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Structural Isoforms of the Circadian Neuropeptide PDF Expressed in the Optic Lobes of the Cricket *Gryllus bimaculatus*: Immunocytochemical Evidence from Specific Monoclonal Antibodies

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ABSTRACT

Pigment-dispersing factor (PDF) is an 18-mer peptide that acts as a principal neurotransmitter of the insect circadian clock. Our previous study, utilizing anti-*Uca* β-PDH polyclonal antibody (pAb) to immunolabel the optic lobe of the cricket *Gryllus bimaculatus*, suggested the existence of an alternative PDF-like peptide in the outer cells of the first neuropile, or lamina (La), which were much less immunoreactive than the inner cells of the second neuropile, the medulla (Me). To obtain structural information about such a PDF-like peptide, we prepared 10 anti-*Gryllus* PDF monoclonal (mAb) and pAb antibodies and analyzed their detailed epitope specificities. The PDFMe and PDFLa inner cells and their axonal projections were clearly immunoreactive to all these antibodies, revealing the widespread immunocytochemical organization of the PDF system in the optic lobe, as seen previously with anti-*Uca* β-PDH pAb and anti-*Gryllus* PDF mAb, the epitope structures of which were also clarified in this study. The lamina outer cells, which we found lacked a target *pdf* mRNA, displayed specific immunoreactivities, indicating that the cells contain a distinct PDF-like peptide possessing both N- and C-terminal structures. These cells were not immunolabeled by some other monoclonal antibodies, however, implying that the PDFLa outer cells have a PDF isoform peptide devoid of Asn at positions 6 and 16. This isoform was also identified in

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a varicose arborization in the lamina. These results suggest not only the structure of the peptide, but also the possibility of additional functions of this novel PDF isoform. J. Comp. Neurol. 499:404–421, 2006.

Indexing terms: mature peptide; monoclonal antibody; peptide isoform; pigment-dispersing factor (PDF); polyclonal antibody; optic lobe

Pigment-dispersing factors (PDFs) are close insect homologs of the β-subfamily of crustacean pigment-dispersing hormone (β-PDH) neuropeptides (Rao et al., 1987; Rao and Riehm, 1988, 1993). PDF is involved in regulating insect circadian rhythms (Stengl and Homberg, 1994; Helfrich-Förster, 1995; Pyza and Meinertzhagen, 1996; Meinertzhagen and Pyza, 1996; Petri and Stengl, 1997), mainly acting as a neuromodulator that functions in the output pathway of the pacemaker to regulate behavior arising from the dorsal central brain (Renn et al., 1999; Park et al., 2000; Helfrich-Förster et al., 2000). Moreover, immunoreactivity to β-PDH exhibits a daily cycling within terminals of axons projecting either from some of the so-called lateral neurons that are required to express circadian rhythms (Ewer et al., 1992), to the fly's optic neuropiles (Pyza and Meinertzhagen, 1997), or from the small ventral lateral neurons (s-LNvs) (Park et al., 2000).

In a previous study (Chuman et al., 2002), we cloned the cDNA for PDF from adult brains of the cricket *Gryllus bimaculatus*, whose behavioral activity manifests nocturnal rhythmicity. The cDNA obtained exhibits a unique preprosequence, including an ordinary PDF sequence, with a very short sequence for the PDF-associated peptide (PAP) consisting of a nuclear localization signal. Electron microscopic observations clearly identify cells, nuclei of which immunolabel with a rabbit polyclonal antibody raised against the β-PDH peptide of the crustacean *Uca pugilator* (designated hereafter as anti-*Uca* β-PDH pAb).

Confocal studies to locate somata showing PDF expression reveal that all the PDFMe (medulla) cells exhibit strong immunoreactivity, yet differing intensities of immunoreactivity are observed among the PDFLa (lamina) dorsal and ventral cells. An inner cell group is strongly immunoreactive, whereas PDFLa cells situated in a more peripheral location show rather faint immunoreactivity (Chuman et al., 2002). We speculated that the lamina outer cells contain a peptide epitope that in some way differs distinctively from the *Gryllus* PDF present in the inner cells. This difference permits additional complexity in the PDF regulatory mechanisms of circadian function in *Gryllus*. A similar complexity has been suggested for PDF immunoreactivities among the PDFMe and PDF calyx (PDFCa) cells in the model species *Drosophila melanogaster* (Helfrich-Förster, 1997). Thus, we sought to analyze this complexity further in *Gryllus*.

To distinguish whether the outer PDFLa cells express an isoform of PDF or a PDF-like peptide, we prepared a complete set of monoclonal antibodies that covered the entire structure of *Gryllus* PDF, using these to explore structural differences between *Gryllus* PDF and the unknown peptide. We adopted this strategy rather than the isolation of the respective peptides themselves because we initially anticipated that there would be very little peptide in the brain. Thus, we adopted a more conservative initial antibody approach, one that would also allow us to identify the postulated PDF isoform with a high level of specificity using a newly prepared antibody. From background experience in the chemical synthesis of peptides (Shimohigashi, 2005), we adopted a strategy to design and synthesize all possible kinds of peptides with which to analyze the epitopes of the antibodies obtained. A major objective was to obtain monoclonal antibodies that independently recognize two particular moieties of the PDF peptide, the N-terminal peptide portion with the NH_2-terminal free amino group, and the C-terminal portion with the $CONH_2$ carboxyl amide group. The results indicate that ordinary *Gryllus* PDF peptide exhibits a general expression profile in the optic lobe, but that a distinct PDF isoform also exists in the outer set of PDFLa cells and in an adjacent varicose arborization.

Abbreviations

BCA	Bicinchoninic acid
Boc	*tert*-butyloxycarbonyl
bThG	Bovine thyroglobulin G
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Fmoc	Fluorenylmethoxycarbonyl
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
HOBt	1-Hydroxybenzotriazole
HRP	Horseradish peroxidase
Ig	Immunoglobulin
La	Lamina
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MBS	*m*-maleimidebenzoyl-*N*-hydroxysuccinimide ester
Me	Medulla
mAb	Monoclonal antibody
NLS	Nuclear localization signal
pAb	Polyclonal antibody
PAP	PDF-associated peptide
PBS	Phosphate-buffered saline
PDF	Pigment-dispersing factor
PDH	Pigment-dispersing hormone
Pmc	2,2,5,7,8-pentamethylchromane-6-sulfonyl
RP-HPLC	Reverse-phase high-performance liquid chromatography
*t*Bu	*tert*-butyl
Trt	Trityl
Tx	Triton X
VA	Varicose arborization
ZT	Zeitgeber time

MATERIALS AND METHODS

Animals

Animals used to prepare monoclonal antibodies were 8-week-old male BALB/c mice (KBT Oriental, Saga, Japan) (Charles River Japan, Tokyo, Japan). The crickets, *G. bimaculatus*, adult males, purchased from Reptiles Bow (Tokyo, Japan), were maintained on a light/dark (L/D) 12:12 cycle at 25°C in a Height incubator (Horikoshi, Tokyo, Japan). To collect samples for immunocytochemistry, *G. bimaculatus* brains and optic lobes were excised at ZT2, 8, 14, or 20 (where ZT, Zeitgeber time, denotes the time entrained by environmental time cues, in which lights "on" is ZT0 and lights "off" ZT12). The studies were carried out in accordance with the "Guidelines for Animal Care and Experimentation" of Kyushu University.

Peptide synthesis

Relatively short (4–8-mer) peptides were synthesized on the 0.025 mmole scale by the manual solid-phase peptide synthesis method using a polystyrene tube (Pierce, Rockford, IL). Peptides of 9–18-mer length were prepared on the 0.1 mmole scale by an automated peptide synthesizer (ABI 433A: Applied Biosystems, Foster City, CA). In both peptide syntheses, the resins used were the Fmoc-NH-SAL-MBHA-resin for C-terminal amide peptides and Fmoc-amino acid-preloaded Alko-resin for C-terminal free peptides. We used the following Fmoc-amino acids with the sidechain protecting groups: Arg(Pmc), Asn(Trt), Asp(*t*Bu), Cys(Trt), Glu(*t*Bu), His(Trt), Lys(Boc), and Ser(*t*Bu). The coupling reaction was carried out with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) in the presence of 1-hydroxybenzotriazole (HOBt). Peptides were liberated from the resin by Reagent K (King et al., 1990) at 20°C for 1–3 hours. Purification was carried out first by gel filtration on a column (1.8 × 80 cm) of Sephadex G-15 (Pharmacia, Uppsala, Sweden) eluted with 3–10% acetic acid. For further purification, reversed-phase high-performance liquid chromatography (RP-HPLC) was carried out on a preparative column (CicaMerk Lichrospher RP-18 (e) (5 μm): 25 × 250 mm) with a linear gradient of 0.1% trifluoroacetic acid and 80% acetonitrile and the fractions containing pure peptides were lyophilized to obtain the final peptide sample. The purity was verified by analytical RP-HPLC (CicaMerk LiChrospher RP-18 (e) (5 μm): 4 × 250 mm). Mass spectra of peptides were measured on a mass spectrometer Voyager DE-PRO (PerSeptive Biosystems, Framingham, MA) by means of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF).

Preparation of monoclonal antibodies

Bovine thyroglobulin G (bThG) and bovine serum albumin (BSA) purchased from Calbiochem (La Jolla, CA) were utilized as carrier proteins. Synthetic peptide was conjugated to bThG or BSA through its component cysteine using a bivalent cross-linking agent *m*-maleimidebenzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce). Carrier protein bThG (10 mg, 15 nmol) or BSA (10 mg, 16 nmol) was dissolved in 1 mL of 10 mM phosphate buffer (PB, pH 7.2), and the solution stirred for 30 minutes at 20°C. A solution of MBS (2.5 mg, 13 μmol) in 9.3 mL of *N,N*-dimethylformamide (DMF) was added, and the reaction mixture further stirred overnight for 12 hours at 20°C. Purification was carried out by gel filtration at 4°C using a prepacked column of Pharmacia PD-10 (Amersham Pharmacia Biotech, Amersham, Bucks, UK). Elution was carried out with 50 mM PB (pH 6.0). Fractions containing the product were pooled and stored at −80°C.

A solution (300 μL) of MBS-linked carrier protein in 0.2 M Na_2HPO_4 (150 μL) was added to a solution (500 μL, 1 mM) of synthetic antigen peptide together with 5 μmol of tris(2-cyanoethyl)phosphine (Molecular Probes, Leiden, Netherlands) as a reductant. The reaction mixture was stirred for 3 hours at 20°C. The solution was then dialyzed with a Spectra/Por membrane (molecular weight cut off: 6,000–8,000) (Spectrum Medical Industries, Los Angeles, CA) in 0.02 M phosphate buffered saline (PBS) (pH 7.4). The concentration of the prepared peptide-linked carrier protein solution was estimated by the bicinchoninic acid (BCA) method using BCA Protein Assay Reagent (Pierce).

The lymph nodes were excised from mice at 9–10 days after we first immunized them with the peptide-linked carrier protein. A fusion operation was performed mainly according to published protocols (Gefter et al., 1977; de St Groth and Scheidegger, 1980; Lane, 1985). Cell fusions between the lymph node and myeloma cells obtained from the mice were initiated and the cells kept in a solution of 50% (w/v) polyethylene glycol (average molecular weight 1,500) in 75 mM HEPES solution (Roche Diagnostics, Mannheim, Germany). The fused cells were then seeded into four 96-well culture plates to deliver 100 μL in each well. When the cells were judged to have grown sufficiently, the supernatants were harvested for enzyme-linked immunosorbent assay (ELISA). Wells showing high titers of antibodies were marked and chosen for further screening. Selected relevant hybridomas were cloned by the limiting dilution method, in which the suspension of a competent hybridoma was diluted and seeded into 96-well culture plates at 0.5 cells/100 μL/well in order to minimize the possibility that more than two cells were included in a well. The antibody titer of cells propagated was monitored by the ELISA method. The hybridoma sustaining the best growth was selected and further cultured. This limiting dilution procedure was repeated at least twice. Cloned hybridomas were eventually stored in liquid nitrogen until use.

Myelomas and hybridomas were maintained in culture medium DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Filtron, Brooklyn, Victoria, Australia), 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO BRL, Grand Island, NY), and 10 mM HEPES (Kishida Chemicals, Osaka, Japan) in a 5% CO_2 atmosphere at 37°C. Each culture supernatant from a hybridoma producing the desired antibody was harvested and stored at −80°C.

Identification of monoclonal antibody isotypes

The isotypes of monoclonal antibodies obtained were determined by the antigen-mediated ELISA procedure using a series of isotype-specific monoclonal antibodies (goat antimouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA; Sigma-Aldrich), mainly according to the manufacturer's protocol. Briefly, the test antibody was first immobilized on an ELISA plate indirectly via antigen peptide-linked bThG or BSA, and a solution of six different isotype-specific antibodies was delivered to the wells. Then peroxidase-conjugated antibody against each isotype-specific antibody was added to detect the peroxidase-based

color reaction. Coloration was observed only in a well in which an isotype-specific antibody bound the test antibody.

Enzyme-linked immunosorbent assay (ELISA)

Antigen peptides, analogs of antigen peptides, antigen peptides conjugated to a carrier protein, and carrier proteins were all examined for their immunoreactivity by the ELISA method. Each of these peptide or protein solutions in 50 mM Na_2CO_3 was delivered to a 96-well ELISA plate and incubated for 2 hours at 20°C to immobilize the test compound at a concentration of 100 ng/50 μL/well. After three washings with 0.02 M PBS containing 0.1% Tween 20 (pH 7.4) (designated hereafter PBST), the plate was further treated with 2% BSA (Wako Pure Chemical Industries, Osaka, Japan) in PBS (200 μL/well) for 2 hours at 20°C. After washing twice the culture supernatant of a resulting hybridoma or serum containing the antibody was added to the wells (50 μL/well) to incubate for 1 hour at 20°C. After five washings, 50 μL of a 1:1,500 solution in PBST of horseradish peroxidase (HRP)-conjugated antimouse Ig (Amersham Pharmacia Biotech), which recognizes both mouse IgG and IgM, was added per well to incubate for 1 hour at 20°C. When the primary antibodies were prepared from rabbits, HRP-conjugated antirabbit IgG was utilized as the secondary antibody. After six repeated washings a mixture of *o*-phenylenediamine (Sigma-Aldrich) and H_2O_2 (1:1, v/v) was added (50 μL/well). The plate was left at 20°C until sufficient color development was observed. To terminate the enzyme reaction, 10% H_2SO_4 was added to each well (50 μL/well), and then the absorbance at 490 nm was measured for all wells, using a microplate reader (Immuno-mini NJ-2300, Intermed, Tokyo, Japan).

Immunocytochemistry

Anatomical analysis using confocal microscopy was performed for brains of adult male crickets, *G. bimaculatus*. Dissected brains that contained the subesophageal ganglion and optic lobes with retina were fixed with 4% formaldehyde, as paraformaldehyde (Polysciences, Warrington, PA), in 0.1 M PB (pH 7.4) for 12 hours. After washings with PBS the tissues were embedded in agarose Type VII-A (Sigma-Aldrich) and cut into a series of 80-μm slices using a vibrating blade microtome (Microslicer DTK-100, Osaka EM, Osaka, Japan). After multiple washings in PBS with 1% Triton X-100 (Sigma-Aldrich) (PBSTx [1%]) and PBSTx [1%] with 0.25% BSA [0.25% BSA-PBSTx, 1%]), the sections were incubated in 0.25% BSA-PBSTx [1%] containing 2% normal donkey serum (NDS) (2% NDS-0.25% BSA-PBSTx [1%]) at 4°C overnight. Then the sections were incubated with the primary antibody for 48 hours at 4°C.

The series of primary antibodies used for incubation were the supernatants of hybridoma cultures containing 1% Triton X-100. The sera obtained from immunized rabbits were diluted at a ratio of 1:500–1:1,000 with 0.25% BSA-PBSTx [1%]. After several washings with 0.25% BSA-PBSTx [1%], the slices were treated for 20 hours at 4°C with a fluorophore-conjugated secondary antibody diluted by 2% NDS-0.25% BSA-PBSTx [1%] (1:200–1:500). The secondary antibodies used were FITC-conjugated antimouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA), FITC-conjugated antirabbit IgG (Jackson ImmunoResearch), FITC-conjugated antimouse IgM (Vector Laboratories, Burlingame, CA), and Texas Red-conjugated antimouse IgM (Vector Laboratories). After 10 repeated washings with 0.25% BSA-PBSTx [1%], labeled preparations were mounted in Vectashield (Vector Laboratories) and observed by confocal laser scanning microscopy using Nikon D ECLIPSE C1 or Zeiss LSM510 microscopes.

For observation of the PDF cells using polyclonal antibodies anti-*Uca* β-PDH pAb and anti-*Gryllus* PDF pAb, tissue slices of 80 μm thickness were immunolabeled in a free-floating reaction by means of the indirect peroxidase-anti-peroxidase technique. Slices were incubated in rabbit polyclonal antibody against β-PDH (1:3,500) for 48 hours at 4°C, and then in swine antirabbit secondary antibody (DAKO, Glostrup, Denmark) followed by rabbit antibody to HRP-conjugated HRP (DAKO). Slices were taken to their final reaction with DAB and H_2O_2 and mounted in glycerol gelatin (Sigma-Aldrich) for observation by light microscopy with an Olympus BX50.

For the whole-mount analysis of cricket brains, preparations were incubated for 96 hours with the primary antibody and for 48 hours with the secondary antibody. After washing out the secondary antibody, the preparations were dehydrated in ethanol, then embedded in methyl salicylate to scan by confocal microscopy. All experiments were performed at least in triplicate.

In situ hybridization

Whole-mount in situ hybridization was performed essentially as described by Wilkinson (1999) with several significant modifications. Briefly, brains of adult male *G. bimaculatus* were dissected under light microscopy and fixed in freshly made 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 12 hours at 4°C. Tissues were washed with PBS with 0.1% Tween 20 (PBT) at 4°C, dehydrated in a series of methanol (MeOH) solutions (25%, 50%, 75%, and 100%) in PBT, and stored at −20°C until use.

All the following procedures were performed at 20°C except as noted. Tissues were rehydrated in a reverse series of MeOH-PBT solutions (75%, 50%, 25% MeOH, and 2× PBT) and then permeabilized with 10 μg/mL proteinase K (Sigma-Aldrich) at 37°C for 15 minutes, washed with a glycine solution (2 mg/mL) for 5 minutes, then washed twice with PBT. They were then fixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBT for 20 minutes, washed twice with PBT, and incubated in PBT at 70°C for 50 minutes. After cooling on ice, tissues were next treated with 6% H_2O_2 for 1 hour, washed three times with PBS, and incubated with prehybridization buffer (50% formamide, 5× SSC [750 mM NaCl and 75 mM sodium citrate, pH 4.5], 1% SDS, 50 μg/mL yeast tRNA, and 50 μg/mL heparin) at 70°C for 1 hour. The prehybridization buffer was then replaced with hybridization buffer, prehybridization buffer containing a digoxigenin (DIG)-labeled complementary RNA (cRNA) probe, for incubation at 70°C for 16 hours. DIG-labeled antisense cRNA probe was prepared in vitro from plasmid linearized with a restriction endonuclease *Bam*HI by using DIG-UTP and T7 polymerase. The plasmid used consisted of almost the full-length *pdf* cDNA corresponding to positions 35–285.

After hybridization, tissues were washed three times at 70°C with solution I (50% formamide, 5× SSC [pH 4.5], and 1% SDS) for 30 minutes and once at 70°C with a 1:1 mixture of solutions I and II (in which solution II con-

tained no formamide) for 10 minutes. Tissues were washed a further three times in solution II for 5 minutes, then incubated in solution II for 20 minutes. Washings were repeated twice in solution III (50% formamide, 2× SSC [pH 4.5], and 1% SDS) for 5 minutes at 70°C, three times in solution III, for 30 minutes each, and in a TBST solution in which Tris-buffered saline (TBS; 150 mM NaCl, and 100 mM Tris-HCl [pH 7.5]) contained 1% Tween 20. After blocking for 1 hour, in order to visualize the mRNA-bound probe immunocytochemically, tissues were treated with Fab fragments of sheep anti-DIG antibody (Roche Diagnostics) directly conjugated to alkaline phosphatase (AP). Tissues were incubated for 16 hours at 4°C and washed four times with TBST for 5 minutes and seven times for 1 hour. After twice washing with NTMT (100 mM NaCl, 100 mM Tris-HCl [pH 9.5], 50 mM $MgCl_2$, and 1% Tween 20) for 5 minutes, the antibody detection reaction was undertaken by incubating the tissues in substrate solution (NTMT with 0.33 mg/mL 4-nitroblue tetrazolium chloride and 0.17 mg/mL 5-bromo-4-chloro-3-indolylphosphate) for 30 minutes while shading from all light, and finally washed with TBST. Eventually, entire tissues were mounted in glycerol-gelatin (Sigma-Aldrich) and observed by light microscopy with an Olympus BX50.

In order to count the number of the mRNA-expressing cells, we prepared serial sections from whole-mount in situ hybridization samples. For this, samples were dehydrated in an ethanol series, embedded in epoxy resin (Epon 812), and the Epon block was sectioned serially at 1.5 μm thickness on an ultramicrotome. All mRNA-expressing cells were photographed digitally with an Olympus BX50 microscope and counted.

Images obtained with both LSM and compound light microscopy were brought into Photoshop (Adobe Systems, San Jose, CA) and modified so that the background without the sample was either black or white, respectively. The images were then formatted and labeled.

RESULTS

Design for synthesis of peptides

Antigen peptides. In this study the specificity of monoclonal antibodies was employed to discriminate the molecular forms of PDF and its related peptides in the cricket *G. bimaculatus*. To explore these forms in the tissue of the cricket's brain, a series of antigens was designed based on the structural composition of particular peptide sites or of the mature PDF peptide. As a neuropeptide, the mature form of PDF arises from a precursor after proteolytic cleavage and processing. To detect the mature PDF specifically, distinct especially from the precleavage forms of this molecule, highly specific antibodies that recognize the unique structures of PDF peptide are required. The α-amino group of the N-terminal Asn is a diagnostic structure because it emerges only after proteolytic processing. If an antibody recognizes the PDF N-terminal sequence Asn-Ser-Glu-Ile-Ile-Asn-Ser (NSEIINS) only when the N-terminus is liberated and free, immunoreactivity to this antibody will reveal immediately that the peptide detected is the mature PDF processed, distinct from its precursor. For preparation of such an antibody we therefore designed and synthesized the peptide H-NSEIINSC-NH_2, in which H- and -NH_2 indicate the presence of the free amino group in the N-terminus and the amide group in the C-terminus, respectively. To conjugate the peptide to a carrier protein, cysteine (Cys or C) was attached to the C-terminus.

The mature PDF has an amide group, -$CONH_2$, at the C-terminus. In order to obtain an antibody that would recognize this amidated PDF structure specifically, a peptide having the entire sequence of *Gryllus* PDF with a cysteine at the N-terminus was used as an antigen peptide. Thus, we synthesized H-CNSEIINSLLGLPKVLNDA-NH_2.

Peptides for screening. In order to prepare an antibody that recognized specifically and exclusively the mature form of PDF, it was necessary to screen a crude batch containing a mixture of antibodies, so as to exclude antibodies that crossreact with α-amino-acylated PDF analog peptides. To this end, we synthesized Ac-NSEIINSC-NH_2, in which Ac- denotes the acetyl group of CH_3CO-, so that the structure of CH_3CO-NH-CH(CH_2CONH_2)CO- is present at the N-terminus.

To obtain an antibody specific to the amide moiety of PDF peptide, it was necessary to screen with the PDF analog NSEIINSLLGLPKVLNDAGRK-OH. In this peptide, the C-terminal "-OH" indicates the presence of a free carboxyl group. This peptide possessed a C-terminal GRK-OH that contained the structure of an amidation signal. We also synthesized the PDF analog NSEIINSL LGLPKVLNDA-OH that does not possess the C-terminal amide group, instead having a carboxyl group (COOH). These peptides were necessary to decide whether the antibodies were specific to the C-terminal PDF amide structure.

Peptides for the analysis of antibody epitopes. To identify the epitope recognized by the monoclonal antibodies we designed and synthesized a series of analogs of antigen peptides. For instance, to characterize an antibody against the PDF N-terminal region with an α-amino group, several different types of PDF analogs were prepared. Those included α-amino-acetylated PDF, PDF with an additional Arg residue at the N-terminal end, a series of PDF analogs lacking a particular residue position, and PDF peptides from other insect sources, such as the flies *Musca* and *Drosophila*. For epitope identification of an antibody against the PDF-amide moiety, we prepared PDF peptide having a C-terminal amidation signal GRK(=Gly-Arg-Lys) and its precursor analog. Peptides possessing Ala-NH_2 or Asp-Ala-NH_2 at the C-terminus were also prepared. These were CGNAARGYTDSD DDAGMGTE**A-NH_2** derived from the *Drosophila* tyramine receptor (TR-A-NH_2), and CKNVVPLYDLLLEM **LDA-NH_2** derived from the human estrogen receptor (ER-DA-NH_2).

A series of Gly-replaced analogs of C-terminal heptapeptides PKVLNDA-NH_2 was also synthesized for precise determination of the antibody recognition site. These included CPGVLNDA-NH_2, CPKGLNDA-NH_2, CPKVGNDA-NH_2, CPKVLGDA-NH_2, CPKVLNGA-NH_2, and CPKVLNDG-NH_2. Since these rather small peptides were suspected to be absorbed onto the plastic surface of the 96-well ELISA plate, we also prepared each derivative conjugated to BSA via N-terminal Cys(=C). Thus, antibodies were tested both for peptides and for BSA-conjugated derivates.

To determine the epitope of antibodies that recognized the internal portion of the PDF peptide, PDF fragments were furthermore prepared that were successively truncated from the N-terminal in a stepwise series, amino-acid-by-amino-acid. Fragments lacking a certain residue,

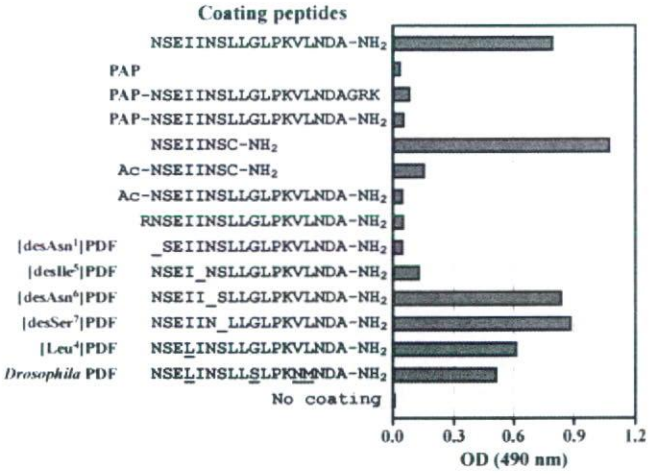

Fig. 1. Epitope analysis of monoclonal antibody anti-H_2N(1-5)PDF mAb. The monoclonal antibody produced by hybridoma NP-G10 was examined by the ELISA-based epitope analysis for a series of chemically synthesized peptides of *Gryllus* PDF and its analogs. No immunoreactivity was found when no peptides were coated to the 96-well polypropylene ELISA plate. Underlined amino acids in *Drosophila* PDF indicate those residues not compatible with those of *Gryllus* PDF. The epitope of this antibody is judged to be H_2N-N^1SEII^5 including the N-terminal α-amino group.

and fragments in which the residue was replaced with Gly were also synthesized.

To prepare antigens and screen and characterize antibodies, more than 30 such peptides were synthesized in this study by Fmoc methods. The peptides were obtained in an average yield of about 30%, but the yields of much longer peptides such as PDF precursor and PAP, which contain a number of Arg residues, were considerably lower (about 5%). For all the peptides synthesized, the mass numbers measured by MALDI-TOF MS coincided exactly with the values calculated for the corresponding molecular formula. The purity of peptides was verified by analytical HPLC, in which each of the peptides emerged with a single peak.

Epitope analysis of antibodies

When antibodies were raised against NSEIINSC-NH_2, only a single usable monoclonal hybridoma was obtained among ~500 lines assessed. This hybridoma was denoted NP-G10. When raised against CNSEIINSLLGL PKVLNDA-NH_2, eight different hybridomas—designated PDF-20, -32, -33, -140, -243, -264, -300, and -432—were obtained. We made a major effort to screen the monoclonal antibodies from these hybridomas, aiming to obtain highly specific clones against the mature PDF peptide. For this purpose, we examined the immunoreactivities to synthetic peptides by ELISA to characterize the epitope more precisely, and so identify the actual peptide structure that an individual monoclonal antibody specifically recognized.

Monoclonal antibody that specifically recognizes the N-terminal PDF structure. NP-G10 was the hybridoma we raised against NSEIINSC-NH_2, the PDF-N-terminal 7-mer peptide having an additional C-terminal Cys amide. Figure 1 shows the results from our attempts to define the peptide segment distinguished by NP-G10, by measuring absorbance at 490 nm in an ELISA-based epitope analysis. The optical density OD_{490} reveals the amount of 2,3-diaminophenazine, the product obtained from *o*-phenylenediamine and H_2O_2 by the action of the HRP conjugated to antimouse Ig secondary antibody. It should be noted that the antibody from NP-G10 showed a high OD_{490} when tested for either the chemically synthesized antigen peptide or *Gryllus* PDF (Fig. 1), indicating that the NP-G10-producing antibody recognized these peptides with high specificity.

For this epitope analysis, we further synthesized the 43-mer *Gryllus* PDF precursor, and also PAP-related peptides of the 22-mer MARRARFEANAAPSPLMCVHKR and 20-mer MARRARFEANAAPSPLMCVH sequences. NP-G10-derived antibody showed only a low level of OD_{490} when tested against these two peptides (Fig. 1), indicating that the antibody exhibits essentially no crossreactivity to either of these peptides. The precursor consists of the full sequence of PDF, and thus contains the segment "NSEIINS" corresponding to the antigen peptide. Nevertheless, the NP-G10-derived antibody exhibited no immunoreactivity to the PDF precursor, clearly implying that the antibody requires the amino group of N-terminal Asn (=N) for recognition to occur. In fact, the NP-G10-derived antibody also did not recognize acetylated analogs of either the antigen peptide or even of PDF itself, namely, Ac-NSEIINSC-NH_2 and Ac-NSEIINSLLGLPKVLNDA-NH_2 (denoted henceforth as "Ac-PDF") (Fig. 1). The acetyl group (CH_3CO) caps the amino group, converting the structure of NH_2- or $^{\oplus}NH_3$- to CH_3CO-NH-. Distinct immunoreactivity to PDF, but not to Ac-PDF, provides a definitive demonstration that NP-G10 requires the amino group of N-terminal Asn for peptide recognition.

In addition to these cases, N-terminal Arg(=R)-attached PDF peptide, RNSEIINSLLGLPKVLNDA-NH_2, also failed to exhibit immunoreactivity to this antibody. Arg is a residue present at the PAP C-terminal, and is thus adjacent to the N-terminal end of PDF peptide in the PDF precursor. This result provides further evidence that the NP-G10-producing antibody distinguishes scrupulously between the PDF precursor and the mature PDF peptide. Thus, immunoreactivity against the NP-G10-derived antibody alone provides discriminating evidence for the endoproteolytic cleavage of the precursor to create the N-terminally matured PDF.

The NP-G10-derived antibody also failed to show reactivity to the PDF analog peptide lacking the N-terminal Asn residue, [desAsn^1]PDF (Fig. 1). Collectively, these results indicate that the epitope of this antibody is restricted to a peptide region comprising the α-amino group of Asn at the PDF N-terminus. It should be noted that the deletion of Asn^6 or Ser^7 from PDF does not affect the immunoreactivity of NP-G10-derived antibody, whereas removing Ile^5 diminishes immunoreactivity almost completely (Fig. 1). Taken together, we conclude that the epitope of this antibody is H-NSEII, including the N-terminal α-amino group.

In addition to this epitope, the NP-G10-derived monoclonal antibody was also found to recognize H-NSELI. Thus, this antibody definitely interacted with *Drosophila* PDF that consists of Leu at position 4 instead of Ile, although its immunoreactivity was diminished to an extent slightly less than that against *Gryllus* PDF (Fig. 1). Furthermore, NP-G10-derived antibody recognized [Leu^4]-*Gryllus* PDF in which Ile^4 was replaced by Leu (Fig. 1). Ile and Leu are structural isomers of each other,

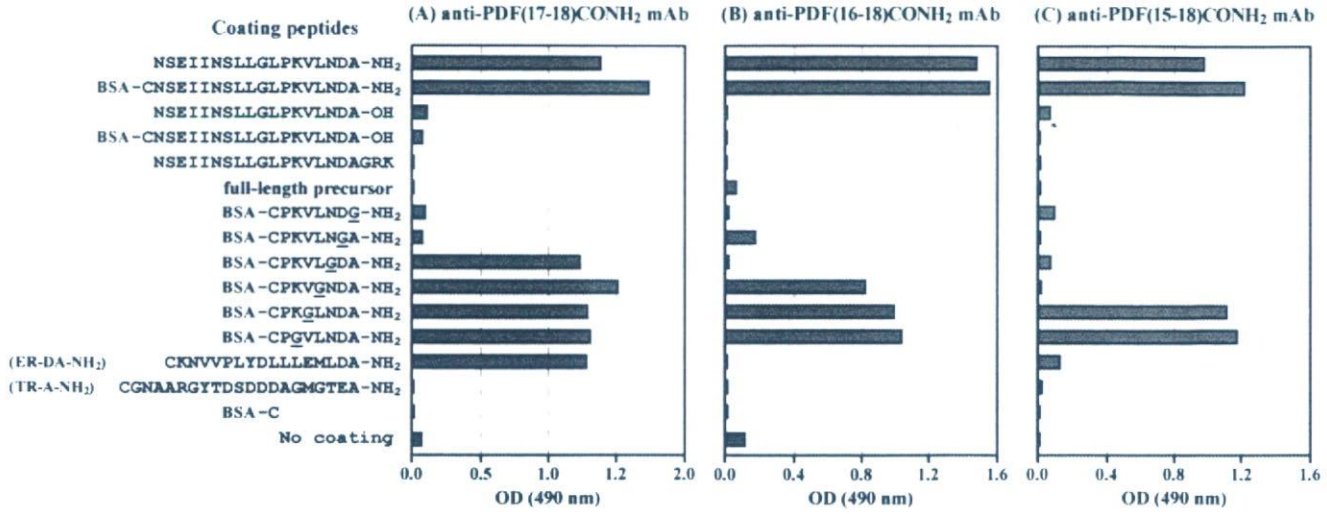

Fig. 2. Epitope analysis of monoclonal antibodies anti-PDF(17-18)$CONH_2$ mAb (**A**), anti-PDF(16-18)$CONH_2$ mAb (**B**), and anti-PDF(15-18)$CONH_2$ mAb (**C**). A series of monoclonal antibodies produced by hybridomas PDF-140, PDF-300, and PDF-432 were examined by the ELISA-based epitope analysis for chemically synthesized *Gryllus* PDF and its analogs. All antibodies were completely inactive to BSA and its derivative MBS-Cys-conjugated BSA. The epitopes are judged to be $D^{17}A^{18}$-NH_2 for the PDF-140-derived monoclonal antibody (therefore denoted anti-PDF(17-18)$CONH_2$ mAb) (A), $N^{16}DA^{18}$-NH_2 for PDF-300-derived monoclonal antibody (anti-PDF(16-18)$CONH_2$ mAb) (B), and $L^{15}NDA^{18}$-NH_2 for PDF-432-derived monoclonal antibody (anti-PDF(15-18)$CONH_2$ mAb) (C).

with side chains of $-C^*H(CH_2CH_3)CH_3$ (where C^* denotes the asymmetric carbon atom) for Ile and $-CH_2-CH(CH_3)_2$ for Leu. Thus, the NP-G10-derived antibody is both highly and exclusively specific to either the N-terminal H-NSEII or H-NSELI moiety of PDF.

Nomenclature of antibodies. Since various types of monoclonal and polyclonal antibodies were eventually prepared in this study, we named these with a systematic nomenclature. First, the name of each antibody denoted the precise epitope structure. PDF is an 18-mer peptide with the sequence H-Asn^1-Ser^2-Glu^3-Ile^4-Ile^5-Asn^6-Ser^7-Leu^8-Leu^9-Gly^{10}-Leu^{11}-Pro^{12}-Lys^{13}-Val^{14}-Leu^{15}-Asn^{16}-Asp^{17}-Ala^{18}-NH_2, denoted in the one-letter code by H-NSEIINSLLGLPKVLNDA-NH_2. The monoclonal antibody (mAb) produced by NP-G10 recognizes the structure H-Asn^1-Ser^2-Glu^3-Ile^4-Ile^5- comprising the α-amino group of Asn^1, and thus is designated "anti-H_2N(1-5)PDF mAb." The antibody "anti-PDF(17-18)$CONH_2$ mAb" recognizes the C-terminal Asp^{17}-Ala^{18}-NH_2 comprising the alanine amide structure. Antibodies that recognize the internal sequence of PDF are designated "anti-PDF(X-Y) mAb" where X and Y give the number of truncated amino acid residues at both N- and C-terminal sides, respectively. For instance, the monoclonal antibody "anti-PDF(6-10) mAb" recognizes Asn^6-Ser^7-Leu^8-Leu^9-Gly^{10}. Polyclonal antibodies were designated as for monoclonal antibodies by using "pAb" instead of mAb as an abbreviation.

Monoclonal antibody against the amide moiety of PDF. Hybridomas PDF-140, PDF-300, and PDF-432 were obtained from the fusion of lymph node cells and myeloma cells obtained after immunization with *Gryllus* PDF, having an N-terminal Cys, as the peptide antigen. A series of monoclonal antibodies produced by these hybridomas was found to recognize the PDF peptide having a C-terminal amide structure. Most important, these antibodies did not react with the PDF analog having a C-terminal free carboxyl group. Since PDF-OH possesses a COOH-carboxyl group rather than a $CONH_2$-carboxyl amide, it is evident that the amide moiety is crucially important as a recognition site for these antibodies. The same antibodies also failed to exhibit an immunoresponse to either the PDF analog having an amidation signal (GRK) at the C-terminus or the GRK-containing PDF precursor. These monoclonal antibodies do recognize the C-terminal amide moiety of PDF, however, and so discriminate between the PDF peptide and its precursor.

To specify the exact epitope structure in the PDF molecule, we designed and synthesized C-terminal PDF 7-mer peptides in which each of the amino acid residues in the Pro^{12}-Lys^{13}-Val^{14}-Leu^{15}-Asn^{16}-Asp^{17}-Ala^{18}-NH_2 sequence was replaced, one by one, by Gly, as shown in Figure 2. The Asn^{16}→Gly substitution was found to drastically diminish the immunoreactivity of the PDF-300-derived monoclonal antibody. The lack of almost all immunoreactivity to PKVLGDA-NH_2 implied that this antibody recognized the C-terminal NDA-NH_2 structure, as confirmed by the lack of immunoreactivity to PKVLNGA-NH_2 and PKVLNDG-NH_2. No such reduction was detected after the (Lys^{13}, Val^{14}, or Leu^{15})→Gly substitution, on the other hand. All the results obtained for Gly-substituted PKVLNDA-NH_2 peptides were also reproduced by the corresponding BSA-conjugated derivatives, as shown in Figure 2. In addition, *Drosophila* PDF, with a C-terminal NMNDA-NH_2 sequence, showed a high level of immunoreactivity to this monoclonal antibody. Thus, the epitope of the monoclonal antibody produced by hybridoma PDF-300 was determined to be the PDF C-terminal NDA-NH_2 structure, for which the antibody was designated "anti-PDF(16-18)$CONH_2$ mAb."

The PDF-432-derived monoclonal antibody was found to have dramatically reduced reactivity to the PKVLNDA-NH_2 analog peptides having Leu^{15}→Gly, Asn^{16}→Gly, Asp^{17}→Gly, and Ala^{18}→Gly substitutions. No reduction was detected by the substitution of Gly for either Lys^{13} or

Val[14]. Furthermore, this PDF-432-produced antibody was not immunoreactive to *Drosophila* PDF that had Met[15] instead of Leu[15], as in *Gryllus* PDF. These results demonstrated that the PDF-432-derived monoclonal antibody recognized the C-terminal sequence LNDA-NH_2 of PDF, for which it was therefore designated "anti-PDF(15-18)$CONH_2$ mAb."

The monoclonal antibodies anti-PDF(16-18)$CONH_2$ mAb and anti-PDF(15-18)$CONH_2$ mAb exhibited no reactivity against chemically synthesized peptides having either an Ala-amide (A-NH_2) or Asp-Ala-amide (DA-NH_2) structure at the C-terminal end. The sequences of these peptides are identical to those in two biological receptor molecules: TR-A-NH_2 (CGNAARGYTDSDDDAGMGTE**A-NH_2**) in the *Drosophila* tyramine receptor; and ER-DA-NH_2 (CKNVVPLYDLLLEML**DA-NH_2**) in the human estrogen receptor. In contrast, PDF-140-derived antibody lacked immunoreactivity only to the latter peptide, suggesting that the epitope of this antibody involves DA-NH_2. By using a series of Gly-substituted analogs of PKVLNDA-NH_2, the epitope of this monoclonal antibody was eventually determined to be DA-NH_2 (Fig. 2). The antibody was designated anti-PDF(17-18)$CONH_2$ mAb and showed distinct reactivity to both *Gryllus* and *Drosophila* PDFs.

Monoclonal antibodies that recognize the internal structure of PDF peptide. Hybridomas PDF-20, PDF-32, PDF-33, PDF-243, and PDF-264, which produce antibodies immunoreactive to both PDF and nonamidated PDF-GRK, were tested next to determine their epitopes. For this epitope screening a series of PDF analogs was designed and chemically synthesized as shown in Figure 3. When PDF-20-derived monoclonal antibody was examined, extremely weak or no immunoreactivity was observed against PDF analogs lacking one of the following amino acid residues: [desAsn[6]]PDF, [desSer[7]]PDF, or [desGly[10]]PDF. Reactivity was also lacking to Gly-substituted PDF analogs such as [Gly[8]]PDF and [Gly[9]]PDF. However, this monoclonal antibody did exhibit distinct immunoreactivity to another series of PDF analogs, including [desAsn[1]]PDF, [desAsn[1]Ser[2]]PDF, [desAsn[1]Ser[2]Glu[3]]PDF, [desAsn[1]Ser[2]Glu[3]Ile[4]]PDF, and [desIle[5]]PDF. These results clearly indicated that the N-terminus of the epitope is the Asn[6] residue. From the finding of immunoreactivity to [desLeu[11]]PDF and of no reactivity to [desGly[10]]PDF, the C-terminus of the epitope was identified as the Gly[10] residue. Thus, the epitope of PDF-20-derived monoclonal antibody was eventually determined to be Asn[6]-Ser[7]-Leu[8]-Leu[9]-Gly[10], and the antibody accordingly designated anti-PDF(6-10) mAb.

In a similar way, the epitope sequences of monoclonal antibodies produced by hybridomas PDF-32, PDF-33, PDF-243, and PDF-264 were also determined (Fig. 3). It should be noted that the epitopes for these antibodies are each very distinctive and well defined, with each antibody recognizing 5–9 consecutive amino acid residues. PDF-32-derived monoclonal antibody recognized Asn[1]-Ser[2]-Glu[3]-Ile[4]-Ile[5]-Asn[6]-Ser[7]-Leu[8]-Leu[9], and was designated anti-PDF(1-9) mAb. This N-terminal-recognizing antibody was found to be immunoreactive to Ac-PDF, indicating that the α-amino group of Asn[1] in NSEIINSLL is not required in the epitope. In addition, the same antibody recognized a PDF analog elongated by the addition of N-terminal Arg as well as synthetic PDF precursor (data not shown). These results imply that anti-PDF(1-9) mAb recognizes the amino acid sequence NSEIINSLL present in both the precursor and the mature structure of PDF.

In the case of three other monoclonal antibodies: PDF-33-derived anti-PDF(3-9) mAb recognized Glu[3]-Ile[4]-Ile[5]-Asn[6]-Ser[7]-Leu[8]-Leu[9]; PDF-243-derived anti-PDF(2-8) mAb recognized Ser[2]-Glu[3]-Ile[4]-Ile[5]-Asn[6]-Ser[7]-Leu[8]; while PDF-264-derived anti-PDF(2-6) mAb recognized Ser[2]-Glu[3]-Ile[4]-Ile[5]-Asn[6].

Drosophila and *Gryllus* PDF peptides have four amino acid differences at positions 4, 10, 14, and 15. A Ser↔Gly difference at position 10 appears to be crucially important, and anti-PDF(6-10) mAb is able to discriminate this Ser/Gly difference very clearly, showing no immunoreactivity to *Drosophila* PDF having Ser[10] instead of Gly[10]. On the other hand, the Leu↔Ile difference at position 4 is not always critical, as described above. Indeed, the NP-G10-derived anti-H_2N(1-5)PDF mAb recognized both Ile[4]-containing *Gryllus* PDF and Leu[4]-containing *Drosophila* PDF. The monoclonal antibodies anti-PDF(1-9) mAb, anti-PDF(3-9) mAb, anti-PDF(2-8) mAb, and anti-PDF(2-6) mAb can all discriminate this difference, however, showing immunoreactivity to *Gryllus* PDF, but not to *Drosophila* PDF (Fig. 3).

Isotypes of monoclonal antibodies

In order to determine the isotypes of monoclonal antibodies that we prepared, the antigen-mediated ELISA procedure was applied using a series of isotype-specific monoclonal antibodies. These included goat antimouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA antibodies. It was found that anti-H_2N(1-5)PDF mAb, anti-PDF(1-9) mAb, anti-PDF(2-8) mAb, and anti-PDF(16-18)$CONH_2$ mAb were all IgG1, while anti-PDF(2-6) mAb, anti-PDF(3-9) mAb, anti-PDF(6-10) mAb, and anti-PDF(17-18)$CONH_2$ mAb were IgM (Table 1). Only anti-PDF(15-18)$CONH_2$ mAb was determined to be IgG2a (Table 1). Thus, most monoclonal antibodies obtained in this study were either IgG1 or IgM. IgM is a pentameric isoform of IgG, and the two have completely different backbone frame structures from each other. This allows us to use these isotypes simultaneously to double-immunolabel tissues, each being differentiated by a different secondary antibody.

Characterization of the polyclonal antibodies

Polyclonal antibodies possess multiple interaction modes because numerous kinds of antibodies occur in the same preparation. In particular, the anti-*Uca* β-PDH pAb is one of the most utilized polyclonal antibodies for detecting insect PDFs (Dircksen et al., 1987; Nässel et al., 1991; Helfrich-Förster et al., 1998; Bloch et al., 2003; Reischig and Stengl, 2003), which we also used in our previous study (Chuman et al., 2002). Additional antibodies have also been raised against insect PDFs, however. For instance, Park et al. (2000) prepared a rat antibody against *Drosophila* PDF, and we have prepared rabbit polyclonal antibodies against PDF in the housefly *Musca domestica* (Sato et al., 2002; Matsushima et al., 2003, 2004). For almost all these antibodies, precise epitope structures have yet to be clarified.

Although it is usually very difficult to determine the epitope structure of a polyclonal antibody, we attempted to depict a general outline of the epitope map of the polyclonal antibodies used in a previous immunocytochemical study on *Gryllus* PDF (Chuman et al., 2002). When these

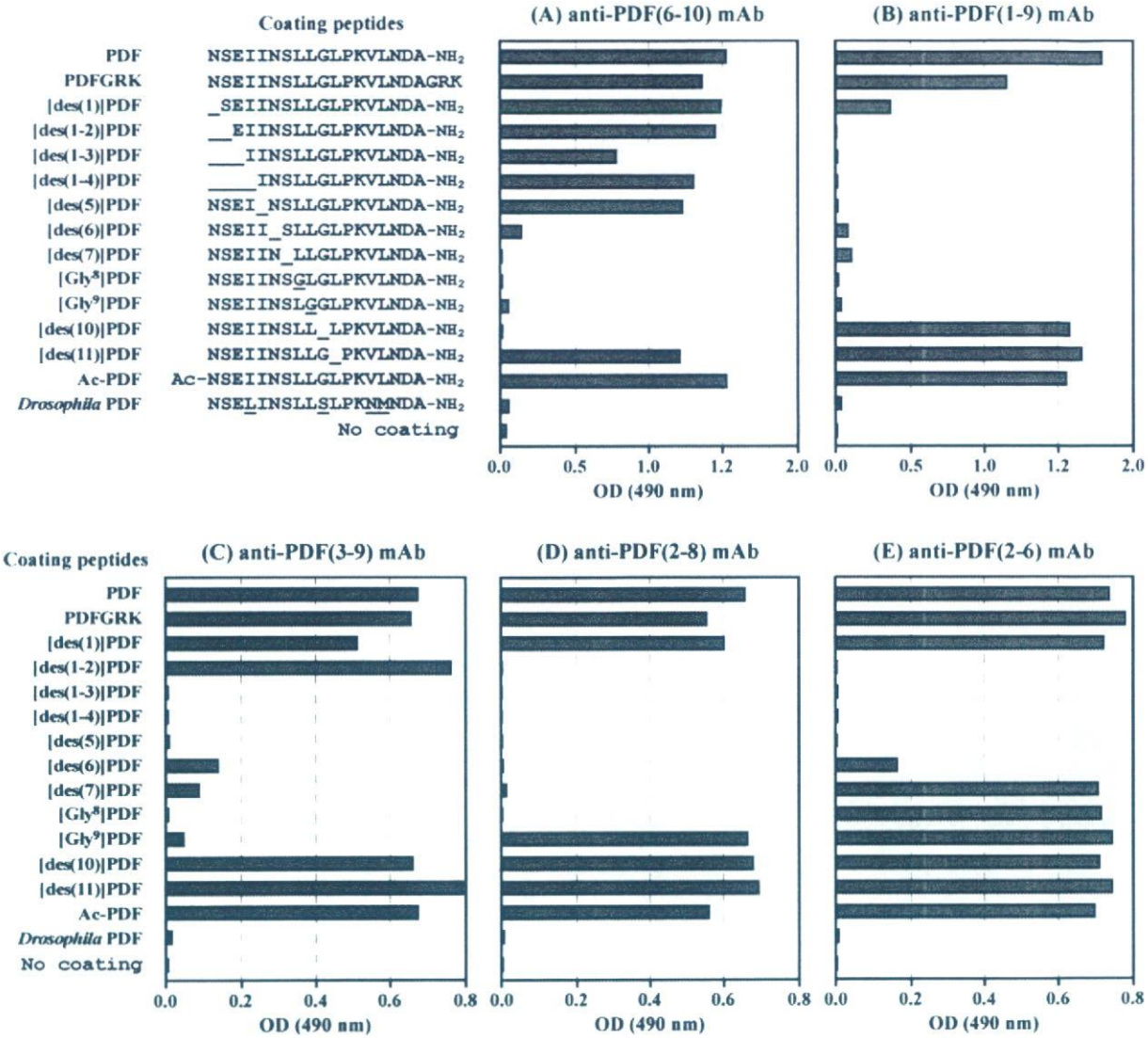

Fig. 3. Epitope analysis of monoclonal antibodies anti-PDF(6-10) mAb (**A**), anti-PDF(1-9) mAb (**B**), anti-PDF(3-9) mAb (**C**), anti-PDF(2-8) mAb (**D**), and anti-PDF(2-6) mAb (**E**). The monoclonal antibodies produced by hybridomas PDF-20, PDF-32, PDF-33, PDF-243, and PDF-264 were examined by the ELISA-based epitope analysis for chemically synthesized *Gryllus* PDF and its analogs. The epitopes of each hybridoma-derived monoclonal antibody are judged to be N^6SLLG^{10} for PDF-20 (therefore denoted anti-PDF(6-10) mAb) (A), $N^1SEIINSLL^9$ for PDF-32 (anti-PDF(1-9) mAb) (B), $E^3IINSLL^9$ for PDF-33 (anti-PDF(3-9) mAb) (C), $S^2EIINSL^8$ for PDF-243 (anti-PDF(2-8) mAb) (D), and S^2EIIN^6 for PDF-264 (denoted as anti-PDF(2-6) mAb) (E).

TABLE 1. Isotypes of Monoclonal Antibodies Prepared against PDF-Like Peptides

Monoclonal antibody	Isotype[1]
anti-H_2N(1-5)PDF mAb	IgG1
anti-PDF(1-9) mAb	IgG1
anti-PDF(2-6) mAb	IgM
anti-PDF(2-8) mAb	IgG1
anti-PDF(3-9) mAb	IgM
anti-PDF(6-10) mAb	IgM
anti-PDF(17-18)$CONH_2$ mAb	IgM
anti-PDF(16-18)$CONH_2$ mAb	IgG1
anti-PDF(15-18)$CONH_2$ mAb	IgG2a

[1]Isotypes were identified by antigen-mediated ELISA using a series of isotype-specific goat antimouse monoclonal antibodies: IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA.

polyclonal antibodies, anti-*Uca* β-PDH pAb (Dircksen et al., 1987) and anti-*Gryllus* PDF pAb (a generous gift from Prof. K. Tomioka), were examined by ELISA, employing peptides used to characterize the monoclonal antibodies described above, they exhibited strong immunoreactivity to *Gryllus* PDF, which was about equal for both mono- and polyclonal antibodies. For analogs of *Gryllus* PDF peptide, both polyclonal antibodies showed very similar immunoreactivity spectra (Fig. 4A,B), although anti-*Uca* β-PDH pAb was ~25–30% less sensitive than anti-*Gryllus* PDF pAb.

When tested with the C-terminal PDF peptides, both polyclonal antibodies showed very much reduced immunoreactivity to PDF-OH, PKVLNGA-NH_2 and PKVLNDG-NH_2, indicating that they contained antibody

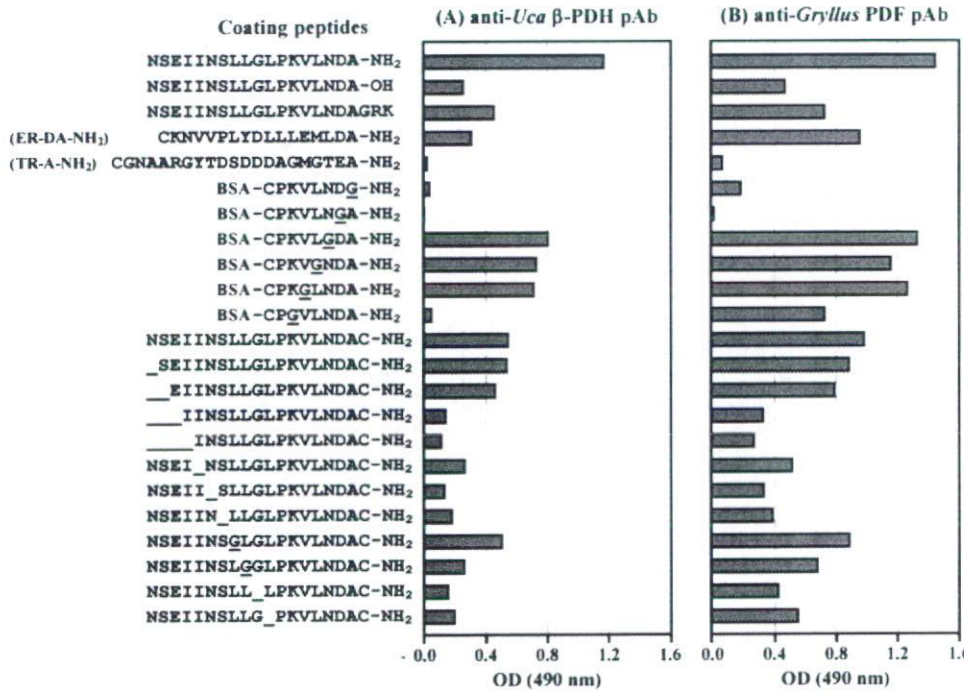

Fig. 4. Epitope analysis of polyclonal antibodies anti-*Uca* β-PDH pAb (**A**) and anti-*Gryllus* PDF pAb (**B**). Polyclonal antibodies anti-*Uca* β-PDH pAb and anti-*Gryllus* PDF pAb were examined by the ELISA-based epitope analysis for a series of chemically synthesized *Gryllus* PDF and its analogs. Both antibodies were completely inactive to BSA. Dilution of antiserum used was 1:12,600 for both antibodies.

components specific for the C-terminal DA amide structure (DA-NH_2). In particular, Asp^{17} is absolutely essential for both polyclonal antibodies. An Asp^{17}→Gly substitution eliminated almost completely the immunoreactivity of both, whereas an Asn^{16}→Gly substitution in PKVLNDA-NH_2 did not alter the reactivity of anti-*Gryllus* PDF pAb, indicating that Asn^{16} is not crucial at all for this antibody to recognize the PDF peptide (Fig. 4B). For anti-*Uca* β-PDH pAb, Lys^{13} is also crucial, since a Lys^{13}→Gly substitution almost completely eliminated immunoreactivity against PKVLNDA-NH_2 (Fig. 4A). Eventually, it was evident that a C-terminal structure of KXXXDA-NH_2 is crucial for anti-*Uca* β-PDH pAb, while DA-NH_2 is crucial for anti-*Gryllus* PDF pAb. This difference was further confirmed by examining immunoreactivity to CKNVVPLYDLLLEMLDA-NH_2 (ER-DA-NH_2), which has the structure LXXXDA-NH_2 instead of KXXXDA-NH_2. Indeed, anti-*Gryllus* PDF pAb exhibited considerable immunoreactivity (~70%) to this non-PDF peptide with a C-terminal DA-NH_2, whereas anti-*Uca* β-PDH pAb exhibited low immunoreactivity (~30%). For the widely used anti-*Uca* β-PDH pAb, Lys^{13} is therefore judged to be one of the most important amino acid residues determining immunoresponses to PDFs and PDHs.

The immunoreactivities of anti-*Uca* β-PDH pAb and anti-*Gryllus* PDF pAb against the N-terminal moiety of *Gryllus* PDF were also tested using a series of PDF analogs based on the structure of NSEIINSLLGLPK VLNDAC-NH_2 (Fig. 4A,B). Both polyclonal antibodies exhibited weak immunoreactivity to PDF analogs lacking an N-terminal tripeptide NSE or tetrapeptide NSEI, indicating that Glu^3 is of crucial importance. Individual elimination of Ile^5, Asn^6, or Ser^7 caused a considerable reduction (80–90% for anti-*Uca* β-PDH pAb and 70–75% for anti-*Gryllus* PDF pAb). When Gly replaced Leu^8 or Leu^9, the antibodies were somewhat less immunoreactive to these analogs (by 40–55% for anti-*Gryllus* PDF pAb and 55–75% for anti-*Uca* β-PDH pAb). A slightly larger reduction was also observed for peptides with the residual elimination of Gly^{10} or Leu^{11}. Collectively, these findings indicate that the N-terminal structure of **EXINSXLGL** at positions 3–11 is of crucial importance for both anti-*Uca* β-PDH pAb and anti-*Gryllus* PDF pAb.

Collectively, the recognition structure of *Gryllus* PDF by anti-*Uca* β-PDH pAb is **XXEXINSXLGLXKXXXDA-NH_2**, the epitope structure of which is represented exactly within *Uca* β-PDH, NS**ELINSILGLPK**VMN**DA-NH_2**. The structure recognized by anti-*Gryllus* PDF pAb has almost the same form, **XXEXINSXLGL**XXXXX**DA-NH_2**, and this antibody shows almost the same immunospecificity as anti-*Uca* β-PDH pAb (Fig. 4). The essential difference between these structures is Lys(=K) at position 13, by which anti-*Uca* β-PDH pAb is able to recognize both PDF and β-PDH. The general conclusion that insect PDF is involved as a principal transmitter in the circadian clock (Dunlap et al., 1999; Shirasu et al., 2003) derives from a growing body of observations obtained using this anti-*Uca* β-PDH pAb; for example, on the brains of the fruit fly *D. melanogaster*, cockroach *Periplaneta americana*, cricket *G. bimaculatus*, and many other species, the amino acid sequences of which contain such consensus residues (Sauman and Reppert, 1996; Renn et al., 1999; Reischig and Stengl, 2003; Chuman et al., 2002; Bloch et al., 2003; Sehadova et al., 2003).

We also examined a rabbit polyclonal antibody that was raised against the *Musca* PDF (Sato et al., 2002; Matsushima et al., 2003, 2004). This polyclonal antibody exhibited high immunoreactivity against *Gryllus* PDF as well as *Musca* PDF, but it showed almost no immunoreactivity against *Gryllus* PDF-GRK (Fig. 5) and none against the

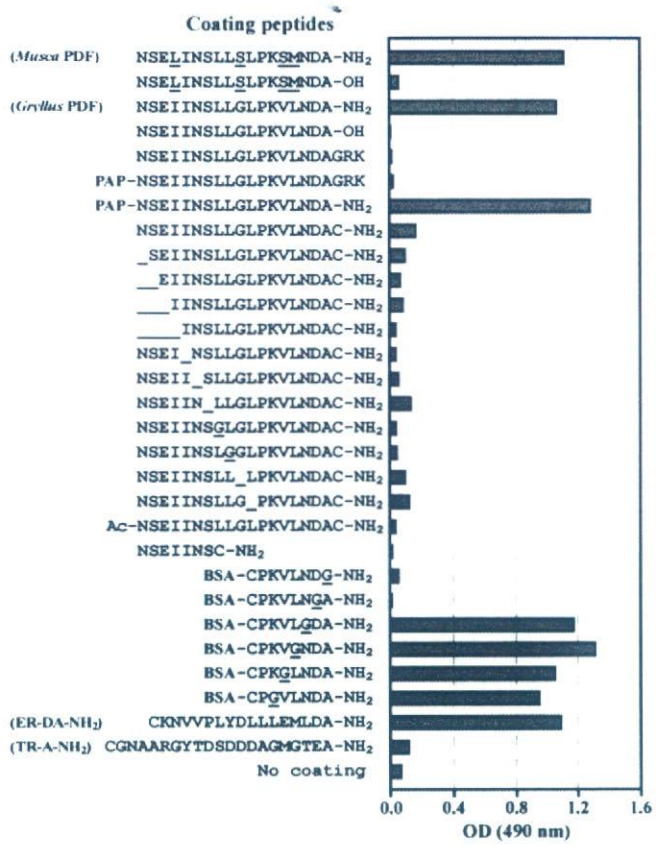

Fig. 5. Epitope analysis of polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb. Rabbit polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb raised against *Musca* PDF was examined by the ELISA-based epitope analysis for chemically synthesized *Gryllus* PDF, its analogs, and also for *Musca* PDF. The antibody 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}-

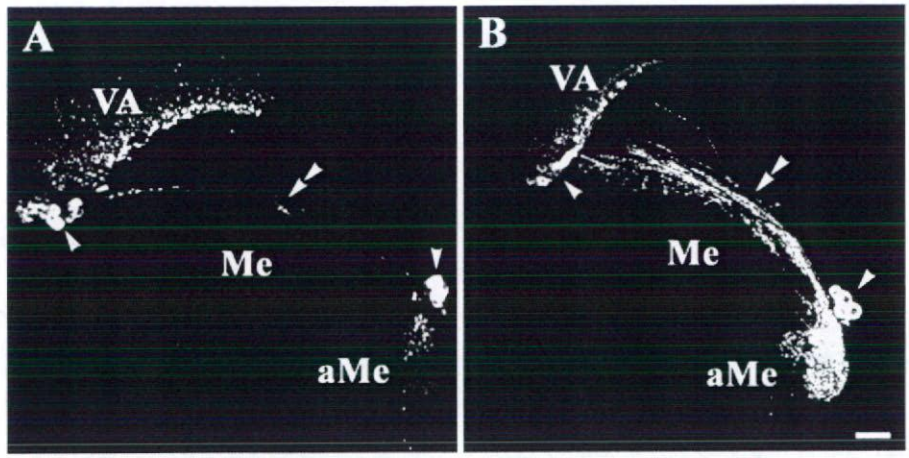

Fig. 6. Horizontal section of the optic lobes of *G. bimaculatus* showing immunoreactivity to anti-H_2N(1-5)PDF mAb (**A**) and anti-PDF(17-18)$CONH_2$ pAb (**B**). Cell bodies (arrowhead) and their axons of a number of neurons are strongly labeled in both sections in the lateral-medial horizontal plane (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.

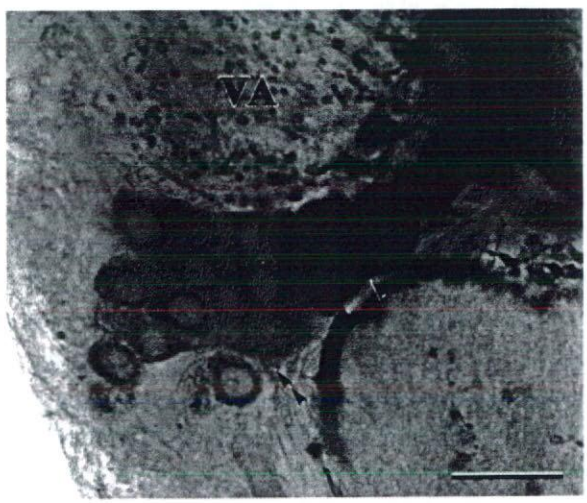

Fig. 7. Horizontal section of the first (lamina) neuropile regions the optic lobes of *G. bimaculatus* showing immunoreactivity to anti-*Uca* β-PDH pAb. Lamina (PDFLa) PDF cells in horizontal Vibratome slices from the optic lobe immunostained by means of the indirect peroxidase-anti-peroxidase technique. 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-

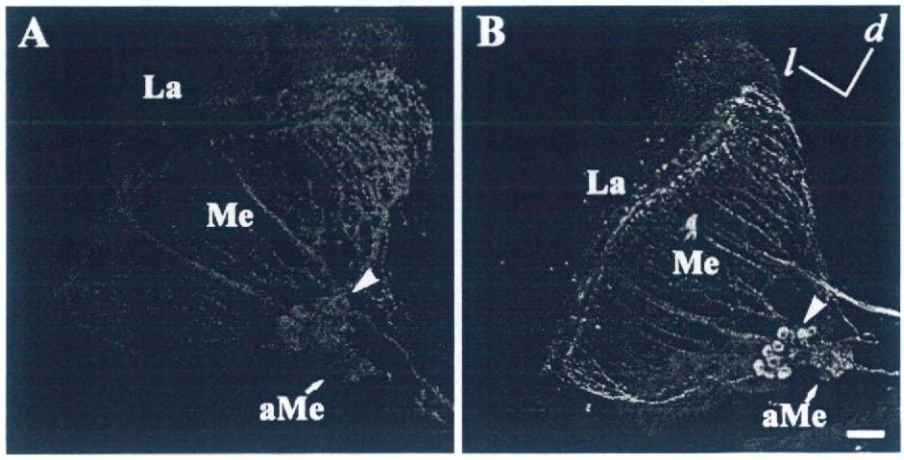

Fig. 8. Frontal image of whole-mount immunocytochemistry of the right optic lobe of *G. bimaculatus* using anti-PDF(17-18)$CONH_2$ mAb (**A**) and anti-PDF(17-18)$CONH_2$ pAb (**B**). PDF medulla cells (PDFMe, arrowheads) and a number of axons in the medulla (Me) are labeled in both samples. The accessory 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.

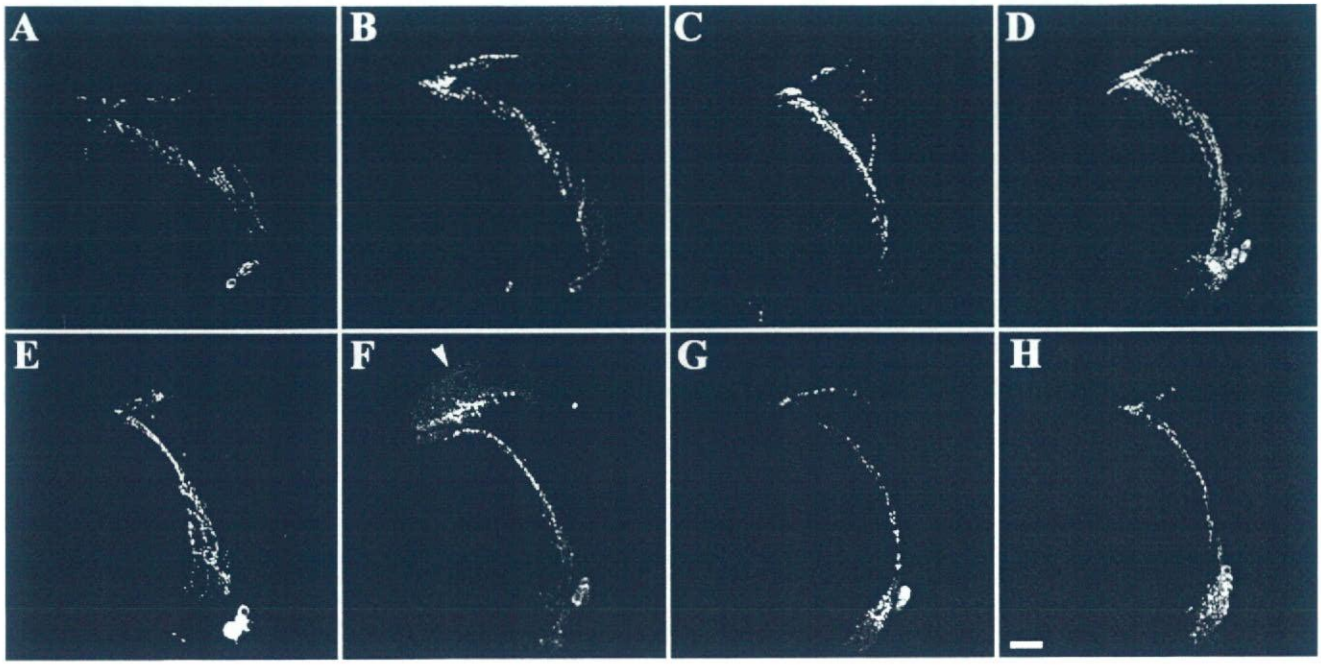

Fig. 9. Immunocytochemistry of a horizontal section of the optic lobes of *G. bimaculatus* by a series of monoclonal antibodies: anti-PDF(6-10) mAb (**A**), anti-PDF(1-9) mAb (**B**), anti-PDF(3-9) mAb (**C**), anti-PDF(2-8) mAb (**D**), anti-PDF(2-6) mAb (**E**), anti-PDF(17-18)$CONH_2$ mAb (**F**), 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.

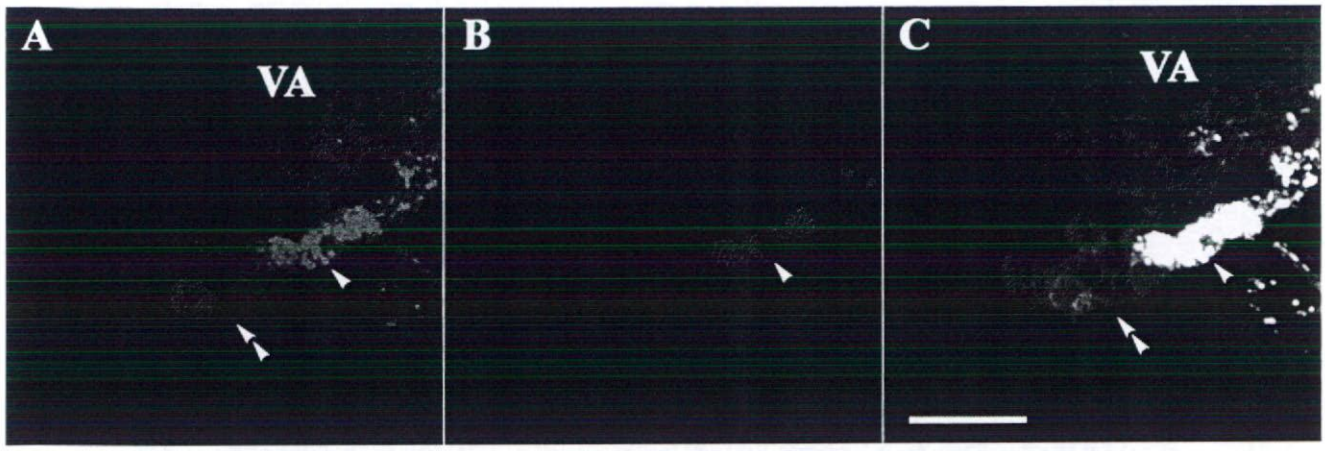

Fig. 10. Immunocytochemistry of horizontal sections of the optic lobes of *G. bimaculatus* by double-labeling with anti-PDF(17-18)$CONH_2$ pAb and anti-PDF(2-8) mAb. Polyclonal antibody anti-PDF(17-18)$CONH_2$ pAb labels the PDFMe cells (not shown in these photographs), both the PDFLa inner (arrowhead) 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.

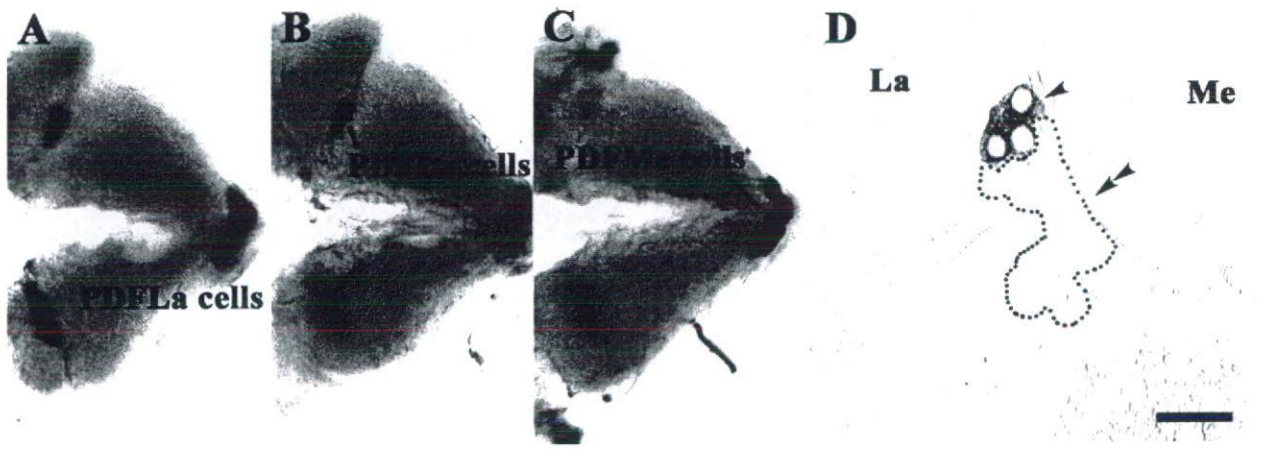

Fig. 11. Expression of *pdf* mRNA observed by whole-mount in situ hybridization in *G. bimaculatus*. The entire brain was mounted in glycerol-gelatin and one of the optic lobes was observed by light microscopy in three focal planes: the cells present at the dorsal region (**A**) and the cells in the 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^5[a]	+++	+++	+++	+++	+++
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-*Gilylus* 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-*Grylus* 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.

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α-Helix Peptides for Bio-Panning in the Phage Display Method to Obtain the Antibodies Specific for Conformation Change in Nuclear Receptors

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Ligand binding to the nuclear receptors (NRs) induces a conformation change. By using a polyclonal antibody to sense such a change, we have established the novel assay procedure to assess simultaneously the activities corresponding to the binding activity and hormonal activity of endocrine disruptors. In order to expand this method to 48 NRs, we attempted to prepare monoclonal antibodies (mAbs) by using phage-display system, and succeeded in the isolation of such mAbs from the Tomlinson I+J library.

Keywords: phage display, bio-panning, antigen peptide, conformation change, estrogen receptor, glucocorticoid receptor.

Introduction

A risk apprehension of endocrine disruptors for the sex hormone receptors has been extensively acknowledged for all of forty-eight human nuclear receptors (NRs) in these several years. It is thus a keen requisite to evaluate comprehensively the chemicals for each NR. We have recently established a novel assay procedure designated as 'conformation-sensing assay', which can estimate simultaneously the activities corresponding to the receptor binding activity and hormonal activity of the chemicals. This method is based on the quantification of a ligand-induced conformation change around the C-terminal domain of NRs, being due mainly to the change in positioning of the amphiphilic α-helix numbered as 12 (H12). The conformation change of H12 is a common feature of most of NRs, and is essential for recruit of coactivator protein [1].

We have recently demonstrated that a polyclonal antibody specific for H12 is able to sense such a conformation change, for example, for the estrogen receptor (ER) and glucocorticoid receptor (GR). A key material for successful conformation-sensing assay is an efficient antibody. In order to expand the methodology to all other NRs, we have attempted to obtain monoclonal antibodies (mAbs) by using phage-display system. This system has a great advantage that human single chain antibodies (scFvs) can be generated without immunization to animals [2]. In this study, to prepare such mAbs, Tomlinson I+J library was subject to bio-panning using

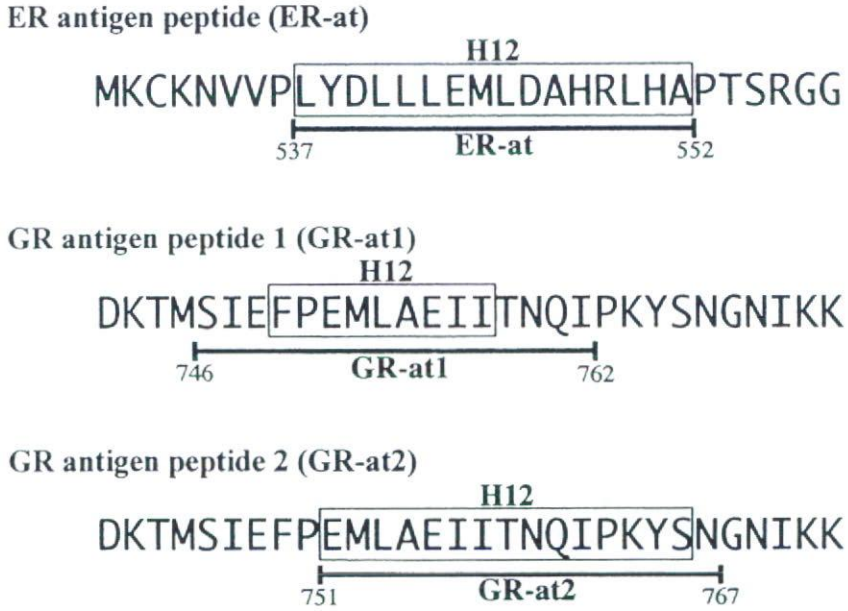

Fig. 1. The amino acid sequence of antigen peptides for bio-panning. Bars truncated indicate the fragment peptides (ER-at, GR-at1, GR-at2) used for preparation of protein KLH-linked antigen. Fragments in the box show the H12 α-helix portion found in each X-ray-clarified 3D-structures.

immobilized antigen peptide containing H12.

Results and Discussion

In order to screen and select the phages desired, antigen peptides containing H12 peptides were synthesized by the Fmoc-based solid phase method. As shown in Fig. 1, two different peptide fragments were chosen as antigens, since GR-H12 was found to adopt α-helix differently against antagonist and agonist (Fig. 1) [3, 4]. To conjugate with a carrier protein keyhole limpet hemocyanin (KLH) or bovine thyroglobulin (BthG), Cys was attached at the N-terminus. Peptides were liberated from the resin by treatment with Reagent K. After gel filtration (Sephadex G-10), peptides were purified by reversed-phase HPLC (LiChrospher 100 RP-18 e (5μm) 4× 250 nm). The mass spectra of peptides were measured to verify their purity on a Voyager™ DE-PRO with the method of MALDI-TOF.

Tomlinson I+J library was kindly provided by Medical Research Council (MRC) (Cambridge, UK). The bio-panning for selection of phages that display scFv specific to antigens was performed repeatedly three times on an immuno-tube coated with purified antigen peptide. ER antigen peptide (ER-at) was immobilized in a 10% TFE/PBS (pH 7.2) solution, whereas GR antigen peptides GR-at1 and GR-at2 were immobilized indirectly as GR-at1 conjugated with KLH and GR-at2 conjugated with BthG. After three-round pannings, phage clones were screened for specific binding to the receptors by ELISA. As the receptors for screening, the ligand-binding domains (LBD) of ER and GR were utilized.

Although no clones specific to ER-LBD were isolated, a number of clones were found to recognize the antigen ER-at (Fig. 2). This result suggests that ER-at lost the appropriate secondary structure owing to directly adsorption onto immuno-tube. For GR, three clones specific to GR-LBD were found and isolated. Sequence analysis of these clones evidenced that two of them (6A and 11B) are identical and do