Differential modulation of androgen receptor transcriptional activity by the nuclear receptor co-repressor (N-CoR)

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Antiandrogens are widely used agents in the treatment of prostate cancer, as inhibitors of AR (androgen receptor) action. Although the precise mechanism of antiandrogen action is not yet elucidated, recent studies indicate the involvement of nuclear receptor co-repressors. In the present study, the regulation of AR transcriptional activity by N-CoR (nuclear receptor co-repressor), in the presence of different ligands, has been investigated. Increasing levels of N-CoR differentially affected the transcriptional activity of AR occupied with either agonistic or antagonistic ligands. Small amounts of co-transfected N-CoR repressed CPA (cyproterone acetate)- and mifepristone (RU486)-mediated AR activity, but did not affect agonist (R1881)-induced AR activity. Larger amounts of co-transfected N-CoR repressed AR activity for all ligands, and converted the partial agonists CPA and RU486 into strong AR antagonists. In the presence of the agonist R1881, co-expression of the p160 co-activator TIF2 (transcriptional intermediary factor 2) relieved N-CoR repression up to control levels. However, in the presence of RU486 and CPA, TIF2 did not functionally compete with N-CoR, suggesting that antagonist-bound AR has a preference for N-CoR. The AR mutation T877A ($Thr^{877} \rightarrow Ala$), which is frequently found in prostate cancer and affects the ligand-induced conformational change of the AR, considerably reduced the repressive action of N-CoR. The agonistic activities of CPA- and hydroxyflutamide-occupied T877A-AR were hardly affected by N-CoR, whereas TIF2 strongly enhanced their activities. These results indicate that lack of N-CoR action allows these antiandrogens to act as strong agonists on the mutant AR.

Key words: antiandrogen, androgen receptor (AR) mutation, nuclear receptor co-repressor (N-CoR), transcriptional intermediary factor 2 (TIF2), transcriptional repression.

INTRODUCTION

As a member of the nuclear receptor superfamily, the AR (androgen receptor) mediates the biological effects of male sex hormones, testosterone and DHT (dihydrotestosterone) [1]. These androgens play a critical role in normal development and maintenance of the male sexual characteristics and are also involved in development and progression of prostate cancer [2]. Unliganded AR exists in an inactive form associated with heat-shock proteins. After ligand binding, heat-shock proteins dissociate from the receptor, and then the AR translocates to the nucleus [3], where it regulates transcription by binding to specific androgen response elements in promoters of target genes. Together with co-regulatory proteins and general transcription factors, a stable transcription initiation complex is formed [4–6]. An important feature of ligand binding is that it induces a conformational change in the AR LBD (ligand-binding domain), providing a surface for interactions with co-regulatory proteins. These co-regulatory proteins have the ability to enhance (co-activators) or repress (co-repressors) the transcriptional activity of nuclear receptors [7,8]. The change in AR protein structure is dependent on the type of ligand that occupies the LBD. In this respect, antiandrogens induce a conformational change in the AR protein different from that induced by androgens [9].

Antiandrogens are pharmaceutical compounds that are used in the treatment of metastatic prostate cancers. They block AR function, which generally results in a reduction of tumour growth [10]. Antiandrogens are either complete or partial inhibitors of AR function, depending on their nature and concentration. At high concentrations, CPA (cyproterone acetate) and RU486 (mifepristone) exhibit partial agonistic activities. In contrast, OHF (hydroxyflutamide) and BIC (bicalutamide) are complete antagonists and reduce AR activity close to that of unliganded AR [11].

In spite of the initial repressive effects, essentially all prostate cancers escape from antiandrogen therapy. In some of these hormone refractory tumours, a hot-spot AR mutation ($Thr^{877} \rightarrow Ala$; T877A) has been reported [12,13]. The mutation, which was initially detected in the prostate tumour cell line LNCaP [14], strongly affects AR steroid binding characteristics and its response to a variety of non-androgenic hormones and antiandrogens [14–16]. In this respect, the antiandrogens OHF and CPA exhibit strong agonistic activities on the T877A mutant AR. The underlying molecular mechanism for the change in response to OHF and CPA is not fully understood. Some clue is given by the observation that, after binding of OHF and CPA, the T877A mutation allows the AR to adopt an active conformation, similar to that found with the agonists R1881 and DHT [9,17].

For the oestrogen receptor and PR (progesterone receptor), the silencing effects of the antihormones, hydroxytamoxifen and RU486, are mediated by N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptor) [18–20]. Transcriptional repression by these co-repressors involves the recruitment of histone deacetylases to

Abbreviations used: AD, activation domain; AR, androgen receptor; BIC, bicalutamide; CHO, Chinese-hamster ovary; CPA, cyproterone acetate; DHT, dihydrotestosterone; LBD, ligand-binding domain; LUC, luciferase; MMTV, murine-mammary-tumour virus; N-CoR, nuclear receptor co-repressor; OHF, hydroxyflutamide; PR, progesterone receptor; PSA, prostate-specific antigen; SMRT, silencing mediator for retinoid and thyroid hormone receptor; TIF2, transcriptional intermediary factor 2; VP16, viral protein 16; wt, wild-type.

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the promoter of target genes [21–24]. More recently, evidence became available that N-CoR and SMRT are also involved in AR action [25–28]. Chromatin immunoprecipitation assays demonstrated that BIC-bound AR in LNCaP cells recruit N-CoR and SMRT to the promoter of the PSA (prostate-specific antigen) gene [25]. SMRT and N-CoR suppress CPA- and flutamide-bound AR, as well as the DHT-stimulated AR activity [26–28]. For a better understanding of the molecular basis of antiandrogen action, we investigated the response of the AR, occupied with different ligands, to overexpression of N-CoR. The results provide new insights into antiandrogen action, with a significant role for N-CoR.

EXPERIMENTAL

Hormones

R1881 (methyltrienolone) was purchased from NEN (Boston, MA, U.S.A.). RU486 (RU38486) was a gift from Roussel Uclaf (Romainville, France), CPA from Schering AG (Berlin, Germany), OHF from Schering USA (Bloomfield, NJ, U.S.A.) and BIC (ICI 176.334, casodex) from AstraZeneca (Macclesfield, U.K.). BIC was dissolved in ethanol freshly before each use.

Plasmid construction

For constructing pSG5-N-CoR, an *Eco*RI–*Not*I–*Bam*HI polylinker was ligated into *Eco*RI–*Bam*HI-digested pSG5 vector. The mouse N-CoR cDNA [29] from pBKS-N-CoR (a gift from Dr C. Glass, Department of Cellular and Molecular Medicine, University of California, San Diego, CA, U.S.A.) was ligated as a *Not*I fragment into this modified pSG5 vector. To obtain wt (wild-type) AR-vp, the AD (activation domain) of VP16 (viral protein 16) (VP16-AD) was fused to the C-terminal end of the AR. To remove the stop codon, PCR was performed on pSVAR [30] with the AR primer 5′-TGAGGCACCTCTCTCAAGA-3′ and with an AR primer containing additional *Cla*I, *Bam*HI and *Eco*RI sequences, 5′-TGGAATTCGGATCCATCGATCCTCCT-CCTCCTCCCTGGGTGTGGTGGAAATAGAT-3′. The PCR fragment was *Eco*RI-digested and exchanged with the *Eco*RI fragment of pSVAR. The resulting construct was digested with *Cla*I–*Bam*HI, and the *Cla*I–*Bam*HI VP16-AD fragment was integrated. T877A-AR-vp was constructed using wt AR-vp and Stratagene's (La Jolla, CA, U.S.A.) Quik Change™ site-directed mutagenesis kit. Mutagenesis was performed according to the manufacturer's instructions. The constructs were verified by sequencing.

LUC (luciferase) assays

CHO cells (Chinese-hamster ovary cells) were plated, in triplicate, in 24-well plates at a density of 2×10^4 cells/well in 0.5 ml of Dulbecco's modified Eagle's medium/F12 medium, supplemented with 5 % (v/v) dextran-coated charcoal-treated foetal calf serum (Hyclone, Logan, UT, U.S.A.). Cells were transfected utilizing FuGENE™ 6 transfection agent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions (0.5 μl/well FuGENE reagent). Reporter plasmids (MMTV-LUC and GRE-TATA-LUC, where MMTV stands for murine-mammary-tumour virus) were constructed as described previously [31]. AR expression plasmids (1 ng/well) were co-transfected with 50 ng/well reporter plasmid and pSG5-N-CoR, pSG5-TIF2 (transcriptional intermediary factor 2) or empty vector pSG5, where indicated. pTZ19 carrier plasmid was added to a total DNA concentration of 250 ng/well. After an overnight incubation with hormones (1 nM R1881 or 1 μM antihormone) or vehicle (ethanol), cells were harvested for LUC measurement as described previously [11]. The results shown are representative of at least three independent experiments.

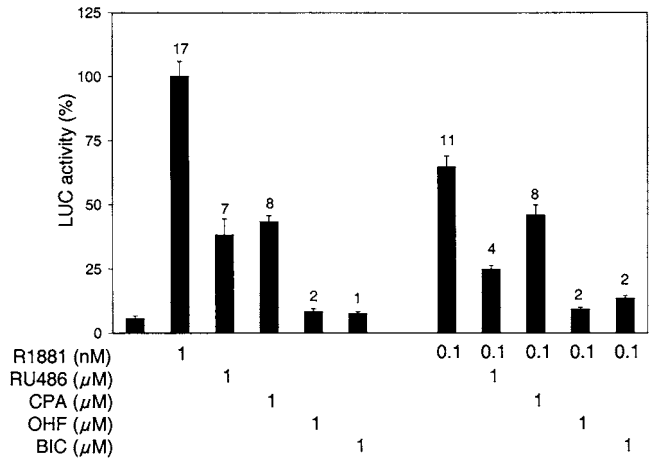

Figure 1 Complete and partial antagonistic activities of antiandrogens

CHO cells were transfected with wt AR (1 ng/well) together with MMTV-LUC reporter construct (50 ng/well) as described in the Experimental section. Cells were incubated overnight with vehicle (ethanol), 1 nM R1881, 1 μM antiandrogen (RU486, CPA, OHF and BIC) or a combination of 0.1 nM R1881 and 1 μM antiandrogen. Cells were lysed and LUC activity was measured. Three independent experiments were performed and a representative experiment (means ± S.E.M.) is shown. Fold induction is displayed above each bar and represents the ratio of the LUC activities in the presence or absence of (anti) hormone.

PC3 cells were plated, in triplicate, in 24-well plates at a density of 2×10^4 cells/well in 0.5 ml of RPMI 1640 medium, supplemented with 5 % dextran-coated charcoal-treated foetal calf serum. Cells were transfected with 0.6 μl/well FuGENE reagent. AR expression plasmids (wt AR or T877A AR, 10 ng/well) were co-transfected with 50 ng/well MMTV-LUC and pSG5-N-CoR (100 ng/well) or empty vector pSG5, where indicated. pTZ19 carrier plasmid was added to a total DNA concentration of 300 ng/well. After an overnight incubation with hormones (1 nM R1881 or 1 μM antihormone) or vehicle (ethanol), cells were harvested and LUC activity was measured.

Western-blot analysis

CHO cells were plated at a density of 8×10^5 cells/80 cm^2 flask and transfected with 40 ng of AR expression plasmid in the absence or presence of 4 μg of pSG5-N-CoR, together with pTZ19 carrier plasmid to a total of 10 μg of DNA and with 20 μl of FuGENE reagent. After an overnight incubation with hormones, cells were washed once with PBS and lysis buffer [32], supplemented with protease inhibitors (Complete™; Roche Diagnostics), was added. Lysates were centrifuged for 10 min at 400 000 ***g*** and AR was immunoprecipitated with monoclonal antibody F39.4.1 [33]. Samples were then subjected to SDS/PAGE and blotted on to a nitrocellulose membrane. AR was immunoblotted with AR polyclonal antibody SP197 [32] and visualized by chemiluminescence detection.

RESULTS

Partial and complete antagonistic action of antiandrogens

Transient transfection studies reveal the antiandrogenic properties of the antiandrogens OHF, CPA, BIC and RU486. Wt AR construct was transfected into CHO cells together with the MMTV-LUC reporter plasmid (Figure 1). The agonist R1881

strongly induced AR activity at low concentrations (0.1 and 1 nM). The activities of AR occupied with the full antagonists OHF and BIC were close to the activity observed in the absence of ligand, whereas the partial agonists RU486 and CPA showed intermediate activities (25 and 40 % respectively). When tested in competition with 0.1 nM R1881, addition of an excess antiandrogen reduced the AR activity to the individual level of each antihormone.

Differential repression of AR activity by N-CoR

To determine the repressive action of N-CoR in the presence of different ligands, co-transfection assays with AR and N-CoR were performed. As shown in Figure 2(A), N-CoR (30 ng/well) repressed the partial agonistic activities of RU486- and CPA-occupied AR. Absolute LUC values as well as induction levels were reduced close to basal level, the activity obtained in the absence of hormone. This suggests that actual expression levels of N-CoR determine the antagonistic potential of RU486 and CPA. In this respect, high expression of N-CoR transforms these partial antiandrogens into complete antagonists. Activities obtained with the pure antagonists OHF and BIC remained at basal levels after co-expression of N-CoR. Repression of AR activity was not restricted to antiandrogens, as the R1881-induced activity was also reduced, to approx. 30 %, by overexpression of N-CoR. To study further the N-CoR response, repression assays with different amounts of co-transfected N-CoR were performed. If for each ligand (R1881, RU486 or CPA) the AR activity obtained in the absence of N-CoR construct was set at 100 % ('C' in Figure 2B), a clear difference in response to N-CoR was observed. At low levels of N-CoR (1 ng/well), the activity of RU486-bound AR was repressed to approx. 60 %. In the presence of CPA, the AR activity was also reduced, although somewhat less when compared with RU486. In contrast, R1881-induced AR activity was not reduced by co-expression of 1 ng/well N-CoR. When larger amounts of N-CoR were transfected (10 and 30 ng/well), AR activities in the presence of RU486 and CPA decreased markedly. The repression curve of R1881 illustrates a different response to N-CoR; the AR activity is only strongly repressed at the highest level of N-CoR.

As determined by Western immunoblotting (Figure 2C), AR expression in CHO cells was not reduced by co-expression of N-CoR. This excludes the possibility that repression of AR activity by N-CoR was due to a decrease in AR protein levels.

Repression of wt AR-vp activity on different promoters

To obtain a more sensitive assay for studying the repressive action of N-CoR, VP16-AD was fused to the C-terminus of the AR. This fusion protein, wt AR-vp, had a 5–10-fold higher transcriptional activity compared with wt AR (Figure 3A; cf. with Figure 1). Similar to wt AR, CPA and RU486 are partial agonists for the AR-vp fusion protein (30 and 50 % respectively). Owing to increased sensitivity, significant wt AR-vp activities with OHF and BIC could be observed (induction values of 7 and 8 respectively).

N-CoR-mediated repression of wt AR-vp activity in the presence of R1881, RU486 or CPA was similar to that observed with wt AR (Figure 3B). Although activities obtained with OHF and BIC were low, a decrease in activity was clearly observed after N-CoR overexepression.

Repression of wt AR-vp activity by N-CoR was also studied for the minimal GRE-TATA promoter. Wt AR-vp was transfected with a GRE-TATA-LUC reporter construct in the absence and presence of N-CoR (Figure 3C). Similar to the results obtained with the MMTV promoter (Figure 3B), wt AR-vp activities were

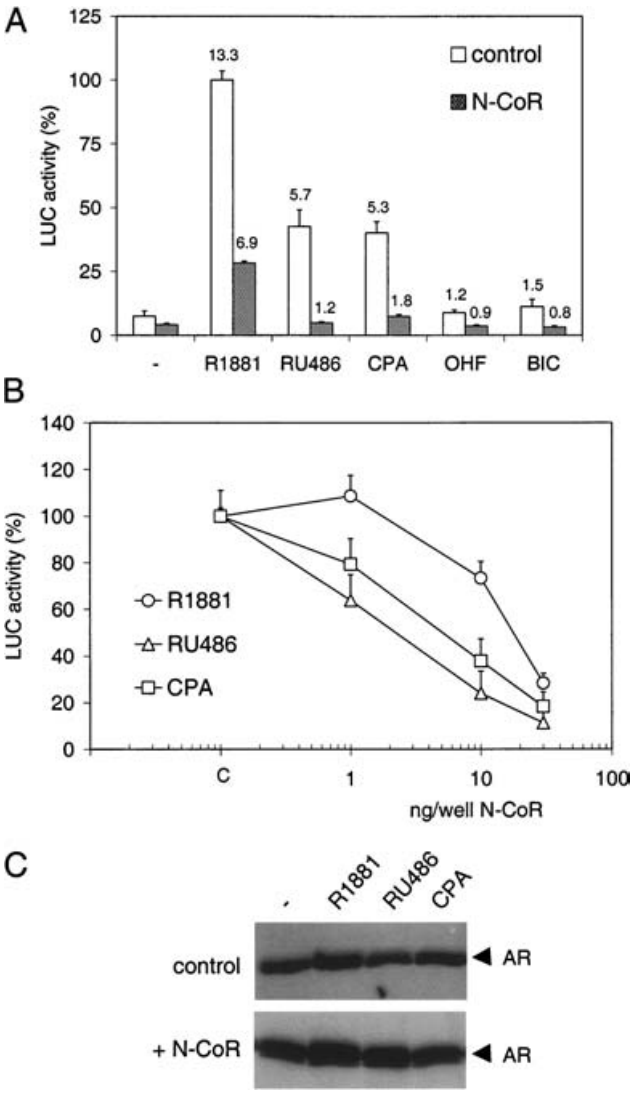

Figure 2 Repression of wt AR activity by N-CoR

(**A**) CHO cells were transfected with MMTV-LUC reporter and wt AR (1 ng/well) in the absence or presence of 30 ng/well pSG5-N-CoR. In the control situation, equal molar fractions of empty vector (pSG5) were transfected. Cells were incubated overnight with vehicle (−), 1 nM R1881 or 1 μM antiandrogen (RU486, CPA, OHF and BIC). LUC activity of wt AR alone with R1881 was set at 100 %. LUC activities were determined from three experiments (means ± S.E.M.). Fold induction is displayed above each bar. (**B**) Wt AR was co-transfected with increasing amounts of pSG5-N-CoR (1, 10 and 30 ng/well). pSG5 vector was added to obtain equal molar fractions of plasmid in each well. For each ligand (R1881, RU486 and CPA), LUC activity of wt AR in the absence of N-CoR was set at 100 %. LUC activities were determined from three experiments (means ± S.E.M.). (**C**) Immunoblot of AR protein expression in CHO cells in the absence or presence of co-transfected N-CoR (4 μg/80 cm^2 flask), as described in the Experimental section. Before AR protein isolation, cells were incubated overnight with vehicle (−), R1881 (1 nM), RU486 (1 μM) or CPA (1 μM).

repressed differentially by N-CoR. Again, the strongest reduction in activity was observed in the presence of RU486. These results indicate that the differential repressive effects of N-CoR do not depend on MMTV-specific promoter elements.

Effect of the T877A mutation on N-CoR repression of AR activities

The ligand-type-dependent effects of N-CoR on AR activity prompted us to study an AR mutant, frequently found in prostate cancer. Due to a mutation in the LBD (T877A), the response of this mutant to antiandrogens is markedly changed. As shown in

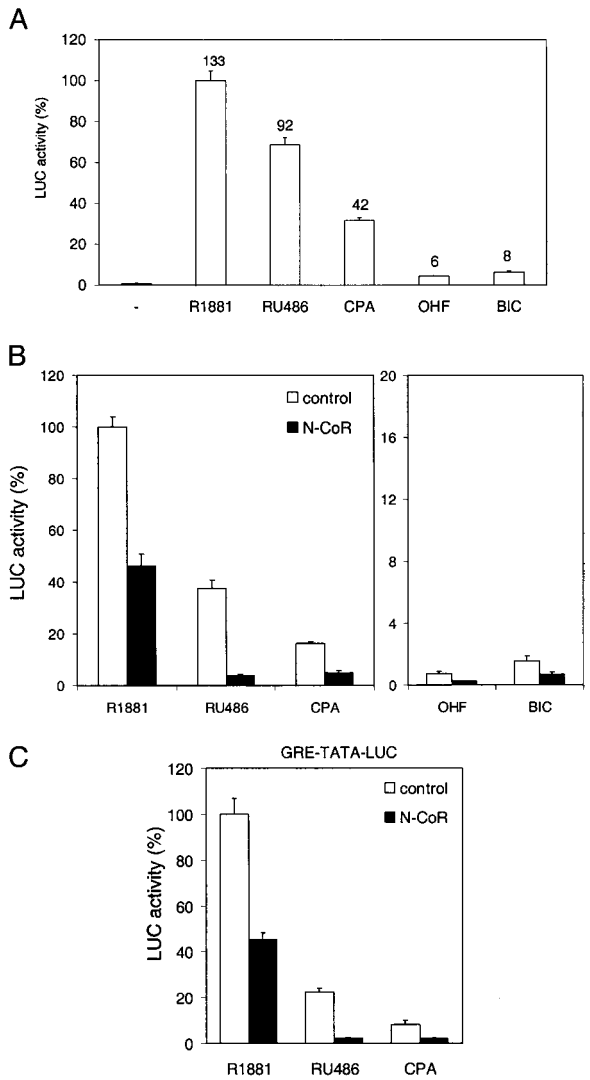

Figure 3 Repression of wt AR-vp activity

(**A**) CHO cells were transfected with MMTV-LUC and wt AR-vp (1 ng/well). After an overnight incubation with vehicle (—), R1881 (1 nM) or 1 μM antiandrogens (RU486, CPA, OHF or BIC), cells were lysed and LUC activity was measured. LUC activities were determined from three experiments (means $\pm$ S.E.M.). Fold induction is displayed above each bar. (**B**, **C**) CHO cells were transfected with wt AR-vp (1 ng/well) and either MMTV-LUC (**B**) or GRE-TATA-LUC (**C**) reporter. In addition, cells were co-transfected with pSG5-N-CoR (100 ng/well) or equal molar fractions of pSG5 vector. LUC activity of wt AR-vp alone in the presence of R1881 was set at 100 %. LUC activities were determined from three experiments (means $\pm$ S.E.M.).

Figure 4, the T877A substitution has differential effects on AR transcriptional activity. The T877A-AR activity in the presence of OHF and CPA was strongly increased when compared with wt-AR. Interestingly, the mutation did not affect the partial agonistic activity of RU486. In addition, BIC remained a pure antagonist for the mutated AR. To investigate the effect of the T877A mutation on recruitment of N-CoR by AR, co-transfection assays were performed with either wt AR-vp or T877A-AR-vp (Figure 5). Wt AR-vp activities induced by increasing concentrations of RU486 and CPA were strongly repressed by co-expression of N-CoR. The R1881-induced activity was reduced to approx. 40 % by N-CoR. Although the activities of OHF- and BIC-bound wt AR-vp were very low, a clear repression by co-transfection of N-CoR could be observed. The T877A-AR-vp activities induced by OHF, CPA and R1881 were not, or only slightly, affected by

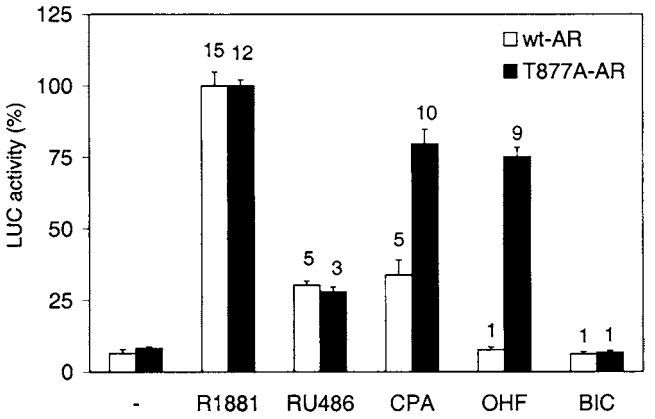

Figure 4 Effect of the T877A mutation on AR transcriptional activity

CHO cells were transfected with 1 ng/well of either wt AR (open bars) or T877A-AR (black bars) together with MMTV-LUC reporter construct. After an overnight incubation with vehicle (—), 1 nM R1881 or 1 μM antiandrogen (RU486, CPA, OHF or BIC), cells were lysed and LUC activity was measured. Three independent experiments were performed and a representative experiment (means $\pm$ S.E.M.) is shown. Fold induction is displayed above each bar.

N-CoR overexpression. This suggests that the relieved N-CoR action can account for the strong agonistic action of OHF and CPA on the T877A-AR. In contrast, the RU486-induced T877A-AR-vp activity was still repressed by N-CoR, and repression in the presence of BIC also seems to remain unaffected by the mutation.

Competition between TIF2 and N-CoR is ligand-type-dependent

The p160 transcriptional co-activator TIF2 has been shown to enhance AR activity through a functional interaction with the activation function 2 (AF-2) region in the LBD [31]. Co-repressors (N-CoR/SMRT) can be recruited to interacting surfaces located on the LBD surface of nuclear receptors, which partially overlaps with that used by P160 co-activators [34–36]. Repression of DHT-activated AR by N-CoR only requires the repressor interaction domains, and is independent of N-CoR domains that can recruit histone deacetylases [27]. Owing to this, N-CoR and TIF2 might be competitors for the same binding site on the liganded AR. The preference for each of these co-regulators might be different for agonistic and antagonistic ligands. To test this, we first determined the effect of TIF2 on AR activity bound to different ligands. TIF2 overexpression enhanced R1881-induced AR activity approx. 2.5-fold (Figure 6A). The complete antagonistic actions of OHF and BIC seemed not to be affected by TIF2. The partial agonists RU486 and CPA responded differently to TIF2 overexpression. Whereas CPA-bound AR was strongly enhanced by TIF2 (approx. 2.5-fold), this was not the case for RU486, whose activity was only slightly increased. The difference in response to TIF2 of the various ligands was even more obvious when smaller amounts of TIF2 were transfected (Figure 6B). Whereas the activity of CPA-bound AR was enhanced (1.8-fold) by 10 ng/well TIF2, RU486 activity remained unaffected. Also in the presence of larger amounts of TIF2 the RU486-occupied AR remained largely unresponsive to TIF2. R1881-bound AR showed the highest sensitivity for TIF2, and was already enhanced at small amounts of TIF2 (1 ng/well). When the largest amount of TIF2 was co-expressed (30 ng/well), CPA and R1881-bound AR showed an identical response (2.5-fold increase).

We next tested whether N-CoR and TIF2 are competitors in controlling AR transcriptional activity. N-CoR was expressed in the absence and presence of co-expressed TIF2 (Figure 6C). AR

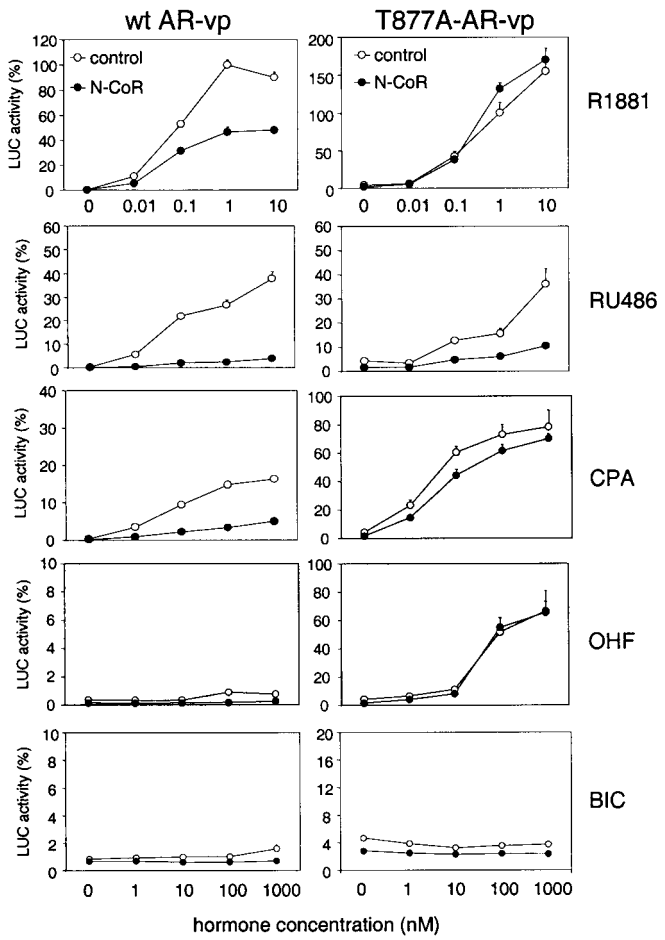

Figure 5 The T877A mutation affects repression of AR activity by N-CoR

CHO cells were transfected with 1 ng/well of either wt AR-vp or T877A-AR-vp, MMTV-LUC and 30 ng/well pSG5-N-CoR (●) or pSG5 vector (○). After an overnight incubation with vehicle or increasing concentrations of R1881, RU486, CPA, OHF or BIC, the cells were lysed and LUC activity was measured. The LUC activity obtained with wt AR-vp or T877A-AR-vp in the control situation with 1 nM R1881 was set at 100%. Note the different scales on the *y*-axis. LUC activities were determined from three experiments (means ± S.E.M.).

activity in the presence of OHF or BIC was insensitive for co-expression of TIF2 and remained at basal levels. In the presence of R1881, the repressive effect of N-CoR was relieved by TIF2 up to control levels. Repression of AR activity in the presence of RU486 was not reverted by co-expression of TIF2. This is consistent with the finding that RU486-occupied AR is very sensitive for overexpression of N-CoR (Figure 2B), but not for TIF2 (Figure 6B). Interestingly, N-CoR repression of AR bound by CPA was also not relieved by co-expression of TIF2. Although TIF2 enhanced CPA-bound AR activity (Figure 6B), it does not effectively compete with N-CoR.

Next, the ligand-dependent action of TIF2 on N-CoR-induced repression on T877A-AR was evaluated. R1881 and CPA showed a similar response to TIF2 overexpression as was found for wt AR (Figure 7A and Figure 6B). In contrast with wt AR, the activity of OHF-bound T877A-AR was strongly enhanced, up to 3.5-fold, by TIF2 (Figure 7A). Although RU486-bound T877A-AR activity was not responsive to small amounts of TIF2, it was strongly increased at high TIF2 levels (30 ng/well). Although the partial agonistic action of RU486 is hardly affected by the T877A mutation (Figure 4), the sensitivity of the mutant AR for TIF2 seems to be increased.

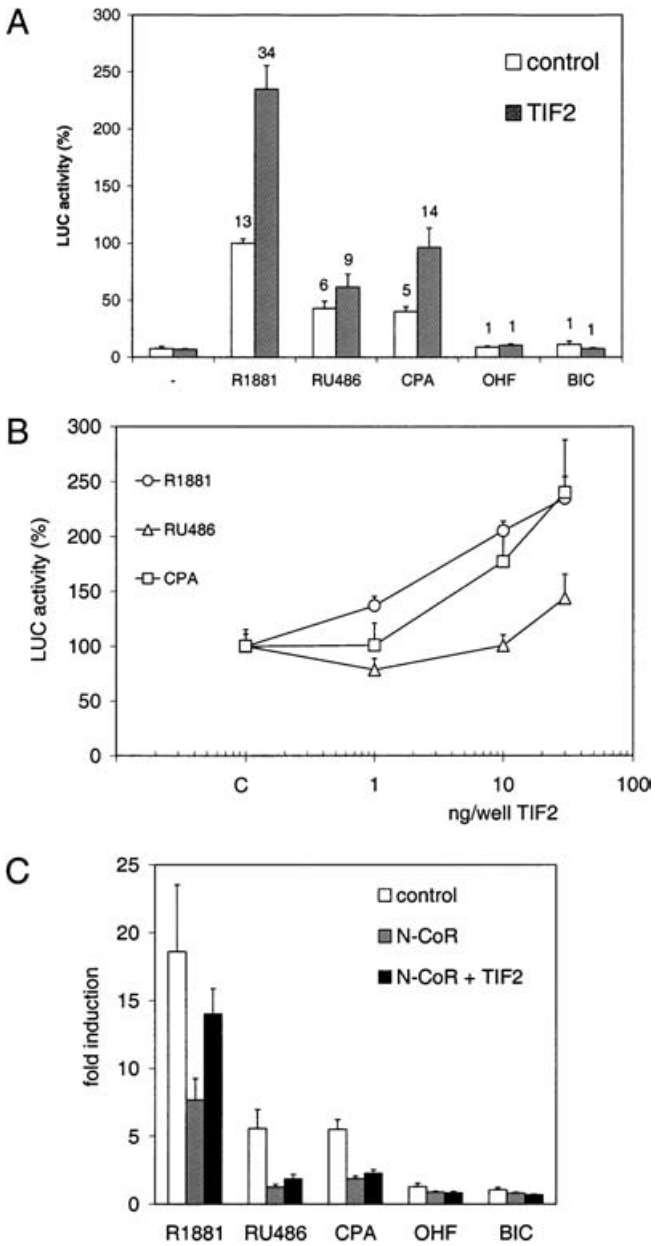

Figure 6 Ligand-dependent effects of TIF2 on wt AR activity

(**A**) CHO cells were transfected with MMTV-LUC reporter and wt AR (1 ng/well) in the absence or presence of 30 ng/well pSG5TIF2. In the control situation, equal molar amounts of empty vector (pSG5) were transfected. Cells were incubated overnight with vehicle (−), 1 nM R1881 or 1 μM antiandrogen (RU486, CPA, OHF and BIC). LUC activity of wt AR alone with R1881 was set at 100%. LUC activities were determined from three experiments (means ± S.E.M.). Fold induction is displayed above each bar. (**B**) Wt AR was co-transfected with increasing amounts of pSG5-TIF2 (110 and 30 ng/well). pSG5 vector was added, where necessary, to obtain equal molar amounts of plasmid in each well. For each ligand (R1881, RU486 and CPA), LUC activity of wt AR in the absence of TIF2 was set at 100%. LUC activities were determined from three experiments (means ± S.E.M.). (**C**) Wt AR was transfected alone (control), in the presence of N-CoR (30 ng/well) or in the presence of both N-CoR and TIF2 (30 ng/well each). pSG5 vector was added, where necessary, to obtain equal molar amounts of plasmid in each well. Average induction values were determined from three experiments.

The T877A mutation also influenced the functional competition of N-CoR with TIF2 (Figure 7B). In the presence of R1881, N-CoR only marginally affected the T877A-AR activity. Co-expression of N-CoR and TIF2 resulted in a strongly enhanced

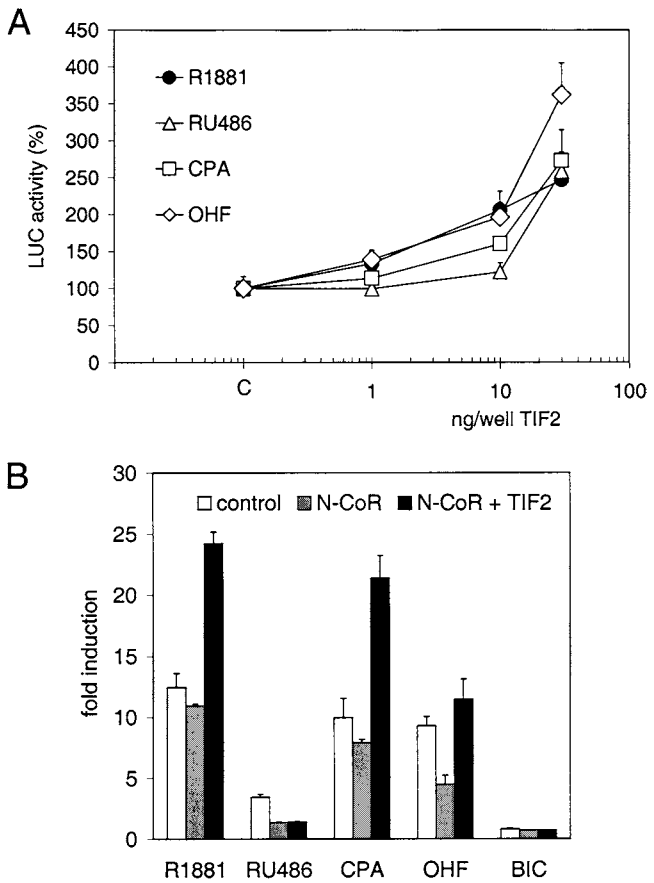

Figure 7 Effects of the T877A mutation on the functional competition of TIF2 and N-CoR

(**A**) T877A-AR was co-transfected with increasing amounts of pSG5-TIF2 as described in Figure 6(B). LUC activities were determined from three experiments (means ± S.E.M.). (**B**) T877A-AR was transfected either alone (control), in the presence of N-CoR (30 ng/well) or in the presence of both N-CoR and TIF2 (30 ng/well each). pSG5 vector was added to obtain equal molar fractions of plasmid in each well. Average induction values were determined from three experiments.

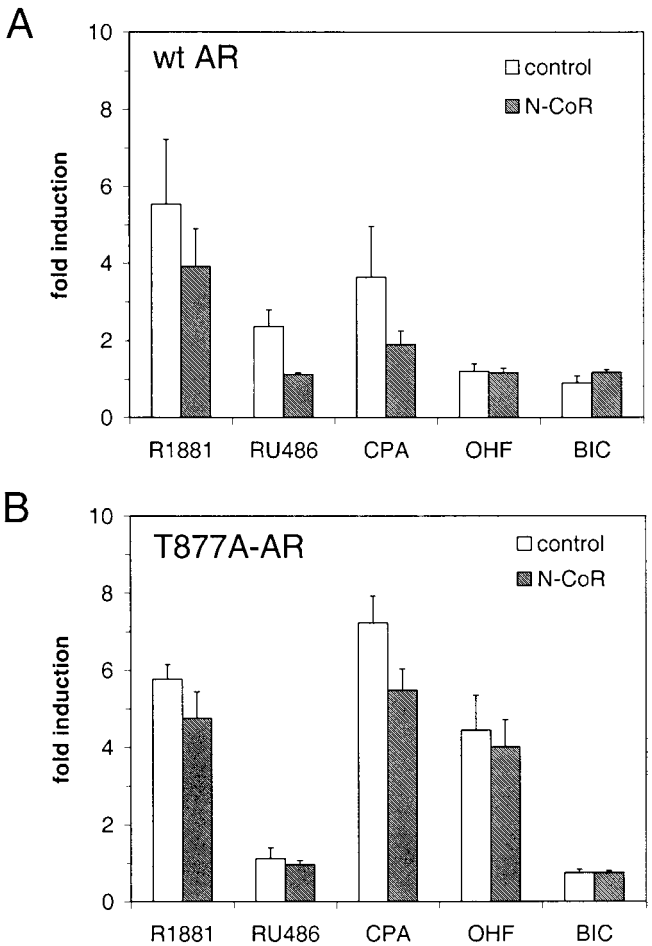

Figure 8 Transcriptional repression in the AR-negative prostate cancer cell line PC3

PC3 cells were transfected with MMTV-LUC reporter (50 ng/well) and 10 ng/well of either wt AR (**A**) or T877A-AR (**B**), in the absence (control) or presence of co-transfected N-CoR (100 ng/well). Empty vector (pSG5) was added, where necessary, to obtain equal molar amounts of plasmid in each well. Cells were incubated overnight with vehicle (−), 1 nM R1881 or 1 μM antiandrogen (RU486, CPA, OHF and BIC), after which LUC activities were measured. Average induction values were determined from three experiments.

receptor activity, up to 2-fold above control level. CPA, a full agonist for the mutant AR, showed the same response to N-CoR and TIF2 as R1881. In addition, TIF2 also enhanced the agonistic action of OHF in the presence of N-CoR. This suggests that the relieved N-CoR action on the mutant AR allows a better recruitment of TIF2, resulting in a higher transcriptional activity. However, this was not observed for RU486. Although the partial agonistic activity of RU486-bound AR was enhanced by a large amount of TIF2 (Figure 7A), an equal amount of TIF2 was not able to alleviate N-CoR repression.

Transcriptional repression of AR activity in PC3 cells

To investigate if the differential effects of N-CoR on AR activity also take place in prostate cells, repression assays were performed in the AR-negative prostate cancer cell line, PC3 (Figure 8). As reflected by low induction values (up to 6-fold), AR activities obtained in PC3 cells were considerably lower than those in CHO cells. However, the induced partial or complete activities of the antiandrogens were comparable with those observed in CHO cells, whereas the wt AR activity was also repressed by N-CoR in a ligand-type-dependent manner (Figure 8A). RU486-induced AR activity was reduced close to basal level and also the activity in the presence of CPA was repressed (approx. 50%). R1881-induced AR activity was slightly reduced (approx. 25%) by N-CoR overexpression.

Similar to the results obtained for CHO cells, the T877A mutation relieved the repressive action of N-CoR (Figure 8B). AR activities induced by CPA or OHF are only slightly affected by N-CoR overexpression (20 and 10% reduction respectively). The T877A-AR activity in the presence of RU486 was close to the activity in the absence of hormone, and remained at this level after co-expression of N-CoR. Similar to our observations in CHO cells, the complete antagonistic action of BIC is not affected by the mutation.

DISCUSSION

The results presented here indicate that the AR conformation induced by both androgens and antiandrogens is suitable for recruiting the N-CoR. However, the sensitivity of liganded AR for N-CoR repression strongly depends on the nature of the ligand. RU486-occupied AR appears to be highly sensitive to

N-CoR repression; its partial agonistic activity was repressed when relatively small amounts of N-CoR were transfected. A slightly weaker response to N-CoR was observed for the partial agonist CPA, whereas the AR activity in the presence of the agonist R1881 was not affected by small amounts of N-CoR. As observed for different promoters and in different cell lines, high N-CoR expression turned the partial agonists RU486 and CPA into strong antagonistic ligands. This implies that variations in endogenous expression levels of N-CoR might influence the antagonistic potential of these antiandrogens. Therefore the ability of these partial agonists to activate transcription might vary among different cell lines and tissues. Tissue-dependent effects of partial antagonists were described previously for RU486-bound PR [37,38]. It was shown that different ratios of endogenous co-activators and co-repressors correlate with differential RU486 effects on PR activity [39]. In this respect, in HeLa cells, with relatively high expression levels of co-repressors, RU486 represses PR activity. In contrast, RU486 has agonistic properties in T47D cells, which show relatively low expression levels of co-repressors [39]. In addition, completely knocking out the N-CoR expression in mouse embryonal fibroblasts from N-CoR gene-deleted mice transforms the anti-oestrogen tamoxifen into a strong oestrogen receptor agonist [40]. Co-transfection of N-CoR in the mouse embryonal fibroblasts restores the antagonistic action of tamoxifen, emphasizing the importance of N-CoR in antihormone action [40].

Although binding of RU486 or CPA results in comparable partial AR activities, limited proteolysis assays revealed that they induce distinct AR LBD conformations [11]. This variation in protein structure might explain why CPA and RU486 responded differentially to overexpression of the co-activator TIF2. Although co-transfection of TIF2 strongly enhanced the activity of CPA-bound AR, it hardly affected RU486-induced AR activity. However, co-expression of the co-activator TIF2 could not reverse the N-CoR-induced transition of both antiandrogens to complete antagonism, suggesting a preference of the AR for N-CoR binding in the presence of RU486 and CPA. Thus, although the activity of CPA-bound AR is strongly enhanced by TIF2, this co-activator does not effectively compete with N-CoR. In the presence of the agonist R1881, the preference for N-CoR, instead of TIF2, was not observed. Although relatively large amounts of N-CoR repressed R1881-induced AR activity, TIF2 co-expression increased the AR activity up to an intermediate level. Thus R1881 induces an AR conformation that allows N-CoR and TIF2 to compete functionally for binding. In addition, it may well be possible that repression of AR, bound to either agonist or antagonist, by N-CoR, occurs through different mechanisms. Repression of agonist (DHT)-activated AR only requires the receptor-interaction domains of N-CoR, possibly through competition for co-activator interaction sites [27]. It might however be postulated that N-CoR repression of antagonist-bound AR does require the N-terminus of N-CoR, and subsequent recruitment of histone deacetylases, resulting in a complete inhibition of transcription.

The recent finding that a majority of recurrent prostate cancers express high levels of nuclear receptor co-activators postulates a role for these co-regulators in the increased AR response to low-affinity steroids, similar to adrenal androgens [41]. Furthermore, it was suggested that AR mutations, frequently found in antihormone refractory prostate cancers, allow the AR LBD to adopt an active conformation and to interact with co-activators in the presence of antiandrogens [17]. Our results, from CHO and PC3 cells, support an additional hypothesis, in which the AR response to antiandrogens is influenced by a change in co-repressor action. AR mutations that affect the ligand-induced AR conformation, similar to T877A, might decrease the recruitment of co-repressors. The predicted three-dimensional structure of the DHT-bound T877A-AR LBD revealed that the threonine to alanine substitution enlarged the hormone-binding pocket [42]. This might explain the binding of hormones that usually do not complex with the AR, and probably permits the antiandrogens OHF and CPA to induce an agonist-like AR conformation. The three-dimensional structure of antagonist-bound T877A-AR, which would provide valuable proof for this assumption, is not yet available. Owing to the AR mutation T877A, N-CoR is no longer able to suppress the activity of CPA- or OHF-complexed AR, whereas TIF2 enhanced their activities. From these results, a model can be proposed in which the altered conformational change induced by CPA or OHF, due to the T877A mutation, prevents a proper recruitment of N-CoR. Subsequently, the lack of N-CoR action on T877A-AR allows these antiandrogens to act as strong agonists. Consistent with this is the observation that the RU486-induced AR conformation was not affected by the T877A mutation [11], and as a consequence could still be repressed by N-CoR, even in the presence of TIF2 (Figures 5 and 7). As expected, BIC-occupied AR remained unaffected by the T877A mutation, and showed no change in response to N-CoR. This is in accordance with the previous finding that the BIC-induced AR conformation is not changed by the T877A mutation [9], and with the ability of T877A-AR from LNCaP cells to recruit N-CoR to the PSA promoter [25] and subsequently inhibit PSA production in the presence of BIC [43].

In summary, these results provide evidence for an equilibrium model in which the liganded AR is bound to either co-activator or co-repressor. The exact position of the AR in this equilibrium, and hence its transcriptional status, depends on the nature of the ligand and on the ratio of co-activator and co-repressor levels in the cell. Furthermore, the transcriptional balance might be influenced by mutations that affect the AR conformation, and hence the recruitment of co-regulators.

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