Potential of androgen receptor transcriptional activity by inhibition of histone deacetylation – rescue of transcriptionally compromised mutants

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Abstract

Androgens are critical in the development and maintenance of the male reproductive system and important in the progression of prostate cancer. The effects of androgens are mediated by the androgen receptor (AR), which is a ligand-modulated transcription factor that belongs to the nuclear receptor superfamily. We and others have previously shown that CREB-binding protein (CBP) can function as a coactivator for AR. Similar to some other nuclear receptor coactivators and/or the proteins that they interact with, CBP has histone acetyl transferase (HAT) activity that is thought to contribute to transcriptional activation by nuclear receptors. We have therefore assessed whether an increase in the histone acetylation status in the cell can influence AR transcriptional activity, by using the histone deacetylase (HDAC) inhibitors (HDACIs) trichostatin A (TSA), sodium butyrate (Na-But) and depsipeptide (FR901228). We found that inhibition of HDAC activity significantly increased the ability of endogenous AR in LNCaP cells, or ectopically expressed AR in HeLa cells, to activate transcription from AR-

dependent reporter constructs. In addition, HDACIs increased the androgen-dependent activation of the prostate-specific antigen (PSA) gene in LNCaP cells, an increase that was not due to an increase in nuclear AR protein levels. Moreover, the viral oncoprotein E1A that inhibits CBP HAT activity fully repressed the ability of HDACIs to stimulate AR-mediated transcription, indicating that CBP is involved in this process. Deletional mutagenesis of AR indicated that whereas the AF-2 domain in the C-terminus is dispensable, the AF-1 domain in the N-terminus is required for augmentation of AR action by HDACIs, an observation which is in concordance with the reduced ability of CBP to activate AR N-terminal deletion mutants. Furthermore, HDACI treatment rescued the deficiency in the transactivation potential of AF-2 mutants. Taken together, our findings suggest that a change in the level of histone acetylation of target genes is an important determinant of AR action, possibly mediated by CBP.

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Introduction

The androgen receptor (AR) is a member of the nuclear receptor family and mediates the effects of androgens, which are important in the development and maintenance of male sexual characteristics and prostate cancer progression (Lindzey *et al.* 1994, Quigley *et al.* 1995). Upon ligand binding, nuclear receptors bind specific DNA response elements through a highly conserved DNA-binding domain and mediate transcriptional activation through transactivating domains, referred to as activation function-1 (AF-1) and activation function-2 (AF-2), localized in the N-terminus (NTD) and the ligand-binding domain (LBD) within the C-terminus respectively (Tsai &

O'Malley 1994, Mangelsdorf *et al.* 1995). Similar to some other nuclear receptors, interactions between the NTD and the LBD, mediated by coactivators, are required for AR transcriptional activity (McInerney *et al.* 1996, Doesburg *et al.* 1997, Ikonen *et al.* 1997). It was previously shown that CREB-binding protein (CBP) acts as a coactivator for AR through interactions with both the NTD and the LBD (Aarnisalo *et al.* 1998, Frønsdal *et al.* 1998). In addition, CBP exhibits histone acetyl transferase (HAT) activity (Bannister & Kouzarides 1996, Ogryzko *et al.* 1996) and is an essential coactivator for many different classes of transcription factors in addition to nuclear receptors (Goodman & Smolik 2000, Chan & La Thangue 2001).

Nuclear receptors have been proposed to regulate gene expression through association with HATs for activation and histone deacetylases (HDACs) for repression (for reviews, see Glass & Rosenfeld 2000, McKenna & O'Malley 2002). It is thought that ligand binding, through the recruitment of HAT-containing coactivators, such as CBP, induces hyperacetylation on the promoter of target genes (Jenster *et al.* 1997). In addition, recent studies indicate that CBP and its close relative p300 can directly acetylate transcriptional regulatory proteins, such as GATA-1 (Hung *et al.* 1999) and p53 (Gu & Roeder 1997, Sakaguchi *et al.* 1998), and modulate their activities. Recent studies suggest that nuclear receptors are directly acetylated themselves by the HAT-containing proteins at critical sites governing hormone-dependent transactivation, thereby modulating their transcriptional activity, including the AR (e.g. Cress & Seto 2000, Gaughan *et al.* 2002, Fu *et al.* 2003).

In order to study the possible role of HAT activity in nuclear receptor function, HDAC inhibitors (HDACIs) have been used (Marks *et al.* 2000), since no HAT inhibitor is yet available. In this study, we utilized three different HDACIs: trichostatin A (TSA) (Tsuji *et al.* 1976, Yoshida *et al.* 1990), sodium butyrate (Na-But) (Newmark *et al.* 1994) and FR901228 (or depsipeptide) (Kramer *et al.* 2001) in order to assess the role of acetylation in AR-mediated transcription. All three HDACIs augmented androgen-mediated transcription and the AR target gene prostate-specific antigen (PSA) mRNA levels in androgen-treated LNCaP cells. We found that the ability of CBP to act as a coactivator for different AR mutants correlated with the response of the mutants to different HDACIs. In addition, E1A, a specific inhibitor of CBP, abolished AR activity in the presence or absence of HDACIs. These results provide evidence that an increase in histone acetylation of target genes is essential for mediating AR transcriptional activity.

Materials and Methods

Cell culture, transient transfection and luciferase assays

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Gibco-BRL), glutamine, penicillin and streptomycin. LNCaP cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in RPMI 1640 medium (Gibco-BRL) which was supplemented with 10% FBS, glutamine, penicillin and streptomycin.

The calcium phosphate coprecipitation method (Frønsdal *et al.* 2000) was used to transfect both the HeLa and LNCaP cells. Transfection of HeLa cells was carried out in 12-well plates (6.0×10^4 cells/well), with 0.25 µg reporter plasmid, indicated amounts of AR expression vector or empty expression vector and pUC18 to a total of

1 µg DNA per well. Transfection of LNCaP cells was carried out in six-well plates in 3.0×10^5 cells/well, with 0.67 µg reporter plasmid and pUC18 to a total of 2 µg DNA. At 1 h before transfection, the medium was changed with DMEM supplemented with 5% FBS. At 4–5 h after transfection, the medium was removed, and cells were permeabilized in 15% glycerol in PBS for 2 min, and then washed with PBS. Cells were then fed with DMEM or RPMI, for HeLa and LNCaP cells respectively, containing 0.5% charcoal-treated FBS. R1881 (NEN), TSA (Sigma), Na-But (Sigma) and/or FR901228 (kindly provided by Dr Robert Schultz, and the National Institutes of Health, Bethesda, MD, USA) were added, as indicated, and after 18 h, cells were washed once with cold PBS and harvested in Tris–MES solution (1 mM DTT, 0.5% TritonX-100, 50 mM Tris–MES pH 7.8), and luciferase activities were determined.

Plasmids

Reporter plasmids -285-PB-LUC and 2XARE-LUC (Ikonen *et al.* 1997) and expression vectors CMV-CBP (Kwok *et al.* 1994) and pBL9-E1A (Deleu *et al.* 2001) have been described. The expression vectors pSG5-AR (Frønsdal *et al.* 1998), the C-terminal deletion mutants (Frønsdal *et al.* 1998), C-terminal point mutations (Slagsvold *et al.* 2000) and N-terminal deletion mutants of AR (Slagsvold *et al.* 2000) have previously been described.

Northern blot analysis

Cells were maintained as above and treated with R1881 (10^{-7} M) together with increasing amounts of TSA, Na-But and FR901228, as indicated, for 18–20 h. Total RNA was extracted and fractionated by standard procedures. An amount of 15 µg total RNA was run per lane. The probes were generated by random priming and had a specific activity of $>3 \times 10^8$ d.p.m./µg. The following probes were used. For PSA, an EcoRI fragment spanning the full-length cDNA (Lundwall & Lilja 1987) was used; for beta-actin cDNA (Clontech, Palo Alto, CA, USA), and for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a BamHI-XhoI fragment of the cDNA was used. The bands were visualized by phosphorimager analysis (Amersham Biosciences).

GST pull-down assay—in vitro

Interactions between AR or its mutants and CBP were examined by the GST pull-down assay, as described previously (Slagsvold *et al.* 2000). Briefly, GST and GST-CBP (amino acids 1–452) fusion proteins were expressed in *Escherichia coli* and purified on glutathione-Sepharose beads (Amersham Pharmacia Biotech). AR and its mutants were translated *in vitro* by the TNT-coupled transcription/translation system (Promega) in the presence

of [35 S]-methionine. The translated proteins were incubated on ice for 30 min in the presence or absence of R1881 (10^{-6} M) before the addition of GST or GST-CBP. The reactions were incubated for 1–2 h on ice in NETN buffer (40 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.01% IGEPAL and 1 mM EDTA) containing protease inhibitors and R1881 (10^{-6} M) with occasional mixing. The beads were then washed three times with NETN buffer, resuspended in SDS-PAGE sample buffer, and size fractionated on an 8% SDS-PAGE. After autoradiography, relative intensity of the bands was determined by densitometry.

Western analysis

Preparation of the whole cell extracts and Western analysis were done as previously described (Xi *et al.* 2004). AR-specific antiserum (used 1:1000 dilution) was raised against the N-terminus of human AR (aa 1–504) fused to GST. After autoradiography, relative intensity of the bands was determined by densitometry. For checking the levels of AR in response to different HDACIs, HeLa cells were cultured and 5 μ g of pSG5-AR was transfected in 10 cm dishes using Fugene (Roche). Cells were then treated with the HDACIs and R1881 as described above for 24 h. Preparation of whole cell extracts and Western analysis were as above.

Results

Histone deacetylase inhibitors potentiate transcription by the AR

To determine whether the histone acetylation status of the cell affects AR function, we first performed a series of transient transfection experiments in the androgen-responsive prostate cancer cell line LNCaP (Horoszewicz *et al.* 1983). An androgen-dependent reporter construct in which a deletion derivative of the rat probasin gene promoter is fused to the luciferase (LUC) gene (-285-PB-LUC) (Ikonen *et al.* 1997) was transfected into LNCaP cells. After transfection, the cells were either left untreated or treated with the synthetic androgen R1881 in the presence or absence of increasing concentrations of TSA, the most commonly used HDACI (Yoshida *et al.* 1990). The cells were harvested 18 h later and LUC activities were determined. As shown in Fig. 1A, R1881 treatment increased reporter expression 70-fold, and this was further potentiated in the presence of TSA, reaching approximately fourfold higher levels than with R1881 alone.

To examine further the effect of acetylation on AR-mediated transcription, we tested another HDACI, sodium butyrate (Na-But), under the same conditions as for TSA. As shown in Fig. 1B, R1881 treatment alone increased -285-PB-LUC activity by 50-fold compared with the basal level. In the presence of increasing amounts

of Na-But, however, the activation by R1881 was further increased in a dose-dependent manner, reaching threefold higher levels than those obtained in the absence of Na-But.

To confirm the validity of the findings above, we tested a third HDACI, a more recently discovered compound, FR901228 (FR) (Kramer *et al.* 2001), that is structurally unrelated to TSA or Na-But. Transient transfection experiments were carried out in LNCaP cells, as described for TSA and Na-But. When LNCaP cells were transfected with -285-PB-LUC (Fig. 1C), similar to the findings above, -285-PB-LUC expression was increased approximately 10-fold in the presence of R1881 alone. In the presence of increasing amounts of FR901228, the R1881-induced levels of -285-PB-LUC expression increased by approximately 2.5-fold, whereas the basal level of reporter expression was not affected.

We next assessed whether the effect of HDACIs on AR function in LNCaP cells could be due to changes in AR expression. LNCaP cells were grown in the presence or absence of the HDACIs and/or R1881, and Western analysis was performed on whole-cell extracts with an AR-specific antiserum. As shown in Fig. 1D, in untreated cells R1881 treatment slightly increased the steady-state levels of AR. However, HDACI treatment did not significantly affect AR levels, suggesting instead that HDACIs influence AR function.

Similar experiments were performed in HeLa cells in the presence of ectopically expressed AR. -285-PB-LUC was cotransfected into HeLa cells either with an empty expression vector (pSG5) or an expression vector encoding AR (pSG5-AR). After transfection, cells were either left untreated or treated with the synthetic androgen R1881 in the presence or absence of increasing concentrations of TSA or Na-But. As shown in Fig. 2A, whereas there was modest activation of the reporter in the presence of AR and R1881, this was dramatically increased in the presence of increasing amounts of TSA, reaching approximately 200-fold activation. In contrast, reporter activity remained low in the absence of AR in response to TSA treatment. When Na-But was used in a similar experiment, -285-PB-LUC was activated by twofold by R1881 treatment, reaching approximately 35-fold higher levels in response to Na-But (Fig. 2B). At the highest level of Na-But used, however, the basal level of -285-PB-LUC expression also significantly increased, although still retaining approximately sevenfold the R1881 response. These data suggest that the effects of HDAC inhibitors are not cell type specific and are likely to be directly mediated through effects on AR function. Similar results were obtained using another AR dependent reporter, 2 XARE-LUC (data not shown).

We next assessed whether the effect of HDACIs on AR function in HeLa cells at the -285-PB-LUC reporter could be due to changes in AR expression levels. HeLa cells were transfected as above and grown in the presence or

Figure 1 Effect of HDACis on AR-dependent transcriptional activation in LNCaP cells. (A) LNCaP cells were transfected with the -285-PB-LUC reporter. Cells were either left untreated or treated with R1881 (10^{-9} M) and/or with the indicated concentrations of TSA and harvested after 18 h, and LUC activities were determined. Activation of reporter gene expression in the presence of R1881, but in the absence of TSA, was arbitrarily set at 100. The differences observed were statistically significant (Mann-Whitney test, $P<0.004$). (B) LNCaP cells were transfected with the -285-PB-LUC reporter. Cells were either left untreated or treated with R1881 (10^{-7} M) and/or with the indicated concentrations of Na-But and harvested after 18 h, and LUC activities were determined. Activation of reporter gene expression in the presence of R1881, but in the absence of Na-But, was arbitrarily set at 100. The differences observed were statistically significant (Mann-Whitney test, $P<0.05$). (C) LNCaP cells were transfected with the -285-PB-LUC reporter. Cells were either left untreated or treated with R1881 (10^{-7} M) and/or with the indicated concentrations of FR901228 and harvested after 18 h, and LUC activities were determined. Activation of reporter gene expression in the presence of R1881, but in the absence of FR901228, was arbitrarily set at 100. The differences observed were statistically significant (Mann-Whitney test, $P<0.05$). (D) LNCaP cells were cultured in the presence or absence of R1881 for 24 h, with or without the HDACis (TSA, 100 nM; Na-But, 1 mM; FR901228, 10 ng/ml) as indicated. Whole-cell extracts were then prepared and used in Western analysis with an AR-specific antibody. Extracts made from Cos1 cells that were either transfected with the empty expression vector pSG5 (–) or transfected with an AR-expression plasmid pSG5-AR (+) were used as controls. The data shown are representative of two independent experiments.

absence of the HDACis and/or R1881. Whole-cell extracts were prepared and Western analysis was performed with an AR-specific antiserum. As shown in Fig. 2C, whereas there was no significant change in AR levels in the presence of TSA compared with nontreated cells, surprisingly, both Na-But and FR901228 significantly increased AR expression. This increase was reduced by approximately 10–30% in the presence of R1881. These data suggest that the effects of Na-But and FR901228 on AR activity may in part be due to effects on AR expression.

Histone deacetylase inhibitors stimulate PSA expression

To examine more directly the possible role of HDAC inhibition on AR activity, we examined the expression of an AR target gene, PSA (Young *et al.* 1995) in the presence or absence of HDACis. LNCaP cells were either left untreated or treated with R1881 and/or increasing concentrations of TSA, Na-But or FR901228 for 24 h. Total RNA was then extracted and used in Northern blot analysis with PSA as probe. As shown in Fig. 3A, whereas the basal level of PSA mRNA accumulation was negligi-

Figure 2 Effect of HDACIs on AR-dependent transcriptional activation in HeLa cells. (A) HeLa cells were transfected with the -285-PB-LUC reporter in the presence of the empty expression vector pSG5 (2.9 ng) or pSG5-AR, which specifies AR production (5 ng). The different amounts for pSG5 and pSG5-AR reflect equalization of molar amounts. Cells were either left untreated or treated with R1881 (10^{-9} M) and/or with the indicated concentrations of TSA and harvested after 18 h, and LUC activities were determined. Activation of reporter gene expression in the presence of pSG5-AR and R1881, but in the absence of Na-But, was arbitrarily set at 10. Note that a suboptimal level of AR was used to clarify the TSA effect. The differences observed were statistically significant (Mann-Whitney test, $P < 0.05$). (B) HeLa cells were transfected with the -285-PB-LUC reporter in the presence of cotransfected pSG5 (5 ng) or pSG5-AR (10 ng). Activation of reporter gene expression in the presence of pSG5-AR and R1881, but in the absence of Na-But, was arbitrarily set at 10. The differences observed were statistically significant (Mann-Whitney test, $P < 0.05$). (C) HeLa cells were transfected with pSG5 or pSG5-AR (5 μ g) and were either left untreated or were treated with R1881 (10^{-8} M) for 24 h in the presence or absence of the HDACIs (TSA, 100 nM; Na-But, 1 mM; FR901228, 10 ng/ml) as indicated. Whole-cell extracts were then prepared and used in Western analysis with an AR-specific antibody. In parallel, LNCaP cells were cultured in the presence or absence of R1881 for 24 h, and whole-cell extracts were prepared and used in Western analysis as above. The experiment was repeated three times with consistent results. The results of densitometric analysis are shown below the Figure as relative intensity (Rel. Int.).

ble, it dramatically increased (approximately by 10-fold) in response to hormone. Upon treatment with increasing concentrations of TSA, hormone-induced levels of PSA mRNA accumulation were increased in a dose-dependent manner, reaching 3.2-fold higher levels than with R1881 alone. Thus, TSA potentiates androgen-mediated PSA gene expression.

The same experiment was also performed in the presence of increasing amounts of Na-But or FR901288. As shown in Fig. 3B and C, both Na-But and FR901288 also increased R1881-induced PSA mRNA accumulation by about twofold. However, at higher concentrations, not only was this activation completely lost, but R1881-

mediated mRNA accumulation was also effectively inhibited. Altogether, these data suggest that, as in the transient transfection experiments described above, HDACIs increase AR-dependent gene activation at its cellular target genes.

E1A inhibits HDACI-mediated potentiation of AR activity

It was previously shown that CBP, which has intrinsic HAT activity, can serve as a cofactor for AR (Aarnisalo *et al.* 1998, Frønsdal *et al.* 1998). To assess whether CBP is involved in the effect of HDACIs on AR activity, we

A

B

C

Figure 3 Histone deacetylase inhibitors increase PSA mRNA accumulation in LNCaP cells. (A) LNCaP cells were either left untreated or treated with R1881 (10^{-7} M) and/or with the indicated concentrations of TSA for 24 h. Total RNA was isolated and was then used in Northern analysis with PSA as probe. EtBr-stained 28S and 18S rRNA bands are shown as controls. (B) Cells were treated similarly to panel A, but Na-But was used instead of TSA. Northern analysis was performed similarly to panel A. EtBr-stained 28S and 18S rRNA bands are shown. (C) Cells were treated with FR901228 instead of TSA as in panel A, and Northern analysis was performed. 28S and 18S rRNA bands are shown as control.

Figure 4 Inhibition of HDACI-potentiated AR-activity by E1A. (A) HeLa cells were transfected with the -285-PB-LUC reporter and pSG5-AR alone, or with the empty expression vector RSV-0 or the expression vector pBL9-E1A. Cells were either left untreated or treated with R1881 (10^{-7} M) and/or with 100 nM of TSA and harvested after 18 h, and LUC activities were determined. Activation of reporter gene expression in the presence of R1881, but in the absence of TSA, was arbitrarily set at 10. Results represent the means of at least three independent experiments with S.E. indicated as error bars. (B) HeLa cells were similarly transfected as in panel A. Cells were either left untreated or treated with R1881 (10^{-7} M) and/or with 1 mM of Na-But and harvested after 18 h, and LUC activities were determined. Activation of reporter gene expression in the presence of R1881, but in the absence of TSA, was arbitrarily set at 100. Results represent the means of at least three independent experiments with S.E. indicated as error bars. (C) HeLa cells were transfected as in panel A. Cells were either left untreated or treated with R1881 (10^{-7} M) and/or with 5 ng/ml of FR901228 and then harvested after 18 h, and LUC activities were determined. Activation of reporter gene expression in the presence of R1881, but in the absence of FR901228, was arbitrarily set at 10. Results represent the means of at least three independent experiments with S.E. indicated as error bars. (D) HeLa cells were transfected with the 5XGAL4-LUC reporter and 10 ng of pSG5-GAL4 expression plasmid. The effect of cotransfection of an empty expression plasmid, RSV-0, or the expression vector for E1A, as indicated at the bottom of the Figure, is shown. Results shown are from an experiment done in triplicate which was repeated two times with comparable results. S.E. is indicated as error bars.

used the viral oncoprotein E1A, which is known to inhibit CBP activity (Chakravarti *et al.* 1999, Li *et al.* 1999, Perissi *et al.* 1999). We performed a series of transient transfection assays in HeLa cells. -285-PB-LUC was cotransfected with AR and either an empty expression vector or a vector encoding E1A, followed by hormone and HDACI treatments at optimal concentrations for the potentiation of AR-mediated transcription. As in the experiments described in Fig. 1 and 2, TSA increased the R1881-induced levels of reporter expression by 60-fold. Whereas in the presence of an empty expression vector, similar results

were obtained, when E1A was ectopically expressed, nearly all of the TSA effect was lost (Fig. 4A). As expected, E1A expression also abrogated the effect of R1881 on the reporter activity, suggesting that CBP directly contributes to AR activity in HeLa cells. Essentially identical results were obtained when Na-But or FR901228 was used (Fig. 4B and C); in contrast, E1A had no effect on 5XGAL4-LUC (Sadowski & Ptashne 1989), a reporter construct known not to be affected by AR or CBP (Fig. 4D). These data suggest that E1A blocks HDACI-mediated increase in AR activity.

Figure 5 Mapping the domains of AR involved in the HDAC1 responses. (A) Schematic depiction of the different mutants of AR that were used in the studies described below. (B) GST pull-down assay, where *in vitro* translated wild-type AR and the AR mutants were tested for their ability to interact with GST-CBP(1–452) or GST as control. Note that there was underloading in the gel for the ARΔ37–503 mutant for the input. (C) HeLa cells were transfected with the -285-PB-LUC reporter, wild-type AR or the different AR mutants. Cells were either left untreated or treated with R1881 (10^{-9} M) and/or with TSA (100 nM) and harvested after 18 h, and LUC activities were determined. Activation of reporter gene expression in the presence of wild-type AR and R1881, but in the absence of TSA, was arbitrarily set at 10%. Results represent the means of at least three independent experiments with s.e. indicated as error bars. (D) HeLa cells were transfected as in panel C. Cells were either left untreated or treated with R1881 (10^{-7} M) and/or with Na-But (1 mM) and harvested after 18 h, and LUC activities were determined. Activation of reporter gene expression in the presence of wild-type AR and R1881, but in the absence of Na-But, was arbitrarily set at 10%. Results represent the means of at least three independent experiments with s.e. indicated as error bars. (E) HeLa cells were transfected with the -285-PB-LUC reporter, wild-type AR or the different AR mutants, in the presence or absence of CMV-CBP (100 ng). Cells were left untreated or treated with R1881 (10^{-7} M) and harvested after 18 h, and LUC activities were determined. Activation of reporter gene expression in the presence of wild-type AR and R1881, but in the absence of CBP, was arbitrarily set at 100. Results represent the means of at least three independent experiments with s.e. indicated as error bars.

Domains of AR that are involved in activation by HDAC1s coincide with those that respond to CBP

We next investigated the regions of AR that contribute to its activation by TSA and Na-But and whether there was a correlation between the domains required for HDAC1 response and activation by CBP. To that end, we used two N-terminal internal deletion mutants (Slagsvold *et al.* 2000) and a C-terminal deletion mutant (Frønsdal *et al.* 1998), as well as two point mutants in the AF-2 core that

have significantly impaired AR function (Slagsvold *et al.* 2000) (Fig. 5A). We first tested the ability of these AR mutants to interact with CBP in the GST pull-down assay, as described previously (Frønsdal *et al.* 1998). As shown in Fig. 5B, all AR mutants bound CBP similarly to wild-type AR, suggesting that any difference in activity is not caused by changes due to lack of CBP interaction.

These mutants were then tested in transient transfection assays in HeLa cells to assess their transactivation properties in response to TSA or Na-But. -285-PB-LUC was

Figure 5 (C-E)

cotransfected into HeLa cells with either the empty expression vector pSG5 or expression vectors specifying wild-type AR or its mutants. After transfection, cells were either left untreated or treated with the synthetic androgen R1881 and/or with TSA or Na-But. After 18 h, cells were harvested and LUC activities were determined. As shown in Fig. 5C, an approximately 150-fold increase from basal level was observed when cells were transfected with wild-type AR and treated with both R1881 and TSA. Significant but lower activation was also observed for all the other mutants, but not for AR Δ 37–503 in which most of the N-terminus of AR has been removed (Fig. 5A). As expected, the AR Δ 715–919 mutant did not respond to R1881, since a big portion of the LBD is missing, but its activity was significantly increased by TSA. The differences in activity were not due to differential expression of the proteins determined by Western analysis (data not shown). These results suggest that an intact N-terminal domain is required for AR to respond to HDACIs, and that the AF-2 domain does not play a significant role in this process.

In a similar experiment using Na-But, qualitatively comparable results were obtained (Fig. 5D). AR Δ 37–503 completely lost the ability to respond to Na-But, whereas the other mutants had approximately 20–30% of wild-type activity. These data corroborate the findings with TSA and point to the N-terminus of AR as the most important region involved in mediating the effects of Na-But on AR activity.

We then tested the ability of these mutants to respond to CBP in the transient transfection assay. –285-PB-LUC was cotransfected into HeLa cells as above, with either an empty expression vector or expression vectors specifying wild-type AR or its mutants in the presence or absence of an expression vector for CBP. After transfection, cells were either left untreated or treated with R1881; after 18 h, cells were harvested and LUC activities were determined. As shown in Fig. 5E, the response of the various mutants to CBP was qualitatively similar to their response to HDACIs. Whereas CBP increased wild-type AR activity by nearly fourfold, no activation could be detected with AR Δ 37–503. For the other mutants, the CBP response ranged between two- and threefold. Altogether, the strong correlation between the response of AR mutants to HDACIs and CBP suggests that the HAT activity of CBP is involved in regulating AR transactivation potential.

Discussion

Recent studies have documented the involvement of histone acetylation of nuclear receptor target promoters in ligand-regulated modulation of transcription. This was first suggested by the identification of nuclear receptor coactivators with intrinsic HAT activity or recruitment by coactivators of proteins with HAT activity, as well as

nuclear receptor corepressors that associate with HDACs (for reviews, see Glass & Rosenfeld 2000, McKenna & O'Malley 2002). Subsequent studies suggested that changes in the histone acetylation status of cells may have an important role in nuclear receptor activity. Consistent with this notion, a recent study analyzing the factors that bind to the PSA gene promoter and enhancer regions *in vivo* identified recruitment of coactivators with HAT activity in the presence of agonists and recruitment of corepressors and HDACs in the presence of antagonists (Shang *et al.* 2002).

We have systematically studied the role of the histone acetylation status of the cell in AR function. All three independent HDACIs used in this study increased endogenous AR target gene expression and the activity of AR-dependent reporter constructs. Mapping of the region in AR that responds to the HDACIs suggests that it is the N-terminal domain that is most important for mediating this response.

The possible role of histone acetylation in AR function was first suggested several years ago when CBP, which has intrinsic HAT activity and can recruit HAT-containing proteins to target promoters (for a review, see Chan & La Thangue 2001), was found to be a coactivator for AR (Aarnisalo *et al.* 1998, Frønsdal *et al.* 1998). In the current study, we found very good correlation between the ability of various mutants of AR to respond to HDACI treatment and CBP coexpression. In addition, ectopic E1A expression, which is known to inhibit CBP activity (Chakravarti *et al.* 1999, Li *et al.* 1999, Perissi *et al.* 1999), essentially annihilated the stimulatory effects on AR transactivation potential of all three HDACIs tested. These results suggest that CBP, or an associated factor, may be involved in providing the HAT activity that is necessary to activate AR target genes. The interactions of AR with CBP may be direct or mediated by an intermediary cofactor bridging the two factors. Further work using HAT mutants of CBP would shed more light on this issue.

Although all three HDACIs used in this study had similar qualitative effects on the endogenous PSA promoter and the AR-dependent reporter constructs, there were quantitative differences. First, TSA was the strongest of the HDACIs in increasing AR activity both for the PSA transcription and at the AR-dependent reporter construct –285-PB-LUC, requiring only nM amounts to give rise to the highest fold activation. Second, whereas at high concentrations of TSA there was only a small decrease in their efficacy, Na-But and FR901228 strongly inhibited endogenous PSA expression when used at concentrations that are optimal for the activation of the –285-PB-LUC. There may be several reasons to explain these observations. First, TSA is known to be significantly more potent than other HDACIs (de Ruijter *et al.* 2003), as consistent with our data. Second, there appear to be differences in the efficiency with which the different HDACIs interact with and inhibit the activity of different HDACs. For example,

whereas TSA effectively blocks HDAC4, Na-But is significantly less efficient in this regard (de Ruijter *et al.* 2003). Thus, the reason for the observed differences not only might lie in the variation in chromatin structure between the PSA promoter in LNCaP cells and the probasin promoter in LNCaP or HeLa cells (giving rise to differences in sensitivity to HDACIs), but could also be related to the HDAC(s) these two promoters are associated with. This is also supported by our finding that whereas FR901228 and, to a lesser extent, Na-But increase the activation of the AR-expression plasmid in transfected cells, especially in the absence of hormone, TSA did not have such an effect. However, this increased AR expression could also be due to the nonspecific effects of Na-But previously reported (Thiagalingam *et al.* 2003). FR901228 could potentially have similar nonspecific effects due to the μM amounts required for this compound in the experiments. Further work is needed to assess these possibilities.

Mutational analysis of AR indicated that the effects of HDACIs were directed primarily at the N-terminus of AR. However, since the HDACI effects are hormone dependent, an intact LBD is also required for maximal activity. Interestingly, hormone-binding-capable, but transcriptionally compromised AF-2 mutants of AR (Slagsvold *et al.* 2000) were rescued almost to wild-type levels by TSA, and up to 30% of wild-type levels by Na-But. This rescue phenomenon coincided with the ability of ectopically expressed CBP to rescue the AR mutants. It has been suggested that the AF-2 core domain, where the mutants that we tested reside, has an important role in the intramolecular interaction between the N-terminal domain and the LBD of AR that is necessary for transcriptional activation by AR (Slagsvold *et al.* 2000, He & Wilson 2002). It has also been suggested that coactivators, such as CBP, could be involved in mediating this intramolecular interaction. It is therefore tempting to speculate that HDACIs may have a role in facilitating this interaction, in addition to their roles in chromatin packing through changes in histone acetylation. Further work would be required to test this possibility.

Previous studies used TSA or Na-But to examine the effect of histone acetylation on AR activity (List *et al.* 1999, Sadar & Gleave 2000). TSA was found to increase activation of the mouse mammary tumor virus (MMTV) promoter in chromatin by AR, at least in part by an increase in AR levels in 29+ L-cell fibroblasts (List *et al.* 1999). In another study, Na-But did not affect the total levels of AR in LNCaP cells, but increased intranuclear localization of AR (Sadar & Gleave 2000). Under the same conditions of the transient transfection experiments that tested the effects of HDACIs on AR activity, we did not find any significant change in AR protein levels, either total, nuclear or cytoplasmic, in the presence or absence of TSA, Na-But or FR901228, independently of the presence or absence of androgens (Fig. 1C and data not

shown). Our findings, therefore, do not support the hypothesis that the effect of HDACIs on AR activity in LNCaP cells is due to an increase in AR protein levels. In the case of the ectopically expressed AR in HeLa cells, the effect of Na-But and FR901228, but not TSA, may partly be mediated by changes in AR levels (Fig. 2C). To determine whether this is due to changes in the nature of proteins associated with the expression plasmid promoter, or is simply due to nonspecific effects of these compounds, will require further analysis.

Our data are most consistent with an alternative hypothesis. The robust increase in AR-transactivation potential in the presence of HDACIs suggests that an increase in histone acetylation may have a direct role in modulating AR activity. It is possible that HDACIs prepare the target promoter to assume a more accessible conformation to which hormone-bound AR and associated proteins can bind and activate transcription to higher levels than when the HDACIs are not present. However, in future studies, it is important to assess any other modifications in the histones, as well as the acetylation state of the AR itself, which has recently been suggested to contribute to its transactivation potential (Fu *et al.* 2000, 2003, Gaughan *et al.* 2002).

HDACIs are being evaluated for use in differentiation therapy of cancer, including prostate cancer (Samid *et al.* 1997, Conley *et al.* 1998, Huang *et al.* 1999). Given the important role of androgens in the initial stages of prostate cancer, as well as the deregulated expression of AR target genes in the advanced, androgen-independent phase of prostate cancer (Gregory *et al.* 1998, Korkmaz *et al.* 2000), the future elucidation of the molecular mechanism of HDACI effects on androgen-regulated genes may provide critical new information on both normal AR function and the progression of prostate cancer.

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