The Orphan Nuclear Receptor SHP Inhibits Agonist-dependent Transcriptional Activity of Estrogen Receptors ERα and ERβ*

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SHP (short heterodimer partner) is an unusual orphan nuclear receptor that contains a putative ligand-binding domain but lacks a conserved DNA-binding domain. Although no conventional receptor function has yet been identified, SHP has been proposed to act as a negative regulator of nuclear receptor signaling pathways, because it interacts with and inhibits DNA binding and transcriptional activity of various nonsteroid receptors, including thyroid hormone and retinoid receptors. We show here that SHP interacts directly with agonist-bound estrogen receptors, ERα and ERβ, and inhibits ER-mediated transcriptional activation. SHP specifically targets the ligand-regulated activation domain AF-2 and competes for binding of coactivators such as TIF2. Thus, SHP may represent a new category of negative coregulators for ligand-activated nuclear receptors. SHP mRNA is widely expressed in rat tissues including certain estrogen target tissues, and subcellular localization studies demonstrate that SHP is a nuclear protein, suggesting a biological significance of the SHP interactions with ERs. Taken together, these results identify ERs as novel SHP targets and suggest that competition for coactivator-binding is a novel mechanism by which SHP may inhibit nuclear receptor activation.

Nuclear receptors comprise the largest superfamily of eukaryotic transcription factors with more than 150 proteins identified (for review, see Refs. 1–3). The capability of many family members to bind structurally diverse hydrophobic ligands is a crucial regulatory element for the transmission of extracellular signals into intracellular transcriptional responses. However, alternative ligand-independent regulation mechanisms have been identified (4–6), and ligands may not exist for all receptors. Nuclear receptors usually modulate transcription of their target genes by binding to cognate promoter response elements. Moreover, cross-talk mechanisms, which do not require DNA binding, may allow nuclear receptors to influence the activity of other transcription factors (7, 8).

Nuclear receptors are modular transcription factors containing a variable N-terminal domain often exhibiting a constitutive transcription activation function (AF-1),¹ a highly conserved zinc finger type DNA-binding domain (DBD), a variable linker region (hinge), and a multifunctional C-terminal domain responsible for ligand binding (LBD), dimerization, and ligand-regulated transcriptional activation (AF-2). Nuclear receptors can be subdivided into steroid receptors that mainly form homodimers and a large diverse subfamily of nonsteroid receptors including receptors for thyroid hormone, retinoids, and vitamin D as well as many orphan receptors, for which natural ligands have not been identified. Whereas certain nonsteroid receptors are monomeric or form homodimers, the majority heterodimerizes with the retinoid X receptor (RXR). These heterodimers have been recognized to function as very dynamic transcription factors in which both subunits influence the other’s capability to interact with ligands and cofactors (9–13). Functional and structural analysis (Refs. 14–17 and references therein) identified a common dimerization surface within the LBDs which primarily is formed by helices 10/11 (dimerization helix).

Estrogen receptors (ERs) are unique steroid receptors because they exist as two different paralogues (encoded by two separate genes), ERα and ERβ (Refs. 18 and 19 and references therein). Both ERs display quite similar ligand binding characteristics (19) and comparable agonist-dependent transcriptional activities on “classical” estrogen response elements (EREs), consistent with the conservation of LBD/AF-2 regions implicated to be critical for these functions. However, differences have been reported with regard to their antagonist-dependent activation properties at AP1 sites (8) which might be related to the lack of conservation in their N termini. These functional differences, together with their distinct expression pattern in tissues, strongly suggest that ERα and ERβ may play different roles in gene regulation. They have been demonstrated to form stable homo- and heterodimers with each other in solution and on DNA (20, 21) indicating that heterodimerization is not a unique feature of nonsteroid receptors forming RXR heterodimers.

Nuclear receptors including ERs function in concert with transcriptional cofactors including basal transcription factors, chromatin-modifying complexes, corepressors, and coactivators (for review see Refs. 22 and 23). Although various receptors in the absence of agonistic ligands bind to corepressors (24, 25), ligand activation is associated with structural rearrangements within the LBD/AF-2 domain, permitting the recruitment of coactivators. The predicted AF-2 coactivator-binding surface includes two highly conserved regions, namely the C-terminal helix 12 (AF-2 core) as well as N-terminal helices 3–5 including.

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* The abbreviations used are: AF, activation function; DBD, DNA-binding domain; LBD, ligand-binding domain; RXR, retinoid X receptor; ER, estrogen receptor; ERE, estrogen-responsive element; SRC-1, steroid receptor coactivator 1; TIF2, transcription intermediary factor 2; AIB1, amplified in breast cancer 1; SHP, short heterodimer partner; WT, wild-type; GST, glutathione S-transferase; GFP, green fluorescent protein; RT-PCR, reverse transcriptase-polymerase chain reaction; PPAR, peroxisome-proliferator activated receptor; TR, thyroid hormone receptor; RIP140, receptor-interacting protein 140; NLS, nuclear localization signal; FCS, fetal calf serum; aa, amino acid(s).
and verified by restriction enzyme analysis and DNA sequencing. Biological evidence for the involvement of p160/SRC-1 coactivators in, for example, ER-mediated gene expression comes from the discovery of AIIB1 gene amplification in ER-positive breast and ovarian cancer cells (36) as well as from recent SRC-1 knock-out studies (43).

In searching for novel proteins interacting with the LBD/AF-2 domain of nuclear receptors, an unusual orphan receptor has been isolated that obviously lacks a nuclear receptor-type DBD but contains a putative LBD (44, 45). Based on its small size and its ability to interact, like RXR, with various non-steroid receptors, this orphan receptor has been designated SHP (short heterodimer partner). Its closest relative within the superfamily is the orphan receptor DAX-1, which contains a new type of DBD, interacts with the orphan receptor SF-1, and plays important roles in both adrenal and gonadal function (Refs. 46–49 and references therein). Although it is unknown whether SHP has ligands and can bind DNA, SHP has been suggested to act as a negative regulator of nuclear receptor signaling pathways by competition with RXR for heterodimerization (44). Furthermore, because SHP has been shown to exert intrinsic repressor activity, active repression mechanisms may contribute to its inhibitory effect (50).

In this report, we have identified ERs as novel receptor targets for SHP. We provide evidence for the existence of a novel mechanism by which SHP inhibits nuclear receptor activation, and we suggest opposing regulatory functions of SHP and AF-2 coactivators in estrogen signaling.

**EXPERIMENTAL PROCEDURES**

**Plasmids**

All plasmids were generated using standard cloning procedures and verified by restriction enzyme analysis and DNA sequencing.

**Yeast Expression Plasmids**—The Gal4 DNA-binding domain fusion constructs Gal-ER LBD/AF-2 (aa 249–595) and Gal-ER/LBD/AF-2 (aa 168–485) were constructed by inserting PCR-generated fragments of the corresponding human ER cDNAs (8) into the BamHI site of AS2–1 (CLONTECH). Gal-PPAR/LBD and Gal-RXRβ/LBD have been described previously (51). The Gal4 activation domain constructs GAD-SHP WT, D1, and D2 (see Fig. 1) were constructed by inserting PCR-generated fragments of the SHP cDNA into the EcoRI site (WT) or into the EcoRI/XhoI sites of pSG5/Stratagene. GST/His Fusion Constructs—GST-ERβ (aa 1–485) was made by recloning the human ERβ cDNA into the XhoI/NolI sites of pBKCMV HA (51). T3-TIF2 (aa 1–1456) was made by cloning the corresponding cDNA into the EcoRI/XhoI sites of pBK-CMV (Stratagene). T3-RIP140 (aa 1–1158) has been described previously (51).

**Mammalian Expression Constructs**—The following plasmids have been described previously: pSG5-based expression vectors for ERα and ERβ (Ref. 8 and references therein), ER reporter constructs 2xERE-tk-luc (20) and 3xERE-TATA-luc (30), pCMXGal4ER/LBD (aa 247–599) (20), and the Gal4 reporter construct UAS-tk-luc (9). pSG5 rSHPWT was a gift from Dr. Peter Brown (Cambridge, U.K.). pSG5-TIF2/GRIP1 was a gift from Dr. Richard Pestell (University of Cambridge, U.K.). pSG5-TIF2/GRIP1 was constructed by PCR cloning of full-length SHP (aa 1–260) into the EcoRI/BamHI sites of pEGFP-N3 (CLONTECH).

**Yeast Two-hybrid Screening and Interaction Assay**

SHP was isolated in a yeast two-hybrid screening for proteins interacting with the rat PPARαLBD/AF-2 (aa 166–485) as described previously for the isolation of hRIP140 (51), except that a rat liver cDNA library (CLONTECH) was used. For the yeast two-hybrid analysis, HFC7 (MATα) transformed with Gal4 DBD plasmids was mated with Y187(MATα) transformed with GAD plasmids. Diploid strains were selected for the presence of both plasmids. Interactions were monitored as growth on selective –His plates using different dilutions of yeast cells in the absence or presence of 1 μM 17β-oestradiol or 1 μM 4-OH tamoxifen, respectively.

**GST Pull-down Assay**

Interaction studies were performed essentially as described (51). Briefly, 35S-labeled proteins, generated in *in vitro* transcription/translation of either plasmids or PCR products containing a 5′-T3 promoter using a TNT kit (Promega), were incubated with approximately 1 μg of GST fusion protein in the absence (MeSO) or presence of 1 μM 17β-oestradiol or 1 μM 4-OH tamoxifen. The proteins were incubated for 2–3 h at 4 °C. For the competition assay, either purified His-SHP, His-RIP140, or TIF2 (which was generated by thrombin cleavage of the purified GST-TIF2 protein) was added to the binding reaction. Protein interactions were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

**Mammalian Cell Transfections**

293 cells were maintained in a 1:1 mixture of F-12 medium with glutamine and Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 100 μM penicillin, and 100 μM streptomycin (Life Technologies, Inc.). MCF-7 cells were maintained in RPMI 1640 (Life Technologies, Inc.) media supplemented with 10% FCS, 1% non-essential amino acids (Life Technologies, Inc.), 100 μM penicillin, and 100 μM streptomycin. Both 293 cells and MCF-7 cells were plated onto 6-well plates in phenol red-free modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% charcoal-stripped FCS, 100 μM penicillin, and 100 μM streptomycin. Twenty-four hours later, cells were transfected with plasmid constructs using Lipofectin (MCF-7 cells and 293 cells transfected with Gal-ERαLBD) as instructed by the manufacturer (Life Technologies, Inc.) or by using DOTAP (293 cells transfected with ERα or ERβ) as instructed by the manufacturer (Boehringer Mannheim). Transfections were performed using 1.0 μg of ERE-tk-luciferase reporter or 0.8 μg of ERE-TATA-luc reporter, together with 0.2 μg of either ERα or ERβ or 50 ng of Gal-ERαLBD. Empty expression vectors were added to equalize total transfected plasmid DNA concentrations. After 12 h, the medium was changed, and fresh medium (without phenol red) containing MeSO or 10 μM 17β-oestradiol was added. 30 h after changing media, the cells were harvested. Cell extracts were analyzed for luciferase activity as described (20, 51).

**RT-PCR for mRNA Expression Analysis**

Total RNA isolation and total cDNA preparation have been described previously (52). For the PCR reaction, 1 μl of the synthesized cDNA was added to the reaction mix and amplified, starting with a preincubation at 94 °C for 2 min, followed by 25 cycles at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 90 s in a PCR 9600 thermocycler (Perkin-Elmer). The oligonucleotides R1051 5′-AGGAAACAGACACGACACGATGACT-3′ and R1031 5′-AGTCCTTGGACGGCAGGAACGG-3′ were used for amplification of a 258-base pair fragment of SHP. The oligonucleotides were used for amplification of actin as were described previously (52). The PCR for actin was performed with an annealing temperature of 50 °C.
After agarose gel electrophoresis and blotting to nitrocellulose filters, the PCR products were hybridized to the labeled 258-base pair SHP oligonucleotide prepared from the original SHP cDNA clone, using the same primers as for the RT-PCR. The actin PCR products were hybridized to an internal actin primer, according to previously described protocol (52). Hybridization of SHP probe was performed at 65 °C for 1 h in ExpressHyb hybridization solution (CLONTECH) followed by four 10-min washes in 2× SSC and 0.05% SDS at room temperature and finally two 20-min washes in 0.1× SSC and 0.5% SDS. Hybridization of actin oligonucleotide was performed at 37 °C for 1 h in ExpressHyb hybridization solution followed by four 10-min washes in 2× SSC and 0.05% SDS at room temperature and finally two 20-min washes in 0.1× SSC and 0.1% SDS.

Analysis of Subcellular Localization of GFP-tagged SHP

293-cells were plated on 6-well plates containing glass coverslips in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Twenty-four hours later, cells were transfected with 2 μg of SHP-GFP or with 2 μg of GFP alone, using Lipofectin as instructed by the manufacturer (Life Technologies, Inc.). Cellular localization was visualized 36 h after transfection. The cells were fixed with 4% formaldehyde in phosphate-buffered saline for 15 min at room temperature and stained with 0.001 mg/ml Hoechst 33342 for 10 min at room temperature. The coverslips were mounted on micro slides with FluorSave Reagent (Calbiochem) followed by viewing in a Zeiss Axiophot epifluorescence microscope. Photographs were recorded on T-max 400 film (Kodak).

RESULTS

Cloning of SHP and Interaction with ERs—The yeast two-hybrid approach was used to identify novel proteins that interact with the LBD of PPARs, beyond the known dimerization partner RXR (for details see Ref. 51). Screening of an activation domain-tagged rat liver cDNA library led to the isolation of independent partial clones encoding RXRβ, dUTPase (a previously characterized PPAR-interacting protein, Ref. 53), and one full-length clone encoding the orphan nuclear receptor SHP (44). The rat SHP is highly conserved compared with its mouse and human orthologues and consists of 260 aa with a predicted molecular mass of 29 kDa, in concordance with recently published data (45). RACE experiments failed to detect extended 5’-coding regions (data not shown), supporting the view that SHP lacks a nuclear receptor-type DNA-binding domain. Structural features of SHP are illustrated in Fig. 1A.

To identify the nuclear receptors with which SHP can interact, we performed two-hybrid interaction assays using GAL4-LBD fusion proteins and the activation domain-tagged SHP (GAD-SHP). We found that SHP interacted with all non-steroid receptors tested, namely PPARs, TR α, RXRβ, and HNF4 (Table I and data not shown), but surprisingly, SHP also interacted with steroid receptors, namely the two ER subtypes ERα and ERβ (Table I). The interaction with the ERα LBD was verified in vitro using the GST pull-down assay (Fig. 1B) and appeared to depend on agonistic ligands, i.e. estradiol, whereas antagonistic ligands, i.e. the anti-estrogen tamoxifen, did not promote interaction of the proteins. The interaction with non-steroid receptors is in agreement with previously reported data (44, 45, 50) and supports the hypothesis that SHP, like RXR, is a heterodimerization partner for nuclear receptors (44). In contrast to RXR, however, SHP also interacts with ERs, indicating major differences between SHP and RXR with regard to receptor specificity and mode of interaction with the LBD.

SHP C Terminus Including the Putative Nuclear Receptor Dimerization Helix Is Not Required for ER Interaction—Previous functional and structural studies have established a requirement of the LBD helices 10/11 for nuclear receptor dimerization (14–16). To investigate the involvement of the predicted SHP helix 10/11 in the interaction with ERs we made two C-terminal SHP deletions (D1 and D2, see Fig. 1A) and tested them for interaction both in vitro and in vivo. First, we expressed and purified SHP WT, D1, and D2 as GST fusion proteins, and we performed pull-down assays using [35S]-radio-labeled in vitro translated wild-type ERα and ERβ, respectively. As seen in Fig. 1C and D, SHP WT and D1 interacted with comparable efficacy with both ERs, whereas further deletion up to an 113 (D2) significantly decreased the interaction. In contrast to the pull-down assay using GST-ERα LBD (Fig. 1B), in vitro translated ERs displayed some ligand-independent interaction with SHP, causing only a weak enhancement...
in the presence of estradiol. However, the “ligand-independent” interaction was decreased in the presence of antagonist (tamoxifen), suggesting that translated ERs were partially activated. Therefore, differences with respect to the folding and activation status between the 

\textit{Escherichia coli} expressed LBD, and the \textit{in vitro} translated wild-type ERs cannot be excluded.

The \textit{in vitro} results could be further supported \textit{in vivo} using the yeast two-hybrid assay (Table I); SHP WT and D1 interacted with both ERs, whereas SHP D2 only interacted with ER\textsubscript{a} but not with ER\textsubscript{b}, perhaps illustrating structural differences between their LBDs. Interestingly, both SHP deletions efficiently interacted with RXR and PPAR, respectively, even in the absence of added ligands, whereas the \textit{in vitro} interaction with the ERs was strictly dependent on the presence of estradiol, and no interaction was seen without hormone or in the presence of tamoxifen. In summary, we conclude the following: 1) that the predicted SHP helix 10/11 does not appear to be involved in the ER interaction, 2) that an N-terminal region encompassing aa 113–209 is required for efficient interaction with ERs, and 3) that agonistic, but not antagonistic, ER ligands significantly enhance the interaction of SHP with the ER LBD.

\textbf{SHP Inhibits Ligand Activation of ERs in Mammalian Cells—}\textbf{SHP has previously been suggested to act as a negative regulator of retinoid receptor signaling in mammalian cells (44). To determine whether SHP also interferes with ER-mediated transcriptional activation in response to estradiol, we performed transient cotransfection studies in 293 cells using expression vectors for the wild-type receptors and an ER-responsive reporter plasmid. 293 cells (derived from human embryonal kidney cells) were selected as a test system because these cells do not express detectable levels of endogenous ER (data not shown). Therefore, reporter gene activation strictly depends on the expression of exogenous ERs. As shown in Fig. 2, A and B, coexpression of increasing amounts of SHP inhibited the ligand-induced transcriptional activity of both ER\textsubscript{a} and ER\textsubscript{b}, respectively. The inhibition was approximately 80% of the ligand-induced activity in the absence of SHP in case of ER\textsubscript{a} and almost complete in case of ER\textsubscript{b}. The residual activity of ER\textsubscript{a} is probably due to transcriptional activity mediated by AF-1, which apparently is not present in ER\textsubscript{b}, although we do not exclude other explanations, e.g. different expression levels of the two ERs. To confirm these results in a cell line expressing endogenous ERs, we cotransfected MCF7 breast cancer cells with the ER-responsive reporter construct and increasing amounts of SHP (Fig. 2C). Under these conditions, SHP was able to down-regulate the estradiol-dependent activity approximately 50%. Compared with the results obtained using 293 cells, the inhibitory effect of SHP appears to be less pronounced in MCF-7 cells for several reasons as follows. (i) The transfection efficiency differed between the two cell lines (data not

\begin{table}
\centering
\caption{Yeast two-hybrid interaction of SHP with ER\textsubscript{a} and ER\textsubscript{b}}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
 & \textbf{GFP-SHP} & \textbf{GAL4-LBD/AF-2} & \textbf{ER\textsubscript{a}} & \textbf{ER\textsubscript{b}} & \textbf{RXR\textsubscript{b}} & \textbf{PPAR\textsubscript{a}} \\
\hline
\textbf{WT} & NH & E2 & OHT & NH & E2 & OHT & NH & 9-cis & NH \\
\textbf{D1} & NH & E2 & OHT & NH & E2 & OHT & NH & 9-cis & NH \\
\textbf{D2} & NH & E2 & OHT & NH & E2 & OHT & NH & 9-cis & NH \\
\textbf{GAD} & NH & E2 & OHT & NH & E2 & OHT & NH & 9-cis & NH \\
\hline
\end{tabular}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Inhibition of ligand-induced ER activity by SHP. 293 cells were cotransfected with the ERE-TATA-luc reporter plasmid and the expression plasmids for either wild-type ER\textsubscript{a} (A) or wild-type ER\textsubscript{b} (B), together with increasing amounts of the expression vector for SHP, either in the absence (Me\textsubscript{2}SO) or presence of 17β-estradiol. All values represent the mean of duplicate samples, and similar results were obtained in at least three independent experiments. C, MCF-7 cells were transfected with the ERE-tk-luc reporter plasmid together with increasing amounts of SHP expression plasmid in the absence or presence of 17β-estradiol. The values shown are the mean of two independent experiments. The luciferase activity observed with the endogenous ERs in the presence of 17β-estradiol (E2), but no added SHP, was set to 100%.

2 L. Johansson and E. Treuter, unpublished results.
Novel Interactions between SHP and ERs

SHP Inhibits ERα AF-2 Activity and Antagonizes TIF2-mediated Coactivation in Mammalian Cells—To elucidate regulatory mechanisms by which SHP antagonizes ER activation, we wanted to distinguish between negative effects of SHP at the level of DNA binding versus DNA-independent inhibition mechanisms, i.e., at the transcriptional level. Because the ER LBD/AF-2 domain was sufficient for interaction with SHP, we made constructs expressing GAL4-ERα LBD/AF-2 fusion proteins and analyzed them in a GAL4-responsive reporter system in 293 cells. As seen in Fig. 3A, coexpression of increasing amounts of SHP clearly inhibited the ligand-dependent activation function AF-2 of ERα, similar to the inhibition observed above with wild-type ERα (see Fig. 2A). This strongly suggests that SHP is able to down-regulate ER activity by inhibiting its ligand-dependent AF-2, without affecting the capacity of ER to bind to DNA.

Considering the interaction characteristics of SHP and its effect on AF-2 activity in mammalian cells, striking similarities are apparent between the action of SHP and negative AF-2 coregulators, for example RIP140 (51) or dominant-negative fragments of AF-2 coactivators such as SRC-1 (29, 30) or TIF2 (32). Thus, SHP might act at the same level as AF-2 coactivators. To test this hypothesis, we asked whether SHP could antagonize the coactivation mediated by one distinct AF-2 coactivator. As illustrated in Fig. 3B, coexpression of SHP together with TIF2/GRIP-1, a member of the p160/SRC-1 family of coactivators previously demonstrated to function as a coactivator for ERα (32, 33, 54), indeed resulted in inhibition of TIF2-mediated coactivation in an SHP concentration-dependent manner. We further reasoned that if SHP is acting as a dominant-negative AF-2 inhibitor, then increasing amounts of TIF2 should overcome SHP-mediated inhibition. The experiment shown in Fig. 3C clearly confirms this assumption.

SHP Competes for Direct Binding of TIF2 to the ERα AF-2 Domain—Our transient transfection studies suggested that SHP and AF-2 coactivators, respectively, may exert antagonistic functions on the ER AF-2. Since SHP has been demonstrated to contain repression domains (50) (DAX-1 homology box, see Fig. 1A), it may utilize dominant repression mechanisms to inhibit AF-2 even in the presence of AF-2 coactivators. Alternatively, a competition model, as recently proposed for RIP140 (51), could explain the dominant-negative effect of SHP in mammalian cells. To investigate whether binding of SHP to the LBD/AF-2 domain occurs simultaneously or competitively with other LBD/AF-2 cofactors, we performed in vitro competition studies based on the GST pull-down assay. Specifically, the binding of radiolabeled in vitro translated cofactors and/or dimerization partners (TIF2, RIP140, SHP, and ERα) to purified GST-ERα LBD/AF-2 fusion protein was assessed in the absence or presence of purified histidine-tagged SHP WT protein, respectively (Fig. 4A–D). The following results were observed: 1) the functionality of the assay is demonstrated in Fig. 4A, as purified SHP almost completely eliminated binding of SHP generated by in vitro translation; 2) purified SHP apparently competed for binding of the ligand-dependent AF-2 cofac-

![Graph A](image1)

**Fig. 3.** SHP inhibits ERα AF-2 activity and antagonizes TIF2 coactivation in vitro. 

A, 293 cells were cotransfected with the UAS-tk-luc reporter plasmid together with the expression plasmid for Gal-ERα and increasing amounts of SHP expression plasmid in the absence (Me2SO) or presence of 17β-estradiol. The values shown are the mean of two independent experiments. The luciferase activity observed with Gal-ERα in the presence of 17β-estradiol (E2), but no added SHP, was set to 100%. B, 293 cells were cotransfected with the UAS- tk-luc reporter, Gal-ERα and TIF2 expression plasmid together with increasing amounts of the expression plasmid for SHP in the absence (Me2SO) or presence of 17β-estradiol. One representative experiment is shown. All values represent the mean of duplicate samples, and similar results were obtained in at least three independent experiments. C, 293 cells were cotransfected with the UAS-tk-luc reporter, Gal-ERα and SHP expression plasmid together with increasing amounts of the expression plasmid for TIF2 in the absence (Me2SO) or presence of 17β-estradiol. One representative experiment is shown. All values represent the mean of duplicate samples, and similar results were obtained in at least three independent experiments.
TIF2, or RIP140 but not in vitro petition assays shown in Fig. 4, equal amounts of GST-ER observed earlier using purified SHP. Importantly, for all com- in the presence of either TIF2 or RIP140, confirming the results translated SHP, but not ER17 (Me2SO) or presence of 17 (HIS)tagged SHP (aa 1–260) and in the absence or presence of His-tagged RIP140 (aa 747–1158) or TIF2 (aa 594–766), respectively, and in the absence (Me2SO) or presence of 17β-estradiol (E2). The input represents 20% of the amount of labeled protein used in each pull down.

FIG. 4. SHP competes for binding of TIF2 or RIP140 to ERα AF-2 in vitro. A–D, purified SHP displaces in vitro translated SHP, TIF2, or RIP140 but not in vitro translated ERα, GST-ERα (aa 249–595) was incubated with in vitro translated proteins as indicated in the absence or presence of His-tagged SHP (aa 1–260) and in the absence (Me2SO) or presence of 17β-estradiol. E and F, purified RIP140 or TIF2, respectively, displaces in vitro translated SHP but not in vitro translated ERα. GST-ERα was incubated with in vitro translated proteins in the absence or presence of His-tagged RIP140 (aa 747–1158) or TIF2 (aa 594–766), respectively, and in the absence (Me2SO) or presence of 17β-estradiol (E2). The input represents 20% of the amount of labeled protein used in each pull down.

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tors TIF2 or RIP140 (Fig. 4, B and C); 3) purified SHP did not compete for binding of the ligand-independent homodimerization partner ERα (Fig. 4D), indicating that the competition seen with TIF2 and RIP140 was not due to nonspecific protein effects and, more importantly, suggesting that SHP does not interfere with LBD-mediated ERα homodimerization; 4) the binding of purified SHP to purified GST-ERα could be confirmed using Western blot analysis (data not shown) and supported the direct character of the interaction between the two proteins.

For verification, the competition assay was additionally performed in the reciprocal arrangement using purified receptor interaction domains of TIF2 or RIP140, respectively, as competitor and using in vitro translated SHP, or for control, ERα. As demonstrated in Fig. 4, E and F, binding of in vitro translated SHP, but not ERα, to GST-ERα LBD-AF-2 was abolished in the presence of either TIF2 or RIP140, confirming the results observed earlier using purified SHP. Importantly, for all competition assays shown in Fig. 4, equal amounts of GST-ERα protein were used, as judged from staining of the SDS gels, and no interaction with purified GST protein alone was seen with any of the ER-interacting proteins (data not shown).

SHP mRNA Is Expressed in ER Target Tissues—Both human and rat SHP have been isolated originally from liver two-hybrid cDNA libraries, and subsequent Northern hybridizations have suggested that SHP mRNA is highly expressed only in liver and, in case of the rat mRNA, also in heart (44, 45). Although these tissues contain high levels of nuclear receptors that may serve as relevant SHP targets (for example PPARα or HNF4), only low levels of ERα mRNA, and no ERβ, are detectable in liver or heart (19). This raised the question whether the interaction of SHP with ERα is biologically relevant with regard to their coexpression in tissues. In this context it is worth considering that current expression data for SHP are derived from Northern blots testing only a limited number of tissues. To analyze SHP mRNA tissue distribution in rat more extensively and, compared with the Northern approach, more sensitively, we prepared mRNA from 19 different rat tissues and performed RT-PCR using primers specific for the unique SHP N terminus. Unexpectedly, by using that approach we could detect SHP mRNA in most of the analyzed rat tissues (Fig. 5), strongly indicating that SHP is much more widely expressed than previously thought. This might, at least in part, be explained by the differences in sensitivity between the RT-PCR and Northern approach.

Although our assay was only semi-quantitative, comparison to the actin signal allowed some estimations about the relative SHP expression in different tissues. SHP appeared to be highly expressed in uterus, lung, liver, heart, adrenals, epididymis, olfactory lobes, and cerebellum; moderate levels could be detected in prostate, small intestine, stomach, thymus, and spinal cord, and low levels seemed to exist in testis, colon, and spleen. SHP could not be detected in kidney and pituitary, thus at the same time serving as a negative control for the RT-PCR. Importantly, these expression data not only demonstrate that SHP mRNA is expressed ubiquitously in rat, they also indicate the possibility for coexpression of SHP with at least one of the two differentially expressed ER subtypes (19) in ER target tissues such as uterus, prostate, and testis but also in bladder, lung, adrenal, and in certain brain regions.

SHP Is Localized in the Nucleus—Our data indicate that SHP inhibits ER activity in transfected cells, suggesting competition for binding of AF-2 coactivators as one possible explanation for this effect. To function as a negative regulator of ER activation, SHP should be coexpressed with ERs also at the subcellular level. ERs and coactivators such as TIF2 are believed to exist mainly in the nucleus, consistent with the direct function of these proteins as transcriptional (co-)factors and further consistent with the presence of nuclear localization signals (NLS) in these proteins. In most nuclear receptors, NLS sequences are located at the C-terminal end of the DBD and lie within the hinge region. However, in case of SHP, which lacks a nuclear receptor-type DBD and apparently also any conventional (basic) NLS-like sequence, the subcellular localization has been uncertain and needed to be determined. To address this issue, we constructed an expression vector for wild-type SHP (aa 1–260) fused to the N terminus of green fluorescent protein (GFP) and transiently transfected this construct into 293 cells. As seen in the photograph of one representative transfected cell (Fig. 6A), the SHP-GFP fusion protein was expressed in and localized to discrete regions of the nucleus (for identification of the nucleus, see Hoechst stain in Fig. 6B), causing a characteristic dot pattern, which was observed in different cell lines (data not shown). Because GFP alone, as predicted, was not specifically localized to any subcellular compartment in transfected cells (Fig. 6C), the nuclear localization
Fig. 5. Rat tissue distribution of SHP mRNA. RT-PCR from the indicated rat tissues was performed as described under “Experimental Procedures.” Shown are the autoradiographs of Southern blots after hybridization with a cDNA probe specific for SHP (top) or a oligonucleotide probe specific for actin (bottom).

Fig. 6. Nuclear localization of SHP. 293 cells were transfected with 2.0 μg of expression vector for either GFP-SHP (A and B) or GFP alone (C and D). 36 h after transfection, cells were fixed, and the nuclei were stained with Hoechst 33342 (B and D). The GFP expression was visualized by fluorescence microscopy (A and C). Photographs in A and B or C and D, respectively, show the same cell.

of the SHP-GFP fusion protein clearly indicates that SHP is a nuclear protein.

DISCUSSION

SHP Represents a New ER-interacting Protein and Putative AF-2 Inhibitor—The interaction of SHP with ERs represents, to our knowledge, the first example of an orphan nuclear receptor directly interacting with and negatively influencing the transcriptional activity of a steroid receptor. The inhibition mechanism we propose ascribes a novel putative function to SHP, which is not related to conventional nuclear receptor functions (see below). The physical and functional interaction of SHP with ERs may be biologically relevant, because we were able to detect SHP mRNA in many target tissues expressing at least one of the two ERs, although these expression data clearly have to be complemented by analysis at the protein level. In support of our RT-PCR based expression data, SHP mRNA was recently detected in several mouse tissues such as adrenal, ovary, and testis (55). Furthermore, since we have demonstrated that SHP is a nuclear protein, the possibility for physical interaction with ERs is given also at the subcellular level. Because SHP lacks any obvious conventional NLS sequence, it will be important to determine which parts of SHP are responsible for its nuclear localization and whether SHP either utilizes unconventional NLS sequences for direct transport to the nucleus or, alternatively, indirect cotransport mechanisms.

Irrespective of the inhibition mechanism (see below), SHP represents a novel negative coregulator for ERs, which might be able to attenuate agonist-dependent transcriptional activation. The envisaged inhibitory function of SHP on ER activation has implications for feedback control mechanisms and for potential therapeutic applications. For example, in ER-positive breast and ovarian cancer cells, SHP would be expected to antagonize dominant ER coactivators such as AIB1 (36). Thus, it might be important to determine whether different SHP expression levels can account for different estrogen responses in cells expressing ERs. Although mechanistically different and only applicable to agonist-mediated effects, SHP-dependent actions may complement recent models suggesting a regulatory involvement of corepressors in certain aspects of ER-dependent carcinogenesis (56, 57).

A Novel Mechanism by Which SHP Inhibits Nuclear Receptor Activity—Previous studies from Moore and co-workers (44) have suggested that SHP may inhibit nuclear receptor signaling by two alternative mechanisms as follows: indirectly by interfering with DNA binding of nuclear receptor dimers, and directly via active repression mechanisms, i.e. recruiting (as yet unknown) corepressors to ligand-activated receptors (50). In this study we have provided evidence for a third mechanism, in which SHP is proposed to interfere directly with AF-2 coactivator function. Although it is uncertain which of the three mechanisms accounts for the inhibition of wild-type ERs, we have demonstrated that SHP is able to directly inhibit AF-2 activity by competition for binding with the coactivator TIF2. This suggests that SHP and AF-2 coactivators may contact a common surface on the LBD/AF-2 or, alternatively, that binding of SHP to the LBD may induce conformational changes leading to the dissociation of AF-2 coactivators. Interestingly, SHP may act similarly to RIP140, an AF-2-binding protein of previously unknown function, which we have recently suggested to act as a negative coregulator by competing for coactivator binding (51). However, unlike RIP140, SHP further contains intrinsic repression activity (50). We are currently investigating whether the putative SHP repression domain is required or whether competition alone is sufficient for ER inhibition by SHP in vivo. In light of previous observations that SHP was able to inhibit VP16-dependent transcriptional activity in cis, but not in trans (50), it is uncertain whether SHP will repress in the presence of simultaneously bound AF-2 coactivators. Thus, the competition we suggest may allow SHP more easily to exert its repressive function.

Similar Interaction Characteristics for SHP and AF-2 Cofactors—Based on the evidence presented here for ERs and in previous studies for RXR (44, 50), we noticed that SHP exhibits certain interaction characteristics similar to those expected for ligand-dependent AF-2 cofactors, including coactivators, but different from those expected for a conventional nuclear receptor dimerization partner. First, SHP interacts efficiently only with the liganded LBD/AF-2 domain. Although dimerization occasionally is enhanced in the presence of ligands (58, 59), both ER and RXR dimers usually associate stably also in the absence of ligands. It is interesting to note that the RXR ligand 9-cis-retinoic acid exerts a negative effect on dimerization of RXR with its heterodimer partners (58, 59) but enhances the interaction with SHP (44, 50). Furthermore, in the case of ERs, we observed significant differences between SHP association in the presence of agonistic or antagonistic ER ligands, respectively, which were not seen for ER dimers (Refs. 15, 20, and 21 and references therein). In addition, SHP is unique with re-
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Despite the presence of an LBD-like domain in both SHP and DAX-1, it has yet to be established that these proteins act as ligand-regulated nuclear receptors. For homology reasons though, the existence of ligands cannot be excluded, and a new cascade of events would be expected upon binding of putative ligands. For example, a ligand-induced conformational change within the LBD may cause dissociation of corepressors converting the former repressor into a transcriptional activator. This is most likely to be the case for DAX-1, because it contains a consensus AF-2 helix 12 motif, whereas in the predicted SHP helix 12 a glutamic acid, which is conserved in all ligand-activatable nuclear receptors (17), is changed to an aspartic acid (see Fig. 1A). Thus, even in the presence of hypothetical ligands, DAX-1 and SHP may exert opposing effects on their gene or receptor targets. This might also be of biological significance, considering the coexpression of DAX-1 and SHP with SF-1 and ERs in some steroidogenic tissues (Refs. 19, 48, and 55 and references therein). In light of our new findings, we would finally like to propose that a liganded SHP will probably not recruit corepressors but still antagonize transcriptional activity of its receptor targets by competition with coactivators for binding to the AF-2 domain.

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REFERENCES

1. Enmark, E. & Gustafsson, J.-Å. (1996) Mol. Endocrinol. 10, 1293–1307
2. Mangelsdorf, D. J. & Evans, R. M. (1995) Cell 83, 841–850
3. Beato, M., Herrlich, P. & Schutz, G. (1995) Cell 83, 851–857
4. Kato, S., Endoh, H., Masuhiru, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masuhiru, S., Gotoh, Y., Nishioka, E., Kawashima, H., Metzger, D. & Champon, P. (1995) Science 270, 1491–1494
5. Bunone, G., Brind, P. A., Miksicek, R. J. & Picard, D. (1996) EMBO J. 15, 2174–2183
6. White, R., Slober, M., Kalkhoven, E. & Parker, M. G. (1997) EMBO J. 16, 1427–1435
7. Kamei, Y., Xu, L., Heintel, T., Torchia, J., Kurokawa, R., Biss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K. & Rosenfeld, M. G. (1996) Cell 85, 403–414
8. Pusch, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J.-Å., Kushner, P. J. & Scanlan, T. S. (1997) Science 277, 1508–1510
9. Forman, B. M., Umesono, K., Chen, J.-H. & Evans, R. M. (1996) Cell 81, 541–550
10. Kurokawa, R., Dillen, J., Boehm, M., Sugarman, J., Biss, B., Rosenfeld, M. G., Heyman, R. A. & Glass, C. K. (1994) Nature 371, 528–531
11. Schulman, I. G., Li, C., Schwabe, J. W. & Evans, R. M. (1997) Genes Dev. 11, 599–608
12. Zamir, I., Zhang, J. & Lazar, M. A. (1997) Genes Dev. 11, 835–846
13. Wiebel, F. F. & Gustafsson, J.-Å. (1997) Mol. Cell. Biol. 17, 3977–3986
14. Bourguet, W., Ruff, M., Champon, P., Gronemeyer, H. & Moras, D. (1995) Nature 375, 377–382
15. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Enström, O., Olman, L., Greene, G. L., Gustafsson, J.-A. & Carlquist, M. (1997) Nature 389, 753–758
16. Tanenbaum, D. M., Wang, Y., Williams, S. P. & Sigler, P. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5996–6003
17. Wutz, J.-M., Bourguet, W., Renaud, J.-P., Vivat, V., Champon, P., Moras, D. & Gronemeyer, H. (1998) Nat. Struct. Biol. 5, 87–94
18. Kuiper, G., Enmark, E., Pelto-Huikko, M., Nilsson, S. & Gustafsson, J.-Å. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5925–5930
19. Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Hagglind, J., Nilsson, S. & Gustafsson, J.-Å. (1997) Endocrinology 138, 863–870
20. Pettersson, K., Grandien, K., Kuiper, G. G. & Gustafsson, J.-Å. (1997) Mol. Endocrinol. 11, 1486–1496
21. Cowley, S. M., Hoare, S., Mosemann, S. & Parker, M. G. (1997) J. Biol. Chem. 272, 19858–19862
22. Herwitz, K. B., Jackson, T. A., Ram, D. L., Richer, J. K., Takimoto, G. S. & Tung, L. (1996) Mol. Endocrinol. 10, 1167–1177
23. Glass, C. K., Rose, D. W. & Rosenfeld, M. G. (1997) Curr. Opin. Cell Biol. 9, 222–232
24. Chen, J.-D. & Evans, R. M. (1995) Nature 377, 454–457

spect to its large number of putative nuclear receptor targets, including nonsteroid receptors such as RXR and its heterodimer partners as well as steroid receptors such as ERs. Significantly, RXR and all other nonsteroid receptors do not directly interact with steroid receptors (and vice versa). Furthermore, the nonrequirement of the SHP C terminus including its putative dimerization helix 10/11 for interaction with other receptors clearly points to the existence of different interaction mechanisms for SHP, compared with conventional nuclear receptor homo- and heterodimerization. Although the interaction of other AF-2 cofactors appears to depend on functional LXXLL (NR-box) motifs (30, 32, 35, 42), the central interaction domain of SHP (an 92–148, 50) does not contain such motifs. Curiously, the SHP-specific N terminus, which contains an LXXLL motif, apparently did not interact with RXR or TR (50). These preliminary data are not necessarily contradictory considering that certain cofactors also display NR box independent interactions with the LBD/AF-2 domain (60, 61). Finally, although the SHP interaction surface on the nuclear receptor LBD/AF-2 has not been mapped yet, our own preliminary results suggest that the conserved AF-2 helix 12 is necessary for interaction with SHP. This is in agreement with the inability of SHP to interact with the antagonist-bound ERα, in which the positional rearrangement of helix 12 is thought to interfere with coactivator binding but not with dimerization (15), and it is further consistent with the notion that all SHP-interacting receptors identified so far possess a conserved helix 12 motif.

SHP and DAX-1 Represent a New Category of Nuclear Receptor Coregulators—Based on structural and functional parallels, we suggest that SHP and its closest relative, the orphan receptor DAX-1, represent a new category of negative coregulators for liganded nuclear receptors. Recent evidence suggests that DAX-1 and the orphan receptor SF-1 cooperate in development of steroidogenic tissues (46–48). Interestingly, the nonconserved N terminus of DAX-1 has been demonstrated to interact directly with SF-1 (47). Thus, as for the interaction of SHP with other nuclear receptors, the interaction of DAX-1 with SF-1 does not require the predicted dimerization helix within the DAX-1 LBD. Strongly supporting our findings about SHP and TIF2, there are new indications for the existence of an overlapping interaction surface for DAX-1 and the TIF2-related SRC-1 on SF-1 (46). Furthermore, both SHP and DAX-1 exhibit intrinsic transcriptional repression activity, in agreement with the presence of a conserved putative repression domain (see Fig. 1A) and the recent observation that at least DAX-1 interacts with the nuclear receptor corepressor N-CoR (46). Thus, both SHP and DAX (in the absence of hypothetical ligands, see below) might function as negative coregulators by recruiting conventional corepressors, which usually bind unliganded receptors, to their transcriptionally active receptor targets. Recently, DAX-1 has been found to bind, although not sequence-specifically, to hairpin secondary structures in the promoter region of putative target genes such as the StAR gene (49). Such a DNA-binding function has yet to be established for the SHP-specific N-terminal domain, which is different from the DAX-1 DBD but with its approximately 50 aa is large enough to act as a separate DNA-binding domain. If this domain functions as DBD, the intriguing possibility exists that a putative SHP-nuclear receptor complex may bind to, as yet unknown, novel binding sites. Additionally, in a hypothetical situation, in which SHP would act like DAX-1 as a DNA-bound receptor, a redirection of ligand (e.g. estrogen) signaling to these novel binding sites could take place. Since the AF-2 domain of the interacting nuclear receptor (e.g. ER), according to our view, may be occupied by SHP and not be available for binding of coactivators, transcriptional activity in such a system would depend on other activation domains, for example on the N-terminal AF-1. This is particularly interesting in case of the ERs, since their N termini are non-conserved and perhaps account for functional differences of ERα and ERβ on AP1 sites (8).
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25. Horlein, A. J., Nair, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K. & Rosenfeld, M. G. (1995) *Nature* 377, 397–404

26. Chakravarti, D., Lamorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montminy, M. & Evans, R. M. (1996) *Nature* 383, 99–103

27. Hanstein, B., Eckner, R., Direnzo, J., Halachmi, S., Liu, H., Searcy, B., Kurokawa, R. & Brown, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11540–11545

28. Kraus, W. L. & Kadonaga, J. T. (1998) *Genes Dev.* 12, 331–342

29. Onate, S. A., Tsai, S. Y., Tsai, M. J. & O'Malley, B. W. (1995) *Science* 270, 1354–1357

30. Kalkhofen, E., Valentine, J. E., Heery, D. M. & Parker, M. G. (1998) *EMBO J.* 17, 232–243

31. Voegel, J. J., Heine, M. J., Zechel, C., Chambon, P. & Gronemeyer, H. (1996) *EMBO J.* 15, 3667–3675

32. Voegel, J. J., Heine, M. J., Tini, M., Vivat, V., Chambon, P. & Gronemeyer, H. (1998) *EMBO J.* 17, 507–519

33. Hong, H., Kohli, K., Garabedian, M. J. & Stallcup, M. R. (1997) *Mol. Cell. Biol.* 17, 2735–2744

34. Chen, H. W., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y. & Evans, R. M. (1997) *Cell* 87, 953–959

35. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K. & Rosenfeld, M. G. (1997) *Nature* 387, 677–684

36. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M. & Meltzer, P. S. (1997) *Science* 277, 965–968

37. Li, H., Gomes, P. J. & Chen, J. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 8479–8484

38. Takeshita, A., Cardona, G. R., Kubachi, N., Suen, C.-S. & Chin, W. W. (1997) *J. Biol. Chem.* 272, 27629–27634

39. Bannister, A. J. & Kouzarides, T. (1996) *Nature* 384, 641–643

40. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. & Nakatani, Y. (1996) *Cell* 87, 953–959

41. Spencer, T., Jenster, G., Bucin, M. M., Allis, C. D., Zhou, J. X., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J. & O'Malley, B. W. (1997) *Nature* 389, 194–198

42. Lee, J. W., Kalkhoven, E., Hoare, S. & Parker, M. G. (1997) *Nature* 387, 733–736

43. Xu, J., Liu, D., H., Denayo, F. J., Tsai, S. Y., Tsai, M. J. & O'Malley, B. W. (1998) *Science* 279, 1922–1925

44. Seol, W., Chung, M. & Moore, D. D. (1997) *Mol. Cell. Biol.* 17, 7126–7131

45. Treuter, E., Albretsen, T., Johansson, L., Leers, J. & Gustafsson, J. (1998) *Endocrinol.* 12, 864–881

46. Spyrou, G., Enmark, E., Miranda-Vizuete, A. & Gustafsson, J. (1997) *J. Biol. Chem.* 272, 2956–2961

47. Li, H.-K., Lee, Y.-K., Park, S.-H., Kim, Y.-S., Park, S. H., Lee, J. W., Kwon, H.-B., Soh, J., Moore, D. D. & Choi, H.-S. (1998) *J. Biol. Chem.* 273, 14398–14402

48. Smith, C. L., Nawaz, Z. & O'Malley, B. W. (1997) *Endocrinol.* 11, 657–666

49. Zhang, X., Jeyakumar, M., Petukhov, S. & Bagchi, M. K. (1998) *Endocrinol.* 12, 513–524

50. Thompson, P. D., Jurutka, P. W., Haussler, C. A., Whitfield, G. K. & Haussler, M. R. (1998) *J. Biol. Chem.* 273, 13060–13065

51. Lee, J. W., Ryan, F., Swaffield, J. C., Johnston, S. A. & Moore, D. D. (1995) *Nature* 374, 91–94

52. Chang, K. H., Chen, Y., Chen, T. T., Chou, W. H., Chen, P. L., Ma, Y. Y., Yang-Peng, T. L., Leng, X., Tsai, M. J., O'Malley, B. W. & Lee, W. H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 9040–9045