Regulation of Androgen Receptor Activity by the Nuclear Receptor Corepressor SMRT

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Androgen receptor (AR) is a hormone-regulated transcription factor that mediates a wide array of biological processes including sexual differentiation, spermatogenesis, and prostate cancer progression. The transcriptional activity of AR and other members of the nuclear receptor superfamily are modulated by coregulatory proteins. In this study, we have investigated the regulation of AR transcriptional activity by the silencing mediator for retinoid and thyroid hormone receptors (SMRT). We found that AR possesses an intrinsic transcriptional repression activity, and AR interacts directly with SMRT. One interacting surface on AR is mapped to the ligand-binding domain, and the presence of a DNA binding/hinge region enhances this interaction. The binding surface on SMRT is mapped to the C-terminal ID2 region, and mutation in the ID2 corepressor motif inhibits the interaction. Overexpression of SMRT inhibits dihydrotestosterone-dependent transcriptional activity by AR and further suppresses the antiandrogen flutamide-mediated inhibition of AR activity. We provide evidence to suggest that the mechanisms of SMRT-mediated inhibition of AR activity involves inhibition of AR N/C interaction and competition with the p160 coactivator. Our data establish a significant role of SMRT in modulating AR transcriptional activity.

Androgen receptor (AR) is a member of the nuclear receptor (NR) superfamily that mediates the biological effects of the male sex hormone androgens in a wide range of physiological processes, including sexual differentiation and maturation, spermatogenesis, and gonadotropin regulation (1). AR is also involved in the development and progression of prostate cancer, which is one of the most frequently diagnosed cancers in males. Indeed, somatic mutations of the AR gene have been found in prostate tumors, which may contribute to androgen-independent growth of the cancer cell (2). Treatment with antiandrogens can partially or completely arrest prostate cancer cell proliferation. Similar to other steroid receptors the unliganded AR is held in an inactive conformation by association with heat shock proteins in the cytoplasm. The heat shock protein complex assists in maintaining an AR conformation that is active to ligand binding. Upon ligand binding, the heat shock protein complex dissociates from the receptor (3), allowing AR to homodimerize and translocate to the nucleus, where it recognizes and binds specific promoter elements to activate gene expression (4).

Like other members of the NR superfamily, AR contains an N-terminal AF-1 transactivation domain (or A/B domain), a centrally located DNA-binding domain (DBD or C domain), a hinge region (or D domain), and a ligand-binding domain (LBD or E domain). The E domain is also involved in receptor dimerization and contains ligand-dependent AF-2 function. The A/B domain is the most variable among NRs, and in the case of AR, it contains two separate transactivation domains that are required for full ligand-inducible transcription activity (5, 6). The A/B region also contains a polyglutamine tract whose expansion may influence AR transcription activity and cause infertility and Kennedy’s disease (7, 8). In addition, mutations in the AF-1 function have also been identified in patients suffering from androgen insensitivity or oligospermia (9–11), indicating the importance of this region in AR function. Interestingly, the A/B domain has been also shown to interact directly with the E domain in a ligand-dependent manner (12, 13). This process is known as N/C interaction, and it is required for full transcription potential of AR. In addition to ligand binding, transcriptional coactivators such as the CREB-binding protein (CBP), the p160 coactivators (14–17), and the proto-oncogene c-Jun (18) have been reported to enhance AR activity by promoting N/C interaction. Two LXXLL-related sequences in the A/B domain have been found responsible for mediating the N/C interaction (19). In addition to binding ligand and mediating N/C interaction, the E domain also mediates AR homodimerization and interactions with heat shock proteins (20, 21) and transcriptional coregulatory proteins (4). X-ray crystallographic studies indicate that the AR LBD adopts a similar structural fold as other NRs (22–24), suggesting that the regulatory mechanisms for AR activity may be conserved among NRs.

The past few years, it has become clear that the transcriptional activity of AR, as well as other members of the NR superfamily, are modulated by cellular coregulatory proteins known as coactivators and corepressors (1, 4, 25–27). The silencing mediator for retinoid and thyroid hormone receptors (SMRT) and the related nuclear receptor corepressor (N-CoR) were originally isolated as RAR- and TR-interacting proteins that form complexes with the unliganded receptors to facilitate...
transcriptional repression of gene expression (28, 29). SMRT and N-CoR were later found to interact with antagonist-bound progesterone receptor (PR) and estrogen receptor (ER) to repress transcription (30–32), and they also serve as corepressors for several additional members of the NR superfamily, including RevErb (33), chicken ovalbumin upstream promoter (COUP)–transcription factor I (34), peroxisome proliferator-activated receptor (PPARα) (35) and δ (36, 37) and the orphan receptor DAX1 (38). In addition, SMRT and N-CoR have also been implicated as corepressors for a variety of unrelated transcription factors involved in a wide array of biological processes (27). The promiscuity of SMRT and N-CoR on interacting with multiple transcription factors is consistent with an essential role in embryonic development as was revealed by studies of N-CoR-null mice, which die in midgestation and exhibit defects in several developmental processes (39).

Despite the established function of SMRT and N-CoR in regulating transcriptional repression activity of several NRs, the role SMRT and N-CoR in regulating AR transcriptional activity is less clear. Recently, it has been shown that AR can interact with N-CoR in HEK293 cells in the presence of DHT (40). Interestingly, this interaction is enhanced substantially by an acetylation site mutation at the hinge/D region of AR that affected transactivation function of AR without affecting its transrepression activity (40). In this study, we demonstrate that the N-CoR-related corepressor SMRT interacts directly with both the unliganded and liganded AR in vivo and in vitro. We demonstrate that this interaction is mediated through the C-terminal NR-interaction domain (ID2) of SMRT. The minimal interacting surface on AR is mapped to the LBD/E domain, while the presence of DBD/hinge region of AR enhances this interaction substantially. We show that SMRT inhibits ligand-dependent transcriptional activation by AR and further suppresses antiandrogen-mediated inhibition of AR activity. Finally, we provide evidence to suggest that the mechanisms of SMRT-mediated inhibition of AR activity are through disruption of AR/CoR interaction and/or competition with the p160 coactivators. These data establish a function role for the corepressor SMRT to regulate the transcriptional activity of AR.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—The human AR cDNA was kindly provided by Dr. Chawnahang Chang (University of Rochester Medical Center, Rochester, NY). The Gal4 DBD (44) fusions of AR fragments (G4-AR A/B, G4-AR D/E, and G4-AR E) were kindly provided by Dr. Lirim Shemshedini (University of Toledo, Toledo, Ohio). The G4s-AR and G4-AR were made by inserting full-length human AR cDNA into the pCMX-Gal vector at the XhoI and AagII sites, respectively. The AR full-length, 1–500, 1–660, 501–660, 501–919, and 661–919 fragments were amplified by PCR and subcloned into the pCMX-HA vector. The human SMRTe (41), GST-cSMRT, GST-SMRT-ID1, and GST-SMRT-ID2 were amplified by PCR and subcloned into the pCMX-HA vector. The slg- and dlb-motifs used for the site-directed mutagenesis were 5′-GGTGTAGTCCTGT-3′ and 5′-GGTGTAGTCCTGTGAGGCCGCACAGGACTACACC-3′, respectively. The AR A/B shows a strong transcriptional activity as expected from the AF-1 function. Similar to the full-length G4-AR, the G4-AR D/E and G4-AR E also show 2.5- and 4-fold repression activity, respectively. We demonstrate that this interaction is mediated through the C-terminal NR-interaction domain (ID2) of SMRT. The minimal interacting surface on AR is mapped to the LBD/E domain, while the presence of DBD/hinge region of AR enhances this interaction substantially. We show that SMRT inhibits ligand-dependent transcriptional activation by AR and further suppresses antiandrogen-mediated inhibition of AR activity. Finally, we provide evidence to suggest that the mechanisms of SMRT-mediated inhibition of AR activity are through disruption of AR/CoR interaction and/or competition with the p160 coactivators. These data establish a function role for the corepressor SMRT to regulate the transcriptional activity of AR.

RESULTS

Transcriptional Repression by AR and Interaction with SMRT in Vivo—To determine if AR contains intrinsic transcriptional repression activity, we first analyzed the transcriptional activity of a series of Gal4 DBD (Gal4)-AR fusion proteins by transient transfection assay in human HEK293 cells. The full-length AR was expressed as G4 (amino acids 1–147) or G4s (amino acids 1–74) fusion proteins, and the AR A/B, D/E, and E fragment were expressed as Gal4 fusions (Fig. 1A). The ability of these Gal4-AR fusion proteins to regulate transcription of a Gal4-dependent MH100-Luc reporter was determined in HEK293 cells (Fig. 1B). In this assay, the Gal4s-AR and Gal4-AR exhibit 5.2- and 3.5-fold repression activities, respectively. The Gal4-AR A/B shows a strong transcriptional activity as expected from the AF-1 function. Similar to the full-length Gal4-AR, the Gal4-AR D/E and Gal4-AR E also show 2.5- and 4-fold repression activities, respectively. These data suggest that AR may contain an intrinsic transcriptional repression function. The ability of AR to repress basal transcription led us to test whether AR might interact with the nuclear receptor corepres-
sor SMRT. To do so, we first determined if the G4-AR fusion proteins could interact with a VP-cSMRT fusion protein in a mammalian two-hybrid assay (Fig. 1C). The VP-cSMRT contains the transactivation domain of VP16 (amino acids 413–490) fused to the C-terminal last 422 amino acids of SMRT that contains two nuclear receptor-interacting domains. B, mammalian one-hybrid assay showing transcriptional activity of G4-AR fusion proteins. The full-length AR, the D/E, and E domains contain moderate transcriptional repression activity. The A/B domain contains constitutive transcriptional activation function. C, mammalian two-hybrid assay showing interactions of AR with SMRT in the absence or presence of DHT (100 nM). The VP-cSMRT interacts weakly with the full-length AR but strongly with the AR A/B, D/E, and E domains. DHT has no effect on these interactions. D, co-immunoprecipitation of endogenous AR with SMRT in prostate cancer cell lysate. The LNCaP human prostate cancer cell lysate were prepared after treatment of the cells with 100 nM DHT (D), 100 nM flutamide (F), or solvent alone (−) for 24 h. The endogenous AR proteins were precipitated by anti-AR polyclonal antibodies or with control IgG (IgG) bound to protein A-agarose beads. The immunoprecipitated complexes were resolved by SDS-PAGE and analyzed for AR and SMRT proteins by Western blotting. Western blot analysis indicates that there are approximately equal amounts of SMRT in the cell lysate before immunoprecipitation (top panel). The anti-AR immunoprecipitation followed by AR Western blot shows an elevation of AR protein level after DHT treatment, and a reduction after flutamide treatment. The co-immunoprecipitation data show that AR can form a stable complex with SMRT in the absence or presence of ligand and that AR ligands, especially, the antagonist flutamide enhance formation of AR-SMRT complex.

Fig. 1. Transcriptional regulation by AR and its interaction with SMRT. A, schematics of the Gal4 DBD (G4)-AR and VP-cSMRT fusion proteins. The G4s (amino acids 1–74) contains the minimal Gal4 DBD. The AR domains are depicted as A/B, the N-terminal domain that contains the AF-1 function. C, the DNA-binding domain; D, the hinge region; and E, the ligand-binding domain that contains the AF-2 function. The VP-cSMRT contains the transactivation domain of VP16 (amino acids 413–490) fused to the C-terminal last 422 amino acids of SMRT that contains two nuclear receptor-interacting domains. B, mammalian one-hybrid assay showing transcriptional activity of G4-AR fusion proteins. The full-length AR, the D/E, and E domains contain moderate transcriptional repression activity. The A/B domain contains constitutive transcriptional activation function. C, mammalian two-hybrid assay showing interactions of AR with SMRT in the absence or presence of DHT (100 nM). The VP-cSMRT interacts weakly with the full-length AR but strongly with the AR A/B, D/E, and E domains. DHT has no effect on these interactions. D, co-immunoprecipitation of endogenous AR with SMRT in prostate cancer cell lysate. The LNCaP human prostate cancer cell lysate were prepared after treatment of the cells with 100 nM DHT (D), 100 nM flutamide (F), or solvent alone (−) for 24 h. The endogenous AR proteins were precipitated by anti-AR polyclonal antibodies or with control IgG (IgG) bound to protein A-agarose beads. The immunoprecipitated complexes were resolved by SDS-PAGE and analyzed for AR and SMRT proteins by Western blotting. Western blot analysis indicates that there are approximately equal amounts of SMRT in the cell lysate before immunoprecipitation (top panel). The anti-AR immunoprecipitation followed by AR Western blot shows an elevation of AR protein level after DHT treatment, and a reduction after flutamide treatment. The co-immunoprecipitation data show that AR can form a stable complex with SMRT in the absence or presence of ligand and that AR ligands, especially, the antagonist flutamide enhance formation of AR-SMRT complex.

To confirm the association of AR and SMRT in vivo, we conducted co-immunoprecipitation assays using LNCaP human prostate cancer cell lysate. The cells were treated with either DHT or flutamide for 24 h before cell lysate preparation. Western blot analysis showed that the SMRT protein levels were similar in different cell lysate (Fig. 1D, top). The endogenous AR proteins were then immunoprecipitated by anti-AR polyclonal antibodies that were conjugated with protein A-agarose beads. The precipitated complexes were resolved by SDS-PAGE and analyzed for AR and SMRT proteins by Western blotting. Western blot analysis indicates that there are approximately equal amounts of SMRT in the cell lysate before immunoprecipitation (top panel). The anti-AR immunoprecipitation followed by AR Western blot shows an elevation of AR protein level after DHT treatment, and a reduction after flutamide treatment. The co-immunoprecipitation data show that AR can form a stable complex with SMRT in the absence or presence of ligand and that AR ligands, especially, the antagonist flutamide enhance formation of AR-SMRT complex.

Physical Interactions between AR and SMRT in Vitro—To determine if the in vivo association between AR and SMRT are
results of direct protein-protein interactions, we analyzed the AR-SMRT interactions in vitro by GST pull-down assays. A series of AR deletion mutants containing various domains (Fig. 2A) were expressed and labeled with 35S-labeled full-length AR (35S-AR). AR binds to SMRT-ID2 but not SMRT-ID1, D, GST pull-down assay showing the effect of androgens on binding of AR to GST-cSMRT. S, solvent (20% dimethyl sulfoxide, 80% ethanol); D, 100 nM dihydrotestosterone; 1, 1 μM flutamide. E, interactions of various AR fragments with GST-cSMRT in the GST pull-down assay. F–K, interactions of various AR fragments with GST-SMRT-ID1 (ID1) and GST-SMRT-ID2 (ID2) domains in the GST pull-down assay.

Fig. 2. The interacting domains between SMRT and AR. A, schematics of AR deletion mutants and their relative binding to SMRT. B, schematics of SMRT mutants and their relative interactions with AR. Numbers indicate starting and ending amino acids based on the human SMRTe sequence (41). Δ indicates an internal deletion of 46 amino acids following residue 2352. The ID1 and ID2 mutants (mt) were generated by site-directed mutagenesis. C, GST pull-down assay showing interactions of GST-cSMRT, SMRT-ID1, and SMRT-ID2 with 35S-labeled full-length AR (35S-AR). AR binds to SMRT-ID2 but not SMRT-ID1. D, GST pull-down assay showing the effect of androgens on binding of AR to GST-cSMRT. S, solvent (20% dimethyl sulfoxide, 80% ethanol); D, 100 nM dihydrotestosterone; 1, 1 μM flutamide. E, interactions of various AR fragments with GST-cSMRT in the GST pull-down assay. F–K, interactions of various AR fragments with GST-SMRT-ID1 (ID1) and GST-SMRT-ID2 (ID2) domains in the GST pull-down assay. wt, wild type; mt, mutant.

but it bound weakly to the AR-(1–660) fragment (A/B/C/D domain). It also bound strongly to the AR-(501–919) fragment (C/D/E) and moderately to the AR-(661–919) fragment (E domain). The AR-(1–500) fragment also did not interact with GST-SMRT-ID1, SMRT-ID2, or ID motif mutants (Fig. 2F). The AR-(501–660) fragment that contains only the DBD and hinge regions (C/D domain) did not bind to GST-SMRT-ID1 or SMRT-ID2 in the absence or presence of DHT (Fig. 2G), suggesting both the A/B and C/D domains alone are not sufficient to bind SMRT in vitro. In contrast, the AR-(661–919) fragment (E domain) alone was sufficient to bind GST-cSMRT (Fig. 2E) and SMRT-ID2 (Fig. 2H). Interestingly, the AR-(501–919) fragment (C/D/E domain) bound both GST-cSMRT (Fig. 2E) and SMRT-ID2 (Fig. 2D) much more strongly, suggesting a role for the C/D domain in regulating AR-SMRT interaction. The interaction of AR-(501–919) with SMRT-ID2 was not affected by DHT treatment in this in vitro assay (Fig. 2J). Finally, although the AR-(1–500) and (501–660) fragments both showed no interaction with SMRT, the AR-(1–660) fragment that combines the A/B and C/D domains displayed some binding to GST-cSMRT (Fig. 2E) and SMRT-ID2 (Fig. 2K), again indicating a role for the C/D domain in regulating SMRT binding. Finally, we show that the interactions of SMRT-ID2 with AR-(661–919), AR-(501–919) and AR-(1–660) fragments were inhibited by mutation of the ID2 corepressor motif (Fig. 2, H, I, and K). Together, these data indicate that SMRT may directly interact with AR in vitro. The AR-SMRT interaction may be mediated through the SMRT-ID2 corepressor motif. The AR E domain appears to be sufficient for SMRT binding, while the C/D domain may play a critical role in regulating AR-SMRT interaction either directly or indirectly.

Subcellular Colocalization of AR with SMRT—To further demonstrate AR-SMRT interaction in mammalian cells, we cotransfected HA-tagged AR and full-length SMRT (SMRTe) in HeLa cells and analyzed potential subcellular colocalization by double immunofluorescence staining. Transfection was conducted under normal serum condition because the AR protein is unstable in the absence of ligand (46). We found that in about 90% of cells where AR and SMRT are coexpressed, the SMRT speckles are greatly reduced in size into microspeckled or uniform patterns (Fig. 3, row A), suggesting an interaction between SMRT and AR in these cells. Strong evidence of AR-SMRT interaction is found in the remaining 10% of transfected cells, where AR and SMRT appear simultaneously in discrete speckles (Fig. 3, row B). These data suggest a close association of AR and SMRT within nuclear speckles in mammalian cells.

To confirm the association of AR and SMRT in mammalian cells, we further analyzed the colocalization of three SMRT-binding AR fragments with SMRT. Coexpression of SMRT with AR-(501–919) or -(661–919) fragment results in clear colocalization at nuclear speckles (Fig. 3, rows C and D), indicating an association of SMRT with these two AR fragments. In fact, unlike full-length AR, the 501–919 and 661–919 fragments alone already localize in nuclear speckles (data not shown). Interestingly, coexpression of the AR-(1–660) fragment with SMRT results in dispersed AR and SMRT localization pattern (Fig. 3, row E), suggesting an association of SMRT with the AR N-terminal domain. These data suggest that the AR N-terminal domain may be able to recruit SMRT from nuclear speckles to nucleoplasm and thus could be responsible for the disruption of SMRT speckles by full-length AR. Together, these data suggest an association between AR and SMRT in cultured mammalian cells.

Inhibition of AR Transcription Activity by SMRT—The interaction between SMRT and AR prompted us to address the role of SMRT in regulating the transcriptional activity of AR.
To address this question, we conducted transient transfection assays to determine the effect of SMRT overexpression on AR activity using the AR-responsive MMTV-Luc reporter in HEK293 cells (Fig. 4A). We found that coexpression of SMRT resulted in a SMRT dose-dependent inhibition of the reporter gene expression that was activated by AR in response to DHT treatment. Importantly, SMRT also had an additive effect with the AR antagonist flutamide to reduce MMTV promoter activity below basal levels. These data suggest that SMRT may inhibit ligand-dependent transcriptional activation by AR, and it also may act together with antagonist to further suppress AR target gene expression.

The transcriptional activity of AR is determined by a strong constitutive AF-1 activity and a relatively weak ligand-dependent AF-2 function. To determine if SMRT can affect either AF-1 or AF-2 function, we analyzed the effect of SMRT overexpression on each of these two activation functions. In the experiment shown in Fig. 4B, the autonomous AF-1 function observed in the G4-AR 1–500 fusion protein was gradually inhibited by SMRT coexpression in a dose-dependent manner, suggesting that SMRT may inhibit the AF-1 activity of AR. Furthermore, we found that SMRT could also inhibit the DHT-dependent AF-2 activity as well (Fig. 4C). The inhibition of AR transcriptional activity by SMRT is specific because overexpression of SMRT had no effect on G4 or G4-VP16 activity (Fig. 4D). These data suggest that SMRT may inhibit the transcriptional activity of AR.

Inhibition of AR N/C Interaction by SMRT—The overall transcriptional activity of AR is determined by a strong constitutive AF-1 activity and a relatively weak ligand-dependent AF-2 function. To determine if SMRT can affect either AF-1 or AF-2 function, we analyzed the effect of SMRT overexpression on each of these two activation functions. In the experiment shown in Fig. 4B, the autonomous AF-1 function observed in the G4-AR 1–500 fusion protein was gradually inhibited by SMRT coexpression in a dose-dependent manner, suggesting that SMRT may inhibit the AF-1 activity of AR. Furthermore, we found that SMRT could also inhibit the DHT-dependent AF-2 activity as well (Fig. 4C). The inhibition of AR transcriptional activity by SMRT is specific because overexpression of SMRT had no effect on G4 or G4-VP16 activity (Fig. 4D). These data suggest that SMRT may inhibit the transcriptional activity of AR.

Inhibition of AR N/C Interaction by SMRT—The overall transcriptional activity of AR is determined by a process known as N/C interaction where two LXXLL-like motifs in the A/B domain interact with the coactivator-binding pocket within the E domain in a ligand-dependent manner (12, 13). To provide insight into the molecular basis of SMRT-mediated inhibition on AR activity, we tested whether SMRT could affect AR N/C interaction. As a result of the N/C interaction, cotransfection of the AR N-terminal 1–500-amino acid fragment with the C-terminal 501–919-amino acid fragment resulted in a strong ligand-dependent activation of the MMTV promoter activity (Fig. 5A). Remarkably, co-expression of SMRT abolished this ligand-dependent activation by 90% (a 10-fold reduction), indicating that SMRT may inhibit AR N/C interaction. Interestingly, cSMRT also had a similar effect, abolishing the N/C interaction by 85% (a 6.7-fold reduction). As control, we found that expression of SMRT and cSMRT had no significant effect on the basal expression of the MMTV-Luc reporter (data not shown). The inhibition of AR N/C interaction by SMRT and cSMRT were further measured at increasing concentrations of SMRT or cSMRT to demonstrate that both SMRT and cSMRT can inhibit AR N/C interaction in a dose-dependent manner (Fig. 5B). These data suggest that SMRT may inhibit the transcriptional activity of AR by disrupting N/C interaction.

Competition with TIF2 by SMRT to Regulate AR Activity—The p160 family coactivator TIF2 has been shown to enhance AR transcriptional activity through stabilization of AR N/C interaction (15, 18). Members of the p160 coactivator family and the SMRT/N-CoR corepressors are known to bind to overlapping surfaces in the AF-2 domain of the receptor (47–51). Therefore, it is reasonable to speculate that SMRT might compete with p160 coactivator for binding to liganded AR. We tested whether SMRT and TIF2 could compete with each other to control the transcriptional activity of AR. Consistent with prior findings (15, 18), we found that co-transfection of TIF2
enhanced DHT-dependent transcriptional activity of AR by about 2-fold (Fig. 6A). As expected, co-transfection with SMRT abolished this TIF2 coactivation effect completely. Interestingly, this inhibition of AR activity by SMRT could be alleviated gradually by increasing TIF2 concentration (Fig. 6A), suggesting that TIF2 may revert SMRT-mediated inhibition on AR activity. Conversely, increasing SMRT concentration could also reduce the enhancement of AR activity by TIF2 (Fig. 6B), suggesting that SMRT mutually competes with TIF2 to inhibit AR transactivation. In contrast, we found that TIF2 was not able to reverse the SMRT-mediated inhibition on AR activity in the presence of flutamide (Fig. 6C). This is consistent with the fact that TIF2 does not bind to antagonist-bound AR. Together, these data suggest that the coactivator TIF2 and the corepressor SMRT may compete with each other to regulate the transcriptional activity of AR.

DISCUSSION

In this study, we have investigated the physical and functional interactions between AR and the corepressor SMRT. We show that SMRT interacts with both the unliganded and liganded forms of AR in vitro and in vivo. The AR-SMRT interaction in vivo appears to be enhanced by agonist or antagonist treatment. We have mapped the interaction surface to the SMRT-ID2 domain and showed that mutation of the ID2 corepressor motif disrupted the interaction. We found that the AR LBD/E domain is sufficient to bind SMRT, while the C/D domain also play a critical role in regulating SMRT binding. In addition, we demonstrate an in vivo colocalization between SMRT and AR in cultured mammalian cells. The biological significance of these in vivo and in vitro interactions between AR and SMRT is supported by transient transfection assay showing that over-

**Fig. 4. Inhibition of AR transcriptional activation by SMRT.** A, SMRT inhibits AR-mediated transactivation and enhances antagonism mediated by flutamide. 293 cells were transiently transfected with AR and increasing concentrations of SMRT. The MMTV-tk-Luc reporter activity was determined from triplicate experiments in the presence of DHT (100 nM) or flutamide (1 μM). B, SMRT inhibits AR AF-1 activity. The G4-AR-(1–500) fusion construct was transiently transfected into 293 cells with increasing concentrations of SMRT and the MH100-tk-Luc reporter. The Gal4 DBD alone basal activity is shown in D. C, SMRT inhibits the ligand-dependent AF-2 activity of AR. The AR-(501–919) construct was transiently transfected into HeLa cells with increasing amounts of SMRT. The MMTV-tk-Luc reporter activity was determined by luciferase assay. D, SMRT has no effect on the basal promoter activity or G4-VP16-activated transcription.
expression of SMRT robotically inhibits AR-mediated transcriptional activation of target promoter. Finally, we provide evidence to suggest that the mechanisms of SMRT-mediated inhibition on AR transcriptional activity may be due to either disruption of the AR N/C interaction and/or functional competition with the p160 coactivator.

Using the Gal4 DBD fusion protein system, we show that AR contains transcriptional repression activity. We localized such repression activity to the E domain of the receptor (Fig. 1B). Consistent with existing knowledge, we show that the AR A/B domain contains a strong transcriptional activation function, and that this activity is masked in the context of full-length AR. The ability of the G4-AR fusion protein to repress transcription is consistent with the interaction of AR with SMRT as first demonstrated by mammalian two-hybrid assay (Fig. 1C). We speculate that the inability of G4-AR A/B to repress transcription despite its interaction with SMRT is due to the presence of a strong AF-1 function. The in vivo interaction between AR and SMRT is further supported by co-immunoprecipitation of endogenous AR and SMRT from the LNCaP human prostate cancer cell lysate (Fig. 1D). Interestingly, we found that the AR ligands, especially the antagonist flutamide, appear to enhance the in vivo association of AR with SMRT. This result is consistent with a previous chromatin immunoprecipitation study showing that the AR antagonist bicalutamide could enhance the recruitment of N-CoR and SMRT to the prostate-specific antigen (PSA) promoter in LNCaP prostate cancer cells (52).

The physical interaction between AR and SMRT was demonstrated by GST pull-down assay, which indicates that SMRT may interact with AR directly in vitro. We have mapped the interacting surfaces on SMRT to the ID2 domain. Interestingly, the SMRT-ID1 domain has no affinity to AR, despite the fact that this domain is the primary interacting surface for RAR and TR (42) and that ID1 and ID2 share a consensus LXXLXXXI/I/L sequence that mediate direct binding of SMRT and N-CoR to several NRs (47–51). Intriguingly, the SMRT-AR interaction is inhibited by mutation of the ID2 corepressor motif, suggesting that the mechanism of SMRT binding to AR may be conserved with other NRs (48, 49). Our data suggest that there might be at least two SMRT binding sites on the AR protein, although the exact binding mechanisms remain to be elucidated. The best AR fragment for SMRT binding is the AR-(501–919) amino acid fragment that contains both the DBD/hinge region and LBD. Since the LBD alone binds to SMRT but DBD/hinge alone shows no interaction with SMRT, we speculate that there may be a SMRT binding site within the LBD. The fact that the AR-(501–919) fragment binds to SMRT much stronger that the AR-(661–919) fragment suggest a critical role for the DBD/hinge region of AR in regulating corepressor binding. It is possible that the presence of the DBD/hinge domain may help to stabilize a conformation of the LBD and A/B domain that is more compatible with SMRT binding. Alternatively, it is equally possible that there might be a weak binding site within the AR DBD or hinge region that may synergize with the other binding site for SMRT binding. Contrary to a recent report (53), we found no interaction between AR A/B alone and SMRT in GST pull-down assays using either GST-cSMRT or SMRT-ID2. However, the interaction between SMRT and AR LBD is readily detectable in our assay. It appears that SMRT utilizes the ID2 corepressor motif for interaction with AR, suggesting that the mechanisms of SMRT binding to these two sites may be related. Since the amino acid sequences of the ID1 and ID2 corepressor motifs are similar, the mechanisms by which AR distinguishes one motif from the other remains to be determined.

Unlike RAR and TR, whose interactions with SMRT are ligand-independent (28, 42, 44), or the PR and ER whose interactions with SMRT are antagonist-dependent (30, 32, 54), we found that the interaction between full-length AR and SMRT is not affected by androgens in the in vitro binding assay (Fig. 2D). These data suggest that SMRT might be able to interact with both the unliganded and liganded forms of AR. The ability of SMRT to interact with liganded AR is consistent with the reporter gene assay demonstrating that SMRT can inhibit ligand-dependent transcriptional activation by AR (Fig. 4). Alternatively, we have previously shown that there are a substantial number of cells containing cytoplasmic SMRT staining (55), implying that SMRT might be involved in the signal transduction pathway of AR in the cytoplasm during its ligand-induced transformation process. It is also possible that SMRT might help to maintain an inactive AR conformation in either the cytoplasm or nucleus.

The subcellular distribution of unliganded AR is cell line-dependent as the AR is predominantly nuclear when expressed in HeLa cells but mainly cytoplasmic in COS-1 cells (56). In the presence of hormone, the AR is translocated into the nucleus and distributed in a microspeckles (57–59). Our data show that coexpression of AR and SMRT in HeLa cells results in apparent
colocalization in the speckles (Fig. 3), thus confirming \textit{in vivo} association of AR and SMRT. Interestingly, unlike the recruitment of RAR to the SMRT speckles (55), AR seems to disrupt the SMRT speckles as a majority of the transfected cells contain microspeckled SMRT staining when AR is coexpressed (Fig. 3). It is possible that AR may be able to recruit SMRT to its nuclear sites. Consistently, SMRT has been detected on the natural AR target promoter by chromatin immunoprecipitation assay in the presence of antiandrogen (52). Likely, when AR binds to the response element in the presence of antiandrogen, it is able to recruit SMRT to the target promoter because of lack of competition from coactivator. In contrast, in the presence of androgen, SMRT will have to compete with coactivator, and only when SMRT is overexpressed will it be able to show binding to the liganded receptor.

Our data suggest that SMRT can inhibit both the AF-1 and AF-2 function of AR in a SMRT dose-dependent manner (Fig. 4). This inhibition is likely due to direct binding of SMRT as demonstrated by \textit{in vivo} and \textit{in vitro} binding assays. We predict that SMRT binding to liganded AR may block recruitment of coactivators or bring in deacetylase activity to the liganded receptor. It is reasonable to speculate that binding of SMRT to the liganded receptor may be sufficient to block AR transcriptional activity. Interestingly, the inhibition of AR activity can be reverted by overexpression of the coactivator TIF2 (Fig. 6). Because TIF2 also binds to the liganded AR, it is conceivable that the reinstatement of AR activity by TIF2 may be due to displacement of SMRT from the liganded AR, resulting in subsequent recruitment of CBP/p300 or other coactivators to the receptor. The existence of a mutual competition between TIF2 and SMRT on regulating AR transactivation suggests that the corepressor SMRT might inhibit transactivation by the liganded receptor. Similarly, we also observed potent inhibition of AR activity by the SMRT-related corepressor N-CoR.2 Because SMRT and N-CoR share highly conserved NR interaction domains, we believe that both SMRT and N-CoR can function independently to negatively regulate the transcriptional activity of liganded AR.

The AR has been shown to undergo several rounds of “recycling” before being subjected to proteasome-mediated degradation (57), indicating that a mechanism of transient AR inactivation must exist. It has been suggested that hormone inactivation or degradation may result in the transient deactivation of AR activity prior to receptor export from the nucleus (57). In contrast, a recent report indicates that HDAC1 may play a key role in the acute repression of AR transcriptional activity (60). We suggest here that SMRT may be another key player in the down-regulation of AR activity. The abilities of SMRT to interact with the liganded AR, to compete with TIF2 coactivator, and to block AR N/C interaction are all consistent with the hypothesis that SMRT is capable of antagonizing the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.pdf}
\caption{Competition between SMRT and TIF2 on regulating AR activity. A, TIF2 can reverse SMRT-mediated inhibition of AR activity. 293 cells were transiently transfected with increasing concentrations of TIF2 (in \mu g), and the full-length AR (0.1 \mu g) and SMRT (0.5 \mu g) where indicated by +, together with the MMTV-tk-Luc reporter. B, SMRT inhibits transcriptional coactivation mediated by TIF2. Where indicated, full-length AR (0.1 \mu g) and TIF2 (0.5 \mu g) were transiently transfected into HeLa cells with increasing concentration of SMRT. C, TIF2 cannot reverse flutamide- and SMRT-mediated inhibition of AR activity. Where indicated, full-length AR (0.1 \mu g) and SMRT (0.5 \mu g) were transiently transfected into HeLa cells with increasing concentrations of TIF2 (in \mu g) in the presence of 100 nM of flutamide.}
\end{figure}

\footnote{G. Liao and J. D. Chen, unpublished data.}
transcriptional activity of AR. Interestingly, SMRT is known to form a stable complex with HDAC1 (61), implying that SMRT and HDAC1 may act synergistically to inhibit AR activity. However, our data also indicate that the primary mechanism for the SMRT-mediated AR inactivation may be due to the blockade of AR N/C interaction. This is supported by the fact that the C-terminal fragment of SMRT, which contains only the receptor-interacting domain but no transcriptional repression activity, is capable of blocking N/C interaction (Fig. 5). The molecular mechanisms by which SMRT blocks AR N/C interaction remain to be defined. However, in light of previous observations that SMRT can function as an activating cofactor for HDAC3 to acetylate histones (62, 63) and that the AR transcriptional activity may be dynamically regulated by acetylation and deacetylation of AR (40, 60), it is possible that SMRT might affect AR activity through enhancing deacetylation of AR.

During preparation of this manuscript, Baniamad and coworkers (53) reported that SMRT could interact with AR in the presence of the natural androgen DHT, the synthetic androgen R1881, or the partial agonist cyproterone acetate (CPA). By using a modified mammalian two-hybrid system on the complex MMTV promoter, that study demonstrated a 4-fold interaction with VP-cSMRT in the absence of ligand and in the presence of the full antigens Casodex or hydroxylflutamide, which is consistent with our findings of a ligand-independent interaction between AR and SMRT. To address the discrepancy between the effects of DHT on the AR-SMRT interaction, we repeated and confirmed in the same modified mammalian two-hybrid system using MMTV-Luc reporter that DHT indeed stimulated the reporter gene expression in the presence of full-length AR and VP-cSMRT. Interestingly, this DHT-mediated enhancement was virtually abolished when the AR N-terminal A/B domain was removed in the same MMTV-based two-hybrid system, indicating a requirement of the AF-1 function for this enhancement effect. It is conceivable that the induction of N/C interaction by DHT might be involved in this process; however, the exact mechanisms remain to be determined. Collectively, these results suggest that SMRT may play an important role in regulating the transcriptional activity of AR and also provide a potential mechanism for down-regulation of AR transcriptional activity. These results may also have implications for antagonizing AR activity in prostate cancer cells.

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