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

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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 (ERRα/ NR3B1; ERRβ/NR3B2) was cloned 15 years ago (Giguere et al. 1988) as orphan receptors and a third member (ERRγ/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 ERRα 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, ERRγ 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. 2001 b). In addition, 4-hydroxytamoxifen binds ERRβ and ERRγ (Coward et al. 2001, Tremblay et al. 2001a) whereas micromolar concentrations of some pesticides bind ERRα (Yang & Chen 1999) and inhibit their transactivation function. Recently, a specific synthetic inverse agonist of ERRα was reported (Mootha et al. 2004).

The expression pattern of ERRα and ERRγ 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). ERRβ is primarily expressed in the embryo...
and the ERRβ-null mouse is embryonically lethal due to placental defects (Luo et al. 1997). The biological function of ERRγ is not clear, but the roles of ERRα in cellular physiology are emerging. ERRα was found to bind to a TCAAGGTGCA element in the human lactoferrin gene promoter and to modulate the estrogen response (Yang et al. 1996). An extensive analysis of ERRα 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, ERRα 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 ERRα 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). ERRα expression after fasting and cold treatment correlates with the expression of physiological stimuli-inducible peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC-1α) (Ichida et al. 2002, Schreiber et al. 2003). The mouse PGC-1α was initially identified in brown fat as a coactivator for PPARγ (Puigserver et al. 1998) and the human homologue was subsequently cloned (Knutti et al. 2000). PGC-1α 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-1α, ERRα was identified as a major interacting partner (Huss et al. 2002, Ichida et al. 2002). More recent evidence demonstrated that PGC-1α and ERRα 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 ERRα gene contains a multi-hormone response element (MHRE) that is a target site for ERα in the estrogen response (Liu et al. 2003). This region also binds ERRα itself and serves as autoregulatory site in the PGC-1α-induced response (Laganiere et al. 2004, Mootha et al. 2004). Since the DNA-binding domain of ERRγ and ERRα 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 ERRγ would bind and regulate ERRα gene expression. In this study, we demonstrated that ERRγ is a potent activator for ERRα gene expression, which is enhanced by PGC-1α. We show that PGC-1α enhances ERRγ binding to the MHRE, suggesting a potential mechanism for PGC-1α regulation of ERRα gene expression.

### Materials and methods

#### Plasmids

ERRγ, ERRγ449 (deletion of the activation function 2 (AF2) domain, AF2Δ), Myc-ERRγ, and glutathione S-transferase (GST)-ERRγ (Hentschke et al. 2002b) were gifts from U Borgmeyer (University of Hamburg, Germany), ERα and ERRβ (Liu et al. 2003), RXRα, RARα and TRα from A Jetten (National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA (NIEHS)), PPARα from C Weinberger (NIEHS), RORα, RORB and RORγ 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-1α and its mutant version, pCDNA3/HA hPGC-1α, 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 (Scrippi Research Institute, La Jolla, CA, USA) and D Kelly (Washington University School of Medicine, St Louis, MO, USA). The human ERRα 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 µg/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 µg/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
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 ERRγ and ERRα mRNA levels in HEC-1B cells under various experimental conditions. The primer pair is as follows: for human ERRγ, forward primer 5'-GCC CAT GAG AAC CTT TCT CGG TCA CGT-3' and the reverse primer 5'-GGC CTC ATC TGC CGA CAT GCC CAC TAC TGC AGA GCG T-3'. For human ERRα, forward primer 5'-GAC AGG ATG CAG AAG GAT CAC 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: ERRγ, 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); ERRα, 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-1α-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 µg/ml aprotinin and 1 µg/ml pepstatin A). Chromatin was sonicated to an average length of DNA of 200–1000 bp as verified by agarose 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 ERα antibody (mouse monoclonal; NeoMarkers, Fremont, CA, USA) were used as a negative (non-specific antibody) control since HEC-1B cells are ERα-negative (Hopfer et al. 1996). The endogenous ERRγ was detected by polyclonal rabbit ERRγ antiserum (a gift from U Borgmeyer). After addition of the antibodies (15 µl), 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 CCA GGA CAG CCC GCG AG-3' (−758/−734) and reverse primer 5'-GAT AGG GCC CCG 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'-GAG CCC TGG CAG TCT GGA TGG-3' (at +85 627) and reverse primer 5'-GCC CTC ATC TGC GGA 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-1α and ERRγ 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 \(^{32}\text{P}\)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\(\gamma\)-expressing plasmids were transformed into \(E.\) coli BL-21 cells and the expression of GST and GST-ERR\(\gamma\) fusion proteins was induced by isopropyl-1-thio-\(\beta\)-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\(\alpha\) and GST-ERR\(\gamma\) protein were incubated with the \textit{in vitro}-translated \(^{35}\text{S}\)-labeled PGC-1\(\alpha\) for 1 h at 4 °C. Binding of the PGC-1\(\alpha\) 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 µl streptavidin-agarose beads (Sigma, St Louis, MO, USA), the \textit{in vitro}-transcribed and -translated \(^{32}\text{S}\)-labeled ERR\(\gamma\) by itself or in combination with the \textit{in vitro}-transcribed and -translated unlabeled PGC-1\(\alpha\) or AAB-streptavidin complex beads. The AAB-bound ERR\(\gamma\) 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 ChemiImager (San Leandro, CA, USA).

**Quantitation of the PCR product or shifted bands in EMSA**

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

**Results**

**MHRE of the ERR\(\alpha\) promoter is a pleiotropic nuclear receptor enhancer**

We have previously shown that the ERR\(\alpha\) 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\(\alpha\) 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\(\alpha\) 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\(\alpha\) promoter is responsive to ligand-bound RXR\(\alpha\) and PPAR\(\alpha\) as either homodimer or heterodimer (Fig. 1B) while RAR\(\alpha\) and TR\(\alpha\) (with or without the presence of their respective ligand) have no effect (data not shown). Interestingly, the ERR\(\alpha\) and ERR\(\gamma\) 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-TF, ROR\(\alpha\), ROR\(\beta\) and ROR\(\gamma\), and found no significant changes under the current assay conditions (data not shown). Taken together, the ERR\(\alpha\) gene is positively regulated by its own gene product and the close family member ERR\(\gamma\). Since ERR\(\alpha\) and ERR\(\gamma\) are coexpressed in many tissues such as skeletal muscle, heart, kidney and pancreas, regulation of ERR\(\alpha\) gene expression in those tissues could be greatly influenced by the presence of ERR\(\gamma\).

**Binding properties of ERR\(\gamma\) to the MHRE**

The MHRE of the human and mouse ERR\(\alpha\) 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. The MHRE in the context of GC-rich ERR\(\alpha\) promoter is responsive to ligand-bound RXR\(\alpha\) and PPAR\(\alpha\) as either homodimer or heterodimer (Fig. 1B) while RAR\(\alpha\) and TR\(\alpha\) (with or without the presence of their respective ligand) have no effect (data not shown). Interestingly, the ERR\(\alpha\) and ERR\(\gamma\) 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-TF, ROR\(\alpha\), ROR\(\beta\) and ROR\(\gamma\), and found no significant changes under the current assay conditions (data not shown). Taken together, the ERR\(\alpha\) gene is positively regulated by its own gene product and the close family member ERR\(\gamma\). Since ERR\(\alpha\) and ERR\(\gamma\) are coexpressed in many tissues such as skeletal muscle, heart, kidney and pancreas, regulation of ERR\(\alpha\) gene expression in those tissues could be greatly influenced by the presence of ERR\(\gamma\).
Figure 1 The MHRE of the ERRα gene is a pleiotropic response element. (A) Diagrammatic presentation of the ERRα promoter-reporter constructs. Different lengths of ERRα 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 ERRα 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 RXRα and PPARα. 9-cis-retinoic acid (9 Cis) at 50 µM and 5,8,11,14-eicosatetraynoic acid (ETYA) at 20 µM. (C) Effect of ERRα and ERRγ. The experiments were repeated three times with duplicated samples. The values are presented as mean ± S.D.
Figure 2 ERRγ binds the MHRE of ERRα 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 ERRγ 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 ERRγ and myc-ERRγ to the AB probe. 32P-labeled probes are indicated. Arrow indicates the ERRγ-DNA complex. SS, super shifted band; γ, ERRγ antibody (Hentschke *et al.* 2002b); LF, lactoferrin antibody (Teng *et al.* 1986); PS, preimmune serum.
Transactivation function of ERγ and PGC-1α on the MHRE

The above test studies (Fig. 1) demonstrated that ERγ strongly transactivates MHRE in the context of its natural promoter. ERγ 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 ERγ’s function, wild-type or mutant MHRE-reporters were co-transfected with ERγ expression constructs into HEC-1B cells and the transactivation activities of these reporters were examined (Fig. 3A). ERγ 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 ERγ transactivation function, such as mutation of the ERRE at m3 position dramatically reduced the transactivation function of ERγ while a lesser effect as the same ERRE was mutated at the m1 position. The transactivation activity of ERγ was least affected by the mutation at m2, the ER core element. When all three sites were mutated (m4), the transactivation capability of ERγ was blocked. Taken together, the data were consistent with the ERγ binding characteristics and showed a correlation between the ability of ERγ to bind and to transactivate the MHRE.

Recently, expression of ERRα in liver and heart was found increased following the induction of PGC-1α (Huss et al. 2002, Ichida et al. 2002, Schreiber et al. 2004). The concerted expression of PGC-1α and ERRα suggests that ERRα is a downstream target of PGC-1α and may be involved in the energy metabolism program. Ectopic expression of the PGC-1α 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 ERγ binding and activation of the MHRE (compare Fig. 2B and Fig. 3A to Fig. 3B).

PGC-1α enhances ERγ transactivation of the MHRE

PGC-1α does not have a typical DNA-binding domain (Puigserver et al. 1998, Knutti et al. 2000); through protein–protein interaction, PGC-1α coactivates a number of nuclear receptors including the ERRα and ERγ (Huss et al. 2002, Schreiber et al. 2003). Therefore, the strong activation of the MHRE-reporters by PGC-1α 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-1α-induced transactivation activity of the MHRE-reporter; especially PGC-1α 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 ERRα gene by the ERγ (Figs 1 and 2) suggested that the ERγ could play a role in PGC-1α-induced reporter activities in HEC-1B cell.

To examine the expression pattern of ERRα and ERγ 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/PRF5 (liver) cell lines (Fig. 4A). ERRα is ubiquitously expressed and the level of expression is comparable in these cell lines (top panel). In contrast, ERγ is selectively expressed with a wide range of expression levels (middle panel). For example, a high level of ERγ was detected in human HEC-1B cells (lane 1) while in the PLC/PRF5 cells (lane 7) it was barely detectable. The ERγ in MCF-7 (lane 4), HEK293 (lane 5) and HepG2 cells (lane 6) was measurable but at a low level. The high level of ERγ in the HEC-1B cells may be important in assessing the MHRE-reporter activity by PGC-1α in transient co-transfection experiments. This cell line could also serve as a model to study the molecular mechanism of ERγ action. To examine

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 ERγ 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 ERγ binding because the mutant oligos (lanes 8 and 19) compete as well as the wild-type oligos (compare to AAAB and AB) in the EMSA. As expected, mutations of all three sites (m4) eliminated the ERγ binding and no competition was found (scan on top, lane 10). These data suggested that ERγ binds to MHRE specifically and preferably to the two ERREs. Binding of ERγ as a homodimer complex with multiple synthetic EREs was shown (Hentschke et al. 2002b, Hupponen & Aarnisalo 2004). Whether ERγ also binds the MHRE of the ERRα gene as homodimer was examined. We transcribed and translated different lengths of ERγ 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-ERγ-DNA complex moved more slowly than the ERγ-DNA complex (compare lanes 2 and 7). When different ratios of ERγ and myc-ERγ expression plasmids were co-transcribed and translated in vitro, a new protein-DNA complex appeared at the intermediate position formed from the heterodimerization of ERγ and myc-ERγ (lanes 3–6). These data are in agreement with the binding study of ERγ from other laboratories (Hentschke et al. 2002b, Hupponen & Aarnisalo 2004).
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 ERRγ in HEC-1B cells affects the transactivation function of PGC-1α, we applied the siRNA technique to reduce the endogenous ERRγ level. After introducing the ERRγ siRNA duplexes into HEC-1B cells for 48 h, the endogenous mRNA levels of both ERRγ and ERRα were significantly reduced as measured by real-time PCR (Fig. 4B, left panel), and the transactivation activity of PGC-1α on MHREs reporter was also reduced (right panel). The control siRNA affected neither the levels of ERRs mRNA nor the transactivation function of PGC-1α. In an opposite experiment, when both PGC-1α and ERRγ were expressed ectopically, the transcriptional activity of the MHRE-reporters was higher than either one alone (Fig. 4C). These experiments demonstrated that ERRγ stimulates the activity of MHRE-reporters and PGC-1α augments its effect. Taken together, PGC-1α enhances but is not required for the ERRγ to transactivate ERRα gene through MHRE.

**Mechanism of PGC-1α and ERRγ 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-1α coactivates the ERRγ 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 ERRα gene. To investigate the functional relationship of ERRγ AF2 domain and PGC-1α LXXLL motifs on the MHRE, we ectopically expressed the ERRγ AF2 deletion mutant (AF2Δ) (Hentschke et al. 2002a) and PGC-1α 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 ERRγ AF2Δ severely reduced the MHRE-reporter activity and the activity stimulated by PGC-1α suggests that the mutant ERRγ acts as a dominant negative receptor (Fig. 5A). The requirement for the three LXXLL motifs (L1, L2 and L3) of the PGC-1α 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-1α 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-1α could coactivate the ERRγ through multiple ways. In this study, we asked whether the binding of ERRγ to the MHRE is affected by PGC-1α (Fig. 6). The in vitro-labeled 35S-PGC-1α interacted strongly with the GST-ERRγ (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-1α and ERRγ. The result agrees with reports from other laboratories that PGC-1α physically interacts with ERRγ. Interaction of ERRγ and PGC-1α 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 ERRγ to the MHRE was assessed in the presence or absence of in vitro-translated PGC-1α (Fig. 6B). The translation condition that produced similar amount of wild-type and mutant PGC-1α 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-1α) was then mixed with the labeled ERRγ. The total protein in every experiment was kept constant. Interestingly, interaction of 35S-ERRγ with biotin-labeled MHRE increased in the presence of PGC-1α, and the amount of 35S-ERRγ bound to the biotin-MHRE was proportional to the concentration of PGC-1α in protein mixture (Fig. 6B). There was no binding in the absence of biotin-DNA. In addition, mutant PGC-1α did not enhance the interaction between ERRγ and the MHRE (Fig. 6B, bottom, L2 L3). This in vitro study was further investigated by the in vivo ChIP assay (Fig. 6C). PGC-1α or empty vector were transfected into HEC-1B cells for 24 h and the endogenous ERRγ that was cross-linked to the MHRE region by formaldehyde treatment was immunoprecipitated by ERRγ antibody. The amount of ERRγ that binds MHRE region was increased 4-fold in cells expressing PGC-1α (Fig. 6C, top), and with no binding detected in the unrelated region (Fig. 6C, bottom). ERRα antibody was used as a negative control, since no ERRα 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-1α increases ERRγ binding to the MHRE, thus providing a mechanism for PGC-1α amplifying the ERRγ transactivation function on the MHRE of the ERRα gene.

**Discussion**

The promoter of the ERRα 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 ERα (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 ERα but not to ERβ 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
element (sequence identical to ERRE) by ERα and ERRα but not by ERβ (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 RXRα and PPARα 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-1α coactivates the ERRγ transactivation function. (A) ERRα and ERRγ 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 ERRα, ERRγ 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 ERRγ mRNA level influences the expression of ERRα and the activation capability of PGC-1α on MHRE. Left panel, the ERRγ siRNA duplex was transfected into HEC-1B cells for 48 h and the endogenous ERRγ and ERRα 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 ERRγ mRNA level from the control siRNA plasmids transfected cells was set as 1. The results are means±S.D. Right panel, the HEC-1B cells were transfected with ERRγ siRNA for 24 h, followed with the transfection of PGC-1α expression constructs (25 ng) and the AAB-TATA-Luc reporters (300 ng). (C) PGC-1α enhances the transactivation activity of ERRγ. PGC-1α (25 ng) and ERRγ (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±S.D.

Figure 5 AF2 of the ERRγ and LXXLL motifs of the PGC-1α are required in functional interaction. (A) Effect of ERRγ AF2 deletion. The 0.8-CAT reporters were co-transfected with expression constructs of the ERRγ wild-type or AF2-deleted (AF2Δ) with or without the PGC-1α. 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±S.D. The activity from the wild-type PGC-1α was set as 100%. (B) Effect of PGC-1α LXXLL motif(s) mutation(s). The AAB-TATA-Luc reporter were co-transfected with expression constructs of the PGC-1α wild-type or LXXLL motif mutated (L2A, L3A and L2A/L3A) with or without the ERRγ. 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±S.D. The activity from the wild-type PGC-1α was set as 100%.
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 (33%). 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 transcripational 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 ERRα gene is estrogen responsive in mouse uterus and heart and the ERα-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 ERRγ to the MHRE and stimulation of its activity by ERRγ and PGC-1α requires the direct repeat at the m1 and m3 positions because mutations at the m2 had no effect on the binding of ERRγ and only mildly affected the transactivation activity in the transient reporter assay.

The ERRα and ERRγ and the coactivator PGC-1α 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-1α and ERRα 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 ERRγ is directly involved in the energy balance program or if it functions indirectly by regulating ERRα gene expression. Our present study demonstrated that the ERRγ and PGC-1α indeed cooperate to stimulate the transactivation activity of the ERRα gene through the MHRE. This finding was further supported by an association of the ERRγ level and the activity of PGC-1α in HEC-1B cells (Fig. 4). PGC-1α coactivates all the nuclear receptors that were examined. Obviously, endogenous nuclear receptors other than ERRγ could influence the function of PGC-1α and the strong activation of MHRE by PGC-1α in HEC-1B cells could be the result of a functional synergy by several nuclear receptors including ERRγ. To understand more of the molecular mechanism of PGC-1α-enhanced transactivation of ERRγ, we performed DNA pull-down assays. This experiment demonstrated that more ERRγ is bound to the MHRE in the presence of wild-type but not mutant PGC-1α, an observation supported by an in vivo ChIP assay (Fig. 6). Whether PGC-1α enhances or stabilizes binding of the ERRγ 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). ERRγ exhibits different coactivator recruitments and transcriptional activity, depending on the response element (Sanyal et al. 2004). Binding of ERRγ to the MHRE could change its conformation and increase interaction with PGC-1α, thus forming a strong activation complex.

In summary, the present work demonstrates that ERRγ and PGC-1α cooperate to stimulate ERRα gene activity. The MHRE of the ERRα gene is the binding site for ERRγ. Furthermore PGC-1α enhances ERRγ binding to the MHRE and increases its transactivation activity. The functional relationship of ERRγ, ERRα and PGC-1α is emerging, and the coexpression pattern of both ERRs and PGC-1α in metabolically active tissues suggests that ERRγ like ERRα could also be involved in adaptive thermogenesis.

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