were washed with the phosphate buffered saline (PBS) with 0.1% Tween 20 (PBT) at 4 °C, dehydrated with a series of MeOH solutions (25%, 50%, 75% and 2x 100%) in PBT, and stored at −20 °C until use.

All the following procedures were performed at room temperature unless a specified temperature is noted. Tissues were hydrated in reverse in a series of MeOH–PBT solutions (75%, 50%, 25% MeOH, and 2x PBT). These rehydrated tissues were permeabilized with 10 μg/ml proteinase K at 37 °C for 15 min, washed with glycine (2 mg/ml) for 5 min, and then washed twice with PBT. They were fixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBT for 20 min, washed twice with PBT, and incubated in PBT at 70 °C for 50 min. After cooling on ice, the tissues were treated with 6% H_2O_2 for 1 h, washed three times with PBS, and incubated with prehybridization buffer (50% formamide, 5x SSC (pH 4.5), 1% SDS, 50 μg/ml yeast tRNA and 50 μg/ml heparin) at 70 °C for 1 h. The prehybridization buffer was then replaced with hybridization buffer, in which the prehybridization buffer contains a DIG-labelled cRNA probe, for incubation at 70 °C for 16 h.

After hybridization, the tissues were washed three times at 70 °C with solution I (50% formamide, 5x SSC (pH 4.5), and 1% SDS) for 30 min, respectively, and once at 70 °C with a 1 : 1 solution of I and II (solution II consists of no formamide) for 10 min. The tissues were washed a further three times with solution II for 5 min, and then incubated with solution II for 20 min. Washings were repeated twice with solution III (50% formamide, 2x SSC (pH 4.5), and 1% SDS) for 5 min at 70 °C, three times with solution III for each 30 min, and with a TBST solution in which Tris-buffered saline (TBS; 150 mM NaCl, and 100 mM Tris-HCl (pH 7.5)) contains 1% Tween 20. After blocking at room temperature for 1 h, in order to visualize mRNA-bound probe by immunocytochemical detection the tissues were treated with Fab fragments of sheep anti-DIG antibody directly conjugated to alkaline phosphatase (AP). This incubation was done for 16 h at 4 °C, and the tissues were washed with TBST for 5 min successively four times and for 1 h seven times. After washing twice with NTMT (100 mM NaCl, 100 mM Tris-HCl (pH 9.5), 50 mM $MgCl_2$, and 1% Tween 20) for 5 min, the antibody detection reaction was performed by incubating the tissues in the 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 min in shaded light, and finally washed with TBST. Eventually, whole tissues were mounted in glycerol–gelatin (Sigma, St Louis, MO, USA) and observed by light microscopy with an Olympus BX50.

RESULTS

Preparation of a *Musca domestica* PDF cDNA

Adult houseflies, *Musca domestica*, were maintained in a daily cycle of L12 : D12. Immediately after the flies were frozen in liquid nitrogen, their heads were collected. To isolate mRNA, the procedure designed for direct isolation of polyadenylated RNA without purifying the total RNA was used. Isolated mRNAs were then all converted to cDNAs by the RT-PCR method, in which the reverse transcriptase catalyses the polymerization of DNA using mRNA as templates.

3′ RACE to Determine the 3′-end Sequence of a *Musca domestica* PDF cDNA

With respect to the nucleotides of insect PDF precursors, no prominent similarities were found except for the *N*-terminal portion of the PDF peptide regions. Thus, in order to screen for the *pdf* cDNA of the housefly *M. domestica*, the 3′ RACE method was carried out. A PDF-specific degenerated sense primer for 3′ RACE was designed with reference to sequence alignment analyses for both amino acid and nucleotide sequences. The *N*-terminal portion of PDF, namely, the residue $-2 \sim 7$ including the dibasic processing site (KR), was chosen as a PDF-specific sense primer (PDF-S1). PCR with a primer of PDF-S1 and an adapter primer Ad lacking $d(T)_{17}$ yielded no distinct gel-bands, and thus the resulting PCR product was used directly as a template for further PCR under the same conditions. Of the several PCR products detected by electrophoresis as bands in the agarose gel, only one PCR product was found to code a polynucleotide of about 250 bp with the characteristic 18-amino acid peptide, PDF. The nucleotide sequence determined also encoded a PDF amidation sequence in addition to the 3′-UTR non-coding region (Figure 1). The open-reading frame encoding PDF was highly conserved among *pdf* and *pdh* cDNAs.

5′ RACE to Determine the 5′-end Sequence of a *Musca domestica* PDF cDNA

To determine the structure of the 5′-end of *pdf* cDNA, the 5′ RACE method was undertaken. Based

Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

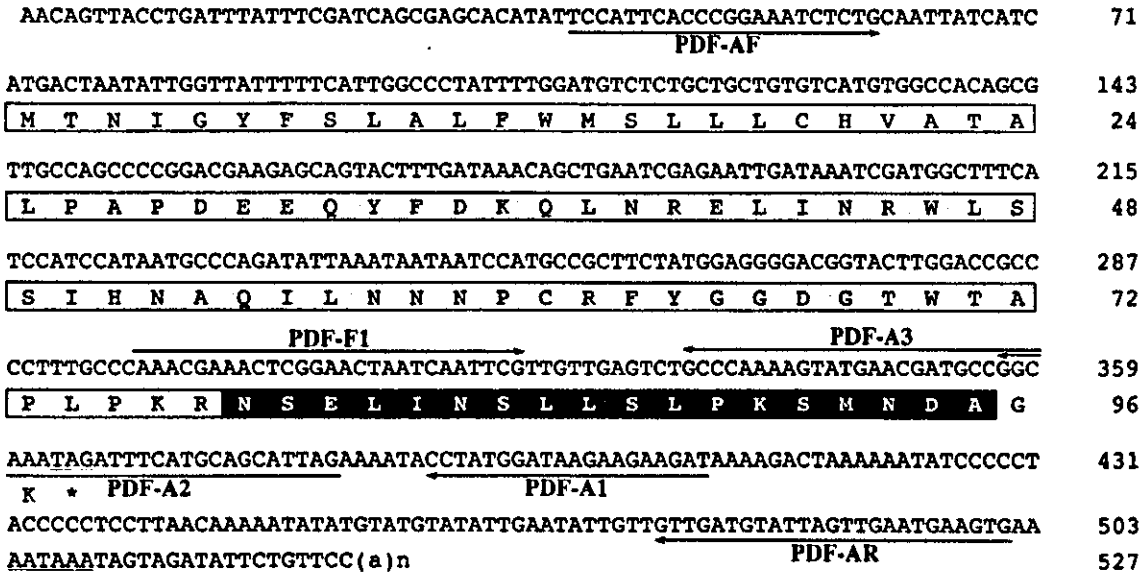

Figure 1 Nucleotide sequence and its deduced amino acid sequence for the *Musca domestica* PDF precursor. Arrows indicate the primers used for the 3′ RACE and 5′ RACE methods. Amino acid sequences in white, grey and black boxes correspond to the signal, PAP and PDF regions, respectively. KR (=Lys-Arg) is a processing cleavage site, while the *C*-terminal GK (=Gly-Lys) is an amidation site. The underlined portion with the asterisk (TAG) is a stop codon. The double-underlined portion (AATAAA) in the 3′-UTR region is a putative poly-adenylation signal site.

on the sequence information obtained from 3′ RACE, the antisense primer for RT-PCR against PDF mRNA was set at the 3′-UTR region (Figure 1). First-strand cDNA was synthesized from mRNA using a PDF-specific antisense primer PDF-A1 and a reverse transcriptase. A characteristic reaction of the 5′ RACE method is an addition of a poly(C) tail to the 3′-end of cDNA obtained. The resulting poly(C)-tailed cDNA was utilized as a template for PCR using an anchor primer AAP, having a poly(I) truncated with deoxyguanosine, G/poly(I)/G, that enables the primer to anneal specifically to poly(C). PCR was carried out by using AAP and PDF-specific antisense primer PDF-A2, which was assigned at the 3′-UTR region. The anchor primer AAP possesses a specific 5′-end anchor sequence, and a subsequent nested PCR was performed using antisense PDF-A3 (assigned at the PDF region) and another anchor primer AUAP lacking G/poly(I)/G. This nested PCR provided a single product that was subsequently subcloned for sequence analysis.

The sequencing analysis afforded complete sequence data including 5′-UTR, signal region and PAP peptide region (Figure 1). The cDNA sequence was further confirmed by RT-PCR using primers set for both 5′-UTR and 3′-UTR to amplify the full-length PDF cDNA. Eventually, the full length of the cDNA clone was found to comprise 527 bp, with a precursor protein of 97 amino acid residues (Figure 1). The initiator codon ATG was assigned as shown in Figure 1, since the selected ATG codon and its adjacent nucleotide sequences fulfilled Kozak's consensus motif [19]. No additional ATG codon was found in the upstream region of any cDNA clones isolated in the RT-PCR analysis. It should be noted that the RLM-RACE method afforded exactly the same oligonucleotide sequence of *pdf* cDNA.

The program for predicting a signal peptide cleavage site, SignalP, showed that 24 amino acids from a selected Met should, according to eukaryote data, be a signal peptide. The *N*-terminal Met-Thr-Asn-Ile-Gly-Tyr-Phe-Ser is unique, showing no sequence similarity to other PDF and β-PDH precursors. Only *Romalea microptera* PDF precursor exhibited an initiating sequence of Met-Thr, although this is followed by the sequence Ala-Met-Ala-Val-Ser-Gly [20].

Identification of *pdf* Expressing Cells

The whole-mount *in situ* hybridization was performed to identify the cells expressing *pdf* mRNA. The *M. domestica pdf* cDNA exhibited a normal segment construction as seen for *Drosophila* PDF

Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

preproprotein, and this *pdf* gene encoded the PDF peptide in an ordinary form. It is important to locate the cells containing mRNA and to analyse the coincidence of the results from the *in situ* hybridization for *pdf* mRNA and immunocytochemistry for PDF peptide. The signals of *in situ* hybridization in *M. domestica* brain were observed only at the single region restricted in the medulla of the optic lobes (both right and left). In each optic lobe, a single cell group called large lateral neurons was found to be positive for hybridization. As shown in Figure 2, a group of immunoreactive cell clusters were identified by anti-DIG antibody conjugated to alkaline phosphatase, showing *pdf* mRNA expression in somata. In each optic lobe, there are two clusters containing at least four cells, respectively.

DISCUSSION

The *Musca* and *Drosophila* PDF Peptides Have a Highly Conserved Primary Structure

The unique amino acid residue of *Drosophila* PDF is Ser^{10}, since Gly^{10} is preserved among the known sequences of all other arthropod species. It is now known that this unusual Ser^{10} is retained in *Musca* PDF. In some cases, Gly → Ser substitutions are evolutionarily significant in attaining a certain characteristic structure or conformation necessary for specific interactions. As described before, peptides and proteins of the fruit fly *Drosophila* are often said to be intermediate between those of vertebrate and invertebrate groups. Insofar as this generalization holds true, the PDF and its precursor protein of the housefly *Musca* must be siblings of those in *Drosophila*. When the amino acid sequence of *Musca*'s PDF peptide, NSELINSLLSLPKSMNDA-amide, was compared with that of *Drosophila* PDF, only a single residue substitution was found at position 14 (Table 1). The Asn^{14} residue in *Drosophila* PDF was replaced by Ser in *Musca* PDF. It should be noted that Ser and Asn are both hydrophilic, while all other insect PDFs and crustacean PDHs contain a hydrophobic Val, Leu, or Phe, at that position. All these features indicate that the PDF peptides in *Musca* and *Drosophila* evolutionarily are highly related to each other and different from all other PDF and PDH peptides.

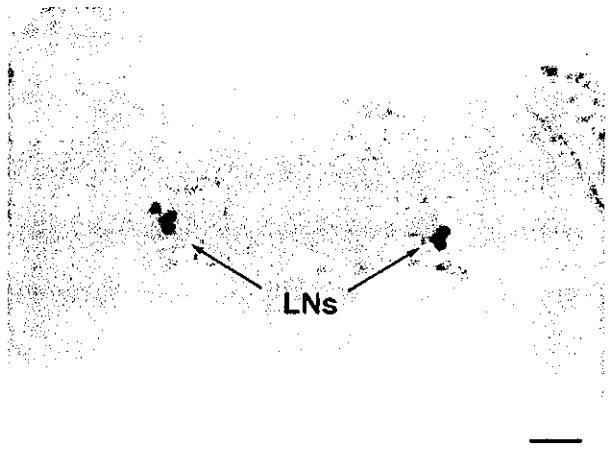

Figure 2 Spatial expression of *pdf* mRNA observed by the whole mount *in situ* hybridization. Only lateral neurons (LNs) in the anterior cortex of the medulla in the optic lobe show positive signals in this *in situ* hybridization. Scale 100 μm.

Despite this difference, PDF in *Musca* is very similar to other ordinary PDFs and β-PDHs (Table 1). The amino acid sequence identity and similarity of *Musca* PDF were calculated to be 78%–94% and 89%–100%, respectively. A two-residue substitution was found between PDF in *Musca* and β-PDH of the crustacean *Penaeus vannamei II*; *i.e.* $Ser^{10} \leftrightarrow$ Gly and $Ser^{14} \leftrightarrow$ Val. On the other hand, one insect PDF and nine crustacean β-PDHs exhibited three-residue substitutions (Table 1). In addition to $Ser^{10} \leftrightarrow$ Gly and $Ser^{14} \leftrightarrow$ Val substitutions, $Leu^{8} \leftrightarrow$ Ile or $Leu^{11} \leftrightarrow$ Ile are found when comparing *Musca* PDF and crustacean β-PDHs. The only exception is $Asn^{16} \rightarrow$Thr seen for *Penaeus japonicus I* β-PDH. As for PDF in the insect *Periplaneta americana*, the third substitution occurs at position 15 with the $Met^{15} \rightarrow$ Leu replacement. Four-residue substitutions were found when comparing four insect PDFs and four crustacean β-PDHs (Table 1). In respect of the amino acid sequence, all crustacean α-PDHs are by contrast quite definitely different from *Musca* PDF.

Insect PDF Precursors Are Classified into Three Groups

It was previously hypothesized that PDF precursors are classified into three different types: the PDF precursors of *Drosophila*, *Romalea* and *Gryllus*. With the recent achievement of the cloning of the cDNA of the PDF precursor from the last-summer cicada, *Meimuna opalifera*, it was found that the PDF precursors of *Meimuna opalifera* and *Romalea microptera* are very similar to each other. Their peptide sizes are $n = 82$ and 89, respectively, with high homology scores for sequence

Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Table 1 Amino Acid Sequences of Insect PDFs and Crustacean α- and β-PDHs

Number of residues different	Species	Peptide sequence	PDF or PDH	Ref.
	Musca domestica	NSELINSLLSLPKSMNDA-NH_2	PDF	
1	*Drosophila melanogaster*	NSELINSLLSLPKNMNDA-NH_2	PDF	[9]
2	*Penaeus vannamei* II	NSELINSLLGLPKVMNDA-NH_2	β-PDH	[16]
3	*Penaeus vannamei* I	NSELINSLLGIPKVMNDA-NH_2	β-PDH	[16]
	Periplaneta americana	NSELINSLLGLPKVLNDA-NH_2	PDF	[21]
	Pandalus jordani[a]	NSELINSLLGLPKVMTDA-NH_2	β-PDH	[22,23]
	Penaeus aztecus	NSELINSLLGIPKVMNDA-NH_2	β-PDH	[24]
	Uca pugilator	NSELINSILGLPKVMNDA-NH_2	β-PDH	[25]
	Cancer magister	NSELINSILGLPKVMNDA-NH_2	β-PDH	[26]
	Pacifastacus leniusculus	NSELINSILGLPKVMNDA-NH_2	β-PDH	[22]
	Carcinus maenas	NSELINSILGLPKVMNDA-NH_2	β-PDH	[13]
	Callinectes sapidus I	NSELINSILGLPKVMNDA-NH_2	β-PDH	[15]
	Penaeus japonicus II	NSELINSLLGLPKFMIDA-NH_2	β-PDH	[27]
4	*Acheta domesticus*	NSEIINSLLGLPKVLNDA-NH_2	PDF	[28]
	Romalea microptera	NSEIINSLLGLPKLLNDA-NH_2	PDF	[20]
	Gryllus bimaculatus	NSEIINSLLGLPKVLNDA-NH_2	PDF	[10]
	Metmuna opalifera	NSEIINSLLGLPKVLNDA-NH_2	PDF	[11]
	Penaeus japonicus I	NSELINSLLGIPKVMTDA-NH_2	β-PDH	[27]
	Procambarus clarkii	NSELINSILGLPKVMNEA-NH_2	β-PDH	[29]
	Orconectes limosus	NSELINSILGLPKVMNEA-NH_2	β-PDH	[14]
	Orconectes immunis	NSELINSILGLPKVMNEA-NH_2	β-PDH	[22]
6	*Callinectes sapidus* II	NSELINSLLGISALMNEA-NH_2	β-PDH	[15]
	Armadillidium vulgare	NSELINSLLGAPRVLNNA-NH_2	β-PDH	[30]
7	*Pandalus jordani*[a]	NSGMINSILGIPKVMADA-NH_2	α-PDH	[22]
8	*Pandalus borealis*	NSGMINSILGIPRVMTEA-NH_2	α-PDH	[31]
	Pandalus jordani[a]	NSGMINSILGIPRVMTEA-NH_2	α-PDH	[22]

Capital alphabetical initials underlined indicate the amino acid residues that are not compatible with those of *Musca* PDF.

[a] Three forms were reported for PDHs from the shrimp *Pandalus jordani*.

identity (60%) and similarity (78%). These results appeared to substantiate the hypothesis mentioned above. *Metmuna — Romalea* PDF precursors, or PAP regions appear to be grouped into a clear subfamily (Figure 3).

Comparing the nucleotide sequences of *pdf* cDNAs, the entire open-reading frame region of *Musca domestica* was found to be very similar to that of *Drosophila melanogaster* (ca. 60% identity). When the entire amino acid sequences were compared for the PDF precursors of the housefly and fruit fly, the homology scores of similarity and identity were calculated to be 62% and 38%, respectively, and thus very high. The number of amino acid residues, 98, for the *M. domestica* precursor was also similar to that of *D. melanogaster* ($n = 102$). Apparently, these two constitute another subfamily of PDF precursors, and this further substantiates the hypothesis that PDF precursors can be considered as different subfamilies.

The peptide sizes of *Metmuna — Romalea* PDF precursors are fairly small, smaller than those of fly PDF precursors (Figure 3). The cDNA cloning of the PDF precursor of the cricket *Gryllus bimaculatus* has also been achieved. Its peptide size was likewise much smaller ($n = 43$) than those of the *Drosophila — Musca* ($n = 98–102$) and the *Metmuna — Romalea* groups ($n = 82–89$). Thus, the *Gryllus* PDF precursor can be considered a third subfamily, and at least for the moment, the PDF precursors are therefore classified into three subfamilies.

The C-terminal region of the precursor proteins is the PDF peptide *per se*. The size of the PDF peptide is fixed at $n = 18$, although the size of the peptide amide precursors can be $n = 20$ or 21. This

Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

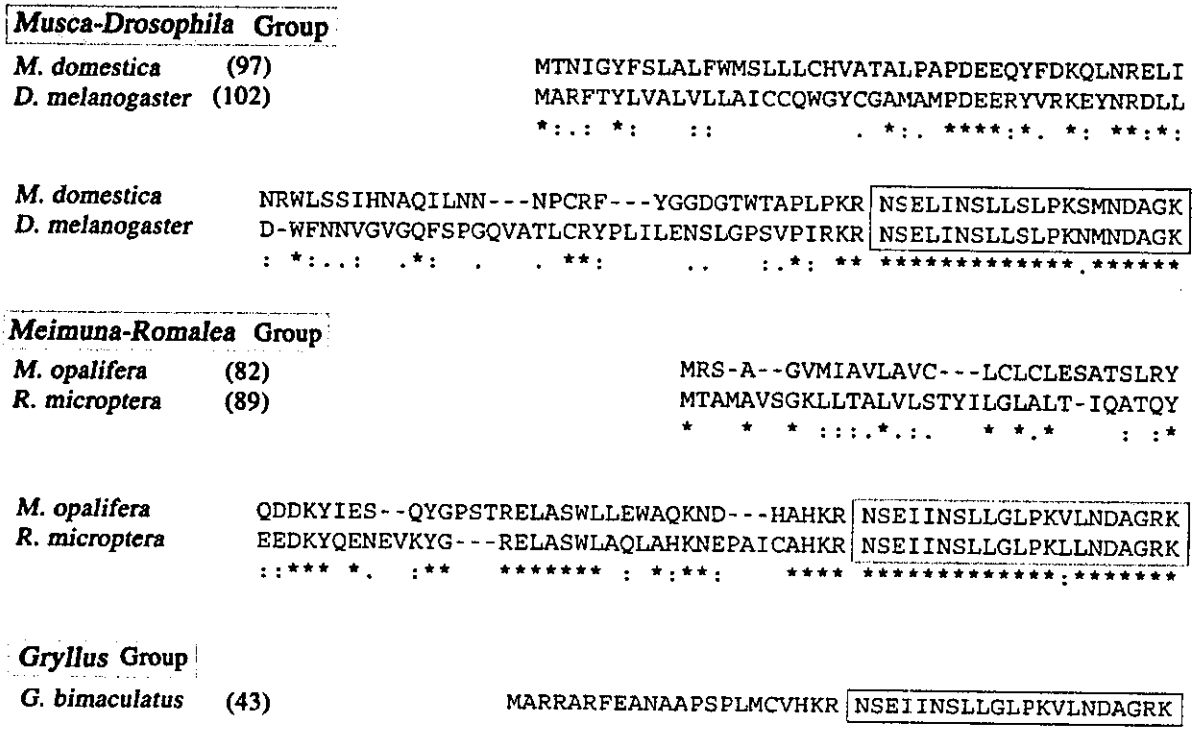

Figure 3 Comparison of the primary structures of insect PDF precursor proteins. The numbers in parentheses indicate the number of total amino acid residues of PDF precursors. The precursor of *Gryllus bimaculatus* has no signal peptide. * Residues in that column are identical in all sequences in the alignment. : Conserved substitutions have been observed. • Semi-conserved substitutions have been observed.

is because the precursors consist of a pre-amide sequence: $n = 2$ (GK) for *Drosophila — Musca* PDFs and $n = 3$ (GRK) for other insect PDFs. The region containing the signal and PAP is the peptide obtained by subtracting the PDF peptide from the entire precursor peptide. Thus, the difference in the size of the entire PDF precursors is the variance in the size of the signal/PAP regions. The peptide size of the signal/PAP regions is 76–80 for the *Drosophila — Musca*, 64–66 for the *Meimuna — Romalea*, and 20 for the *Gryllus* subfamilies. Such differences in peptide size might reflect the different functions of the PAP peptides in the different species.

The region of the signal peptides has been analysed by the computer-assisted signal peptide prediction method for eukaryotic sequences [32,33]. Assuming the result of this prediction, the peptide size of PAP was determined to be $n = 52$ for *Musca domestica* PDF precursor. Those of other insect PAPs were 56 for *Drosophila*, 34 for *Meimuna* and 32 for *Romalea*. No signal peptide was found for *Gryllus bimaculatus* PDF precursor, which, instead, consists of a nuclear localization signal (NLS) at the *N*-terminal portion.

PDF peptide in *Drosophila* has recently been reported to transmit the rhythm of daily locomotor activity via output pathways from the circadian clock [6–8]. Our immunohistochemical study using a polyclonal antibody specific for *Musca* PDF reveals immunoreactivity in the axons and terminals of the *M. domestica* PDF cells, suggesting a similar role for PDF in this species too (unpublished data). To elucidate such a function for PDF, however, investigations to identify its specific receptor and to understand the details of the molecular mechanisms of peripheral circadian systems are first necessary.

pdf Expressing Cells

Pyza and Meinertzhagen have reported the results of immunocytochemistry for PDF peptide, using an antibody raised for the crustacean *Uca pugilator* PDH [3,4]. It was found that the cell bodies, namely somata in exactly the same places in the optic lobes are immunostained by both *in situ* hybridization

Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

and immunocytochemical methods, suggesting that PDF is produced in the neurons where it functions. Figure 2 shows two groups of cell clusters of lateral neurons (LNs) in each optic lobe, and each group is likely to contain at least four cells, respectively. It should be noted that these groups appear to correspond to the large lateral neurons (l-LNs) and the small lateral neurons (s-LNs). These l-LNs and s-LNs cells in *Drosophila* express the *period* clock gene and PERIOD clock protein and produce PDF [34]. Thus, *Musca* PDF would reveal similar projections of circadian pacemaker cells within the brain.

Acknowledgements

The authors thank Professor Ian A. Meinertzhagen (Dalhausie University, Halifax, Canada) for his critical reading of this manuscript.

REFERENCES

1. Stengl M, Homberg U. Pigment dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. *J Comp. Physiol. [A]* 1994; **175**: 203–213.
2. Petri B, Stengl M. Pigment-dispersing hormone shifts the phase of the circadian pacemaker of the cockroach *Leucophaea maderae*. *J. Neurosci.* 1997; **17**: 4087–4093.
3. Pyza E, Meinertzhagen IA. Neurotransmitters regulate rhythmic size changes amongst cells in the fly's optic lobe. *J. Comp. Physiol. [A]* 1996; **178**: 33–45.
4. Pyza E, Meinertzhagen IA. Neurites of *period*-expressing PDH cells in the fly's optic lobe exhibit circadian oscillations in morphology. *Eur. J. Neurosci.* 1997; **9**: 1784–1788.
5. Helfrich-Fõrster C, Stengl M, Homberg U. Organization of the circadian system in insects. *Chronobiol. Int.* 1998; **15**: 567–594.
6. Renn SCP, Park JH, Rosbash M, Hall JC, Taghert PH. A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* 1999; **99**: 791–802.
7. Park JH, Helfrich-Fõrster C, Lee G, Liu L, Rosbash M, Hall JC. Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc. Natl Acad. Sci. USA* 2000; **97**: 3608–3613.
8. Helfrich-Fõrster C, Täuber M, Park JH, Mühlig-Versen M, Schneuwly S, Hofbauer A. Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. *J. Neurosci.* 2000; **20**: 3339–3353.
9. Park JH, Hall JC. Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster*. *J. Biol. Rhythms* 1998; **13**: 219–228.
10. Chuman Y, Matsushima A, Sato S, Tomioka K, Tominaga Y, Meinertzhagen I, Shimohigashi Y, Shimohigashi M. cDNA cloning and nuclear localization of the circadian neuropeptide designated as pigment-dispersing factor PDF in the cricket *Gryllus bimaculatus*. *J. Biochem.* 2002; **131**: 895–903.
11. Sato S, Chuman Y, Matsushima A, Tominaga Y, Shimohigashi Y, Shimohigashi M. A circadian neuropeptide, pigment-dispersing factor — PDF, in the last-summer cicada *Meimuna opalifera*: cDNA cloning and immunocytochemistry. *Zool. Sci.* 2002; **19**: 821–828.
12. Frohman MA, Dush MK, Martin GR. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl Acad. Sci. USA* 1988; **85**: 8998–9002.
13. Klein JM, de Kleijn DPV, Keller R, Weidemann WM. Molecular cloning of crustacean pigment dispersing hormone precursor. *Biochem. Biophys. Res. Commun.* 1992; **189**: 1509–1514.
14. de Kleijn DPV, Linck B, Klein JM, Weidemann WM, Keller R, van Herp F. Structure and localization of mRNA encoding a pigment dispersing hormone (PDH) in the eyestalk of the crayfish *Orconectes limosus*. *FEBS Lett.* 1993; **321**: 251–255.
15. Klein JM, Mohrherr CJ, Sleutels F, Riehm JP, Rao KR. Molecular cloning of two pigment-dispersing hormone (PDH) precursors in the blue crab *Callinectes sapidus* reveals a novel member of the PDH neuropeptide family. *Biochem. Biophys. Res. Commun.* 1994; **205**: 410–416.
16. Desmoucelles-Carette C, Sellos D, Van Wormhoudt A. Molecular cloning of the precursors of pigment dispersing hormone in crustaceans. *Ann. NY Acad. Sci.* 1998; **839**: 395–396.
17. Schaefer BC. Revolutions in rapid amplification of cDNA ends: New strategies for polymerase chain reaction cloning of full-length cDNA ends. *Anal. Biochem.* 1995; **227**: 255–273.
18. Wilkinson DG (ed.). *In Situ Hybridization: A Practical Approach*. IRL Press: Oxford, 1992.
19. Kozak M. Initiation of translation in prokaryotes and eukaryotes. *Gene* 1999; **234**: 187–208.
20. Rao KR, Mohrherr CJ, Riehm JP, Zahnow CA, Norton S, Johnson L, Tarr GE. Primary structure of an analog of crustacean pigment-dispersing hormone from the lubber grasshopper *Romalea microptera*. *J. Biol. Chem.* 1987; **262**: 2672–2675.
21. Mohrherr CJ, Rao KR, Riehm JP. Characterization of a pigment-dispersing factor from the american cockroach. *Soc. Neurosci. Abstr.* 1991; **17**: 276.
22. Rao KR, Kleinholz LH, Riehm JP. Characterization of three forms of pigment-dispersing hormone from the

Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

shrimp *Pandalus jordani*. *Soc. Neurosci. Abstr.* 1989; **15**: 26.
23. Rao KR, Riehm JP. Pigment-dispersing hormones. *Ann. NY Acad. Sci.* 1993; **680**: 78–88.
24. Philips JM, Rao RK, Riehm JP, Morgan WT. Isolation and characterization of a pigment-dispersing hormone from the shrimp *Penaeus aztecus*. *Soc. Neurosci. Abstr.* 1988; **14**: 534.
25. Rao KR, Riehm JP, Zahnow CA, Kleinholz LH, Tarr GE, Johnson L, Norton S, Landau M, Semmes OJ, Sattelberg RM, Jorenby WH, Hintz MF. Characterization of pigment-dispersing hormone in the eyestalks of the fiddler crab *Uca pugilator*. *Proc. Natl Acad. Sci. USA* 1985; **82**: 5319–5322.
26. Kleinholz LH, Rao KR, Riehm JP, Tarr GE, Johnson L, Norton S. Isolation and sequence analysis of a pigment-dispersing hormone from eyestalks of the crab, *Cancer magister*. *Biol. Bull.* 1986; **170**: 135–143.
27. Yang W-J, Aida K, Nagasawa H. Characterization of chromatophorotropic neuropeptides from the kuruma prawn *Penaeus japonicus*. *Gen. Comp. Endocrinol.* 1999; **114**: 415–424.
28. Rao KR, Riehm JP. The pigment-dispersing hormone family: Chemistry, structure-activity relations, and distribution. *Biol. Bull.* 1989; **177**: 225–229.
29. MaCallum ML, Rao KR, Riehm JP, Mohrherr CJ, Morgan WT. Primary structure and relative potency of an analog of β-PDH (pigment dispersing hormone) from the crayfish *Procambarus clarkii*. *Pigment Cell Res.* 1991; **4**: 201–208.
30. Knowles AC. Master thesis. 1992; The University of West Florida, Pensacola, FL.
31. Fernlund P. Structure of a light-adapting hormone from the shrimp *Pandalus borealis*. *Biochim. Biophys. Acta* 1976; **439**: 17–25.
32. Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 1997; **10**: 1–6.
33. Nielsen H, Brunak S, von Heijne G. Machine learning approaches for the prediction of signal peptides and other protein sorting signals. *Protein Eng.* 1999; **12**: 3–9.
34. Helfrich-Förster C. The period clock gene expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 1995; **92**: 612–616.

Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Peptide Science 2004 : Y. Shimohigashi (Ed.)
The Japanese Peptide Society (2005)

Structural Characteristics of *Drosophila* Estrogen-related Receptor Ligand Binding Domain to Capture the Peptide and Non-peptide Ligands

Xiaohui Liu[1], Ayami Matsushima[1], Naoto Shirasu[1], Yoshiya Tominaga[2], Miki Shimohigashi[2], Yasuyuki Shimohigashi[1], and Takeru Nose[1]

[1]Laboratory of Structure-Function Biochemistry, Kyushu University, Fukuoka 812-8581, Japan, and [2]Laboratory of Biology, Fukuoka University, Fukuoka 814-0810, Japan
e-mail: xiaohuiscc@mbox.nc.kyushu-u.ac.jp

In order to establish the binding assay system for Drosophila *estrogen-related receptor (dERR), we examined a few triturated ligands for expressed dERR-ligand binding domain (LBD). None of those compounds that were reported as binders for mammalian ERR revealed binding activity. When constructed a 3D model of the dERR-LBD, its ligand-binding pocket exhibited a very limited space, suggesting a rather tiny size of ligand for receptor activation.*

Keywords: computational analysis, estrogen receptor, estrogen-related receptor, ligand binding domain.

Introduction

A series of estrogen-related receptors (hERRα, β, γ) have recently been found in human as components to modulate the ordinary estrogen receptors (hERα, β), and they appeared to be a novel target of endocrine disruptors. No ER-like receptors have been found in the genome of the fruit fly *Drosophila melanogaster*, but instead an ERR-like nuclear receptor was revealed. When the amino acid sequences were compared, this termed as the *Drosophila* estrogen-related receptor (dERR) was found to resemble most closely the hERRs at both the DNA binding domain (DBD) and the ligand-binding domain (LBD) [1]. Thus, this receptor likely belongs to the steroid hormone subfamily NR3 of nuclear receptor.

In the present study, we carried out the cDNA cloning of full-length dERR, expression of dERR-LBD, and the receptor-binding assay. In addition, we carried out a computational analysis of dERR-LBD to understand the result of the receptor binding assay.

Results and Discussion

We first amplified dERR cDNA clone by PCR using dERR-gene-specific primers (ERR-*EcoR*I:5'CCGGAATTCATGTCCGACGGCGTCAGCAT3', ERR-*Sal*I: 5'CGAGTCGACTCACCTGGCCTGGCCAGCGGCTCGA3') for five different

cDNA libraries derived from *Drosophila* embryo, larvae-pupae, adult head, ovary, and adult testis. Eventually, from the testis cDNA library, we obtained a full-length of dERR cDNA clone, which is comprised of 1,455 bp oligonucleotides with a mature protein of 482 amino acid residues.

The amino acid sequence of clarified dERR was aligned together with hERRs and hERs for analysis of domains. A cDNA fragment (residues 734-1455, n=241) corresponding to the LBD was subcloned into pGEX-6p-1 vector for its expression in *E. coli* BL-21 cells as a GST-fusion protein. For screening of the ligands of dERR, we used purified GST-dERR-LBD fusion protein and newly developed the radio-ligand binding assay system. In this binding assay, we utilized polyethylene glycol for receptor protein precipitation followed by rapid filtration. While natural ligand is not known yet, the ER agonist diethylstilbestrol (DES) and the selective ER modulator 4-hydroxytamoxifen (4-OHT) have been identified as hERRγ antagonists [2-4]. Thus, we used triturated analogues of these compounds as tracers. However, no obvious specific binding was observed, suggesting that the structure of dERR binding site is different from that of hERRγ.

Homology modeling using four different ERR-LBD structural data as templates was carried out to construct a 3D model structure of dERR. The binding site analysis revealed that dERR-ligand binding pocket (LBP) has a binding pocket rather smaller than mouse ERRγ-LBD-LBP (data not shown). When compared the structure of mERRγ-LBD containing DES or 4-OHT, it was found that dERR-LBD places the residual indole group of Trp-459 in the LBP. Apparently, this makes DES and 4-OHT to be unable to bind to this small pocket of dERR (Fig. 1).

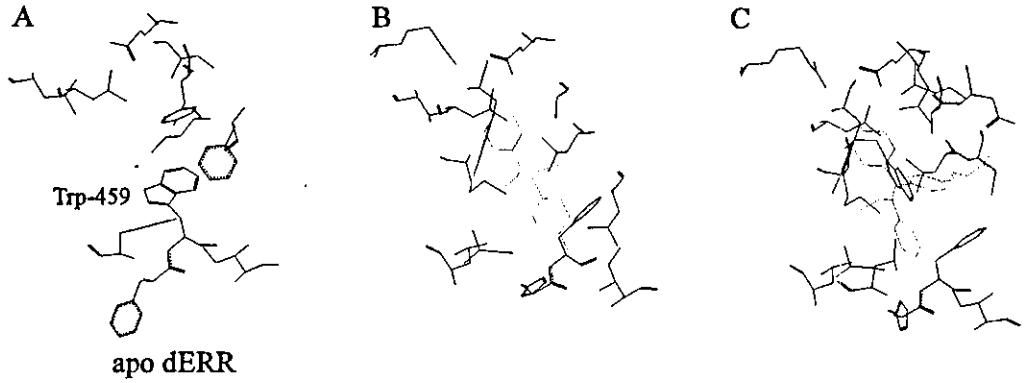

Figure 1. Comparison of ligand binding pockets of dERR (A: by homology modeling), mERRγ with DES (B: 1S9P), and mERRγ with 4-OHT (C: 1S9Q). Ligand molecules of DES (B) and 4-OHT (C) were depicted in gray scale.

References

1. Östberg, T., Jacobsson, M., Attersand, A., Urquiza, AM., and Jendeberg, L. (2003) *Biochemistry,* **42,** 6427-6435.
2. Tremblay, GB., kunath, T., Bergeron, D., Lapointe, L., Champigny, C., Bader, J.A., Rossant, J., and Giguère, V. (2001) *Genes Dev.* **15,** 833-838.
3. Tremblay, GB., Bergeron, D. and Giguère, V. (2001) *Endocrinol.* **142,** 4572-4575.
4. Coward, P., Lee, D., Hull, M.V., and Lehmann, J. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 8880-8884.

Peptide Science 2004 : Y. Shimohigashi (Ed.)
The Japanese Peptide Society (2005)

Highly Sensitive Monoclonal Antibodies to Distinguish Conformational Changes Induced by Endocrine Disrupting Chemicals in the Estrogen Receptor

Osamu Kuwata, Takeshi Honda, Daisuke Asai, Takatoshi Tokunaga, Ayumi Shibuya, Naoto Shirasu, Takeru Nose, and Yasuyuki Shimohigashi

Laboratory of Structure-Function Biochemistry, Department of Chemistry, Faculty and Graduate School of Sciences, Kyushu University, Fukuoka 812-8581, Japan
e-mail: kuwatascc@mbox.nc.kyushu-u.ac.jp

Estrogen receptor (ER) changes its conformation upon ligand binding. Mouse monoclonal antibodies (mAbs) were raised against an ER C-terminal 17-mer peptide involved in such a conformation change, and a set of mAbs were demonstrated to detect efficiently the receptor conformation change. The results indicate that these mAbs can be used as a sensitive molecular tool to evaluate the potential risk of environmental xenoestro-genic chemicals.

Keywords: conformation change, conformation-sensing, endocrine disruptors, estrogen receptor, monoclonal antibody.

Introduction

Rapid and effective evaluation of the biological effects of suspected environmental chemicals has become increasingly important to shed light on the critical issue of endocrine disruption in humans and animal wildlife. Xenoestrogenic effects caused by environmental chemicals cause most threat to mammalian hormonal activities, and the estrogen receptor (ER) is the major target of endocrine disrupting chemicals (EDCs). The ER and other nuclear receptors are known to change their conformation after binding ligands, as evidenced by X-ray crystallographic analyses. The resulting relocation of the C-terminal α-helix numbered 12 (H12) within the ligand-binding domain of the receptors is the most prominent and common feature of ligand-induced conformation change, which leads to an ultimate hormonal action. We have previously demonstrated that a rabbit antiserum raised against human ERα-H12 peptide was able to detect this conformation change in ER [1]. Here we present the preparation of a number of mouse monoclonal antibodies (mAbs) that detect such changes even more efficiently.

Results and Discussion

A 17-mer peptide corresponding to the ERα H12 moiety was synthesized and immunized into the footpads of Balb/c mice. Lymph cells were isolated ten days

after immunization and fused with mouse myeloma cells to obtain hybridomas. Among hundreds of antibody-secreting hybridomas tested, a number exhibited affinities towards both the peptide and ERα in the early rounds of screening by competitive ELISA. From among these, antibodies were selected that could detect the conformation change induced by 17β-estradiol (E_2), an agonist of ER. This selection assay (*conformation sensing assay*) was carried out using the competitive ELISA method to detect and differentiate the different affinities toward ligand-bound and -free ERs. Several mAbs showed conformation-sensing capabilities similar to those of the previous polyclonal antibodies. Eventually, a few were found to possess higher efficiency in sensing, because they required smaller amounts of the ER as the competitor and of H12 peptide as the immobilized antigen.

The conformation-sensing mAbs were further applied to assay several tens of known chemicals. The effective concentrations of chemicals to elicit a half maximal immunological response from the mAb, the EC_{50} value, correlated well with, but were lower than those of the polyclonal antibody (Fig. 1). These results clearly indicate that the mAbs were > 10 times more sensitive. The fact that their EC_{50} values are almost compatible with those obtained in the competitive receptor binding assays suggests that the conformation change detected by our mAbs reflects appropriately the ligand-binding.

For mAbs that displayed conformation-sensing capabilities, the epitopes were analyzed using sequence analogs of antigen peptides that were additionally synthesized. In a conformation sensing assay using different ligands, one set of mAbs was sensitive to the conformation change induced only by E_2, while another mAb displayed a differential sensitivity toward an antagonist-induced change as well. The difference in the ligand sensitivity among these sets of mAbs is relevant to the difference in the location of their respective epitopes within the H12 moiety, indicating that the mAbs are sufficiently specific to distinguish the different conformation changes induced by agonists and antagonists. In conclusion, the use of these mAbs should allow a highly effective and accurate evaluation of the latent risk of EDCs.

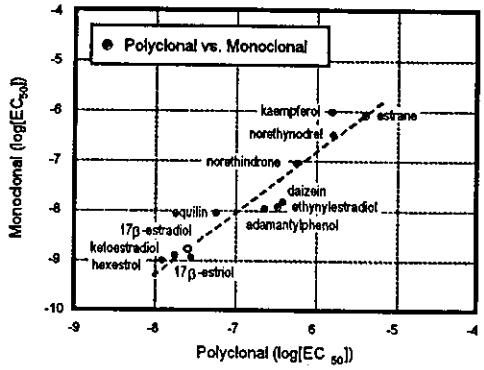

Figure 1. Correlation of the conformation sensing assays using polyclonal and monoclonal antibodies against ERα. Chemicals were assayed and their effective concentrations to elicit half-maximal immunological responses were plotted against each other. Correlation coefficient was 0.96, and regression coefficient was 0.63.

Reference

1. Asai, D., Koizumi, O., Mohri, S., Nakai, M., Yakabe, Y., Tokunaga, T., Nose, T., and Shimohigashi, Y. (2003) *Peptide Science 2002*, 127-130.

Peptide Science 2004 : Y. Shimohigashi (Ed.)
The Japanese Peptide Society (2005)

Ligand-inducing Conformation Changes in the Estrogen Receptor C-Terminal Tail Moiety and Their Sensing by Polyclonal Antibodies

Ayumi Shibuya[1], Takatoshi Tokunaga[1], Daisuke Asai[1], Osamu Koizumi[2], Shirou Mohri[3], Makoto Nakai[4], Yoshikuni Yakabe[4], Takeru Nose[1], and Yasuyuki Shimohigashi[1]

[1]Laboratory of Structure-Function Biochemistry, Kyushu University, Fukuoka 812-8581, Japan, [2]Faculty of Human Environmental Science, Fukuoka Women's University, Fukuoka 813-8529, Japan, [3]Department of Laboratory Animal Science, Kyushu University, Fukuoka 812-8582, Japan, and [4]Chemicals Evaluation and Research Institute, Saitama 345-0043, Japan
e-mail: ayushibuscc@mbox.nc.kyushu-u.ac.jp

Ligand binding to the estrogen receptor induces a conformation change. By using a polyclonal antibody to sense such a change, we have established the assay method to assess simultaneously the binding ability and hormonal activity of endocrine disruptors. In order to improve the immunoreactivity, we prepared four varieties of antibodies in this study. It was revealed that the ability of antibody to sense the conformation change is related to the structural characteristics of each antigen peptide.

Keywords: antigen peptide, conformation change, endocrine disruptors, estrogen receptor, polyclonal antibody.

Introduction

The estrogen receptor (ER) is a member of the nuclear receptor family which functions as a transactivation factor. The conformations of the ligand-bound (holo-ER) and ligand-free (apo-ER) forms of the ER are intrinsically different from each other [1]. This is due mainly to the change in positioning of the amphiphilic α-helix numbered as 12 (H12) present in the receptor C-terminal portion. This ligand-induced conformation change of the receptor is essential to bind the coactivator protein [2]. Antibodies provide a feasible tool to differentiate between these conformations, provided they could recognize specifically and selectively either the apo or holo conformation. In fact, an anti-ER H12 antibody was already found to discriminate apo-ER and holo-ER. In addition, this antibody was found to quantify the amount of ligand-bound and ligand-free ERs [3]. When compared with H12 *per se*, ER possesses a C-terminal tail including H12 which is about four times longer. It is highly likely that this tail moiety would also be involved in some conformation change. Thus, the purpose of the present study is to test the structural availability of this tail region as an antigen.

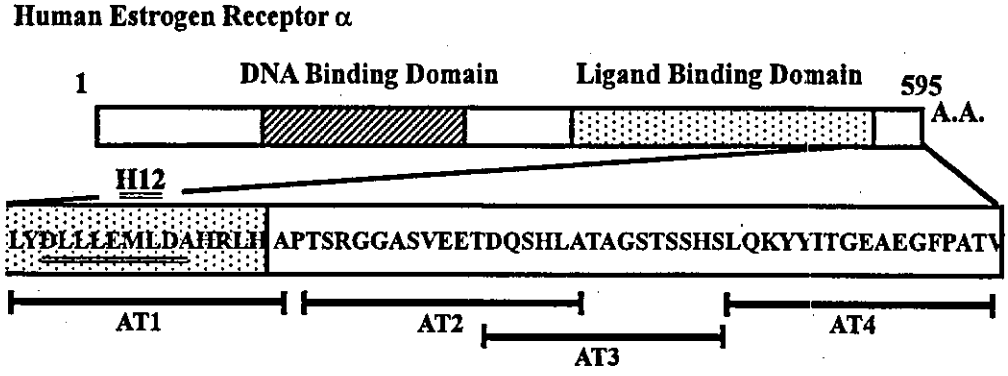

Figure 1. Design of antigen peptides for preparation of conformation-sensing antibody.

Results and Discussion

The C-terminal tail of ER was segmented into four peptide fragments (Fig. 1). The fragment AT1 contains H12. These peptides were synthesized by the Fmoc-based solid phase method. To conjugate to a carrier protein Keyhole Limpet Hemocyanin (KLH), Cys was incorporated into these peptides at the N-terminus. Peptides were liberated from the resin by treatment with Reagent K and purified by gel filtration (Sephadex G-25, 1.8 x 72 cm) followed by preparative reversed-phase high performance liquid chromatography (RP-HPLC) (Lichrospher RP-18 (e), 25 x 250 mm, 5 μm). The mass spectra of peptides were measured to verify their purity on a mass spectrometry Voyager™ DE-PRO with the method of matrix assisted laser desorption ionization time-of-flight (MALDI-TOF).

The peptides conjugated to KLH were injected into a rabbit, respectively. About three months later from the first immunization, blood was collected. The serum was purified successively by KLH immunoprecipitation, affinity chromatography with antigen-linked agarose gel, and then with a protein A-linked agarose gel. The specificity of antibody was analyzed by the enzyme-linked immunosorbent assay (ELISA) method. Competitive ELISA was employed to evaluate the ability of antibody to bind to apo-ER and/or holo-ER. The production of antibody was checked by preparative ELISA using ER and the antigen peptide. In this titer checking it was found that the serum contains enough amount of antibodies sensitive to both the receptor and peptide. As shown in Fig. 2, the serum obtained from the immunization by AT4 peptide interacted with the antigen peptide and the receptor ER almost equally well. Similar results were obtained for AT2 and AT3 peptides.

When the relative immunoactivity of the antibody was estimated under the certain concentrations of ER (10^{-12} - 10^{-6} M), a dose-dependent reduction was observed against the antigen peptide (10^{-12} - 10^{-11} mol) coated on the plate (Fig. 3). This assay was carried out under the presence of 10^{-5} M 17β-estradiol (E2), and the results were depicted in the same figure. As shown in Fig. 3A, a dose-dependent curve became much more gentle, making a certain deviation between the curves. This deviation (about 30%) corresponds to the sensing based on the conformation change. Thus, it is concluded that anti-AT1 exhibits a high ability to distinguish between holo-ER and apo-ER (Fig. 3). A similar result was obtained for anti-AT3 antibody, although the extent of deviation is much smaller than for anti-AT1 antibody. For anti-AT4

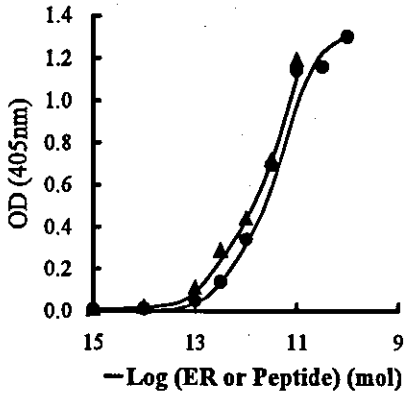

Figure 2. Indirect ELISA using anti-AT4 antibody for titer checking against recognition to antigen peptide (●) and ER (▲). The antibody used is the preparation purified by affinity chromatography with a protein A-linked agarose gel.

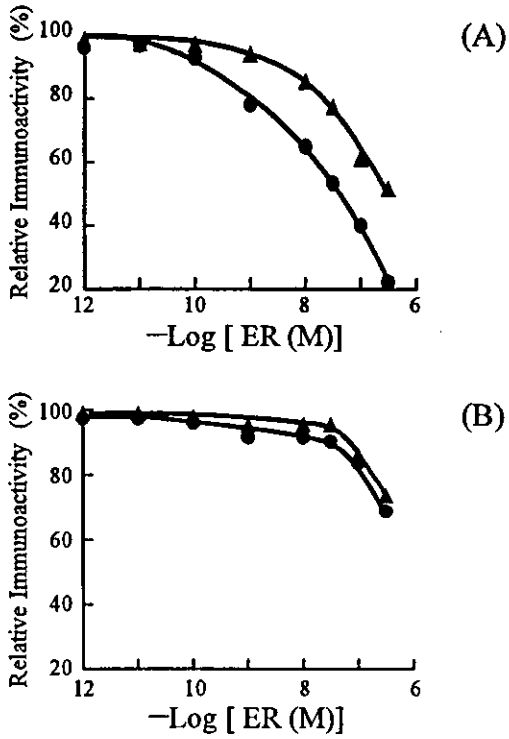

Figure 3. Immunoresponse of anti-AT1 (A) and anti-AT4 (B) antibodies against apo-ER (●) and holo-ER (▲). The antibodies used are the preparation purified by affinity chromatography with a protein A-linked agarose gel. Holo-ER was prepared by treatment with 10 μM 17β-estradiol.

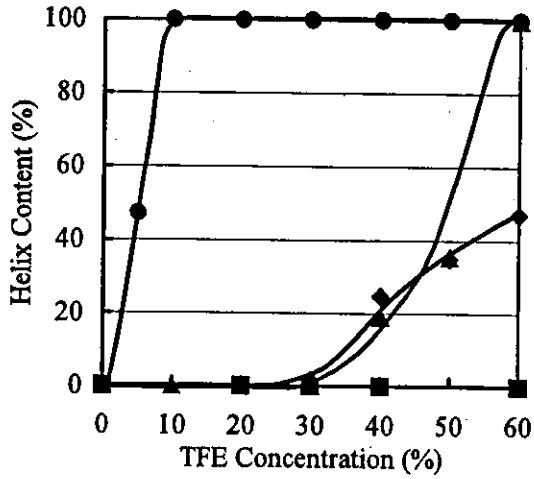

Figure 4. The helical characteristics of each peptide; AT1(●), AT2(■), AT3(▲),and AT4(◆)

antibody, almost no deviation was observed (Fig. 3B). A similar result was shown by anti-AT2 antibody (data not shown).

The CD spectra were masured on a J-725 Spectropolarimeter (Jasco), and the % contents of the secondary structures were calculated by SSE-338W protein secondary structure analysis program (Jasco). It was found that AT1 peptide is easy to adopt an α-helix structure. AT1 exhibited an extremely high content of α-helix (100% even in 10% TFE). AT3 peptide was found to adopt also a 100% α-helix structure, but only in 60% TFE. Almost no helical content was observed for AT2 peptide and a very low for AT4 peptide. These results indicated that the ability of antibody to sense the conformation change is well-related to the structural characteristics of each peptide fragment to adopt an α-helical conformation.

References

1. Gould, J.C., Leonard, L.S., Maness, S.C., Wagner, B.L., Conner, K., Zacharewski, T., Safe, S., McDonnell, D.P., and Gaido, K.W. (1998) *Mol. Cell Endocrinol.*, **142**, 203-214.
2. Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., and Carlquist, M. (1997) *Nature*, **389**, 753-757.
3. Asai, D., koizumi, O., Mohri, S., Nakai, M., Yakabe, Y., Tokunaga, T., Nose, T., and Shimohigashi, Y. (2003) *Peptide Science 2002*, 127-130.