Differential Regulation of the Orphan Nuclear Receptor Small Heterodimer Partner (SHP) Gene Promoter by Orphan Nuclear Receptor ERR Isoforms*

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The orphan nuclear receptor small heterodimer partner (SHP; NROB2) interacts with a wide array of nuclear receptors and represses their transcriptional activity. SHP expression is regulated by several other members of the nuclear receptor superfamily, including the orphan receptors SF-1 and LRH-1, and the bile acid receptor FXR. We have found that the SHP promoter is also activated by the estrogen receptor-related receptor γ (ERRγ) but not the related ERRα and ERRβ isoforms. SHP and ERRγ mRNAs are coexpressed in several tissues, including pancreas, kidney, and heart, confirming the potential relevance of this transactivation. ERRγ transactivation is dependent on only one of five previously characterized DNA-binding sites for SF-1, and this element differs from previously reported ERR response elements. However, treatment with the histone deacetylase inhibitor trichostatin A significantly increased SHP activity on this element indicating that the lack of activity of ERRα and -β may depend on their association with co-repressor in vivo. Furthermore, using protease sensitivity assays on DNA bound receptors it was demonstrated that DNA sequence of different response elements may cause allosteric modulation of ERR proteins, which in turn may be responsible for the differential activities of these receptors on different response elements. SHP inhibits ERRγ transactivation and physically interacts with all three members of ERR subfamily, as demonstrated by both yeast two-hybrid and biochemical assays. As with other SHP targets, this interaction is dependent on the AF-2 coactivator-binding site of ERRγ and the previously described N-terminal receptor interaction domain of SHP. Several recently described SHP mutations associated with moderate obesity in humans block the inhibition of ERRγ activity. Overall, these results identify a new autoregulatory loop controlling SHP gene expression and significantly extend the potential functional roles of the three ERRs.

The orphan nuclear receptor superfamily is a diverse group of transcription factors that includes both conventional receptors with known ligands and orphan nuclear receptors that lack them (for reviews, see Refs. 1 and 2). Both conventional and orphan nuclear receptors share a very similar structure. At the N terminus is a domain that is not conserved in sequence in different receptor families, but in many cases contains a ligand independent transcriptional activation domain termed activation function 1. The central DNA-binding domain (DBD) is strongly conserved and includes two zinc-binding units based on invariant cysteine residues. A conserved helical region within the DBD termed the P box (3) makes base specific contacts with hormone response elements. The sequence of this motif serves as one of the main criteria for dividing the nuclear receptor superfamily into subgroups. The C-terminal ligand-binding domain (LBD) functions not only in ligand binding, but also dimerization and ligand mediated transcriptional activation. This transactivation is based on an additional activation function referred to as AF-2. In most receptors, allosteric effects of ligand binding result in the formation of an appropriate binding site for a series of transcriptional coactivators. In a number of orphans, however, coactivator binding is not dependent on the presence of a ligand. Members of this subset of orphans, which includes HNF-4, and ERRγ, function as constitutive transactivators (4, 5).

SHP is an atypical member of nuclear receptor superfamily that lacks a DBD. Various studies have reported SHP to be a repressor of transcriptional activities of a number of nuclear receptors, including both ligand responsive receptors like, ER, TR, RAR, and RXR, and orphan receptors like CAR, HNF-4, and FTF (6–10). The very broad range of receptors sensitive to inhibition by SHP suggests a central role for SHP in modulation of nuclear receptor signaling pathways. Although the mechanisms underlying this repressive function remain unclear, recent results (7, 11) demonstrate that SHP can compete with coactivators for binding to the AF-2 surface. In addition, a direct transcriptional repressor domain contributes significantly to the inhibitory function of SHP. The SHP gene is structurally conserved in all the species from which it has been characterized. It consists of 2 exons

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The abbreviations used are: DBD, DNA-binding domain; SHP, small heterodimer partner; ERR, estrogen receptor-related receptor; TR, thyroid hormone receptor, GR, glucocorticoid receptor; ERRE, ERR response element; SF-1, steroidogenic factor 1; LRH1, liver receptor homolog 1; SF-1RE, SF-1 response element; ER, estrogen response element; TRE-Pal, palindromic thyroid hormone response element; GRIP1, glucocorticoid receptor interacting protein 1; TSA, trichostatin A; LBD, ligand-binding domain; ER, estrogen receptor; GST, glutathione S-transferase; HA, hemagglutinin.
interrupted by an intron and is located on human chromosome 1 at position 1p36.1 (12). Initial studies indicated that SHP is expressed predominantly in the liver, spleen, small intestine, and pancreas (8, 12). However, later studies with more sensitive approaches have demonstrated the presence of SHP mRNA in a wide variety of tissues, with highest expression in heart, brain, liver, lung, and adrenal gland (11). Although very little is known about SHP gene regulation, some recent reports have suggested important roles for the bile acid receptor FXR, and AP-1 transcription factors in regulating SHP gene expression and subsequent regulation of cholesterol homeostasis (13–15). A recent report also demonstrated the activation of the SHP promoter by SF-1 and its close relative LRH-1, both of which bind five distinct sites present in the region spanning from 453 to 68 base pairs upstream of the transcriptional start (16). SHP is coexpressed with SF-1 or LRH-1 only in gonads, adrenal gland, and liver, however, indicating that other transcription factors are involved in SHP gene expression in other tissues, such as pancreas, heart, and brain.

The ERR subfamily includes the first orphan receptors described (17). These proteins form a distinct subgroup within the superfamily with striking sequence similarity in the DBD with estrogen receptors (ER). The similarity in the LBD is more limited, and the ERs do not bind natural estrogen. However, a recent report has demonstrated the binding of the synthetic estrogen analogue diethylstilbestrol to the ERR subfamily members (18). In this case, diethylstilbestrol acts as an inverse agonist by disrupting ERR-coactivator interaction (18). Three different ERR genes have been characterized (5, 17, 19, 20). The ERα, ERβ, and ERRγ isoforms show considerable homology among themselves, with the highest similarity between ERRβ and ERRγ. The hypervariable N-terminal A/B domain of ERRβ shares 58.2% amino acid identity with ERRγ, and in the DBD and the LBD they share 98.9 and 73.1% identity, respectively (5). All three isoforms have been reported to bind and transactivate both estrogen response elements (ERE) and SF-1 response elements (SF-1RE) (5, 20–27). ERα has been implicated in various roles in bone and muscle development, modulation of aromatase expression in breast tissues, regulation of thyroid hormone receptor α (TRα) expression, overall development of mouse embryos, and fatty acid metabolism (21, 23, 28–30). Inactivation of the murine ERRγ gene caused severe placental abnormalities and embryonic mortality at 10.5 days post-coitum, and ERRγ has also been implicated in repression of glucocorticoid receptor-mediated transcriptional activities (31, 32). The biological roles of ERRγ, the newest member of the subfamily, remain to be elucidated. The expression profiles of the three ERR isoforms show an overlapping pattern. ERRα mRNA is primarily expressed in tissues associated with fatty acid metabolism such as kidney, heart, and brown adipocytes. ERRγ is expressed at a high level in heart, brain, kidney, pancreas, and at a lower level in liver (5, 20, 26), while the expression of its closer relative ERRβ is barely detectable in few tissues in adult rat including kidney, testis, heart, brain, and prostate. In the mouse ERRβ expression has been demonstrated in a subset of extraembryonic tissues in a small window between 5.5 days post-coitum and 8.5 days post-coitum (17).

Since the SHP gene promoter contains several SF-1 response elements that are predicted to be ERR response elements, we investigated whether ERR subfamily members can regulate the SHP gene promoter. Unexpectedly, transient transfection studies demonstrated a preferential effect of ERRγ on this promoter. Mapping studies revealed that only one of the five previously reported SF-1REs on SHP gene promoter is responsible for the ERRγ-mediated activation of the SHP promoter. Electrophoretic mobility shift assays, and mutational studies confirmed the binding of ERRγ to this element. As expected, SHP coexpression inhibits ERRγ transactivation of its own promoter, and SHP competes with the coactivator GRIP-1/SRC-2 for binding to ERRγ. We conclude that ERRγ is a component of a new potential autoregulatory loop controlling SHP gene expression.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pcMX ERRα and pcMX ERRβ were kind gifts from Dr. Vincent Giguerre. pSG5A ERR3, pSG5A GRIP1, and ERR2 Luc were kind gifts from Dr. Michael R. Stalleur (to avoid confusion with the nomenclature, ERR3 will be indicated by ERRγ in this report henceforth). Five copy SF-1RE Luc was a kind gift from Dr. Jean Mark Vanacker. B42AD fused yeast expression constructs of ERRα and ERRβ were made by inserting PCR products encoding the open reading frame of ERRα or ERRβ containing a 5’ EcoRI and a 3’ Xhol sites into pJG4-5 (CLONTECH). B42AD fused construct of ERRγ was constructed by inserting a PCR product encoding the complete open reading frame of ERRγ with flanking Xhol sites into pJG4-5. LexA DNA-binding domain fused ERRγ LB was created by inserting a PCR product encoding the ligand-binding domain in the appropriate restriction sites in a pEG202 vector (33). For bacterial expression, GST-fused full-length ERRα and ERRβ were constructed by inserting EcoRI-Xhol fragments of ERRα or ERRβ from B42 ERRα or B42 ERRβ in-frame into pGEX4T-1 vector (Amersham Bioscience). GST-fused full-length ERRγ was constructed by inserting a Xhol fragment encoding complete open reading frame from B42ERRγ in-frame into pGEX-4T-1. GST fused ERRα-, β-, and γ-ligand-binding domains were created by inserting PCR products into appropriate restriction sites in pGEX-4T-1 vector. To put all the ERR isoforms into same mammalian expression vector pcDNA3ERRα, pcDNA3ERRβ, and pcDNA3ERRγ were constructed by inserting fragments from B42 constructs of ERRα, ERRβ, and ERRγ as described elsewhere (35). Yeast expression constructs of naturally occurring SHP mutants were created by PCR-based site-directed mutagenesis from pcDNA3ERRγ using a proofreading Vent polymerase (New England Biolabs), the resulting mutant PCR product was confirmed by restriction digest as well as by sequencing and cloned into pCdNA3 at HindIII-EcoRV sites. ERRγ-DAP2 construct was created by PCR from pcDNA3 ERRγ and cloned into pcDNA3, the pcDNA3 ERRγ DAP2 was then digested with HindIII and NotI and the resulting fragment was cloned in-frame into pYesTRP2 (Invitrogen), a B42 yeast vector. SHP mutants are described elsewhere (35). Yeast expression constructs of naturally occurring SHP mutants were created by inserting respective EcoRI-Xhol fragments into pEG202 (33). pcDNA3 HA ERRα and ERRβ were constructed by subcloning EcoRI-Xhol fragments from respective B42 constructs in-frame into a pcDNA3 vector containing an HA coding sequence. Three-copy sf4 reporter element was created by adding ligase ligation at BamHI and BgII sites followed by cloning into BgII site of pGL2 promoter vector (Promega). All the clones were verified by sequencing.

**In Vitro Translation**—SHP or ERRγ S-N cDNAs in pcDNA3 were transcribed and translated in vitro by using a coupled rabbit reticulocyte system (TNT, Promega) in the presence or absence of [35S]methionine (Amersham Bioscience, Inc.) according to the manufacturer’s instructions.

**GST Pull-down Assay**—A GST pull-down assay was performed according to the method described previously (10). Briefly the GST fusion proteins or GST protein only were expressed in Escherichia coli BL21(DE3) pLys bacterial culture and recovered on glutathione-Sepharose 4B beads (Amersham Bioscience). The GST fusion proteins bound to glutathione-Sepharose 4B beads were incubated in a 100-μl reaction for 2 h at 4 °C with 35S-labeled receptors expressed by coupled in vitro transcription and translation (TNT, Promega). Specifically bound proteins were eluted from beads with 15 μM reduced glutathione in 50 mM Tris (pH 8.0) and analyzed by SDS-polyacrylamide gel electrophoresis and visualized by a PhosphorImager analyzer (Bas1500, Fuji).

**Yeast Two-hybrid Interaction Assay**—Yeast two-hybrid interaction assays were performed as described elsewhere (10). Briefly LexA fused full-length murine SHP, or deletions (Fig. 8A) (7) and B42 ERRα, β-, γ-, or ERRγ were transformed into Saccharomyces cerevisiae dAF-2 strain containing the β-galactosidase reporter plasmid 8H18-34, and the transformants were selected on plates with appropriate selection markers. The β-galactosidase assay on plates was carried out as described elsewhere (10).

**Gel Mobility Shift Assays**—Bacterially expressed GST-fused ERRα, ERRβ, and ERRγ, were purified using GST-Sepharose beads (Amersham Bioscience).
Fig. 1. ERRγ activates SHP gene promoter. HeLa (A), CV-1 (B), and HEK293 (C) cells were co-transfected with 100 ng of 2.2-kb mouse SHP gene promoter fused with a luciferase reporter (−2.2SHP-Luc) with indicated doses of ERRα, ERRγ, or ERRγ. D, CV-1 cells were co-transfected with 100 ng of ∼2.2-kb human SHP gene promoter with 500 ng of ERRα, ERRγ, or ERRγ. F, HeLa cells were co-transfected with 100 ng of ERE II Luc, and 500 ng of ERRα, ERRγ, or ERRγ. Approximately 40 h after transfection the cells were harvested, the luciferase activity was measured and normalized against β-galactosidase activity. One representative experiment is shown. All values represent the mean of duplicate samples, and similar results were obtained in at least three independent experiments. RLU indicates normalized relative luciferase activity. E, HA-tagged ERR isoforms were isofomed and expressed in HeLa cells, 48 h later, the cell lysates were prepared and 20 μg of protein from each lysate were analyzed by anti-HA monoclonal IgG, in the Western blot analysis. Numbers on the left indicate molecular mass in kilodaltons (kDa).

Results

Activation of the SHP Gene Promoter by ERRγ, but Not ERRα or ERRβ—Since a previous report demonstrated that SF-1 activates the SHP gene promoter via five characterized SF-1REs (16) and SF-1 and ERR family members share similar DNA binding characteristics (23, 34), we investigated whether ERR family members can also regulate the SHP gene promoter. Transient transfection studies using ∼2 kb pairs of either mouse or human SHP promoter sequence fused to a luciferase reporter demonstrated a strong activation of SHP gene promoter by ERRγ, whereas ERRα and ERRβ did not show a significant effect. ERRγ showed a strong transactivation of SHP promoter in both HeLa (Fig. 1A) and CV-1 (Fig. 1B) cells. However, ERRγ showed a moderate activity in HEK-293 cells, inducing the promoter by 5-fold at the highest dose of receptor DNA (Fig. 1C). Unexpectedly SF-1, the positive control for the transfection assays, failed to activate the SHP gene promoter in vitro, using a coupled rabbit reticulocyte system. Gel mobility shift assays (20 μl) contained 10 mM Tris (pH 8.0), 40 mM KC1, 0.05% Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, and 1 μg of poly(dI-dC). Either 1 μg of GST purified proteins, or 25 μl of programmed/mock-programmed reticulocyte lysates were used in each reaction. Competitor oligonucleotides were included at a 10–250-fold molar excess as indicated in the figure legends. After 10 min incubation on ice, 10,000 cpm of end labeled oligonucleotide probes were added and the incubation continued for another 10 min. DNA-protein complexes were analyzed on 5% polyacrylamide gel in 1× TBE (1× TBE = 90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were dried and analyzed by autoradiography. The sequences of the oligonucleotides used are described elsewhere (16).

Limited Proteolysis Analysis by Gel Shift Assay—Protease sensitivity assays were performed as described earlier (37) with minor modifications. Briefly, 5,000 cpm of end labeled oligonucleotides corresponding to sft4, (GATCCACATGACTTCTGGAGTCAAGGTCATTT-GGA) or consensus SF-1RE (GATCCACATGACTTCTGGAGTCAAGGTCATTT-GGA) were incubated on ice with GST purified ERRγ in DNA-binding buffer in a reaction volume of 10 μl. After 20 min incubation on ice, 0, 1, 2.5, 5, 10, or 50 ng of proteinase K (Promega) was added to the binding reaction and the reaction was incubated for a further 15 min at room temperature. The reactions were stopped by mixing 10 μl of 1× DNA binding buffer and putting the samples on ice. The samples were then immediately subjected to electrophoresis through a 8% nondenaturing polyacrylamide gel, and analyzed as described in the case of gel mobility shift assays.

Limited Proteolysis Analysis by SDS-PAGE—Six μl of in vitro transcribed and translated ERRγ was incubated with 40 pmol of sft4 or SF-1RE oligonucleotides in the presence of DNA binding buffer in a reaction volume of 10 μl. After incubation on ice for 20 min, indicated amounts of proteinase K was added, and the incubation was continued for a further 15 min at room temperature. The reactions were stopped by mixing with equal volume of 2× SDS loading buffer, heated at 95 °C for 5 min, and subjected to electrophoresis on a 12% denaturing polyacrylamide gel. The gels were then dried and analyzed by autoradiography.

Transient Transfection Assays—HeLa (Human cervical carcinoma), CV-1 (green monkey kidney), and HEK 293 (human embryonic kidney) cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) in the presence of 10% fetal bovine serum (Invitrogen). For Luciferase assays, 24 h before transfection cells were plated in 24-well plates and transfected, transfections were carried out with Superfect reagent (Qiagen) according to the manufacturer’s instructions. Total DNA used in each transfection was adjusted to 1 μg by adding appropriate amount of pcDNA3 vector. Approximately 48 h post-transfection, cells were harvested, and the luciferase activity was measured as described (33) and normalized against β-galactosidase activity.

Western Blot Analysis—HA-tagged ERRα, β, or γ (5 μg) were transfected into HeLa cells, in 10-cm dishes using Superfect reagent. The cell lysates were prepared 48 h after transfection. 20 μg of protein from each cell lysates were loaded and separated on a 12% denaturing polyacrylamide gel. The proteins were blotted onto the Hybond-C extra nylon membranes (Amersham Bioscience, Inc.), and visualized with monoclonal HA antibody (Roche Molecular Biochemicals) and ECL detection kit (Amersham Bioscience, Inc.).
promoter in CV-1 cells, although it did so in HeLa and HEK-293 cells (Fig. 1, A and C). It is possible that CV-1 cells lack a specific signaling pathway or coactivator required for SF-1 activity. Consistent with the ERRγ-dependent response of the mouse promoter, ERRγ also substantially activated the human SHP gene promoter (Fig. 1D), demonstrating the conservation of ERRγ responsiveness.

To ensure that all the ERR constructs were effectively expressed, Western blot analysis was performed with whole cell extracts from HeLa cells transfected with the HA epitope-tagged ERR isoforms. As demonstrated in Fig. 1E, comparable levels of the three ERR isoforms were expressed, indicating that their relative expression levels were not responsible for their differential activity on SHP promoter. To further ensure that all the ERR constructs are transcriptionally active, HeLa cells were transfected with a two-copy estrogen response element-driven reporter element (ERE II Luc) (Fig. 1F) along with the ERR expression vectors. In agreement with other observations (18) ERRα showed a modest activity on an estrogen response element-driven reporter (ERE-II-Luc) (Fig. 1F) along with the ERR expression vectors. In agreement with other observations (18) ERRα showed a modest activity on an estrogen response element-driven reporter (ERE-II-Luc), whereas ERRβ and γ showed a stronger activity. These results indicate that the differences in the activity of the ERR isoforms exhibited on SHP promoter are not dependent on their relative expression, but may depend on the sequences of respective DNA response elements.

The DBDs of ERRβ and ERRγ differ at only a single residue. To determine whether this variation is involved in the differential response observed, an ERRγ construct (ERRγ S-N) in which this serine residue was converted to the asparagine residue of ERRβ was generated. Transient transfection assays demonstrated ERRγ S-N still retained the capacity to activate SHP promoter (data not shown). Thus, the lack of activity of ERRβ must be dependent on sequences outside the DBD. This could be due to a lack of transactivation function or to indirect effects of other domains on DNA binding or interaction with other factors. Taken together, these results demonstrate that ERRγ specifically induces SHP promoter.

Identification of the Sequences Responsible for ERRγ Response on SHP Gene Promoter—To determine the sequences required for ERRγ mediated activation, a previously described series of 5′ deletions of the mouse SHP promoter were used. ERRγ responsiveness was retained with a deletion retaining 139 bp upstream of the putative transcriptional start site, but was lost with the construct containing only 68 bp upstream of the putative transcriptional start point (Fig. 2A). Thus, sequences required for ERRγ response lie in the region between −139 and −68, which contains a single SF-1 responsive site previously designated sft4 (16).

To specifically identify whether the ERRγ response requires sft4, a series of mutant constructs containing individual GG to TT double substitution mutations in the core of the five SF-1-binding sites in the −0.453 promoter (17) were tested for ERRγ activity (Fig. 2B). As shown in Fig. 2B, the sft4 mutation completely abolished ERRγ response, whereas mutations of other SF-1-binding sites did not significantly alter response, indicating that sft4 is the only one of these sites required for ERRγ response.

To further clarify whether ERRγ specifically binds sft4, a series of gel mobility shift assays were conducted using oligonucleotides for all the five SF-1REs as probes. As demonstrated in Fig. 3A, both ERRγ and ERRγ S-N formed specific complexes with sft4 (lanes 2, 5, and 12) and a 250-fold molar excess of unlabeled sft4 oligonucleotide successfully competed with the DNA-protein complex (lanes 3, 9, and 13). As expected a 250-fold molar excess of msft4 did not affect the DNA-protein interaction (lanes 4 and 14). To identify whether ERRγ binds to other sft sequences, a series of competition assays were performed with 250-fold molar excess of unlabeled sft sequences. Excess unlabeled oligonucleotides corresponding to sft1, −2, −3, and −5 had no effect on ERRγ-sft4 interaction (lanes 6, 9, and 10, respectively). Direct binding assays using labeled sft1, −2, −3, and −5 confirmed that ERRγ forms specific DNA-protein complex with sft4 only (data not shown). Since all the ERR isoforms exhibit a highly homologous DBD sequence we examined whether ERRα and ERRβ also bind to sft4 sequence, and whether their apparent lack of activity on SHP promoter is a result of different binding affinity of these two proteins on sft4 sequence. Surprisingly, both ERRα and ERRβ formed specific complex with sft4 sequence and their affinity for sft4 sequence was similar to ERRγ (Fig. 3B). In all the cases at least
100-fold molar excess of unlabeled sft4 was required to successfully compete with the DNA-protein complex (compare lanes 6, 12, and 18) whereas purified GST protein alone did not form any complex with the probes (lanes 1 and 14). Intriguingly, ERRα and ERRβ did not bind to other sft sequences as evidenced by both direct binding and competition analysis (data not shown). In agreement with a previous report (20), all three isoforms produced two specific complexes. We assume the upper complex may be a result of dimerization between two ERR molecules, and both yeast two-hybrid and biochemical interaction assays indicated that all the ERR family members can form homodimeric complexes.2

To specifically determine whether the sft4 element by itself is sufficient to confer complete ERRγ activity, a reporter construct containing three copies of the sft4 element cloned upstream of a luciferase gene was transiently transfected with ERR isoforms in CV-1 and HeLa cells. As expected, ERRγ

2 S. Sanyal and H-S. Choi, unpublished observation.

Fig. 3. All the ERR isoforms demonstrate specific DNA-protein complex with sft4 only. A, purified GST-ERRγ or in vitro transcribed and translated ERRγ-S-N, was incubated with end labeled sft4 as indicated. In competition assays 250-fold molar excess of unlabeled wild type or mutant sft sequences were used as indicated. B, GST-ERRα, GST-ERRβ, or GST-ERRγ were incubated with end labeled sft4 as indicated in the figure. GST, purified GST protein only, 5, 10, 50, 100, and 250 indicate the -fold of molar excess of unlabeled specific competitors. Msft4 indicates 250-fold molar excess of mutant sft4 (msft4). Lys, mock programmed reticulocyte lysate. C, HeLa and CV-1 cells were co-transfected with a three-copy sft4 luciferase and 500 ng of ERRα, ERRβ, or ERRγ. The luciferase activity was measured and normalized against β-galactosidase activity. One representative experiment is shown, and similar results were obtained from at least three independent experiments. D, a comparison of human, mouse, and rat SHP promoter sequences, the sft4 motif is in bold letters. Numbers indicate corresponding nucleic acid positions.
substantially induced this reporter whereas, both ERRα and ERRβ failed to do so (Fig. 3C).

Taken together, we conclude ERRγ confers its response solely through sfr4. The high degree of sequence identity of the sfr4 site and surrounding sequences in mouse, rat, and human SHP promoters (Fig. 3D) indicates that this ERRγ response is conserved.

Potential Mechanism for the Differential Activation of SHP Promoter by ERR Isoforms—Differential corepressor recruitment is one mechanism that could explain why all the three ERR isoforms bind the sfr4 DNA element with comparable affinity but only ERRγ activates it significantly. The effects of corepressors can be blocked by TSA, a selective inhibitor of histone deacetylases as well as a disruptor of histone deacetylase corepressors. Thus, the observation that TSA treatment significantly increased ERRα and ERRβ activity on sfr4, but failed to potentiate ERRγ activity (Fig. 4A), is consistent with the possibility that ERRα and ERRβ but not ERRγ, associate with corepressors in vivo.

Since ERRγ can bind to a consensus SF-1RE (20, 29) we tried to determine whether ERRγ can also transactivate other gene promoters containing such sites. Unexpectedly, ERRγ demonstrated no significant activity on TRα gene promoter (data not shown), which contains a consensus SF-1RE (23) that is reported to be a bona fide target of ERRα (23) and binds ERRγ efficiently (Ref. 20, and see below). To determine whether these discrepancies in ERRγ mediated activities on different promoters depend on the sequence of the available binding sites, a comparison of ERRγ activity on the tandem repeats of such binding sites was executed. Interestingly, ERRγ demonstrated distinctly different activities on these elements. Thus, although ERRγ activated a three-copy sfr4-Luc in HeLa and CV-1 cells by 12–15-fold and 35–40-fold, respectively, it activated the five-copy SF-1 RE Luc by 2–3-fold in HeLa and 4–5-fold in CV-1 cells (Fig. 4B).

These results indicate that binding of ERRγ to different DNA elements may induce specific allosteric modulations of the tertiary structure of this protein as reported previously in cases of GR (36), ER (37, 38), and TR (39). To investigate this possibility, a protease sensitivity experiment was performed. GST purified ERRγ was incubated with 32P-labeled DNA fragments containing either sfr4 or consensus SF-1RE followed by digestion with increasing concentrations of proteinase K for a fixed time period as described under “Experimental Procedures” and resolved on a nondenaturing polyacrylamide gel. As demonstrated in Fig. 4C, ERRγ formed specific DNA-protein complexes with both sfr4 and SF-1RE (lanes 1 and 8). Surprisingly the proteinase K-digested sfr4 and SF-1RE bound ERRγ demonstrated different patterns, with additional partial protease products observed with the latter (arrows, Fig. 4C). This result was also confirmed by a different approach in which 35S-labeled in vitro transcribed and translated ERRγ were incubated with
with a mouse ERR followed by stripping and hybridization in a dependent manner, as expected (Fig. 6). This inhibition was also observed in pancreas and heart, as well as submaxillary gland; Sk. Muscle, skeletal muscle; Sm. muscle, smooth muscle.

In agreement with the gel shift results the labeled ERRy demonstrated distinctly different digestion patterns when bound to sf4 or SF-1RE (Fig. 4D). These results demonstrate that the sequences of response elements may act as a determining factor for the tertiary conformation of the DNA-bound ERRy. It remains to be determined whether such DNA induced conformational changes can alter co-activator or co-repressor binding.

**CoeXpression of SHP and ERRy**—Based on previous reports, SHP and ERRy are apparently coexpressed in several tissues, including pancreas and heart (5, 6, 20). To further explore their expression patterns, Northern blot analyses were performed using SHP or ERRy probes. As demonstrated in Fig. 5A, among the human tissues examined both SHP and ERRy are expressed relatively highly in pancreas, as expected, and also in the stomach. SHP was coexpressed with relatively lower level of ERRy in adrenal medulla, adrenal cortex, and small intestine. To examine expression of ERRy and SHP in mouse tissues, a quantitative dot blot (CLONTECH Inc.) was hybridized with either ERRy or SHP cDNAs, and the respective signals were measured by densitometry and plotted as relative density. As demonstrated in Fig. 5, B and C, ERRy and SHP coexpression was observed in pancreas and heart, as well as submaxillary gland, kidney, epididymus, and prostate.

**SHP Represses ERRy Transactivation of Its Own Promoter**—Since SHP is known to interact with ERRα and ERRβ and repress their activity (6, 10), we investigated whether SHP can also repress ERRy mediated transactivation of its own promoter. Co-transfection studies in CV-1 cells demonstrated that SHP inhibits the ERRy response of the SHP promoter in a dose-dependent manner, as expected (Fig. 6). This inhibition was also observed in HER2393 cells, and ERRα and ERRβ mediated transactivation of an ERE was also found to be repressed by SHP (data not shown).

The inhibitory effect of SHP on its various nuclear receptor targets is based on a direct interaction with their AF-2 surface. To determine whether SHP can interact directly with ERR family members, a yeast two-hybrid interaction study was performed. As demonstrated in Fig. 7A LexA fusion proteins including either human or mouse SHP strongly interacted with B42 activation domain fused to ERRy. ERRα also showed a significant interaction with mouse SHP, but its interaction with human SHP was comparatively weak. Both human and mouse SHP showed a much weaker interaction with ERRβ in this assay. A similar result was obtained by a biochemical approach, which demonstrated interaction of in vitro translated mouse SHP with GST fusions to each of the three ERR subfamily members (Fig. 7B). In agreement with the yeast two-hybrid results, SHP showed a somewhat weaker interaction with ERRβ in comparison to other ERR family members.

To investigate whether the interaction between SHP and ERRy is also dependent on the AF-2 pocket of ERRy, an AF-2 domain deletion construct of ERRy was prepared. As expected from results with other receptors, the interaction of LexASHP with B42 fused wild type ERRy is lost with B42 ERRy dAF-2 (ERRy minus the conserved C-terminal helix 12 motif) (Fig. 8A).

To determine whether the SHP sequences required for interaction with ERRy are the same as those required for interaction with other receptors, a series of previously described LexA fused deletion constructs of SHP were used as outlined in Fig. 8B. Yeast two-hybrid interactions revealed that dN-148, which contains the entire interaction domain of SHP, was sufficient for the interaction, whereas, d210 which contains the repressor
domain only, failed to interact with ERRγ and thus did not increase the reporter activity (Fig. 8C). Overall, these data are consistent with a number of previous reports. As with its other targets, we conclude that the SHP receptor interaction domain interacts directly with the AF-2 surface of ERRγ.

To explore the role of coactivator competition in the repression of ERRγ activity, a co-transfection assay was performed in HEK293 cells, which were found to exhibit significant coactivator mediated increase in ERRγ activity. As demonstrated in Fig. 8D, GRIP–1/SRC-2, a previously described coactivator for all the ERR family members (5, 26, 27), significantly increased ERRγ activity on the SHP promoter, and coexpression with SHP repressed this induction in a dose-dependent manner. This result suggests that GRIP-1 and SHP compete for binding the AF-2 pocket of ERRγ, as demonstrated for other receptors (6, 10, 11).

Recently, a series of mutations affecting the human SHP protein were identified in Japanese subjects with mild obesity (35). These include deletion mutants removing large portions of the protein. An apparent polymorphism present in both normal and obese subjects was also identified. All of the mutant proteins, but not the polymorphic variant, showed significantly decreased ability to inhibit transactivation by the orphan nuclear receptor HNF-4 (35). As expected the truncated SHP mutants H53 and L98, did not inhibit ERRγ activity, whereas both wild type SHP, and the polymorphic form R216H substantially reduced ERRγ activity on SHP promoter (9). To identify whether this inability of the truncated SHP mutants to inhibit ERRγ activity was due to any defect in interaction between the mutants and ERRγ, yeast two-hybrid interactions were performed. As demonstrated in Table I, although wild type SHP and the polymorphic form R216H strongly interacted with ERRγ, both L98 and H53 showed no interaction with ERRγ confirming that the receptor interacting domain of SHP is essential for its interaction with ERRγ.

FIG. 7. SHP physically interacts with the ERR isoforms. A, a yeast strain EGY48 which contains an integrated β-galactosidase reporter gene controlled by the LexA-binding site was transformed with the indicated LexA and B42 plasmids. The transformants were selected on plates containing appropriate selection markers, and assayed for β-galactosidase activity. The result shown is the mean of β-galactosidase value from six independent transformant colonies. The error bars indicate standard deviation. B, GST fused ERRα, ERRβ, and ERRγ were isolated from bacterial culture and immobilized on glutathione-Sepharose beads. In vitro transcribed and translated [35S]methionine-labeled SHP was incubated with purified GST fused receptors or GST alone as indicated in the figure. The interaction complexes were resolved by a 12% denaturing polyacrylamide gel electrophoresis, and analyzed by autoradiography.

Several recent results have identified three nuclear receptors, SF-1, LRH-1, and FXR, as potential regulators of SHP expression. This is consistent with the relatively high levels of expression of SHP in tissues that express these three receptors including liver, adrenal, and pancreas. However, SHP is expressed in additional tissues, including heart, small intestine, stomach, epididymus, and prostate, several of which also express the orphan receptor ERRγ. Thus, it has been previously reported that murine ERRγ is expressed relatively highly in heart, brain, and kidney, with a lower expression in liver (5). Human ERRγ transcripts were found in heart, brain, placenta, kidney, pancreas, with a lower level of expression detected in spleen, thymus, prostate, testis, and small intestine (20). In our study of human endocrine tissues, we found that both ERRγ and SHP are strongly expressed in pancreas, with a lower level of expression for both observed in adrenal medulla and cortex, stomach, and intestine. In mouse tissues, ERRγ expression was highest in the submaxillary gland, which also showed relatively high levels of SHP. Coexpression of ERRγ and SHP was also observed in heart, kidney, epididymus, prostate, and pancreas. Based on both this coexpression and its ability to transactivate the SHP promoter, we conclude that ERRγ is an additional potential regulator of SHP expression. As a consequence of the broad inhibitory functions of SHP, such regulation by ERRγ could have significant implications for nuclear receptor signaling pathways in a number of tissues.

In addition to identifying a novel target gene for ERRγ, to our knowledge the first for this as yet poorly characterized receptor, these results also define unexpected DNA binding and functional properties for ERRγ. SF-1 requires at least three high affinity binding sites to efficiently transactivate the SHP promoter (16). Although such SF-1 sites have been proposed to be targets for ERR family members, only the sft4 element showed such binding. Unexpectedly, although all three ERR family members bind this element with comparable affinity, only ERRγ significantly transactivated the SHP promoter in several different cell lines examined. Somewhat similar differential effects have been described in other contexts. Thus, a previous report demonstrated that ERRγ could efficiently bind a consensus SF-1RE/ERRE, TCAAGGTCA, but could only transactivate it weakly (20). In agreement with this, we found that ERRγ exhibited insignificant activity on the TRα promoter, which contains a perfect ERRE (29), whereas, on a five-copy SF-1RE reporter ERRγ demonstrated modest activity relative to that on a three-copy sft4 Luc (Fig. 4B). An additional study demonstrated ERRγ could not activate a palindromic thyroid hormone response element (TRE-Pal) (5), whereas both ERRα and ERRβ are known to bind this element and efficiently activate a reporter containing it (26). In our hands, ERRγ shows a modest activity on TRE inducing it by 1.5–2-fold in HeLa cells and DNA binding studies indicate that ERRγ binds TRE-Pal with a similar affinity as it binds sft4 (data not shown). The basis for the differential activities of the three
ERR isoforms observed on SHP promoter is yet to be completely elucidated, but our results demonstrate differential effects of different DNA sequences on ERRγ tertiary structure. Protease sensitivity experiments performed on consensus SF-1RE, sf4, and TRE-Pal bound ERRγ or ERRα (Fig. 4, B and C, and data not shown) confirm this conclusion and suggest that DNA sequences may also act as allosteric modulator of ERRα structure. These effects are similar to those previously described for the GR and other nuclear receptors (36–39).

Although on sf4 TSA induced the activities of ERRα and -β but not ERRγ, we found that the activities of all three isoforms could be augmented by TSA treatment on ERR and TRE-Pal (Fig. 4A, and data not shown). The allosteric effects of DNA-binding sites on ERR structure suggest a specific mechanism for such differential transactivation. In this hypothesis, the structure of ERRα and -β bound to sf4 would be relatively permissive for corepressor binding, while the ERRγ-sf4 would be nonpermissive. It is also possible, of course, that allosteric effects of the DNA-binding site could alter interactions with coactivators, or with other DNA bound transcription factors. Detailed further studies will be required to test the potential importance of the differential effects of DNA sequence on ERR functions suggested by the initial results described here.

The current study also demonstrates that SHP inhibits ERRγ transactivation. As with other receptors (5, 6) this inhibition is a consequence of a direct physical interaction of SHP with the AF-2 surface of ERRγ (Fig. 4, A and B).

Interaction of ERRγ with naturally occurring SHP mutants in yeast

The indicated B42- and LexA-plasmids were transformed into a yeast strain containing an appropriate LacZ reporter gene, as described in "Experimental Procedures." At least six separate transformants from each transformation were transferred to indicator plates containing 5-bromo-4-chloro-3-indolyl β-galactosidase, and reproducible results were obtained using colonies from two separate transformations. SB, strongly blue colonies after 6–8 h of incubation; W, white colonies. N indicates empty vectors. Wt, wild type human SHP; R216H, replacement of arginine codon 216 by histidine; H53, deletion of 10 bases starting at codon 53 for histidine; L98; deletion of 9 bases and insertion of a dinucleotide AC at codon 98 for leucine.

| B42 fusions | LexA SHP mutants |
|-------------|------------------|
| N           | Wt              |
| R216H       | H53             |
| L98         |                 |

For such differential transactivation. In this hypothesis, the structure of ERRα and -β bound to sf4 would be relatively permissive for corepressor binding, while the ERRγ-sf4 would be nonpermissive. It is also possible, of course, that allosteric effects of the DNA-binding site could alter interactions with coactivators, or with other DNA bound transcription factors. Detailed further studies will be required to test the potential importance of the differential effects of DNA sequence on ERR functions suggested by the initial results described here.

The current study also demonstrates that SHP inhibits ERRγ transactivation. As with other receptors (5, 6) this inhibition is a consequence of a direct physical interaction of SHP with the AF-2 surface of ERRγ. Thus, in tissues where ERRγ is active, SHP should autoregulate its own gene expression. This mechanism should limit the response of not only SHP, but other ERRγ target genes to signals that increase ERRγ transactivation.
activation. The identification of such signals and such gene targets will be necessary to test this hypothesis.

While this report was under review two reports demonstrated an antiestrogenic therapeutic drug, 4-hydroxytamoxifen, as a ligand for ERRβ and ERRγ (40, 41). These reports, together with our report indicate that the SHP gene promoter may be a bona fide target of 4-hydroxytamoxifen. Further studies are needed to characterize such a possibility.

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Differential Regulation of the Orphan Nuclear Receptor Small Heterodimer Partner (SHP) Gene Promoter by Orphan Nuclear Receptor ERR Isoforms

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