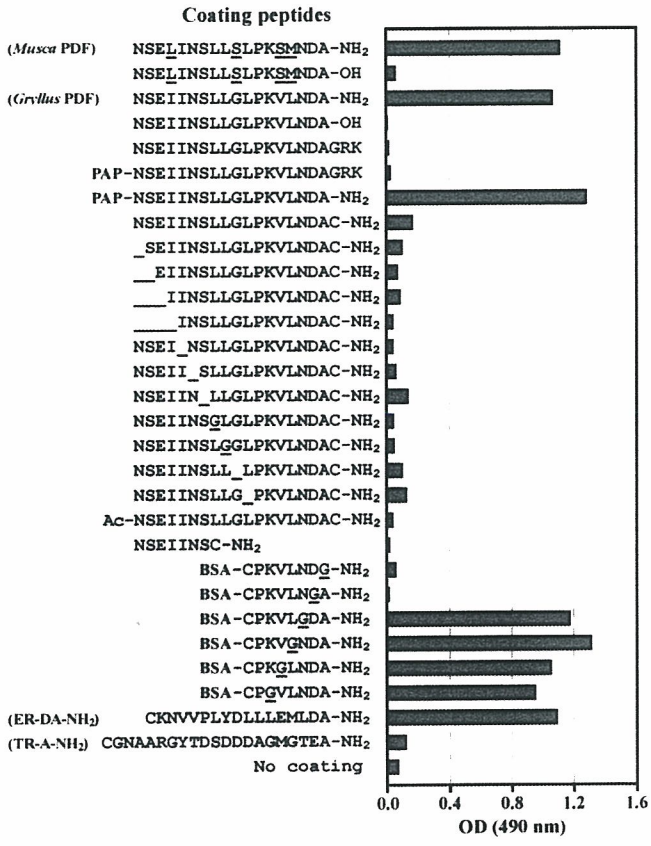

Fig. 5. Epitope analysis of polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb. Rabbit polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb raised against *Musca* PDF was examined by the ELISA-based epitope analysis for chemically synthesized *Gryllus* PDF, its analogs, and also for *Musca* PDF. The antibody was completely inactive to BSA. Dilution of antiserum used was 1:31,600.

PDF analog having a C-terminal free carboxyl group, PDF-OH, either (Fig. 5). Moreover, the antibody exhibited no immunoreactivity against the peptide TR-A-NH_2, which has a Glu-Ala-amide structure at the C-terminus, but exhibited high reactivity against ER-DA-NH_2. Furthermore, it showed clear immunoreactivity to PKVGNDA-NH_2 and PKVLGDA-NH_2, but to neither PKVLNGA-NH_2 nor PKVLNDG-NH_2. All these findings indicate that this antibody recognizes the structure of DA-NH_2, for which this highly specific polyclonal antibody is designated "anti-PDF(17-18)$CONH_2$ pAb."

Immunocytochemistry to localize PDF

Using this series of monoclonal and polyclonal antibodies to discriminate specifically the mature form of PDF from related peptide structures, we undertook immunocytochemical investigations of the optic lobes in *Gryllus* to localize this form of the peptide to particular neurons. The chief purpose was to identify sites of genuine PDF expression, as well as to seek the expression of PDF-like peptide molecules in the *Gryllus* brain.

Detection of a PDF N-terminal pentapeptide with an Asn[1]-α-amino group. Vibratome slices of the optic lobes from *Gryllus* brains dissected and fixed at particular times of the day (ZT2, ZT8, ZT14, and ZT20) were immunolabeled using anti-H_2N(1-5)PDF mAb as the primary antibody and FITC-conjugated antimouse IgG as the secondary. In the medulla, confocal microscopic examination revealed the locations of somata of neurons that expressed mature PDF N-terminal pentapeptide. These PDFMe cells give rise to a dense arborization in a region anterior to the medulla neuropile, in the so-called accessory medulla (aMe), and to neurites in the chiasma between the lamina and medulla neuropiles (Fig. 6A). The cells also extended axons into the central brain. This general pattern of labeling was also revealed by anti-PDF(17-18)$CONH_2$ mAb and the rabbit anti-PDF(17-18)$CONH_2$ pAb (Fig. 6B). The coincidence of these results obviously indicates that the substance labeled by these antibodies is a peptide having the structures of the N-terminal H_2N-Asn-Ser-Glu-Ile-Ile and the C-terminal Asp-Ala-NH_2, both of which form part of the PDF molecule. As discussed further below, this provides evidence for the detection of the mature PDF molecule.

The monoclonal antibody anti-H_2N(1-5)PDF mAb also labeled the somata of neurons present in the lamina (PDFLa cells). Such cells comprise two groups, one in the dorsal region and one in the ventral. Each group further comprises two subgroups, those at the inner and those at the outer lamina margin. The cells present at both inner and outer margins were labeled almost equally strongly by anti-H_2N(1-5)PDF mAb, in contrast to the immunolabeling obtained with the polyclonal antibody raised against *Uca* β-PDH, which gave intense immunoreactivity in the inner cells, but only faint immunoreactivity for the outer ones (Fig. 7).

From both dorsal and ventral regions, the PDFLa cells extend their axons beneath the lamina and up into the overlying neuropile. Another group of axons from the PDFLa cells gather in the equatorial region at the edge of the lamina and extends further proximally. They were all also labeled by anti-H_2N(1-5)PDF mAb. No daily variation in the labeling intensities of the cell cluster and its axons was evident among samples collected at 6-hour intervals (data not shown), confirming our previous report for samples collected at 4-hour intervals (Chuman et al., 2002). The general morphology of the PDF-immunoreactive cells in the PDFLa cell group is almost completely compatible with that observed using the polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb (Fig. 6).

In the analysis of immunolabeled whole-mounts, we found that anti-H_2N(1-5)PDF mAb labeled the cell bodies of 14 of the PDFMe cells located along the proximal rim of the medulla; these were 12–15 μm in diameter for seven small PDFMe cells and 18–24 μm in diameter for seven large PDFMe. The size of the PDFMe cells was confirmed by the analysis of whole-mounts labeled with anti-*Gryllus* PDF pAb. By contrast, the cell clusters labeled with this monoclonal antibody at both the inner and outer margins of each lamina had much smaller cell bodies, 7–10 μm in diameter. From three independent counts, the numbers of labeled PDFLa cells recorded per optic lobe were as follows: 70–75 cells in the dorsal region, where ~35 each are present at the inner and outer margins; and 70–75 cells in the ventral region, with again ~35 each present at the inner and outer margins.

Detection of the PDF C-terminal dipeptide amide Asp-Ala-NH_2. Two antibodies we used recognize the PDF carboxyl terminal dipeptide amide structure Asp[17]-

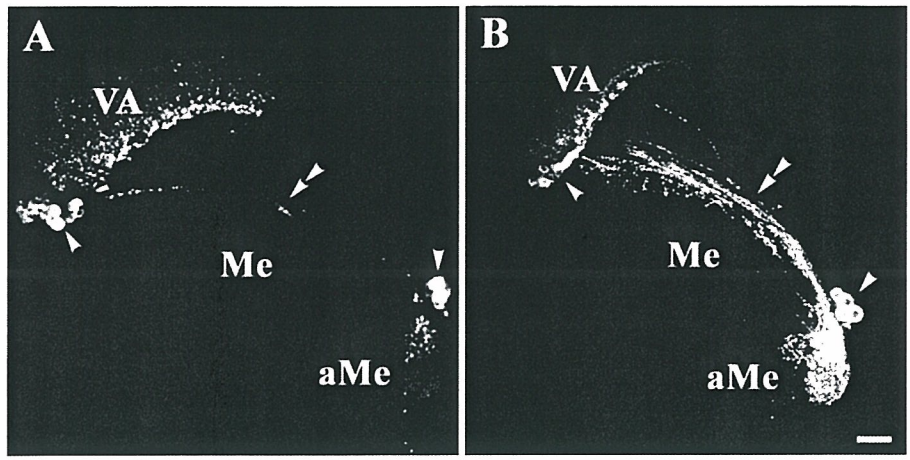

Fig. 6. Horizontal section of the optic lobes of *G. bimaculatus* showing immunoreactivity to anti-H_2N(1-5)PDF mAb (**A**) and anti-PDF(17-18)$CONH_2$ pAb (**B**). Cell bodies (arrowhead) and their axons of a number of neurons are strongly labeled in both sections in the lateral-medial horizontal plane (see Fig. 8). In the medulla (Me), the PDF-immunoreactive cells give rise to a dense arborization in the accessory medulla (aMe) and to neurites (double arrowhead) in the first chiasma between the lamina and medulla. They also extend axons into the central brain. PDFLa cells extend their axons beneath the lamina to form a varicose arborization (VA) in the overlying neuropile, lamina. Similarly labeling profiles were obtained by monoclonal antibody anti-PDF(17-18)$CONH_2$ mAb. All brain samples were fixed at ZT8. FITC-conjugated antimouse IgG (1:200 dilution) and antirabbit IgG (1:500 dilution) were used as secondary antibodies against anti-H_2N(1-5)PDF mAb and anti-PDF(17-18)$CONH_2$ pAb, respectively. Scale bar = 50 μm.

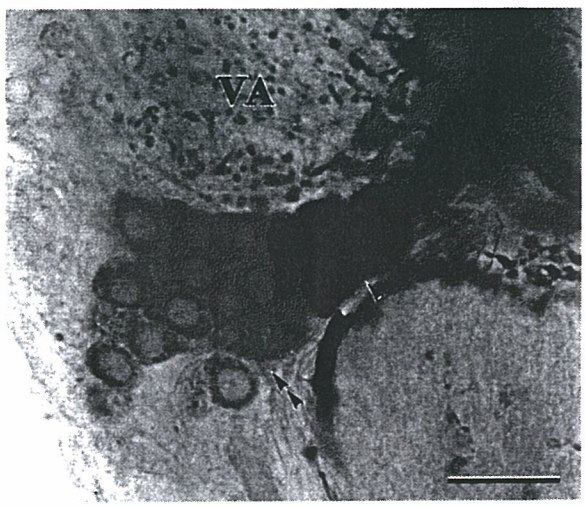

Fig. 7. Horizontal section of the first (lamina) neuropile regions the optic lobes of *G. bimaculatus* showing immunoreactivity to anti-*Uca* β-PDH pAb. Lamina (PDFLa) PDF cells in horizontal Vibratome slices from the optic lobe immunostained by means of the indirect peroxidase-anti-peroxidase technique. An inner cell group (arrowhead) is strongly immunoreactive, whereas PDFLa cells situated in a more peripheral location (double arrowhead) shows rather faint immunoreactivity. Immunoreactivities in the varicose arborization (VA) in the lamina are also weak. The antibody used was a rabbit polyclonal antibody raised against the β-PDH peptide of the crustacean *Uca pugilator* (anti-*Uca* β-PDH pAb, 1:3,500). Scale bar = 25 μm.

Ala^{18}-NH_2: the monoclonal antibody anti-PDF(17-18)$CONH_2$ mAb and the polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb. When the optic lobes of the cricket *G. bimaculatus* were examined using these antibodies, as shown in Figure 8, almost the same immunolabeled profiles were observed as those seen with the monoclonal antibody anti-H_2N(1-5)PDF mAb (Fig. 6). The numbers of PDFMe and PDFLa cells observed with these two antibodies were also in good agreement with those observed with anti-H_2N(1-5)PDF mAb.

Detection of the internal structure of PDF peptide with different antibodies. Two different groups of monoclonal antibodies were used to immunolabel the *Gryllus* optic lobes. The first included five different antibodies, anti-PDF(1-9) mAb, anti-PDF(2-6) mAb, anti-PDF(2-8) mAb, anti-PDF(3-9) mAb, and anti-PDF(6-10) mAb. These recognize the internal structure of PDF peptide. A second group, anti-PDF(16-18)$CONH_2$ mAb and anti-PDF(15-18)$CONH_2$ mAb, recognizes the C-terminal 3–4-mer peptide amide structure. In the PDFMe cells, all seven monoclonal antibodies exhibited the same distinct pattern of immunoreactivity to cell bodies, axons, and the accessory medulla. The results together with those from the study using anti-H_2N(1-5)PDF mAb, anti-PDF(17-18)$CONH_2$ mAb, and anti-PDF(17-18)$CONH_2$ pAb indicated that these PDFMe cells contain mature PDF peptide.

In the lamina region we found that the PDFLa inner cells and axons are also immunoreactive to these seven monoclonal antibodies. Surprisingly, therefore, the PDFLa outer cells were immunonegative. Neither anti-PDF(1-9) mAb, anti-PDF(2-6) mAb, anti-PDF(2-8) mAb, anti-PDF(3-9) mAb, nor anti-PDF(6-10) mAb exhibited immunoreactivity to the lamina outer cells and the same was also true for anti-PDF(16-18)$CONH_2$ mAb and anti-PDF(15-18)$CONH_2$ mAb. In addition, the PDFLa cells (PDFLa-VA) give rise to a varicose arborization of neurites, which lie between the lamina neuropile and the compound eyes, and none of these antibodies exhibited an immunosignal in the varicose arborization either (Fig. 9). It was therefore noteworthy, on the other hand, that the monoclonal antibodies anti-H_2N(1-5)PDF mAb and anti-PDF(17-18)$CONH_2$ mAb and pAb did exhibit immunore-

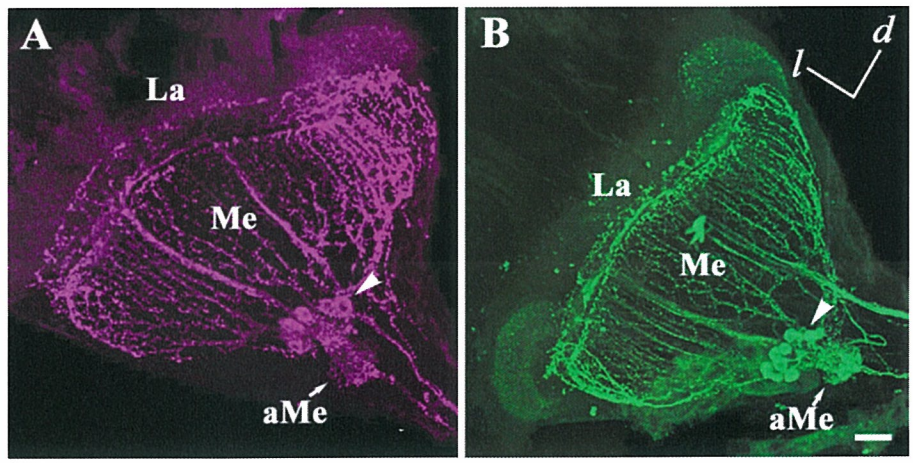

Fig. 8. Frontal image of whole-mount immunocytochemistry of the right optic lobe of *G. bimaculatus* using anti-PDF(17-18)$CONH_2$ mAb (**A**) and anti-PDF(17-18)$CONH_2$ pAb (**B**). PDF medulla cells (PDFMe, arrowheads) and a number of axons in the medulla (Me) are labeled in both samples. The accessory medulla (aMe) adjacent to the cell bodies of PDFMe neurons is also labeled. Varicose arborization is clearly labeled at both the dorsal region and the ventral region of the lamina (La) in both panels. Texas Red (shown by the magenta color)-conjugated antimouse IgM (1:200 dilution) and FITC-conjugated antirabbit IgG (1:500 dilution) were used as secondary antibodies against anti-PDF(17-18)$CONH_2$ mAb (A) and anti-PDF(17-18)$CONH_2$ pAb (B), respectively. All optic lobe samples were fixed at ZT8. Axes labeled in (B) indicate the lateral (l) and dorsal (d) directions. Scale bar = 50 μm.

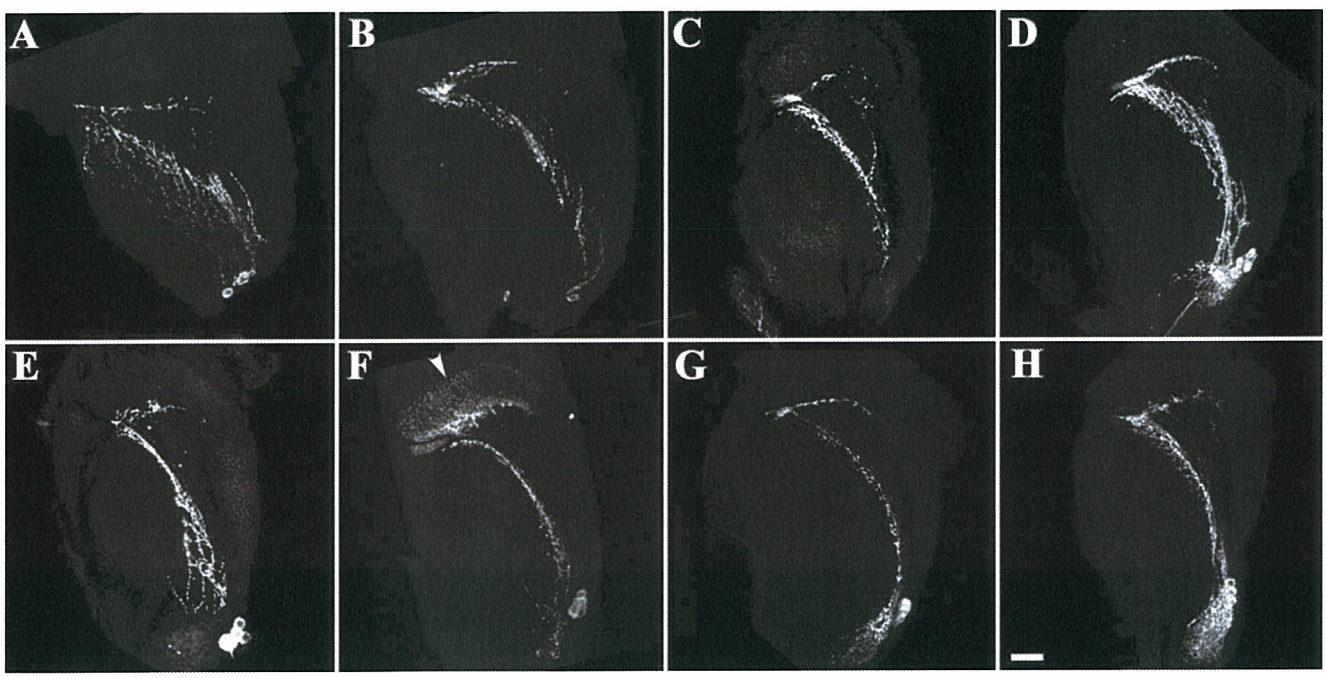

Fig. 9. Immunocytochemistry of a horizontal section of the optic lobes of *G. bimaculatus* by a series of monoclonal antibodies; anti-PDF(6-10) mAb (**A**), anti-PDF(1-9) mAb (**B**), anti-PDF(3-9) mAb (**C**), anti-PDF(2-8) mAb (**D**), anti-PDF(2-6) mAb (**E**), anti-PDF(17-18)$CONH_2$ mAb (**F**), anti-PDF(16-18)$CONH_2$ mAb (**G**), and anti-PDF(15-18)$CONH_2$ mAb (**H**). Only anti-PDF(17-18)$CONH_2$ mAb (F) shows strong immunoreactivity in the varicose arborization in the lamina (arrowhead) in addition to the PDFLa outer cell group, while all other monoclonal antibodies showed no such labeling. All brain samples were fixed at ZT8. Secondary antibodies used at 1:200 were FITC-conjugated antimouse IgG for anti-PDF(1-9) mAb, anti-PDF(2-8) mAb, anti-PDF(16-18)$CONH_2$ mAb, and anti-PDF(15-18)$CONH_2$ mAb, and FITC-conjugated antimouse IgM for anti-PDF(6-10) mAb, anti-PDF(3-9) mAb, anti-PDF(2-6) mAb, and anti-PDF(17-18)$CONH_2$ mAb. Scale bar = 50 μm.

activity in these PDFLa outer cells as well as the PDFLa-VA. These results clearly imply that the PDFLa outer cells and PDFLa-VA contain a peptide having the structure H-Asn-Ser-Glu-Ile-Ile- and -Asp-Ala-NH_2, and not the PDF internal structure detected by anti-PDF(1-9) mAb, anti-PDF(2-6) mAb, anti-PDF(2-8) mAb, anti-PDF(3-9) mAb, anti-PDF(6-10) mAb, anti-PDF(16-18)$CONH_2$ mAb, or anti-PDF(15-18)$CONH_2$ mAb.

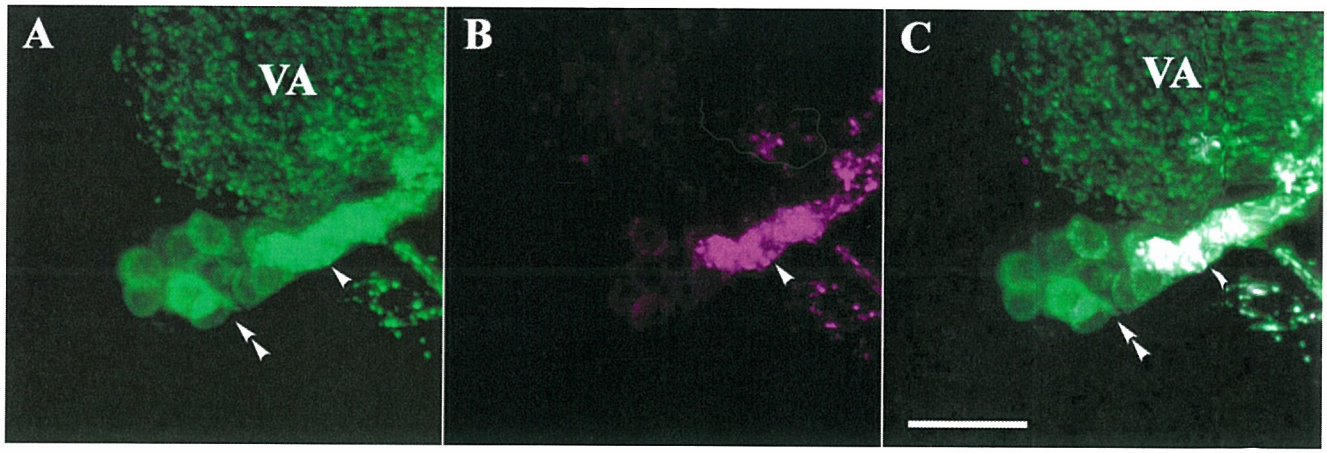

Fig. 10. Immunocytochemistry of horizontal sections of the optic lobes of *G. bimaculatus* by double-labeling with anti-PDF(17-18)$CONH_2$ pAb and anti-PDF(2-8) mAb. Polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb labels the PDFMe cells (not shown in these photographs), both the PDFLa inner (arrowhead) and outer cells (double arrowhead), and also the varicose arborization (VA) in the lamina (**A**). In the same sample of the optic lobe, the monoclonal antibody anti-PDF(2-8) mAb labels neither the PDFLa outer cells nor the varicose arborization (**B**). Merged coimmunolabeling images (**C**) show a distribution of two distinct PDF isoforms, namely, the mature PDF (white) labeled by both antibodies and the isoform (green) labeled only by anti-PDF(17-18)$CONH_2$ pAb. Similar results were obtained with many other pairs of antibodies, indicating that there are two different types of PDF-like peptides. FITC-conjugated antirabbit IgG (1:500 dilution) and Texas Red (shown by the magenta color in B)-conjugated antimouse IgG (1:100 dilution) were used as secondary antibodies against anti-PDF(17-18)$CONH_2$ pAb and anti-PDF(2-8) mAb, respectively. Scale bar = 25 μm.

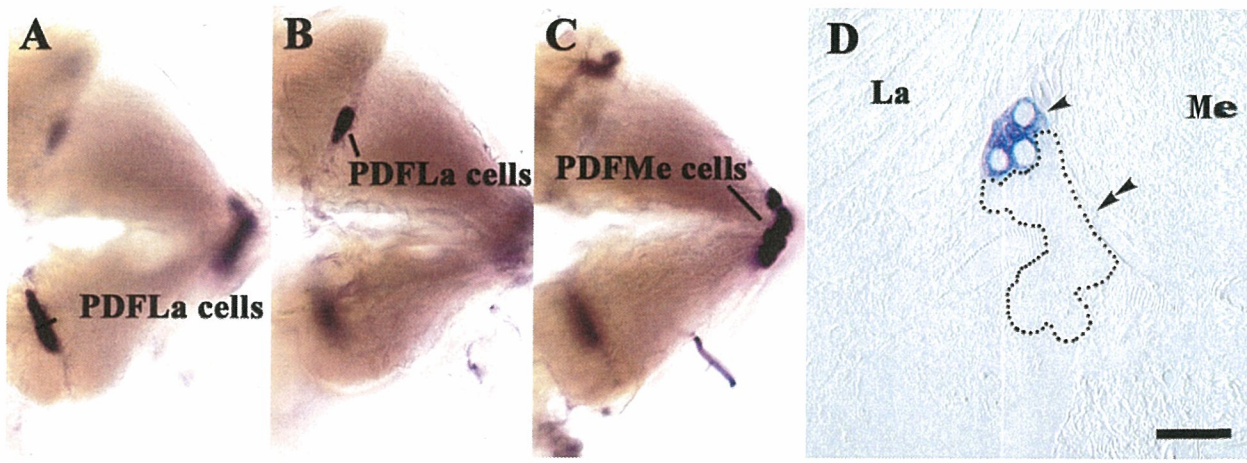

Fig. 11. Expression of *pdf* mRNA observed by whole-mount in situ hybridization in *G. bimaculatus*. The entire brain was mounted in glycerol-gelatin and one of the optic lobes was observed by light microscopy in three focal planes: the cells present at the dorsal region (**A**) and the cells in the ventral region (**B**) in the lamina (PDFLa cells), and the cells in the medulla (PDFMe cells) (**C**). Serial sections obtained from the sample of the whole-mount in situ hybridization (**D**) revealed that only the inner cells (arrowhead) are positive at both the dorsal and ventral regions at the lamina, whereas negative cells (double arrowhead) are found in the outer area (dotted line). Negative control by sense cRNA is not shown. Scale bar = 20 μm.

Colabeling the lamina cells to identify the PDF isoform

The antibodies anti-H_2N(1-5)PDF mAb and anti-PDF(17-18)$CONH_2$ mAb, and anti-PDF(17-18)$CONH_2$ pAb label all the PDF-expressing cells in the lamina. In contrast, anti-PDF(15-18)$CONH_2$ mAb, anti-PDF(16-18)$CONH_2$ mAb, anti-PDF(2-6) mAb, anti-PDF(2-8) mAb, anti-PDF(3-9) mAb, anti-PDF(6-10) mAb, and anti-PDF(1-9) mAb all require the full sequence of *Gryllus* PDF peptide. Double-immunolabeling of the lamina cells expressing PDF peptides should therefore provide a means to demonstrate the precise expression site of the PDF isoform. There are two possible ways to coimmunolabel the cells: using a combination of polyclonal and monoclonal antibodies (of which there are seven possible combinations) or using a combination of IgG and IgM antibodies (of which seven combinations are also possible; see Table 1). As a representative example, a concluding figure shows the double-labeling obtained using anti-PDF(17-18)$CONH_2$ pAb and anti-PDF(2-8) mAb (Fig. 10).

The anti-PDF(17-18)$CONH_2$ pAb labels both the PDFLa inner and outer cells, and also the axon projections beneath the varicose arborization (Fig. 10A). Numerous neurites form a wide flame-like arborization of varicose neu-

rites. Almost the same pattern of immunolabeled profiles was obtained with the monoclonal antibody anti-H_2N(1-5)PDF mAb (Fig. 6A). In contrast, anti-PDF(2-8) mAb labeled only the PDFLa inner cells and their projections (Fig. 10B), whereas immunolabeling in the PDFLa outer cells and varicose arborization was not obvious. Images of double-immunolabeled preparations (Fig. 10C) revealed distinct distributions for the two different PDF isoforms, the mature PDF (in white) labeled by both antibodies and the isoform (in green) labeled only by one, anti-PDF(17-18)$CONH_2$ pAb. All other set-3 antibodies exhibited similar results. These results strongly suggest that there are two different PDF peptides in the optic lobes of the cricket *G. bimaculatus*.

In situ hybridization to identify *pdf* mRNA

The expression of *pdf* mRNA was monitored by in situ hybridization using a cRNA probe we prepared, labeled with DIG. The immunocytochemical detection by anti-DIG antibody detected a clear alkaline phosphatase signal in the PDFLa cells (Fig. 11A,B) and PDFMe cells (Fig. 11C). When we counted the PDFMe expressing cells in serial sections, their number was judged to be 14, consistent with the results from immunocytochemistry using the series of antibodies. When the lamina region was carefully examined from horizontal serial sections, it was found that only the PDFLa inner cells (about 35 each cluster) showed any signal for *pdf* mRNA expression (Fig. 11D), with no immunosignal detected in the PDFLa outer cells.

DISCUSSION

Epitope-defined monoclonal and polyclonal antibodies for detecting PDF peptide

In the present study we eventually obtained three different sets of monoclonal and polyclonal antibodies against the PDF peptide of *Gryllus*. These included: set 1, the monoclonal antibody anti-H_2N(1-5)PDF mAb that recognizes the N-terminal structures of H-N^1SEII^5 comprising the α-amino group of Asn^1; set 2, the monoclonal antibodies anti-PDF(15-18)$CONH_2$ mAb, anti-PDF(16-18)$CONH_2$ mAb, and anti-PDF(17-18)$CONH_2$ mAb and the polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb, that all recognize the PDF C-terminal amide structures of $L^{15}NDA^{18}$-NH_2, $N^{16}DA^{18}$-NH_2, $D^{17}A^{18}$-NH_2, and $D^{17}A^{18}$-NH_2, respectively; and set 3, the five monoclonal antibodies anti-PDF(1-9) mAb, anti-PDF(2-6) mAb, anti-PDF(2-8) mAb, anti-PDF(3-9) mAb, and anti-PDF(6-10) mAb that recognize the PDF peptide internal structure N^1SEIINSLL^9, S^2EIIN^6, S^2EIINSL^8, E^3IINSLL^9, and N^6SLLG^{10}, respectively.

All these antibodies, nine monoclonal and one polyclonal, exhibited very high selectivity and specificity for *Gryllus* PDF peptide. In addition, they reacted with the PDF peptides of other species having the same structure in ways that were specific for each antibody (data not shown). It should be noted that *Gryllus* PDF peptide has the epitope for all these antibodies, so that the use of all three sets is required to provide defining evidence of PDF structure. The best combination of antibodies in that case, one from each set, would be anti-H_2N(1-5)PDF mAb, anti-PDF(15-18)$CONH_2$, and anti-PDF(6-10) mAb, which give the most complete recognition of 14 of the constituent amino acid residues within the 18-mer PDF peptide. On the other hand, we could not obtain any monoclonal antibody that recognizes the PDF intermediate internal structure -Leu^{11}-Pro^{12}-Lys^{13}-Val^{14}-. This is probably because of the presence of the Pro^{12} residue, given that the α-amino acid Pro, known as an epitope-killer, usually breaks the specific stereo-structure of peptides. It should finally be noted that polyclonal antibodies anti-*Uca* β-PDH pAb and anti-*Gryllus* PDF pAb both recognize Leu^{11}, while anti-*Uca* β-PDH pAb also discriminates Lys^{13}.

PDFLa outer cells and varicose arborization are negative to monoclonal antibodies specific for the internal structure of PDF peptide

The PDFLa outer cells were definitely immunoreactive to anti-H_2N(1-5)PDF mAb and anti-PDF(17-18)$CONH_2$ mAb. These cells were also labeled by the rabbit polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb. Apparently, they have a peptide with an N-terminal H-Asn-Ser-Glu-Ile-Ile- and a C-terminal Asp-Ala-NH_2. In general, these findings would be sufficient to conclude that there is a PDF peptide in the PDFLa outer cells. Most surprisingly, however, the cells were not labeled by a series of monoclonal antibodies, all of which recognize the internal structure of the PDF peptide. Table 2 shows the record of immunoreactivity of each region of the optic lobe against a series of anti-PDF peptide antibodies. The PDFLa outer cells were immunonegative to the third set of five monoclonal antibodies, anti-PDF(1-9) mAb, anti-PDF(2-6) mAb, anti-PDF(2-8) mAb, anti-PDF(3-9) mAb, and anti-PDF(6-10) mAb. Since Asn^6 is the only amino acid residue shared among the epitopes of all these antibodies (Fig. 12), this finding strongly suggests that a PDF-like peptide expressed in the PDFLa outer cells does not contain the normal Asn^6 residue.

The PDFLa outer cells were also immunonegative to anti-PDF(15-18)$CONH_2$ mAb and anti-PDF(16-18)$CONH_2$ mAb, even though the same outer cells were labeled with anti-PDF(17-18)$CONH_2$ mAb and anti-PDF(17-18)$CONH_2$ pAb. When the epitope amino acid sequences of these antibodies were compared, the critical residue was found to be Asn^{16} (Fig. 12). It thus appears that the PDF-like peptide in the PDFLa outer cells does not contain the normal Asn^{16} residue, so that this peptide is devoid of both Asn^6 and Asn^{16} residues, having a substituting amino acid other than Asn.

In addition to the PDFLa outer cells, their varicose arborization (PDFLa-VA) exhibited exactly the same profile of immunoreactivity as that shown by the cell bodies. PDFLa-VA was intensely labeled by the monoclonal antibodies anti-H_2N(1-5)PDF mAb and anti-PDF(17-18)$CONH_2$ mAb, and by the polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb (Table 2) and by none of anti-PDF(15-18)$CONH_2$ mAb, anti-PDF(16-18)$CONH_2$ mAb, anti-PDF(2-6) mAb, anti-PDF(2-8) mAb, anti-PDF(3-9) mAb, anti-PDF(6-10) mAb, and anti-PDF(1-9) mAb (Fig. 12, Table 2). These results again imply that the PDFLa-VA contains a PDF-like peptide that possesses the structure H-Asn-Ser-Glu-Ile-Ile^5- and -Asp^{17}-Ala-NH_2, but does not have Asn^6 and Asn^{16}. This, then, is the prediction: that the PDFLa outer cells and their neurites contain a PDF-like peptide isomer, which should be discriminable by the antibodies used in our study.

TABLE 2. Epitope Amino Acid Sequences of Monoclonal Antibodies and Their Immunolabeling Characteristics

		Immunoreactivity				
		Lamina			Medulla	
Antibody	Epitope sequence	Inner	Outer	VA	PDFMe	aMe
anti-H_2N(1-5)PDF mAb	(free H_2N-)^{1}NSEII^{5a}	+++	+++	+++	+++	+++
anti-PDF(1-9) mAb	^{1}NSEIINSLL^9	+++	−−−	−−−	+++	+++
anti-PDF(2-6) mAb	^{2}SEIIN^6	+++	−−−	−−−	+++	+++
anti-PDF(2-8) mAb	^{2}SEIINSL^9	+++	−−−	−−−	+++	+++
anti-PDF(3-9) mAb	^{3}EIINSLL^9	+++	−−−	−−−	+++	+++
anti-PDF(6-10) mAb	^{6}NSLLG^{10}	+++	−−−	−−−	+++	+++
anti-PDF(15-18)$CONH_2$ mAb	^{15}LNDA^{18}-NH_2	+++	−−−	−−−	+++	+++
anti-PDF(16-18)$CONH_2$ mAb	^{16}NDA^{18}-NH_2	+++	−−−	−−−	+++	+++
anti-PDF(17-18)$CONH_2$ mAb	^{17}DA^{18}-NH_2	+++	+++	+++	+++	+++
anti-PDF(17-18)$CONH_2$ pAb	^{17}DA^{18}-NH_2	+++	+++	+++	+++	+++
anti-*Uca* β-PDH pAb[b]		+++	+	+	+++	+++
anti-*Gllylus* PDF pAb[c]		+++	+	+	+++	+++

VA, varicose arborization; PDFMe, pigment-dispersing factor medulla; aMe, accessory medulla.
[a]The monoclonal antibody anti-H_2N(1-5)PDF mAb requires the α amino group of PDF-Asn^1.
[b]The epitope structure of anti-*Uca* β-PDH pAb is XXEXINSXLGLXKXXXDA-NH_2.
[c]The epitope structure of anti-*Gryllus* PDF pAb is XXEXINSXLGLXXXXXDA-NH_2.

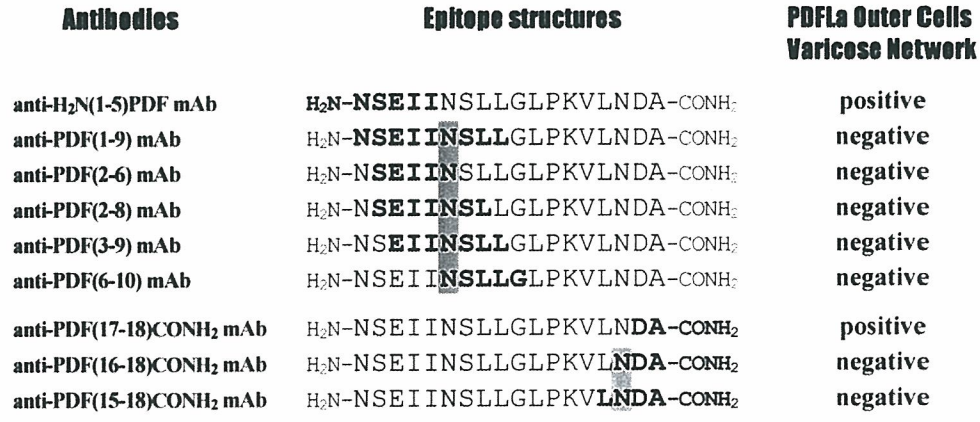

Fig. 12. Epitope structures of a series of monoclonal antibodies prepared for specific recognition of the PDF peptide of *G. bimaculatus*. The epitope sequences of the monoclonal antibodies are shown in bold letters. Asn(=N) at positions 6 and 16 (boxes), are shared among monoclonal antibodies that are negative to the PDFLa outer cells and varicose arborization.

Molecular outline and physiological significance of putative PDF isoform

The results of immunolabeling *Gryllus* brains with a series of antibodies are summarized in Table 2, and further demonstrated by double-labeling examples. By overlaying each epitope sequence for the series of antibodies, we could check what kind of PDF peptide is present in particular neurons and their neurites. The PDFLa inner cells and PDFMe cells, for example, contain a mature peptide with almost the entire amino acid sequence of PDF. The axons of these cells are also characterized by the presence of mature PDF, as is the accessory medulla. The general structure of these PDF cells in *G. bimaculatus* is similar to that previously reported (Homberg et al., 1991; Chuman et al., 2002).

By contrast, the peptide present in the PDFLa outer cells and the varicose arborization to which these give rise is not mature PDF. Given that the peptide structure is judged from the immunoreactivities of cells containing the peptide, the peptide reveals the following structural conditions: 1) An N-terminal structure of H-Asn^1-Ser^2-Glu^3-Ile^4-Ile^5- with an Asn-1-α-NH_2 group and a C-terminal structure of Asp^{17}-Ala^{18}-NH_2; and 2) neither Asn^6 nor Asn^{16} in the usual structure. Since, in addition Ile, anti-H_2N(1-5)PDF mAb recognizes Leu at position 4, there is a possibility that the peptide contains Leu^4. Together, these facts indicate that there is an isoform of PDF peptide in the PDFLa outer cells and varicose arborization.

From the in situ hybridization evidence, a *pdf* mRNA that could supply the mature PDF peptide does not exist in the cell bodies of PDFLa outer cells (Fig. 11). Our preliminary analysis of the *pdf* gene indicates moreover a gene structure that yields probable alternative mRNA splice variants (unpubl. data). Thus, it is possible that the outer cells have an alternative *pdf* gene to produce the second PDF peptide molecule, one that might possess amino acid residues other than Asn at positions 6 and 16. It is therefore interesting that Helfrich-Förster (1997) has suggested that the PDFCa neurons in the dorsal brain of the fruit fly *Drosophila* contain either a slightly different PDF peptide, with a lower affinity to anti-*Uca* β-PDH pAb, or a smaller amount of PDF, than the PDFMe cells. Using a more recently prepared rat anti-*Drosophila* PDF pAb, Park et al. (2000) found a lack of immunoreactive cells in the dorsal brain of adult flies, including the PDFCa neurons. They also demonstrated that there is no mRNA for the normal PDF (Park and Hall, 1998; Park et al., 2000). We also confirmed that the ordinary *Drosophila pdf* mRNA is expressed only in the large and small lateral neurons in each optic lobe (Matsushima et al., 2003, 2004). Although it is possible that a faint immunoreactivity of the PDFCa neurons is simply due to crossreactivity

against the antibodies used, the results suggest as well that the dorsal brain calyx cells in *Drosophila*, like the PDFLa outer cells and VA in *Gryllus*, contain a PDF isoform, and that they lack the ordinary PDF peptide and fail to express the *pdf* gene. To date, there have been no reports announcing the presence of any *pdf* mRNA isoforms, either for *Drosophila* or for any other insect, and thus it now becomes important to identify such isoforms, which may function differently from the normal PDF peptide. Final confirmation of the existence of the novel PDF isoform predicted from our antibody studies will come only by directly isolating the peptide from cricket brains, in experiments we are undertaking.

In a previous study, de Kleijn et al. (1993) reported that a certain cell cluster in the eyestalk of the crayfish *Orconectes limosu* was labeled with the PDH antiserum, but not with a PDH cRNA probe. They suggested from this evidence the presence of a PDH-like peptide in the lamina ganglionaris. Among crustacean β-PDH neuropeptides, PDH isoforms have been reported, for example, for the Pacific white shrimp *Penaeus vannamei* (Desmoucelles-Carette et al., 1998), the blue crab *Callinectes sapidus* (Klein et al., 1994), and the kuruma prawn *Penaeus japonicus* (Yang et al., 1999). All these species have two PDH isoforms, PDH I and PDH II, with respective residue differences between the species, as follows: 1 (Leu/Ile^{11}), 6 (Ile/Leu^{8}, Leu/Ile^{11}, Pro/Ser^{12}, Lys/Ala^{13}, Val/Leu^{14}, and Asp/Glu^{17}), and 3 (Ile/Leu^{11}, Val/Phe^{14}, and Thr/Ile^{16}). Apparently, there is no isoform having an Asn substitution. The existence in *G. bimaculatus* of an alternative PDF peptide with amino acid residues other than Asn at positions 6 and 16 raises the difficult question of whether the mature PDF peptide might itself cause a structural change at both Asn^{6} and Asn^{16} residues. This possibility arises because the asparagine (Asn) residue in proteins often undergoes deamination to form either α- or β-isomeric aspartate (Reissner and Aswad, 2003). The mechanism is characterized by the cyclization of Asn to form a succinimide intermediate. The Asn^{6}-Ser^{7} sequence is one of the most labile structures that undergoes such deamination. To examine this possibility and distinguish whether there may be posttranslational modification of PDF, or alternatively, whether there is translation of a related but different *pdf* gene, we are now endeavoring to directly isolate the PDF isoform from the cricket optic lobe.

The important question remaining to be answered is the physiological function of this novel PDF isoform. The varicose arborization lies over the face of the lamina neuropile. As a neuropeptide, release of the PDF isoform from this arborization would be strategically placed to modulate photic information from the external environment that emanates from the overlying compound eyes, or to modulate photoreceptor or neuron sensitivities to light. In *Musca*, injecting PDF causes the lamina axons of specific visual interneurons to swell (Pyza and Meinertzhagen, 1996), leading to the suggestion that PDF helps mediate rhythmicity in such changes (Meinertzhagen and Pyza, 1996). Possibly a PDF isoform mediates a related function in *Gryllus*, but in that case it is not clear whether the lamina varicose arborization would necessarily be the source of endogenous PDF, nor why an isoform of PDF, and not PDF itself, should be the modulator. It is also not clear what interrelationships may exist between the varicose arborization and the PDFLa outer and inner cells, and the different PDF peptides these apparently release. Equally unclear is whether both peptides act at the same receptor, or whether different receptor mechanisms also exist that might totally segregate the function of the two peptide signals. Such a duality of receptor mechanisms, however, would require that the two PDF isoforms be recognized as different ligands, and this in turn would require that residues 6 and 16 be substantially different from Asn. To answer these and related questions, identification of the PDF receptor(s), corresponding to the recently reported *Drosophila* PDFR gene (Mertens et al., 2005), and determination of its expression and ligand specificity, will all be required. Regardless, the identification of a new PDF isoform in *Gryllus* indicates a further level of sophistication in the mechanisms of the circadian clock system, in this case in its output pathways, in insects. Alternatively, the lamina neurons may have completely different roles, for example, in visual physiology.

LITERATURE CITED

Bloch G, Solomon SM, Robinson GE, Fahrbach SE. 2003. Patterns of PERIOD and pigment-dispersing hormone immunoreactivity in the brain of the European honeybee (*Apis mellifera*): age- and time-related plasticity. J Comp Neurol 464:269–284.

Chuman Y, Matsushima A, Sato S, Tomioka K, Tominaga Y, Meinertzhagen IA, Shimohigashi Y, Shimohigashi M. 2002. cDNA cloning and nuclear localization of the circadian neuropeptide designated as pigment-dispersing factor PDF in the cricket *Gryllus bimaculatus*. J Biochem 131:895–903.

de Kleijn DPV, Linck B, Klein JM, Weidemann WM, Keller R, van Herp F. 1993. Structure and localization of mRNA encoding a pigment dispersing hormone (PDH) in the eyestalk of the crayfish *Orconectes limosus*. FEBS Lett 321:251–255.

de St Groth SF, Scheidegger D. 1980. Production of monoclonal antibodies: strategy and tactics. J Immunol Methods 35:1–21.

Desmoucelles-Carette C, Sellos D, Van Worhmoudt A. 1998. Molecular cloning of the pigment dispersing hormone in a crustacean. Ann N Y Acad Sci 839:395–396.

Dircksen H, Zahnow CA, Gaus G, Keller R, Rao KR, Riehm JP. 1987. The ultrastructure of nerve endings containing pigment-dispersing hormone (PDH) in crustacean sinus gland: identification by an antiserum against synthetic PDH. Cell Tissue Res 250:377–387.

Dunlap JC. 1999. Molecular basis for circadian clocks. Cell 96:271–290.

Ewer J, Frisch B, Hamblen-Coyle MJ, Rosbash M, Hall JC. 1992. Expression of the period clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. J Neurosci 12:3321–3349.

Gefter ML, Margulies DH, Scharff MD. 1977. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. Somatic Cell Genet 3:231–236.

Helfrich-Förster C. 1995. The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 92:612–616.

Helfrich-Förster C. 1997. Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of *Drosophila melanogaster*. J Comp Neurol 380:335–354.

Helfrich-Förster C, Stengl M, Homberg U. 1998. Organization of the circadian system in insects. Chronobiol Int 15:567–594.

Helfrich-Förster C, Täuber M, Park JH, Muhlig-Versen M, Schneuwly S, Hofbauer A. 2000. Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. J Neurosci 20:3339–3353.

Homberg U, Würden S, Dircksen H, Rao KR. 1991. Comparative anatomy of pigment-dispersing hormone-immunoreactive neurons in the brain of orthopteroid insects. Cell Tissue Res 266:343–357.

King DS, Fields CG, Fields GB. 1990. A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. Int J Pept Protein Res 36:255–266.

Klein JM, Mohrherr CJ, Sleutels F, Riehm JP, Rao KR. 1994. Molecular cloning of two pigment-dispersing hormone (PDH) precursors in the

blue crab *Callinectes sapidus* reveals a novel member of the PDH neuropeptide family. Biochem Biophys Res Commun 205:410–416.

Lane RD. 1985. A short-duration polyethylene glycol fusion technique for increasing production of monoclonal antibody-secreting hybridomas. J Immunol Methods 81:223–228.

Matsushima A, Yokotani S, Lui X, Sumida K, Honda T, Sato S, Kaneki A, Takeda Y, Chuman Y, Ozaki M, Asai D, Nose T, Onoue H, Ito Y, Tominaga Y, Shimohigashi Y, Shimohigashi M. 2003. Molecular cloning and circadian expression profile of insect neuropeptide PDF in black blowfly, *Phormia regina*. Lett Pept Sci 10:419–430.

Matsushima A, Sato S, Chuman Y, Takeda Y, Yokotani S, Nose T, Tominaga Y, Shimohigashi M, Shimohigashi Y. 2004. cDNA cloning of the housefly pigment-dispersing factor (PDF) precursors protein and its peptide comparison among the insect circadian neuropeptides. J Pept Sci 10:82–91.

Meinertzhagen IA, Pyza E. 1996. Daily rhythms in cells of the fly's optic lobe: taking time out from the circadian clock. Trends Neurosci 19:285–291.

Mertens I, Vandingenen A, Johnson EC, Shafer OT, Li W, Trigg JS, De Loof A, Schoofs L, Taghert PH. 2005. PDF receptor signaling in *Drosophila* contributes to both circadian and geotaxic behaviors. Neuron 48:213–219.

Nässel DR, Shiga S, Wikstrand EM, Rao KR. 1991. Pigment-dispersing hormone-immunoreactive neurons and their relation to serotonergic neurons in the blowfly and cockroach visual system. Cell Tissue Res 266:511–523.

Park JH, Hall JC. 1998. Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster*. J Biol Rhythms 13:219–228.

Park JH, Helfrich-Förster C, Lee G, Liu L, Rosbash M, Hall JC. 2000. Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. Proc Natl Acad Sci U S A 97:3608–3613.

Petri B, Stengl M. 1997. Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. J Neurosci 17:4087–4093.

Pyza E, Meinertzhagen IA. 1996. Neurotransmitters regulate rhythmic size changes amongst cells in the fly's optic lobe. J Comp Physiol [A] 178:33–45.

Pyza E, Meinertzhagen IA. 1997. Neurites of *period*-expressing PDH cells in the fly's optic lobe exhibit circadian oscillations in morphology. Eur J Neurosci 9:1784–1788.

Rao KR, Riehm JP. 1988. Pigment-dispersing hormones: a novel family of neuropeptides from arthropods. Peptides 9:153–159.

Rao KR, Riehm JP. 1993. Pigment-dispersing hormones. Ann N Y Acad Sci 680:78–88.

Rao KR, Mohrherr CJ, Riehm JP, Zahnow CA, Norton S, Johnson L, Tarr GE. 1987. Primary structure of an analog of crustacean pigment-dispersing hormone from the lubber grasshopper *Romalea microptera*. J Biol Chem 262:2672–2675.

Reischig T, Stengl M. 2003. Ultrastructure of pigment-dispersing hormone-immunoreactive neurons in a three-dimensional model of the accessory medulla of the cockroach *Leucophaea maderae*. Cell Tissue Res 314: 421–435.

Reissner KJ, Aswad DW. 2003. Deamidation and isoaspartate formation in proteins: unwanted alterations or surreptitious signals? Cell Mol Life Sci 60:1281–1295.

Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH. 1999. A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell 99:791–802.

Sato S, Chuman Y, Matsushima A, Tominaga Y, Shimohigashi Y, Shimohigashi M. 2002. A circadian neuropeptide, pigment-dispersing factor, PDF, in the last summer cicada *Meimuna opalifera*: cDNA cloning and immunocytochemistry. Zool Sci 19:821–828.

Sauman I, Reppert SM. 1996. Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of period protein regulation. Neuron 17:979–990.

Sehadova H, Sauman I, Sehnal F. 2003. Immunocytochemical distribution of pigment-dispersing hormone in the cephalic ganglia of polyneopteran insects. Cell Tissue Res 312:113–125.

Shimohigashi Y. 2005. Peptide Science 2004: the proceedings of the joint meeting of the first Asia-Pacific International Peptide Symposium and the forty-first Japanese Peptide Symposium (APIPS-JPS 2004). Osaka: Japanese Peptide Society.

Shirasu N, Shimohigashi Y, Tominaga Y, Shimohigashi M. 2003. Molecular cogs of the insect circadian clock. Zool Sci 20:947–955.

Stengl M, Homberg U. 1994. Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. J Comp Physiol [A] 175:203–213.

Wilkinson DG. 1999. In situ hybridization: a practical approach. Oxford: Oxford University Press.

Yang WJ, Aida K, Nagasawa H. 1999. Characterization of chromatophorotropic neuropeptides from the kuruma prawn *Penaeus japonicus*. Gen Comp Endocrinol 114:415–424.

Peptide Science 2006: H. Ishida and H. Mihara (Eds.)
The Japanese Peptide Society (2006)

Conformation Change of α-Helix Peptide for Sensing of Deactivation of Nuclear Receptor: Immunoassay Using Polyclonal Antibody Specific for the C-terminal α-Helix 12 of Estrogen-related Receptor γ (ERRγ)

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Introduction

Nuclear receptors (NRs) constitute a large group of transcription factors that are involved in many important biological processes and the regulation of cholesterol metabolism. Forty-eight members of this protein family are known to date in mammals and about thirty possess known ligands or activators [1]. We have recently established the assay system for these NRs to evaluate simultaneously the ligand binding and the hormonal activity of exogenously administered chemicals. This method named as "conformation-sensing assay" requires a specific antibody that discriminates the conformation of ligand-bound holo-NR and ligand-free apo-NR, predominantly at the C-terminal site of α-helix 12 (H12).

Approximately twenty NRs are so-called orphan receptors, the ligands of which are unknown. It is now well acknowledged that some of these NRs retain H12 in an active conformation, resulting in eliciting extremely high basal constitutive activity. Thus, it seemed to be difficult to introduce a sensing assay for such NRs because of their lack of natural ligands. One of such orphan NRs is estrogen-related receptor γ (ERRγ). It was recently found that 4-hydroxytamoxifen (4-OHT) deactivates ERRγ by repositioning its H12 [2], and thus we intended to screen a polyclonal antibody that discriminates this H12's repositioning. In order to evaluate the ability of the chemicals to bind ERRγ, the antibody was utilized to measure their potency in reversing the repositioning of H12 by 4-OHT.

Results and Discussion

To prepare the antibody against H12 in ERRγ, antigen peptide (GKVPMHKLFLEMLEAKV) was synthesized by the Fmoc synthetic strategy. For conjugation to career protein KLH, Cys was attached at the N-terminus of this antigen peptide. After immunization into a rabbit, the serum collected was first purified by immunoprecipitation with KLH. Further purification was carried out by affinity chromatography using agarose gel conjugating the antigen peptide. Purified antibody was found to recognize equally well the receptor and the antigen peptide.

To establish the conformation-change sensing assay, bovine thyroglobulin-linked H12-peptide was coated onto the polypropylene 96-well immunoplate as a competitor. The immunoreactivities of the antibody against the receptor or the immobilized peptide were measured under the varying receptor concentrations. The immunoreactivity of the purified antibody was examined with or without 4-OHT for ERRγ. 10 μM 4-OHT was applied to construct a holo-form of the receptor, and the reactivity of the apo-fprm was tested just without the chemical. Anti-ERRγ-H12 antibody was found to exhibit a sufficient ability to differentiate the apo- and holo-forms of ERRγ (Fig. 1).

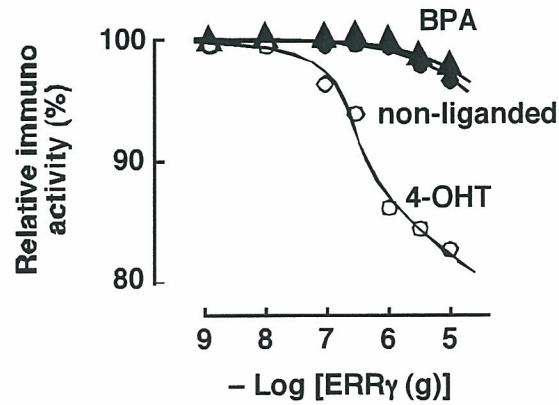

Fig. 1. Conformation-sensing assay.

H12 in 4-OHT-bound ERRγ does not occupy the coactivator groove and shifts from the constitutive-active position [3]. It was thought that prepared anti-H12 polyclonal antibody can distinguish this position change, recognizing 4-OHT-bound ERRγ better than ligand-free ERRγ.

Bisphenol A (BPA) was found to bind to ERRγ very strongly in the receptor binding assay. In the reporter gene assay, BPA retained a high constitutive activity of ERRγ. 4-OHT deactivates ERRγ in a dose-dependent manner, but BPA was found to reverse this 4-OHT's inverse agonist activity. This reversal effect of BPA on ERRγ was also demonstrated in the conformation change sensing assay.

References

1. Robinson, R. M., Carpentier, A. S., Duffraisse, M., and Laudet, V. (2001) *Trends Genet.*, **17**, 554-556.
2. Coward, P., Lee, D., Hull, M. V., and Lehmann, J. M. (2001) *Proc. Natl. Acad. Sci.USA*, **98**, 8880–8884.
3. Greschik, H., Flaig, R., Renaud, J. -P., and Moras, D. (2004) *J. Biol. Chem.*, **279**, 33639-33646.

Peptide Science 2006: H. Ishida and H. Mihara (Eds.)
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Functional Analysis of F-domain Peptides Important for the Basal Constitutive Activity of Human Nuclear Receptor

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Introduction

Nuclear receptors (NRs) are essential transcriptional regulators involved in widely diverse physiological functions such as control of embryonic development, cell differentiation, and homeostasis. 48 human NRs are classified into seven groups (NR1-NR6 and NR0) along with their homology of amino acid sequences. These receptor proteins possess structurally highly conserved domains; a DNA binding domain in the zinc finger motif structure (DBD), and a ligand binding domain (LBD) (Fig. 1). LBD is in the α domain structure, which is also highly conserved to retain NRs in a common molecular mechanism as a transcription factor. Interestingly, we found that there are several NRs lacking the F-domain completely or partly, in spite of the presence of NRs having very long F-domain peptides. Apparently, this appeared to relate with their basal levels of constitutive activity.

Approximately 30 NRs possess distinct ligands or activators, while others are orphan receptors, the ligand of which is unknown. Some of orphan receptors are believed not to have an endogenous ligand. To convey different transcription activities, these NRs might evolve especially for ligand recognition, while the structure essential for transcription mechanism might be maintained. In this study, to demonstrate the structural requirement for all human NRs or specific for each NR group, we analyzed structural essentials by means of the evolutionary trace (ET) method. ET is an algorithm that proves the evolutionary record that resides in the divergent amino acid sequences of a large gene family and determines the importance of each amino acid position by correlating their variations with evolutionary divergences [1,2].

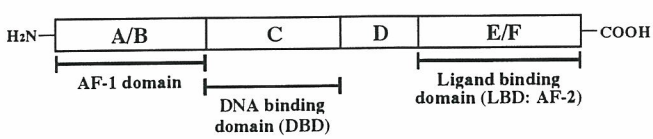

Fig. 1. The basic structure of nuclear receptors

Results and Discussion

When we applied the ET method for all of 48 NR-LBD, thirty amino acid residues were found to be preserved. We then mapped and highlighted them onto the 3D-structures of four different NRs, namely the representatives of NR1-NR4 groups. Those include TRβ (PDB-code = 1XZX), RXRα (1FBY), ERα (1GWR), and Nurr1 (1OVL). Two distinct clusters were clearly evidenced for all four NRs. One is a cluster in the interior of coactivator interaction site, and the other is a cluster in the interior of dimer interface. Both sites are highly conserved among almost all NRs, suggesting that the coactivator interaction and the dimer formation are crucially important for their basal functions.

In interior of dimmer interface, TRβ has the Arg residue at position 429, where its guanidino group makes a hydrogen bond with Ser380. This interaction would stabilize strongly the dimer interface, but apparently only in the NR1 group. In other NR groups, the corresponding Arg side chain orientates to an opposite direction. In coactivator binding site, Glu422 in Nurr1 is specific for NR4 group. Glu422 makes a hydrogen bond with Arg418 backbone, and this interaction would stabilize the coactivator binding site to retain a high constitutive activity. Other NR groups possess Lys instead of Glu at the position.

The F-domain is a C-terminal tail moiety tethered to helix-12, and constitutes a part of coactivator interaction site. It cannot be analyzed by the ET method, because the amino acid sequences of F-domain are completely different from each other. To obtain the information about structure-activity relationship (SAR), we attempted to catalog by aligning all NRs by their lengths. A good linear correlation was found between the size of F-domain peptide fragment and the level of basal constitutive activity. All these results strongly suggested that the molecular function of NRs is largely dependent upon their ordered structures in the C-terminal moiety.

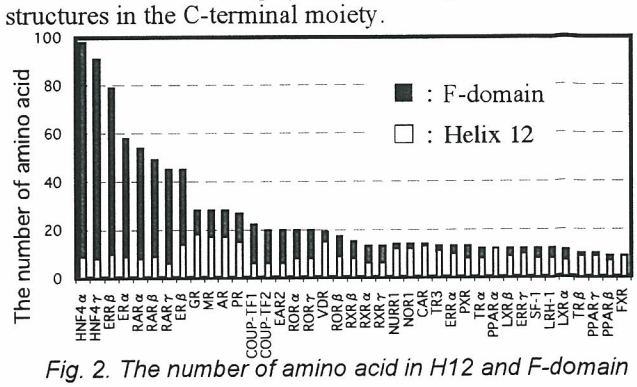

Fig. 2. The number of amino acid in H12 and F-domain

References

1. Lichtarge, O., Bourne, H. R., and Cohen, F. E. (1996) *J. Mol. Biol.*, **257**, 342-358.
2. Mihalek, I., Res, I., and Lichtarge, O. (2004) *J. Mol. Biol.*, **336**, 1265-1282