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Cytochrome P450 Enzymes Belonging to the CYP4 Family from Marine Invertebrates

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Six new cytochrome P450s assigned to the CYP4 family were identified from marine invertebrates belonging to the arthropod, mollusc, and echinoderm phyla. These are the first reported members of the CYP4 gene family from marine invertebrates, and extends the finding of the CYP4 family of cytochrome P450s to molluscs and echinoderms. Members of each phyla (echinodermata, arthropoda (crustacea), and mollusca) expressed genes belonging to the CYP4C subfamily in their respective digestive tissues. A mollusc, the mussel *Mytilus galloprovincialis*, expressed a gene belonging to a new CYP4 subfamily, CYP4Y. In Northern blotting experiments with digestive tissues of *M. galloprovincialis*, the expression of the CYP4Y1 gene was found to be inhibited by increasing concentrations of the hydrocarbon β-naphthoflavone. Thus, the potential use of marine invertebrate CYP4 genes as biomarkers of xenobiotic exposures may be warranted. © 1998 Academic Press

Cytochrome P450 enzymes constitute the largest protein family yet found in nature. The latest published account lists 481 genes and 22 pseudogenes representing 74 families (1), although many more genes and families are being described and this current information can be obtained from Dr. D. Nelson's homepage (http://drnelson.utmem.edu/nelsonhomepage.html). P450s are found in organisms ranging from bacteria to humans, and exhibit diverse isoforms within many given species. There are estimates of between 60-200 different genes in a single mammalian species (2). For invertebrates, P450s have been best studied in insects from which genes belonging to 5 families have been identified (CYP4, CYP6, CYP9, CYP18, and CYP28). Two other invertebrate groups also have P450 sequences represented in the Genbank database (crustacean CYP2L (3); crustacean CYP45 (4); mollusc CYP10 (5) and CYP30 (Genbank AF014795).

Cytochrome P450 enzymes metabolize a wide range of endogenous compounds (i.e. fatty acids, steroids, eicosanoids) and xenobiotics (i.e. hydrocarbons, plant chemicals, pesticides). The function of these enzymes in marine invertebrates has been questioned since early studies claimed that P450s were present, though activities were low compared to mammalian enzymes (6-8). Problems with marine invertebrate P450 biochemical measurements may also explain the apparent lack of enzymatic inductions in species exposed to xenobiotics (9-10), since others have reported such inductions in crustacean microsomal preparations following treatment of crabs, crayfish, and copepods with xenobiotics (11-13). Conclusive evidence of NADPH cytochrome P450 reductase destruction during microsome preparation from crustacean tissues has been documented (14). This may explain many of the reported low P450 activities noted by many researchers (reviewed in 15-16) as resulting from reduced or inactive P450 electron donors.

The aforementioned biochemical inconsistencies in marine invertebrate P450 studies can be overcome using molecular tools to identify new CYP genes and study their expression patterns following chemical treatments. This approach has been largely successful in the identification of new P450 genes belonging to the CYP4, CYP6, CYP9, and CYP28 families from terrestrial invertebrates (17-23). Here, this approach was successfully utilized to obtain new P450 genes from crustaceans, molluscs, and an echinoderm. In a series of Northern blotting studies, mussel digestive gland CYP4Y1 expression is negatively affected by exposure to β-naphthoflavone, a typical hydrocarbon inducer of cytochrome P450.

MATERIALS AND METHODS

Identification of P450 cDNAs. Total RNA was isolated from juvenile lobster (*Homarus americanus*) hepatopancreas, adult shrimp

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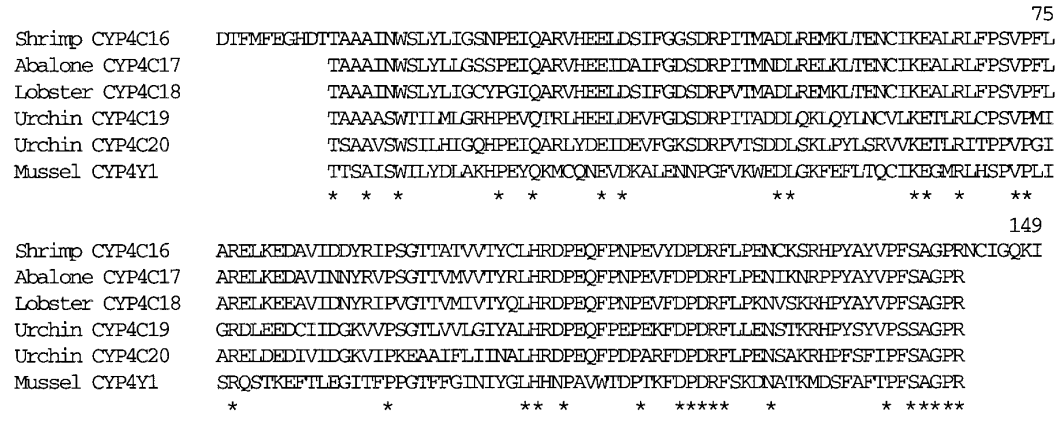

FIG. 1. Deduced amino acid sequence alignment of the partial new CYP4 P450s obtained from the following marine invertebrate species: shrimp *Penaeus setiferus* (CYP4C16), abalone *Haliotis rufescens* (CYP4C17), lobster *Homarus americanus* (CYP4C18), sea urchin *Lytechinus anamesis* (CYP4C19; CYP4C20), and mussel *Mytilus galloprovincialis* (CYP4Y1). All partial CYP4 cDNAs were generated by RT-PCR of digestive tissue RNA from each species.

(*Penaeus setiferus*) hepatopancreas, adult mussel (*Mytilus galloprovincialis*) digestive gland, juvenile abalone (*Haliotis rufescens*) digestive gland, and adult sea urchin (*Lytechinus anamesis*) pyloric caeca using the Totally RNA kit (Ambion). Reverse transcription was performed using 5 μg of RNA, and an oligo-dT primer with Superscript reverse transcriptase at 37^{O}C according to the Stratagene protocol. PCR was performed using TAQ DNA polymerase (PCR buffer with 2.5 mM Mg^{2+}) and the following conditions: 94°C 1 min, 54°C 1 min, 72°C 1 min for 35 cycles and the following degenerate primers designed against CYP4 sequences (18): 1) forward, GA(C/T)ACITT (C/T)ATGTT(C/T)GA(A/G)GGCATGCA(C/T)GA(C/T)AC; 2) reverse, GC(A/G)AT(C/T)TT(C/T)TG(A/C/G/T)AT(AG/T)AT(A/G)CA(A/G)TT. PCR products were gel purified and cloned into the pCR II vector (Invitrogen). Sequencing of PCR products was done by the RBI sequencing facility at the University of California, Davis. Identification of P450 clones was done by NETBLAST searching (NCBI). Percent identities between these sequences and other CYP4′s was done using the Distances program (GCG) and an algorithm comparing the partial cDNA sequence identity from the I helix to the heme-binding region to that of the entire P450 protein (17).

Mussel CYP4Y1 expression. Adult mussels (*M. galloprovincialis*) were maintained in running seawater (14°C) at the Bodega Marine Laboratory. Mussels were exposed in individual static, aerated glass jars, to 0.5 mg or 5.0 mg/1.5 liter of β-naphthoflavone (BNF, or methyl cellosolve vehicle alone) for two days. Mussels were not fed during the exposures. Total RNA was prepared from digestive glands using the above protocols and Northern blots were obtained from denaturing gels (1%) using 30 μg of RNA/lane. Mussel CYP4Y1 cDNA was random primed (^{32}P-dCTP) using the Stratagene Kit, and blots were probed at 42°C in 50% formamide, 5x Denhardt's, 2X SSPE, 0.5% SDS, and 100μg/ml denatured herring sperm DNA. Final washes were in 0.1X SSPE, 0.1% SDS at 60°C. Resulting blots were scanned to determine band intensities, and CYP4Y1 mRNA levels were compared with those of lobster actin probes of the same blots to correct for equal RNA loading.

RESULTS AND DISCUSSION

Identification of marine invertebrate CYP4. Six partial cDNAs for new marine invertebrate CYP4 family members were initially obtained by RT-PCR. Figure 1 shows an alignment of the deduced amino acid sequences for shrimp, *Penaeus setiferus*, CYP4C16 (Genbank accession # AF072854), abalone, *Haliotis rufescens*, CYP4C17 (Genbank accession # AF072853), lobster, *Homarus americanus*, CYP4C18 (Genbank accession # AF072852), sea urchin, *Lytechinus anamesis* (formerly *L. pictus*), CYP4C19 and CYP4C20 (Genbank accession #s AF074850 and AF072851), and mussel, *Mytilus galloprovincialis*, CYP4Y1 (Genbank accession # AF072855). These new marine invertebrate CYP4 sequences add considerable information about the diversity of cytochrome P450 families in eucaryotes. The result from mussels confirms earlier reports of the possible presence of a CYP4-like gene in *M. edulis* on the basis of digestive gland mRNA binding of a rat CYP4A1 probe (24). The mussel CYP4Y1 appears most similar to vertebrate CYP4A , CYP4F, and CYP4T subfamily members, while all of the other marine invertebrate CYP4C sequences belong in an invertebrate-specific CYP4C subfamily (D. Nelson, pers. comm.). It will be of interest to compare the gene structures and chromosomal localization of these sequences with known clusters of CYP genes in other invertebrates. For example, clusters of P450 genes have been described in insects, including CYP6 genes in the housefly *M. domestica* (25), and those of CYP4, CYP6, and CYP9 families in *Drosophila melanogaster* (20, 21, 27).

Overall sequence identities (calculated using the algorithm of Scott et al. (17) with the cockroach *Blaberus discoidalis* CYP 4C1 (27) CYP4 family members are given in Table 1. The algorithm gives an accurate depiction of approximate overall sequence identities (r^2 = 0.983) based on knowledge of the portion of the sequence between the I helix and the heme-binding regions of P450s. The percentage identities of the CYP4 sequences are: Shrimp CYP4C16 (60%); Abalone CYP4C17 (53%); Lobster CYP4C18 (58%); Sea Urchin CYP4C19 (50%); and Sea Urchin CYP4C20 (43%); Mussel CYP4Y1 (35%).

The only reported P450 sequences to date from marine/aquatic invertebrates are those of the CYP45 (lobster *H. americanus*, (4), CYP10 (snail, *Lymnaea stagnalis*, (5), CYP30 (clam *Mercenaria mercenaria*, Genbank AF014795), and CYP2L (spiny lobster, *Panulirus argus*, (3). Molecular evidence (binding of nucleotide probes to RNA blots) for the presence of additional P450 families in marine invertebrates are CYP1, CYP3, and CYP11 in molluscs (28-29). In addition, the presence of P450s similar to the CYP1, CYP2, and CYP3 families in cnidarians (30), and CYP2 in molluscs (31) was insinuated on the basis of immunological relatedness on western blots using antisera against vertebrate P450s.

Mussel CYP4Y1 expression studies. A single band of 2.2 kd representing the *M. galloprovincialis* CYP4Y1 was found by Northern blotting (not shown). Currently, the function of these new CYP4 family members in the mussel or in any of these marine invertebrates is unknown. Cytochrome P450s of the CYP4 gene family are involved in fatty acid and prostaglandin metabolism (reviewed in 32). Some genes in this family are inducible by hydrocarbons, polychlorinated biphenyls, and peroxisome-proliferating chemicals such as clofibrate. Bradfield et al. (27) previously determined that the insect peptide hypertrehalosemic hormone, involved in carbohydrate mobilization, induces expression of CYP4C1 in the fat body of the cockroach *Blaberus discoidalis*. Likewise, some mammalian CYP4A genes are induced by androgens in certain tissues (32).

Expression of CYP4Y1 in digestive glands was determined after two days of exposure to BNF (Fig. 2). A 30% fold decrease in CYP4Y1 mRNA was found following exposure to vehicle alone (0.03% by volume). Exposure to 0.5-5.0 mg BNF for 2 days resulted in 58-90% decrease in CYP4Y1 expression. The reason for this dramatic reduction in CYP4Y1 expression by relate either to direct effects of a hydrocarbon receptor on gene transcription although the evidence for such a receptor in marine invertebrates is still in question (34-35). Information on specific CYP gene expression patterns is currently lacking for other aquatic and marine species with significant P450 enzyme activity alterations following xenobiotic treatment (for example 11-13). Michel et al. (33) showed BP hydroxylase (CYP1A-type activity) and laurate hydroxylase (CYP4-type activity) P450 activities in mussel digestive glands were inducible by hydrocarbons and phenobarbital. In contrast, hydrocarbon treatment can result in inhibition of benzo[a]pyrene hydroxylase activity in mussels, and BNF exposure resulted in lowered total cytochrome P450 and reduced P450 enzymatic activities in *Octopus pallidus* (36). In the lobster, *H. americanus*, BNF exposure had no effect on hepatopancreas CYP45 expression (4). These results support the potential for both reduced specific CYP4 mRNA levels and P450 enzyme activities as potential biomarkers of xenobiotic exposures in molluscs.

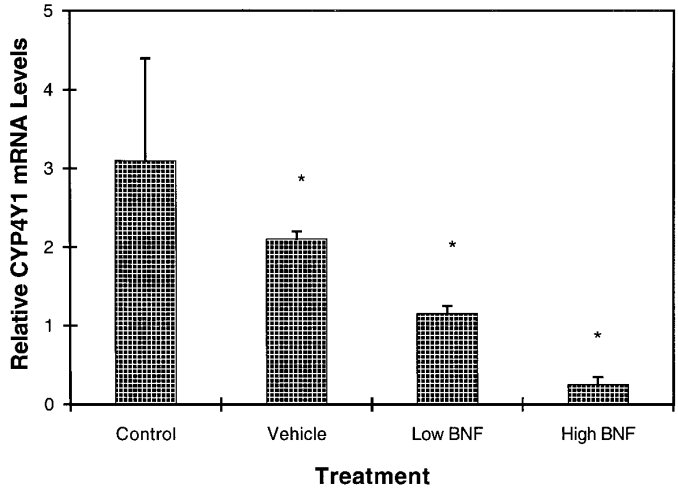

FIG. 2. Mussel (*M. galloprovincialis*) CYP4Y1 mRNA levels in digestive glands following β-naphthoflavone exposure. Bars represent the mean $\pm$ 1 standard deviation of 4 individuals. Asterisks indicate significant difference from controls. Control = seawater control, vehicle = 0.03% methyl cellosolve alone, low BNF = 0.5 mg/1.5 l β-naphthoflavone, high BNF = 5.0 mg/1.5 l β-naphthoflavone.

TABLE 1

Sequence Homologies of Marine Invertebrate CYP4 Family Members with Insect CYP4C1

Species	CYP4	Partial cDNA identity	Predicted overall identity[a]
P. setiferus	CYP4C16	65%	60%
H. rufescens	CYP4C17	61%	53%
H. americanus	CYP4C18	64%	58%
L. anamesis	CYP4C19	58%	50%
L. anamesis	CYP4C20	54%	43%
M. galloprovincialis	CYP4Y1	43%	35%

[a] The algorithm of Scott *et al.* (1994) was used to predict the overall amino acid sequence identity of each new marine invertebrate CYP4 from the partial identity of the RT-PCR product.

The results reported in the present work demonstrate specific marine mollusc CYP4 gene expression changes as a result of chemical treatment. Cytochrome P450-mediated testosterone metabolism in snail digestive gland microsomes is significantly altered by increasing concentrations of tributyl tin (TBT, 37). This finding presents a strong argument that TBT-induced genital defects (imposex, 38) in molluscs may result from its direct effects on as yet unknown P450(s). The potential involvement of mollusc CYP4 genes in these physiological responses to certain xenobiotics remains to be studied.

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