Molecular Characterization of Three Peroxisome Proliferator-Activated Receptors from the Sea Bass (*Dicentrarchus labrax*)

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ABSTRACT: Peroxisome proliferator-activated receptors (PPAR) are nuclear hormone receptors that control the expression of genes involved in lipid homeostasis in mammals. We searched for PPAR in sea bass, a marine fish of particular interest to aquaculture, after hypothesizing that the physiological and molecular processes that regulate lipid metabolism in fish are similar to those in mammals. Here, we report the identification of complementary DNA and corresponding genomic sequences that encode three distinct PPAR from sea bass. The sea bass PPAR are the structural homologs of the mammalian PPARα, β/δ, and γ isotypes. As revealed by RNase protection, the tissue expression profile of the fish PPAR appears to be very similar to that of the mammalian PPAR homologs. Thus, PPARα is mainly expressed in the liver, PPARγ in adipose tissue, and PPARβ in all tissues tested, with its highest levels in the liver, where it is also the dominant isotype expressed. Like mammalian PPAR, the sea bass isotypes recognize and bind to PPAR response elements of both mammalian and piscine origin, as heterodimers with the 9-*cis* retinoic acid receptor. Through the coactivator-dependent receptor ligand assay, we also demonstrated that natural FA and synthetic hypolipidemic compounds can act as ligands of the sea bass PPARα and β isotypes. This suggests that the sea bass PPAR act through similar mechanisms and perform the same critical lipid metabolism functions as mammalian PPAR.

Paper no. L9546 in *Lipids 39*, 1085–1092 (November 2004).

In all species, energy homeostasis is achieved through the efficiencies of caloric intake, storage of excess calories, and fuel mobilization. In higher vertebrates under normal conditions, two classes of biological molecules, lipids and carbohydrates, provide essentially all of the necessary fuel. In fish and especially in the carnivorous species, which includes most marine fish, dietary lipids are the major provider of energy while carbohydrates play only a minor role due to their low abundance in natural diets.

Interest in fish FA and lipid metabolism has grown in the last decade as a result of the rapid expansion of intensive aquaculture. As a result, issues relating to physiology [e.g. excess deposition of fat in the carcass and liver of farmed fish (2)] or nutrition [e.g. the substitution of fish oils in fish diets by vegetable oils (3)], have become areas of considerable interest (see review in Ref. 1).

It is generally accepted that the biosynthesis and catabolism of FA involve equivalent pathways in mammals and fish (4). However, it remains to be determined whether the same molecular mechanisms control these pathways. In mammals, peroxisome proliferator-activated receptors (PPAR) have emerged as central factors in sensing FA levels and in regulating FA metabolism. PPAR are ligand-induced transcription factors belonging to the nuclear hormone receptor superfamily. Three PPAR isotypes, termed α, β/δ, and γ, have been identified from different organisms, including mammals, birds, and amphibians. PPAR activate the transcription of target genes by binding as heterodimers with the 9-*cis* retinoic acid receptor (RXR) to specific enhancer elements of the DR-1 (direct repeat of the hexanucleotide sequence 5′-AGCTCA, spaced by one nucleotide) type, known as peroxisome proliferator or PPAR response elements (PPRE). Ligand binding induces conformational changes to the receptor, resulting in both the recruitment of transcriptional coactivators in the promoter region of the target gene and in increased transcription. Specific ligands for each PPAR isotype have been identified and include a number of unsaturated FA, eicosanoids, and hypolipidemic and antidiabetic agents (5,6).

In contrast to the large amount of information available on mammalian PPAR, reports for PPAR in fish are scarce and, thus far, limited to the identification of the PPARγ isotype in two species (7,8). The presence of even a single PPAR isotype in lower vertebrates raises intriguing questions for the evolution of the structure and function of these receptors. In addition, the possibility that PPAR in fish fulfill the same functions as in mammals presents possibilities for manipulating FA metabolism in fish by interventions targeted specifically at these key regulatory factors. To begin to address these

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Abbreviations: bp, base pair; CARLA, coactivator-dependent receptor ligand assay; DR-1, direct repeat of the hexanucleotide sequence 5′-AGCTCA, spaced by one nucleotide; EMSA, electrophoretic mobility assay; GST, glutathione-*S* tranferase; nt., nucleotide(s); LBD, ligand binding domain; PPAR, peroxisome proliferator-activated receptors; PPRE, PPAR response elements; RACE, rapid amplification of cDNA ends; RT, reverse transcription; RXR, retinoic acid receptor; SRC-1, steroid receptor coactivator-1.

possibilities, we searched for PPAR in the sea bass (*Dicentrarchus labrax*), a species of particular interest to aquaculture. We report here the isolation of cDNA (the Genbank accession numbers of cDNA sequences reported here are AY590300 for sea bass PPARα, AY590302 for sea bass PPARβ, and AY590303 for sea bass PPARγ) and genes encoding three PPAR isotypes from this species. We also provide a preliminary account of the functions of these receptors.

EXPERIMENTAL PROCEDURES

PPAR gene isolation. Sea bass genomic DNA was prepared from muscle tissue with the DNeasy tissue kit (Quiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. PPAR genomic clones were isolated by direct PCR amplification of the DNA using isotype-specific primers. A partial genomic PPARα clone was obtained with primers 5′-CCA AAA GAA GAA CCG CAA CAA G and 5′-TTG CAG GAG CGG GTG CAA CGA CG. A PPARβ partial clone was obtained with primers 5′-ATG GAA GGG TTT CAA CAA ACT G and 5′-CTA ATA CAT GTC TTT GTA GAT CTC CTG. For PPARγ, primer pairs 5′-GTC GAC ATG GTG GAC AC and 5′-TGT AAT CCA TGT TCG TCA GG were used to amplify the genomic sequences encoding for the A/B domain; primers 5′-TTC AAT CAA GAT GGA GCC and 5′-TCA CAG GCA TGG ACG CC were used to amplify the exon encoding the first Zn finger of the C domain; and primers 5′-GCT GCA AGG GTT TCT TCA G-3′ and 5′-CGT TGT GTG ACA TGC CG-3′ were used to amplify the genomic sequences encoding the second Zn finger of the receptor's C domain. In all of the PCR reactions, high fidelity polymerase (Expand High Fidelity PCR system; Roche Applied Science, Mannheim, Germany) was used to minimize errors in the amplified products.

PPAR cDNA isolation. Total sea bass liver RNA was reverse transcribed with Expand Reverse Transcriptase (Roche). The resulting cDNA was used as a template for RACE PCR for the amplification of the 5′-ends of the receptors, with primers derived from partial genomic sequences. Either the SMART RACE kit (Clonetech) or the 5′/3′ RACE kit (Roche) was used in these experiments. The gene-specific primers 5′-GCC ACC TCT TTC TCC ACC A and 5′-CGG CCC TCT TCT TGG TCA T for PPARα and β, respectively, were used. Isolation of the entire coding sequences of the PPAR isotypes was done with RT-PCR (Expand Reverse Transcriptase; Roche) on total liver RNA with primers 5′-ACG CGG GTG GTA TTT ATC TTC T and 5′-TCA GTA CAT GTC CCT GTA GAT TTC TTG C for PPARa; 5′-ATG GAA GGG TTT CAA CAA ACT G and 5′-CTA ATA CAT GTC TTT GTA GAT CTC CTG for PPARβ; and 5′-GTC GAC ATG GTG GAC AC and 5′-CTA ATA CAA GTC TTT CAT GAT CTC for PPARγ (initiation and termination codons underlined). All cDNA were cloned into the pCR Script vector (Stratagene, La Jolla, CA) for further analyses.

Riboprobes and RNase protection assay. For the synthesis of sea bass PPAR isotype-specific riboprobes, the fragment encoding the D domain of each isotype was amplified by PCR. For PPARα, primers 5′-TTG GAT CCG CCA TTC GGT TTG GTC and 5′-AGA ATT CCT TGC TTG TCT TTC C were used to amplify a 197 base pair (bp) fragment [nucleotides (nt.) 554–751 of cDNA)]. For PPARβ, primers 5′-TTG GAT CCG CGA TCC GAT ACG GAC and 5′-AGA ATT CGA TGC TGC GGG CCC T were used to amplify a 175 bp fragment (nt. 631–806 of cDNA). For PPARγ, primers 5′ TTG GAT CCG CTA TTC GTT TTG and 5′-AGA ATT CCG CGT TAT CTC CGG T were used to amplify a 201 bp fragment (nt. 598–799 of cDNA). For directional cloning into the pBluescript KS vector (Stratagene), all upstream primers contained a *Bam*HI restriction enzyme site and all downstream primers an *Eco*RI site (underlined in the preceding primer sequences). A 203 bp β-actin fragment (nt. 228–431 of Genbank accession number AY148350) was amplified by reverse transcription (RT)-PCR from sea bream liver total RNA, with primers 5′-GAC CAA CTG GGA TGA CAT GG and 5′-GCA TAC AGG GAC AGC ACA GC and was cloned into the pCR Script vector (Stratagene).

Antisense PPAR riboprobes were synthesized by T3 RNA polymerase (Promega, Madison, WI) transcription on the *Bam*HI-digested plasmids. The β-actin plasmid construct was digested with *Not*I and the antisense riboprobe was synthesized by T7 RNA polymerase (Promega) transcription.

Total RNA from sea bass tissues, eggs, and larvae was extracted with the RNeasy tissue kit (Qiagen) according to the manufacturer's instructions. Intestine refers to the gut region adjacent to the cecum. Adipose is mesenteral adipose.

Sea bass PPAR mRNA expression was assessed by the RNase protection assay using a commercial kit (RNase protection kit; Roche), according to the manufacturer's instructions. All riboprobes were labeled with [α-^{32}P]CTP (800 Ci/mmol; Amersham Biosciences Europe GmbH, Freiburg, Germany) and their specific activity was quantified as described in the manual of the RNase protection kit. For PPAR expression, 8 μg of total RNA from each tissue sample were hybridized with the isotype-specific probes (approximately 3 fmol of each) before being subjected to digestion by RNases. For β-actin expression, 5 μg of total RNA were used with approximately 80,000 cpm of the riboprobe. The protected fragments were separated on a 6% polyacrylamide gel containing 7 M urea. Signals were visualized by autoradiography.

Electrophoretic mobility shift assay. Sea bass and mouse retinoic acid receptor (RXR)β (mRXRβ, 9) PPAR proteins were obtained by *in vitro* transcription and translation using the TNT coupled reticulocyte lysate system (Promega). Electrophoretic mobility assay (EMSA) was performed as previously described (9). The acyl-CoA oxidase and Cyp4A6-Z PPRE have been previously described (10). The GSTA1.1-3 probes correspond to the presumed PPRE elements of the plaice GSTA1 promoter (11). Specifically, these are between nucleotide positions 3713 and 3734 (GSTA1.1), 3718 and 3740 (GSTA1.2), and 3771 and 3793 (GSTA1.3) of Genbank accession number X95199. For antibody-induced supershifts, 2 μL of PPARγ antibody or pre-immune serum were intro-

duced to the reaction mix simultaneously with the proteins and probe.

Coactivator-dependent receptor ligand assay (CARLA) screen. The sequences encoding the D and E domains of the sea bass PPAR (nt. positions 504–1510 and 631–1533 of the cDNA for α and β isotypes, respectively) were amplified by PCR and cloned into the pGEX-2TK vector (Amersham Biosciences Europe GmbH) for high expression as glutathione-*S* transferase (GST) fusion proteins in *Escherichia coli* (BL21-pLysS; Stratagene). The correct sequence of all recombinant constructs was confirmed by sequencing. Recombinant proteins were purified and the CARLA screen was performed as previously described (12). The concentration of all compounds tested was 10^{-4} M. All compounds were obtained from Cayman Chemical (SPI-BIO; Massy, France), except for Wy14,643 (pirinixic acid) and CLA, mixed isomers (Sigma Aldrich, St. Louis, MO). Signals were quantified either by phosphor analysis (Molecular Imager FX system; Bio-Rad Laboratories, Richmond, CA) or image analysis (Gel-Pro, Media Cybernetics, Silver Springs, MD). Statistical analysis of data (ANOVA) was done using the STATGRAPHICS*Plus*, version 5, package (Manugistics, Inc., North Reading, MA).

RESULTS

Genomic sequences and cDNA encoding three distinct PPAR from sea bass. Using primers designed from sequences conserved in PPAR from other phyla, as well as from other fish species (7), PCR was performed on sea bass DNA. With this approach, distinct genomic fragments were isolated and, upon sequence analysis, revealed regions with significant homology to known PPAR. Specifically, the genomic regions amplified included all of the coding exons of the presumed PPARβ gene and the exons encoding the carboxy terminus, that is, part of the C domain and the D and E domains of the presumed PPARα receptor were amplified (Fig. 1). For the PPARγ gene, we were successful in amplifying only two individual exons, namely, those encoding the A/B domain and the second Zn finger of the C domain of the receptor. Notable is the small size of the introns in both the PPARβ gene and in the characterized portion of the PPARα gene. Therefore, these two sea bass genes are several times smaller than their mammalian counterparts. Interestingly, the proposed gene structures suggest that the E domain of the fish receptors is encoded by three exons. This is in contrast to mammalian or amphibian PPAR, where this domain is encoded by only two exons (13,14). Also of interest is that, with the exception of the additional exon in the E domain, all exon/intron boundaries in the characterized regions of the sea bass PPAR genes are in essentially identical positions in the mammalian genes (Fig. 2).

To confirm that the isolated genomic fragments constitute parts of functional genes, we performed 5′ RACE and RT-PCR

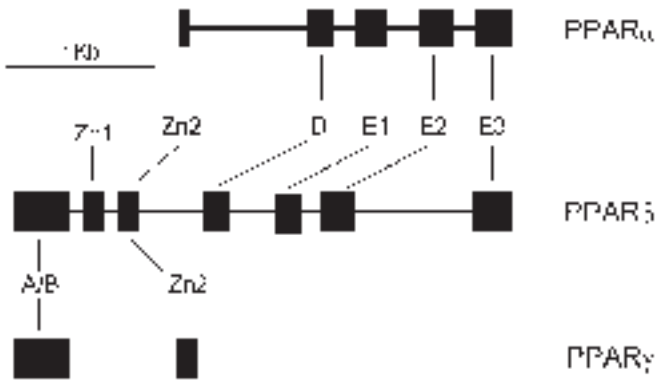

FIG. 1. Schematic representation of the sequenced portions of the peroxisome proliferator-activated receptor (PPAR) genes. Equivalent exons between isotypes are indicated by lines. Zn1 and Zn2 are the exons of the DNA-binding domain and E1–E3 are the exons of the ligand-binding domain.

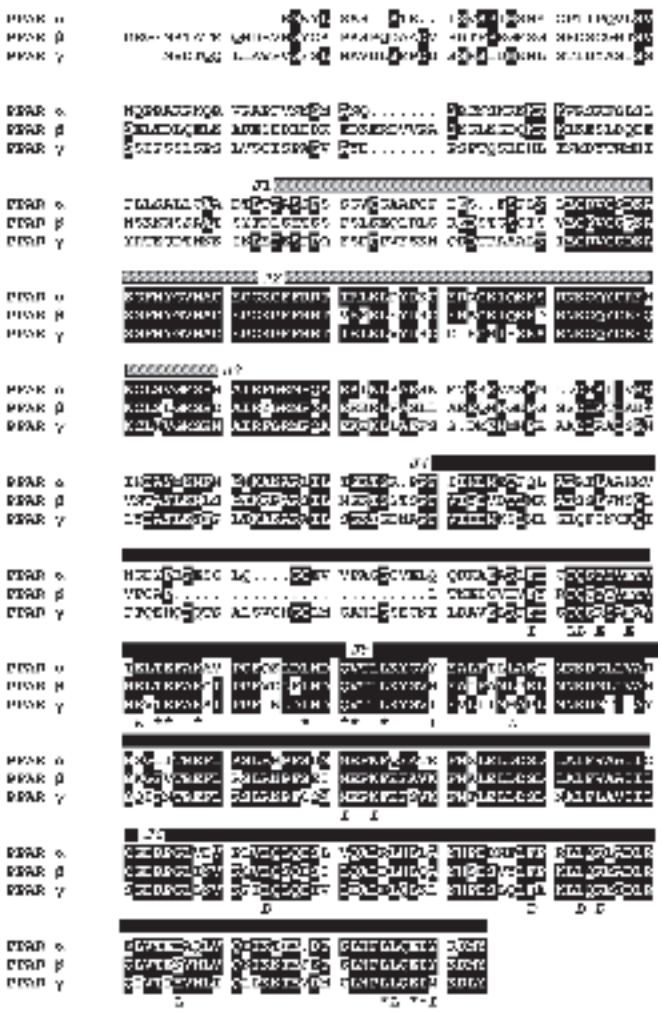

FIG. 2. Amino acid sequence alignment of the three sea bass PPAR isotypes. Identical residues between the three sequences are shaded. The DNA- and ligand-binding domains are indicated by gray and black bars, respectively. *J1–J6* indicate positions of corresponding introns (see Fig. 1). Italicized letters below a sequence indicate corresponding residues involved in ligand binding (*L*), ligand entry (*E*), and dimerization (*D*) in mammalian PPAR (20). Similarly, asterisks indicate residues involved in PPAR–SRC-1 (steroid receptor coactivator-1) interactions. See Figure 1 for abbreviation.

on sea bass liver-derived cDNA, using PPAR isotype-specific primers that were designed according to the distinct genomic sequences. These experiments resulted in the isolation of three cDNA, each encoding a distinct PPAR isotype. The cDNA corresponding to the PPARβ and γ isotypes apparently contained the entire coding sequence and, in the case of PPARβ, the 5′ untranslated region. For PPARα, none of the 5′ RACE products that we analyzed contained the complete 5′-end sequence, as no translation initiation codon conforming to the Kozak consensus could be located within the sequences obtained. Therefore, the cDNA for the sea bass PPARα must be considered partial.

The deduced amino acid sequences of the sea bass PPAR are shown in Figure 2. The encoded proteins have a length of 510 and 522 amino acids for the β and γ isotypes, respectively, while the obtained open reading frame for PPARα encodes a protein of 502 residues. The alignment in Figure 2 also demonstrates that the highest identity between isotypes is observed within the C and E domains.

When the sea bass PPAR proteins are compared to their human counterparts (Fig. 3), the region of highest identity is the C domain, where ≥90% of the residues are common among the corresponding homologs. Identity is lower at the E domain, where 67, 78, and 66% of the residues are common in the α, β, and γ isotypes, respectively. It should be noted that this reduced identity results in part from the insertion of 20 and 25 amino acid residues at the amino terminus of the domain in the sea bass α and γ isotypes, respectively. This insertion may be related to the fact that the E domain of the sea bass receptors is encoded by three exons, as opposed to two exons for the corresponding region in the human receptors. However, it is notable that the sea bass PPARβ E domain, which is also encoded by three exons, contains exactly the same number of residues as its human counterpart.

The region with the lowest identity between the sea bass and human receptors is the A/B domain, which is considerably longer in the isotypes of the fish species than in mammals.

Sea bass PPAR are differentially expressed in tissues and during development. The expression of PPAR in different tissues of sea bass was determined by the RNase protection assay. As the results in Figure 4A demonstrate, relatively abundant transcripts of PPARα are detected in liver, gills, heart, red muscle, and brain. The highest expression, relative to the other two subtypes, was observed in red muscle. PPARα is weakly expressed in intestine and spleen, while no expression is detected in either kidney or adipose tissue. In general, the tissue expression profile of this isotype is very similar to its mammalian counterpart in tissues with high β-oxidation capacity (15). Also, like its mammalian homolog, PPARβ is expressed in all the tissues of sea bass that were tested, with the liver being the site where mRNA for this receptor is most abundant. Like the α isotype, PPARγ exhibits a restricted tissue distribution and, like its mammalian homolog, is most abundantly expressed in adipose tissue. Relatively high amounts of PPARγ transcripts are also detected in the gills and lower amounts in red muscle and the intestine. Only trace amounts of PPARγ are detected in the liver. Notable is the absence of expression of this isotype in spleen and kidney, two tissues known to express PPARγ in mammals and amphibians (16,17).

PPAR expression is differentially regulated during development in both mammals and amphibians (18,19). Therefore, we also examined the expression of these receptors at stages considered critical during the development of sea bass. These included the fertilized egg (mostly, the neurula stage in our sample), the day following the first feeding (day 6, post-hatch), and different stages leading to metamorphosis (day 50 post-hatch).

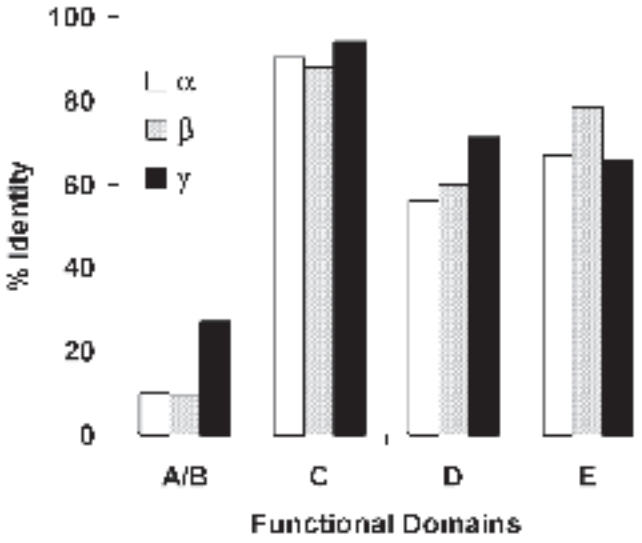

FIG. 3. Amino acid sequence identity between the functional domains of the sea bass and human PPAR isotypes. Open, gray, and black bars correspond to the α, β, and γ isotypes, respectively. For the C domain, only the residues in the two Zn fingers and the carboxy-terminal extension are compared. See Figure 1 for abbreviation.

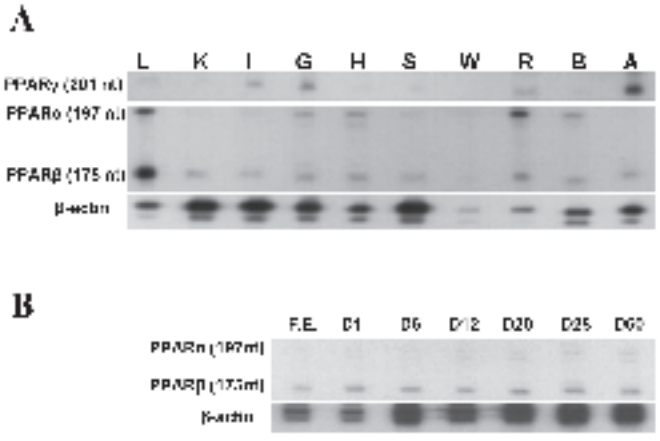

FIG. 4. Tissue and developmental stage expression of the sea bass PPAR. (A) Expression of PPAR and β-actin in the liver (L), kidney (K), intestine (I), gills (G), heart (H), spleen (S), white muscle (W), red muscle (M), brain (B), and adipose tissue (A) of sea bass, as determined by RNase protection. The length in nucleotides (nt) of the corresponding riboprobe for each PPAR isotype is indicated. (B) Expression of the sea bass PPARα and β isotypes during development. F.E. is fertilized egg and D indicates the number of days post-hatch of the larvae tested. See Figure 1 for abbreviation.

As demonstrated in Figure 4B, PPARβ transcripts were detectable in fertilized eggs and persisted at an approximately constant level in all the larval stages that we examined. The early expression of this receptor is in agreement with observations made in *Xenopus* sp. (16,18) and also in mammals (19).

Also like the expression in other species, sea bass PPARα zygotic transcripts appeared to accumulate at later embryonic stages as compared to PPARβ. Thus, PPARα mRNA was detected in one day post-hatch larvae, albeit at low levels, and in all subsequent larval stages (Fig. 4B).

With the approach used, we were not able to detect PPARγ transcripts in any of the larval stages examined. However, it should be noted that these experiments were performed in whole larvae and it is possible that PPARγ transcripts, which in the adult fish exhibit the most restricted tissue distribution (Fig. 4), have been discriminated against.

The sea bass PPAR bind to DR-1 elements. As previously mentioned (Fig. 3), the DNA binding domains of the three sea bass PPAR exhibit high levels of identity with their homologs from other species. Furthermore, residues shown to contribute to the PPAR:RXR dimerization interface in the human PPARγ (20) are apparently conserved in the sea bass receptors. Thus, it is expected that the sea bass PPAR also form heterodimers with RXR and that the sea bass PPRE are also of the DR-1 type. To test this, we performed EMSA with *in vitro* translated sea bass PPAR and mRXRβ. As probes, we used the well-characterized mammalian PPRE of the acyl-CoA oxidase and Cyp4A6 promoter (Cyp4A6-Z) (13), as well as potential PPRE derived from the promoter of the plaice GSTA1 gene, a piscine gene previously shown to be positively regulated by peroxisome proliferators (11). The sequences of these PPRE are shown in Figure 5A. In contrast to the sea bass PPARα, which lacks a proper initiation codon, both the β and γ isotypes were efficiently translated *in vitro* (not shown) and were used in EMSA. As shown in Figure 5B, both receptors bound efficiently to all the PPRE that were

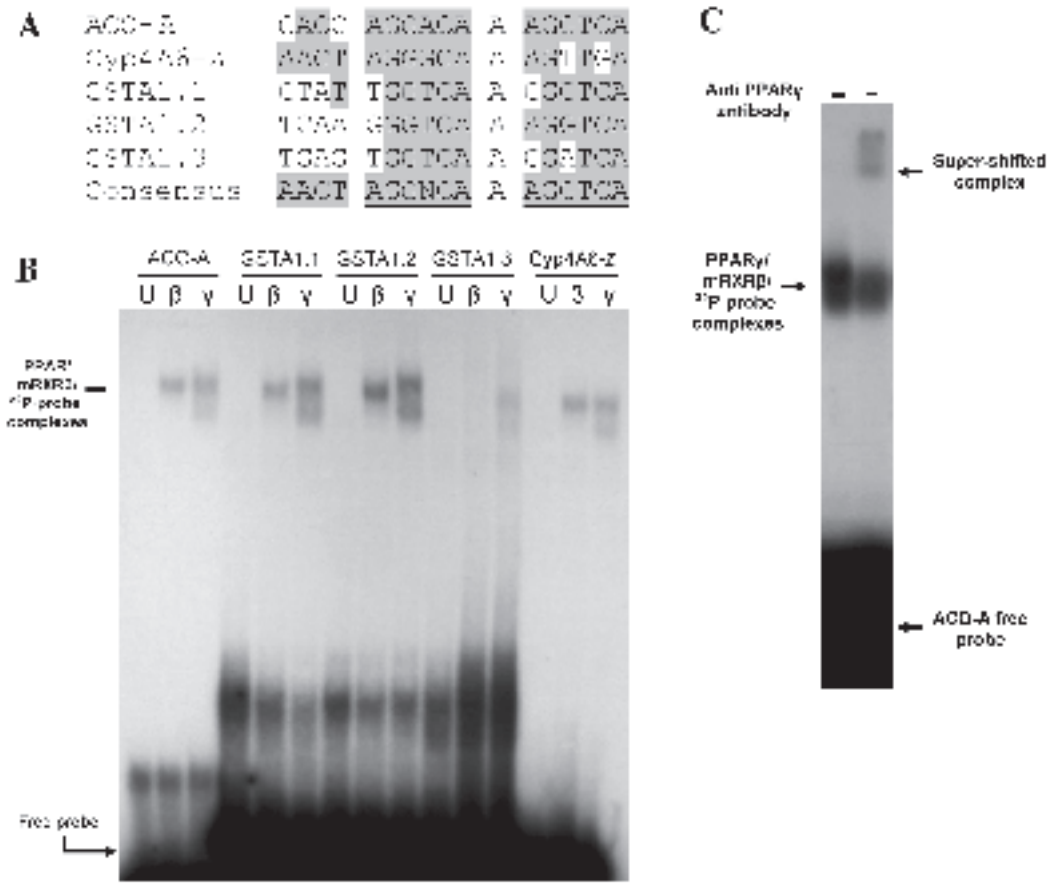

FIG. 5. The sea bass PPAR bind to PPAR response elements (PPRE) as heterodimers with retinoic acid receptors (RXR). (A) The sequences, including the 5′ flanking sequences, of the different PPRE tested. Residues identical to the consensus element, which is a direct repeat of the hexanucleotide sequence 5′-AGCTCA, spaced by one nucleotide (DR-1) (10), are shaded and the hexanucleotide repeats are underlined. (B) Specific complex formation with *in vitro* translated sea bass PPARβ and γ and mRXRβ on the ^{32}P-labeled PPRE indicated. Arrows indicate the position of the specific complexes and of the free probe. U is unprogrammed reticulocyte lysate. (C) The PPARγ/mRXRβ heterodimer on the acyl-CoA oxidase (ACO-A) probe is supershifted with the PPARγ -specific antibody (+) but not with the preimmune serum (–). Cyp46-z, z element in the promoter of the cyp4A6 gene; GST, glutathione-*S* transferase. See Figure 1 for other abbreviation.

tested, with the exception of the GSTA1.3 element. Indeed, the GSTA1.3 element exhibited the least identity with the consensus PPRE sequence (Fig. 5A) and, according to this assay's criteria, it does not constitute a functional DR-1 element. However, a detailed analysis of the plaice GSTA1 promoter is required in order to conclude whether this element is functional or not. The presence of PPAR in the above EMSA complexes was confirmed, in the case of PPARγ, by the use of a marine fish PPARγ-specific antibody (Diez, A., and Bautista, J.M., unpublished data) (Fig. 5C). For the β isotype, competition experiments with specific and nonspecific probes were performed and confirmed that the observed complexes require both the presence of the PPAR protein and the DR-1 element (results not shown).

FA and hypolipidemic drugs are ligands of sea bass PPARα and β. Residues that have been implicated both in ligand binding and in interactions with coactivator proteins in well-characterized PPAR (20,21) appear to also be conserved in the sea bass PPAR (Fig. 2). Therefore, we applied the CARLA screen, an assay based on the ligand-induced interactions of the receptor's ligand-binding domain (LBD) with coactivators (12), to determine whether the sea bass PPAR share common ligands with their mammalian and amphibian counterparts.

Sea bass PPARα and β LBD were expressed in *E. coli*, as GST fusions and the CARLA screen were performed with these two proteins, immobilized on glutathione-sepharose beads, ^{35}S-radiolabeled human steroid receptor coactivator-1 (SRC-1), and a variety of natural and synthetic compounds (22). As shown in Figure 6, statistically significant SRC-1 interactions with either the PPARα or β LBD were observed with the FA linoleic, linolenic, and CLA. Regarding CLA, all three isomers tested, in addition to a mixture of isomers, acted as ligands of PPARβ, while only the 10E, 12Z and 9E, and 11E isomers in addition to the mixed isomers, promoted interactions of the PPARα LBD with SRC-1. Similarly, the arachidonic acid analog eicosatetraynoic acid (ETA), a known ligand of both PPARα and β from other species (12,23,24), also interacted with the two sea bass isotypes. As is the case in other species (12,23,24), the hypolipidemic drug pirinixic acid (Wy-14,643) was able to interact only with the sea bass PPARα.

Neither of the FA phytanic, EPA, or DHA, nor the prostaglandin J_2 metabolite 15d-$\Delta^{12,14}$-PGJ_2, a well characterized mammalian PPARγ-specific ligand (25), were efficient in promoting LBD–SRC-1 interactions. Thus, from these results and at least for the compounds tested, the sea bass α and β PPAR isotypes appear to share the ligand binding properties of their mammalian and amphibian homologs.

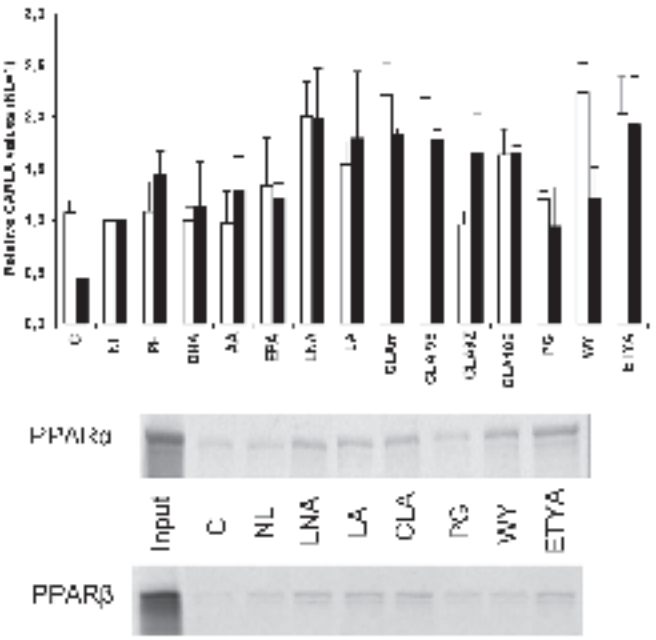

FIG. 6. Coactivator-dependent receptor ligand assay (CARLA) screen for the identification of ligands for the PPARα and β isotypes of sea bass. Upper panel: quantification of interactions (averages, with SD, of at least three independent experiments) of the PPARα (empty bars) and PPARβ (black bars) ligand binding domains (LBD) with SRC-1. A relative CARLA value of 1 is the amount of SRC-1 retained by the glutathione-*S* tranferase (GST)-PPAR LBD in the absence of ligand (NL). The compounds tested were: phytanic acid (PH); DHA; arachidonic acid (AA); eicosapentaenoic acid (EPA); linolenic acid (LNA); linoleic acid (LA); mixed isomers of CLA (CLAm); the 9*E*,11*E* (CLA9*E*), 9*Z*,11*E* (CLA9*Z*), and 10*E*,12*Z* (CLA10*E*) isomers of CLA; 15d-$\Delta^{12,14}$-PGJ_2 (PG); pirinixic acid-Wy-14,643 (WY); and eicosatetraynoic acid (ETYA). C is the control (immobilized GST in the presence of 10^{-4} M CLAm. Statistically significant interactions ($P < 0.05$) of the PPARα LBD with SRC-1 were observed with LNA, LA, CLAm, CLA9*E*, CLA10*E*, WY, and ETYA. The same compounds resulted in significant interactions of the PPARβ LBD with SRC-1 with the addition of CLA9*Z* and the exception of WY. Lower panel: Autoradiogram of ^{35}S-labeled SRC-1 retained by the PPAR LBD in the presence of selected compounds. See Figures 1 and 2 for abbreviations.

The genes of the sea bass PPAR are organized in a manner similar to that of their mammalian homologs in terms of different exons encoding distinct functional regions of the receptors, implying a modular organization of the proteins' domains. Thus, as with other species, the A/B and D domains of the receptors are encoded by individual exons, while two exons—one for each of the Zn fingers—encode the C domain. The main difference in the gene structure between the fish and mammalian receptors lies within the exons coding for the E domain. In the sea bass PPARα and β genes, this domain is encoded by three exons, while in all other known PPAR genes, it is encoded by only two exons (5).

The deduced protein products of the sea bass genes and cDNA show extended amino acid sequence identity with their mammalian homologs, especially at the C and E domains. Regarding the E domain, it is important to note that residues involved in ligand binding, heterodimerization with RXR, and

DISCUSSION

We have isolated genes and cDNA encoding three distinct PPAR from sea bass, and we have provided evidence that these receptors are the structural and functional homologs of the mammalian PPARα, β, and γ.

association with coactivators in the mammalian receptors (20,21) are also present in the sea bass PPAR. However, as a result of sequence insertion in the region corresponding to the additional exon present in the fish genes, both the sea bass PPARα and γ E domains are longer than their mammalian counterparts. Whether this insertion affects the overall structure of the domain, and therefore its function, remains to be determined.

The structure–function relationships between sea bass PPAR and PPAR from other species were assessed by comparing two fundamental properties of these receptors: DNA binding and ligand recognition. The sea bass PPAR recognize and bind to DR-1 elements as heterodimers with RXR. This implies that the sea bass receptors are expected to regulate transcription of piscine genes, which contain this type of elements in their promoter regions (6). Furthermore, sea bass PPAR-mediated transcription must involve similar mechanisms to those operating in mammals since, in terms of transcription complex assembly, we have shown that these receptors are capable of ligand-induced interactions with SRC-1, a mammalian PPAR coactivator (22).

This latter property, as is applied in the CARLA screen, allowed us to demonstrate that the sea bass receptors share common ligands with their mammalian or amphibian counterparts. Thus, both the α and β isotypes selectively associate with FA and hypolipidemic compounds that have been shown to be *bona fide* ligands of PPAR in other species (12,23,24). In addition, we have confirmed that the specificity of the hypolipidemic compound pirinixic acid for PPARα extends to this fish species.

Finally, the results presented herein demonstrate that the sea bass PPAR genes exhibit a tissue expression pattern resembling that observed in other species (17,18). Thus, the sea bass PPARα isotype is mainly expressed in tissues with increased β-oxidation activity, implicating this receptor in the regulation of FA catabolism. Similarly, PPARγ, which as a key factor in adipogenesis in mammals mainly expressed in adipose tissue (26), is also expressed principally in this tissue in sea bass. PPARβ, which has been recently proposed to function as a widespread regulator of fat burning in mammals (27), also exhibits a ubiquitous expression pattern in sea bass tissues. Furthermore, the expression of the β isotype during early development in sea bass suggests its involvement in the mobilization of energy stored in the yolk sac and other critical functions (e.g., differentiation, membrane lipids synthesis and turnover), as these have been proposed for mammals (19).

Although a more detailed analysis is required to fully elucidate the function of the sea bass PPAR, our results provide sufficient evidence that PPAR structure and function evolved before the divergence of the fish, amphibian, and mammalian lines. Thus, the ancestral PPAR gene and the duplication events that led to the appearance of distinct PPAR isotypes are likely to precede the osteicthyan lineage and must be located in the early vertebrates or the provertebrates. Interestingly, partial PPAR gene sequences have been reported from both lamprey (an early vertebrate) and amphioxus (a cephalocord), although no information is available concerning the presence of distinct PPAR isotypes in these organisms (28).

It should also be noted that we have recently isolated and functionally characterized the three PPAR isotypes from additional marine fish species (Boukouvala, E., Leaver, M.J., Antonopoulou, E., Diez, A., Favre-Krey, L., Bautista, J.M., Tocher, D.R., and Krey, G., manuscript in preparation). This will allow us to confirm the observations made with the sea bass PPAR, as far as the gene structure, expression, DNA binding, and transcriptional activation properties are concerned. The availability of these genes and cDNA will provide useful tools for the study of lipid metabolism in fish species.

ACKNOWLEDGMENTS

We thank W. Wahli and M.G. Parker for gifts of plasmids. This work was supported by the European Union (5FP, Project Q5RS-2000-30360).

Competing interests statement

The authors declare that they have no competing financial interests.

REFERENCES

1. Sargent, J.R., Tocher, D.R., and Bell, J.G. (2002) The Lipids, in *Fish Nutrition*, 3rd edn. (Halver, J. E. and Hardy, R.W, eds.), pp. 181–257, Academic Press, San Diego.
2. Hillestad, M., Jonsen, F., Austreng, E., and Asgard, T. (1998) Long Term Effects of Dietary Fat Level and Feeding Rate on Growth, Feed Utilization and Carcass Quality of Atlantic Salmon, *Aquacult. Nutr. 4*, 89–97.
3. Caballero, M.J., Lopez-Calero, G., Socorro, J., Roo, F.J., Izquierdo, M.S., and Fernandez, A.J. (1999) Combined Effect of Lipid Level and Fish Meal Quality on Liver Histology of Gilthead Sea Bream (*Sparus aurata*), *Aquaculture 179*, 277–290.
4. Buzzi, M., Henderson, R.J., and Sargent, J.R. (1996) The Desaturation and Elongation of Linolenic Acid and Eicosapentaenoic Acid by Hepatocytes and Liver Microsomes from Rainbow Trout (*Oncorhynchus mykiss*) Fed Diets Containing Fish Oil or Olive Oil, *Biochim. Biophys. Acta 1299*, 235–244.
5. Desvergne, B., and Wahli, W. (1999) Peroxisome Proliferator-Activated Receptors: Nuclear Control of Metabolism, *Endocr. Rev. 20*, 649–688.
6. Berger, J., and Moller, D.E. (2002) The Mechanisms of Action of PPAR, *Annu. Rev. Med. 53*, 409–435.
7. Leaver, M.J, Wright, J., and George, S.G. (1998) A Peroxisomal Proliferator-Activated Receptor Gene from the Marine Flatfish, the Plaice (*Pleuronectes platessa*), *Mar. Env. Res. 46*, 75–79.
8. Andersen, O., Eijsink, V.G., and Thomassen, M. (2000) Multiple Variants of the Peroxisome Proliferator-Activated Receptor (PPAR) γ are Expressed in the Liver of Atlantic Salmon (*Salmo salar*), *Gene 255*, 411–418.
9. Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., and Wahli, W. (1993) Fatty Acids and Retinoids Control Lipid Metabolism Through Activation of Peroxisome Proliferator-Activated Receptor-Retinoid X Receptor Heterodimers, *Proc. Natl. Acad. Sci. USA 90*, 2160–2164.
10. Ijpenberg, A., Jeannin, E., Wahli, W., and Desvergne, B. (1997) Polarity and Specific Sequence Requirements of Peroxisome

Proliferator-Activated Receptor (PPAR)/Retinoid X Receptor (RXR) Heterodimer Binding to DNA, *J. Biol. Chem. 274*, 20108–20117.
11. Leaver, M.J., Wright, J., and George, S.G. (1997) Structure and Expression of a Cluster of Glutathione S-Transferase Genes from a Marine Fish, the Plaice (*Pleuronectes platessa*), *Biochem. J. 321*, 405–412.
12. Krey, G., Braissant, O., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M.G., and Wahli, W. (1997) Fatty Acids, Eicosanoids, and Hypolipidemic Agents Identified as Ligands of Peroxisome Proliferator-Activated Receptors by Coactivator-Dependent Receptor Ligand Assay, *Mol. Endo. 11*, 779–791.
13. Krey, G., Keller, H., Mahfoudi, A., Medin, J., Ozato, K., Dreyer, C., and Wahli, W. (1993) *Xenopus* Peroxisome Proliferator-Activated Receptors: Genomic Organisation, Response Element Recognition, Heterodimer Formation with Retinoid X Receptor and Activation by Fatty Acids, *J. Steroid Biochem. Mol. Biol. 47*, 65–73.
14. Zhu, Y., Qi, C., Korenberg, J.R., Chen, X.N., Noya, D., Rao, M.S., and Reddy, J.K. (1995) Structural Organisation of Mouse Peroxisome Proliferator-Activated Receptor γ, mPPARγ Gene: Alternative Promoter Use and Different Splicing Yield Two mPPAR Isoforms, *Proc. Natl. Acad. Sci. USA 92*, 7921–7925.
15. Braissant, O., Foufelle, F., Scotto, C., Dauça, M., and Wahli, W. (1996) Differential Expression of Peroxisome Proliferator-Activated Receptors, PPAR: Tissue Distribution of PPARα, β, and γ in the Adult Rat, *Endocrinology 137*, 354–366.
16. Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G., and Wahli, W. (1992) Control of the Peroxisomal β-Oxidation Pathway by a Novel Family of Nuclear Hormone Receptors, *Cell 68*, 879–887.
17. Escher, P., Braissant, O., Basu-Modak, S., Michalik, L., Wahli, W., and Desvergne, B. (2001) Rat PPAR; Quantitative Analysis in Adult Rat Tissues and Regulation in Fasting and Refeeding, *Endocrinology 142*, 4195–4202.
18. Dreyer, C., Keller, H., Mahfoudi, A., Laudet, V., Krey, G., and Wahli, W. (1993) Positive Regulation of the Peroxisomal β-Oxidation Pathway by Fatty Acids Through Activation of Peroxisome Proliferators-Activated Receptors (PPAR), *Biol. Cell 77*, 67–76.
19. Braissant, O., and Wahli, W. (1998) Differential Expression of Peroxisome Proliferator-Activated Receptor-α, -β, and –γ During Rat Embryonic Development, *Endocrinology 139*, 2748–2754.
20. Nolte, R.T., Wisely, G.B., Westin, S., Cobb, J.E., Lambert, M.H., Kurokawa, R., Rosenfeld, M.G., Willson, T.M., Glass, C.K., and Milburn, MV. (1998) Ligand Binding and Coactivator Assembly of the Peroxisome Proliferator-Activated Receptor-γ, *Nature 395*, 137–143.
21. Xu, H.E., Lambert, M.H., Montana, V.G., Plunket, K.D., Moore, L.B., Collins, J.L., Oplinger, J.A., Kliewer, S.A., Gampe Jr, R.T., McKee, D.D., Moore, J.T., and Willson, T.M. (2001) Structural Determinants of Ligand Binding Selectivity Between the Peroxisome Proliferator-activated Receptors, *Proc. Natl. Acad. Sci. USA 98*, 13919–13929.
22. Kalkhoven, E., Valentine, J.E., Heery, D., and Parker, M.G. (1998) Isoforms of Steroid Receptor Coactivator 1 Differ in their Ability to Potentiate Transcription by the Oestrogen Receptor, *EMBO J. 17*, 232–243.
23. Forman, B.M., Chen, J., and Evans, R.M. (1997) Hypolipidemic Drugs, Polyunsaturated Fatty Acids, and Eicosanoids are Ligands for Peroxisome Proliferator-Activated Receptors α and δ, *Proc. Natl. Acad. Sci. USA 94*, 4312–4317.
24. Kliewer, S.A., Sundseth, S.S., Jones, S.A., Brown, P.J., Wisely, G.B., Koble, C.S., Devchand, P., Wahli, W., Willson, T.M., Lenhard, J.M., and Lehmann, J.M. (1997) Fatty Acids and Eicosanoids Regulate Gene Expression Through Direct Interactions with Peroxisome Proliferator-Activated Receptors α and γ, *Proc. Natl. Acad. Sci. USA 94*, 4318–4323.
25. Kliewer, S.A., Lenhard, J.M., Willson, T.M., Patel, I., Morris, D.C., and Lehmann, J.M. (1995) A Prostaglandin J2 Metabolite Binds Peroxisome Proliferator-Activated Receptor γ and Promotes Adipocyte Differentiation, *Cell 83*, 813–819.
26. Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., and Spiegelman, B.M. (1994) mPPARγ2: Tissue Specific Regulator of an Adipocyte Enhancer, *Genes Dev. 8*, 1224–1234.
27. Wang Y.X., Lee, C.H., Tiep, S., Yu, R.T., Ham, J., Kang, H., and Evans, R.M. (2003) Peroxisome Proliferator-Activated Receptor δ Activates Fat Metabolism to Prevent Obesity, *Cell 113*, 159–170.
28. Robinson-Rechavi, M., Marchand, O., Escriva, H., Bardet, P.L., Zelus, D., Hughes, S., and Laudet, V. (2001). Euteleost Fish Genomes are Characterised by Expansion of Gene Families, *Genome Res. 11*, 781–788.

[Received July 19, 2004; accepted November 20, 2004]