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The Amino Terminus of the Human AR Is Target for Corepressor Action and Antihormone Agonism

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Antiandrogens inhibit the ligand-induced transactivation by the androgen receptor (AR) and have a widespread use in the treatment of prostate cancer but their mode of action is not fully understood. Here we show that the ability of the antiandrogen cyproterone acetate (CPA) to inhibit transactivation by the human AR (hAR) involves the corepressor SMRT (silencing mediator for retinoic acid and thyroid hormone receptor). We detect binding of SMRT to hAR when treating with the antiandrogen CPA, but not with the antihormones casodex or hydroxyflutamide. Interestingly, we find that SMRT binds to the N terminus of the hAR. Thereby, SMRT modulates the activity of hAR in receptor-negative CV1 cells. In addition, we have used receptor point mutants that exhibit normal transactivation potential and unchanged partial agonistic activity when treated with CPA, but lack both SMRT binding and SMRT-mediated inhibition of CPA-bound AR. This indicates that mechanisms involved in hAR-mediated transactivation are distinct from antihormone-induced receptor inactivation. Furthermore, we show that treatment of transfected cells with a cAMP analog or coexpression of the catalytic subunit of PKA, known to activate hAR, inhibits the binding of SMRT to the AR.

This suggests that the association of SMRT with hAR is regulated at the level of cross-talk mechanisms and that ligand-independent receptor activation is due to corepressor dissociation. Taken together, we provide novel insights in AR regulation, antihormone action, and functional nuclear receptor-corepressor interaction. (*Molecular Endocrinology* 16: 661–673, 2002)

PROSTATE CANCER IS one of the most frequently diagnosed cancers in males (1). This cancer is initially androgen dependent and, as a result, reduction in the level of circulating androgens or treatment with antiandrogens leads to a partial or a complete arrest in proliferation of the cancer. Later on in therapy, almost all prostate cancers become androgen independent but the mechanism that brings about this change is not well understood (2–5). Androgen-independent cancers, however, retain the receptor for androgens (AR) and express the androgen-inducible protein, prostate-specific antigen (PSA).

It has been suggested that amplification of the AR gene, activation of the signal transduction machinery, or changes in coactivator binding to AR lead to androgen-independent cancer cell growth. Furthermore, mutations in the AR gene have been implicated in the progression of prostate cancer from androgen-dependent to androgen-independent growth (6, 7). Signal transduction mechanisms that regulate the action of the AR in a ligand-independent manner, such as elevated levels of PKA, have also been reported (8–10), and these may play a role in the transition into androgen-independent growth of prostate cancer.

Thus, a general goal in prostate cancer therapy is to inhibit the AR-mediated transactivation in a long-term manner.

The AR is a member of the nuclear hormone receptor superfamily, which represents a large family of hormone-regulated transcription factors. Members of this superfamily share a common tripartite structure with a central DNA binding domain, which separates the receptor amino (N) terminus from the carboxy (C) terminus. The C terminus harbors the hormone binding domain (HBD) and the ligand-dependent transcriptional activation function (11, 12). The activity of nuclear receptors, in general, is modulated by associating proteins termed coactivators and corepressors. Whereas coactivators enhance the action of the liganded receptor, corepressors are thought to bind to the silencing domain, a region overlapping the hormone-binding domain (13), and to keep the receptor in an inactive state in the absence of hormone. Upon hormone binding, the corepressors dissociate, allowing the coactivators to bind and to exert their positive regulatory action. This model of corepressor action has been established mainly for the RARs and TRs. A

Abbreviations: aa, Amino acids; 8-Br-cAMP, 8-bromo-cAMP; CAS, casodex; CPA, cyproterone acetate; DHT, dihydrotestosterone; GRE, glucocorticoid response element; GST, glutathione-*S*-transferase; HA, hemagglutin; hAR, human AR; HBD, hormone-binding domain; MMTV-luc, mouse mammary tumor virus-luciferase; OH-F, hydroxyflutamide; PSA, prostatespecific antigen; SMRT, silencing mediator for retinoic acid and thyroid hormone receptor.

typical example is the corepressor SMRT (silencing mediator for RA and TR) that was originally identified as a corepressor for the unliganded TR or RAR (14).

In contrast to other members of the nuclear hormone receptor superfamily, the major transactivation domains of the AR are localized at its N terminus (2, 15–17). The C terminus that serves as a transactivation domain for other steroid receptors has a very weak activity in the AR. It is therefore curious how binding of antiandrogens to the C terminus of the receptor can reduce the transactivation function at the N terminus of the AR.

In this communication, we demonstrate that the corepressor SMRT plays a role in the inhibition of androgen action by the antiandrogen cyproterone acetate (CPA). SMRT binds the N terminus of the AR inhibiting the transactivation function of the receptor by CPA. Receptor point mutants show that corepressor binding to the AR N terminus can be separated from the receptor-mediated transactivation. We also show that enhancement of the transactivation function of the AR achieved by signal transduction factors, such as PKA, correlate with the dissociation of SMRT from AR. These results together show the involvement of a corepressor in the regulation of the activity by the human AR (hAR).

RESULTS

SMRT Interaction with the hAR Can Be Detected in the Presence of the Antihormone CPA Leading to an Enhanced Antagonism by CPA

We first analyzed the effect of various androgens and antiandrogens on transactivation by AR in the AR-negative simian kidney CV1 cell line. Cells were cotransfected with an expression vector encoding the AR cDNA and the reporter gene MMTV-luc (mouse mammary tumor virus-luciferase). Transfected cells were then treated with the natural androgen dihydrotestosterone (DHT) and the synthetic androgen methyltrieolone (R1881), as well as with the antiandrogens casodex (CAS), hydroxyflutamide (OH-F), and cyproterone acetate (CPA). Although DHT and R1881 enhanced transactivation by the AR, the antiandrogens had no effect, with the exception of CPA, which exhibited a partial agonistic activity (Fig. 1A).

Because corepressors are known to bind to certain steroid receptors, as shown for the ERs and PRs (18–20), we next analyzed whether the corepressor SMRT is able to bind to the AR in the presence of the various agonists and antagonists. We performed modified mammalian two-hybrid experiments using full-length wild-type hAR and VP16, as a control, or the VP16-SMRT fusion protein with MMTV-luc as a reporter gene. VP16-SMRT represents a fusion of the C-terminal 422 aa (21) that harbors the two receptor-interacting domains (22) fused to the transactivation domain of VP16. Interaction of SMRT with hAR is measured as higher reporter activity (Fig. 1B). In the absence of hormone VP16-SMRT led to an 4-fold increase in reporter gene activity. This induction is dependent on the presence of AR in the cells (not shown). In the presence of DHT and R1881, transactivation was increased a further 12- and 21-fold, respectively. Of the three antihormones tested, OH-F, CAS (bicalutamide), and CPA, an interaction of the AR with SMRT was observed only in the presence of CPA. This is shown by a further 25-fold increase in reporter activity when compared with the VP16 control. These data suggest that the corepressor SMRT interacts with the AR both in the presence of agonists and in the presence of the partial agonist CPA.

We then analyzed whether the overexpression of the corepressor SMRT might have an inhibitory effect on the transactivation induced by R1881 and CPA. For this purpose we transfected a full-length SMRT (14) expression vector into CV1 cells, together with the AR expression vector and the reporter MMTV-luc gene. As expected, addition of R1881 and CPA led to an increased reporter gene expression in the absence of coexpressed SMRT (Fig. 2A). Although coexpression of SMRT hardly affected basal promoter activity compared with an empty expression vector in the absence of R1881 or CPA, it reduced transactivation by both R1881 and CPA. Note that while SMRT strongly reduced CPA-mediated activation of the AR, its effect on R1881-induced transactivation was only mediocre. This suggests that the repressive effect of SMRT depends on the type of ligand, and the partial agonistic activity of CPA on the AR may be due to the low levels of SMRT in the recipient CV-1 cells (23).

We speculated that because overexpression of SMRT more strongly reduced transactivation induced by CPA than by R1881, expression of SMRT might increase the efficacy of CPA as an androgen antagonist. To test this hypothesis, we performed an experiment in which we assayed the effect of different concentrations of CPA on R1881-induced transactivation of AR, in the presence and absence of SMRT. In the absence of SMRT, a high molar excess, of up to 1,000-fold, of CPA is required to act efficiently as an antagonist of R1881. However, coexpression of SMRT led to a dramatic enhancement of CPA-mediated antagonism (Fig. 2B). In the presence of SMRT, an equimolar concentration of 10^{-9} M CPA resulted in a significant reduction of AR-mediated transactivation, and at 10^{-7} M CPA the R1881-induced transactivation mediated by AR was completely abrogated. The expression of AR is not changed by coexpression of full-length SMRT, by VP16 alone, or by the VP16-SMRT fusion determined by Western blotting (data not shown).

Taken together, these results suggest that the level of CPA-mediated partial agonism of AR is regulated by the corepressor SMRT, and that CPA acts as a more efficient androgen antagonist in the presence of SMRT.

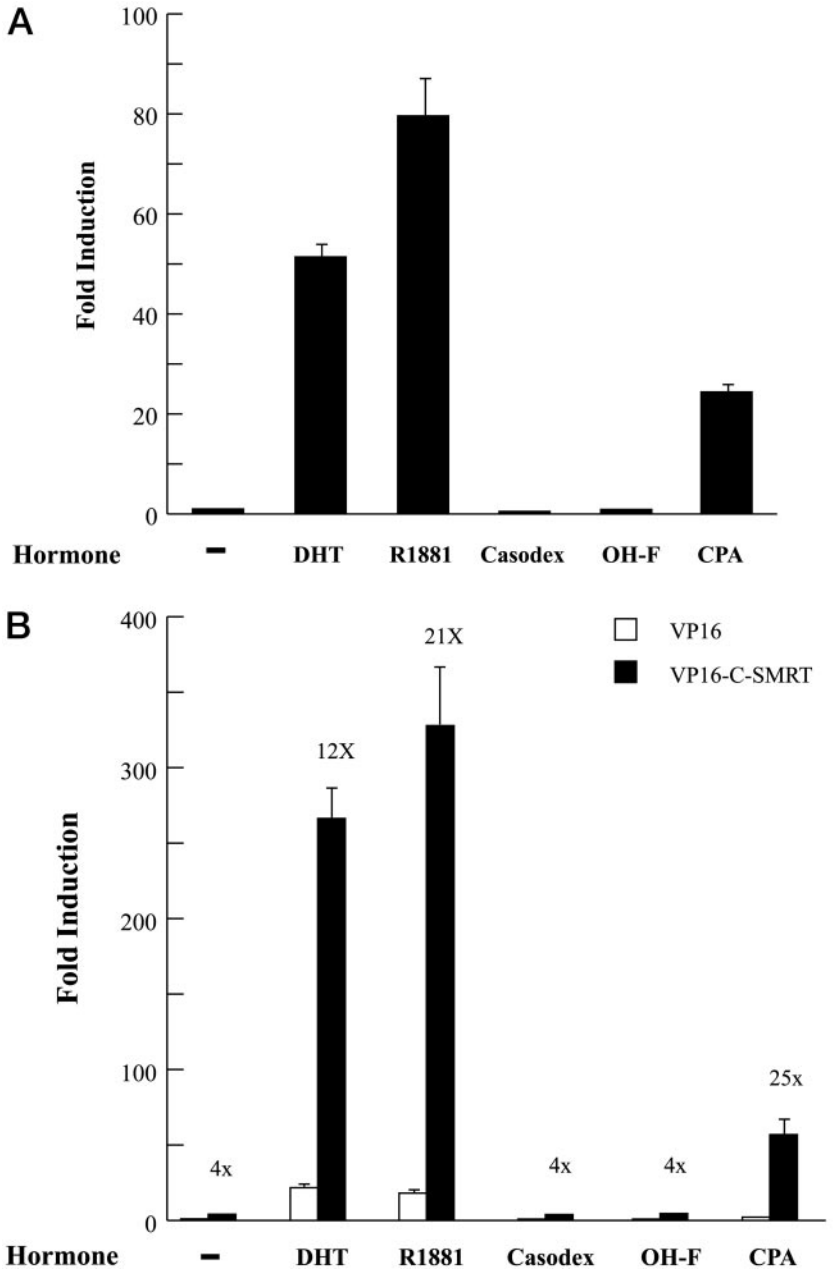


Fig. 1. Ligand Specificity of SMRT-hAR Interaction

The expression plasmid for the full-length wild-type hAR was cotransfected with the MMTV-luc (MMTV long terminal repeat) reporter and tested for ligand-dependent activation in CV1 cells. The following ligands were used: the agonists DHT and the synthetic ligand R1881; the antagonists CAS, OH-F, and CPA. Results shown are the average of two independent experiments with the SEM. A, The luciferase-units obtained in the absence of ligand were set arbitrarily as 1. The values obtained upon treatment with 10^{-7} M ligand are shown as fold induction. B, Modified mammalian two-hybrid experiment cotransfecting full-length wild-type AR, the reporter MMTV-luc, and the expression plasmid coding for VP-SMRT, a fusion of the C-terminal 422 aa of SMRT with the activation domain of VP16 or as control VP16 alone. Interaction is indicated as enhanced promoter activity. *Numbers* indicate the fold activation of VP-SMRT compared with the control VP16.

Response Element Specificity of AR-SMRT Interaction

PSA and probasin are used as important markers of prostate differentiation and prostate cancer growth, as both PSA and probasin expression are indicators of androgen action and AR functionality (24–26). We therefore wanted to analyze whether SMRT interacts with the AR in the context of natural promoters active in prostate such as the PSA and probasin promoters. Using the modified two-hybrid system we analyzed the interaction of VP16-SMRT with the AR on the

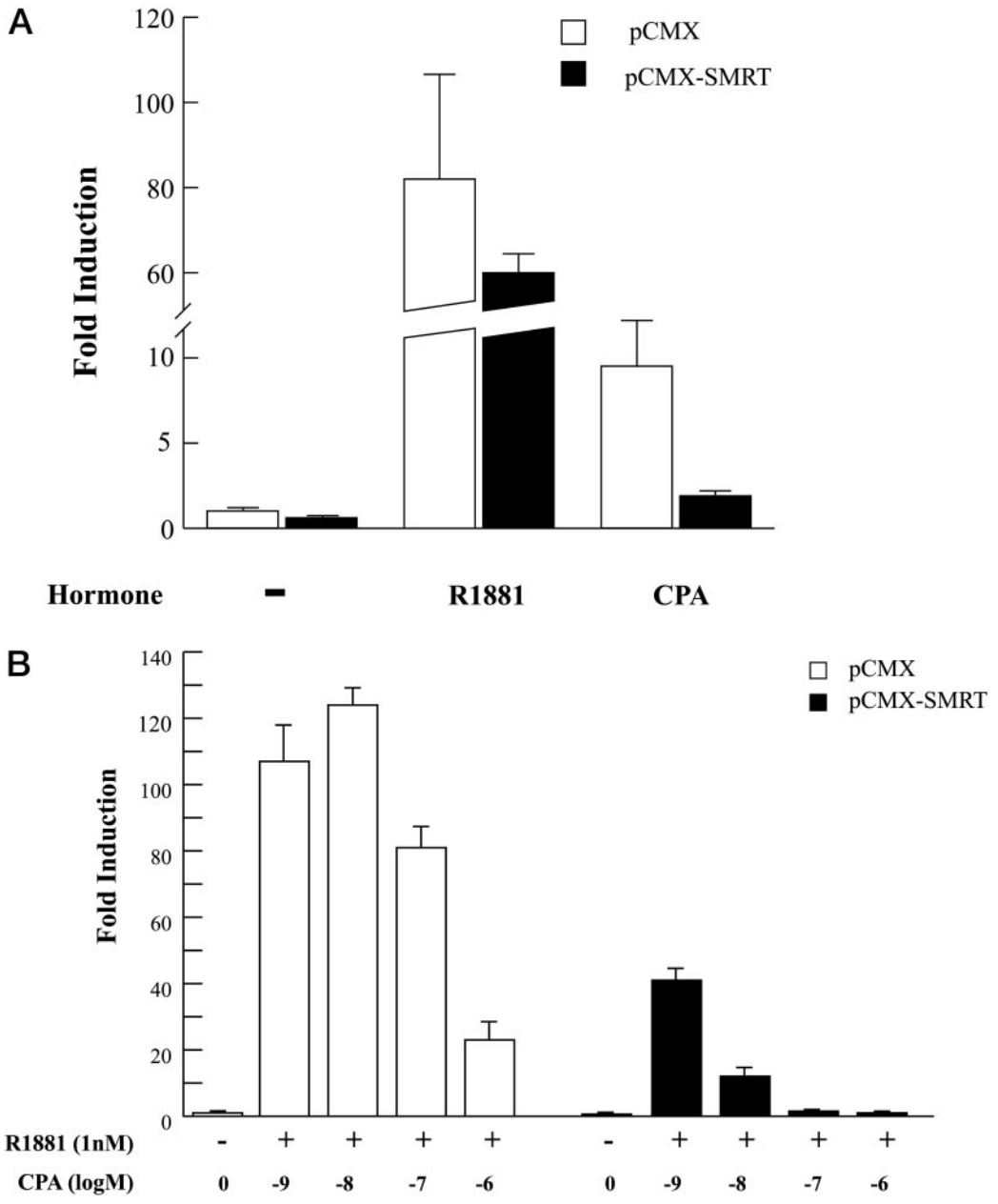


Fig. 2. SMRT Coexpression Renders the Partial Agonist CPA into a Complete Antagonist

The hAR was cotransfected with the expression vector for SMRT (pCMX-SMRT) or as control the empty vector (pCMX) together with MMTV-luc as reporter in CV1 cells. Results shown are the average of two independent experiments with the SEM. A, Effect of the expression of SMRT (*black bars*) on MMTV-luc reporter activity in the presence of R1881 or CPA. Fold induction is calculated relative to the untreated vector control. B, Transfected CV1 cells were treated with both ligands: the agonist R1881 at a concentration of 10^{-9} M and increasing concentrations ranging from 10^{-9} to 10^{-6} M of the antagonist CPA with cotransfected SMRT (*black*) or empty vector (*white bars*). Fold induction is calculated relative to the untreated vector control (pCMX).

following reporters, all of which contain response elements for the AR: PSA-luciferase, GRE2-tk-luciferase, ARR3-luciferase (probasin), and TAT-luciferase, and compared these with the MMTV-luc reporter (Fig. 3). Interestingly, we observed great differences in AR-SMRT interaction using these different response elements. Interaction of SMRT with AR is indicated as an enhancement of CPA-AR-mediated transactivation, comparing the values obtained with and without CPA using VP16 or VP-SMRT. A strong enhancement of transactivation was obtained using the natural promoters of the ARR3 (probasin) and the PSA genes in the presence of VP-SMRT. This effect is dependent on the presence of AR (not shown). Interestingly, both the double glucocorticoid response elements (GREs) in the vector GRE2-tk-luc and the TAT sequence exhib-

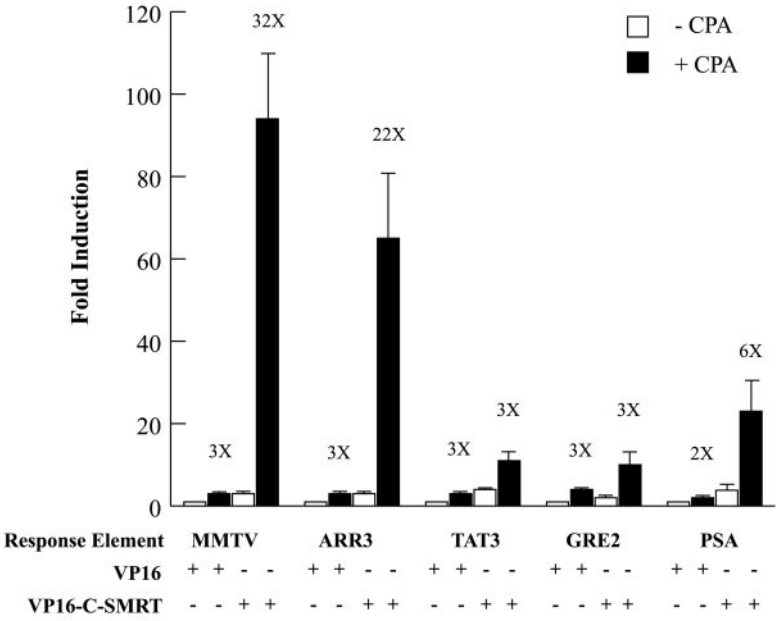


Fig. 3. Response Element Specificity of SMRT-hAR Interaction

Various response elements for AR were used in transient cotransfections in CV1 cells with hAR in the absence or presence of CPA and tested for transactivation and interaction with SMRT in the one-hybrid system (see Fig. 1). Numbers indicate fold activation mediated by CPA, where the basal level in the presence of the VP16 control is arbitrarily set as 1. The response element ARR is derived from the probasin promoter, TAT is derived from the tyrosine amino transferase promoter, GRE represents a palindromic classical GRE, and the PSA reporter harbors the promoter of the PSA. Results shown are the average of two independent experiments with the SEM.

ited no significant enhancement in the presence of the VP-SMRT fusion. The fold CPA-mediated induction is similar in the presence of VP or VP-SMRT, suggesting that CPA-bound AR does not recruit SMRT when AR is bound to these AR-responsive promoters.

This indicates that the gene expression of the prostate markers probasin and PSA may be used to elucidate both antihormone- and corepressor-AR functionality. Thus, our data suggest that the interaction of the corepressor SMRT with the AR is influenced by different AR-responsive promoters, and that SMRT can interact with the AR on natural prostate marker gene promoters.

Detection of SMRT-hAR Complex by Coimmune Precipitation

To verify the interaction of SMRT with AR in the presence of CPA, we performed a coimmune precipitation using wild-type hAR and hemagglutin (HA)-tag alone or HA-tagged full-length SMRT coexpressed in HEK 293 cells in the presence of CPA. Anti-HA antibody was used for immune precipitation and the anti-AR antibody was used for Western detection (Fig. 4). We were able to detect coimmune-precipitated hAR only in the presence of SMRT. As controls the empty pSG5 vector was used in combination with the HA-SMRT expression vector and the empty HA vector in combination with the hAR expression vector. The expression of SMRT was controlled using HA antibody (not shown). A slower migrating unspecific band is present in the immune-precipitations (*asterisk*). LNCaP cell extracts were used as migration and detection control for hAR. Furthermore, we compared the effect of CPA on the AR-SMRT complex in coimmune precipitation experiments. The *lower panel* of Fig. 4 shows a moderate CPA-induced SMRT-AR interaction comparing the treatment with and without CPA, supporting the idea that CPA induces binding of SMRT to AR. As controls, we used both the empty HA vector and HA-SMRT alone. As input control AR-transfected HEK 293 cell extracts were used (input lane).

The N Terminus of hAR Is the Target for Corepressor Interaction *in Vivo*

To identify the interaction site of AR with SMRT we used different deletion mutants of the AR and tested for their interaction with SMRT in the presence of CPA in the modified two-hybrid system (Fig. 5A). Truncation of the AR C terminus [ΔHBD; hAR amino acids (aa) 1–682] resulted in a hormone-independent transcriptionally active receptor. VP-SMRT expression led to a higher transactivation (compare VP16 with VP-SMRT and note the different scaling), suggesting that surprisingly the N terminus of AR is able to interact with SMRT. The N-terminal deletion (ΔN-Term; hAR aa 505–919) lacked significant transcriptional activity in the presence of ligand, which confirms that the major transactivation function of AR is localized in its N ter-

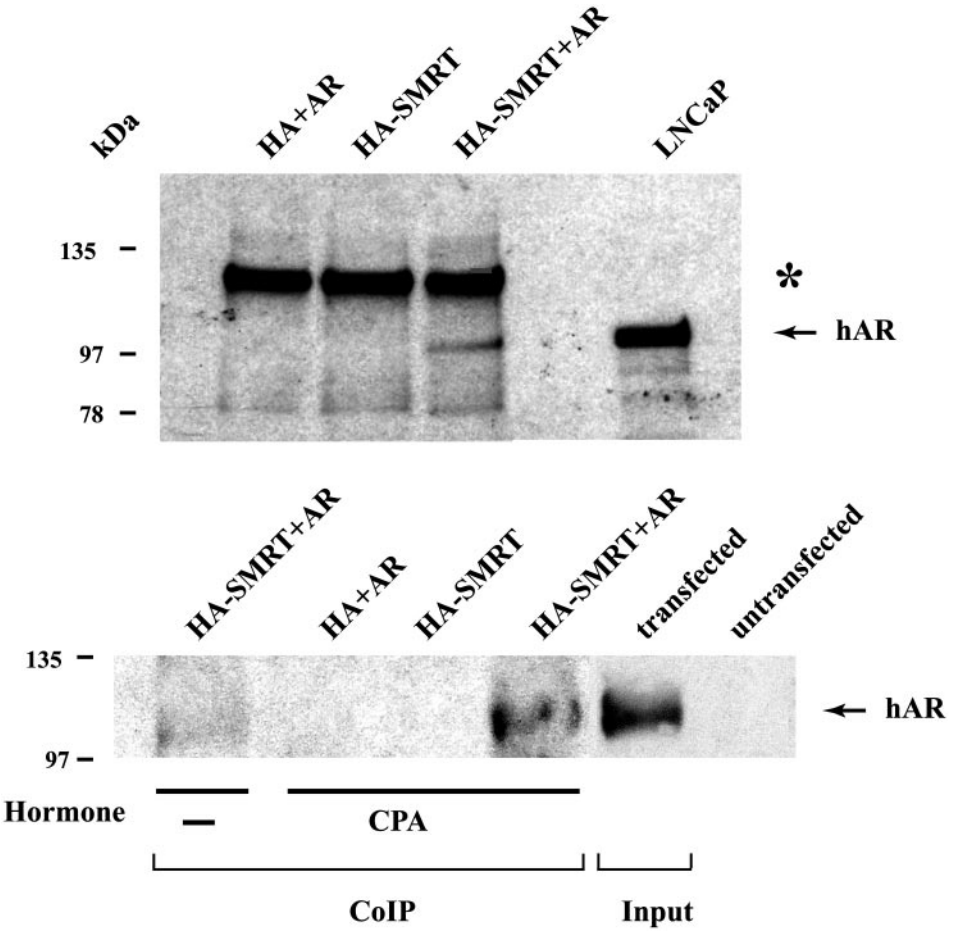


Fig. 4. hAR Interacts with SMRT *in Vivo*

Coimmune precipitation of HA-tagged full-length SMRT with wild-type hAR. Human endothelial kidney 293 cells were transfected with expression plasmids coding for HA or HA-SMRT together with hAR or the empty expression vector. Immune precipitation was performed using HA antibody and the Western was performed using AR antibody. An unspecific band migrating at 130 kDa is recognized by the anti-AR antibody (*asterisk*). As migration control for hAR we used cell extracts from human LNCaP prostate cancer cells. In the *lower panel* anti-hAR antibody Western was performed from coimmune precipitation of transfected 293 cells treated with or without CPA. The *input lane* shows hAR from transfected 293 cells.

minus. This deletion also lacked interaction with SMRT. To control the functionality of this AR deletion, we generated an N-terminal VP16 fusion protein, replacing the AR N terminus with VP16 (VP-AR aa 505–919). This fusion exhibits hormone-dependent transcriptional activation but no effect of SMRT was observed (Fig. 5A). This confirms that the AR N terminus, and not the C terminus, of the hAR is the target for SMRT.

To delineate the sequences in the N terminus of the AR required for interaction with SMRT, we used deletion mutants in this region of hAR. An N-terminal mutant deleting the first 171 aa (171–919; 171-E) bound to SMRT, whereas the deletion of the first 328 aa of hAR abrogated interaction with SMRT (Fig. 5B). Because the first 33 aa of hAR were shown to be involved in intramolecular N- and C-terminal interaction (27), we wanted to know whether they influence SMRT-AR interaction. Therefore, we used internal deletions of hAR (Fig. 5B), which harbor the N-terminal interaction motif required to interact with its C terminus. A mutant containing an internal deletion from aa 39–171 bound SMRT in mammalian cells. However, the deletion mutant Δ39–328 did not bind SMRT (Fig. 5B). Thus, a similar observation was made using internal deletions. This indicates that the first 39 aa are not involved in SMRT binding and that the N-terminal sequences required for AR to interact with SMRT are localized between aa 171 and 328.

We then analyzed the effect of two point mutations localized in recently identified negative regulatory regions in the N-terminal region of AR (28) on their ability to interact with SMRT using the modified mammalian two-hybrid experiment. We analyzed mutant K385E or K518E either singly or in combination. In experiments with the mutant constructs individually or combined, we observe a strongly reduced interaction with SMRT compared with the wild-type AR (Fig. 5C).

In agreement with this finding, we examined the effect of full-length SMRT on various AR deletions and

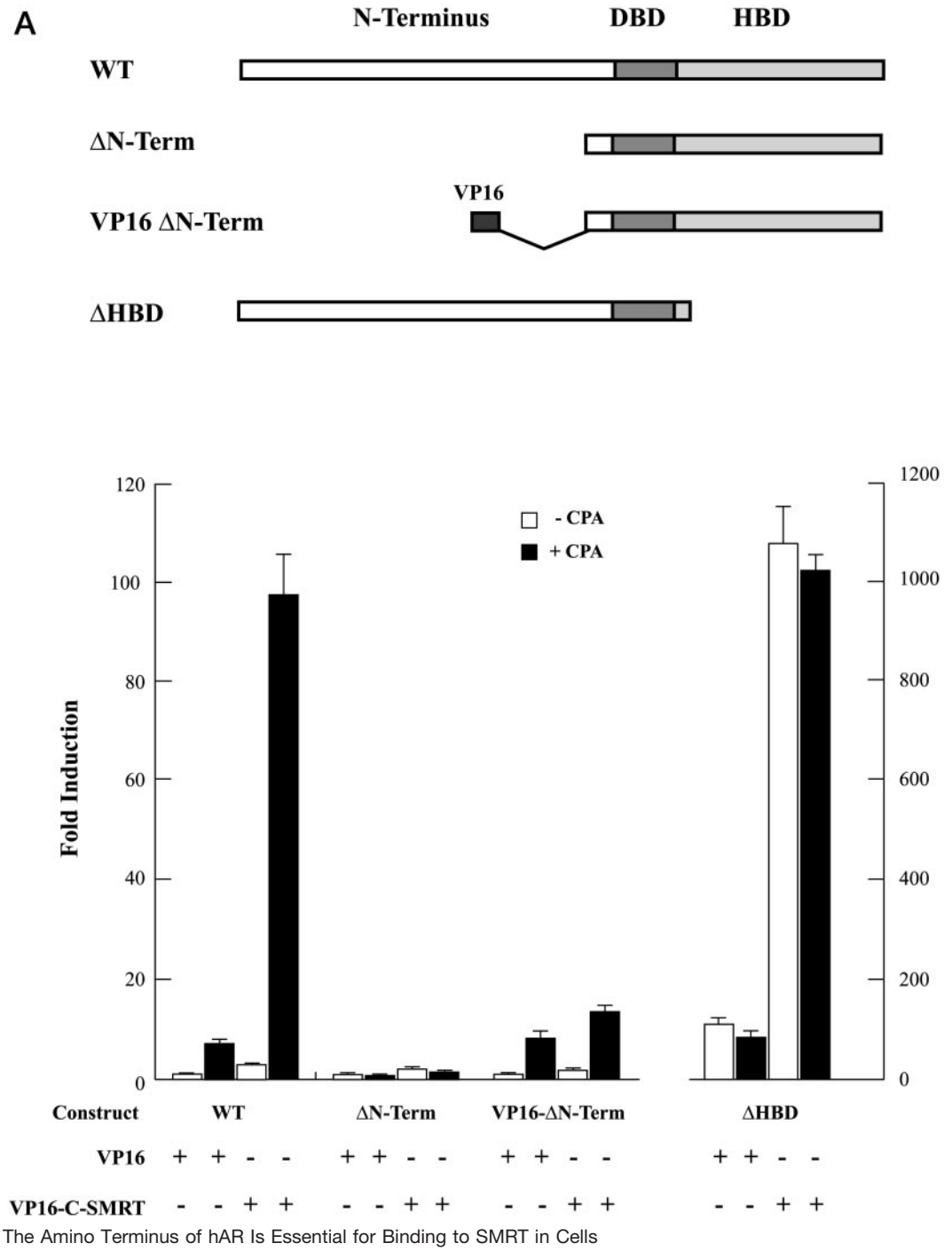


Fig. 5. The Amino Terminus of hAR Is Essential for Binding to SMRT in Cells

Various deletions of hAR were tested for interaction with SMRT in the presence of CPA with the modified mammalian two-hybrid system using MMTV-luc as reporter in CV1 cells. The expression plasmid VP-SMRT or as control VP16 alone (A, B, and C), or full-length SMRT (D) was cotransfected. *White bars* represent values obtained in the absence of ligand and *black bars* represent those with CPA, where the untreated control vector value is arbitrarily set as 1. Results shown are the average of two independent experiments with the SEM. A, Schematic representation and results of wild-type AR and deletions used for the modified mammalian two-hybrid assay. ΔHBD represents a deletion of the HBD including amino acids from 1–682 of hAR; the N-terminal deletion ΔN-Term includes the aa 505–919. VP16 was fused to the transcriptionally inactive ΔN-Term-mutant to regain ligand-dependent transactivation. B, Schematic view and results of N-terminal truncated AR mutants in the modified mammalian two-hybrid analysis in the presence or absence of CPA. C, Point mutants of AR N terminus were tested for interaction with VP-SMRT. Modified mammalian two-hybrid experiment comparing wild-type hAR and the amino acid exchanges K385E and K518E and the double mutant K385E/K518E. *Numbers on top of the bars* show the fold induction mediated by CPA-treated hAR, compared with the untreated, control vector. D, Coexpression of full-length SMRT with wild-type hAR, the various AR deletions, or the double mutant K385E/K518E. Hormone induction is shown in the presence or absence of CPA.

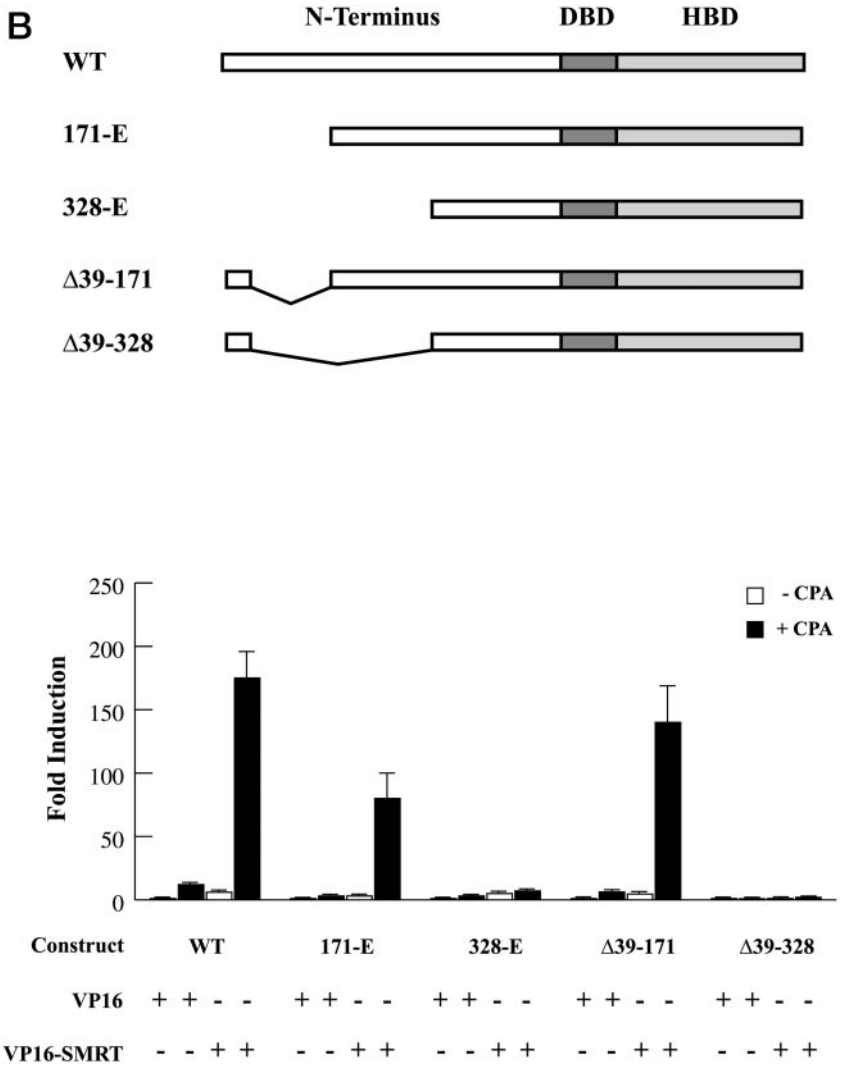
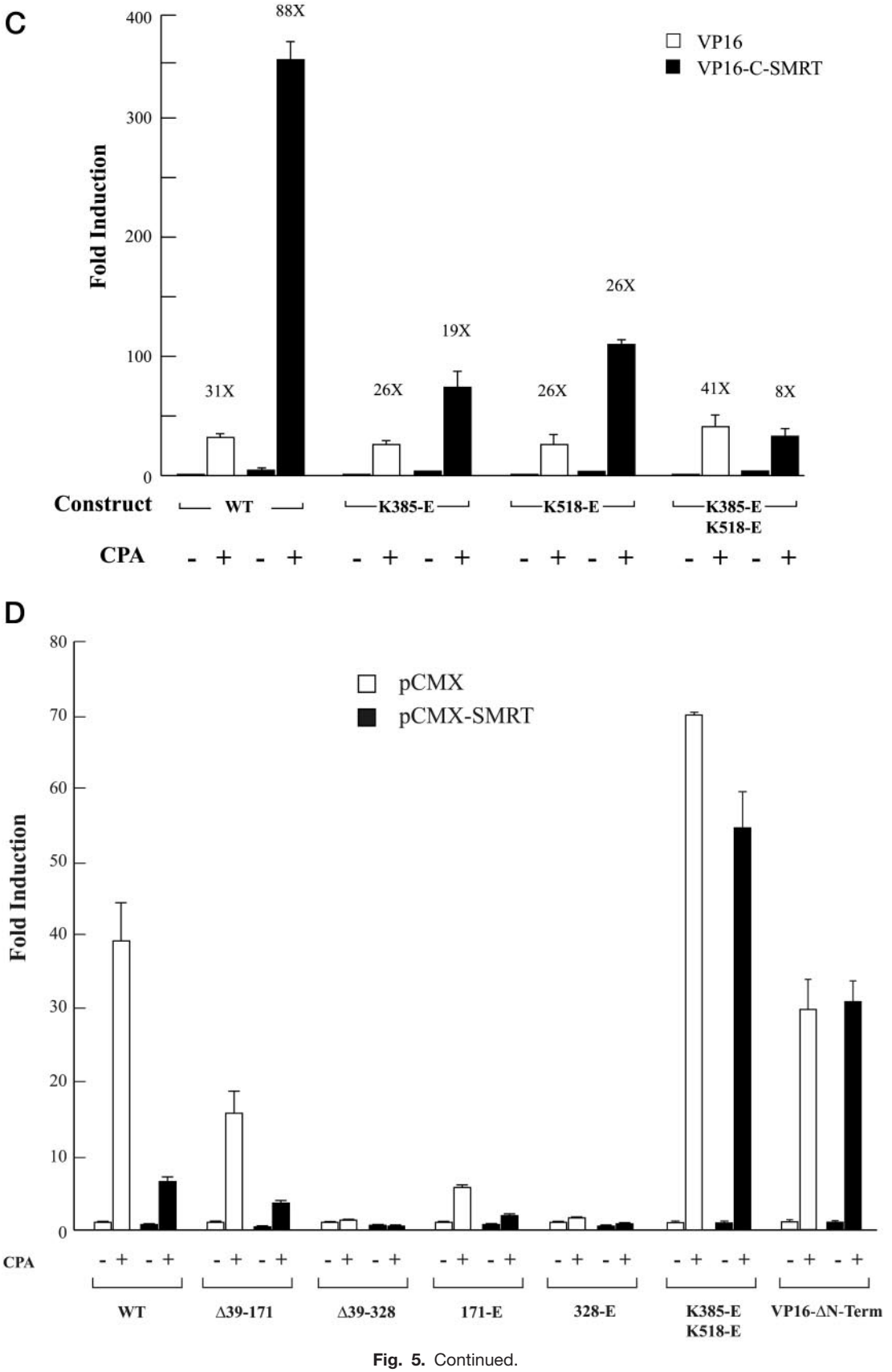


Fig. 5. Continued.

found that SMRT reduced the agonistic activity of CPA on those AR deletions that showed interaction with SMRT. Although the expression of full-length SMRT strongly reduced the agonistic activity of CPA on the wild-type AR, ARΔ39–171, and AR 171-E, only a marginal reduction of the agonistic activity of CPA was observed with the double mutant K385E/K518-E and the N-terminal AR mutants lacking the interaction with SMRT (Fig. 5D). Taken together, this indicates that the binding and inhibitory effect of SMRT is dependent on the AR N terminus and at least overlaps the negative regulatory regions of AR, with the region between 385 and 518 also contributing to the functional interaction with SMRT.

SMRT Binds to the AR N-Terminus *in Vitro*

To analyze the interaction of AR with SMRT *in vitro* and to verify our observation that SMRT binds to the N terminus of hAR, we performed glutathione-*S*-transferase (GST) pull-down experiments. Bacterially expressed GST or GST-C-SMRT, a fusion of the C-terminal 422 aa of SMRT, were incubated with *in vitro* translated and ^{35}S-methionine labeled full-length hAR in the presence of CPA or N-terminal receptor deletions (Fig. 6). In this assay the wild-type hAR bound specifically to GST-SMRT but not to GST alone. If instead of the wild-type AR, only the N terminus (1–505) was radioactively labeled and used in this assay, an interaction with GST-SMRT was still observed, which confirmed our previous data. A fragment of the N terminus encompassing aa 1–328 showed a significant but weaker interaction with SMRT. Neither the mutant AR 1–171, nor, as a negative control, the unrelated protein luciferase interacted with SMRT (Fig. 6, *lower panel*). Taken together, this suggests that the first 328 aa of hAR are sufficient to interact with SMRT but further sequences of the AR N terminus may be required to strongly enhance the interaction of SMRT with the AR N terminus.



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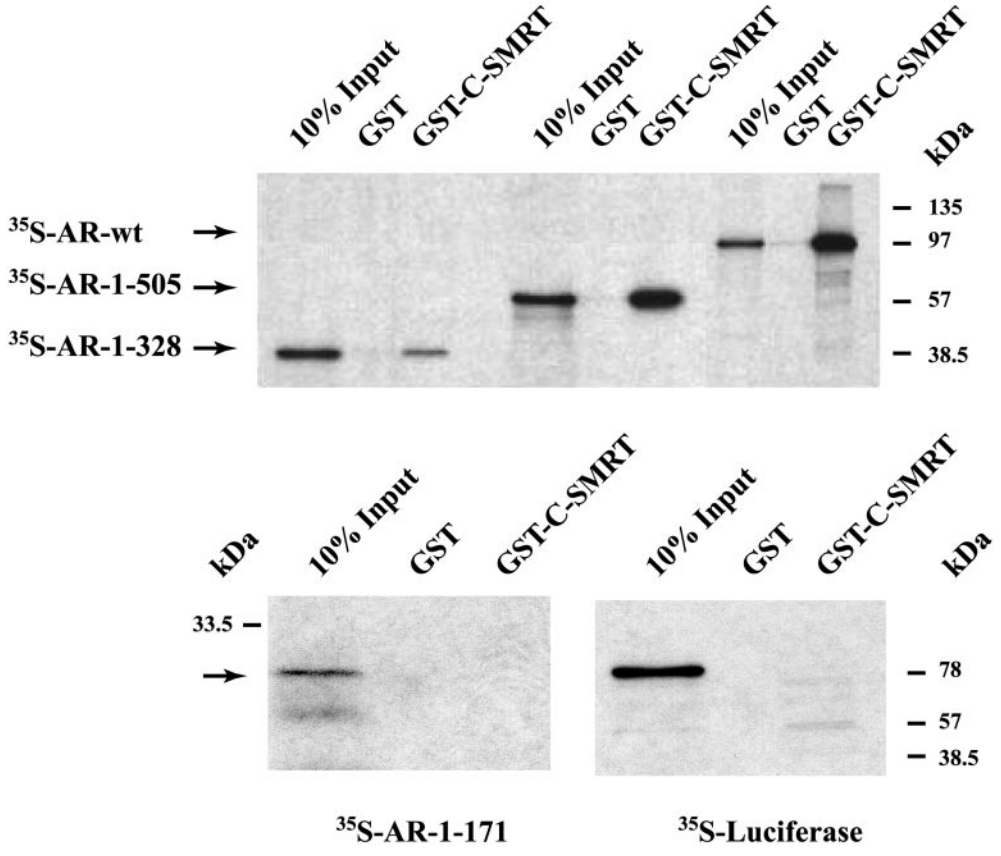

Fig. 6. SMRT Binds to the N Terminus of hAR *in Vitro*

In vitro GST pull-down experiments were performed using bacterially expressed GST-C-SMRT, a fusion of the C-terminal 422 aa of SMRT to GST, or GST alone as control. Full-length wild-type hAR or indicated deletions were *in vitro* translated, ^{35}S-methionine labeled and incubated with immobilized GST or GST-C-SMRT. As negative controls, hAR aa 1–171 or luciferase are shown in the *lower panel*. The *input lane* represents 10% of total radiolabeled protein added. Bound proteins were visualized by fluorography.

Thus, AR binds to SMRT *in vivo* and *in vitro,* suggesting a direct interaction through the involvement of the AR N terminus.

The Interaction of hAR with SMRT in the Presence of CPA Is Controlled by cAMP

Because it is known that factors that increase the intracellular levels of cAMP positively modulate the transcriptional activity of AR (8, 9), we investigated whether cAMP can relieve the binding of SMRT to AR. We first added 8-bromo-cAMP (8-Br-cAMP), a cAMP analog, in the modified mammalian two-hybrid system with hAR (Fig. 7A). Addition of 8-Br-cAMP does not affect the basal promoter activity in the absence of CPA. However, treatment with 8-Br-cAMP resulted in a significant reduction in the fold activation in the presence of VP16-SMRT as compared with in the absence of 8-Br-cAMP, which indicates that treatment with 8-Br-cAMP leads to a decrease in SMRT-AR interaction.

As it is known that cAMP exerts its effects through activating PKA, we tested the effect of the catalytic subunit of PKA on SMRT interaction with AR (Fig. 7B). In cotransfection experiments with the catalytic subunit of PKA, we made similar observations as we did with 8-Br-cAMP. PKA did not have a significant effect on basal promoter activity. However, coexpression of PKA resulted in a strong reduction in reporter activity in the presence of VP16-SMRT, suggesting that overexpression of PKA leads to the dissociation of SMRT from the CPA-bound AR.

Taken together, these results show that increasing the intracellular levels of cAMP or coexpression of PKA down-regulates the interaction of SMRT with AR.

DISCUSSION

Prostate Cancer and Receptor Inactivation

Antiandrogens have found a widespread use in prostate cancer therapy because of their ability to reduce or completely abolish transactivation by AR and, as a result, to reduce or completely block cancer cell proliferation. The molecular basis of action of antiandro-

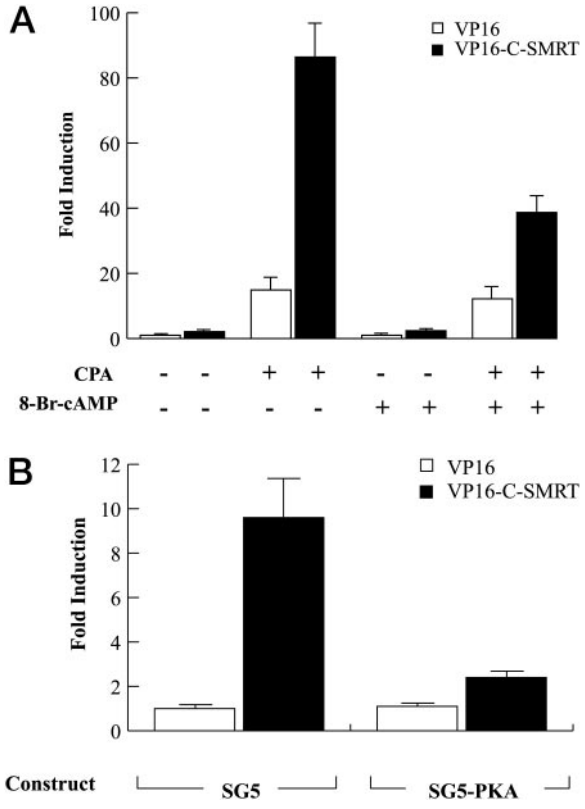

Fig. 7. cAMP Mediators Influence the Interaction of SMRT with hAR

The interaction of hAR with SMRT was tested using the modified mammalian two–hybrid system with expression vectors coding for VP16 or VP-SMRT (each 1 μg) fusions. *Black bars* represent values obtained with expression vectors for hAR and VP-SMRT fusion; *white bars* show the control using expression vector for VP16 alone on the MMTV promoter. Results shown are the average of two independent experiments with the SEM. A, Effect on CPA-mediated MMTV-luc reporter activity of 10^{-4} M of the cAMP analog 8-Br-cAMP on hAR-SMRT interaction. Fold induction is calculated relative to the untreated, empty vector control (pCMX). B, Effect on CPA-mediated MMTV-reporter activity by cotransfection of the expression vector coding for the catalytic subunit of PKA on AR-SMRT interaction, using the modified mammalian two-hybrid system. Fold induction is calculated relative to the untreated control vector.

gens is, however, not fully understood. Moreover, the usefulness of antiandrogen therapy is limited in that a large number of prostate cancers progress to hormone insensitivity and no longer respond to treatment with antiandrogens, despite expressing the AR. One explanation for this resistance could be that the level of a specific corepressor decreases. A reduced corepressor-AR interaction would decrease the repressive effect of the antiandrogen and might subsequently lead to partial agonism by the applied antiandrogen. This, in turn, would then render the AR into a stronger transcriptional activator and lead to tumor proliferation.

Here, we show that at least one of the antagonists, CPA, functions as an androgen antagonist by inducing the association of the corepressor SMRT with the AR. This corepressor strongly reduces the transcriptional activity of the receptor when bound to the AR in the presence of CPA. Similar observations were made for the PR using NCoR and specific antiprogestins such as RU486 and ZK 98299, as well as for the ER using SMRT and the antiestrogen hydroxytamoxifen (18–20, 29).

In our studies we showed that the partial agonistic activity of CPA in CV1 cells is most likely due to a reduced level of SMRT in these cells. Accordingly, coexpression of SMRT resulted in a decrease of CPA-mediated agonism in these cells. Importantly, coexpression of SMRT leads to an enhancement of the antagonistic activity of CPA in the presence of androgen. We therefore conclude that the level of SMRT regulates the action of the antiandrogen CPA. SMRT does not interact with AR in the presence of the antiandrogens OH-F and CAS, indicating that their regulatory activities are based on other mechanisms.

Another proposed mechanism for antiandrogen resistance and tumor regrowth is the activation of the signal transduction machinery (3, 7–10). On the molecular level the activation of the signal transduction pathway and phosphorylation cascades may regulate corepressor-nuclear receptor binding. Our results showing that PKA induces the dissociation of AR from SMRT support this notion.

Mechanisms of SMRT-AR Functionality

Our data show that the C terminus of SMRT interacts with the N terminus of AR. Although both R1881 and CPA induce interaction of hAR with SMRT, our data indicate that there is a functional difference of the corepressor SMRT bound either to the agonist- or antagonist-liganded AR. Either the interaction leads to a functional difference of SMRT itself, or the presence of coactivators modulate the effect mediated by SMRT. This is in agreement with our finding (Fig. 2A) that the corepressor SMRT only weakly affects the transcriptional activity of agonist-bound AR. This may also indicate that the agonist-induced transcriptional activity of AR by recruitment of coactivators overrides the repressive effect of SMRT (30–32).

For both the *in vitro* GST pull-down and the mammalian interaction system we found that the C terminus of SMRT, which harbors the two receptor interaction domains (22), is sufficient for the interaction with AR. Interestingly, both receptor interacting domains of SMRT were shown to be required to bind to nonsteroid receptors, such as TRs and RARs as well as to orphan receptors, such as Rev-Erb (33). In these cases, however, it is the HBD of the receptors that interacts with the C terminus of SMRT. Our finding that the C terminus of SMRT interacts with the N terminus of the AR is therefore unique.

Recently, it was shown that several steroid receptors harbor negative regulatory regions in their N termini when mutated, leading to an increase in receptor-mediated transactivation (28, 34, 35). A small sequence

motif encompassing the following consensus amino acid sequence P-X $_{(0-4)}$ -I/V-K-X-E-X$_{(0-4)}$-P is conserved among receptors for steroids and other transcription factors. Mutations of these sequences in AR led to an enhancement of AR-mediated transcriptional activity. Our findings that SMRT functionally interacts with the AR N terminus and that mutations in this negative regulatory region of the AR abolish interaction with SMRT make these sequences functionally relevant also in negative regulation by antiandrogens. As several coactivators have been shown to mediate AR transactivation function through interaction with the N terminus of AR, binding of SMRT to this region presumably either competes for coactivator binding or the equilibrium of activation, and repression is shifted to a more repressive state when corepressors bind.

MATERIALS AND METHODS

Plasmids

GST Fusion Expression Vectors

The bacterial expression vectors for GST and GST-C-SMRT were described previously (23).

In Vitro Translation Vectors

The AR was translated using pSG5-AR (16) with the coupled *in vitro* transcription-translation TNT kit (Promega Corp., Madison, WI). The vectors pSGAR 1–171, pSG-AR 1–328, and pSG-AR 505 were generated by deleting the coding sequences from *Afl*II to *Bgl*II, Kpn2I to *Bgl*II, and *Acc*65I to *Bgl*II, respectively.

Mammalian Expression Vectors

The expression vectors for AR [pSG-AR; pSG-ARΔHBD; (16)] and all AR deletions were generated using standard cloning techniques with restriction enzyme digests. The internal deletions of AR were generated by deleting AR sequences with *Cfr*9 and *Afl*II (aa 39–171) or with *Cfr*9I and *Eco*47III (aa 39–328) and insertion of a small oligonucleotide to keep the reading frame intact. Clones were verified by *in vitro* translation. The mammalian expression vectors for VP16, VP-SMRT, and pCMX-SMRT (21) were kindly provided by Ron Evans (The Salk Institute, La Jolla, CA). Full-length pHA-SMRT was constructed by in-frame insertion of SMRT cDNA (14) in the pMTII-HA vector. The reporters MMTV-luc (16), ARR3-Luc kindly provided by Dr. R. J. Matusik (Vanderbilt University, Nashville, TN) (24), TAT3-Luc (28), GRE2-Luc (16), and PSA-Luc (25) contain the luciferase gene as reporter gene.

Cell Culture

CV1 and HEK-293 cells were grown in DMEM with 10% FCS at 37 C/5% CO_2. Cotransfections were carried out using the $CaPO_4$-method as described earlier (36). hAR expression vector (1 μg) was cotransfected with 5 μg of indicated reporter plasmid. For the modified mammalian two-hybrid experiments, 1 μg of either VP16-C-SMRT, VP16 expression plasmids, full-length SMRT, or empty expression plasmid was used. For PKA coexpression studies, 1 μg of PKA expression plasmid was cotransfected, and charcoal-treated 10% FCS was used. All transfection assays shown were done in independent duplicate or triplicate sets. Each set was repeated at least two more times. At least two different double CsCl gradient-purified plasmid preparations were used. The error bars represent the deviation of the mean value. For hormonal studies 10^{-7} M hormone was added except when otherwise stated. Where indicated, cells were treated with 10^{-4} M 8-Br-cAMP. pCMV-β-galactosidase was cotransfected as internal control for transfection efficiency.

Coimmune Precipitation

HEK-293 cells were cotransfected with full-length hAR expression vector together with either HA or HA-SMRT expression vector coding for the HA-tagged full-length SMRT. Hormone was added (10^{-7} M CPA) immediately after transfection. After 48 h, cells were lysed on ice in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 1% Nonidet P-40, 1%, Triton X-100, and 10^{-7} M CPA. Cell debris was pelleted at 13,000 rpm at 4 C for 10 min. Purified HA monoclonal antibody (1.5 μg) (12C5 m-αHA) was added to each cell extract and incubated at 4 C overnight. Cell extracts were incubated for 2 h with 15 μl of protein A/G-agarose beads (Sigma, St. Louis, MO) at 4 C. Beads were washed five times with washing buffer [50 mM Tris-HCl, (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1% Nonidet P-40, and 10^{-7} M CPA], and subjected to SDS-PAGE. Western analysis was performed by using anti-AR antibody (F39.4.1, BioGenex Laboratories, Inc., San Ramon, CA) and the enhanced chemiluminescence detection method (Amersham Pharmacia Biotech, Arlington Heights, IL).

GST Pull-Down Experiments

Bacterial expression of GST or GST-C-SMRT, encompassing the C-terminal 422 aa, was performed by induction of gene expression with 0.2 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 C in the BL21(lys) bacterial strain. Purification of the GST fusion proteins and interaction studies with *in vitro* translated, ^{35}S-methionine-labeled nuclear hormone receptors (TNT kit from Promega Corp.) were as described previously (36). In each experiment, the amount of the input lane was 10% of that incubated with the glutathione beads (Pharmacia Biotech, Piscataway, NJ). The SDS-PAGEs were stained with Coomassie brilliant blue to ensure equal loading of GST fusion proteins. The bound and labeled proteins were visualized by fluorography. For full-length AR, 10^{-7} M CPA was used during translation and interaction studies.

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