied that such cerebral opsin exist in the insect brains generally.

In the silkworm, the photoperiodic signals are received by the mother moth during her embryonic and

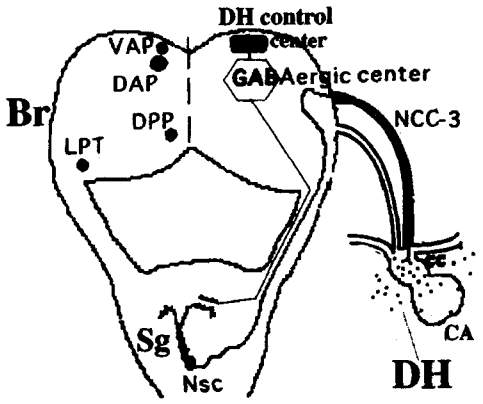

Fig. 2. Schematic drawing of the physiological process underlying diapause induction in the silkworm.

larval stages and the information is kept in yet undefined neural region until the pupal stage when diapause hormone should be secreted. In this connection, microsurgical operation at the early pupal stage of the cortex of the anterolateral area on the ventral side of protocerebrum, which is in the vicinity of DAP and VAP cells, disturbed the control of diapause hormone secretion. These observations suggested that the components responsible for the photoperiodic response concerning the diapause phenomenon reside in this confined region and strengthened the idea that DAP and/or VAP cells are the photoperiodic receptors

Hypothetical mechanism for the photoperiodic induction of diapause in the silkworm is as follows (Fig. 2): the photoreceptive cells containing Boceropisn, probably DAP and/or VAP cellls, receive photoperiodic information and the photoperiodic signal is translated to the diapause hormone (DH) control center of diapause induction. Finally the accumulated information is translated from the center to GABAergic center by which secretion of diapause hormone (DH) from CC and CA is regulated [10].

In some insects circadian photoreceptors are condisered to be brain-centered as well the photoperiodic receptor. In the silkworm it was found that carotenoid is not indispensable for the entrainment, formation and sustainment of circadian rhythm [11].

This study also suggested that there is a circadian photoreceptive molecule other than carotenoid- or retinal-based pigments. Recent studies using mutants and molecular techniques on circadian rhythms in fruit flies (*Drosophila*) have revealed that a Vitamin B2-based photopigment (cryptochrome: CRY) is involved in photoreception for entrainment of the circadian clock in insects [12]: Boceropsin is considered not to be involved in the circadian photoreception.

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