Characterization of the Interaction between Androgen Receptor and a New Transcriptional Inhibitor, SHP†

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ABSTRACT: SHP (short heterodimer partner) is an orphan nuclear receptor, first described for its interaction with nuclear receptors. This study explores a new way of inhibiting the androgen-signaling pathway. We demonstrated that SHP inhibited up to 97% of AR-induced activity. Characterization of AR/SHP interaction provided evidence of a clear ligand dependency. We also showed that the LXXI/LL motifs previously found on SHP mediated the interaction with the AR ligand-binding domain (AR-LBD), the motif responsible for the interaction being slightly different from that found with ER. The AR N-terminal domain (AR-NTD), in contrast to that of other nuclear receptors, accounts for most of the entire receptor transactivation potential. SHP also interacted with AR-NTD, thus stabilizing the interaction with AR. We demonstrated that SHP inhibited both AR-LBD and NTD-dependent transactivation, which evidenced for the first time a protein capable of inhibiting a steroid receptor amino-terminal-dependent transactivation. We further characterized the SHP mechanism of action by showing that SHP reversed AR coactivator-mediated activation. Conversely, FHL2 and TIF2 counteracted SHP-mediated inhibition of AR. SHP evidences a new way of inhibiting AR activity by competing with AR coactivators. This new type of inhibitor could dictate the activity of nuclear receptors, depending on the equilibrium between activators and inhibitors.

The androgen receptor is a ligand-activated transcription factor. As a member of the steroid receptor subfamily, it possesses a modular structure (Figure 1A), and its structural organization shows a high level of molecular identity with other members of the subfamily. Functional domains have been characterized. The variable N-terminal domain (NTD) is implicated in transactivation and contains the transcriptional activation region AF-1 (aa 51−211), which is essential for transactivation activity in the full-length receptor, and AF-5 (aa 370−494), which shows a strong constitutive activity in the LBD deletion mutant AR (1−660) (*1*). NTD accounts for most of the AR-dependent transcription activation. The highly conserved zinc finger-type DNA-binding domain (DBD) mediates the interaction with a DNA response element. The ligand-binding domain (LBD) contains the strictly ligand-dependent transcriptional activation region AF-

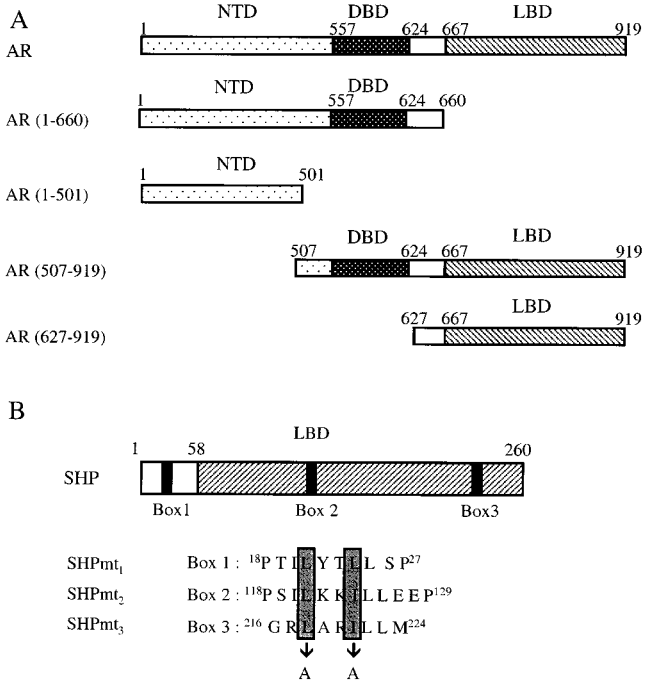

FIGURE 1: Schematic representation of the constructs used in the study. (A) Functional domains of the AR deletion mutants. (B) SHP mutants. The three SHP nuclear boxes were mutated. Amino acids, +1 and +4, of each box were substituted by alanine in boxes 1, 2, and 3, respectively.

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[1] Abbreviations: AR, androgen receptor; ER, estrogen receptor; NTD, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain; SHP, short heterodimer partner; TIF2, transcription intermediary factor 2; FHL2, four and an half lim-only protein 2; TR4, TR4 orphan receptor; HBO1, human origin recognition complex interacting protein 1; R1881, methyltriolenone; NR box, nuclear receptor interacting box; AF-2, activation function domain 2; GST, gluthatione *S*-transferase; PCR, polymerase chain reaction.

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2, which is very weak as compared with that of other steroid receptors. The LBD can functionally interact with intermediary factors and nuclear cofactors (*2*). Recent studies suggest that interaction between NTD and LBD results in the formation of surfaces that allows interaction with NR cofactors (*3*).

Cofactors are transcriptional adapters with no specific DNA-binding affinity. They are organized in multiprotein complexes with various and distinct enzyme activities which either stimulate (co-activators) or repress (co-inhibitors) the transcriptional activity of target genes. Most of the cofactors to the AR isolated so far were co-activators, including FHL2 which was recently described as a specific activator of AR (*4*). The situation regarding AR co-repressors is somehow different since only a few modulators and inhibitors, ARIP3 (*5*), TR4 (*6*), and HBO1 (*7*), are characterized. Our main interest is to shed light on the mechanism of AR activity modulation by cofactors.

An atypical protein, SHP, was described to inhibit some nuclear receptors. SHP was first isolated for its interaction with RXR (retinoid X receptor) (*8*). It is an orphan NR with no NTD and no standard DBD, but it has a putative LBD like its closest relative in the NR superfamily, DAX-1 (*9*). SHP is a nuclear protein of 260 amino acids with a molecular mass of 29 kD, an intrinsic repression domain, and three LXXLL-like domains (Figure 1B). It was named SHP (short heterodimer partner) on the basis of its ability to interact with a large variety of NRs. SHP has been shown to repress RXR heterodimers by a mechanism of competitive dimerization. SHP was also shown to interact with the activated estrogen receptor (ER) via both NR boxes 1 and 2 and to inhibit ER-dependent activation (*10*−*12*). In other studies, SHP was found to play a major role in the feedback loop of biliary acid in the liver (*13*, *14*). Recently, mutations of SHP were reported to be involved in pathological obesity (*15*). Moreover, SHP mRNA was shown to be expressed in androgen target tissues (*10*). Therefore, we questioned the putative implication of SHP in the regulation of AR activation.

Herein, we present evidence that SHP is a transcriptional repressor of AR. We demonstrate that SHP interacts both in vitro and in vivo with the full-length receptor. It can repress AR activation in the different cell types studied. In addition, we provide evidence that SHP targets not only AF-2-dependent transactivation but also NTD-dependent activation. Moreover, SHP is able to reverse the action of AR coactivators such as FHL2 or TIF2. SHP seems to mimic the presence of coactivators on AR and compete with them, providing a new mechanism of androgen-dependent inhibition.

EXPERIMENTAL PROCEDURES

Plasmids. Mammalian Expression Plasmids. pCMV5-AR was a generous gift of Terry Brown (*16*). pCMV5-AR (1−660) was previously described (*17*). pCMV5-AR (507−919) was obtained by digestion of pCMV5-AR with *Bgl*II and *Kpn*I and insertion of the resulting fragments into the pCMV5 plasmid using a blunt ligation method. pSG5-SHP and pSG5-SHPmt$_1$, -mt$_2$, and -mt$_3$ were generous gifts of Lotta Johansson. pCMX-FHL2 was a generous gift of Roland Schule, and pSG5-TIF2 was a generous gift of Hinrich Gronemeyer.

Yeast Expression Plasmids. pGBKT7 and pGADT7, allowing respectively the expression of GAL4DBD- and GAL4AD-fused proteins, were purchased from CLONTECH. pGBK-hAR (627−919) was constructed by inserting the PCR-generated fragment of hAR cDNA into the *Bam*HI/*Sal*I sites of pGBKT7. pGBK-SHP and pGAD-SHP were obtained by digestion of pSG5-SHP with *Eco*RI and insertion of the resulting fragments into the *Eco*RI site of pGBKT7 and pGADT7, respectively. pGBK-SHPmt$_1$, pGAD-SHPmt$_1$, pGBK-SHPmt$_2$, pGAD-SHPmt$_2$, pGBK-SHPmt$_3$, and pGAD-SHPmt$_3$ were constructed by inserting into the *Eco*RI/*Bam*HI sites of, respectively, pGBKT7 and pGADT7 the fragments resulting from digestion of pSG5-SHPmt$_1$, pSG5-SHPmt$_2$, and pSG5-SHPmt$_3$ with *Eco*RI/*Bam*HI. Full-length AR was obtained by digesting pGBK-AR with *Nde*I/*Bam*HI and inserted into the *Nde*I/*Bam*HI sites of pGAD to create pGAD-AR.

Bacterial Expression Plasmids. pGEX vectors allowing expression of glutathione *S*-transferase (GST) were purchased from Pharmacia. SHP was obtained by digestion of pSG5-SHP with *Eco*RI and inserted into pGEX4T1 at the same site to create pGEX-SHP. pGEX-SHPmt$_1$, pGEX-SHPmt$_2$, and pGEX-SHPmt$_3$ were constructed by inserting into the *Eco*RI/*Sal*I sites of pGEX4T1 the fragments resulting from digestion of, respectively, pGBK-SHPmt$_1$, pGBK-SHPmt$_2$, and pGBK-SHPmt$_3$ with *Eco*RI/*Sal*I.

Plasmids for In Vitro Translation. AR (36−919) was obtained by digesting pCMV-AR with *Xma*I and *Bam*HI and then inserted into pGBKT7 at the same sites to obtain pGBK-AR (36−919). The cDNA encoding the first 36 amino acids of AR was amplified by PCR and then inserted into the *Nde*I/*Xma*I sites of pGBK-AR (36−919) to create pGBK-AR. AR (1−501) was amplified by PCR and then cloned into the *Nde*I/*Eco*RI sites of pGADT7 to create pGBK-AR (1−501).

β-Galactosidase Assays. Yeast strain AH109 was transformed with the appropriate recombinant pGBKT7 and pGADT7 vectors and grown overnight in an SD medium lacking tryptophan and leucin. Two milliliters of the overnight culture were transferred to 9 mL of YPD, and the fresh culture was incubated at 30 °C until the cells reached the mid-log phase. Next, 1.5 mL of culture was centrifuged, washed with Z buffer, and resuspended in 300 μL of Z buffer, of which 100 μL was placed in liquid nitrogen until cells were frozen. Cells were then thawed at 37 °C. This freeze/thaw cycle was repeated two more times. Next, 0.7 mL of Z buffer + β-mercaptoethanol + 160 μL of ONPG were added to the lysate, and after the yellow color developed, 0.4 mL of 1 M Na_2CO_3 was added. After centrifugation, the OD_{420} of the supernatant was measured, and the β-galactosidase units were calculated using the formula: β-galactosidase units = $1000 \times OD_{420}/(0.5t \times OD_{600})$, where τ is the elapsed time (in min) of incubation, and $OD_{600} = A_{600}$ of 1 mL of culture. All values represent the mean (±SD) from triplicates and were reproduced in at least three independent experiments.

Cell Lines, Transfection, and Luciferase Assay. CV-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), and CHO cells were cultured in DMEM-F12, both supplemented with 10% fetal bovine serum. CV-1 cells were transiently transfected using the calcium phosphate method in 12-well dishes with 0.1 μg pCMV-hAR [or AR mutant pCMV-hAR (1−660)]; 1 μg p-mouse-mammary-tumor-

virus-luciferase (MMTV-luc), used as an androgen-regulated gene; 0.5 μg pCMV-β-galactosidase; various concentrations of pSG5-SHP or mutants; and various concentrations of pCMX-FHL2. CHO cells were transfected using the FUGENE-6, according to the manufacturer's instructions (ROCHE), in 12-well dishes with 0.05 μg pCMV-hAR [or 0.1 μg AR mutant pCMV-hAR (507−919) with 0.2 μg pSG5-TIF2]; 0.25 μg p-mouse-mammary-tumor-virus-luciferase (MMTV-luc), used as an androgen-regulated gene; 0.125 μg pCMV-β-galactosidase; and various concentrations of pSG5-rSHP or mutants. The amount of plasmid vector (pSG5, pCMX) was kept constant in all experiments.

Twelve hours after transfection, the cells were incubated with 1 nM R1881. After 24 h at 37 °C, the cells were harvested in a lysis buffer: 25 mM Tris-H_3PO_4 (pH 7.8), 2 mM DTT, 2 mM EDTA, 1% TritonX-100, and 10% glycerol. Aliquots were used in the β-galactosidase activity assay. The luciferase activity was measured by the reaction of lysate with the luciferin solution: 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP, 20 mM Tris-H_3PO_4, 1.05 mM $MgCl_2$, 2.7 mM $MgSO_4$, 0.1 mM EDTA, and 33 mM DTT. Luciferase activity was measured as relative light units (RLU) on a luminometer (LKB Instruments, Rockville, MD). Luciferase activity in the presence of 1 nM R1881 was set as 100%. All values represent the mean RLU/β-galactosidase (±SD) from triplicates and were reproduced in at least three independent experiments.

In Vitro Transcription, Translation, and Activation. Expression plasmids [pGBK-hAR, pGBK-AR (1−501), pGBK-AR (627−919)] were transcribed and translated with the TNT^{T7}-coupled reticulocyte Lysate System (Promega, Charbonieres, France) in the presence of [^{35}S] methionine (1000 Ci/mmol, ICN, Orsay, France), according to the manufacturer's instructions, for 1.5 h at 30 °C. Each aliquot was divided in two, and R1881 was added to a final concentration of 10^{-6} M to one aliquot. Both were incubated for 30 min at 37 °C.

GST Pull-Down Assays. GST-SHP and GST-SHP mutants were expressed in *Escherichia coli*, BL21 strain. Bacteria were transformed with pGEX-SHP and grown overnight at 37 °C. The ON culture was diluted to 1/100 and grown at 37 °C until OD_{600} reached 0.6. IPTG was then added to a final concentration of 0.5 mM, and induction continued at 37 °C for 3 h. Bacteria cells were collected by centrifugation, resuspended in a lysis buffer (PBS containing 1 mM PMSF, 0.5 mg/mL lysosyme, 20 μg/mL DNaseI, 10 μg/mL RNaseI, 10 mM $MgCl_2$, and 1 mM $MnCl_2$) and incubated at 4 °C for 30 min. The lysate was then incubated for 45 min at 4 °C with GSH-sepharose beads in PBS. The beads were washed 4 times with 10 mL of PBS and one more time with PDB (PBS containing 20 mM Hepes-KOH, pH 7.9, 10% glycerol, 100 mM Kcl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF). Similar extracts with GST alone were performed. To each aliquot of 100 μL containing 30 μL of GSH-sepharose was mixed either [^{35}S]hAR or [^{35}S]AR (1−501) or [^{35}S]AR (627−919). It was then incubated for 3 h at 4 °C and washed 4 times with PDB. SDS buffer (30 μL) was added to the beads and boiled for 5 min. Proteins were separated on a 12% SDS-PAGE. Gels were dried, colored with Coomassie Blue, and autoradiographies were performed with Kodak biomax films. The figures are representatives of at least three independent experiments.

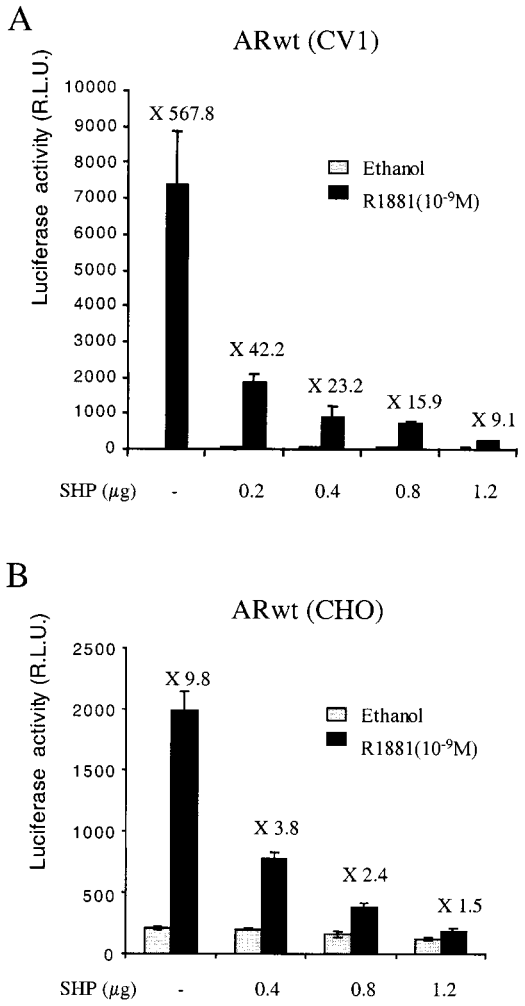

FIGURE 2: SHP inhibits AR activity and interacts with AR both in vivo and in vitro. (A) SHP inhibits AR activation in a dose-dependent manner. CV1 cells were transiently transfected using the calcium phosphate method. (B) SHP inhibits AR activation in a dose-dependent manner in CHO cells. They were transiently transfected with FUGEN-6. The luciferase activity was normalized with the β-galactosidase value and expressed in relative light units (RLU). The value above each set of SHP treatment represents the fold induction by R1881. All values represent the mean from triplicate transfections and were reproduced in at least three independent experiments. In this study, we determined the luciferase value of ARwt as 100% of activity.

RESULTS

SHP Inhibits AR Activation in Mammalian Cells. The different AR and SHP constructs we used in our study are summarized in Figure 1. To determine whether SHP interferes with AR transcriptional activation in response to R1881, we performed transient cotransfection studies in CV1 and CHO cells. These cell types, CV1 (derived from monkey kidney cells) and CHO (Chinese hamster ovary cells), were selected because they do not express endogenous AR. Therefore, reporter gene activation depends only on the expression of exogenous AR. Transfection of increasing amounts of SHP in the two cellular types represses AR ligand-induced activity in a dose-dependent manner (see Figure 2A and B). No variation of the basal activity was observed in either CV1 or CHO cell types. SHP inhibited 97% of the AR ligand-induced activity in CV1 cells. In CHO cells, the inhibition was similar, with 87% inhibition of the activity. Variations between the two cell types may be due

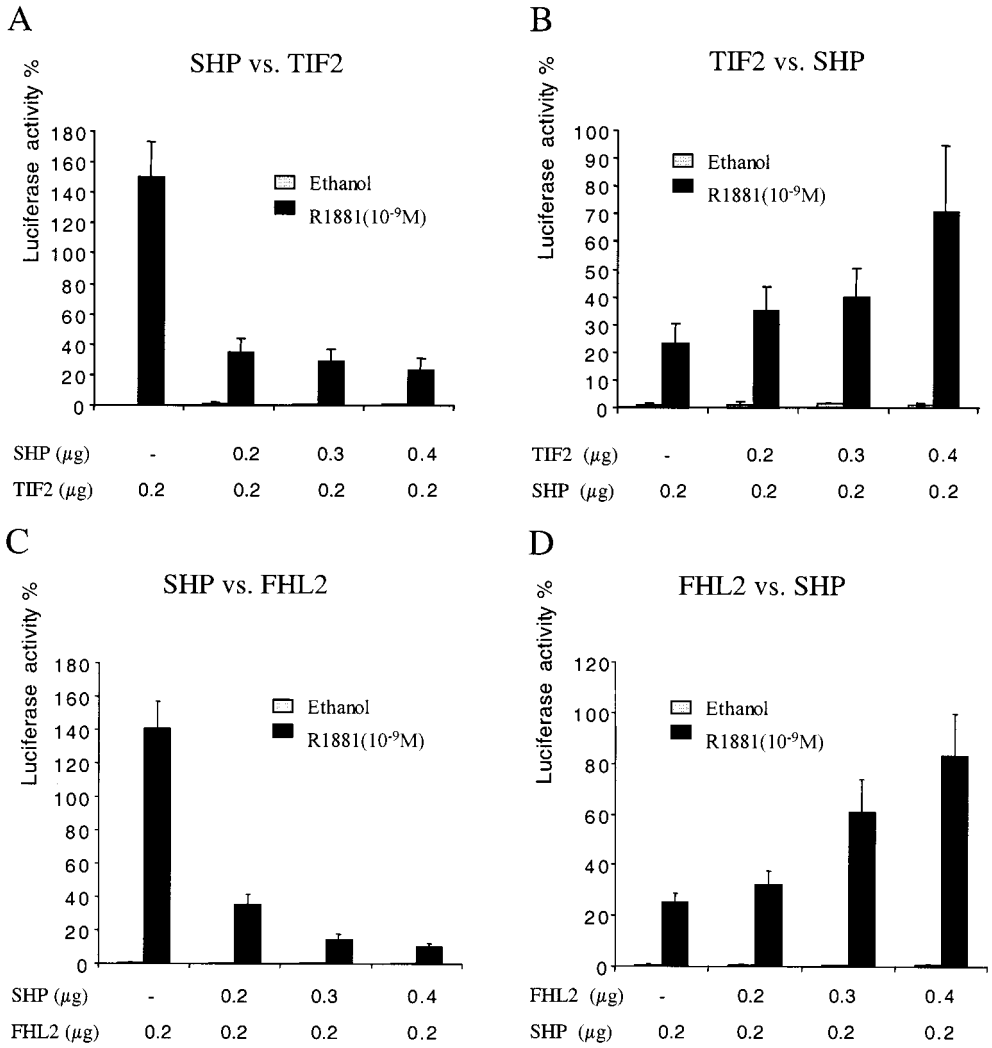

FIGURE 3: SHP reverses AR coactivator-induced activity. (A) SHP reverses AR overactivation due to TIF2, a coactivator that belongs to the p160 family, in a dose-dependent manner. (B) TIF2 restores AR activity, previously repressed by SHP, in a dose-dependent manner. (C) In the same way, SHP reverses overactivation due to FHL2, a specific coactivator of AR that enhances its activity in the presence of ligand. (D) FHL2 reverses the SHP inhibition of AR activity and restores it in a dose-dependent manner. These experiments were performed in a transiently transfected CV1 cell line, in which TIF2, FHL2, and SHP were cotransfected by the calcium phosphate method as described in Figure 2.

to a difference in the endogenous cofactor expression. Binding assays allowed us to conclude that SHP did not down-regulate the AR expression in transfected cells (data not shown). A CV1 cell line was chosen for the next experiment because SHP seems to be a better inhibitor in this cell type.

SHP and AR Coactivators Act Conversely on AR Ligand-Dependent Activation. To further characterize the mechanism by which SHP inhibits ligand-dependent AR transcriptional activity, we performed transient cotransfection experiments in CV1 cells according to methods described in the experimental procedures. We used two different coactivators, TIF2 and FHL2, that allowed us to up-regulate the ligand-dependent AR activation. TIF2 is a p160 protein that presents no specificity for AR and possesses LXXLL domains implicated in NR regulation. TIF2, in the presence of R1881, up-regulated AR activation up to 150% of its activation with the ligand alone (Figure 3A). We showed that SHP reversed the TIF2-mediated AR activation and, conversely, that TIF2 was able to counteract SHP-mediated inhibition; both did so in a dose-dependent manner (Figure 3A and B). FHL2 is a 32 kDa protein specific to AR that does not possess any LXXLL domain. Like TIF2, FHL2, in the presence of R1881, up-regulated AR activation up to 140% of its activation with the only ligand (Figure 3C). In a similar manner, SHP reversed the FHL2-mediated AR activation and, conversely, FHL2 was able to counteract SHP-mediated inhibition; both did so in a dose-dependent manner (Figure 3C and D). These results support the hypothesis that SHP inhibits AR-mediated gene activation by competing with AR coactivators.

SHP Inhibits Both AR-LBD and NTD-Dependent Activity. We then investigated which AR domains were implicated in the repression. We first performed transient transfection assays in CV1 cells to determine whether SHP represses the AR-LBD-dependent activation. In these assays we used the deletion mutant AR (507−919) that lacks NTD. This mutant does not show any transcriptional activity per se. Therefore, we cotransfected it with a determined amount of the coactivator TIF2 that allowed us to restore its transactivation ability up to 80% of the full-length receptor (Figure 4A). TIF2 may permit the recruitment of the core transcriptional machinery. This activation is strictly ligand-induced. All the

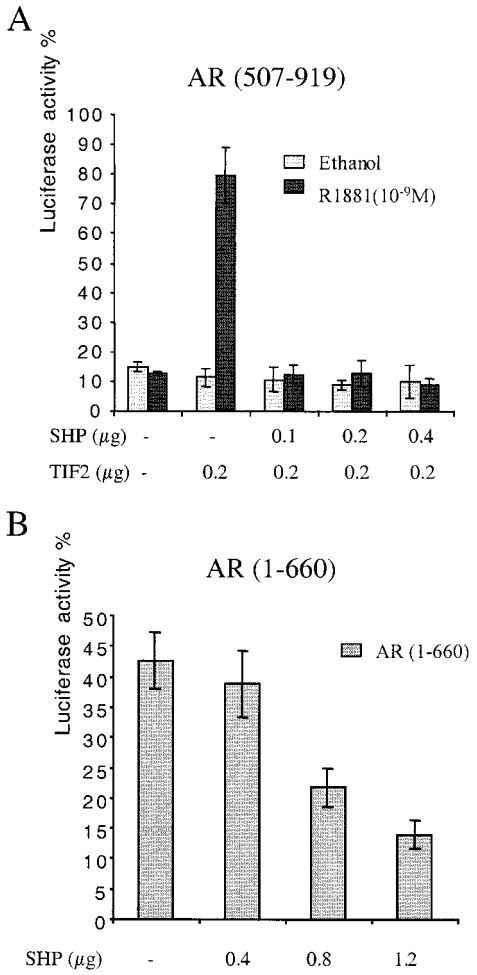

FIGURE 4: AR inhibits both LBD and NTD of AR. A In CHO cells, SHP inhibits AR (507−919) + TIF2 transcriptional activity in the presence of R1881. (B) In CV1 cells, SHP represses AR (1−660) constitutive activity in a dose-dependent manner. Transient transfections were performed as described in Figure 2.

amounts of tested SHP completely abolished the TIF2-induced AR (507−919) activity (Figure 4A). We then examined the effect of SHP on AR-NTD-dependent activation. We used the mutant AR (1−660) which shows a strong constitutive transcriptional activity. Increasing amounts of SHP were transfected and inhibited AR (1−660) up to 70% of AR (1−660) activity, which also represents 30% of the full-length receptor activity (Figure 4B). These results support the conclusion that SHP represses both the AR carboxyl and the amino-terminal domains' transcriptional-dependent activity in a dose-dependent manner.

SHP Interacts with AR Both In Vivo and In Vitro and Targets Both AR-LBD and NTD. To examine the potential interaction between SHP and activated AR, we performed a yeast two-hybrid assay using Gal4DBD-SHP and Gal4AD-AR. Gal4DBD-p53 and Gal4AD-Large T were used as a control for the interaction. As shown in Figure 5A, SHP clearly interacted, in vivo, with AR. Here we can observe a basal interaction between SHP and AR (compare lane 3 to lane 2), but this interaction was significantly enhanced by R1881 (compare lane 3 to lane 4). To confirm this interaction, we performed GST pull-down assays using GST-SHP and in vitro translated and ^{35}S labeled AR. GST-SHP bound specifically to AR (Figure 5B), which failed to interact with GST. We noted that AR interacted directly with SHP both in the absence and in the presence of saturating R1881 concentrations. Similar observations have been described for other cofactors (*3*, *18*). Thereafter, to specify the AR domains mediating the protein−protein interaction with SHP in vitro, we used AR deletion mutants, AR (627−919) (AR-LBD) and AR (1−501) (AR-NTD) (see Figure 1A). Mutants were translated and labeled with ^{35}S in the same conditions as the full-length receptor. GST pull-down allowed us to show that GST-SHP binds both AR (627−919) and AR (1−501) (Figure 5C D). Of note, on the contrary to full-length AR or AR (1−501), in vitro translation of AR(627−919) resulted in two bands probably due to instability of this AR fragment. The lower band may be a degradation product of AR (627−919), although existence of an alternative start codon cannot be excluded. As for the full-length receptor, AR-LBD interacted in a similar manner with GST-SHP both in the presence and in the absence of ligand at saturating concentration. SHP interacted with AR and targeted both its amino- and carboxyl-terminal domains.

Effect of SHP Mutants on Interaction with AR and Receptor-Dependent Activation. SHP has been described to have three LXXI/LL motifs, named NR boxes, as shown for cofactors of the p160 family (*19*). To determine whether these three motifs are implicated in a functional interaction with AR, we used three SHP mutants in which amino acids +1 and +4 of the LXXI/LL motifs were mutated into alanines (see Figure 1B). In vivo, analysis of the AR/SHP mutant interaction was performed in yeast. $SHPmt_1$ and $SHPmt_3$ interacted with the full-length receptor (AR) (Figure 6A, compare lanes 6 and 7 to lanes 3 and 4 and compare lanes 12 and 13 to lanes 3 and 4). Remarkably, mutation of NR box 2 ($SHPmt_2$) was sufficient to completely abolish the ligand effect on the interaction between AR and SHP (Figure 6A, compare lanes 9 and 10 to lanes 3 and 4). It is interesting to note that the basal interaction between AR and SHP was not affected by the $SHPmt_2$ or the other mutants (Figure 6A, compare lanes 3, 6, 9, and 12). We then investigated the effect of mutants on AR-dependent transactivation. We performed transient transfection into CV1 cells to analyze SHP mutants' inhibitory capacity on the full-length AR-dependent activation. Inhibition by $SHPmt_1$ and $SHPmt_3$ of AR activation was the same as with the wild-type SHP (see Figure 6B, compare lanes 5, 6, 7 and 11, 12, 13 to lanes 2, 3, 4, respectively). $SHPmt_2$ clearly showed a weaker inhibitory effect on the AR ligand-dependent activation than the wild type (see Figure 6B, compare lanes 8, 9, 10 to lanes 2, 3, 4) but maintained a partial ability to inhibit the full-length receptor. Thus, SHP NR box 2 is implicated in the action of SHP on AR activity.

SHP NR Box 2 Is Necessary for Interaction with AR-LBD and Inhibition of AR-LBD-Dependent Activity. The LXXLL motifs of the p160 coactivator family are differentially implicated in the interaction with AR-LBD and the LBD of other NRs (*20*). To narrow down which domains of AR are contacted by SHP mutants, we performed yeast two-hybrid assays. Interaction between AR-LBD and SHP was strictly dependent on the presence of R1881 (Figure 7A, lanes 4 and 5). $SHPmt_1$ interacted with AR-LBD as did the full-length receptor, whereas $SHPmt_3$ presented a weak decreased interaction. As for mutation of NR box 2, it completely abolished the interaction (Figure 7A, lane 9). It is of particular interest to note that there was no basal interaction between AR-LBD

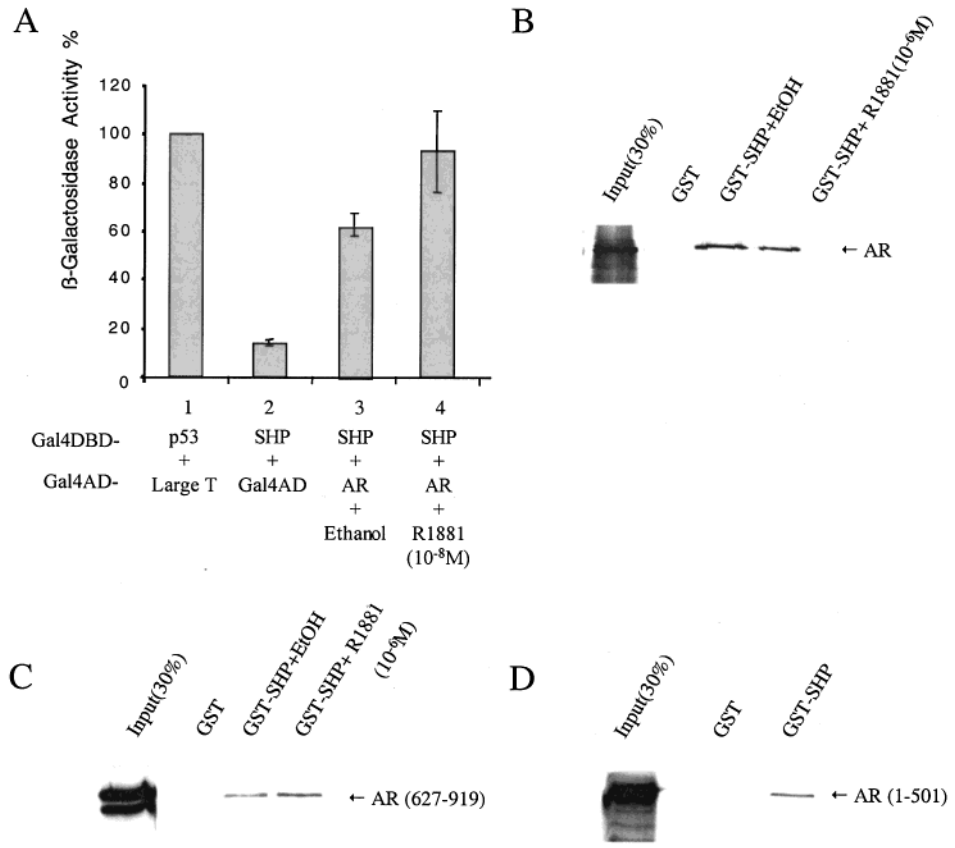

FIGURE 5: SHP interacts with AR and targets both AR-LBD and NTD. (A) SHP interacts with AR in a ligand-dependent manner. β-galactosidase activity in yeast is expressed as the percentage of the positive control, that is, the interaction between Gal4DBD-p53 and Gal4AD-Large T. (B) In vitro, AR interacts with SHP. GST pull-down assays were performed with in vitro translated ^{35}S labeled AR and GST-SHP protein in the presence or absence of its cognate ligand. GST protein was used as a control. (C) AR-LBD interacts with GST-SHP in vitro. AR (627−919) was expressed as described above. GST protein was used as a control for the interaction. (D) AR-NTD interacts with GST-SHP in vitro. AR (1−501) was expressed as described above. GST protein was used as a control for the interaction. The input represents 30% of the amount of labeled protein used in each pull-down.

and SHP, indicating that the basal signal observed with full-length AR (see Figure 6A) was probably due to the interaction between AR-NTD and SHP. To parallel interaction and effect on AR transactivation, we examined the involvement of the SHP mutations in the inhibition of AR-LBD ligand-dependent activity by transient transfection in CHO cell lines. AR-LBD activity was restored as previously described with the coactivator TIF2. SHPmt$_1$ and SHPmt$_3$ were as effective as the wild type to inhibit AR-LBD activity in the presence of R1881 (Figure 7B, lanes 3 and 5). SHPmt$_2$, in contrast, showed a clear decrease in its capacity to inhibit AR-LBD activity in the presence of R1881, with only 50% of the LBD ligand-dependent activity inhibited by the mutant (Figure 7B, lane 4). SHP NR box 2 is necessary for the interaction with AR and is strongly implicated in its ligand-dependent transcriptional activity repression.

Interaction with and Inhibition of AR-NTD Is Not Dependent on SHP NR Boxes. In contrast to other steroid receptors, AR-NTD plays a major role in receptor-dependent activation. Therefore, it was interesting to determine whether SHP NR boxes had any effect on the interaction with AR-NTD. We performed GST pull-down assays using GST-SHP, GST-SHP mutants, and in vitro translated and ^{35}S labeled AR (1−501). GST alone was used as a control. In Figure 8A, we show that GST-SHP mutants interacted exactly as the GST-SHP with AR (1−501). SHPmt$_2$ was the only mutant to affect both full-length AR and AR-LBD-dependent transactivations; we thus questioned whether the mutation of NR box 2 would be implicated in AR-NTD-dependent activity inhibition. We performed transient cotransfection studies in CV1 cells using SHPmt$_2$ and AR (1−660). SHPmt$_2$ repressed the AR (1−660) transcriptional activity as did SHP (compare Figure 8B and Figure 4B) in a dose-dependent manner. In conclusion, NR boxes are not implicated in the AR-NTD/SHP interaction, and SHP NR box 2 does not participate in the inhibition of AR-NTD-dependent activity.

DISCUSSION

In this study, we characterized a new inhibitory protein of the androgen receptor transcriptional activity. SHP, which is expressed in androgen target tissues, inhibited both carboxyl-terminal-dependent and amino-terminal-dependent transactivations. We also evidenced that SHP can inhibit and reverse the effect of AR coactivator proteins, TIF2 and FHL2. We could evidence that the second LXXIL motif was necessary to mediate the interaction between AR-LBD and SHP, whereas these nuclear boxes' motifs were not implicated in the interaction between AR-NTD and SHP.

The two coactivators, FHL2 and TIF2, show strong differences in their nuclear receptor interacting motifs and specificity. TIF2 belongs to the p160 family of proteins and is not specific for AR. It was described to be both an AF-2 interacting protein via its LXXLL motifs and an NR-NTD

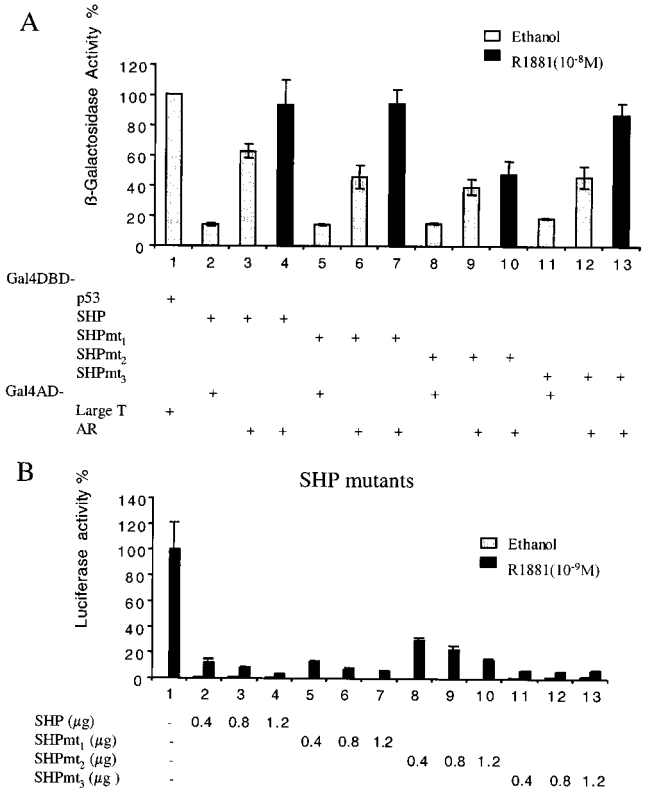

FIGURE 6: Differential effects of SHP mutations on AR/SHP interaction and AR repression. (A) The $SHPmt_2$ shows a clear decrease in its interaction with AR. As for Figure 5, β-galactosidase activity in yeast is expressed as the percentage of the positive control, that is, the interaction between Gal4DBD-p53 and Gal4AD-Large T. (B) In CV1 cells, the mutant, $SHPmt_2$, presents a clear decrease in its capacity to inhibit AR activity. Transient transfections were performed as described in Figure 2.

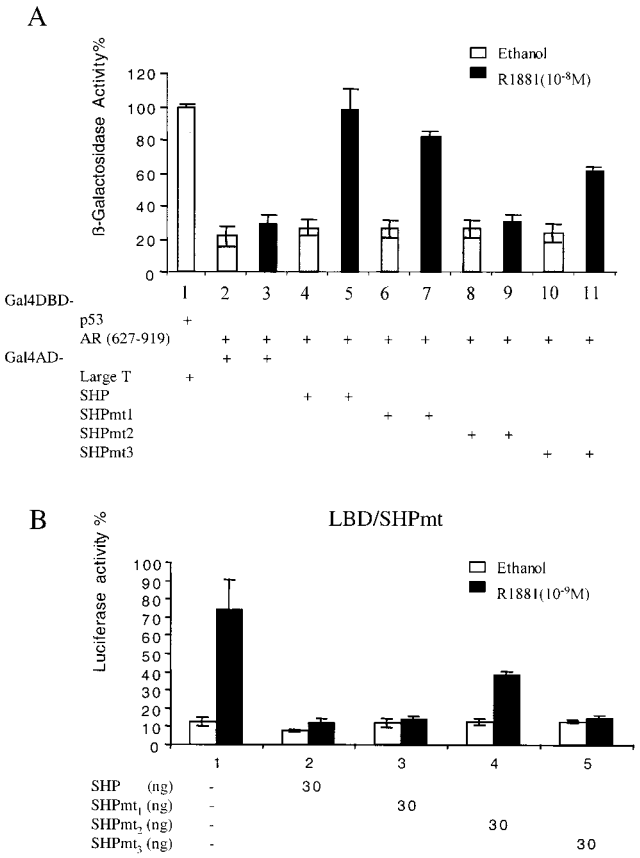

FIGURE 7: SHP box 2 is implicated in the interaction with AR-LBD. (A) Interaction between AR ligand-binding domain and SHP mutants. The AR-LBD, that is, AR (627−919), was fused to GAL4DBD, and the different SHP mutants were fused to GAL4AD. AR (627−919)-dependent activation is given in lanes 2 and 3. β-galactosidase activity is expressed as the percentage of the positive control, that is, the interaction between Gal4DBD-p53 and Gal4AD-Large T. SHP interacts in vivo with AR-LBD in the presence of R1881. $SHPmt_2$ is unable to interact with AR-LBD in vivo. (B) In CHO cells, the $SHPmt_2$ presents a clear decrease in its capacity to inhibit AR (507−919) + TIF2 activity. Transient transfections were performed in the same conditions described in Figure 2.

interacting protein (*21*, *22*). FHL2, which lacks LXXLL motifs, was described as a specific AR coactivator that interacts only with the full-length receptor (*3*). In addition, in vivo, AR activity is mainly governed by N/C interactions (*23*). They may also permit the generation of new interacting surfaces allowing interaction with coactivators (*3*). The FXXLF and WXXLF motifs located in AR-NTD and AF-2 located in the LBD were found to be directly involved in this interaction (*24*). Still, the functional significance of the N/C interaction remains unclear. The mutant including the AR amino-terminal and DNA-binding domain, but lacking the LBD, has a strong constitutive transcriptional activity on a reporter gene (*25*). In this context, we demonstrated in Figure 4A that SHP was able to fully counteract TIF2-induced activity of the LBD, whereas SHP inhibition of the amino-terminal-induced transactivation (Figure 4B) was not as complete. In addition, it was previously reported that the affinity of p160 coactivators for AR-LBD is not as strong as for other nuclear receptors LBD (*23*). Furthermore, a previous study noted that, in vitro, SHP competed directly with two AF-2 interacting peptides on ER-LBD. These peptides contained, respectively, the NID (nuclear receptor interacting domain) of TIF2 (aa 594−766) and RIP140 (aa 747−1158) (*10*). This supports the hypothesis that SHP can compete directly with AR coactivators on AR-LBD. Therefore, it is tempting to hypothesize that SHP would destabilize the interaction between coactivators and AR AF-2 and allow a synergistic interaction with the AR amino-terminal domain.

In terms of recognition domains, we demonstrated that SHP interacted with both AR-NTD and -LBD. SHP was described to possess three LXXI/LL NR boxes (*12*), and we showed that the second NR box was directly implicated in both AR-LBD/SHP interaction and inhibition of AR-LBD-dependent activity. In a previous study, SHP NR boxes 1 and 2 were reported to be involved in a synergistic manner in the ER-LBD/SHP interaction; mutation of one of these NR boxes was not sufficient to abolish the ER/SHP interaction, whereas mutations of both motif 1 and motif 2 completely abolished the ER/SHP interaction (*12*). Like SHP, LXXLL-containing coactivators show a redundancy in their NR box motifs. In a recent study, it was shown that the four LXXLL motifs of SRC-1 interacted differentially with nuclear receptors. Motif 2 preferentially interacted with ERα and ERβ, while motif 4 preferentially interacted with GR, AR, PPARα, and PPARγ (*20*). SHP NR boxes seem to be differentially implicated in interaction with AR and ER. In addition, the interaction between SHP and AR-NTD may also participate in the specificity of SHP targets such as the three LXXI/LL motifs. This interaction seems to be specific to AR. Indeed, no similar interaction was described between SHP and ER or HNF-4. Moreover, as previously described,

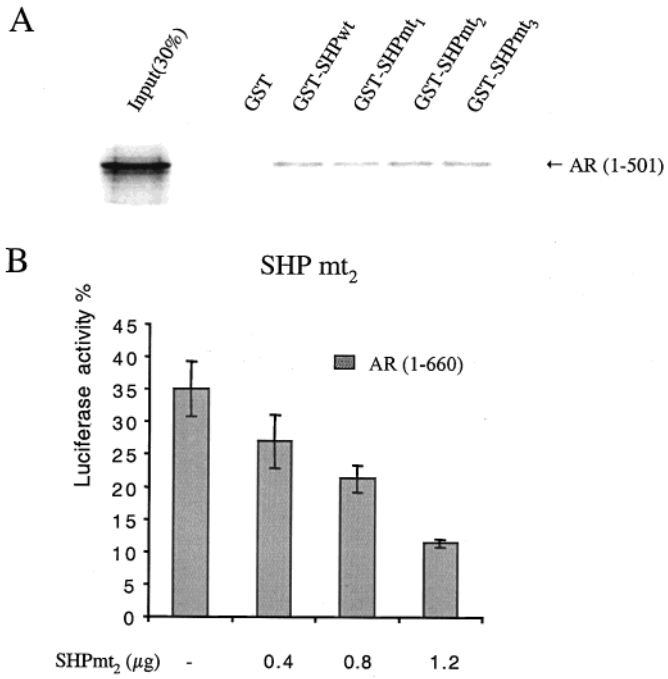

FIGURE 8: SHP NR boxes are not implicated in the interaction with AR−NTD. (A) Interaction between GST-SHP mutants and AR (1−501) is the same as wild-type GST-SHP. AR (1−501) is expressed as described in Figure 5. GST alone was used as a control for the interaction. The input represents 30% of the amount of labeled protein used in each pull down. (B) $SHPmt_2$ inhibits AR (1−660) constitutive activity like the wild-type SHP (Figure 4B). These experiments were performed in a transiently transfected CV1 cell line as described in Figure 2.

the interaction between AR-LBD and LXXLL-containing cofactors such as p160 proteins is weaker than interactions with other receptor LBDs. Interaction with AR-NTD could therefore stabilize the interaction between cofactor and AR, as may be the case with SHP (*26*).

An alternative scheme may be envisaged to explain SHP inhibition of AR-induced activation. SHP was described to possess an intrinsic repression domain located in its C-terminal domain (*27*). Like SHP, DAX-1, its closest relative in the NR superfamily, is an LXXLL-containing repressor that presents a carboxyl-terminal repression domain (*9*, *28*). DAX-1 is implicated in steroid receptor regulation. It interacts with steroidogenic factor 1 (SF-1) and represses its transcriptional activity by recruiting N-CoR, which harbors a strong histone deacetylase activity (*29*). In contrast, SHP, which does not interact with N-CoR (*27*), may be able to recruit, like DAX-1, a transcriptional inhibitor with a strong repressive activity. SHP could recruit HDAC to nuclear receptors to inhibit their activity. It will be interesting to study the implication of HDAC activity in the NR inhibition by SHP. At this stage of the study, we can propose a two-step mechanism of AR activity inhibition by SHP. In the first step, SHP may compete with AR coactivators on both AR-LBD and -NTD. In the second step, SHP may inhibit the receptor by its inhibitory domain. This concept is supported by similar mechanisms, previously described for HNF-4-LBD- (*30*) and ER-LBD-dependent activity inhibition (*12*).

Until now, little is known about AR transcriptional repressors. As in the present study, two other proteins have been described to recognize an active structure and repress it, TR4 (testicular orphan nuclear receptor 4) and HBO1 (histone acetyltransferase binding to ORC 1). TR4 was demonstrated to be a competitive inhibitor for the AR that acts as a heterodimer (*6*), and HBO1 inhibits AR-dependent gene activation by inhibiting elements of the core transcriptional machinery (*7*). These two repressors cannot, on their own, reflect the complexity of the numerous cross-talks that exist between the AR-signaling pathway and other NRs. It is now widely acknowledged that cofactors can play a balancing role between the different NR-signaling pathways. SHP seems to participate like the other cofactors in AR and NR activity regulation. Moreover, SHP may be regulated by natural ligand(s), yet not identified, that modulate its regulatory role of the NR-signaling pathways. As a result, the implication of SHP in NR physiology remains unclear. Recently, new clues regarding the involvement of SHP in NR signaling were revealed by two elegant works that described the implication of SHP in the regulation of catabolism of cholesterol into bile acids. SHP clearly participates in a feedback control of FXR/LRH-1 activities, which permits the catabolism of cholesterol (*13*, *14*). In light of these studies, it can be hypothesized that SHP is involved in a regulatory loop where activation of the AR directly or indirectly changes the intranuclear balance between activators and inhibitors, thus allowing SHP to repress AR and stop the activation signal. We cannot exclude that AR could either directly or indirectly regulate SHP expression and therefore affect AR activation level.

The diversity of SHP involvement in NR pathway regulation was underlined by a study of natural mutants directly linked to mild pathological obesity probably affecting the HNF-4 and the peroxisome proliferator-activated receptor pathways. SHP was suggested to be associated with maturity-onset diabetes of the young (MODY), and subjects showing the mutations were mildly or moderately obese at the onset of diabetes (*15*). These studies suggest that SHP may play a role in the regulation of a specific target, thereby acting as a modulator of implicated NR-signaling pathways in tissues in which SHP is expressed. The two other AR inhibitors described, TR4 and HBO1, like SHP, are expressed in these tissues as well (*7*, *31*). These three repressors may orchestrate a negative regulation of AR in androgen target tissues. After ligand binding, AR activity may result in a subtle equilibrium between activators and inhibitors. In that respect, natural mutations of SHP could affect the AR-signaling pathway in androgen-dependent diseases.

In this study, it is emphasized that the effect of AR coactivators may be reversed by SHP. Therefore, it will be necessary to study a potential regulation of SHP expression levels by the androgen receptor. Indeed, either a down-regulation or up-regulation of this expression could dictate AR activation. Naturally occurring mutations of SHP could also impede the effect of SHP on activated AR and lead to AR-associated pathologies. Characterization of SHP domains or motifs implicated in the interaction with AR could help in diagnosing the effects of SHP mutations.

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