CREB Binding Protein Is a Coactivator for the Androgen Receptor and Mediates Cross-talk with AP-1*

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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 through the androgen receptor (AR), which is a ligand-modulated transcription factor that belongs to the nuclear receptor superfamily. In addition to its ability to activate transcription from androgen response elements, AR can inhibit activator protein-1 (AP-1) activity, composed of Jun and Fos oncoproteins, in a ligand-dependent manner. Conversely, when activated, AP-1 can block AR activity. We found that CREB (cAMP response element-binding protein) binding protein (CBP) had a direct role in both of these activities of AR. CBP significantly increased the ability of endogenous AR in LNCaP cells to activate transcription from an AR-dependent reporter construct. On the other hand, repression of AR activity by treatment of LNCaP cells with an activator of AP-1 was largely relieved when CBP was ectopically expressed. AR and CBP can physically interact in vitro as was shown in glutathione S-transferase pulldown assays. Whereas both the N terminus and ligand-binding domain of AR can interact with CBP, a short region in the N terminus of CBP is required for these interactions. As opposed to the interaction of CBP with other nuclear receptors studied so far, CBP-AR interactions were not affected by ligand binding to AR in vitro. These data suggest that CBP is a coactivator for AR in vivo and that the transcriptional interference between AR and AP-1 is the result of competition for limiting amounts of CBP in the cell.

Androgens have a pivotal role in the development and maintenance of the male reproductive system (1, 2). The actions of androgens are mediated through an intracellular receptor, the androgen receptor (AR), which belongs to the nuclear receptor superfamily (3, 4). Nuclear receptors are ligand-activated transcription factors that possess highly conserved DNA-binding domains and moderately conserved ligand-binding domains (LBDs), whereas they are quite divergent in the N-terminal domain (NTD) (for reviews, see Refs. 3 and 4). Transactivation function of nuclear receptors is primarily mediated by sequences in both the NTD and a short region in the LBD, referred to as the activator function-1 (AF-1) and AF-2 domains, respectively. Recent studies suggest that an interaction between the NTD and the LBD may play a role in the transcriptional activities of some nuclear receptors, including AR (5–7).

The activity of nuclear receptors is modulated by interactions with other proteins. These could be mediated through heterodimeric interactions within the nuclear receptor family, such as those between retinoid X receptors and thyroid hormone, retinoic acid, and vitamin D receptors in which the heterodimer has an increased ability to activate transcription (for a review, see Ref. 8). On the other hand, activator protein-1 (AP-1) complexes, composed of either Jun homodimers or Jun-Fos heterodimers (for a review, see Ref. 9), interfere with ligand-dependent transactivation by some nuclear receptors including AR (for a review, see Ref. 10). Reciprocally, liganded nuclear receptors, as first described for the glucocorticoid receptor, interfere with AP-1 activity (for a review, see Ref. 10). The molecular mechanisms of this cross-talk have not been definitively established. AR and other nuclear receptors can also cross-talk with other transcription factors, but these have not been studied in as much detail (e.g. Refs. 11 and 12).

Most recently, proteins that act as putative coactivators (13–21) or corepressors (22–25) and that physically interact with nuclear receptors have been identified (for a review, see Ref. 26). Most of these cofactors are expressed in a wide variety of cell types and can interact with more than one type of nuclear receptor. Furthermore, it appears that multiple cofactors may regulate nuclear receptor function at any one time. Therefore, the exact contribution of these cofactors to the activities of different receptors in vivo is still not well understood.

In this study we considered the possibility that one of the putative coactivators for nuclear receptors, CREB (cAMP response element-binding protein) binding protein (CBP), originally identified as a coactivator for genes that are responsive to cyclic AMP and mitogens (27, 28), may function as a coactivator for AR. We found that CBP can increase the transcriptional activity of endogenous AR in LNCaP cells. This is most likely because of a direct physical association of CBP, through its N terminus, with both the AR NTD and LBD, based on in vitro binding assays. Furthermore, we provide evidence that the transcriptional interference between AR and AP-1 may be mediated through competition for limiting amounts of CBP in the cell.

EXPERIMENTAL PROCEDURES

Cell Culture, Transient Transfection, and Luciferase Assays—LNCaP cells were cultured in RPMI 1640 medium (Life Technologies, Inc.), which was supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin. The polyethyleneimine method (29) was used for transfection. Briefly, 50 μl of 150 mM NaCl solution containing
The following primers: AR-N5, 5'-CAGTTAGG-3' and AR-N3, 5'-TAATAAGATCTTGGATGGAAGTG-3' were used to amplify the fragments by polymerase chain reaction using pSG5. 

For the generation of the human AR expression vector, the AR cDNA from CMV-hAR (30) was inserted between the EcoRI and BamHI sites of pSG5. For the generation of glutathione S-transferase (GST) fusion constructs of the AR N terminus and the ligand-binding domain (C terminus), the fragments were amplified by polymerase chain reaction using the following primers: AR-N5, 5'-TAATAAGATCTTGGATGGAAGTG-3' and AR-C1, 5'-TAATAAGATCTTGGGACTGAGA-3'; AR-N3, 5'-AGTCGTCAGAAGCCCGAC-3' and AR-C5, 5'-TAATAAGATCTTGGGACTGAGA-3'. The polypeptide chain reaction products were cleaved with BglII and BamHI and inserted into the BamHI site in pGEX-3X (Amersham Pharmacia Biotech) to generate GST-AR-N and GST-AR-C.

To generate the C-terminal deletion mutants of AR, pSG5-AR was digested with BamHI and one of the restriction enzymes, PmlI, HindIII, KpnI, or SacI. After filling in with Klenow polymerase, the plasmids were religated to generate AR-(1–714), AR-(1–566), AR-(1–503), and AR-(1–333), respectively.

To generate pGEX-CBP-(1–452), the N-terminal BamHI-EcoRI fragment of RSV-CBP (28) was inserted into the same sites in pGEX-4T1 (Amersham Pharmacia Biotech). The purified proteins were incubated in the absence or presence of 10^2 M R1881, 10^2 M DHT, and 10 mM dithiothreitol, 0.5% Triton X-100, and 50 mM Tris-MES, pH 7.8, and luciferase activities were determined.

Plasmids—Reporter plasmids −286PB-LUC and 2XARE-LUC (5) and CBP expression vectors RSV-CBP and CMV-CBP (28) have been described.

For the generation of the human AR expression vector, the AR cDNA from CMV-hAR (30) was inserted between the EcoRI and BamHI sites of pSG5.

Results

CBP Stimulates Activation of Transcription by the Androgen Receptor—To test the possible role of CBP in AR-dependent transactivation, we performed transient transfection experiments in LNCaP cells. An androgen-dependent reporter construct in which a deletion derivative of the rat probasin gene promoter was fused to the luciferase gene (−286PB-LUC) (5) was transfected into LNCaP cells in the presence of either an empty expression vector or an expression vector encoding CBP (28). In the absence of androgen, cells were either left untreated or treated with the synthetic androgen R1881. After 18 h, cells were harvested and luciferase activities were determined. As shown in Fig. 1A, in the absence of R1881 there was no significant level of −286PB-LUC activation when the empty expression vector was cotransfected with the reporter. In the presence of R1881, −286PB-LUC expression was increased by about 15-fold. When an expression vector encoding CBP was cotransfected, the basal levels in the absence of R1881 did not change, but the −286PB-LUC expression in the presence of R1881 was increased approximately 4–5-fold more than observed in the absence of the empty expression vector. Similar results were obtained when AR and CBP were expressed in CV-1 cells, which do not have endogenous AR (data not shown).

To assess the validity of these findings using another androgen-dependent reporter, we performed a similar experiment with 2XARE-LUC in which two copies of an androgen response element (ARE) drove expression of the luciferase gene in front of the thymidine kinase promoter (11). LNCaP cells were transfected with 2XARE-LUC in the presence of either an empty expression vector or an expression vector encoding CBP. After transfection, cells were either left untreated or treated with...
synthetic androgen R1881 and after 18 h, cells were harvested and luciferase activities were determined. As shown in Fig. 1, in the absence of R1881 there was no significant level of 2XARE-LUC activation when the empty expression vector was cotransfected with the reporter. In the presence of R1881, 2XARE-LUC expression was increased by approximately 3-fold. When an expression vector encoding CBP was cotransfected, the basal levels of 2XARE-LUC expression in the absence of R1881 increased by about 2-fold. In the presence of R1881, reporter expression increased approximately 3-fold further compared with that observed in the presence of the empty expression vector. These data show that the CBP effect observed on −286PB-LUC activity (Fig. 1A) is not specific for this reporter and suggest that CBP can function as a coactivator for AR in vivo.

Ectopic Expression of CBP Can Reverse TPA-induced Inhibition of AR Activity—Previous work has documented an inhibition of AR-mediated activation of transcription by the tumor promoter TPA, most likely through the activation of AP-1 (32). CBP was also shown to serve as a coactivator for c-Jun (27), a component of the AP-1 complex, and it has been suggested as having a role in the cross-talk between AP-1 and some nuclear receptors (19). Therefore, we tested the effect of CBP expression on TPA-induced inhibition of AR activity. To that end, LNCaP cells were transiently transfected with the −286PB-LUC reporter construct. After transfection, cells were either left untreated or treated with R1881 and/or TPA. After 18 h, cells were harvested and luciferase (LUC) activities were determined. As shown in Fig. 2A, R1881 treatment increased −286PB-LUC expression by approximately 15-fold, and TPA treatment alone did not significantly affect expression. However, when cells were simultaneously treated with R1881 and TPA, most of the activation by R1881 alone was lost. However, when the same experiment was repeated in the presence of an expression vector encoding CBP, TPA-induced block of R1881-mediated activation of −286PBLUC was restored to levels induced by R1881 alone. These data suggest that CBP is present in limiting amounts in LNCaP cells and that the ability of TPA to inhibit AR activity results from the engagement of CBP by other transcription factors induced by TPA, such as AP-1.

To substantiate these findings, the same experiment was repeated with the 2XARE-LUC reporter. LNCaP cells were transiently transfected with the 2XARE-LUC construct. After transfection, cells were either left untreated or treated with R1881 and/or TPA. After 18 h, cells were harvested and luciferase activities were determined. As shown in Fig. 2B, R1881 treatment increased 2XARE-LUC expression by approximately 10-fold. However, when cells were simultaneously treated with R1881 and TPA, about 90% of the activation by R1881 alone was lost. When the same experiment was repeated in the presence of an expression vector encoding CBP, block of R1881-mediated activation induced by TPA on the activity of 2XARE-LUC was restored to approximately 90% of that induced by R1881 alone. These data are consistent with the data described above obtained with −286PB-LUC and suggest that competition for CBP is the mechanism for the inhibition of AR activity by TPA.

CBP and AR Physically Interact in Vitro—It was previously shown that CBP can interact with a number of transcription factors including CREB, AP-1, and some nuclear receptors (for reviews, see Refs. 33 and 34). To assess the possibility that CBP may physically interact with AR, we used the GST pulldown assay. As shown in Fig. 3A, various fragments of CBP were fused to GST and were expressed in E. coli. These proteins were purified on glutathione-agarose beads and then used as affinity matrices for possible interactions with in vitro translated, 35S-labeled AR. As shown in Fig. 3B, whereas no significant binding was observed with GST alone, AR was efficiently retained by GST-CBP-(1–452), corresponding to the N-terminal 452 amino acids of CBP fused to GST. Interestingly, this interaction was equally strong both in the absence and presence of R1881. C-terminal deletion derivatives GST-CBP-(1–271) and GST-CBP-(1–117), or GST-CBP-(720–1678), which spans the middle third of CBP, did not display any significant binding to AR that was different from GST alone. These results suggest that the region between residues 271 and 452 of CBP is required for interaction with AR and that this interaction is not dependent on ligand binding in vitro.

To more conclusively assess the interactions between AR and CBP, we performed the GST pulldown experiment in “reverse,”
where the AR sequences were fused to GST and used as the affinity matrix. The N-terminal and ligand-binding domains of AR were expressed in E. coli as GST fusion proteins (GST-AR-N and GST-AR-C). These proteins were purified on glutathione-agarose beads and then used as affinity matrices for possible interactions with in vitro translated, 35S-labeled CBP. The in vitro translation of CBP resulted in multiple bands in addition to the expected full-size protein, presumably because of premature termination reactions giving rise to C-terminal truncations, because of the large size of CBP. Western blot analysis of cell extracts expressing CBP with an N-terminal-specific antiserum supports this conclusion. As shown in Fig. 3C, whereas no significant binding was observed with GST alone, most of the CBP-specific bands were retained on both GST-AR-N and GST-AR-C. These interactions were not dependent on R1881 because preincubation of the GST fusion proteins with R1881 did not significantly alter CBP binding. These data suggest that CBP can physically associate with AR through both the N-terminal and C-terminal domains in vitro.

To determine which region(s) in AR is sufficient for binding to CBP, we generated C-terminal deletion mutants of AR (Fig. 4A). These mutants were first tested in a transient transfection assay to assess their transactivation properties from an ARE. To that end, −286PB-LUC was cotransfected into PC-3 cells either with wild-type AR or its C-terminal deletion derivatives. Cells were either left untreated or treated with R1881. After 18 h, cells were harvested and LUC activities were determined. As shown in Fig. 4B, wild-type AR efficiently activated −286PB-LUC expression 10-fold in the presence of R1881. The AR-(1–714) deletion mutant increased the basal level of expression of −286PB-LUC by 5-fold even in the absence of R1881, and addition of ligand did not significantly change this activation. On the other hand, the deletion mutants AR-(1–566), AR-(1–503), and AR-(1–333) did not significantly alter expression of the reporter, as expected, because they lack a functional DNA-binding domain.

These deletion mutants of AR were then used in the GST pulldown assay with GST-CBP-(1–452) as shown in Fig. 3B. As shown in Fig. 4C, when most of the ligand-binding domain or the DNA-binding domain was deleted, AR still interacted efficiently with GST-CBP-(1–452). However, when we used AR-(1–503), in which the C-terminal end of the N-terminal domain of AR is deleted, interaction was substantially decreased, and a further deletion toward the N terminus (AR-(1–333)) resulted in the loss of almost all CBP-AR interactions. These data suggest that the N-terminal domain of AR is sufficient to interact with CBP in vitro, consistent with the data presented in Fig. 3C.

**DISCUSSION**

In light of the ability of CBP to functionally interact with some members of the nuclear receptor superfamily, we assessed its role in AR function. Similar to findings with glucocorticoid receptor, estrogen receptor, progesterone receptor, thyroid hormone, retinoic acid, and retinoid X receptors (for a review, see Ref. 26), we found that the transcriptional activity of the endogenous AR in LNCaP cells is significantly increased when CBP is overexpressed. To our knowledge, this is the first case in which CBP or another putative coactivator for nuclear receptors has been shown to affect the activity of an endogenous nuclear receptor rather than receptors that are ectopically expressed.

The effect of CBP on AR function is probably the result of direct physical interactions between the two proteins as supported by the data obtained in GST pulldown assays. Using either GST-CBP or the GST-AR as the affinity matrix, we observed specific interactions between AR and CBP (Figs. 3 and 4). In contrast to previous work on the interactions of other nuclear receptors with CBP in which primarily the LBD has

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been implicated (for reviews, see Refs. 33 and 34), we have found that CBP can bind to both the NTD and LBD of AR. In light of the observed intramolecular interactions between the N and C termini of some nuclear receptors (5–7), it will be interesting to study the possible role of CBP in these interactions for AR.

Interestingly, in contrast to the ligand-induced increase in the association of CBP or various other putative coactivators with other nuclear receptors, hormone binding did not affect CBP-AR interactions in vitro (Figs. 4 and 5). We did not observe any ligand-mediated effects under different binding conditions tested (data not shown). The ligand-independent interaction between AR and CBP is unexpected because the transcriptional activity of AR is increased only in the presence of R1881 when CBP is ectopically expressed (Fig. 1).

There are several possibilities that can account for these data. First, it is possible that the conditions we have used for binding assays do not mimic the in vitro conditions that may be required for the recruitment of another cofactor(s), yet to be identified, that is necessary for optimal transcriptional activation by AR. In this regard, it is important to note that other putative cofactors for nuclear receptors (e.g., Refs. 19–21 and 35) have been shown to interact both with CBP and a number of nuclear receptors. It has therefore been suggested that a trimeric complex composed of the receptor, CBP, and another coactivator may be at work when the receptor is in a liganded and activated conformation (for reviews, see Refs. 33 and 34).

The deletion analysis of GST-CBP-(1–452) in the GST pull-down assay (Fig. 4) indicates that the region of CBP that is required for interaction with AR lies between residues 271 and 452. This is different from findings on the interaction of other nuclear receptors with CBP that have been studied to date, which may be due to the different experimental approaches employed: the yeast two-hybrid system in the earlier studies versus the GST pull-
down assay in our current work. Further investigation is required to clarify the basis for these differences.

Conversely, deletion analysis of AR suggests that its N terminus is sufficient for binding to CBP. Similar to other steroid receptors, AR has a large NTD in which a hormone-independent activation function, AF-1, resides (37). If AF-1 is deleted, AR loses the ability to transactivate (5, 37). Therefore, at least for the N-terminal CBP interaction domain, the sequences important for transactivation overlap with those required for interactions with CBP in vitro. It will be interesting to determine whether the activity of AF-1 is mediated through CBP. Further work is needed to clarify the role of CBP on AF-1 activity of AR and whether CBP plays an integrative role between the NTD and LBD of AR.

An important way to regulate AR function is through its interactions with other transcription factors. In this regard, the interaction with AP-1 has been studied most extensively. AP-1 is an oncoprotein complex composed of Jun and Fos proteins, and its activity is increased by a variety of extracellular stimuli, including phorbol esters, UV irradiation, cytokines, etc. (9). Based on a large amount of data from many different laboratories and involving different nuclear receptors, it has been suggested that the cross-talk between nuclear receptors and AP-1 may be because of competition for limiting amounts of cofactors in the cell (for a review, see Ref. 10). A later study provided evidence for this hypothesis, showing that overexpression of CBP and the related protein p300 overcomes the transcriptional interference with AP-1 by liganded retinoic acid and glucocorticoid receptors (19). However, a recent study on AP-1 and AR cross-talk suggested that the mutual transcriptional inhibition was because of direct protein-protein interactions between AR and AP-1 (32). In contrast, our data provide evidence that CBP plays a direct role in this cross-talk and that direct physical contacts between AR and AP-1 may not be necessary (Fig. 2). We have also found that the reverse is true: R1881 treatment of LNCaP cells blocks TPA-induced expression of an AP-1-dependent reporter. Therefore, these data are consistent with the hypothesis that transcriptional interference between AP-1 and AR is because of competition for a limited amount of CBP in the cell (see Fig. 5 for the proposed mechanism). It is possible, on the other hand, that a ternary complex composed of components of AP-1, AR, and CBP may exist in the cell because Jun and Fos associate with different regions of CBP than AR and other nuclear receptors (for reviews, see Refs. 33 and 34). Further work is needed to determine whether indeed such complexes are found in the living cell.

CBP was originally identified as a CREB binding protein (27, 28) and subsequently shown to bind a large number of proteins involved in transcriptional regulation (for reviews, see Refs. 33 and 34). CBP has intrinsic histone acetyltransferase activity and can associate with other proteins that also have histone acetyltransferase activities (for reviews, see Refs. 33 and 34). It is thought that acetylation of histones brings about an “open” conformation of chromatin that is more conducive to the interaction of the transcriptional initiation complex with DNA and therefore results in activation of transcription. However, further work is needed to determine the in vivo significance of the histone acetyltransferase activity that is associated with CBP for nuclear receptor function and whether it is required for modulation of AR transactivation potential.

CBP may have a role in tumorigenesis because a large number of proto-oncogene products can interact with CBP (33, 34). Moreover, patients with Rubinstein-Taybi syndrome, who lack one copy of the CBP gene, have a higher incidence of tumors compared with normal people, and rearrangements have been observed in the CBP gene in leukemias (38, 39). Given the important role of androgens and AR in the initiation and progression of prostate cancer and the role of CBP in modulating AR function, which is documented in this study, it will be important to determine whether there are changes in the CBP structure, expression, and/or activity profiles at different stages of prostate carcinogenesis.

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Note Added in Proof—Aarnisalo et al. also found recently that CBP modulates AR action (Aarnisalo, P., Palvimo, J. J., and Janne, O. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2122–2127).

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