Functional interference between estrogen-related receptor alpha and peroxisome proliferator-activated receptor alpha/9-cis-retinoic acid receptor alpha heterodimer complex in the nuclear receptor response element-1 of the medium chain acyl-coenzyme A dehydrogenase gene

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Abstract

We undertook a study of molecular interference of nuclear orphan receptors. Nuclear receptor response element-1 (NRRE-1) from the human medium-chain acyl coenzyme A dehydrogenase (MCAD) gene promoter was shown to contain three hexamer elements (site 1 through 3) that are known to interact with a number of nuclear receptors including chicken ovalbumin upstream promoter transcription factor (COUP-TF) and estrogen-related receptor alpha (ERR α). We demonstrated that the peroxisome proliferator-activated receptor alpha/9-cis-retinoic acid receptor alpha (PPAR α /RXR α) heterodimer complex can also bind to the two hexamer repeat sequences (between site 1 and site 3) arranged as an everted imperfect repeat separated by 14 bp (ER14). Mutations of the putative core elements have shown that these three sites are differentially involved in ERR α and PPAR α /RXR α binding. Homodimer of ERR α was shown to interact between site 1 and site 3 (ER14). To date, no nuclear receptor is known to bind to response elements over such long intervals. Interestingly, site 1 was shown to be essential for ERR α binding while site 3 supports its binding only in the presence of site 1. Furthermore, it was shown that the binding profile of ERR α and PPAR α /RXR α are competitive rather than making a high order complex within NRRE-1. At the cellular level, transcriptional activation driven by the PPAR α /RXR α complex was counteracted by the expression of ERR α in HeLa cells. These results suggest that ERR α and PPAR α /RXR α could interfere with each other's function through binding to similar DNA elements, thereby finetuning the transcriptional outcome of the target gene. Our findings suggest a mechanism whereby multiple nuclear receptors can activate or repress DNA binding or transcription via a single pleiotropic regulatory element.

Journal of Molecular Endocrinology (2003) **31**, 47–60

Introduction

The nuclear hormone receptors are a family of ligand-induced transcription factors that bind to specific DNA response elements and play crucial roles in a wide variety of cellular processes (excellent reviews are found in Mangelsdorf *et al.* 1995, Aranda & Pascual 2001, Willson & Moore 2002). Transcriptional regulation by nuclear

hormone receptors involves participation of basal transcription factors, including TATA-binding protein and TFIIB, and other cofactors, known as nuclear transcriptional coactivators or corepressors, that form bridges between nuclear receptors and the basal transcription machinery (Polak 1997, Edwards 1999, Robyr *et al.* 2000). Many nuclear hormone receptors possess bimodal transcriptional properties and are capable of either repressing or

activating target gene transcription, depending on the status of the ligand, the promoter and the nature of the host cell (Polak 1997, Gottlicher *et al.* 1998, Gay *et al.* 2002). In the absence of ligand, many receptors actively repress transcription via direct interactions with co-repressors such as SMRT (silencing mediator for retinoid and thyroid receptors), N-CoR (nuclear HDAC) or Sin3A (Rosenfeld & Glass 2001). Upon ligand binding, these co-repressor complexes dissociate and the agonist-bound receptors interact with multiprotein coactivator complexes that contribute to the transmission of activating signals to the general transcription machinery. It is hypothesized that the ability of this complex to deacetylate histones results in an altered chromatin state that is inhibitory to transcription (Rosenfeld & Glass 2001, Hsiao *et al.* 2003).

The nuclear receptors bind either as monomers or as homo- or heterodimers to response elements composed of a single core motif A/GGGTCA or to direct, palindromic or inverted repeats of the core motif spaced by one or more nucleotides (Forman & Evans 1995, Khorasanizadeh & Rastinejad 2001). Target gene recognition is specified by the DNA binding domain (DBD) which is composed of two zinc finger motifs that fold into two helical domains and a third helix extending from the second zinc finger (Freedman & Luisi 1993). Response elements for these receptors generally consist of paired hexamer sites that bind receptor homo- or heterodimers, with specificity determined, at least in part, by the relative orientation and spacing of hexamers (Forman & Evans 1995, Khorasanizadeh & Rastinejad 2001). However, some members of these receptors are known to bind to the single hexamer sites as monomers (Kutogh *et al.* 2000, Lee & Moore 2002).

The estrogen-related receptor α (*ERRα*) is an orphan member of the nuclear hormone receptor superfamily. A cDNA encoding this protein was originally isolated on the basis of sequence homology in its DNA-binding domain with estrogen receptor α (*ERα*, Giguere *et al.* 1988, Giguere 2002). While *ERRα* displays significant homology to the estrogen receptor, it does not bind estrogens *in vitro* nor is its transcriptional activity modulated by estrogens (Yang *et al.* 1996). *ERRα* binds as a monomer to the extended half-site TNAAGGTCA (core element underlined; Johnston *et al.* 1997, Sladek *et al.* 1997, Vanacker

et al. 1998) or as a heterodimer or homodimer to the consensus estrogen response element (Pettersson *et al.* 1996, Vanacker *et al.* 1999). In the adult mouse, *ERRα* is most highly expressed in kidney, heart, and brown and white adipocytes—tissues which preferentially metabolize fatty acids and show a high capacity of fatty acid β -oxidation. These results suggest that *ERRα* may play a role in determining the metabolic potential of these tissues. *ERRα* was shown to interact with the nuclear receptor response element-1 (NRRE-1) of the medium-chain acyl coenzyme A dehydrogenase (MCAD) gene that is the key enzyme of the mitochondrial fatty acid β -oxidation cycle (Vega & Kelly 1997). Given this background, *ERRα* appears to play a role in lipid metabolism. The transcriptional property of *ERRα* is still controversial. *ERRα* has been shown to have no effect or to repress transcription of the MCAD gene (Sladek *et al.* 1997) and SV40 major promoter (Wiley *et al.* 1993, Johnston *et al.* 1997). However, *ERRα* was also reported to activate transcription through the SFRE in a cell-specific manner (Bonnelye *et al.* 1997, Vanacker *et al.* 1999).

Peroxisome proliferator-activated receptors (PPARs) are also members of the nuclear hormone receptor superfamily. Their ligand-activated transcription factors respond to a class of chemical agents termed peroxisome proliferators that include the fibrate family of hyperlipidemia drugs, phthalate ester plasticizers, herbicides, pesticides, anti-diabetic thiazolidinediones as well as certain fatty acids (reviewed by Takahashi & Kawada 2001). PPARs heterodimerize with the 9-cis-retinoic acid receptor, *RXRα*, and bind to specific peroxisome proliferator-response elements (PPREs) found up-stream of target genes. PPREs have been identified in the promoter region of a number of genes involved in fatty acid metabolism. PPREs consists of a direct repeat of the hexameric motif TGACC(T/C) separated by one nucleotide (referred to as DR1), and serve to bind not only PPARs but also several other nuclear hormone receptors that differentially modulate PPAR function. Nuclear hormone receptors shown to modulate PPAR function include chicken ovalbumin upstream promoter transcription factor (COUP-TF) (Miyata *et al.* 1993), hepatocyte nuclear factor-4 (Winrow *et al.* 1994), TR (Hunter *et al.* 1996), *LXRα* (Miyata *et al.* 1996), and *RZRα* (Winrow *et al.* 1998). Therefore, transcriptional

regulation from PPREs is a net aggregate response generated, in part, by the availability of PPARs and other factors that bind PPREs, the complexity of response elements, and the interplay of PPARs with other nuclear hormone receptors and cofactors, including corepressors and coactivators. This complex interaction of various factors and elements ensures that the correct transcriptional response to extra- and intracellular stimuli will be elicited from appropriate target genes.

In this study, we studied the interference of DNA binding and transcriptional properties of *ERRα* and *PPARα/RXRα* within the NRRE-1 from the human MCAD gene. Our findings suggest a mechanism whereby multiple nuclear receptors can activate or repress transcription via a single pleiotropic element.

Materials and methods

All buffers and solutions were prepared and standard molecular biology techniques were used as described in Sambrook *et al.* (1989).

Plasmid constructs

Rat *PPARα*, human *RXRα*, mouse *ERRα* expression vectors (Ijpenberg *et al.* 1997, Sladek *et al.* 1997, Kassam *et al.* 1999, kind gifts from Drs Rachubinski and Giguere) were used for *in vitro* expression in reticulocyte lysate and transfection assays. For NRRE1/pBLCAT2 reporter plasmid, double stranded NRRE-1 wild-type (WT) as well as mutated (M) oligonucleotides designed to carry the HindIII (5') and BamHI (3') restriction sites at each end were subcloned into the HindIII/BamHI sites present upstream of the thymidine kinase (TK) promoter of the pBLCAT2 plasmid (Luckow & Schutz 1987). These plasmid DNAs were prepared using Qiagen EndoFree columns (Qiagen, Düsseldorf, Germany) for subsequent transfection experiments.

In vitro transcription/translations

Transcription/translation of cDNAs encoding *PPARα*, *RXRα* and *ERRα* was performed using the TNT T7-coupled rabbit reticulocyte lysate system according to the manufacturer's protocol

(Promega, Madison, WI, USA). Synthesized proteins were used for gel retardation assays.

Oligonucleotides

The following complementary synthetic oligonucleotides including the original flanking sequences were synthesized (Hitachi, Kawagoe, Japan), annealed and used for the gel retardation assay. One pmol of each double-stranded oligonucleotide was labeled with [³²P]dGTP (Amersham, Amersham, Bucks, UK) using Kenow DNA polymerase (Takara, Kyoto, Japan), and purified with a Nick column (Pharmacia, Uppsala, Sweden). A fill-in reaction was performed by adding the HindIII (5' end) and BamHI (3' end) at each end. The radiolabeled probes were used for gel retardation assays. Sequences of the sense strands of the annealed oligonucleotides are shown below. Consensus sequences are underlined. Mutations are shown with bold, small letters.

NRRE1WT: GGGTTTGACCTTTCTCTCC
GGGTAA AGGTGAAGG; NRRE1 M1: GGGT
TTG**ctt**TTTCTCTCCGGGTAA AGGTGAAGG;
NRRE1 M2: GGGTTTGACCTTTCTCTCCG
ttcAA AGGTGAAGG; NRRE1 M3: GGGTT
TGACCTTTCTCTCCGGGTAA **Attc**GAAGG;
NRRE1 M4: GGGTTTG**ctt**TTTCTCTCCG**ttc**
AA AGGTGAAGG; NRRE1 M5: GGGTT
TG**ctt** TTTCTCTCCGGGTAA **Attc**GAAGG;
NRRE1 M6: GGGTTTGACCTTTCTCTCCG
ttcAA **Attc**GAAGG; SFRE: AGCTAGCTGA
AGGTCATG; AoxPPRE: AGCTTAACGTGAC
CTTTGTCCTGGTTCG; Histone H2B octamer:
CTTCACCTTATTTCGATAAGCG.

Gel retardation assays

In vitro synthesized proteins in reticulocyte lysates as above were prepared and the gel retardation assays were performed as described previously (Kutoh *et al.* 1992). Briefly, binding reactions of 20 µl were carried out in buffer containing 10 mM Hepes (pH 7.9), 2.5 mM MgCl₂, 10% glycerol (v/v), 1 mM dithiothreitol, 1 µg poly (dl-dC), 50 mM KCl, 2.5 fmol ³²P-labeled oligonucleotides and 1 µl reticulocyte lysate. Competitors (unlabeled probes) were added at the same time as the radiolabeled probes. Incubation was carried out at room temperature for 15 min. Free and bound DNA were separated by electrophoresis on

non-denaturing 4% polyacrylamide gels (acrylamide bis 29:1, cross linked) with $0.5 \times$ TBE ($1 \times$ TBE: 90 mM Tris-borate, 1 mM EDTA) as running buffer. The gels were dried and analyzed by autoradiography. For some experiments, intensities of the signal were quantified using Adobe Photoshop 5.0 image analyzing software (Vero Beach, FL, USA).

Cell culture, transfection and reporter gene assays

HeLa cells (purchased from ATCC, Manassan, VA, USA and RIKEN, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies). The medium was supplemented with sodium bicarbonate 50 mM, penicillin 100 U/ml, streptomycin 50 µg/ml and 10% fetal calf serum (FCS) and the cells were grown in a 95% air/ 5% CO₂ atmosphere at 37 °C. Ten micrograms of each CAT reporter plasmid together with varying amounts of expression vector (*ERRα*, *PPARα* and *RXRα*) and 1 µg of the β-galactosidase (pSV-βgal, Promega, Madison, WI, USA) plasmid were transfected using the calcium phosphate transfection method (Protocols and Application Guide, Promega). The cells were harvested 48 h after the transfection and the CAT was measured using a CAT ELISA kit (Roche Diagnostics, Basel, Switzerland). To check the transfection efficiency, β-gal activities were measured with a β-gal ELISA kit (Roche Diagnostics).

Preparation of RNA and RT-PCR

Total RNAs of the transfected cells were prepared using Trizol reagent according to the manufacturer's protocol (Life Technologies, Paisley, Scotland). One microgram RNA was reverse-transcribed by 500 ng oligo-dT primer, 1 µl 10 mM dNTPs and 200 units of SuperScript II reverse transcriptase (all from Life Technologies) in a total volume of 20 µl according to the manufacturer's protocol. After the reaction, the mixture was heated at 65 °C for 10 min and 80 µl H₂O were added. Subsequently, PCR reactions were performed in a total volume of 100 µl containing 3 µl cDNA, 1 µl of 20 mM of each specific *ERRα* primers, 1 µl of 10 mM dNTPs and 2.5 U Ex-Taq DNA polymerase (Takara, Kyoto, Japan).

	site1	site2	site3
NRRE-1 WT:	GGGTTTGACCTTTCTCTCCGGGTAAAGGTGAAGG		
M1:	GGGTTTG g acCTTTCTCTCCGGGTAAAGGTGAAGG		
M2:	GGGTTTGACCTTTCTCTCCGGTAAAGGTGAAGG		
M3:	GGGTTTGACCTTTCTCTCCGGTAAAGGTGAAGG		
M4:	GGGTTTG g acCTTTCTCTCCG ttc AAAGGTGAAGG		
M5:	GGGTTTG g acCTTTCTCTCCGGGTAAAG ttc GAAGG		
M6:	GGGTTTGACCTTTCTCTCCG ttc AAAG ttc GAAGG		
	site1	site2	site3
NRRE-1 WT:	_____	_____	_____
M1:	_____X_____		
M2:		_____X_____	
M3:			_____X_____
M4:	_____X_____	_____X_____	
M5:	_____X_____		_____X_____
M6:		_____X_____	_____X_____

Figure 1 Consensus and mutational sequence of NRRE-1 from the human MCAD gene. The core hexamer elements are underlined and the mutations are shown with small, bold letters. In the bottom panel, mutations within the potential half-binding sites (site 1 through site 3) are shown with an X.

PCR primers used were *ERRα* forward: ATAACCTCCAAGCAGGCTCCA; *ERRα* reverse: CAAAAGCGGTTTCTCTTTGTC. For control, glyceraldehyde phosphate dehydrogenase (GA, Kutoh *et al.* 1998) specific primers were used.

After an initial denaturation at 93 °C for 2 min, PCR was carried out in 30 cycles (from step 1 to step 3) using Gene Amp 9700 (Perkin Elmer, Norwalk, CT, USA) as follows: step 1: 93 °C for 1 min (denaturation); step 2: 60 °C for 1 min (annealing); step 3: 72 °C for 2 min (reaction); step 4: 72 °C for 7 min (final elongation). The PCR products were loaded on 1.5% agarose $0.5 \times$ TBE gel and the images of the ethidium bromide stained gels were obtained.

Results

ERRα and *PPARα/RXRα* complex bind to the NRRE-1

NRRE-1 is a pleiotropic element capable of conferring transcriptional properties by other nuclear receptors including *ERRα*, *RARα*, or COUP-TFs (Vega & Kelly 1997 and references therein). NRRE-1 is composed of three potential nuclear receptor half binding site sequences (Fig. 1). Specifically, the 5' hexamer (site 1, AGGTCA) is in the antisense orientation 8 bp and 14 bp upstream

of an imperfect sense repeat (site 2 and site 3, GGGTAA and AGGTGA respectively). The architecture of NRRE-1 is unique compared with those of other previously identified nuclear receptor response elements. The receptor hexamer binding sites within this element do not correspond to prototypical receptor response elements such that sequences contributing to receptor function are not predictable from previous reports. Accordingly, we next directly explored the receptor–DNA interactions in detail.

$PPAR\alpha$ and $RXR\alpha$ are known to form a heterodimer complex and this complex was shown to bind to the response element named PPRE (Kliwer *et al.* 1992, Gearing *et al.* 1993). We then addressed the question of whether, in addition to $ERR\alpha$, the $PPAR\alpha/RXR\alpha$ complex could interact with the NRRE-1, although NRRE-1 is clearly distinct from a conventional PPRE. To answer this question, an *in vitro* gel retardation assay was performed using a radiolabeled NRRE-1 together with *in vitro* expressed $ERR\alpha$ or $PPAR\alpha/RXR\alpha$ complex. As shown in Fig. 2A, both $ERR\alpha$ and the $PPAR\alpha/RXR\alpha$ protein complex also bind to the wild-type NRRE-1. As expected, $PPAR\alpha$ or $RXR\alpha$ alone failed to bind to the DNA (Fig. 2A). To check the specificity of the DNA–protein complex, a molar excess of unlabeled competitors (SFRE, AoxPPRE or OCT) were included (Fig. 2B). SFRE is a known copy of the $ERR\alpha$ binding site from the SF-1 gene (Bonnelye *et al.* 1997) and AoxPPRE is a known $PPAR\alpha/RXR\alpha$ binding site from the fatty acyl-CoA oxidase (Kassam *et al.* 1999). A 50-fold molar excess of specific competitors (SFRE or AoxPPRE) was able to eliminate the DNA–protein complex whereas the same amounts of non-specific competitors (OCT) were unable to do so. These experiments confirmed that both $ERR\alpha$ and $PPAR\alpha/RXR\alpha$ specifically interact with the NRRE-1.

Binding site analysis

NRRE-1 contains three hexamer binding sequences separated by 8 bp (site 1 and site 2), 14 bp (site 1 and site 3) or 0 bp (site 2 and site 3) which may be the target sites for nuclear receptor binding as indicated in Fig. 1. Since the nuclear hormone receptor binding to elements with 8 or 14 bp hexamer spacing is currently unknown, we examined the role of these sites by mutational analysis. As a first step in identifying the receptor

binding sites in this complex element, systematic mutations were introduced within the potential nuclear receptor half binding sites (M1 through M6, see Fig. 1). As presented in Fig. 3A, mutations in site 1 (M1) had completely lost $ERR\alpha$ binding while mutations in site 3 (M3) had a partial reduction. Mutations in site 2 (M2) had little, if any, effect on $ERR\alpha$ binding. One interesting observation is that site 1 appears to be essential for $ERR\alpha$ binding to NRRE-1, since mutations within site 1 had completely lost the binding activity, irrespective of the presence of an intact site 2 and site 3 (M1, M4, M5). The presence of an intact site 1 with mutated site 2 and/or site 3 (M2, M3 and M6) still had efficient, but lower levels of DNA binding activity in comparison to the wild type (WT). This phenomenon was confirmed by using these mutated as well as WT oligonucleotides as cold competitors (Fig. 3B). The $ERR\alpha$ /NRRE-1-dependent DNA–protein complex was strongly inhibited with a 50-fold molar excess of WT, M2 or SFRE while other competitors (M1, M3, M4, M5, M6, OCT) had little effect. These results show the differential importance of these sites for $ERR\alpha$ binding. In order to find out the importance of this phenomenon, we undertook gel retardation assays using WT as well as mutated NRRE-1 (M1 through M6) with increasing amounts of $ERR\alpha$ (Fig. 3C i and ii). The intensity of the $ERR\alpha$ -dependent DNA–protein complex was measured and its graphic display is shown in Fig. 3D i and ii. These results indicate that site 1 is essential for $ERR\alpha$ binding while site 3 supports $ERR\alpha$ binding only in the presence of an intact site 1. Site 2 appears to be involved in little, if any, $ERR\alpha$ binding.

In the case of $PPAR\alpha/RXR\alpha$, mutations in either site 1 or site 3 (M1 and M3) had reduced DNA binding activities while mutations in site 2 (M2) had little, if any, effect (Fig. 4A). However, mutations in both site 1 and site 3 lost their DNA binding activity (M5) whereas mutations in either site 1 (M4) or site 3 (M6), irrespective of the integrity of site 2, had reduced levels of DNA binding activity. This phenomenon was confirmed by using these mutated as well as WT oligonucleotides as cold competitors (Fig. 4B). The $PPAR\alpha/RXR\alpha$ -dependent DNA–protein complex was strongly inhibited with a 50-fold molar excess of WT, M2, or AoxPPRE, while competitors harboring mutations in either site 1 or site 3 (M1,

Figure 2 *ERRα* and *PPARα/RXRα* heterodimer complex can bind to NRRE-1. *In vitro* transcribed/translated *ERRα*, *PPARα*, and *RXRα* were tested for their ability to bind NRRE-1. ³²P-labeled double-stranded NRRE-1 probes were incubated with 2 μ l of *in vitro* transcribed/translated *ERRα* or *PPARα/RXRα* in reticulocyte lysate and the DNA binding was analyzed by gel retardation assay as described in Materials and methods. In some experiments (B), a 50-fold molar excess of unlabeled competitor oligonucleotides (comp) (SFRE, AoxPPRE, or OCT) was added to the reaction. The DNA–protein complex is indicated with an arrow. Free probes are shown at the bottom of the gel (F).

Figure 3 Binding site for $ERR\alpha$. (A) ^{32}P -labeled double stranded NRRE-1 WT and mutation probes (M1 through M6) were incubated with 2 μ l of *in vitro* transcribed/translated $ERR\alpha$ in reticulocyte lysate and the DNA binding was analyzed by gel retardation assay as described in Materials and methods. Free probes are shown at the bottom of the gel (F). (B) A 50-fold molar excess of unlabeled competitor oligonucleotides (site 1 through 3, OCT) was added to the reaction. Free probes are shown at the bottom of the gel (F). (Ci and ii) Indicated probes (NRRE-1, WT, M1, M2, M3, M4, M5, M6) were incubated with increasing amounts (0, 1, 2, 4, 8 μ l) of *in vitro* transcribed/translated $ERR\alpha$. (Di and ii) The intensity of the DNA-protein complex (shown with an arrow in C) is displayed graphically. The X axis indicates the increasing amounts (0, 1, 2, 4, 8 μ l shown as 1, 2, 3, 4, 5) of *in vitro* transcribed/translated $ERR\alpha$. The Y axis indicates the intensities of the signal.

M3, M4, M6) reduced the intensity of the DNA-protein complex. However, M5 (mutations in both site 1 and site 3) had no such effect. These results show the differential importance of three

sites for $PPAR\alpha/RXR\alpha$ binding. In order to further analyze this phenomenon, we undertook gel retardation assays using WT as well as the mutated NRRE-1 (M1 through M6) with increasing

Figure 4 Binding site for PPARα/RXRα. (A) ³²P-labeled double-stranded NRRE-1 WT and mutation probes (M1 through M6) were incubated with 2 μl of *in vitro* transcribed/translated PPARα/RXRα in reticulocyte lysate and the DNA binding was analyzed by gel retardation assay as described in Materials and methods. Free probes are shown at the bottom of the gel (F). (B) A 50-fold molar excess of unlabeled competitor oligonucleotides (site 1 through 3, OCT) was added to the reaction. Free probes are shown at the bottom of the gel (F). (Ci and ii) Indicated probes (NRRE-1 WT, M1, M2, M3, M4, M5, M6) were incubated with increasing amounts (0, 1+1, 2+2, 4+4, 8+8 μl) of *in vitro* transcribed/translated PPARα/RXRα (P/R). (Di and ii) The intensity of the DNA–protein complex (shown with an arrow in C) is displayed graphically. The X axis indicates the increasing amounts (0, 1+1, 2+2, 4+4, 8+8 μl, shown as 1, 2, 3, 4, 5) of *in vitro* transcribed/translated PPARα/RXRα. The Y axis indicates the intensities of the signal.

amounts of PPARα/RXRα (Fig. 4C i and ii). The intensity of PPARα/RXRα-dependent DNA–protein complex was measured and its graphic display is shown in Fig. 4D i and ii. These results

indicate that site 1 and site 3 are involved in PPARα/RXRα binding while, analogous to the case of ERRα, site 2 appears to have little, if any, activity in PPARα/RXRα binding.

Figure 5 Binding property of *ERRα* or *PPARα/RXRα* to NRRE-1. (A) Radiolabeled SFRE or NRRE-1 was incubated with increasing amounts (0, 1, 2, 4 μ l) of *in vitro* transcribed/translated *ERRα*. The DNA–protein complex is indicated with an arrow. Free probes are shown at the bottom (F). (B) Radiolabeled AoxPPRE or NRRE-1 was incubated with increasing amounts (0, 1, 2, 4 μ l) of *in vitro* transcribed/translated *PPARα/RXRα* (P/R). The DNA–protein complex is indicated with an arrow. Free probes are shown at the bottom (F).

Do *ERRα* or *PPARα/RXRα* bind to NRRE-1 as multimers?

In the next step, we tested the possibility that *ERRα* and/or *PPARα/RXRα* could bind to the NRRE-1 as multimers. To answer this question, we performed gel retardation assays using SFRE or NRRE-1 together with increasing amounts of *ERRα* or *PPARα/RXRα* protein expressed in reticulocyte lysates. As shown in Fig. 5A, the position of the DNA–protein complex was different with SFRE or NRRE-1. Although it is not clear, *ERRα* appears to bind to this extended half-site as a monomer (Johnston *et al.* 1997). Thus it is likely that *ERRα* binds to NRRE-1 as a homodimer or as two independent monomers. In the case of *PPARα/RXRα*, the position of the DNA–protein complex was similar with both AoxPPRE and NRRE-1 (Fig. 5B). However, the *in vivo* relevance and significance of this observation are still unanswered and will need more investigations.

Competitive binding between *ERRα* and *PPARα/RXRα*

From the DNA binding analysis using mutations (Figs 3 and 4), it appears that the binding sites for *ERRα* and *PPARα/RXRα* overlap (site 1 and site 3). We then addressed the question of whether they

can compete with each other for the same response element or whether they form a high order complex. To answer this question, *in vitro* translated *PPARα/RXRα* was then added in a dose-dependent manner to the NRRE-1-*ERRα*-dependent DNA–protein complex. As shown in Fig. 6, the addition of *PPARα/RXRα* could dose-dependently compete with *ERRα* binding to NRRE-1. This result implies that *PPARα/RXRα* can compete with *ERRα* for the same response element present in NRRE-1.

ERRα counteracts the *PPARα/RXRα*-mediated transcriptional activation

To gain an insight into the cellular significance of the *in vitro* observation above, we performed transient transfections of HeLa cells with the *ERRα* and/or *PPARα/RXRα* expression vectors and the CAT reporter gene plasmid containing the NRRE-1 response element upstream of the TK-promoter (NRRE-1/pBLCAT2) or the TK-promoter alone (pBLCAT2). In advance, to certify that *ERRα* is expressed in this experimental system, we checked its expression after transfection. RNA was prepared and quantitative RT-PCR was performed using *ERRα* specific primer. As shown in Fig. 7A, transfection of increasing amounts of

Figure 6 Competition between the *PPARα/RXRα* complex and *ERRα* to *NRRE-1*. Gel retardation assay was performed using the radiolabeled *NRRE-1* probe together with fixed amounts of *in vitro* translated *ERRα* (3 μ l) and increasing amounts of *PPARα/RXRα* complex (0+0, 1+1, 2+2, 3+3, 4+4 μ l). Free probes are shown at the bottom (F)

ERRα expression vectors did indeed increase its mRNA levels. In contrast to this, expression levels of glyceraldehyde phosphate dehydrogenase (shown as GA; Kutoh *et al.* 1998) were not influenced by *ERRα* expression vectors. This pre-experiment showed that *ERRα* is indeed expressed in our system.

Transfection of *ERRα* expression vector slightly increased the TK-promoter activity (Fig. 7B). *ERRα* had little, if any, effect on the reporter activity driven by *NRRE-1* while *PPARα/RXRα* with its agonist, Wy14,643 (Takahashi & Kawada 2001), increased the same activity by approximately sevenfold (Fig. 7C). Then, increasing amounts of *ERRα* expression vectors with fixed amounts of *PPARα/RXRα* dose-dependently suppressed the reporter gene activity driven by *PPARα/RXRα* complex with Wy14,643 (Fig. 7D). These results suggest that *ERRα* counteracts the *PPARα/RXRα*-mediated transcriptional activation within the *NRRE-1*. Taken together, these *in vitro* (Fig. 6) and cellular (Fig. 7) experiments suggest that *ERRα* can compete with *RXRα/PPARα* for

the *NRRE-1* and repress the *RXRα/PPARα*-mediated transcriptional activity.

There are conflicting data regarding the transcriptional activity of *ERRα*. As mentioned in the Introduction, some groups showed that *ERRα* is a transcriptional repressor while others reported that it is an activator. Differential corepressor recruitment is one mechanism that could explain why *ERRα* can bind its response element without significant transcriptional activities. Thus, we tested the effect of trichostatin A (TSA), a selective inhibitor of histone deacetylase (HDAC) as well as a disruptor of HDAC associated co-repressor complexes (Rosenfeld & Glass 2001), on the transcriptional repression observed by the expression of *ERRα*. We added TSA in an effort to re-activate the *ERR*-repressed conditions. However, no consistent data have been obtained (results not shown).

Discussion

DNA binding properties of *ERRα* and *PPARα/RXRα* complex to the *NRRE-1* of the human *MCAD* gene promoter

In the first place, we showed that the *PPARα/RXRα* complex as well as *ERRα* could bind to *NRRE-1* which was shown to be essential for high levels of expression of *MCAD* gene (Vega & Kelly 1997). The three potential hexamer half-binding sites (core elements) for nuclear receptors were separated by 8, 14 and 0 bp respectively. Comparison of the DNA sequences and arrangement of *NRRE-1* binding sites with the list of other known naturally occurring and artificial *ERRα* or *PPAR/RXRα* response elements (Ijpenberg *et al.* 1997, Juge-Aubry *et al.* 1997, Sladek *et al.* 1997) demonstrates that only site 1 matches the proposed consensus binding sequence AGGTCA, whereas site 2 (GGGTAA) and site 3 (AGGTGA) contain a mismatch in the penultimate residue. The hexamer orientation and spacing requirements for elements conferring *ERRα* or *PPARα/RXRα* have not been completely established, although the majority of elements are direct repeats. The mutational analysis of the *NRRE-1* shown here demonstrated that the active sites for nuclear receptor binding (site 1 and site 3) have an everted repeat motif with site 1 separated from site 3 by 14 bp (Figs 3 and 4, site 2 appears to be non-functional in binding to

Figure 7 Effect of $ERR\alpha$ on $PPAR\alpha/RXR\alpha$ -mediated transcription. Ten micrograms pBLCAT2 reporter gene with or without NRRE-1 before the TK-promoter were co-transfected with 10 μ g $PPAR\alpha/RXR\alpha$ and increasing amounts (0, 5, 10, 20 μ g) of $ERR\alpha$ expression vectors in HeLa cells in the presence of 10 μ M $PPAR\alpha$ agonist, Wy14,643. One microgram pSV- β -gal control plasmid was also co-transfected to check the transfection efficiency. After 24 h, the cells were lysed and either RNA was prepared and semi-quantitative RT-PCR was undertaken (A) or CAT activity was calculated by ELISA (B, C and D). The bar graphs represent the mean values with S.E. obtained from four independent experiments. (A) Expression of $ERR\alpha$ after transfection (5, 10, 20 μ g). PCR primers used were $ERR\alpha$ and control GA. Negative controls were taken from RNA without reverse transcriptase (-RT). Mw, molecular weight ladder. (B) Effect of $ERR\alpha$ on the constitutive TK (thymidine kinase) promoter. 1, pBLCAT2; 2, pBLCAT2+10 μ g $ERR\alpha$ expression vector. (C) Effect of $ERR\alpha$ on NRRE-1. 1, NRRE-1/pBLCAT2; 2, NRRE-1/pBLCAT2+10 μ g $ERR\alpha$; 3, NRRE-1/pBLCAT2+10 μ g $PPAR\alpha/RXR\alpha$. (D) Effect of $ERR\alpha$ on NRRE-1. 1, NRRE-1/pBLCAT2+0 μ g $ERR\alpha$; 2, NRRE-1/pBLCAT2+5 μ g $ERR\alpha$; 3, NRRE-1/pBLCAT2+10 μ g $ERR\alpha$; 4, NRRE-1/pBLCAT2+20 μ g $ERR\alpha$.

$ERR\alpha$ or $PPAR\alpha/RXR\alpha$). This binding half-site arrangement (ER14) is novel compared with other previously reported PPREs or ERR-response elements (Ijpenberg *et al.* 1997, Juge-Aubry *et al.* 1997, Sladek *et al.* 1997). Based on the results from gel retardation assays using mutated NRRE-1 (Figs 4 and 5), our conclusions regarding the importance of these hexamer sites are as follows: site 1– $ERR\alpha$: essential, $PPAR\alpha/RXR\alpha$: involved; site 2– $ERR\alpha$: not involved, $PPAR\alpha/RXR\alpha$: not involved; site 3– $ERR\alpha$: partially involved only in the presence of intact site 1, $PPAR\alpha/RXR\alpha$: involved.

The schematic presentation of this conclusion is illustrated in Fig. 8.

It is of interest that $ERR\alpha$ binds to site 3 only in the presence of an intact site 1, implying a possible dimerization formation with site 1 and site 3. That dimerization is present on the everted repeat with 14 inverted bp (ER14), as distinct from dual monomer co-occupation, is evidenced by the fact that site 3 is occupied only in the presence of an intact site 1. Thus, $ERR\alpha$ most probably binds exclusively to site 1 and the dimer binding is necessary for simultaneous occupation of half-sites (site 1 and site 3). In the case of the $PPAR\alpha/RXR\alpha$ heterodimer complex, it appears to bind site 1 and site 3, everted repeat with 14-interval bp (ER14). One of the explanations of why $PPAR\alpha/RXR\alpha$

Figure 8 Schematic representation of *ERRα* and *PPARα/RXRα* interactions with NRRE-1.

can still bind to NRRE-1, which is mutated either in site 1 or site 3, is that the binding site requirement of this nuclear receptor complex might be rather permissive. Anyhow, it is rather unusual that heterodimer (*PPARα/RXRα*) or homodimer (*ERRα*) receptors interact with such long intervals.

Competitive binding and effect of transcription of *ERRα* and *PPARα/RXRα* within NRRE-1

Although *ERRα* and *PPARα/RXRα* may use overlapping but distinct determinants on the NRRE-1, we did not observe a higher order complex containing all three receptors *in vitro*. Instead, we observed competitive binding between *ERRα* and *RXRα/PPARα* for the same sites within the NRRE-1 (Fig. 6). This observation is reflected in the cellular experiment showing that expression of *ERRα* can counteract the transcriptional activation by the *RXRα/PPARα* complex (Fig. 7). *ERRα* may also form inactive and non-binding heterodimers with *PPARα*, *RXRα* or other nuclear factors, effectively sequestering these receptors and preventing them from forming heterodimers that normally induce transcriptional activation, leading to an overall repression of transcription. However, the latter mechanism is unlikely, since inhibition by *ERRα* required the integrity of the NRRE-1 (Fig. 7B,C,D). It is possible, *in vivo*, that one of the receptors may constitute a predominant component of the complex, because these nuclear receptor expression patterns are distinct among different tissues.

Characterization of individual binding profiles may provide clues to the compositions of the heterogeneous complex.

At the same time, isolation and characterization of endogenous nuclear proteins that bind to NRRE-1, apart from the ones that have already been identified (Carter *et al.* 1994, Vega & Kelly 1997), will be necessary to fully define this transcriptional regulatory mechanism and to delineate the precise upstream regulatory pathways involved in the control of fatty acid oxidation enzyme expression.

Transcriptional and physiological properties of *ERRα* on MCAD gene: an hypothesis

ERRα was reported to regulate the promoters of lactoferrin (Yang *et al.* 1996), MCAD (Vega & Kelly 1997), osteopontin (Bonnelye *et al.* 1997) or *TRα* (Vanacker *et al.* 1998) in transient transfection assays. *ERRα* generally represses gene transcription in these assays (Sladek *et al.* 1997) as well as in cell-free systems (Wiley *et al.* 1993, Johnston *et al.* 1997), and has also been shown to antagonize the action of GR via an unknown mechanism (Trapp & Holsboer 1996). The lack of *ERRα* transcriptional activity may be due to the absence of its cognate ligand. However, under certain conditions, *ERRα* was reported to activate transcription (Vanacker *et al.* 1998). These authors (Vanacker *et al.* 1998) reported that the transcriptional activity observed is dependent on a serum compound that was withdrawn by charcoal treatment. We were not able to reproduce this data of transactivation in HeLa, HepG2 or SK-N-MC cells (results not shown). It may be that *ERRα* ligand is present in certain serum preparations but not in others. Taken together, it appears that *ERRα* can exert pleiotropic transcriptional controls depending on the targeted promoter and on the cell type considered. However, its mechanism remains completely unknown.

NRRE-1 is crucial for MCAD expression in transgenic models (Vega & Kelly 1997). Transcriptional regulation via NRRE-1 appears to be a net aggregate response manifested, in part, by the availability of *ERRα* and other factors that bind to NRRE-1, the complexity of response elements and the interplay of *ERRα* with other nuclear hormone receptors. It can be proposed that *ERRα* can influence the MCAD expression through

interaction with these nuclear receptors. Our hypothesis is that $ERR\alpha$ acts as a transcriptional repressor by competing with the $RXR\alpha/PPAR\alpha$ complex (or other activators that interact with NRRE-1) for the same sequence within the NRRE-1. Physiological evidence for this model comes from the $ERR\alpha$ -deficient mice (Giguere 1999). They are smaller than the wild-type and show decreased fat content throughout their bodies. Probably due to the absence of $ERR\alpha$ that acts as a repressor of the MCAD gene through NRRE-1, other activators (e.g. $RXR\alpha/PPAR\alpha$) could occupy the NRRE-1 and drive out high rates of transcription of MCAD gene, thereby inducing the high rate of β -oxidation. Our data of this comparative profile demonstrate that this receptor may interact with elements possessing a wide variety of architectures, a phenomenon consistent with the hypothesis that it acts as a general suppressor of many genes. In this hypothesis, the structure of $ERR\alpha$ bound to NRRE-1 or its response element would be relatively permissive for corepressor binding. It is also possible that allosteric effects of the DNA binding site could alter interactions with co-activators or with other DNA-bound transcription factors. Although we failed to show re-activation of the $ERR\alpha$ -dependent repression with the addition of TSA, it remains to be determined whether such DNA-induced conformational changes can alter co-activator or co-repressor binding. As yet, the transcriptional regulation of $ERR\alpha$ remains largely unknown, despite the fact that this was the first nuclear orphan receptor identified many years ago (Giguere *et al.* 1988). Ligands of this receptor may change its transcriptional activity thereby influencing the expression of the target genes by $ERR\alpha$, including the MCAD gene which is an important regulator of the rate of fatty acid β -oxidation and lipid metabolism. Selective ligands for $ERR\alpha$ may change the net transcriptional activity of the MCAD gene through NRRE1, thereby influencing lipid metabolism through β -oxidation. To this end, it is important to monitor the effect of $ERR\alpha$ on the whole MCAD gene promoter.

Acknowledgements

We thank Drs Rachubinski and Giguere for the expression plasmids.

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Received in final form 5 February 2003

Accepted 2 April 2003