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A Circadian Neuropeptide, Pigment-Dispersing Factor–PDF, in the Last-Summer Cicada *Meimuna opalifera*: cDNA Cloning and Immunocytochemistry

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ABSTRACT—Pigment-dispersing factor (PDF), an 18-amino acid neuropeptide, is a principal circadian neuromodulator functioning downstream of the insect brain's circadian clock, modulating daily rhythms of locomotor activity. Recently, we found that PDF precursors of the cricket *Gryllus bimaculatus* comprise a nuclear localization signal (NLS). Moreover, the nuclear localization of PDF immunoreactivity and the translocation of GFP-fused PDF precursor into the nucleus have both been demonstrated. These suggest a fundamental role for PDF peptide in the circadian clock system within the nucleus, in addition to its role in downstream neural events. In the present study, we carried out the cDNA cloning of PDF from adult brains of the last-summer cicada *Meimuna opalifera*, and found that an isolated clone (545 bp) encodes an ordinary PDF precursor protein. PDF peptide itself shows a high sequence identity (78–94%) and similarity (89–100%) to insect PDFs and also to the crustacean β-PDH peptides. The computer-assisted sequence analysis of PDF precursor revealed a possible translocation into the nucleus, despite the lack of a definite NLS-like sequence. Using immunocytochemistry, the optic lobes of *M. opalifera* revealed PDF-immunoreactive neurons in both the medulla and lamina neuropiles. All these PDF cells exhibited prominent immunolabeling of both their perikarya and axons, but not their nuclei. Our results provide the first structural and immunocytochemical identification of PDF neurons in Hemiptera.

Keywords: circadian rhythm, neuropeptide, pigment-dispersing factor, cDNA cloning

INTRODUCTION

Pigment-dispersing factor (PDF) is involved in regulating insect circadian rhythms (Stengl and Homberg, 1994; Pyza and Meinertzhagen, 1996, 1997; Petri and Stengl, 1997), acting as a neuromodulator that functions in the output pathway of the brain's circadian pacemaker, and thereby regulating behavior arising from the dorsal central brain (Helfrich-Förster *et al*., 1998, 2000; Renn *et al*., 1999; Park *et al*., 2000). Many lines of evidence have been obtained aimed at clarifying the function of PDF in the circadian system of the fruit fly *Drosophila melanogaster*. To date, PDF has been thought to be a neuropeptide functioning as a principal circadian modulator downstream of the brain's clock machinery, organizing daily locomotion or other behaviors.

Recently we have carried out the cDNA cloning of PDF from adult brains of the cricket *Gryllus bimaculatus*, in which rhythmic behavioral activity manifests a nocturnal peak. The cDNA obtained exhibits a unique preprosequence including an ordinary PDF sequence, but with very short PDF-associated peptide (PAP) sequence consisting of a nuclear localization signal (NLS) at the N-terminus (Chuman *et al*., 2002). Immunoreactivity to *Gryllus bimaculatus* PDF was identified in all the axons of the PDF neurons. Electron microscopic observation of cells with nuclei that show PDF immunoreactivity and microscopic observation of cells with nuclei that contain GFP-fusion PDF precursor protein, both demonstrate however the translocation of PDF peptide into the nucleus of PDF neurons. These results strongly suggest that, in addition to its role as a neuromodulator at output pathways from the insect's circadian clock, PDF is also

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involved in the clock mechanism itself. In this study, we attempted to clone the *pdf* cDNAs from other insect species in order to detect similar types of PDF precursors.

Insect PDFs are close homologues of the β-subfamily of crustacean pigment-dispersing hormone (β-PDH) neuropeptides (Rao and Riehm, 1993). Among 16 β-PDH precursors deduced from *pdh* cDNAs, the molecular sizes are notably similar to each other. For insect PDF precursors, only three *pdf* cDNAs have so far been elucidated: the precursors of *D. melanogaster* (Park and Hall, 1998), *Romalea microptera* (GenBank: U42472), and *G. bimaculatus* (Chuman *et al*., 2002). Although the C-terminal PDF region possesses exactly the same peptide size, the region containing the signal and PAP in *D. melanogaster*, *R. microptera*, and *G. bimaculatus* obviously differed, with 80, 66, and 20 amino acid residues, respectively. Such differences in peptide sizes might reflect different functions of the PAP peptides. In the present study, we carried out the cDNA cloning of PDF from adult brains of the last-summer cicada, *Meimuna opalifera*, a member of the suborder Homoptera of Hemiptera, and thus representing a different order of insects from the previous three, *D. melanogaster* (Diptera), *R. microptera* (Orthoptera) and *G. bimaculatus* (Orthoptera). We here report the sequence analyses of nucleotides and deduced amino acids of *M. opalifera* PDF, and the results from immunocytochemistry using an antibody directed against this peptide sequence.

MATERIALS AND METHODS

Animals

Last-summer cicadas, *M. opalifera*, were collected in the suburbs near Fukuoka and maintained at 25°C in an incubator which was illuminated for the same photoperiod as the day/night cycle as that in the field in Fukuoka. Phases of morning and evening peaks are given in Zeitgeber time (ZT), whereby lights on is ZT0 and lights off is ZT12. The brains were dissected at 4 hr intervals over 24 hr starting with ZT0 and were then immediately frozen in liquid nitrogen and stored directly at –80°C within a day. For immunocytochemistry, cicadas were dissected at 06:00, 30 min before lights 'on' (ZT23:30).

3' RACE for identification of 3' end of PDF mRNA

mRNAs were purified using a QuickPrep® *Micro* mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the Manufacturer's instructions. To extract mRNAs, the brains of cicadas frozen at each time point were mixed and homogenized in the extraction buffer. The mRNAs obtained (1 μg) were reverse-transcribed by the polymerase chain reaction (PCR) using AMV reverse transcriptase (Promega, Madison, WI) and $d(T)_{17}$-adapter primer (5'-GGCCACGCGTCGACTAGTAC-T_{17}-3') as previously described (Frohman *et al*., 1988). After phenol extraction followed by ethanol precipitation, the resulting cDNA pellet was used as a template for 3' RACE. Primers were all obtained from Hokkaido System Science (Sapporo) and those for 3' RACE were as follows: PDF specific primer PDF-SP (5'-AAGCGCAACTCN-GARMTVATCAA-3') and adapter primer Ad (5'-GGCCACGCGTC-GACTAGTAC-3'). In these primers, the letters M, N, R, and V denote the nucleotides (A or C), (any nucleotides), (A or G), and (not T), respectively. A PDF-specific primer was designed based on the nucleotide sequence homology among mRNAs clarified for β-PDH and PDF (Klein *et al*., 1992, 1994; de Kleijn *et al*., 1993; Desmoucelles-Carette *et al*., 1996; Park and Hall, 1998). The PCR mixture (100 μl) included 1 μl of cDNA, 2 pmol of Ad primer, 20 pmol of PDF-SP primer, 200 μM dNTP, 2.5 mM $MgCl_2$, and 2.5 units of PLATINUM *Taq* DNA polymerase (GIBCO BRL). PCR (30 cycles) was performed as follows: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 90 sec, and final extension for 7 min at 72°C, using a Geneamp PCR system 2400 (Perkin Elmer). Single-primer control PCRs were also carried out in parallel. Two primer-specific PCR products were identified on the 2% agarose gel, and recovered by phenol extraction followed by ethanol precipitation. The gel-purified PCR products were subcloned into pBluescript II SK+ vector. Sequencing analysis on a Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit (Amersham Pharmacia Biotech) identified plasmid DNA containing *pdf*.

5' RACE for identification of 5' end of PDF mRNA

To amplify the 5' end of the *M. opalifera pdf* cDNA, we carried out the cDNA cloning by using a 5' RACE System for rapid amplification of cDNA ends (version 2.0: GIBCO BRL) according to the Manufacturer's protocol. The antisense primers were designed from the cDNA sequence clarified by the 3' RACE method, and these included MSP-RT (5'-TATGAGGTCTTTGTAGTT-3'), MSP-1A (5'-AGTGTGGTTCAAGGTTTGTATAATTT-3'), and MSP-2A (5'-TTCA-AGGTTTGTATAATTTGTTGTATG-3').

First-strand cDNA was synthesized from a mRNA using a single gene specific primer (MSP-RT) and a reverse transcriptase SUPER SCRIPT™ II (GIBCO BRL). The original mRNA template was removed by treatment with the enzyme RNase Mix (a mixture of RNase H and RNase T_1). Unincorporated dNTPs, MSP-RT, and proteins were separated from synthesized cDNA using an adsorbent GLASSMAX® Spin Cartridge. A homopolymeric tail, poly(C), using terminal deoxytransferase (TdT) and a substrate dCTP, elongated the 3'-end of the purified cDNA. Since this tailing reaction was performed in a PCR-compatible buffer, the entire contents of the reaction mixture were directly amplified by PCR without intermediate organic extraction and ethanol precipitation.

PCR amplification was accomplished using PLATINUM *Taq* DNA polymerase (GIBCO), gene-specific primers (MSP-1A and MSP-2A), and deoxyinosine-containing anchor primers. PCR product using MSP-1A and Abridged Anchor Primer AAP (5'-GGCCACGCGTCGACTAGTAGTACGGGIIGGGIIGGGIIG-3', GIBCO) was further amplified with MSP-2A and Abridged Universal Anchor Primer AUAP (5'-GGCCACGCGTCGACTAGTAC-3', GIBCO). Respective PCR was carried out for 30 cycles as described above. The PCR products were separated in an agarose gel and cloned into pBluescript II SK^+. The clones were verified by sequencing each separated cDNA as mentioned above.

Immunocytochemistry

For anatomical analysis of PDF immunoreactive cells, confocal microscopy was first used on whole-mount immunolabeled preparations. Dissected brains containing the optic lobes with attached retina were fixed with 4% paraformaldehyde for 16 hr. After washing with PBS, brains were incubated for 96 hr at 4°C in a rabbit polyclonal antibody raised against *G. bimaculatus* PDF (Tomioka *et al*., in preparation) (1:1500). Fluoresein isothiocyanate (FITC) conjugated goat anti-rabbit secondary antibodies (Jackson Immunoresearch, Westgrove, PA) were used. For washings between all steps, PBS with 1% Triton X-100 was used. Preparations were dehydrated in graded ethanol, cleared in 100% methyl salicylate, and coverslipped in methyl salicylate prior to imaging in a Zeiss LSM510 laser scanning confocal microscope.

To localize the PDF cells and their neurites precisely in the neuropile, horizontal Vibratome slices of 80 μm thickness were also immunolabeled, using the same rabbit polyclonal antibody against

G. bimaculatus PDF but at 1:3500 for 48 hr at 4°C in a free-floating reaction (Sternberger, 1979). Sections were then incubated with FITC-conjugated secondary antibody to rabbit IgG. Sections were mounted in Vectashield (Vectoo Lab., Bmlingame, CA).

RESULTS AND DISCUSSION

Isolation of a *Meimuna opalifera* PDF cDNA

To be sure of obtaining target mRNAs, heads of adult cicadas *Meimuna opalifera* were collected every 4 hr, namely six times a day. The last-summer cicadas only appeared during a very limited period, and thus those that were collected were taken within about two weeks at a particular area where they appeared in a large population. The total number of collected adults was about 700.

All animals were maintained in a daily cycle of L12:D12, that was almost the same as the natural day/night cycle, in September in Fukuoka. We adopted the procedure designed for direct isolation of polyadenylated RNA without purifying the total RNA. Isolated mRNAs are all converted to cDNAs by the RT-PCR method, in which AMV reverse transcriptase catalyzes the polymerization of DNA using mRNA as templates. This enzyme has an intrinsic RNase H activity to degrade mRNA, and thus the method eventually affords a mixture of single-strand cDNAs.

RACE methods to determine the full sequence of a *Meimuna opalifera* PDF

To screen for the *pdf* cDNA of the cicada *M. opalifera*, we first carried out the 3' RACE method. There is a clear reasoning to carry out the 3' RACE method as an initiating cloning procedure. With respect to the nucleotide sequence of insect PDF precursors, no prominent similarities have been found except for the N-terminal portion of the PDF peptide regions. Thus, if we had first carried out 5' RACE, it would have been difficult to judge whether or not a cloned cDNA were the one for PDF. This is not the case, however, for 3' RACE, which would yield the entire structure of the PDF peptide region. Thus, we first screened a *pdf* cDNA clone from the cDNA mixture by the 3' RACE method.

PDF-specific sense primer for 3' RACE was designed with reference to sequence alignment analyses for both amino acid and nucleotide sequences. The N-terminal portion of PDF, including the dibasic processing site (KR) -- the residues –2 ~ 6, was chosen as a PDF-specific sense primer (PDF-SP). The first PCR with a primer of PDF-SP and an adapter primer Ad lacking $d(T)_{17}$ yielded no distinct gel-bands, while the second PCR using the resulting PCR product provided several products. A dim gel-band, a two primer-specific PCR product of about 270 bp was subsequently isolated for sequence analysis. The nucleotide sequence so determined encoded a PDF peptide with the amidation sequence in addition to the 3'-UTR non-coding region (Fig. 1). The open reading frame encoding PDF was found to be highly conserved among *pdf* and *pdh* genes.

Compared with the 3'-UTR regions of other insect *pdf* genes, the 3'-UTR region of *M. opalifera pdf* was found to be very similar to that of *R. microptera* both in length (190 and 197 bp, respectively) and sequence (ca. 50% identity). The 3'-UTR regions of both *M. opalifera* and *R. microptera* pdf are unique in length and sequence as compared with those of *Drosophila* (492 bp) and *G. bimaculatus* (113 bp).

To determine the structure of the 5'-end of *pdf* cDNA, we undertook the 5' RACE method. Based on the sequence information obtained from 3' RACE, the antisense primer for RT-PCR against PDF mRNA was set at the 3'-UTR region (Fig. 1). First-strand cDNA was synthesized from mRNA using a gene-specific antisense primer MSP-RT and a reverse transcriptase SUPER SCRIPT^{TM} II. A characteristic reaction of the 5' RACE method is an addition of poly(C) tail to the 3'-end of cDNA obtained. The resulting poly(C)-tailed cDNA was utilized as a template for PCR using an anchor primer AAP, having a poly(I) truncated with deoxyguanosine, G/poly(I)/G, that enables the primer to anneal specifically to poly(C). PCR was carried out by using AAP and gene-specific antisense primer MSP-1A, which was assigned at the 3'-UTR region. The anchor primer AAP possesses a specific 5'-end anchor sequence, and subsequent nested PCR was performed using antisense MSP-2A (assigned at the 3'-UTR region) and another anchor primer AUAP lacking G/poly(I)/G. MSP-1A and MSP-2A are adjacent, being separated by only 9 nucleotides.

The sequencing analysis afforded complete sequence data including 5'-UTR, signal region, and PAP peptide region. The cDNA sequence was further confirmed by RT-PCR using primers set for both 5'-UTR and 3'-UTR to amplify the full-length PDF cDNA. Eventually, the full length of the cDNA clone was found to comprise 545 bp, with a precursor protein of 82 amino acid residues (Fig. 1). We assigned the initiator codon as shown in Fig. 1 according to the following sequence data. (i) The selected ATG codon and its adjacent nucleotide sequences fulfilled Kozak's consensus motif (Kozak, 1999). (ii) We found no additional ATG codon in the upstream region of any cDNA clones isolated in the RT-PCR analysis. (iii) The four-amino-acid sequence at the putative N-terminal (Met-Arg-Ser-Ala) was exactly the same as those of *Carcinus maenas* (Klein *et al*., 1992), *Orconectes limosus* (de Kleijn *et al*., 1993), and *Penaeus vannamei* (Desmoucelles-Carette *et al*., 1996), all crustacean β-PDH precursors. Finally, (iv) the full-length PDF cDNA was isolated by RT-PCR using a primer set for both 5'-UTR and 3'-UTR.

Comparing the sequences of insect *pdf* cDNAs, the entire open-reading frame region of *M. opalifera* was found to be very similar, as for the 3'-UTR region, to that of *R. microptera* in sequence (ca. 60% identity). Furthermore, the number of amino acid residues (n), 82, of the PDF precursor was also similar to that of *R. microptera* (n = 89), although it was considerably different from those of other insects (102 for *D. melanogaster* and 43 for *G. bimaculatus*) (Fig. 2). When analyzed by the computer-assisted signal peptide prediction method for eukaryotic sequences (Nielsen *et al*.,

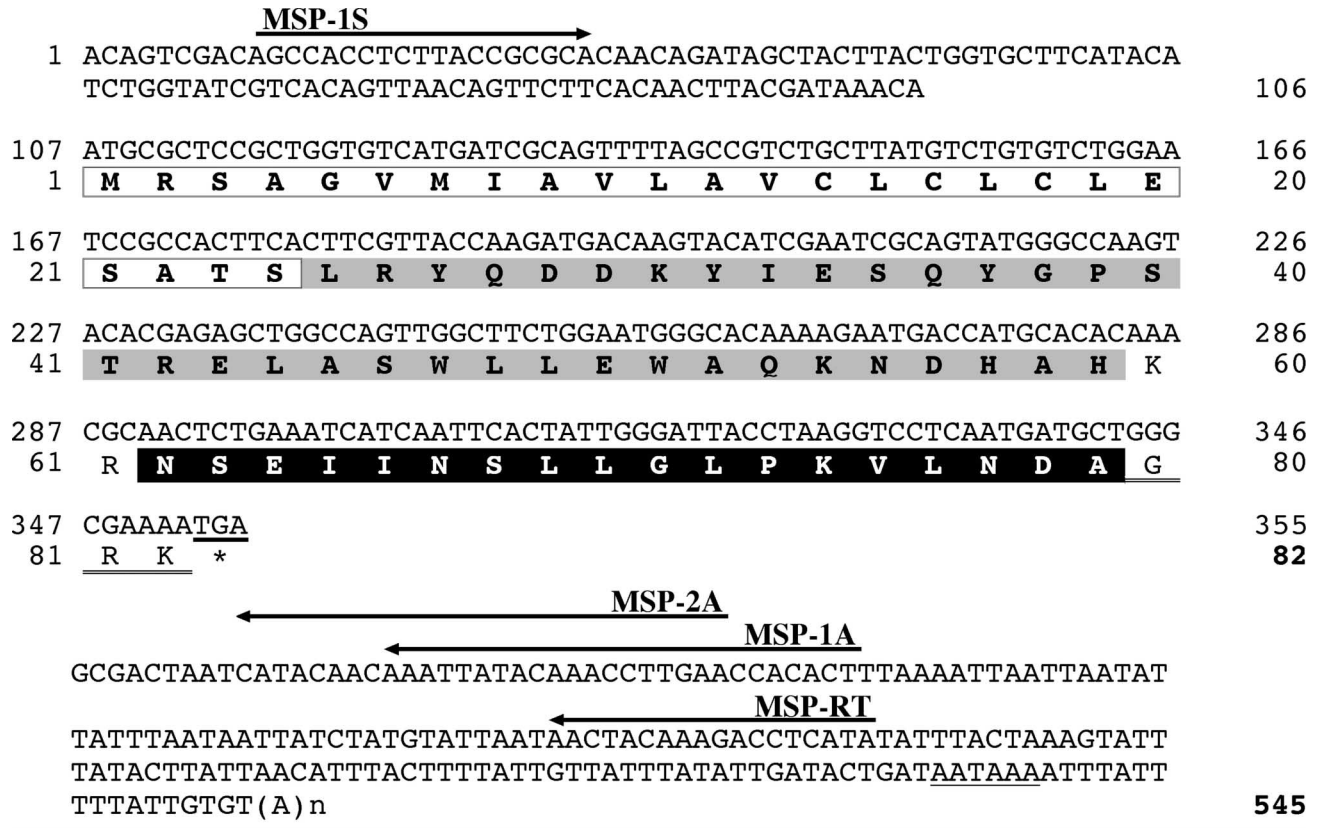

Fig. 1. Nucleotide and deduced amino acid sequences of the *Meimuna opalifera* PDF-precursor. Arrows indicate primers used for the 3' RACE or 5' RACE method. Amino acid sequences in white, gray, and black boxes correspond to the signal, PAP, and PDF regions, respectively. KR (=Lys-Arg) is a processing cleavage site, while the C-terminal GRK(=Gly-Arg-Lys) double-underlined is an amidation site. An underlined portion (AATAAA) in the 3'-UTR region is a putative poly-adenylation signal site (Conway and Wickens, 1985; Gil and Proudfoot, 1987; Takagaki *et al*., 1988). The underlined TGA trinucleotide sequence, shown by the asterisk, is the stop codon.

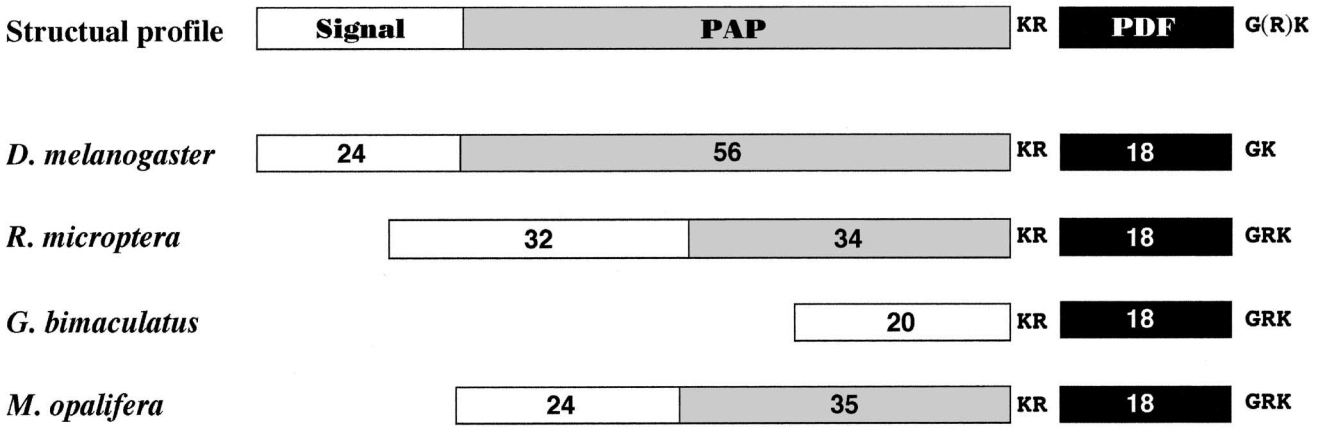

Fig. 2. Schematic comparison between the primary structures deduced from the nucleotide sequences of insect PDF precursor proteins. White, gray, and black rod boxes correspond to the signal, PAP, and PDF regions, respectively. Each number in the box indicates the number of amino acid residues. KR (=Lys-Arg) is a processing cleavage site, and the C-terminal GRK(=Gly-Arg-Lys) or GK(=Gly-Lys) is an amidation site.

1997, 1999), *M. opalifera* PDF precursor was assigned to possess 24-peptide sequences for the signal regions and 35-peptide sequences for the PAP region. This structural profile appears to be just normal when compared with those of *D. melanogaster* and *R. microptera*. Although the *G. bimaculatus* PDF precursor consisted of a nuclear localization signal (NLS) at the N-terminal portion, no NLS-like sequence was found in the PAP region of the *M. opalifera*

PDF precursor.

Highly Conserved Primary Structure of the *M. opalifera* PDF Peptide

When the sequences of insect PDF peptides were compared, it was found that *M. opalifera* PDF, NSEIINSLLGLP-KVLNDA-amide, is completely identical to those reported in *G. bimaculatus* (Chuman *et al*., 2002) and *Acheta domesticus* (Rao and Riehm, 1988) (Fig. 3). The sequence identity and similarity of *M. opalifera* PDF peptides were calculated to be 78–100% and 89–100%, respectively, considerably higher compared with PDFs of other insect species. Only a sole residue substitution was found, for PDFs of *Periplaneta americana* (Mohrherr *et al*., 1991) and *R. microptera*; i.e., $Ile^4 \leftrightarrow Leu$ and $Val^{14} \leftrightarrow Leu$, respectively. As for *Drosophila* PDF (Park and Hall, 1998), four residue substitutions were found; *i.e*., $Ile^4 \leftrightarrow Leu$, $Gly^{10} \leftrightarrow Ser$, $Val^{14} \leftrightarrow Asn$, and $Leu^{15} \leftrightarrow Met$. The Ser^{10} residue of *Drosophila* PDF is uncommon, since Gly^{10} is preserved among the known sequences of all other arthropod species.

Prediction of translocation of *M. opalifera* PDF precursor

When we analyzed the *pdf* cDNA of the nocturnal cricket *G. bimaculatus*, it was found that PDF precursor consists of a nuclear localization signal (NLS)-like sequence. Nuclear localization and translocation of *G. bimaculatus* PDF were demonstrated, by both electron microscopic observation of PDF immunocytoreactivity and biochemical translocation of GFP-fused PDF precursors (Chuman *et al*., 2002). Nuclear localization was also predicted by means of the network computer-assisted system running the PSORT II program (Nakai and Kanehisa, 1992; Reinhardt and Hubbard, 1998; Nakai and Horton 1999). The *G. bimaculatus* PDF precursor was judged to have a higher likelihood of being in the nucleus (about 48%) than in the cytoplasm (about 22%) (Table 1).

Using a combined sequence of the signal and PAP regions, we calculated the score that distinguishes the tendency of a peptide to translocate either to the nucleus or to other cellular sites. The cicada's PDF precursor was judged to be present mainly at the extracellular site (about 44%) (Table 1). The possibility of this extracellular translocation was even greater for PDF precursors of *R. microptera* (about 56%) and *D. melanogaster* (about 67%). These results strongly suggest that all these PDF peptides are secretory and not nuclear. Even though the PDF precursor proteins of *D. melanogaster* and *R. microptera* are unlikely to translocate into the nucleus (Table 1), the program PSORT II has calculated for *M. opalifera* that the PDF precursor is likely to be located in the nucleus with a finite possibility of about 22% (Table 1). It is however difficult to distinguish and identify the NLS-like sequence in the signal

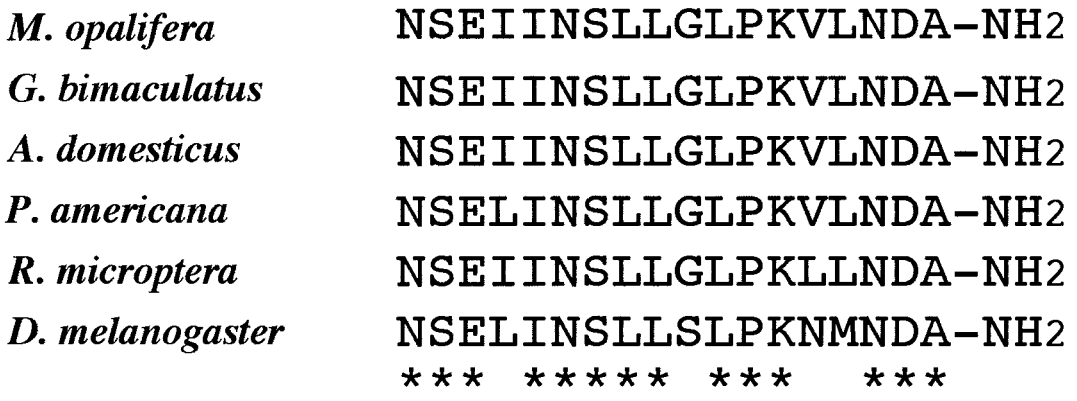

Fig. 3. Amino acid sequences of insect PDFs. PDFs from insects: *Meimuna opalifera*, *G. bimaculatus* (Chuman *et al*., 2002), *Acheta domesticus* (Rao and Riehm, 1988), *R. microptera* (Rao *et al*., 1987), *P. americana* (Rao and Riehm, 1993), and *D. melanogaster* (Park and Hall, 1998). Asterisk indicates the residues conserved among all PDFs.

Table 1. Prediction of the subcellular localization sites of insect PDF precursor proteins from their amino acid sequences by the computer Program PSORT II.

Species expressing PDF precursors	Subcellular localization sites (%)					
	Extracellular	Cytoplasmic	Nuclear	Vacuolar	Mitochondrial	Endoplasmic reticulum
D. melanogaster	66.7[a)]	11.1	0	11.1	0	11.1
R. microptera	55.6	22.2	0	11.1	0	11.1
G. bimaculatus	0	21.7	47.8	0	30.4	0
M. opalifera	44.4	0	22.2	11.1	22.2	0

[a)] Extracellular site involves the cell wall.

and PAP regions of *M. opalifera* PDF precursor protein.

Immunocytochemical examination of *M. opalifera* PDF in brain

The relatively high score for the possible nuclear translocation of the *M. opalifera* PDF precursor prompted us to investigate the cicada's brain immunocytochemically with anti-PDF. Since the amino acid sequence of *M. opalifera* PDF is exactly the same as that of the cricket *G. bimaculatus*, we utilized a rabbit polyclonal antibody raised against *G. bimaculatus* PDF (Tomioka *et al*., in preparation). Confocal analysis revealed two cell groups in the optic lobe clearly labeled by this antibody. One of them was located at the proximal-frontal region in the optic lobe, while the other was in the posterior cleft between the lamina and the medulla. The latter cell cluster was elongated from the dorsal to the ventral rim. In addition, the same antibody labeled clearly the fibers of these cells in the optic lobes and midbrain. The whole profile also revealed PDF immunoreactive fibers in the midbrain of *M. opalifera* similar to those previously reported in the orthopteroid insects (Homberg *et al*., 1991; Stengl and Homberg, 1994; Okamoto *et al*., 2001; Chuman *et al*., 2002), namely the posterior optic commissure and the ramification in the superior lateral protocerebrum (data not shown).

Immunolabeled Vibratome slices revealed precisely the location of the PDF cells and their arborizations in the optic lobe and midbrain. Fig. 4 shows the LSM image of a virtual section from such a Vibratome slice of the optic lobe. The numerous PDFLa cells in the cleft between the lamina and the medulla from the preparation which was dissected at ZT23:30 are strongly immunoreactive, and give rise to a dense arborization in the first optic chiasma and thence to the lamina and the medulla. In the medulla, these axons extend along the module structure to make the ramification in the middle layer of the medulla. On the other hand,

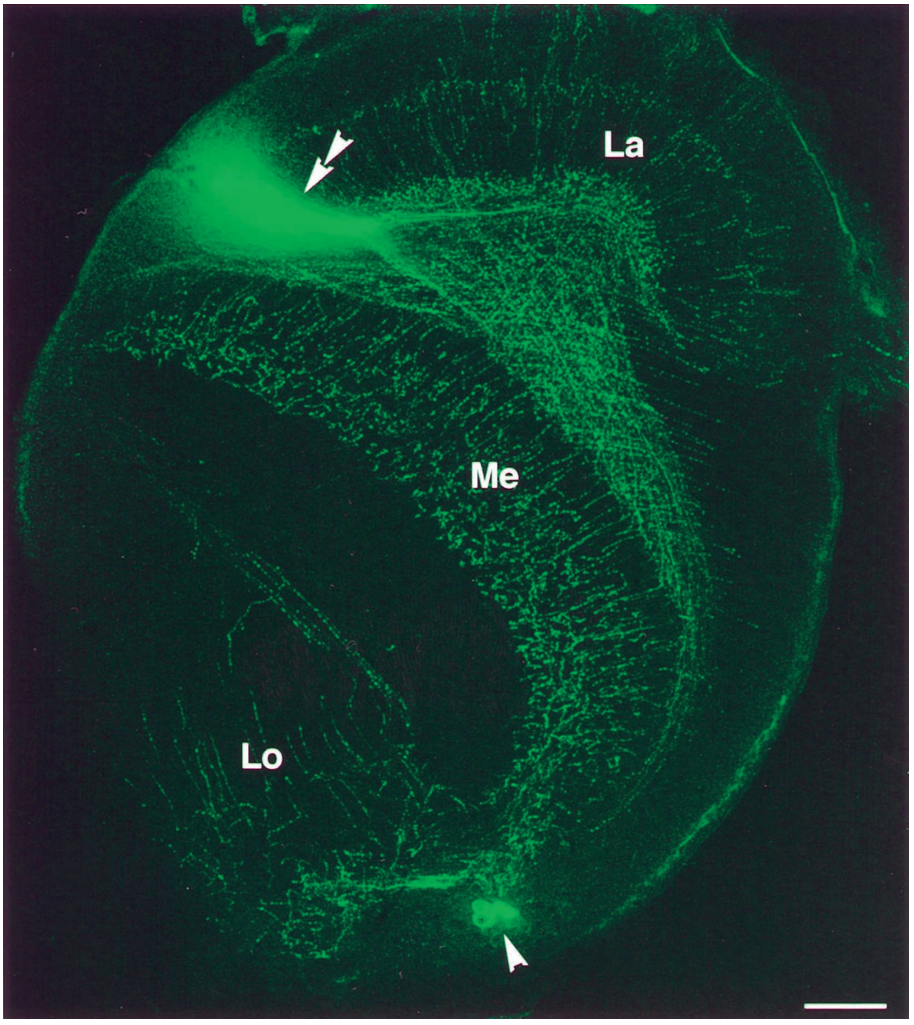

Fig. 4. Medulla (PDFMe) and lamina (PDFLa) PDF cells and their neurite network in the optic lobe from the horizontal Vibratome slice immunolabeled with anti-PDF antiserum (ZT 23:30). PDFMe cells (arrow) are located in the anterior cortex of the medulla, and PDFLa cells (double arrows) in the posterior cleft between the lamina and medulla. Strongly immunoreactive PDFLa cells give rise to the neurite in the first chiasma and thence into the lamina and medulla. PDFMe cells give rise to the neurite proximally, and partially into the medulla La: lamina, Me: medulla, Lo: lobula. Scale bar, 100 μm.

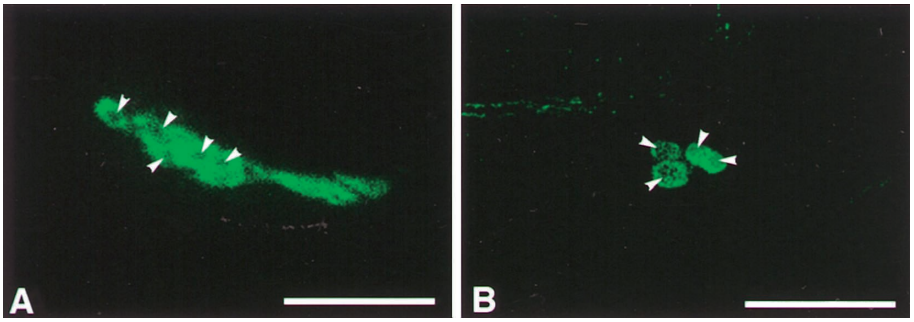

Fig. 5. LSM image of the PDFLa cells (A) and PDFMe cells (B) immunolabeled with anti-PDF antiserum. All PDF cells showed the nucleus (arrowhead) unlabeled in the series of images by 1 μm steps. Scale bars, 100 μm.

PDFMe cells (which comprise at least four cells) are less immunoreactive than PDFLa cells at this time of day. Immunoreactive fiber from PDFMe cells enter into the middle layer of the medulla, and make the network. Immunolabeled fibers in the lobula are seen sparsely. These results imply that PDF precursor or PDF itself was secreted from the particular PDF-expressing cells. In hemimetabola insects previously described, PDFMe cells are generally reported to be much immunoreactive rather than PDFLa cells. In *M. opalifera*, however, PDFLa cells are shown to be strongly immunoreactive at just before dawn in this study. In *G. bimaculatus*, the distal region of the medulla is reported to be important for the persistence of rhythmicity (Okamoto *et al*., 2001). In cockroach, anti-PER antiserum labeled the cells clusters located in the outer optic chiasm (Takeda *et al*., 2000). These results suggest that the expression of PDF in the PDFLa cells is regulated by some clock mechanism to elicit the daily fluctuation.

When the immunolabeled cells were carefully examined for their nuclei, no cells with an immunolabeled nucleus were found. Fig. 5 is the LSM images of a virtual section from the series of images scanned by 1 μm steps. As shown in these figures, all immunolabeled cells in the lamina and medulla exhibited a nucleus that was unlabeled. It is thus concluded that *M. opalifera* PDF neurons have no PDF-related peptides in the nucleus, at least at ZT23:30. This is in agreement with the sequence profile, which shows no cluster of basic amino acids, and thus no candidate for a NLS. Since the calculation using the PSORT II program indicated the finite (22%) possibility that the *M. opalifera* PDF precursor might be localized in the nucleus, a signal might therefore be in the precursor sequence that is unknown. Examination of this possibility may require a careful examination of the cells at various time intervals throughout the entire day.

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