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Steroid Receptor Structure

CLONING OF NOVEL MEMBERS OF THE STEROID HORMONE RECEPTOR SUPERFAMILY

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Summary—We describe the cloning of four novel members of the steroid hormone receptor superfamily of ligand-activated transcription factors. Using chimaeric receptors we demonstrate that one of the receptor homologues is activated by the diverse peroxisome proliferator class of rodent hepatocarcinogens that includes hypolipidaemic drugs and industrial plasticisers. The identification of this receptor homologue should help elucidate the hypolipidaemic mechanism of these compounds and aid our evaluation of their potential carcinogenic risk to man.

INTRODUCTION

Peroxisome proliferators are a diverse group of chemicals that includes the hypolipidaemic compounds nafenopin and clofibrate as well as industrial plasticizers used in the production of food wrapping and PVC blood dialysis bags; see Refs [1, 2] for reviews. Administration of these compounds to rodents results in a dramatic proliferation of hepatic peroxisomes and liver hyperplasia [3]. Coincidently there is increased transcription of the genes for a number of peroxisomal enzymes important for the β-oxidation of long-chain fatty acids including fatty acyl-CoA-oxidase, bifunctional enzyme (enoyl-CoA-hydratase/3-hydroxy acyl-CoA-dehydrogenase) and thiolase [4]. There is also increased transcription of the cytochrome *P*450 IV gene family that encode microsomal enzymes with ω-hydroxylase activity [5].

Clofibrate [6] represents a class of pharmaceuticals that predominantly lower plasma triglycerides but also cholesterol levels; see Refs [7, 8] for reviews. In addition clofibrate mobilizes cholesterol from tissue pools leading to a marked improvement of xanthomatosis in some patients [7, 8]. Although beneficial in the prevention of ischaemic heart disease in patients with elevated cholesterol levels [7, 8] the pharmaceutical use of these compounds has been questioned by a single study indicating an increased morbidity of patients receiving clofibrate [9] and by the demonstration that clofibrate is a rodent hepatocarcinogen [10]. Peroxisome proliferators represent a novel class of rodent hepatocarcinogens [11] that are termed non-genotoxic carcinogens since they fail to directly cause DNA damage when tested using, for example, the *Ames salmonella* mutagenesis assay [12]. The number of non-genotoxic carcinogens, that also includes 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the herbicide DDT and steroid analogues [13], is growing. Furthermore, there are no suitable short-term predictive tests for these compounds and our knowledge concerning their mechanism(s) of action is poor. Traditionally, evaluation of the potential risk that these chemicals pose to man and the environment has depended upon data extrapolated from rodent bioassays with little understanding of their mechanism of action or possible species differences. We are therefore interested in using the peroxisome proliferator class of non-genotoxic rodent hepatocarcinogens as a model system to help identify their role in the tumourigenic process.

The basic mechanism(s) by which peroxisome proliferators induce tumours in rodents is unknown. It has been proposed that hydrogen peroxide, produced by the increase in peroxisomal fatty acid β-oxidation, results in oxidative stress leading to DNA damage and possibly tumour initiation [1]. However, since the carcinogenicity of the plasticizer di-(2 ethylhexyl) phthalate (DEHP) and the hypolipidaemic drug Wy 14,643 also correlates with their mitogenic

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effects in the liver it has been suggested that peroxisome proliferators could act as tumour promoters [14]. Indeed the administration of Wy 14,643 increased the number of liver tumours in rats given an initiating dose of the genotoxic carcinogen diethylnitrosamine [15]. Since peroxisome proliferators appear to act as complete carcinogens in animal models it is possible that they are able to act both as liver tumour initiators as well as liver tumour promoters. Further work is required, however, to elucidate their true role in tumourigenesis.

We were intrigued by the report of a nafenopin binding protein in rat liver [16, 17]. This, together with the known ability of peroxisome proliferators to rapidly modulate specific gene transcription [4, 5], suggested to us that peroxisome proliferators could act by a mechanism similar to that of steroid hormones [18]. We therefore attempted to clone and identify a putative peroxisome proliferator receptor by screening a mouse liver cDNA library using oligonucleotide probes based on a highly conserved region within the DNA binding domain of the nuclear receptor superfamily. We report here the cloning of four novel members of this family and identify one that is activated by peroxisome proliferators.

EXPERIMENTAL

Optimization of hybridization conditions

Bacteria containing receptor or vector plasmids were grown in duplicate onto agar plates and transferred to Hybond N nylon filters (Amersham). Hybridization was performed for 24 h at 42°C with 0–50% formamide, 1 × Denhardt's solution, 6 × NET (1 × NET is 0.15 M NaCl, 1 mM EDTA, 15 mM Tris–Cl, pH 7.5), 0.2% SDS and 100 mg/ml denatured salmon sperm DNA. The filters were washed 4 times for 20 min at 25°C in 2 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7), 0.1% SDS.

Cloning

Oligo-dT primed cDNA was synthesized using mouse (Alpk:APfCD-1) liver polyadenylated RNA and cloned into the EcoRI site of λ Zap or ZapII (Stratagene). After amplification the library was plated, transferred to duplicate Hybond N nylon filters (Amersham) and the DNA fixed with u.v. light. The filters were screened with the ^{32}P end-labelled consensus oligonucleotide probe mixture (0.5 pmol/ml; 5×10^6 cpm/pmol) using low stringency conditions of hybridization (42°C for 24 h with 0 or 10% formamide and other conditions as described above). After washing (see above) the filters were autoradiographed for 3 days at −70°C with intensifying screens.

Receptor probing

λ Clones that hybridized consistently with the oligonucleotide probe were spotted as an array onto an agar plate and grown overnight. The DNA was transferred to a Hybond N nylon filter and hybridized with cDNA probes encoding the DNA binding domain of either the glucocorticoid (GR), oestrogen (ER), thyroid hormone (TR) or retinoic acid (RAR) receptors, using moderate stringency conditions: 42°C for 16 h with 30% formamide, 1 × Denhardt's solution, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM Na2HPO4, 1 mM EDTA, pH 7.4), 0.2% SDS and 100 mg/ml denatured salmon sperm DNA. The filters were washed for 10 min at ambient temperature in 2 × SSC, 0.1% SDS followed by 4 washes at 37°C. Autoradiography was overnight at −70°C with intensifying screens.

Trans-activation assay

ER-peroxisome proliferator activated receptor (PPAR) and GR-PPAR were constructed essentially as described previously [19, 20]. Briefly, site-directed mutagenesis was used to insert an XhoI restriction enzyme site into clone 54 between codons 166 and 167. To generate ER-PPAR, the XhoI–EcoRI fragment, encoding the putative ligand binding domain of mouse-PPAR (mPPAR), was ligated to the EcoRI–XhoI fragment of HE28, that encodes the ER DNA binding domain [20], and inserted into the EcoRI site of the expression vector pSG5 [21]. The fragment containing the GR DNA binding domain was constructed by insertion of an XhoI site into HG1 between codons 486 and 487 using 100 ng of plasmid DNA and 5 cycles of PCR. GR-PPAR was constructed by ligating this fragment to the XhoI–EcoRI fragment of clone 54 and insertion into pSG5. Vit-G-chloramphenicol acetyltransferase (CAT) was constructed by replacing the thymidine kinase (tk) promoter of vit-tk-CAT with that of the rabbit β-globin promoter from 17M2-G-CAT [19]. COS1 cells (3×10^5) were seeded into 9 cm dia plates in phenol red-free medium (Gibco) supplemented with 5% foetal

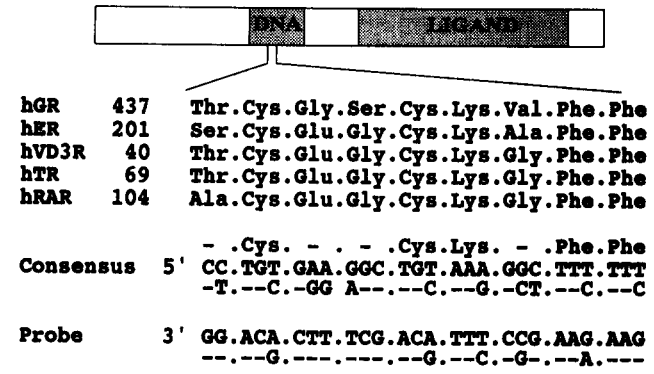

Fig. 1. Nucleotide sequence of the oligonucleotide receptor probe. The schematic structure of a steroid hormone receptor is shown at the top of the figure with the DNA and ligand binding domains shaded. Below is shown the amino acid sequence alignment within the DNA binding domain of the GR [28], hER [29], vitamin D3 (hVD3R [30]), hTR [31] and hRAR [32, 33] receptors. The consensus DNA and amino acid sequence for this region and the sequence of the derived degenerate oligonucleotide probe mixture are also shown. Note that the antisense strand of the consensus sequence was used to design the probe so as to maximize G:T base pairing in the event of a mismatch.

calf serum (Gibco) stripped of endogenous steroids by treatment with dextran coated charcoal. After 3 days the medium was replaced and CsCl purified plasmid DNA (10 μg) added by calcium phosphate precipitation. Each plate contained a CAT reporter gene (1 μg), an expression vector (pSG5, HE0, HG1, ER-PPAR, GR-PPAR; 1 μg), the β-galactosidase expression vector, pCH110 (3 μg; Pharmacia) and pBluescribe M13+ DNA (Stratagene) as a carrier (5 μg). Ligands (1000 × stocks in DMSO) were added 30 min after transfection. After a further 20 h the cells were washed and fresh ligand added. After an additional 24 h cell extracts were prepared by 3 cycles of freeze–thawing and assayed for β-galactosidase activity which was used to normalize the CAT assays [19].

RESULTS

Comparison of the primary amino acid sequence of several cloned nuclear receptors indicated a highly conserved region within the DNA binding domain. A mixture of 32 different 26-mer oligonucleotides was synthesized based upon the consensus sequence of the non-coding

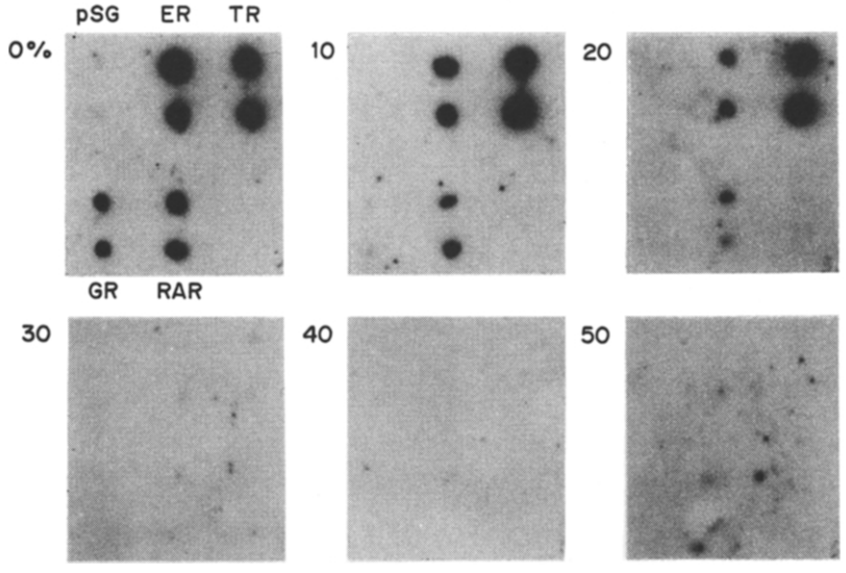

Fig. 2. Optimization of hybridization conditions. The oligonucleotide probe mixture was hybridized to duplicates of plasmids containing receptor cDNA sequences. The stringency of hybridization was adjusted by increasing the percentage of formamide in the hybridizations solution (see Experimental). pSG5 is the wild type plasmid that does not contain any receptor sequences.

strand of this region (Fig. 1). A number of hybridization conditions were tested to determine those that were optimal for the oligonucleotide probe mixture to hybridize specifically to plasmid DNA that contained sequences of either the GR, ER, RARα or TR receptors (Fig. 2). Approximately 700,000 phage plaques from a mouse liver cDNA library were screened with the oligonucleotide probe using low stringency conditions (0 or 10% formamide) and 64 plaques that consistently hybridized were grouped into 26 classes on the basis of cross-hybridization and restriction mapping data. The clones were then screened using moderate stringency conditions with cDNA probes encoding the DNA binding domain of either the TR, RAR, ER or GR (Fig. 3). Clone 9 hybridized strongly to the TR probe, moderately to the RAR probe and weakly to the ER probe. The sequence of clone 9 indicated that it was the murine TRβ. Clone 54, representing a class that also includes clones 45, 47, 56 and 73, hybridized strongly to the RAR probe but to none of the other probes (Fig. 3). A third clone, 71, hybridized with both the TR and RAR probes. A fourth class, represented by clone 74, failed to hybridize with any of the DNA binding domain probes but hybridized strongly with the oligonucleotide probe. The sequence of clones 54, 71 and 74 demonstrated them to be novel members of the steroid hormone receptor superfamily (data not shown). The cDNA inserts of clones 54, 71 and 74 were used as probes to re-screen the mouse liver cDNA library using moderate stringency conditions and resulted in the isolation of clone 15 that is closely related to clone 71. The sequence and analysis of clones 15, 71 and 74 will be presented elsewhere (I. Issemann, J. Tugwood, T. Aldridge, S. Thorneycroft and S. Green, in preparation).

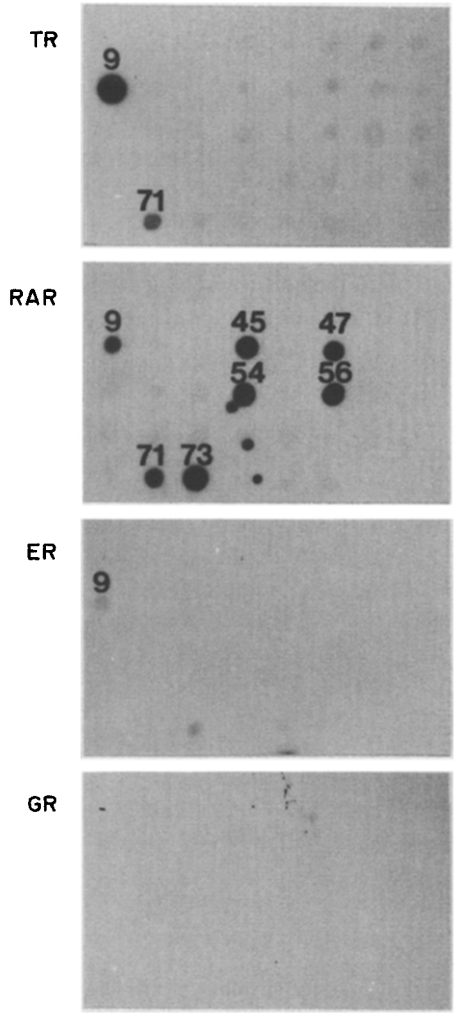

Fig. 3. Hybridization of selected clones using receptor DNA binding domain probes.

A second mouse liver cDNA library was screened using the insert of clone 54 resulting in the isolation of clone 3 that extends further 5′ than clone 54. Nucleotide sequence analysis of clones 3 and 54 indicated an open reading frame of 468 amino acids that is expected to encode a protein of 52,400 molecular mass (hereafter referred to as the mouse peroxisome proliferator activated receptor, mPPAR). The sequence surrounding the first ATG conforms closely to the Kozak consensus sequence and is preceded by an in-frame translation termination codon suggesting it to be the initiator methionine codon.

To test the ability of peroxisome proliferators to activate mPPAR we constructed chimaeric receptor expression vectors that contain regions encoding the putative ligand binding domain of mPPAR and the N-terminal sequence and DNA binding domain of either hER or hGR [Fig. 4(A), ER-PPAR and GR-PPAR]. A transient *trans*-activation assay was established in COS1 cells using the ER-PPAR chimaeric receptor expression vector and the vit-G-CAT reporter plasmid that contains the bacterial CAT gene under the transcriptional control of the ER. The assay was used to screen a number of potential ligands. Notably, a 100 μM concentration of the peroxisome proliferator nafenopin stimulated CAT activity approx. 10–20-fold [Fig. 4(B), compare lanes 6 and 7]. Importantly, nafenopin at this concentration had no effect upon CAT activity when using the parent expression vector pSG5 that does not contain ER-PPAR sequences (lane 2), nor upon the wild

type hER expression vector, HEO (lane 4). Similar results were observed when using the GR-PPAR expression vector together with the glucocorticoid-responsive reporter plasmid MMTV-CAT (lanes 9–16). In this system 100 μM nafenopin induced CAT activity approx. 40-fold (compare lanes 14 and 15). Thus, the putative ligand binding domain of mPPAR is able to confer nafenopin inducibility to either the ER or GR in the appropriate chimaeric receptor. Importantly, nafenopin failed to stimulate CAT activity in the absence of ER-PPAR suggesting that mPPAR mediates its effect. In addition, a number of other compounds including oestradiol [Fig. 4(B), lane 8], dexamethasone [Fig. 4(B), lane 16] did not stimulate CAT activity (data not shown). Activation of ER-PPAR therefore appears to be specific for peroxisome proliferators.

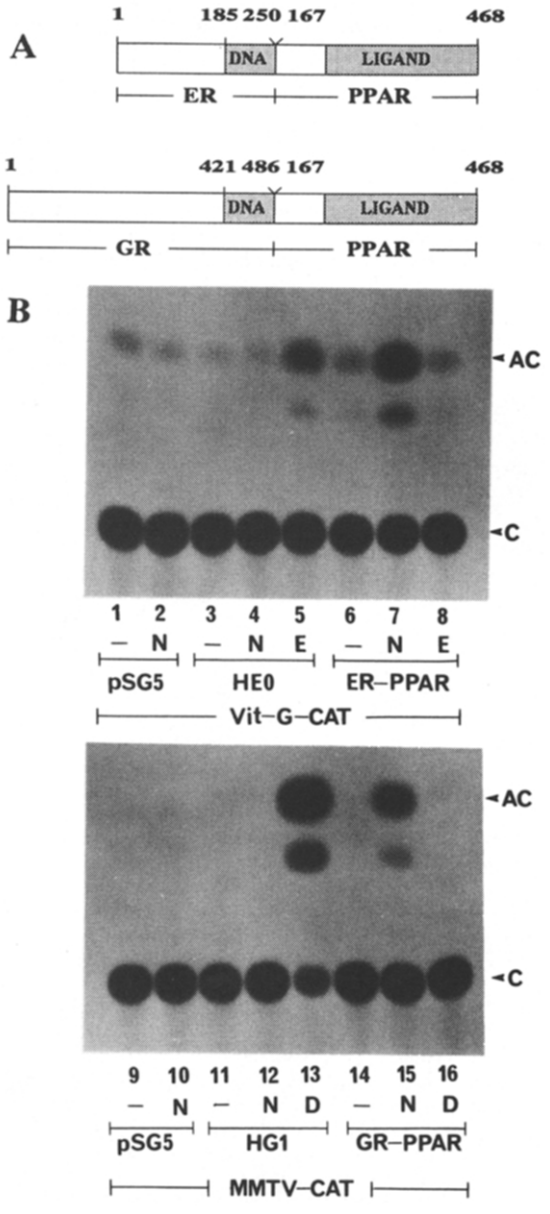

Fig. 4. Stimulation of CAT reporter genes by ER-PPAR and GR-PPAR chimaeric receptors in the presence of nafenopin. (A) Schematic structure of the ER-PPAR and GR-PPAR chimaeric receptors with numbers referring to the amino acid sequence. (B) The oestrogen-responsive (Vit-G-CAT, lanes 1–8) or glucocorticoid-responsive (MMTV-CAT [19], lanes 9–16) reporter genes were introduced into COS1 cells together with either the parent expression vector pSG5 [21] (Stratagene), the hER expression vector HEO [29], the hGR expression vector HG1 [34] or the chimaeric receptors ER-PPAR and GR-PPAR. The cells were maintained either in DMSO vehicle (0.1% v/v) alone(—), 10^{-8} M oestradiol (E), 10^{-7} M dexamethasone (D) or 100 μM nafenopin (N). The position of [^{14}C]-chloramphenicol (C) and acetylated [^{14}C]-chloramphenicol (AC) after TLC is indicated.

DISCUSSION

The mechanism by which peroxisome proliferators regulate gene expression and induce liver tumours in rodents is unknown. The identification of mPPAR as a member of the steroid hormone receptor superfamily of ligand-activated transcription factors that is specifically activated by peroxisome proliferators suggests that mPPAR may directly mediate the effects of this class of chemical.

The physiological role and putative natural ligand of mPPAR are unknown. The expression of mPPAR in brown adipose tissue, liver, kidney and heart, together with the increased peroxisomal fatty acid β-oxidation seen in these tissues after administration of peroxisome proliferators [22, 23], may indicate a role for mPPAR in providing a source of energy by utilizing fatty acids. Additional possibilities include a role for mPPAR in the regulation of peroxisomal function or cholesterol metabolism for example by increasing the conversion of cholesterol into bile acids; a key step of which is performed mainly, if not exclusively, in the peroxisomes.

mPPAR shares 38% amino acid identity in the putative ligand binding domain with the h-erbA related gene product (ear1) that is also expressed in liver, kidney and brown adipose tissue [24, 25]. We were therefore interested to determine whether ear1 was also activated by peroxisome proliferators. However, a chimaeric receptor, ear1-ER.CAS, constructed by replacement of the mouse ear1 DNA binding domain with that of the ER, was not activated by 100 μM nafenopin indicating that ear1 does not respond to peroxisome proliferators (I. Issemann, P. Leroy, P. Chambon and S. Green, unpublished observations).

Interestingly, other non-genotoxic carcinogens that appear to be liver tumour promoters may act through additional members of the steroid hormone receptor family. Indeed, such a model has been proposed for the elusive TCDD receptor; see Ref. [26].

In conclusion, the identification of a PPAR suggests the existence of a novel hormone-regulated system important for triglyceride and cholesterol homeostasis that may play a role in the development of liver tumours in rodents. Understanding the function of this receptor could help determine the basis for the variable response to peroxisome proliferators observed amongst species [27] as well as aid our understanding of how these chemicals act as rodent carcinogens.

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