clarify the mechanisms by which exogenous estrogen affect development of teleost fish.

To isolate the fhER, we used a cDNA library from mRNA of the sexually mature female liver during the spawning season. cDNA screening using RT-PCR product resulted in two cDNA clones containing complete open reading frame. Phylogenetic analysis of ER families indicates that fhER belongs to the ERα clusters consisting of tetrapod and fish ERα. Specifically, the fhER was clonally related to medaka ERα, tilapia type-1 ER, red seabream ERα, and gilthead seabream ERα (Fig. 2). These results indicate that the receptor we isolated and cloned in the mummichog should be classified as an ERα.

The comparison between the deduced amino acid sequence of fhER and ERs of four species ER shows high levels of identity in the C and E domains. The C (DNA binding) domain is the most highly conserved (90–95%) among ERs and our observation on the fhERα is consistent with this conclusion. This region is well conserved in the ER family, containing two zinc-finger motifs (Schwabe et al., 1993). These structures are necessary for recognizing and binding to the target sites in DNA. These results suggested that fhER might interact with the target sites on DNA by a similar mechanism as reported for other species.

The E (ligand binding) domain has high identity with other species' ER amino acid residues, especially with the medaka ER, as it has 92% amino acid sequence identity. Several amino acid residues of this E domain have been identified as important for ligand binding, dimerization, and transcriptional activation functions (AF-2) (Lees et al., 1990; Danielian et al., 1992). The estradiol-binding studies of human ERα identified a number of amino acid residues (A350, E353, L387, I424, G521, H524, L525 and M628) as involved in ligand binding (Brzozowski et al., 1997; Ekena et al., 1996, 1998). These amino acids were conserved in the fhER (A356, E359, L393, R400, I430, G527, H530, L531 and M534). The His513, which plays an important role for dimerization in human ER, was conserved in the fhER (H519). In transactivation, the AF-2 region is conserved in fhER (L545 to L550), equivalent to the fhER region L539 to L544.

The A/B domain shared a high identity with medaka ER (77%), compared with other ERs (15–23%). The A/B domain has a cell type and promoter specific transactivation function (AF-1) (Tora et al., 1989). Pakdel et al. (2000) indicated 45 amino acids differences in the A/B domain between short and long isoforms of rainbow trout ERα. These sequence differences in the A/B domain suggested the presence of species or isoform specific mechanisms in the transactivation of ERs.

The Northern blot analysis indicated the presence of two transcripts (5.5 and 4 kb) in the mummichog liver (Fig. 4). The presence of several transcripts of ER has been reported in many other mammals and fishes (Weiler et al., 1987; Pakdel et al., 1990; Todo et al., 1996; Tchoudakova et al., 1999; Socorro et al., 2000). Moreover, two functional ERα isoforms, which differ by 45 amino acids in the A/B domain, were reported in trout (Pakdel et al., 2000). In Northern blot analysis of time course, the 4 kb transcript was rapidly observed (within 8 h) after exogenous treatment with E_2, followed by the 5.5 kb transcript 12 h after E_2 stimulation. At 24 h after E_2 stimulation, the 5.5 kb transcript seemed to be expressed strongly. VTG mRNA was expressed from 8 h. Then, it tended to be expressed strongly at 24 h after E_2 stimulation. Many studies showed that ER levels have been regulated by ligand in various animals and tissues. In trout's hepatocyte culture, ER mRNA had increased after 2 h, and reached maximum expression 24 h after E_2 stimulation. VTG mRNA was induced 8 h after E_2 stimulation (Flouriot et al., 1996). In this study, the 4.5 kb transcript was expressed before the inducement of 5.5 kb transcripts and VTG mRNA expression. The 5.5 kb transcript was expressed from 8 h after E_2 stimulation, and then VTG mRNA seemed to be expressed strongly. These results suggested that the both of 4 and 5.5 kb transcripts may be functional isoforms and play important roles in transcription of fhER and VTG mRNAs.

It has been reported that alkylphenols are xenoestrogens (Soto et al., 1991; White et al., 1994). However, binding affinities of alkylphenols to various ERs from different species showed wide spectrum of responses, i.e. 4-*t*-octylphenol bound to rainbow trout ERα stronger than human ERα (Matthews et al., 2000), and binding affinity of nonylphenol for the ERα from testicular and liver cytosol of Atlantic croaker was comparable to human ERα (Loomis and Thomas, 1999; Tabira et al., 1999). RBA values of 4-*t*-octylphenol and 4-nonylphenol for fhERα were approximately 0.0067- and 0.0042-fold of E_2, respectively, and these values were 20 and 7 times greater in affinity when compared with the interaction between these chemicals and human ERα (Tabira et al., 1999). The binding affinities for the fhERα were similar to those reported for rainbow trout ERα using the same chemicals.

Benzophenone exhibited weak binding affinity, approximately 0.008% of E_2. Phthalates also showed weak binding affinities and they bound to fhERα at 0.01–0.02% the strength of E_2. Di-*n*-butyl phthalate showed a slightly higher receptor binding ability for fhERα when compared with human ERα, however, its RBA value was no more than three times the RBA for human ERα (Nakai et al., 1999).

Octachlorostyrene was inactive in both the human ER binding assay (Nakai et al., unpublished data) and immature rat uterotrophic assay (Yamasaki et al., 2002). However, its binding potency to fhERα was detectable (0.02% of E_2). These results suggested there

are species differences in receptor binding abilities of various environmental pollutants. To resolve the ecotoxic effects of chemicals based on endocrine disruption mediated by receptor binding, it is necessary to measure the binding potencies of chemicals to various receptors, and evaluate the differences among species.

Binding affinities of nonylphenol and octylphenol with fish ERα were higher in medaka (*O. latipes*) than in the humans (Ministry of Environment, 2001). Breeding experiments performed with medaka exposed to nonylphenol levels reported in natural aquatic system in Japan, revealed increased incidences of ovotestis and decreases in the numbers of males produced. VTG expression was observed in fish exposed to nonylphenol levels of 11.6, 23.5 and 22.5 μg/l, respectively. Recent studies report that the maximum concentration of nonylphenol in Japanese river water is 21 μg/l. Based on these results, the Ministry of the Environment restricted the release of nonylphenol into the aquatic environment in 2001 (Iguchi et al., 2002b). We do not know whether chemicals used present study have a possibility to affect mummichog in the wild, since we have no wild mummichog in Japan. However, some of the chemicals may affect the medaka in Japan. There are species differences in ER binding to chemicals, therefore, we need to consider the species difference in the risk assessment of chemicals especially in fish species (Iguchi et al., 2002b).

In conclusion, cDNA of fhERα was cloned, and the protein of the LBD of the fhERα binds E_2 as well as alkylphenols which previously have been reported to have estrogenic activity. Interestingly, fhERα binds nonylphenol and octylphenol more than 50 times greater than that observed for the human ERα, suggesting species-specific differences in ligand binding of ERα.

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Structure-function Studies on the Ligand/ Receptor π Interactions

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For the molecular recognition, the most important structural characteristics of bioactive peptides are the side chain of each amino acid. The aromatic amino acid phenylalanine (Phe) is often to be such a structural essential. In order to elucidate the role of the side chain of Phe, two different types of structure-function studies have been carried out. Consequently, we have shown that the π-π interaction of the Phe-phenyl group plays important roles in the molecular recognition by thrombin receptor and chymotrypsin.

Introduction

Biologically active peptides consist of amino acid residues essential for their molecular interaction. In general, the most important for the molecular recognition by such peptides is the structural characteristics of each amino acid side chain. The aromatic amino acid phenylalanine (Phe) is often to be such a structural element, for example, of neuropeptides, hormone peptides, and enzyme inhibitors. The side chain of Phe, namely, the phenyl group, is characterized by its aromaticity and/or hydrophobicity. We have elucidated recently that the CH/π or π/π interaction of the Phe-phenyl group plays important roles in various kinds of molecular recognition. Here two different types of structure-function studies on the π interactions are discussed for better understanding of the functional roles of the Phe-phenyl group.

Thrombin Receptor Tethered-ligand Peptide

Serine protease thrombin plays central roles in blood coagulation and possesses a specific receptor on the platelet surface. When thrombin binds to the receptor to cleave the peptide bond between Arg-41 and Ser-42 in the N-terminal, newly exposed N-terminal segment being a tethered ligand which activates the receptor [1]. We have found that synthetic heptapeptide SFLLRNP corresponding to this tethered-ligand can activate the receptor without thrombin. In this ligand peptide, there is only one aromatic amino acid residue at the second position. We have assigned this phenylalanine (Phe)-2 to be a most important residue for receptor activation by means of alanine scanning. Furthermore, we have found that the replacement of this Phe-2 by *para*-fluorophenylalanine induces several times activity enhancement in the phosphoinositide (PI) turnover of the SH-EP cells and human platelet aggregation

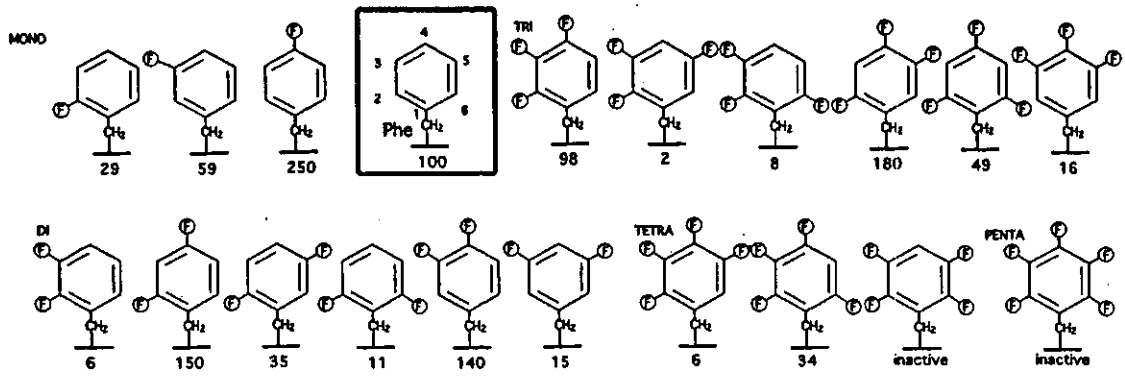

Fig. 1. Fluorine-substitutions on the Phe-2-phenyl group in thrombin receptor tethered-ligand peptide SFLLRNP. Receptor activation was estimated in the human platelet aggregation assay. The activity of the parent peptide SFLLRNP was refereed as a standard (EC_{50} = 2.7 μM).

assay [2].

In order to identify a detailed interaction mode between this essential Phe-2-phenyl group and the receptor, we synthesized a series of fluorophenylalanine-containing S/Phe/LLRNP analogs and tested their ability to induce a human platelet aggregation [3]. To this end, we have synthesized all of fluorophenylalanines (Fn)Phe (n=1~5) by our hands. The syntheses was started from the coupling between diethyl acetamidomalonate and fluorinated benzyl bromides, and finished up with the enzymatic optical resolution by acylase. The results of activity evaluation for a series of S/(Fn)Phe/LLRNP are summarized in Fig. 1, showing the relative potencies against the parent SFLLRNP.

The substitution of the benzene hydrogen atoms with fluorine has provided a powerful methodology to analyze directly the function of benzene hydrogen *per se*. Benzene-fluorine can reinforce the CH/π interaction of hydrogen present at other position by enhancing its acidity. Increased activities of analogs containing (4-F)Phe, (2,4-F_2)Phe, (3,4-F_2)Phe, and (2,4,5-F_3)Phe were explained by this reinforcement of the CH/π interaction. The H→F substitution at the *para* position elicits certainly such an effect. The necessity of benzene hydrogen for this kind of interaction could be also assessed by the H→F substitution. Direct substitution decreases the activity drastically. The substitution at the *ortho* and *meta* positions demonstrated such a function of benzene hydrogens. In reverse, occupation of fluorine atom at the positions where the hydrogen atom is not in the interaction dose clear the direct involvement of the hydrogen in the CH/π interaction. The results from (F_3)Phe- and (F_4)Phe-containing analogs are prominent to declare the importance of *meta*-hydrogen(s) and *ortho*-hydrogen(s). The involvement of *meta*-hydrogen(s) in the CH/π interaction was further evidenced by the H→Cl or H→Br substitution. Based on these analyses, the putative binding mode of the Phe-2-phenyl group for the receptor aromatic groups is illustrated in Fig. 2.

Recently, a three-dimensional structure of bovine rhodopsin has clarified by Palczewski *et al.* [4]. Based on this structural analysis, computer modeling of other GPCRs became feasible by the homology modeling procedure. For finding the

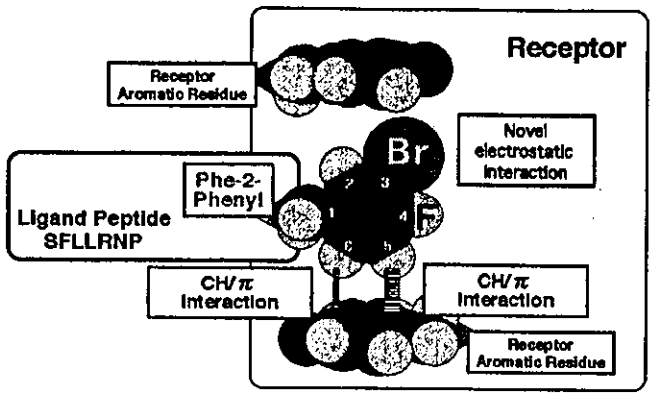

Fig. 2. Schematic illustration showing putative CH/π interactions between ligand (3-Br,4-F)Phe-2 phenyl and receptor Phe-phenyl group.

binding site of the Phe-2-phenyl group in the thrombin receptor, we attempted to construct a three-dimensional structure of the receptor. It was found that, in the fifth transmembrane helix (TM5) of the receptor model, there is a cluster of aromatic amino acids (*YYAYYFSAFSAVFFF*). These aromatic amino acids including Tyr at positions 266, 269 and 270 construct a cavity-like pocket. This pocket was thought to be a binding site of Phe-2-phenyl.

All these results have suggested the involvement of edge-to-face π/π interactions (CH/π interaction) in TM5 in the receptor activation. Thus, the CH/π interaction of the Phe-2-phenyl group is a determinant in the molecular recognition by thrombin receptor.

Chymotrypsin inhibitor: D-Leu-Phe-NHBzl(*p*-F)

In an attempt to design a novel type of enzyme inhibitors, we found the dipeptides having a structure D-Xxx-Phe-OBzl or -NHBzl as a powerful chymotrypsin inhibitor. They construct a unique conformation to inhibit chymotrypsin very strongly in a competitive manner. From the NOE experiment on ^{1}H-NMR, their conformation was characterized by a formation of the CH/π interaction between side chains of D-Xxx-1 and Phe-2. [5-6]. During our efforts to obtain strong enzyme inhibitors, D-Leu-Phe-NH-CH_2-C_6H_5(*p*-F) emerged as one of the strongest chymotrypsin inhibitors (Ki = 0.61 μM).

From various types of structure-activity studies, we have suggested that the C-terminal benzyl-phenyl group interacts with chymotrypsin at the S1 site, while a side chain hydrophobic core fits the S2 or S1' site. For clarification of a genuine structure of the dipeptide inhibitor, we analyzed a three-dimensional structure of the complex of D-Leu-Phe-NH-CH_2-C_6H_5(*p*-F) and chymotrypsin [7]. In the chymotrypsin–inhibitor complex, D-Leu-1 and Phe-2 residues fold together to form a hydrophobic core. This folded conformation is probably caused by the CH/π interaction between D-Leu-isobutyl and Phe-2-phenyl groups. This structural feature is in good agreement with what has been suggested from the ^{1}H NMR experiments [6]. The δ-methyl proton signals of D-Leu exhibited a large upfield shift due to the shielding effect from Phe-phenyl in close proximity. On the other hand, the C-terminal *para*-fluorobenzyl amide moiety in the inhibitor molecule was found to orient towards the opposite direction against the Phe-2-phenyl group.

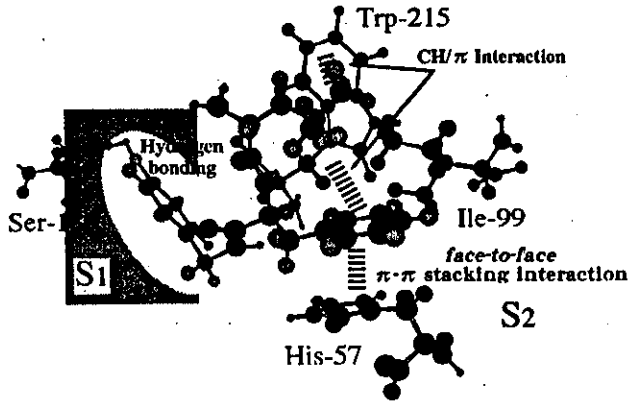

Fig.3. Schematic diagram of chymotrypsin–dipeptide inhibitor complex at the enzyme active center.

The most remarkable structural feature in the chymotrypsin–inhibitor complex is the face-to-face π-π stacking interaction between the Phe-phenyl group and the chymotrypsin His-57 imidazolyl group. His-57 is one of the constituents of catalytic triad at the active center. The distance observed is 3.75Å from the imidazolyl ring of His-57 to Phe-2-phenyl. In this inhibitor–enzyme complex structure, the inhibitor Phe-phenyl group is in a unique environment. Together with the side chains of inhibitor D-Leu-1 and chymotrypsin His-57, the indole ring of Trp-215 and the *sec*-butyl group of Ile-99 form a kind of hydrophobic surroundings for the inhibitor Phe-2-phenyl group. The presence of a π-π stacking interaction between the Phe-phenyl and His-imidazolyl groups should be emphasized as a characteristic structural element for chymotrypsin inhibition.

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Biochemical Evaluation of Hormonal Activity of Endocrine Disruptors by Sensing the Estrogen Receptor Conformation Changes

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Ligand binding to the estrogen receptor induces a critical conformation change. We produced the polyclonal antibody which recognizes such a ligand-induced conformation change. This antibody was found to quantitate the amount of ligand-bound and ligand-free estrogen receptors. We did establish the assay system to assess simultaneously the receptor binding ability and hormonal activity of a series of endocrine disruptors.

Introduction

Environmental chemicals, so-called endocrine disruptors bind to nuclear receptors, which function as transcription factors essential for specific gene expression [1, 2]. This receptor binding induces a critical conformation change for further binding of the complex to the coactivator protein [3]. From the results of X-ray crystallographic analyses, it was proven that the conformations of ligand-bound and ligand-free forms are considerably different from each other. This is due mainly to the change in positioning of the H12 α-helix in the receptor C-terminal moiety of the nuclear receptors.

In the present study, we produced the anti-rabbit polyclonal antibody which discriminates the ligand-induced conformation changes of estrogen receptor. Preliminary results have been reported in part previously [4]. We here evaluated in detail the binding abilities of the antibody with and without various chemicals by enzyme linked immunosorbent assay (ELISA)-based experiments.

Materials and Methods

The C-terminal tail domain of estrogen receptor ligand binding domain was

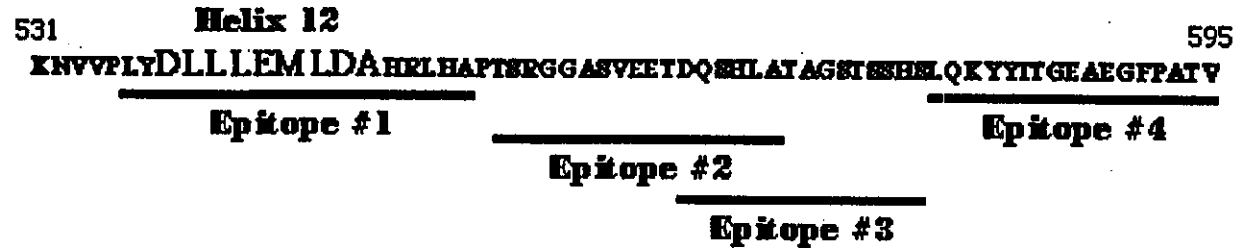

Fig. 1. The C-terminal tail domain of estrogen receptor ligand binding domain with designed epitope regions.

segmented into four peptide fragments (n = 15-17) and these peptides were synthesized by the solid phase method. To conjugate to Keyhole limpet hemocyanin (KLH), Cys was added at the N-terminus. Peptides were cleaved from the resin by trifluoroacetic acid (TFA), and side chain protecting groups except for Cys(Acm) were removed by Reagent K. The Acm group of Cys(Acm) was removed by $AgNO_3$/DIEA/DMSO treatment as described [5]. Peptides were purified by gel filtration followed by reversed-phase (RP) HPLC with 0.05% TFA/H_2O/CH_3CN. The purity was verified by analytical RP-HPLC and mass spectroscopy.

Peptide synthesized was conjugated to KLH protein by crosslinking with bifunctional linker agent of *m*-maleimidebenzoyl-*N*-hydroxysuccinimide ester (MBS). Antibody was raised by immunizating against New Zealand white rabbits. After the final boost, blood was collected from the ear artery for serum preparation. The serum was treated with KLH overnight at 4°C, and then centrifuged. The resulting supernatant was further purified by affinity chromatography using a peptide-linked agarose gel column. The specificity of antibody was analyzed by ELISA.

Competitive ELISA was employed to evaluate the abilities of antibodies to bind to ligand-bound and ligand-free estrogen receptors. Before adding the antibody, 17β-estradiol of various concentrations was added to the solution of estrogen receptor (40 nM), and the mixtures were incubated at room temperature for 1 hr. The solutions were transferred to each well of peptide-coated immunoplate prepared beforehand, and the antibody in 1% BSA solution was added. The solution was incubated at 4°C overnight. After six repeated washings, peroxidase conjugated anti-rabbit IgG (H+L) in 1% BSA solution was added, and the plate was incubated at room temperature for 1 hr. A solution of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and H_2O_2 was added after six washings. 1% SDS solution was added to terminate the enzyme reaction, and the plate was measured at 405 on a microplatereader.

Results and Discussion

The C-terminal tail moiety of estrogen receptor ligand binding domain was segmented into four peptide fragments as in Fig. 1. Peptide 1 consists an α-helix named H12. The results of X-ray crystallographic analyses have shown that H12 is in an amphiphilic α-helix, positioning on the ligand binding cavity in the ligand-bound estrogen receptor. The difference in the receptor structures between ligand-bound and ligand-free forms is very clear, since they differ only in positioning of H12.

The binding ability of antibody produced to the estrogen receptor was evaluated by the ELISA method. It was found that the antibody binds strongly to a ligand-free receptor as well as the antigen peptide 1 (Fig. 2). When the competitive ELISA was carried out using this antibody in the presence of 10 μM 17β-estradiol (E2), a native ligand of estrogen receptor, the binding ability of the antibody reduced considerably

(about 30%). This clearly indicated that the antibody consists of the constituent that binds to ligand-free receptor, but hardly binds to ligand-bound receptor. When the ligand binds to the receptor, the hydrophobic surface of the H12 α-helix orients to the ligand molecule in the receptor cavity just as a lid. This conformation change of the receptor should cause an imbalance in antibody constituents. A certain proportion of the antibody would not bind to the receptor. Thus, if we could capture this proportion of the antibody and quantify immunochemically, we would know the amount of receptors that caused a conformation change. To this end, we prepared the immunoplate with the wells in which antigen peptide was coated on thyroglobulin absorbed on the plastic surface.

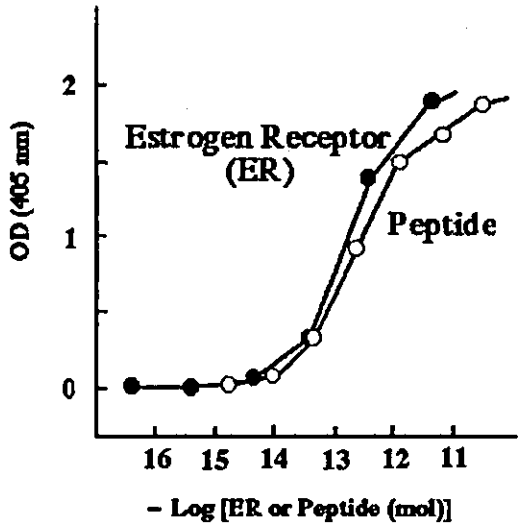

Fig. 2. The antibody responses to the estrogen receptor and antigen peptide.

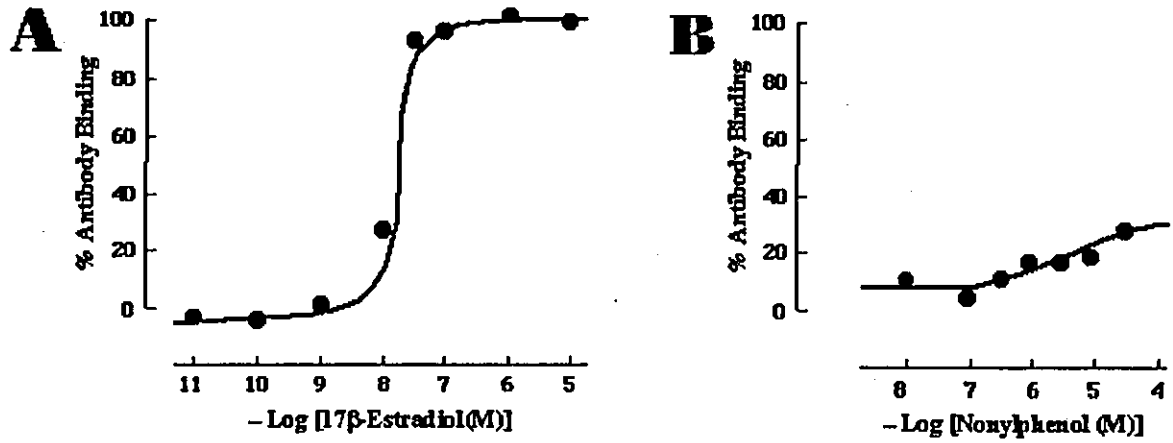

Fig. 3. Antibody titration of estrogen receptor/ligand complex. 17β-Estradiol (A) and nonylphenol (B).

Table 1. The results of conformation-sensing assay.

	EC50 (nM)	Immunoresponse (%)
Estrone (E1)	16	64
17β-Estradiol (E2)	23	100
Estriol (E3)	14	110
Diethylstilbestrol (DES)	14	79
Ethynylestradiol (EE2)	22	86
4-OH Tamoxifen	99	20
4-Nonylphenol	6,900	35
4-*tert*-Ocylphenol	1,700	32
4-Cyclohexylphenol	1,400	33
4-(1-Adamantyl)phenol	156	55
2,2-Bis(4-hydroxyphenyl-4-methyl-*n*-pentane	934	11
Chlorotrianisene	—	0

When the receptor was treated with various concentrations of E2, it was found that the proportion of antibody remaining varies, depending upon the concentration of E2 used. This depicted an ideal sigmoidal curve as shown in Fig. 3. Apparently, this curve gives the estimation of effective concentration of E2, which would correspond to the binding affinity of E2 to the receptor. On the other hand, the vertical axis exhibits the percentage of antibody binding to the receptor. This receptor response would represent a hormonal activity, since an appropriate conformation change induces a coupling of coactivator protein for completion of transcriptional activity. These afford a great advantage that we are able to evaluate the ligand binding activity and hormonal activity simultaneously.

Using such assay principles, we carried out the examination of a series of hormones and chemicals. Table 1 show the results obtained from the assay curves as shown in Fig. 3. For instance, 4-nonylphenol afforded a weak, but distinct curve depicting the EC50 value of 6.90 μM and the immunoresponse of 35% (Fig. 3, Table 1). These are very much compatible with the values reported. Table 1 shows the results for all the compounds tested.

The present study provides a very useful screening system to assess the chemicals for their latent ability as endocrine disruptors. Using this method, we are evaluating a number of chemicals listed as putative disruptors.

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The Structure-activity Studies of *Drosophila* FMRFamide-related Peptides

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DPKQDFMRFamide is one of Drosophila *FMRFamide-related peptides (FaRPs). It was found that, with Ala-scanning experiment, the C-terminal tetrapeptide amide element plays a crucial role in activating the receptor in* Meretrix lusoria *heart muscle. A role of N-terminal element was assesed by constructing a series of hybrid analogs between DPKQD and C-terminal tetrapeptides from other FaRPs. The results suggested that the N-terminal element reinforces a contractile activity in the heart muscle.*

Introduction

FMRFamide is a neuropeptide originally isolated from the clam *Macrocallista nimbosa* [1]. FMRFamide exhibited a contractile activity specific, for example, for the muscles of mollusk. A number of neuropeptides having FMRF-like sequences has been isolated in a diverse range of species, and those peptides have been denoted as FMRFamide-related peptides (FaRP) [2]. Several different FaRPs have recently been isolated from brains of the fruit fly *Drosophila melanogaster* [3], although their gene had been already clarified as a single clone encoding thirteen copies of eight different FaRPs [4]. All these FaRPs are 7~9-amino acid peptides consisting of FMRFamide or FMRFamide-like sequences at the C-terminus. We synthesized eight different FaRPs and assessed their contractile active in the heart muscle of *Meretrix lusoria*. The results indicated that five *Drosophila* FaRPs having FMRFamide sequence in their C-terminus are highly active, although other FaRPs were very weak or inactive. This suggested an intrinsic role of C-terminal FMRFamide structure in receptor activation.

In the present study, we examine the functional importance of DPKQD-FMRFamide, one of FMRF-containing *Drosophila* FaRPs which exhibited the highest activity for *M. lusoria* heart muscle. We first synthesized and biologically

Table 1. The primary structure of FMRFamide-related peptides deduced from the gene mRNA isolated from the brain of Drosophila melanogaster.

Peptides	Number of amino acid	Copy numbers in the gene
SAPQDFMHFamide	9	1
DPKQDFMRFamide	9	5
TPAEDFMRFamide	9	2
SDNFMRFamide	7	1
SPKQDFMRFamide	9	1
PDNFMRFamide	7	1
SVQDNFVRSamide	9	1
MDSNFIRFamide	8	1

evaluated Ala-substituted analogs of C-terminal tetrapeptide amide element. In order to evaluate the importance of N-terminal structural element (DPKQD), we synthesized various hybrid analogs between DPKQD and C-terminal tetrapeptides from other FaRPs.

Material and Methods

Peptides were synthesized by either Fmoc or Boc-based solid phase synthesis method and purified by gel filtration (Sephadex G-15) followed by preparative RP-HPLC. Purified peptides were verified MALDI-TOF mass spectroscopy and amino acid analysis.

The contractile activity was evaluated using the heart muscle of *M. lusoria*. The muscle mounted in a chamber was kept in a solution (pH 7.8) consisting of 445 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 20 mM $MgCl_2$, and 10 mM Tris-HCl. Tension changes of the heart muscle were profiled on the recorder in conjunction with the force displacement transducer. Samples were injected directly into the experimental chamber. Dose-response curve was analyzed by the computer program ALLFIT. The contractile activity was estimated as EC_{50}, the value of which exhibits the concentration for the half maximal effect.

Results and Discussion

In order to evaluate the residual importance of the C-terminal FMRFamide element, we carried out Ala-scanning examination for *Drosophila* FaRP, DPKQ-DFMRFamide. Each amino acid residue was substituted with Ala and the resulting Ala-substituted analogs were assayed for their contractile activity in *M. lusoria*. Although DPKQDFMRFamide itself showed a strong contractile activity, all of analogs, namely, DPKQD*A*MRFamide, DPKQDF*A*RFamide, DPKQDFM*A*Famide, and DPKQDFMR*A*amide, were found to be completely inactive. None of Ala-substituted analogs showed any contraction even at extremely high concentrations. This result was extractly reproduced for tetrapeptide FMRFamide. All of four Ala-substituted FMRFamide analogs were completely inactive. These results certainly demonstrate that C-terminal tetrapeptide is a major structural element to elicit a contractile activity in the heart muscle of *M. lusoria*.

FLRFamide is an endgenous ligand of the receptor for a contractile activity in the heart of *M. lusoria*. It should be noted that FLRFamide is considerably stronger

Table 2. Contractile activity of Ala-substituted analogs and hybrid analogs.

Peptides	EC_{50} (μM)
(*Ala-substituted analogs of Drosophila FaRP*)	
DPKQDFMRFamide	10.4 ± 6.92
DPKQDAMRFamide	inactive
DPKQDFARFamide	inactive
DPKQDFMAFamide	inactive
DPKQDFMRAamide	inactive
(*Ala-substituted analogs of tetrapeptide FaRPs*)	
FLRFamide	2.76 ± 2.39
FMRFamide	242 ±82.8
AMRFamide	inactive
FARFamide	inactive
FMAFamide	inactive
FMRAamide	inactive
(*Hybrid analogs*)	
DPKQDFLRFamide	1.76 ± 0.706
DPKQDFIRFamide	106 ±60.7
DPKQDFMHFamide	inactive
DPKQDFVRSamide	inactive

than FMRFamide (Table 2). Since DPKQDFMRFamide is about 23 times more potent than FMRFamide *per se*, it appeared to be intriguing to produce a hybrid analog between DPKQDFMRFamide and FLRFamide. Thus, we synthesized a hybrid analog of DPKQD*FLRFamide*. As shown in Table 1, this hybrid DPKQD*FLRFamide* was found to be almost 6 times more potent than the parent DPKQDFMRFamide. Since about 60% increased contractile activity was attained by this hybridization, DPKQD*FLRFamide* emerged as a strongest FaRP among the peptides tested in the present study.

We also synthesized a hybrid analog between DPKQDFMRFamide and MDSNFIRFamide. MDSNFIRFamide was very weak (EC_{50} = 322 μM) in the *M. lusoria* heart muscle assay. The resulting hybrid DPKQD*FIRFamide* was about 3 times more potent than MDSNFIRFamide. This clearly indicate that DPKQD reinforces the contractile activity. Although the activity of tetrapeptide FIRFamide was not evaluated in this study, it must be extremely weak.

When hybrid was produced between DPKQDFMRFamide and SAPQDFMHFamide or SVQDNFVRSamide, the resulting hybrid analogs DPKQD*FMHFamide* and DPKQD*FVRSamide* were found to be inactive. This result indicated that DPKQD moiety can not elicit directly any contractile activity. The present study has shown clearly the different roles of N-terminal DPKQD and C-terminal FMRFamide peptide structures in interacting with the receptors in the *M. lusoria* heart muscle. N-terminal moiety functions to reinforce perhaps supportively the direct receptor activation by the C-terminal FMRFamide. However, this is just for the contractile activity in the heart muscle of *M. lusoria*. *Drosophila* FaRPs may function differently in the tissues in the fruit flies. To elucidate such genuine

functions of FaRPs, physiological examinations of the peptides are in progress in our laboratory.

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The Effect of the Peptide Corresponding to the No.12 α-Helix on the Estrogen Receptor Activation

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An α-helix designated H12 present in the estrogen receptor is a major structural element for the receptor activation. The change in positioning of H12 after ligand binding is a key step to differentiate the agonist and antagonist. We examined the effects of H12 on the estrogen receptor activation induced by 17β-estradiol. It was found that this peptide affects negatively the 17β-estradiol-induced receptor activation without any influences in the receptor binding of 17β-estradiol.

Introduction

Estrogen receptor is a transcriptional regulatory factor present in the cell nucleus. Recent X-ray crystallographic analysis of the nuclear receptors revealed that the structures of ligand-free and ligand-bound structures are considerably different from each other [1]. It was further demonstrated that this conformational difference is due mainly to the lid-effect of an amphiphilic α-helix present at the C-terminal portion of the estrogen receptor. When the ligand binds to the receptor, this α-helix designated as H12 alters its positioning as to situate on the ligand in the binding cavity. This recruits a coactivater protein to bind to the complex for induction of the transcriptional activity [2].

In the present study, in order to elucidate a functional contribution of the peptide corresponding to an α-helix H12 in the receptor activation, we investigated the effects of the peptide on the activation of estrogen receptor by 17β-estradiol (E2).

Results and Discussion

The C-terminal region of the estrogen receptor was divided into four consecutive sections (15-17 amino acid residues), and these four peptides with N-terminal Cys(Acm) were synthesized by the solid-phase method with Fmoc chemistry. Peptide 1 consists of H12. Peptides were first assayed for the estrogen receptor to evaluate their binding ability using [^{3}H]17β-estradiol as a tracer. All peptides tested were completely inactive, showing absolutely no binding even at their extremely high concentrations. Even excess amount of peptides exhibited no effect for the binding of

17β-estradiol.

peptide **1**	C(Acm)LYDLLLEMLDAHRLHAa	(17aa)
peptide **2**	C(Acm)TSRGGASVEETDQSHLAa	(18aa)
peptide **3**	C(Acm)DQSHLATAGSTSSHSa	(16aa)
peptide **4**	C(Acm)LQKYYITGEAEGFPATVa	(18aa)

Fig. 1. The Fragment peptides of the C-terminal region of the estrogen receptor.

When these peptides were examined in the assay using anti-H12 antibody as described [3], it was found that the antibody response induced by 17β-estradiol is clearly inhibited by peptide **1** (Fig. 1). This is certainly due to the interruption of the binding of antibody to the receptor by peptide **1**. It should be noted that the antibody possesses a very high specificity, not interacting with other peptide segments of estrogen receptor C-terminal moiety.

All the peptides were also examined in the reporter gene assay, in which a protein luciferase is produced to evaluate the enzyme amount as a result of transcriptional activation. Estrogen receptor expression plasmid and luciferase reporter plasmid (a kind gift of Dr. M. Takeyoshi, Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita) were transiently transfected into HeLa cells by the liposome method. HeLa cells were maintained in Eagle's Minimal Essential Medium containing 10% dextran coated charcoal-treated fetal calf serum and appropriate antibiotics without phenol red. Details about the assay were described [4]. When peptides were added directly in the assay system, no effects were observed for all peptides. Thus, we utilized dimethyl sulfoxide (DMSO) as a permeabilizing agent for permitting the delivery of the peptides into the cell [5]. It was found that only peptide **1** diminishes the transcription activity induced by 17β-estradiol. This suggested that H12 recruits the coactivator by interacting at the site where the receptor binds. The present result is a demonstration that H12 is a genuine structural element essential for receptor activation.

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JOURNAL OF PEPTIDE SCIENCE

cDNA Cloning of the Housefly Pigment-Dispersing Factor (PDF) Precursor Protein and Its Peptide Comparison among the Insect Circadian Neuropeptides

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Abstract: Pigment-dispersing factor (PDF), an 18-amino acid neuropeptide, is a principal circadian neurotransmitter for the circadian rhythms of the locomotor activity in flies. Recently, two completely different types of PDF precursor were clarified; that of the cricket *Gryllus bimaculatus* and that of the last-summer cicada *Meimuna opalifera*. The *G. bimaculatus* PDF precursor is extraordinarily short and comprises a nuclear localization signal (NLS), while the *M. opalifera* PDF precursor is of ordinary length, comparable to that seen for the precursors of crustacean β-PDH homologues. Although their PDF peptide regions were exactly the same, the regions containing a signal peptide combined with a PDF-associated peptide (PAP) were remarkably different from each other. Such a grouping suggested a fundamental role for the PAP peptide in the circadian clock, perhaps associated with PDF function. In the present study, the cDNA cloning of PDF from the adult brains of the housefly *Musca domestica* was carried out and it was found that an isolated clone (527 bp) encodes a PDF precursor protein of ordinary length. The PDF peptide shows a high sequence identity (78%–94%) and similarity (89%–100%) to insect PDFs and also to the crustacean β-PDH peptides. In particular, there is only a single amino acid difference between the PDFs of *Musca* and *Drosophila*; at position 14 Ser for *Musca* PDF and Asn for *Drosophila* PDF. A characteristic Ser^{10} in *Drosophila* was retained in *Musca*, indicating the presence of a structural profile unique to these PDFs. The results of sequence analyses suggest that *Musca* and *Drosophila* PDFs are to be considered members of a single group that has evolved structurally. When the primary structure of the PAP regions was compared, the *Musca* PDF precursor also belonged to the same group as that to which the *Drosophila* PDF precursor belongs. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

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

Abbreviations: AAP, abridged anchor primer; AUAP, abridged universal anchor primer; La, lamina; LNs, lateral neurons; Me, medulla; NLS, nuclear localization signal; PAP, PDF-associated peptide; PCR, polymerase chain reaction; PDF, pigment-dispersing factor; PDH, pigment-dispersing hormone; RACE, rapid amplification of cDNA ends; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends; RT-PCR, reverse transcription PCR.

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The nucleotide sequence datum reported in this article has been submitted to the DDBJ/EMBL/GenBank Data libraries under accession number AB095922.

INTRODUCTION

Pigment-dispersing factor (PDF) is an 18-amino acid insect neuropeptide with the sequence of NSE(L/I)INSLL(G/S)LPKX(L/M)NDA-NH_2, where X represents a variable amino acid comprising either Asp, Val or Leu. PDF is involved in regulating insect circadian rhythms [1–4]. From many lines of evidence aimed at clarifying the function of PDF in the circadian system of the fruitfly *Drosophila melanogaster*, PDF has been thought to be a neuropeptide functioning as a principal circadian modulator downstream of the brain's clock machinery, organizing daily locomotion or other behaviours [5–8].

Until recently, only three *pdf* cDNAs have been elucidated for the structure of insect PDF precursors: the precursors of *D. melanogaster* [9], *Romalea microptera* (GenBank) and *Gryllus bimaculatus* [10]. The *C*-terminal PDF regions of these possess almost the same peptide size and sequence (number of amino acid residues (n) = 20 or 21). However, the region containing the signal sequence and PDF-associated peptide (PAP) region obviously differ, with 80, 66 and 20 amino acid residues, respectively. This is in contrast to the structures of the precursor proteins of the crustacean β-PDH (β-pigment-dispersing hormone) peptides. The latter are homologues of PDF and the size of their precursor proteins is almost the same, about 70 amino acids, similar to that of the *Romalea* PDF precursor. The size differences between the PDF precursors might reflect different functions of the PAP peptides. In pursuit of a further understanding of such functions it is necessary to accumulate molecular information about PDF and its precursor.

The cDNA cloning of the PDF precursor of the last-summer cicada *Meimuna opalifera* has been achieved and it was found that the signal and PAP peptide region were very similar to that of *Romalea microptera* [11]. Thus, it was hypothesized that PDF precursors are classified into at least three different types: *Drosophila*, *Meimuna-Romalea* and *Gryllus*. In the present study, the cDNA cloning of PDF from adult brains of the housefly *Musca domestica* (Diptera) was carried out. It is often said that the gene products of flies resemble those of mammals and especially the products of the fruit fly *Drosophila*, an invertebrate, are said to be intermediate between those of the vertebrate and invertebrate groups. In this regard, the peptides and proteins of the housefly *Musca* must be siblings of those in *Drosophila*. The housefly is a useful and important alternative to the fruit fly because of the size difference, for example, for *in vivo* studies. The sequence analyses of nucleotides and the deduced amino acids of *M. domestica* PDF are reported here. In order to characterize the PDF peptide and its precursor protein, further structural analyses were carried out to clarify the sequence.

MATERIALS AND METHODS

Animals

Animals, the housefly *Musca domestica* (a kind gift from Professor Ian A. Meinertzhagen, Dalhausie University, Halifax, Canada), were maintained at 25 °C under a day : night cycle of L12 : D12. To collect samples for cDNA cloning, the flies were frozen in liquid nitrogen and their heads were immediately harvested. The heads were stored at −80 °C until use. For *in situ* hybridization, male adults older than 24 h were used.

3′ RACE for Identification of 3′ end of PDF mRNA

The cDNA cloning of *Musca pdf* was carried out essentially as previously described [10]. mRNAs were extracted using a QuickPrep® *Micro* mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The mRNAs (350 ng) were reverse-transcribed by Super Script™ II reverse transcriptase (Life Tech., Rockville, MD, USA) using $d(T)_{17}$-adapter primer (5′-GGCCACGCGTCGACTAGTAC-T_{17}-3′) as previously described [12]. The resulting cDNA were subjected to the polymerase chain reaction (PCR) using the adapter primer (5′-GGCCACGCGTCGACTAGTAC-3′) and the degenerate primer PDF-F1 (5′-AAGCGCAACTCNGARMTVA-TCAACTCN-3′). In these primers, the letters M, N, R and V denote the nucleotides (A or C), (any nucleotides), (A or G) and (not T), respectively. The primer was designed based on the nucleotide sequence homology among mRNAs clarified for β-PDH and PDF [9,13–16]. PCR was performed using PLATINUM *Taq* DNA polymerase (Life Tech.) with the following conditions: 3 min at 94 °C followed by 3 cycles of 94 °C for 30 s, 72 °C for 90 s, 3 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 60 s, 3 cycles of 94 °C for 30 s, 64 °C for 30 s, 72 °C for 60 s, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, and a final extension for 7 min at 72 °C. The

PCR products were subcloned into pBluescript II SK+ and the sequence was analysed by a Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit (Amersham Pharmacia Biotech). The sequence analysed was confirmed by sequencing several other clones simultaneously.

5′ RACE for Identification of 5′ end of PDF mRNA

To amplify the 5′ end of the *M. domestica pdf* cDNA, 5′ RACE was carried out using Rapid amplification of cDNA ends (version 2.0: Life Tech.) mainly according to the manufacturer's protocol. First-strand cDNA was synthesized from a mRNA by Super Script™ II reverse transcriptase (Life Tech.) with a PDF-specific antisense primer (PDF-A1) (5′-ATCTTCTTCTTATCCATAGG-3′). The product was digested by RNase H/T_1 to remove the original mRNA template. A homopolymeric tail poly(C) was elongated by using terminal deoxynucleotidyl transferase and a substrate dCTP. The resulting tailed cDNA was amplified by the Abridged Anchor Primer AAP (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′, Life Tech.) of the kit and PDF antisense primer (PDF-A2) (5′-CTAATGCTGCATGAAA TCTATTTGCC-3′). The PCR product was further amplified with an Abridged Universal Anchor Primer AUAP (5′-GGCCACGCGTCGACTAGTAC-3′, Life Tech.) and PDF antisense nested primer (PDF-A3) (5′-GCCGGC-ATCGTTCATACTTTTGGGC-3′). Respective PCR and sequence analyses were carried out as described above, and the sequence analysed was confirmed by sequencing several different clones simultaneously.

Based on the sequence information obtained on 3′ RACE and 5′ RACE analyses, reverse transcription PCR (RT-PCR) was performed to amplify the full-length *pdf* cDNA by PfuTurbo® DNA polymerase (Stratagene, CA, USA). The PCR conditions were as for PCR in the 3′ RACE method, except for the primers, PDF-AF (5′-TCCATTCACCCGGAAATCTCTG-3′) and PDF-AR (5′-CACTTCATTCAACTAATACATCAACAAC-3′). These PCR products were treated as described above for sequence analysis.

To confirm the oligonucleotide sequence of the 5′ end, the RLM-RACE method was applied to the *M. domestica pdf* cDNA [17]. This RNA ligase-mediated rapid amplification of cDNA ends is used to define the transcription start site of the *pdf* gene. RLM-RACE was performed using the Gene Traer™ RLM-RACE Kit (Invitrogen, CA, USA) according to the manufacturer's instructions. After reverse transcription using the PDF-specific primer PDF-A1, the 5′ end of *pdf* cDNA was amplified using the outer adaptor primer of the kit and PDF-specific primer PDF-A2. The nested PCR was performed using the inner adaptor primer of the kit and PDF-A3. All these PCR were carried out under the same conditions as for 5′ RACE and amplified products were analysed as described above.

Whole Mount *in situ* Hybridization

RNA probes. Using T7 RNA polymerase, an antisense RNA probe labelled with digoxigenin (DIG)-UTP was produced *in vitro* from plasmid linearized with *Bam*HI. The plasmid used consisted of almost the full-length of *pdf* cDNA (463 bp in total) in the vector pBluescript II SK+. This *pdf* cDNA is a product of PCR using PDF-AF and PDF-AR. The reaction mixture of plasmid (1 μg), 5x buffer (4 μl; 0.2 M Tris•HCl (pH 8.0), 40 mM $MgCl_2$, 10 mM spermidine-$(HCl)_3$, 125 mM NaCl), 10x DIG RNA labelling mix (2 μl; 10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP (pH 7.5): Roche Diagnostics, Mannheim, Germany), 5 mM dithiothreitol (DTT) (2 μl), an RNase inhibitor RNasin® (1 μl; Promega, Madison, WI, USA), and T3 RNA polymerase for sense primer or T7 RNA polymerase for antisense primer (2 μl each (100 U) stored in 20 mM potassium phosphate (pH 7.7), 0.1 M NaCl, 0.1 mM DTT and 50% glycerol: Life Tech.) was incubated at 37 °C for 2 h. The reaction was terminated by adding 0.5 M EDTA (2 μl; pH 8.0). In order to precipitate DIG-labelled cRNA product, 4 M LiCl (2 μl) and ethanol (75 μl) were added and the solution was centrifuged at 17000 **g** for 20 min at 4 °C. The residual pellet was washed with 70% ethanol and dried in air. The pellet was eventually dissolved in 50% formamide (50 μl). The quality of the transcript was analysed by electrophoresis on 1.5% agarose gel stained with ethidium bromide.

In order to prepare a sense RNA probe for the assay of negative control, essentially the same method was utilized except for the use of T3 RNA polymerase instead of T7 RNA polymerase. *Hin*dIII was used as a restriction enzyme.

Tissue hybridization. Whole mount *in situ* hybridization was performed essentially as described by Wilkinson [18] with several significant modifications. Briefly, the brains of the housefly *Musca domestica* were dissected under microscopy and fixed in freshly made 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4 °C. Tissues