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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

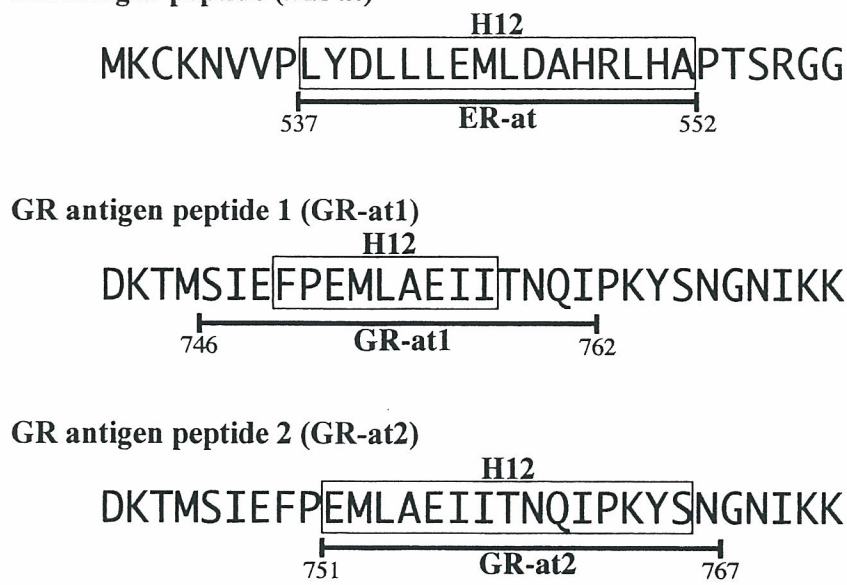

Fig. 1. The amino acid sequence of antigen peptides for bio-panning. Bars truncated indicate the fragment peptides (ER-at, GR-at1, GR-at2) used for preparation of protein KLH-linked antigen. Fragments in the box show the H12 α-helix portion found in each X-ray-clarified 3D-structures.

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

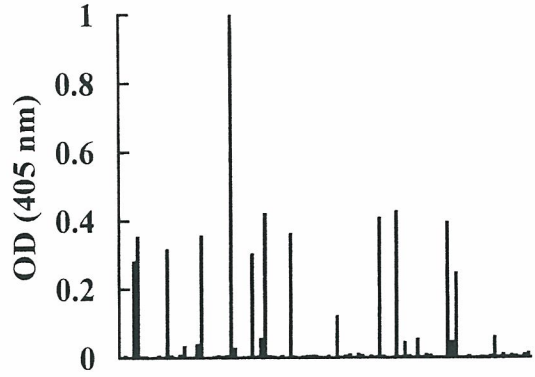

Fig. 2. Screening phage clones in ELISA to evaluate the specific binding to ER-at. The assay was carried out in 10% TFE. A number of clones were found to recognize ER-at, but none of them did bind to ER-LBD.

not contain potential V_HCDR2 and V_HCDR3 regions. The clone 6A was eventually used as a phage-antibody for the conformation-sensing assay.

After titer checking (Fig. 3), competitive ELISA was employed to evaluate the ability of the resulting phage antibody in binding to the ligand-free GR (apo-GR) and ligand-bound GR (holo-GR). Holo-GR was prepared by treatment with 10 μM dexamethasone. It was found that 6A-based phage antibody binds to apo-GR. The capability to differentiate between apo-GR and holo-GR was estimated to be approximately 10%. This result is compatible with our result from the preparation of sensing polyclonal antibody for GR [5].

The present results indicate that the phage display system permit a rapid preparation of monoclonal antibodies (scFvs), which recognize the conformation change of H12. It is essential for bio-pannings to take the conformation of immobilized antigen peptide into consideration.

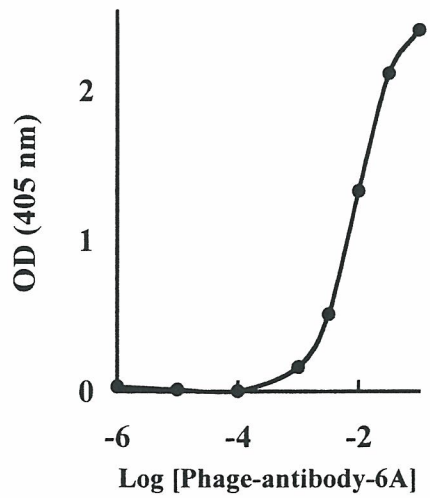

Fig. 3. GR-at1 binding curve for phage-antibody-6A by ELISA. GR-at1 was immobilized at 2.5 μg/ml. GR-at 2 binding curve was depicted almost as above.

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Conformation Sensing Assay Using Polyclonal Antibody Specific for the C-terminal α-helix of Glucocorticoid Receptor and Progesterone Receptor

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We have prepared polyclonal antibodies that discriminate the ligand-bound and ligand-free conformations of the glucocorticoid receptor (GR) and progesterone receptor (PR). These antibodies were raised against the C-terminal H12 α-helix of GR and PR, which conducts a conformation change along with the ligand binding. Using these antibodies, we established the conformation change-sensing assay that affords the receptor activity parameters corresponding to the receptor binding activity and the hormonal activity.

Keywords: conformation-sensing assay, endocrine disruptors, glucocorticoid receptor (GR), polyclonal antibody, progesterone receptor (PR)

Introduction

Endocrine disruptors are the chemicals that cause the interference or disorder in the endocrine system. Estrogen receptor (ER) is one of the nuclear receptors and a target binding protein of such chemicals. It is known that a number of synthetic chemicals produce undesirable effects on the reproduction system, especially in the process of fetal development in animals and humans. For efficient risk assessment of such undesirable effects, many efforts have been done to develop novel methodolgies. For instance, we did establish a quite novel procedure designated 'conformation-sensing assay.' Preparing a polyclonal antibody that discriminates the conformations of the C-terminal α-helix (designated as H12) of ER, we constructed an assay method to estimate the parameters corresponding to the receptor binding activity and the hormonal activity. H12 is in different positionings, depending upon either the ligand-free or ligand-bound form of ER.

Endocrine disruptors are now aknowledged to have a damaging influence upon not only estrogen receptor, but also all other nuclear receptors including glucocorticoid receptor (GR) and progesterone receptor (PR). Glucocorticoids have a wide range of functions including the regulation of plasma glucose concentration, fat and protein metabolism, and have effects on anti-inflammatory and immunosuppressive actions. On the other hand, progesterone plays an important role in reproductive physiology, as well as bone metabolism and neurotrophic functions. In the present study, we

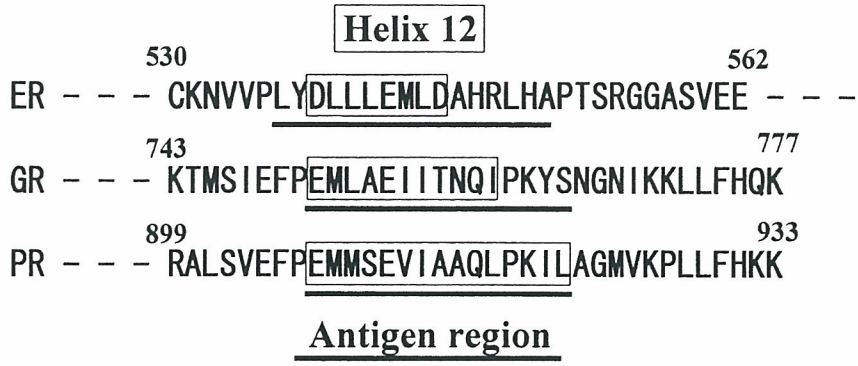

Fig. 1. Peptide fragments selected for preparation of conformation-sensing antibody. Cysteine was attached at the N-terminus of antigen peptides.

attempted to establish the efficient conformation-sensing assay system to endocrine disruptors for GR and PR.

Results and Discussion

To prepare the antibody against H12 in GR and PR, antigen peptides were synthesized by the Fmoc synthetic strategy. To conjugate to career protein KLH, Cys was attached at the N-terminus of each antigen peptide (Fig 1). After immunization into rabbit, the serum collected was first purified by immunoprecipitation with KLH. Further purfication was carried out by affinity chromatograghy using agarose gel conjugating the antigen peptide. Purified antibodies were examined for their ability to bind to the receptor and the target peptide. Both anti-GR and anti-PR antibodies were found to recognize the receptor more strongly than the peptide.

To establish the conformation-sensing assay, bovine thyroglobulin-linked H12-peptide was coated onto the polypropylene 96-well immunoplate as a competiter. Each antibody was examined with or without agonist for the GR and PR, and the agonist used were dexamethasone (Dex) for GR and progesterone for PR. 10 μM of these chemicals were used to construct a ligand-bound holo-form of the receptor. Ligand-free apo-form was examined just without the chemicals. Anti-GR antibody was found to exhibit a sufficient ability to differenciate the apo- and holo-forms of GR (Fig. 2). Also, anti-PR antibody showed such apporopriate immunoresponses under the conditions with or without progesterone (Fig. 2).

At the constant concentration (1 nM for PR, 0.1 nM for GR) of the receptor, the immunoreactivities of each antibody against either the receptor or the peptide fixed on the well was measured under the varying concentrations of chemicals. This was analyzed by plotting the % immunoresponses at each concentration of the chemicals. The maximal immunoresponse R_{max} (%) and the concentration (EC_{50}) to yield a half-maximal immunoresponse $R_{max}/2$ were estimated from this plotting. The R_{max} values were estimated by calculating the immunoresponses relative to the maximal responses of Dex for GR and progesterone for PR. Based on these basal analyses, a series ofchemicals were evaluated their affinity for GR and PR.

As shown in Table 1, cortisol, a natural agonist of GR, exhibited considerably

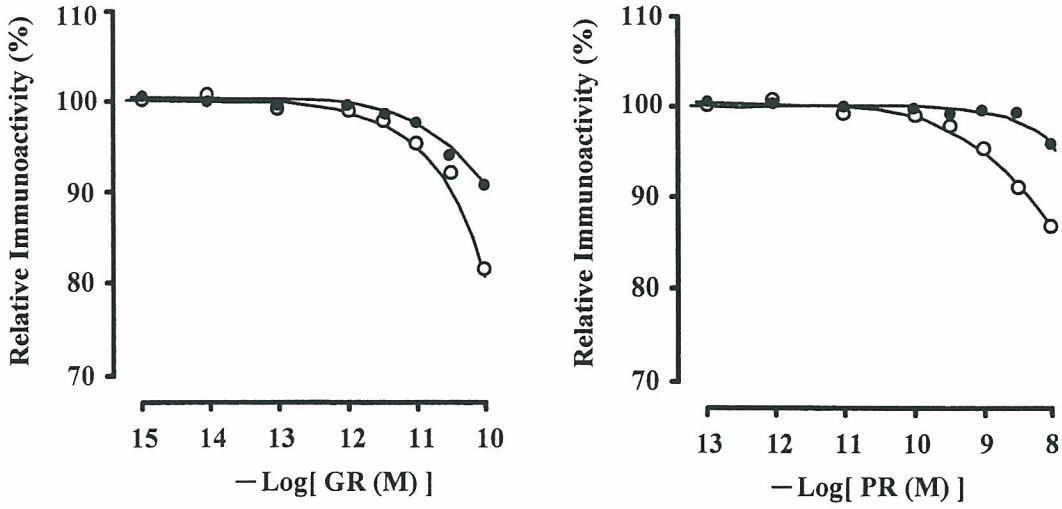

Fig. 2. Immunoresponses of anti-GR (A) and anti-PR (B) antibodies against apo-form (○) and holo-form (λ) of each nuclear receptor. Holo-form was with 10 μM deamethasone (A) and progesterone (B).

Table 1. Immunoresponses of chemicals against glucocorticoid receptor.

Chemicals	EC_{50} (nM)	R_{max} (%)
dexamethasone	32	100
cortisol	860	52
mifepristone	N.B.	N.B.
aldosterone	N.D.	N.D.
cyproterone acetate	N.B.	N.B.

N.B. means that the chemical did not show the immunoresponse. N.D. means that the chemical showed the weak immunoresponse and its EC_{50} value was not able to be determined.

Table 2. Immunoresponses of chemicals against progesterone receptor.

Chemicals	EC_{50} (nM)	R_{max} (%)
norethisterone	33	97
progesterone	87	100
levonorgestrel	220	88
11-ketoprogesterone	N.D.	N.D.
mifepristone	N.B.	N.B.
cyproterone acetate	N.B.	N.B.

N.B. means that the chemical did not show the immunoresponse. N.D. means that the chemical showed the weak immunoresponse and its EC_{50} value was not able to be determined.

weak activity (EC_{50} = 860 nM) as compared to that of Dex (32 nM). Although The result correlated with the data of the receptor binding assay [1], this weak activity may indicate that the antibody obtained is somehow insufficient to discriminate the conformation change induced by cortisol. However, this antibody did not respond to the antagonist mifepristone and cyproterone, indicating that it distinctly discriminates between the agonists and antagonists.

Similar results were also obtained for anti-PR antibody as shown in Table 2. Agonist progesterone was found to be slightly weaker than another agonist norethindrone. Antagonist mifepristone and cyproterone exhibited no responses against this anti-PR antibody. The results clearly show that the antibody discriminates between the agonist- and antagonist-bound conformations of PR.

It is known that PR ligands also bind to androgen receptor (AR) and GR. For example, levonorgestrel was synthesized as a progestin, but it is highly active also to GR and AR [2,3]. Mifepristone is known as antagonist against AR, GR and PR [4,5]. Nonetheless, we could establish the conformation sensing assay for nuclear receptors GR and PR.

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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.

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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,

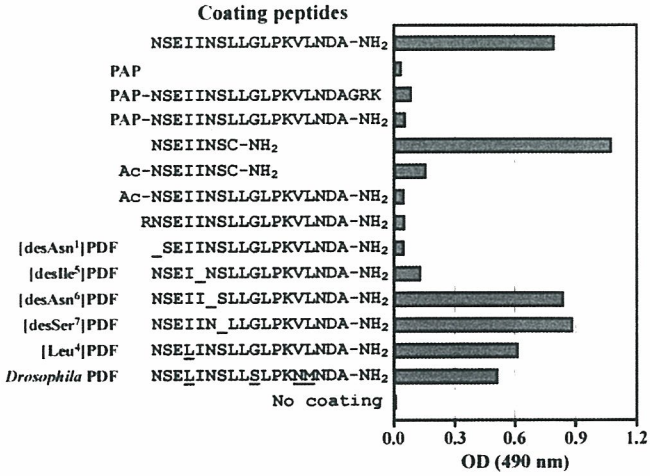

Fig. 1. Epitope analysis of monoclonal antibody anti-$H_2N(1\text{-}5)$PDF mAb. The monoclonal antibody produced by hybridoma NP-G10 was examined by the ELISA-based epitope analysis for a series of chemically synthesized peptides of *Gryllus* PDF and its analogs. No immunoreactivity was found when no 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 $H_2N\text{-}N^1SEII^5$ 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,

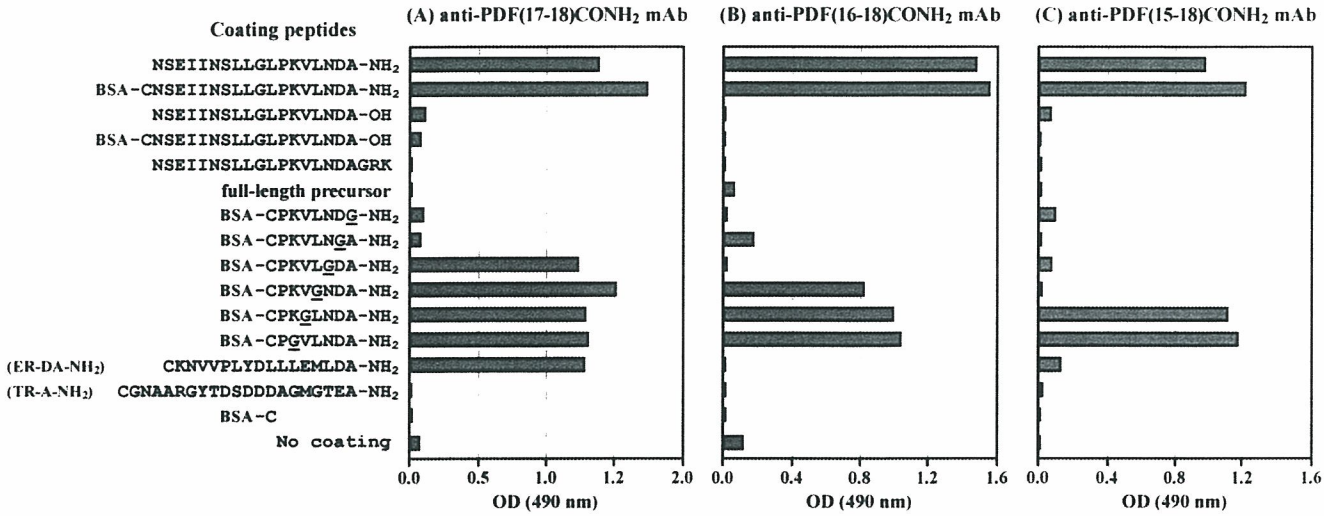

Fig. 2. Epitope analysis of monoclonal antibodies anti-PDF(17-18)$CONH_2$ mAb (**A**), anti-PDF(16-18)$CONH_2$ mAb (**B**), and anti-PDF(15-18)$CONH_2$ mAb (**C**). A series of monoclonal antibodies produced by hybridomas PDF-140, PDF-300, and PDF-432 were examined by the ELISA-based epitope analysis 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

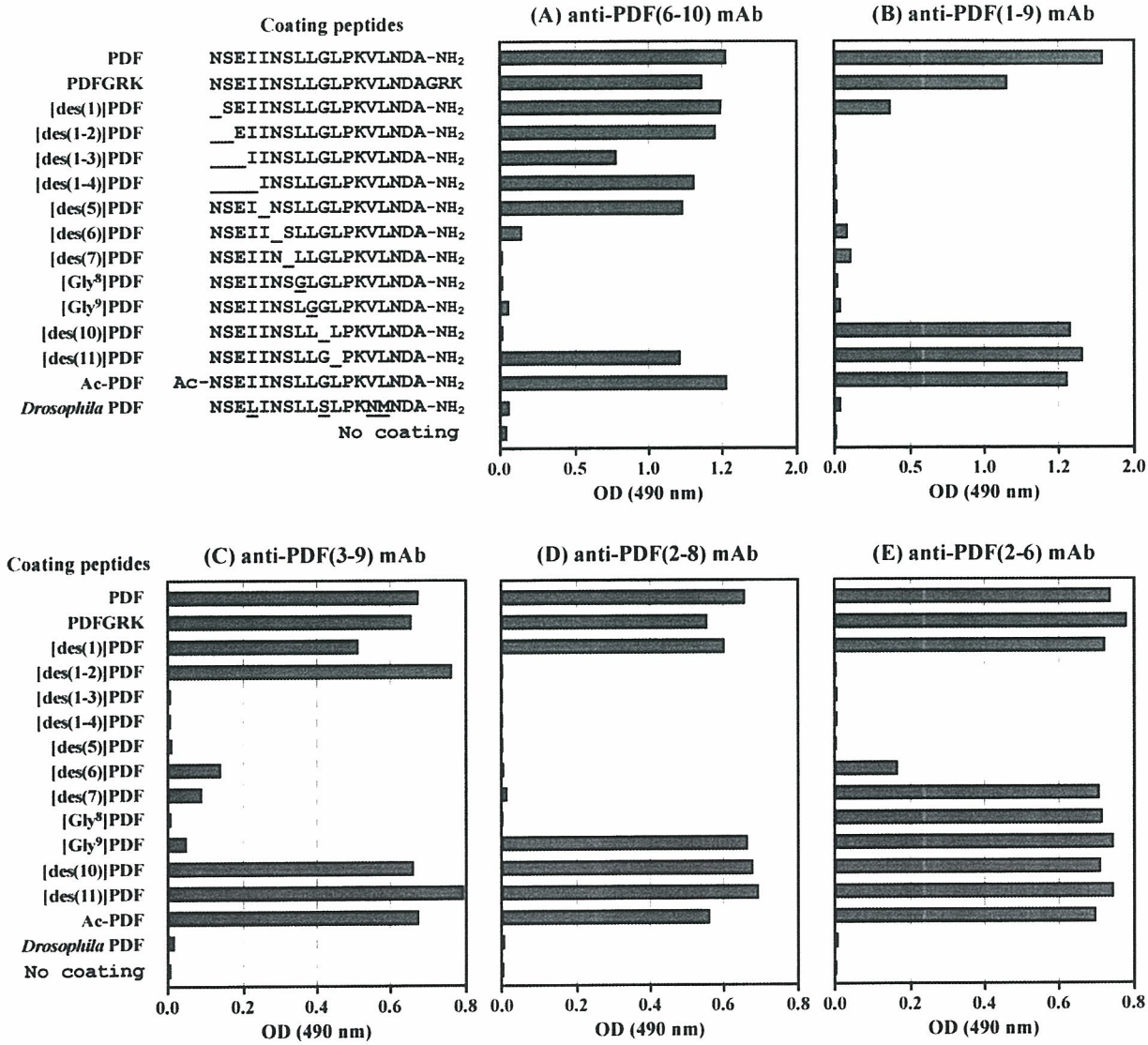

Fig. 3. Epitope analysis of monoclonal antibodies anti-PDF(6-10) mAb (**A**), anti-PDF(1-9) mAb (**B**), anti-PDF(3-9) mAb (**C**), anti-PDF(2-8) mAb (**D**), and anti-PDF(2-6) mAb (**E**). The monoclonal antibodies produced by hybridomas PDF-20, PDF-32, PDF-33, PDF-243, and PDF-264 were examined by 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

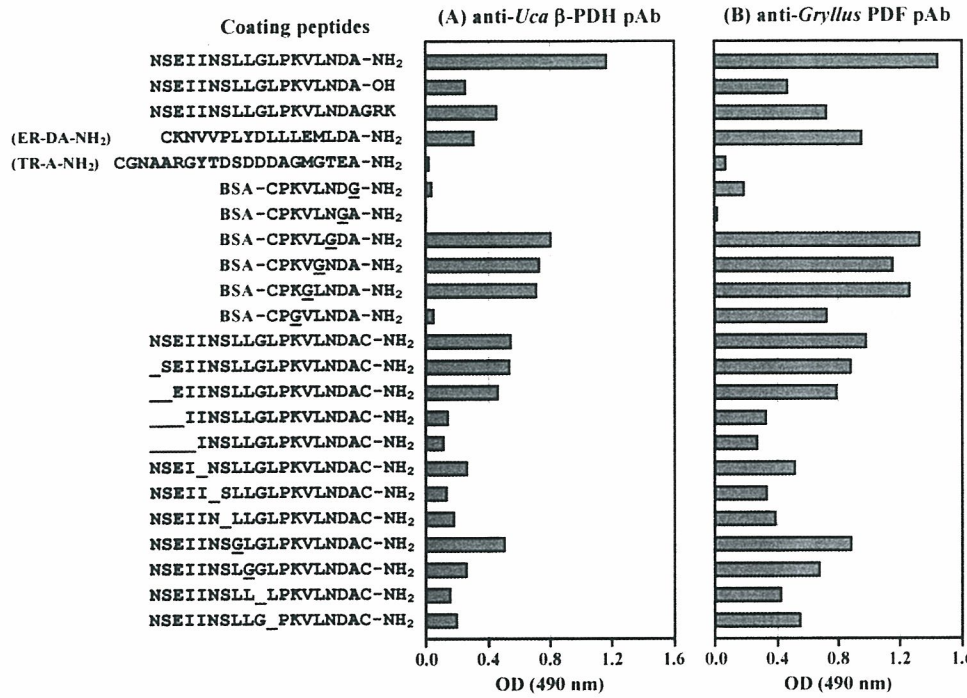

Fig. 4. Epitope analysis of polyclonal antibodies anti-*Uca* β-PDH pAb (**A**) and anti-*Gryllus* PDF pAb (**B**). Polyclonal antibodies anti-*Uca* β-PDH pAb and anti-*Gryllus* PDF pAb were examined by the ELISA-based epitope analysis for a series of chemically synthesized *Gryllus* PDF and its analogs. 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, NSE**LINSI**LGLPKVMN**DA-NH_2**. The structure recognized by anti-*Gryllus* PDF pAb has almost the same form, **XXEXINSXLGLXXXXXDA-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