Estrogen-related receptor- γ and peroxisome proliferator-activated receptor- γ coactivator-1 α regulate estrogen-related receptor- α gene expression via a conserved multi-hormone response element

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Abstract

The expression of estrogen-related receptor- α (ERR α) is stimulated by estrogen in selective tissues. Recently, a correlation between ERR α expression and the induction of peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) in the liver of fasting animals and in cold-stressed brown-fat tissues and skeletal muscle was shown. To explore the molecular mechanisms of ERR α regulation by diverse signals, the promoter of the human ERR α gene was cloned and characterized. Mutation and deletion analyses revealed that a 53 bp region containing repeated core element AGGTCA motifs of the ERR α gene serves as a multi-hormone response element (MHRE) for several nuclear receptors in transient co-transfection studies of human endometrial carcinoma (HEC-1B) cells. Among the nuclear receptors tested, ERR γ bound to and robustly stimulated the transcription of reporters containing at least two AGGTCA motifs. Ectopic expression of PGC-1 α in HEC-1B cells strongly activated the reporter containing the MHRE, presumably via the endogenous nuclear receptor binding to the element. Reducing the endogenous level of ERR γ by small interfering RNA, and increasing the ERR γ level by ectopic expression, substantially decreased and increased respectively the transactivation capability of PGC-1 α . The activation function 2 domain of the ERR γ and the L2 and L3 motifs of PGC-1 α were essential to transactivate the MHRE. Additionally, PGC-1 α increases the amount of endogenous ERR γ bound to the MHRE region as determined by a chromatin immunoprecipitation assay. The present study demonstrates that the MHRE of the ERR α gene is a target for ERR γ transactivation, which is enhanced by PGC-1 α .

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Introduction

Nuclear receptors constitute an important group of transcription factors that regulate diverse biological functions through multiple signaling pathways (see reviews by Tsai & O'Malley (1994) and Mangelsdorf *et al.* (1995) and references therein). The activities of many nuclear receptors are controlled by their respective ligands; however, most members have either no or unidentified ligands, and these have been collectively named orphan receptors (see review by Giguere (1999) and references therein). A subfamily close to the estrogen receptor (ER), estrogen-related receptors (ERR α /NR3B1; ERR β /NR3B2) was cloned 15 years ago (Giguere *et al.* 1988) as orphan receptors and a third member (ERR γ /NR3B3) was cloned recently (Hong *et al.* 1999, Heard *et al.* 2000). Whether or not a ligand is required for this subfamily remains unclear and controversial. The ERR α has been reported to function in the absence of ligand (Xie *et al.* 1999, Zhang & Teng

2000) as well as in the presence of a ligand such as serum factors (Vanacker *et al.* 1999a,b) or protein ligand (Kamei *et al.* 2003). In contrast, ERR γ consistently functions as a positive activator without exogenous ligand in transient co-transfection experiments (Hong *et al.* 1999, Coward *et al.* 2001, Sanyal *et al.* 2002). Nonetheless, inverse agonists for the ERRs were found. The synthetic estrogen, diethylstilbestrol binds all three ERRs, interrupts the receptor–coactivator interaction and antagonizes the ERRs' transactivation activities (Tremblay *et al.* 2001b). In addition, 4-hydroxytamoxifen binds ERR β and ERR γ (Coward *et al.* 2001, Tremblay *et al.* 2001a) whereas micromolar concentrations of some pesticides bind ERR α (Yang & Chen 1999) and inhibit their transactivation function. Recently, a specific synthetic inverse agonist of ERR α was reported (Mootha *et al.* 2004).

The expression pattern of ERR α and ERR γ in human and mouse is both overlapping and different (Shi *et al.* 1997, Shigeta *et al.* 1997, Hong *et al.* 1999, Heard *et al.* 2000). ERR β is primarily expressed in the embryo

and the ERR β -null mouse is embryonically lethal due to placental defects (Luo *et al.* 1997). The biological function of ERR γ is not clear, but the roles of ERR α in cellular physiology are emerging. ERR α was found to bind to a TCAAGGTCA element in the human lactoferrin gene promoter and to modulate the estrogen response (Yang *et al.* 1996). An extensive analysis of ERR α binding preference suggests that this receptor could bind a variety of estrogen-response elements (EREs) (Johnston *et al.* 1997, Sladek *et al.* 1997) and regulate similar ER target genes, thus implicating a modulatory role in ER-mediated signaling pathways (Zuo & Mertz 1995, N Yang *et al.* 1996, C Yang *et al.* 1998, Giguere 2002, Teng 2002). In addition, ERR α is involved in bone morphogenesis (Vanacker *et al.* 1998a, Bonnelye *et al.* 2002) and in energy balance (Sladek *et al.* 1997, Vega & Kelly 1997, Vanacker *et al.* 1998b, Vega *et al.* 2000, Huss *et al.* 2002, Luo *et al.* 2003).

Recently, the expression of ERR α was found to be upregulated in mouse uterus and heart by estrogen (Liu *et al.* 2003) as well as in liver by fasting (Ichida *et al.* 2002) and in brown fat by exposure to cold (Schreiber *et al.* 2003). ERR α expression after fasting and cold treatment correlates with the expression of physiological stimuli-inducible peroxisome proliferator-activated receptor- γ (PPAR γ) coactivator-1 α (PGC-1 α) (Ichida *et al.* 2002, Schreiber *et al.* 2003). The mouse PGC-1 α was initially identified in brown fat as a coactivator for PPAR γ (Puigserver *et al.* 1998) and the human homologue was subsequently cloned (Knutti *et al.* 2000). PGC-1 α interacts with an array of nuclear receptors by enhancing their transactivation function (Kressler *et al.* 2002) and importantly, it coordinately regulates genes involved in adaptive thermogenesis and serves as a 'master' regulator of cellular energy metabolism (see reviews by Knutti & Kralli (2001) and Puigserver & Spiegelman (2003) and references therein). While searching for proteins that interact with PGC-1 α , ERR α was identified as a major interacting partner (Huss *et al.* 2002, Ichida *et al.* 2002). More recent evidence demonstrated that PGC-1 α and ERR α work in concert to regulate mitochondrial biogenesis (Schreiber *et al.* 2004) and the oxidative phosphorylation program (Mootha *et al.* 2004), by directly influencing the expression of subsets of these genes.

The promoter of ERR α gene contains a multi-hormone response element (MHRE) that is a target site for ER α in the estrogen response (Liu *et al.* 2003). This region also binds ERR α itself and serves as autoregulatory site in the PGC-1 α -induced response (Laganiere *et al.* 2004, Mootha *et al.* 2004). Since the DNA-binding domain of ERR γ and ERR α are highly conserved (93%) and both receptors are coexpressed in high-energy demanding tissues such as skeletal muscle, heart and kidney, it seems likely that ERR γ would bind and regulate ERR α gene expression. In this study, we

demonstrated that ERR γ is a potent activator for ERR α gene expression, which is enhanced by PGC-1 α . We show that PGC-1 α enhances ERR γ binding to the MHRE, suggesting a potential mechanism for PGC-1 α regulation of ERR α gene expression.

Materials and methods

Plasmids

ERR γ , ERR γ 449 (deletion of the activation function 2 (AF2) domain, AF2 Δ), Myc-ERR γ , and glutathione S-transferase (GST)-ERR γ (Hentschke *et al.* 2002b) were gifts from U Borgmeyer (University of Hamburg, Germany), ER α and ER β (Liu *et al.* 2003), RXR α , RAR α and TR α from A Jetten (National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA (NIEHS)), PPAR α from C Weinberger (NIEHS), ROR α , ROR β and ROR γ from V Giguere (McGill University, Canada, Montreal, Quebec) and chicken ovalbumin upstream promoter-transcription factor-1 (COUP-TFI) from M Tsai (Baylor College of Medicine, Houston, TX, USA). Coactivator PGC-1 α and its mutant version, pcDNA3/HA hPGC-1 α , pcDNA3/HA hPGC-1 L2A, pcDNA3/HA hPGC-1 L3A, and pcDNA3/HA hPGC-1 L2A/3A (Huss *et al.* 2002, Schreiber *et al.* 2003) were obtained from A Kralli (Scripps Research Institute, La Jolla, CA, USA) and D Kelly (Washington University School of Medicine, St Louis, MO, USA). The human ERR α promoter reporter constructs (0-6-CAT and 0-8-CAT) and the MHREs and its mutant versions (AAB, AB, A, m1, m2, m3, m4 in SV40-CAT) were described before (Liu *et al.* 2003). The AAB-TATA-Luc was constructed by excising out the AAB element from the AAB-SV40-CAT construct with NheI/XhoI digestion and then cloning into pLuc-MCS (Stratagene, La Jolla, CA, USA) reporter at the XhoI site by blunt-end ligation.

Cell culture and transient transfection

HEC-1B (ATCC# HTB-113, endometrial), HepG2 (ATCC# CRL-8024, liver), PLC/PRF/5 (ATCC# HB-8065, liver) and HeLa (ATCC# CCL-2, cervical) and MCF-7 (ATCC# HTB-22, mammary gland) cells were maintained in Eagle's MEM. The MCF-7 cells were supplemented with 10 μ g/ml insulin. The HEK293 (ATCC# CRL-1573, kidney) cells were cultured in Dulbecco's MEM medium. All cells were cultured in the presence of 10% fetal bovine serum, 100 IU/ml penicillin and 100 μ g/ml streptomycin at 37 °C under 5% CO₂. To investigate the reporter activities in HEC-1B cells, the transfections were carried out with Qiagen Effectene transfection reagent (Qiagen, Valencia, CA, USA) according to the provider's instruction. The total DNA transfected in each experiment was kept

constant with reporter constructs (300 ng/well), internal control PCH 110 plasmid (100 ng/well), expression plasmids (specified in individual experiments) and the carrier DNA to make the total amount of 500 ng. Prior to transfections, cells were plated in six-well plate and grown overnight in medium containing 10% dextran-coated charcoal-stripped serum. Cells were collected 36 h after transfection and the CAT or Luc activities measured (Liu *et al.* 2003). The reporter activities were normalized by β -galactosidase activities. Qiagen TransMessenger Transfection reagent (Qiagen) was used in the experiments intended for quantitative real-time PCR determination, ERR γ mRNA reduction, and the chromatin immunoprecipitation (ChIP) assays.

Transient transfection of small interfering RNA (siRNA)

Synthesized siRNA was ordered from Qiagen-Xeragon (Qiagen, Germantown, MD, USA). The siRNA duplex (500 ng) was mixed with TransMessenger transfection reagent and transfected into the cells for 48 h. The sequence of the control siRNA (non-silencing) is 5'-AAT TCT CCG AAC GTG TCA CGT-3' and the ERR γ siRNA (specific silencing) is 5'-AAT GGC CAT CAG AAC GGA CTT-3'. The effect of ERR γ mRNA reduction on the ERR α gene activity was determined by first introducing the siRNA (500 ng) to the cells for 24 h and again with the transfection mixture (300 ng reporter plasmid, 100 ng internal control plasmid) for 36 h before the cells were collected and the Luc activity measured.

Quantitative real-time PCR and RT-PCR

The total RNA was extracted with Qiagen RNeasy Mini Kit according to the supplier's protocol (Qiagen). Quantitative real-time PCR was used to measure the ERR γ and ERR α mRNA levels in HEC-1B cells under various experimental conditions. The primer pair is as follows: for human ERR γ , forward primer 5'-GGC CAT CAG AAC GGA CTT G-3' and reverse primer 5'-GCC CAC TAC CTC CCA GGA TA-3' (67 bp amplicon); for human ERR α , forward primer 5'-GGC CCT TGC CAA TTC AGA-3' and the reverse primer 5'-GGC CTC GTG CAG AGC TTC T-3' (79 bp amplicon). The 144 bp amplicon of human β -actin was detected with the forward primer 5'-GAC AGG ATG CAG AAG GAG ATC AC-3' and the reverse primer 5'-GCT TCA TAC TCC AGC AGG-3'. The quantitative real-time PCR method has been previously described in detail (Liu *et al.* 2003). For standard RT-PCR, 200 ng total RNA were used with the following primers: ERR γ , the forward primer 5'-ATG TCA AAC AAA GAT CGA CAC-3' and reverse primer 5'-GAC AGG CCC GCT GCC TCC CAG GA-3' (222 bp); ERR α , the forward primer 5'-AGA TGT

CAG TAC TGC AGA GCG T-3' and reverse primer 5'-CGG CTT CAT ACT CCA GCA-3' (322 bp). The PCR reaction was run for 25 cycles.

ChIP assay

The ChIP assay was performed according to the instructions of the ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA) with minor modifications. Twenty-four hours after transfection of either empty vectors or PGC-1 α -expressing vectors, protein and DNA were cross-linked with 1% formaldehyde overnight at 4 °C. Cells were washed with cold PBS twice and disrupted in SDS lysis buffer containing a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin and 1 μ g/ml pepstatin A). Chromatin was sonicated to an average length of DNA of 200–1000 bp as verified by agarose gel electrophoresis (data not shown). The sheared chromatin was diluted in ChIP dilution buffer and an aliquot of the solution reserved for input control. Fifteen microliters of ER α antibody (mouse monoclonal; NeoMarkers, Fremont, CA, USA) were used as a negative (non-specific antibody) control since HEC-1B cells are ER α -negative (Hopfer *et al.* 1996). The endogenous ERR γ was detected by polyclonal rabbit ERR γ antiserum (a gift from U Borgmeyer). After addition of the antibodies (15 μ l), the chromatin solutions were gently rotated overnight at 4 °C. The Protein A agarose slurry (containing sonicated salmon sperm DNA) was added to the antibody-bound chromatin solution and incubated for 1 h at 4 °C with constant rotation. The agarose beads were collected by centrifugation, washed and the antibody-bound chromatin was released from the agarose beads according to the supplier's specification. Finally, the DNA was purified by phenol/chloroform extraction and ethanol precipitation. The MHREs region was detected with forward primer 5'-GTC AGT GCA GGA CAG CCC GCG AG-3' (–758/–734) and the reverse primer 5'-GAT AGG GCC CGG ACG GAG AAA GC-3' (–649/–627) in PCR reaction. As control, an 8.5 kb region downstream from the MHRE (human genomic sequence AP001453, gi 31790751) was selected and detected with the forward primer 5'-CAG CCC TGG CAG TCT GGA TGG-3' (at +85 627) and reverse primer 5'-GCC CTC ATC TGC CGA CAT CAA-3' (at +85 881). The PCR conditions for ChIP assay were 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s for a total of 35 cycles.

In vitro transcription and translation, and electrophoretic mobility shift assay (EMSA)

PGC-1 α and ERR γ were transcribed and translated *in vitro* with either unlabeled or ³⁵S-labeled L-methionine (Amersham Biosciences, Piscataway, NJ, USA) using the

TNT Coupled Reticulocyte Lysate Systems (Promega, Madison, WI, USA). The proteins were used in the EMSAs, the biotin-labeled DNA pull-down and the GST pull-down assays. Double-stranded DNA elements (AAB, AB, A, m1, m2, m3, m4) were cut out from the SV40-CAT reporters by *NheI* and *XhoI*, gel purified and used in EMSAs. The AAB and AB double-stranded oligos were labeled with [³²P]dGTP by fill-in with Klenow large fragment of DNA polymerase I and the dNTP mixture. The unlabeled AAB, AB, A, m1, m2, m3, m4 elements were used as the competitors. The EMSA has been previously described (Yang *et al.* 1996, Shigeta *et al.* 1997).

GST and biotin-DNA pull down assays

The GST- and GST-ERR γ -expressing plasmids were transformed into *E. coli* BL-21 cells and the expression of GST and GST-ERR γ fusion proteins was induced by isopropyl-1-thio- β -D-galactopyranoside. The bacteria were disrupted by sonication, and the GST and its fusion protein were isolated with a 50% slurry of glutathione-Sepharose beads. Equal amounts of GST, GST-ERR α and GST-ERR γ protein were incubated with the *in vitro*-translated ³⁵S-labeled PGC-1 α for 1 h at 4 °C. Binding of the PGC-1 α to the GST-fusion protein was examined with SDS-PAGE and visualized by autoradiography. Two micrograms of biotin-labeled AAB elements (purchased from Sigma Genosys, The Woodlands, TX, USA) from each strand in 200 mM NaCl were heated at 95 °C for 5 min and then slowly cooled down to room temperature. After the double-stranded biotin-labeled AAB elements were bound to 20 μ l streptavidin-agarose beads (Sigma, St Louis, MO, USA), the *in vitro*-transcribed and -translated ³⁵S-labeled ERR γ by itself or in combination with the *in vitro*-transcribed and -translated unlabeled PGC-1 α was added to the biotin-AAB-streptavidin complex beads. The AAB-bound ERR γ was examined with SDS-PAGE and visualized by autoradiography. The intensity of the band was determined by the spot-density analysis program of an Alpha Innotech ChemImager (San Leandro, CA, USA).

Quantitation of the PCR product or shifted bands in EMSA

Scanning was done in an Innotech ChemImager 5500 with signal spot densitometry according to the User's Manual.

Results

MHRE of the ERR α promoter is a pleiotropic nuclear receptor enhancer

We have previously shown that the ERR α gene is estrogen responsive and the MHRE is a major site

responsible for the ER-mediated transactivation (Liu *et al.* 2003). The MHRE does not resemble any typical nuclear receptor response element yet it consists of three TCAAGGTCA (ERRE), an element originally identified to bind ERR α and steroidogenic factor (SF)-1 (Yang *et al.* 1996, Bonnelye *et al.* 1997, Johnston *et al.* 1997), and two AGGTCA motifs of nuclear receptor binding core element. These motifs are arranged in various spacing and orientation within the MHRE that could be recognized by different nuclear receptors. Using a transient transfection approach, we tested a number of nuclear receptors for their ability to activate the ERR α promoter-reporter with or without the MHRE present in the HEC-1B cells (Fig. 1A). Consistent with the earlier findings, the MHRE in the context of GC-rich ERR α promoter is responsive to ligand-bound RXR α and PPAR α as either homodimer or heterodimer (Fig. 1B) while RAR α and TR α (with or without the presence of their respective ligand) have no effect (data not shown). Interestingly, the ERR α and ERR γ in the absence of exogenous ligand enhanced the reporter activity driven by MHRE 10- and 25-fold respectively (Fig. 1C, 0-8-CAT). We have also tested COUP-TFI, ROR α , ROR β and ROR γ , and found no significant changes under the current assay conditions (data not shown). Taken together, the ERR α gene is positively regulated by its own gene product and the close family member ERR γ . Since ERR α and ERR γ are coexpressed in many tissues such as skeletal muscle, heart, kidney and pancreas, regulation of ERR α gene expression in those tissues could be greatly influenced by the presence of ERR γ .

Binding properties of ERR γ to the MHRE

The MHRE of the human and mouse ERR α gene is conserved (Fig. 2A) except for a 23 bp region which appears twice in the human gene. The 23 bp region ('A'), contains one ERRE and one core AGGTCA element and an 11 bp region ('B'), has a single ERRE (Fig. 2A). Binding of ERR γ to the MHRE of human form (AAB) or the mouse form (AB) was examined by EMSA. The *in vitro*-transcribed and -translated ERR γ protein binds MHRE of both human (AAB, Fig. 2B, lane 3) and mouse (AB, lane 14), while the reticulocyte lysate protein did not bind (mix, lanes 2 and 13). The specificity of binding was demonstrated by an effective competition with unlabeled wild-type AAB and AB oligos (lanes 4, 5 and 15, 16) and by supershifting the protein-DNA complex with ERR γ antibody (lanes 11 and 20). Preimmune serum (PS, lane 22) and non-relevant antibody (LF, lane 21) had no effect on the mobility of the complexes. To further characterize the binding requirement for ERR γ , several mutant oligos were tested in the competition study. Deletion of the B region from the MHRE (A) or mutation of the A region

Figure 1 The MHRE of the ERR α gene is a pleiotropic response element. (A) Diagrammatic presentation of the ERR α promoter-reporter constructs. Different lengths of ERR α gene 5'-sequences linked to CAT-reporter. The 70% GC region contains 11 Sp1 sites (Liu *et al.* 2003). The location of the MHRE and the primers used to detect this region in the ChIP assay are marked by arrows. The positions of the 0.6 and 0.8 kb 5'-flanking region of the ERR α gene in relation to the initiation start site (Shi *et al.* 1997) are indicated. For the transient co-transfection, HEC-1B cells were cultured for 24 h in charcoal-stripped serum before transfection with 300 ng of reporters and 100 ng of expression constructs. Cells were either treated with vehicle or ligand as indicated. (B) Effect of RXR α and PPAR α . 9-cis-retinoic acid (9 Cis) at 50 μ M and 5,8,11,14-eicosatetraynoic acid (ETYA) at 20 μ M. (C) Effect of ERR α and ERR γ . The experiments were repeated three times with duplicated samples. The values are presented as means $\pm$ S.D.

Figure 2 ERR γ binds the MHRE of ERR α gene promoter in an EMSA. (A) Diagrammatic presentation of the wild-type and mutant version of the MHRE. The sequences were linked to either CAT-reporter or Luc-reporter constructs for functional study or made into double-stranded oligos for EMSA. AAB, the human MHRE consists of a 23 bp ('A') repeats and an 11 bp ('B') sequence; AB, the mouse MHRE; m1, m2 and m3, mutations of GG to AA at the indicated location; m4, all three sites were mutated; A, the 23 bp sequence only. (B) Binding of *in vitro*-transcribed and -translated ERR γ to AAB or AB probes in an EMSA. Scan of the shifted bands (arrow) is presented on top of the gel. (C) Binding of *in vitro*-transcribed and -translated ERR γ and myc-ERR γ to the AB probe. ³²P-labeled probes are indicated. Arrow indicates the ERR γ -DNA complex. SS, super shifted band; γ , ERR γ antibody (Hentschke *et al.* 2002b); LF, lactoferrin antibody (Teng *et al.* 1986); PS, preimmune serum.

at the ERRE (CC to AA, m1) reduced but did not abolish the competition (lanes 6, 7, 17 and 18). Interestingly, the same mutation at ERRE of the B region (m3) severely affecting the ERR γ binding (lane 9) and the mutant oligos could only compete at 50% efficiency (scan on top, lane 9). In contrast, mutation of the middle core element (GG to AA, m2) has no obvious effect on ERR γ binding because the mutant oligos (lanes 8 and 19) compete as well as the wild-type oligos (compare to AAB and AB) in the EMSA. As expected, mutations of all three sites (m4) eliminated the ERR γ binding and no competition was found (scan on top, lane 10). These data suggested that ERR γ binds to MHRE specifically and preferably to the two ERREs. Binding of ERR γ as a homodimer complex with multiple synthetic EREs was shown (Hentschke *et al.* 2002b, Huppunen & Aarnisalo 2004). Whether ERR γ also binds the MHRE of the ERR α gene as homodimer was examined. We transcribed and translated different lengths of ERR γ protein (full length and myc-tagged full length) *in vitro* and examined their binding patterns in the EMSA (Fig. 2C). Due to the extra myc sequence, the myc-ERR γ -DNA complex moved more slowly than the ERR γ -DNA complex (compare lanes 2 and 7). When different ratios of ERR γ and myc-ERR γ expression plasmids were co-transcribed and translated *in vitro*, a new protein-DNA complex appeared at the intermediate position formed from the heterodimerization of ERR γ and myc-ERR γ (lanes 3–6). These data are in agreement with the binding study of ERR γ from other laboratories (Hentschke *et al.* 2002b, Huppunen & Aarnisalo 2004).

Transactivation function of ERR γ and PGC-1 α on the MHRE

The above test studies (Fig. 1) demonstrated that ERR γ strongly transactivates MHRE in the context of its natural promoter. ERR γ also transactivated the MHRE in heterologous promoters (SV40-CAT or TATA-Luc) in a dose-dependent manner (data not shown). To identify elements within the MHRE that are required for ERR γ 's function, wild-type or mutant MHRE-reporters were co-transfected with ERR γ expression constructs into HEC-1B cells and the transactivation activities of these reporters were examined (Fig. 3A). ERR γ strongly transactivated the AAB and AB reporters, but weakly with the A reporter. Interestingly, mutation of various elements within the MHRE makes a significant difference in ERR γ transactivation function, such as mutation of the ERRE at m3 position dramatically reduced the transactivation function of ERR γ while a lesser effect as the same ERRE was mutated at the m1 position. The transactivation activity of ERR γ was least affected by the mutation at m2, the ERE core element. When all three sites were mutated (m4), the transactivation capability of ERR γ was blocked. Taken together, the data were

consistent with the ERR γ binding characteristics and showed a correlation between the ability of ERR γ to bind and to transactivate the MHRE.

Recently, expression of ERR α in liver and heart was found increased following the induction of PGC-1 α (Huss *et al.* 2002, Ichida *et al.* 2002, Schreiber *et al.* 2004). The concerted expression of PGC-1 α and ERR α suggests that ERR α is a downstream target of PGC-1 α and be may involved in the energy metabolism program. Ectopic expression of the PGC-1 α in HEC-1B cells vigorously stimulated the transcriptional activity of the MHRE-reporters (Fig. 3B, AAB and AB) and the required element for this activity mimics the requirement for ERR γ binding and activation of the MHRE (compare Fig. 2B and Fig. 3A to Fig. 3B).

PGC-1 α enhances ERR γ transactivation of the MHRE

PGC-1 α does not have a typical DNA-binding domain (Puigserver *et al.* 1998, Knutti *et al.* 2000); through protein–protein interaction, PGC-1 α coactivates a number of nuclear receptors including the ERR α and ERR γ (Huss *et al.* 2002, Schreiber *et al.* 2003). Therefore, the strong activation of the MHRE-reporters by PGC-1 α expression in HEC-1B cells (Fig. 1B) has to rely on the endogenous nuclear receptors or transcription factors that bind the MHRE. Many nuclear receptors and transcription factors in HEC-1B cell could participate in the PGC-1 α -induced transactivation activity of the MHRE-reporter; especially PGC-1 α was found to coactivate diverse nuclear receptors and transcription factors (Puigserver & Spiegelman 2003, Schreiber *et al.* 2003). Our data on the binding and transactivation of ERR α gene by the ERR γ (Figs 1 and 2) suggested that the ERR γ could play a role in PGC-1 α -induced reporter activities in HEC-1B cell.

To examine the expression pattern of ERR α and ERR γ in HEC-1B cells as well as several other human cultured cell lines, we performed limited RT-PCR (25 cycles) of total RNA prepared from the HEC-1B (endometrial), HeLa (cervical), MCF-7 (mammary gland), HEK293 (kidney), HepG2 (liver) and PLC/PRF/5 (liver) cell lines (Fig. 4A). ERR α is ubiquitously expressed and the level of expression is comparable in these cell lines (top panel). In contrast, ERR γ is selectively expressed with a wide range of expression levels (middle panel). For example, a high level of ERR γ was detected in human HEC-1B cells (lane 1) while in the PLC/PRF5 cells (lane 7) it was barely detectable. The ERR γ in MCF-7 (lane 4), HEK293 (lane 5) and HepG2 cells (lane 6) was measurable but at a low level. The high level of ERR γ in the HEC-1B cells may be important in assessing the MHRE-reporter activity by PGC-1 α in transient co-transfection experiments. This cell line could also serve as a model to study the molecular mechanism of ERR γ action. To examine

Figure 3 *ERRγ* and *PGC-1α* transactivate the MHRE. (A) Effect of ectopic expression of *ERRγ*. (B) Effect of ectopic expression *PGC-1α*. The wild-type or mutant versions of MHRE were linked to the SV40-CAT reporter (300 ng) and the effect of *PGC-1α* (25 ng) or *ERRγ* (100 ng) on the reporter activities was measured 48 h after transfection of the HEC-1B cells. Descriptions of the mutant reporters are the same as in Fig. 2. The experiments were repeated three times with duplicated samples. The results are presented as mean±s.d.

whether changing the level of ERR γ in HEC-1B cells affects the transactivation function of PGC-1 α , we applied the siRNA technique to reduce the endogenous ERR γ level. After introducing the ERR γ siRNA duplexes into HEC-1B cells for 48 h, the endogenous mRNA levels of both ERR γ and ERR α were significantly reduced as measured by real-time PCR (Fig. 4B, left panel), and the transactivation activity of PGC-1 α on MHREs reporter was also reduced (right panel). The control siRNA affected neither the levels of ERRs mRNA nor the transactivation function of PGC-1 α . In an opposite experiment, when both PGC-1 α and ERR γ were expressed ectopically, the transcriptional activity of the MHRE-reporters was higher than either one alone (Fig. 4C). These experiments demonstrated that ERR γ stimulates the activity of MHRE-reporters and PGC-1 α augments its effect. Taken together, PGC-1 α enhances but is not required for the ERR γ to transactivate ERR α gene through MHRE.

Mechanism of PGC-1 α and ERR γ activation of the MHRE

The AF2 domain of the nuclear receptor and the LXXLL motifs of the coactivator/corepressor are involved in protein–protein interaction and the transactivation function (Glass *et al.* 1997, Lanz *et al.* 1999, McKenna *et al.* 1999). PGC-1 α coactivates the ERR γ on a synthetic ERE and the medium-chain acyl-CoA dehydrogenase response element (Hentschke *et al.* 2002a, Huss *et al.* 2002); however, it has not yet been examined with the MHRE of ERR α gene. To investigate the functional relationship of ERR γ AF2 domain and PGC-1 α LXXLL motifs on the MHRE, we ectopically expressed the ERR γ AF2 deletion mutant (AF2 Δ) (Hentschke *et al.* 2002a) and PGC-1 α L2 and L3 mutant constructs (Huss *et al.* 2002, Schreiber *et al.* 2003) in the HEC-1B cells and the MHRE activities were measured (Fig. 5). Expression of ERR γ AF2 Δ severely reduced the MHRE-reporter activity and the activity stimulated by PGC-1 α suggests that the mutant ERR γ acts as a dominant negative receptor (Fig. 5A). The requirement for the three LXXLL motifs (L1, L2 and L3) of the PGC-1 α in nuclear receptor interaction and function has been carefully analyzed (Puigserver *et al.* 1998, Huss *et al.* 2002, Schreiber *et al.* 2003). While the L2 is essential for most of the receptors examined, both the L2 and L3 are needed for ERRs interaction and function (Huss *et al.* 2002, Kressler *et al.* 2002, Schreiber *et al.* 2003). As expected, the coactivation activity of PGC-1 α on the MHRE was diminished but not eliminated with either L2 or L3 mutation and completely abolished with double mutations (L2/L3) (Fig. 5B).

PGC-1 α could coactivate the ERR γ through multiple ways. In this study, we asked whether the binding of

ERR γ to the MHRE is affected by PGC-1 α (Fig. 6). The *in vitro*-labeled ³⁵S-PGC-1 α interacted strongly with the GST-ERR γ (Fig. 6A, upper panel, lanes 3 and 4) fusion protein (compare the intensities with the 10% input in lane 1). There was no binding with the GST protein by itself (lane 2), demonstrating the binding specificity and the proper interaction of the *in vitro*-translated PGC-1 α and ERR γ . The result agrees with reports from other laboratories that PGC-1 α physically interacts with ERR γ . Interaction of ERR γ and PGC-1 α was further investigated by including the MHRE in a DNA pull-down assay. The MHRE was labeled with biotin and immobilized onto streptavidin beads. Binding of ³⁵S-labeled ERR γ to the MHRE was assessed in the presence or absence of *in vitro*-translated PGC-1 α (Fig. 6B). The translation condition that produced similar amount of wild-type and mutant PGC-1 α protein was first established and verified by ³⁵S labeling (Fig. 6A, lower panel). An equal amount of the translation reaction mixture (with or without the unlabeled wild-type or mutant PGC-1 α) was then mixed with the labeled ERR γ . The total protein in every experiment was kept constant. Interestingly, interaction of ³⁵S-ERR γ with biotin-labeled MHRE increased in the presence of PGC-1 α , and the amount of ³⁵S-ERR γ bound to the biotin-MHRE was proportional to the concentration of PGC-1 α in protein mixture (Fig. 6B). There was no binding in the absence of biotin-DNA. In addition, mutant PGC-1 α did not enhance the interaction between ERR γ and the MHRE (Fig. 6B, bottom, L2 L3). This *in vitro* study was further investigated by the *in vivo* ChIP assay (Fig. 6C). PGC-1 α or empty vector were transfected into HEC-1B cells for 24 h and the endogenous ERR γ that was cross-linked to the MHRE region by formaldehyde treatment was immunoprecipitated by ERR γ antibody. The amount of ERR γ that binds MHRE region was increased 4-fold in cells expressing PGC-1 α (Fig. 6C, top), and with no binding detected in the unrelated region (Fig. 6C, bottom). ER α antibody was used as a negative control, since no ER α was detected in HEC-1B cells by either Western blotting or RT-PCR (C T Teng, unpublished observations). Together, these results demonstrated that the PGC-1 α increases ERR γ binding to the MHRE, thus providing a mechanism for PGC-1 α amplifying the ERR γ transactivation function on the MHRE of the ERR α gene.

Discussion

The promoter of the ERR α gene is TATA-less and highly rich in Gs and Cs (Shi *et al.* 1997). Embedded within the GC-rich region are 11 consensus Sp1-binding sites and this region is estrogen responsive even though there is no typical ERE (Liu *et al.* 2003). The estrogen response may be mediated through a protein–protein

interaction of Sp1 and ER α (Pipaon *et al.* 1999, Saville *et al.* 2000). This GC-rich region, however, is not responsive to other ligand-dependent or -independent nuclear receptors examined in this study (Fig. 1). An MHRE functioning as a pleiotropic nuclear receptor

response element is present upstream from the GC-rich region. Interestingly, the MHRE is very responsive to ER α but not to ER β in the presence of ligand (Liu *et al.* 2003). This observation is similar to the osteopontin gene promoter that is stimulated through an SF response

Figure 5 AF2 of the ERR γ and LXXLL motifs of the PGC-1 α are required in functional interaction. (A) Effect of ERR γ AF2 deletion. The 0.8-CAT reporters were co-transfected with expression constructs of the ERR γ wild-type or AF2-deleted (AF2 Δ) with or without the PGC-1 α . CAT activities were measured 48 h after transfection of the HEC-1B cells. The experiments were repeated three times with duplicated samples and results are presented as means $\pm$ s.d. The activity from the wild-type PGC-1 α was set as 100%. (B) Effect of PGC-1 α LXXLL motif(s) mutation(s). The AAB-TATA-Luc reporter were co-transfected with expression constructs of the PGC-1 α wild-type or LXXLL motif mutated (L2A, L3A and L2A/L3A) with or without the ERR γ . The Luc activities were measured 36 h after transfection of the HEC-1B cells. The experiments were repeated three times with duplicated samples and results are presented as means $\pm$ s.d. The activity from the wild-type PGC-1 α was set as 100%.

element (sequence identical to ERRE) by ER α and ERR α but not by ER β (Vanacker *et al.* 1999a). Based on the composite nature of MHRE, it could be recognized by a variety of nuclear receptors and our initial test results supported the predication. The RXR α and

PPAR α heterodimer showed strong activation of the MHRE in the presence of their respective ligands. Although the MHRE is pleiotropic, it is also selective in responding to nuclear receptor stimulation. A computer search for motif sequence identity suggested that the

Figure 4 PGC-1 α coactivates the ERR γ transactivation function. (A) ERR α and ERR γ mRNA levels. Total RNA was prepared from the indicated human cultured cell lines. A limited RT-PCR was performed and the PCR product analyzed on the gel. The intensity of each band was determined by image analysis and normalized to the product of HEC-1B cells. Specific primer sets for ERR α , ERR γ and β -actin mRNA detection were described in the Materials and Methods. Molecular markers at lane 1 are included to verify the product size. (B) Endogenous ERR γ mRNA level influences the expression of ERR α and the activation capability of PGC-1 α on MHRE. Left panel, the ERR γ siRNA duplex was transfected into HEC-1B cells for 48 h and the endogenous ERR γ and ERR α mRNA levels were determined by the quantitative real-time PCR as described in the Materials and Methods. The experiments were repeated three times and the ERR γ mRNA level from the control siRNA plasmids transfected cells was set as 1. The results are means $\pm$ s.d. Right panel, the HEC-1B cells were transfected with ERR γ siRNA for 24 h, followed with the transfection of PGC-1 α expression constructs (25 ng) and the AAB-TATA-Luc reporters (300 ng). (C) PGC-1 α enhances the transactivation activity of ERR γ . PGC-1 α (25 ng) and ERR γ (50 ng) expression constructs were co-transfected with the AAB-TATAT-Luc reporters (300 ng) individually or together into the cells for 36 h and the activities measured. The experiments were repeated three times with duplicated samples and results are presented as means $\pm$ s.d.

Figure 6 PGC-1α enhances ERRγ interaction with the MHRE both *in vitro* and *in vivo*. (A) GST pull-down assay. Binding of ³⁵S-PGC-1α to GST-ERRα or GST-ERRγ was analyzed with SDS-PAGE and visualized by X-ray film exposure. (B) Biotin-labeled DNA pull-down assay. Double-stranded biotin-AAB was interacted with ³⁵S-ERRγ alone or in the presence of different concentrations of unlabeled *in vitro*-translated PGC-1α (5 and 10 μl) and the control samples contain 10 μl of reaction mixture. The amount of ³⁵S-labeled ERRγ pulled down by the beads was resolved in the SDS-PAGE and exposed to X-ray film with an intensifying screen at -70 °C. Top, a representative gel picture. Bottom, relative fold of increase in ³⁵S-ERRγ binding in the presence of wild-type (wt) and mutant (L2/L3) PGC-1α. Results are presented as means±s.d. of three independent experiments. (C) The ChIP assay. PGC-1α or empty vector was ectopically expressed in the HEC-1B cells for 24 h. The ChIP assay was performed with ERRγ or ERα antibodies. Top, detection of the MHRE region by PCR with specific primers (indicated in Fig. 1 and described in Materials and Methods). Bottom, PCR detection of the 8.5 kb region downstream from the MHRE (according to the human chromosome 11 genomic sequence: AP001453; gi 31790751). The intensity of the PCR band detected by the ERα antibody is set to 1 (at bottom of the top graph).

MHRE closely resembles the RORE. Several ROR expression vectors (ROR α, β, γ) were examined, none of which showed a significant response in the present study. Surprisingly, the MHRE was dramatically stimulated by its own family member ERRα and especially ERRγ.

The protein sequence of ERRα and ERRγ is highly conserved at the DNA-binding domain (93%) and shows moderate homology at the potential ligand-binding domain (53%). The AF2 regions of these receptors are identical with a major difference between these proteins in the N-terminal region (18%) (Shigeta *et al.* 1997, Hong *et al.* 1999). The close sequence homology of the ERRα and ERRγ proteins suggests that they could bind and activate similar target genes. However, differential regulation of target genes by these proteins was reported. For example, the small heterodimer partner SHP is constitutively activated by ERRγ and not by ERRα, whereas the thyroid receptor response element is activated by ERRα but not by ERRγ (Vanacker *et al.* 1999a, Heard *et al.* 2000, Sanyal *et al.* 2002). Depending on the response element, ERRγ binds differentially and recruits different cofactors, thus exhibiting different

transcriptional activity (Sanyal *et al.* 2002). These studies demonstrated that the ERRα and ERRγ may recognize similar response elements, but subtle differences in the sequence of a composite response module such as the MHRE could elicit diverse responses by the two proteins and the protein complexes which they assembled. By EMSA, the *in vitro*-transcribed and -translated ERRα (data not shown) and ERRγ (Fig. 2) bind the MHRE in a similar fashion as a homodimer, in agreement with reports from other laboratories (Hentschke *et al.* 2002b, Huppunen & Aarnisalo 2004). However, the ERRγ transactivates the MHRE more effectively than the ERRα. In general, ERRα is not a strong activator by itself, it requires PGC-α or another ligand for activity (Vanacker *et al.* 1999a, Kamei *et al.* 2003, Mootha *et al.* 2004). On the other hand, ERRγ is possibly a constitutive activator and could function as a true orphan receptor, which is consistent with the crystal structure of its AF2 domain showing it to be in an active conformation in the absence of ligand (Hong *et al.* 1999, Greschik *et al.* 2002). It is reasonable to expect strong transactivation activity by ERRγ when it binds to the response element of the target gene. In a recent study,

we showed that the ERR α gene is estrogen responsive in mouse uterus and heart and the ER α -mediated transactivation is via the MHRE (Liu *et al.* 2003). All three AGGTCA motifs (at m1, m2 and m3 positions) in the mouse MHRE are equally important in estrogen response and mutation at any one site causes similar reduction of estrogen response. In this study, we found that the binding of ERR γ to the MHRE and stimulation of its activity by ERR γ and PGC-1 α requires the direct repeat at the m1 and m3 positions because mutations at the m2 had no effect on the binding of ERR γ and only mildly affected the transactivation activity in the transient reporter assay.

The ERR α and ERR γ and the coactivator PGC-1 α are coexpressed in adult tissues with a high mitochondrial content which utilize fatty acid oxidation as the primary energy source (Shi *et al.* 1997, Shigeta *et al.* 1997, Hong *et al.* 1999, Knutti *et al.* 2000, Huss *et al.* 2002, Ichida *et al.* 2002, Sanyal *et al.* 2002, Luo *et al.* 2003). The PGC-1 α and ERR α link in regulation of oxidative phosphorylation (Mootha *et al.* 2004) and biogenesis of mitochondria (Schreiber *et al.* 2004) has been recently established. It is not known whether ERR γ is directly involved in the energy balance program or if it functions indirectly by regulating ERR α gene expression. Our present study demonstrated that the ERR γ and PGC-1 α indeed cooperate to stimulate the transcriptional activity of the ERR α gene through the MHRE. This finding was further supported by an association of the ERR γ level and the activity of PGC-1 α in HEC-1B cells (Fig. 4). PGC-1 α coactivates all the nuclear receptors that were examined. Obviously, endogenous nuclear receptors other than ERR γ could influence the function of PGC-1 α and the strong activation of MHRE by PGC-1 α in HEC-1B cells could be the result of a functional synergy by several nuclear receptors including ERR γ . To understand more of the molecular mechanism of PGC-1 α -enhanced transactivation of ERR γ , we performed DNA pull-down assays. This experiment demonstrated that more ERR γ is bound to the MHRE in the presence of wild-type but not mutant PGC-1 α , an observation supported by an *in vivo* ChIP assay (Fig. 6). Whether PGC-1 α enhances or stabilizes binding of the ERR γ to MHRE is not clear; it is known that the receptor conformation can be modified and its activity modulated upon binding to the response element and by interaction with the coactivators (Klinge *et al.* 2001, Loven *et al.* 2001, Wood *et al.* 2001, Hall *et al.* 2002, Sanyal *et al.* 2002). ERR γ exhibits different coactivator recruitments and transcriptional activity, depending on the response element (Sanyal *et al.* 2004). Binding of ERR γ to the MHRE could change its conformation and increase interaction with PGC-1 α , thus forming a strong activation complex.

In summary, the present work demonstrates that ERR γ and PGC-1 α cooperate to stimulate ERR α gene

activity. The MHRE of the ERR α gene is the binding site for ERR γ . Furthermore PGC-1 α enhances ERR γ binding to the MHRE and increases its transactivational activity. The functional relationship of ERR γ , ERR α and PGC-1 α is emerging, and the coexpression pattern of both ERRs and PGC-1 α in metabolically active tissues suggests that ERR γ like ERR α could also be involved in adaptive thermogenesis.

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