Transactivation functions (AF2) in the ligand-binding domains (LBD) of many steroid receptors are well characterized, but there is little evidence to support such a function for the LBD of the androgen receptor (AR). We report a mutant AR, with residues 628–646 in the hinge region deleted, which exhibited transactivation activity that was more than double that of the wild type (WT) AR. Although not androgen-dependent AF2 activity could be observed for the WT ARLBD fused to a heterologous DNA-binding domain, the mutant ARLBD(Δ628–646) was 30–40 times more active than the WT ARLBD. In the presence of the p160 coactivator TIF2, AR(Δ628–646) was significantly more active than similarly treated WT AR. Deletion of residues 628–646 also enhanced TIF2-ARLBD activity 8-fold, an effect not present when the LBD-interacting LXXLL motifs of TIF2 were mutated, suggesting that the negative modulatory activity of residues 628–646 were exerted via coactivator pathways. Although the AP-1 (c-Jun/c-Fos) system and NcoR have been reported to interact with and repress the activity of some steroid receptors, c-Jun, c-Fos, c-Jun/c-Fos, nor NcoR function was consistently affected by the absence or presence of residues 628–646, implying that the AR hinge region exerts its silencing effects in a manner independent of these corepressors. Our data provide evidence for the novel finding that strong androgen-dependent AF2 exists in the ARLBD and is the first report of a negative regulatory domain in the AR. Because mutations in this region are commonly associated with prostate cancer, it is important to characterize the mechanisms by which the hinge region exerts its repressor effect on ligand-activated and coactivator-mediated AF2 activity of the ARLBD.
Hinge Region Inhibits AF2 of the AR LBD

Fig. 1. Structure of the AR and homology comparison of the bipartite NLS and hinge regions of human AR, PR, GR, ERα, and THRβ. The vertical line demarcates the well-conserved DBD from the poorly conserved hinge region. Two lines forming the bipartite NLS in the AR are underlined, and the 19 residues, which are deleted in AR(Δ628–646), are boxed. Underlined residues in PPARα (166–179) and THR-β (206–214) have been described to interact with the corepressor NCoR (2, 3). The black box in the AR is the hinge region (residues 628–669), and the shaded box is the DBD (residues 557–627).

to why the prominent AF2 activity present in other steroid receptors cannot be elicited in the ARLBD.

Although the LBD and the DBD of the AR have been characterized in some detail (29), the hinge region in between these major domains, defined by residues 628–669, is less well understood (Fig. 1). Although this region is poorly conserved among steroid receptors, several lines of evidence indicate that key functional elements may reside in this AR domain. For example, 7 of 10 reported amino acid substitutions affecting residues 619–672 in the AR hinge region are associated with the androgen-dependent tumor, prostate cancer (30). Furthermore, a sequence located between amino acids 628 and 657 within the hinge region contains a short stretch of basic amino acids that resemble the nuclear targeting signals of the glucocorticoid receptor and the SV-40 large T antigen (31) and has been described to form part of a bipartite nuclear localization signal (NLS) (32). In addition, transactivation activity of the ARLBD in yeast, but not in mammalian cells, appears to be modulated when the hinge region is attached (33), and coexpression of Ubc9, a ubiquitin-conjugating enzyme that interacts with the hinge region, can enhance AR transactivation activity (34). In this study we explored the effects of deleting key residues in the hinge region and observed that the ligand-activated and coactivator-augmented transcriptional activity of the deletion mutant was unexpectedly higher than the WT. Experiments were performed to elucidate the mechanism whereby the deleted residues exert an inhibitory effect on ARLBD function.

EXPERIMENTAL PROCEDURES

**Construction of Plasmids**—The full-length AR deletion mutant, AR(Δ628–646), was created by site-directed mutagenesis using two internal primers: Sense (dF), 5′-gaagcagggatgactctgg-3′, and the reverse primer 5′-cagcaccaccaagcccagtagggagaa-3′ and the antisense (dR), 5′-gaagcagggatgactctgg-3′, and antisense (QE3), 5′-cagcaccaccaagcccagtagggagaa-3′. Initially, two primary PCRs were performed using the AR expression plasmid pSV-AR as the template. Primers DNA-B and QE3 were used to generate fragment 1, and primers dF and QE3 for fragment 2. These overlapping ends. The recessed ends of the heteroduplexes were extended with T4 polymerase to produce a fragment that is the sum of the two overlapping products. A subsequent reamplification was performed for 30 cycles using primers DNA-B and QE3 to generate the cDNA fragment with a deletion of AR codons 628–646 (Δ628–646). The second primary PCR was double-digested with HindIII and XhoI and ligated into pSV-AR, with the equivalent fragment excised, to generate AR(Δ628–646). The pGAL4DBD-ARLBD chimeric vector comprised the AR hinge and LBD regions (codons 628–919) fused in-frame to the GAL4DBD residues 1–147 (23). pGAL4DBD-ARLBD Δ628–646 was generated by using AR(Δ628–646) as the template and amplifying the cDNA fragment encoding the ARLBD(Δ628–646) with the forward primer 5′-cagcaccaccaagcccagtagggagaa-3′ and the reverse primer 5′-gtttccaaagcttcactgggtggaa-3′. The PCR product, including the stop codon in exon 8 was digested with HindIII and ligated in-frame into the Smal/HindIII site of pST containing the GAL4DNA-binding domain. The plasmid pSV-AR(N727K,M886V), containing the double mutations M886V and N727K, was derived from pSV-AR(N727K) containing the N727K mutation (17) into the M886V AR expression vector (18). pGAL4DBD-ARLBD(N727K,M886V) was generated by using pSV-AR(N727K,M886V) as the template and amplifying the LBD with the forward primers 5′-gcgccggaaggagaactc-3′ and the reverse primer 5′-gtttccaaagcttcactgggtggaa-3′. The PCR product, including the stop codon was digested with HindIII and ligated into the Smal/HindIII site of pM containing the GAL4DNA-binding domain pGAL4DBD-ARLBD(Δ628–646), and pGAL4DBD-ARLBD(Δ628–646,N727K,M886V) were generated by amplifying the relevant fragments of pSV-AR or pSV-AR(N727K,M886V) and fused in-frame into GALADBD. pVP16-NcoRC was generated by digesting the cDNA encoding NcoR (kind gift of Dr. G. Jenster, Erasmus University, Rotterdam) with EcoRI and BamHI to obtain a 1.9-kb fragment containing residues 1818–2453 of the C-terminal fragment of NcoR, and ligating it in-frame to the VP16 transactivation domain. This NcoRC fragment both have repression domains removed, but included amino acids 1859–2142 and 2238–2300 of steroid-receptor-interacting domains I (3) and II (35), respectively. The pCMV-cJun encoding human c-Jun driven by the cytomegalovirus promoter (36), and the pSV-t-cfos containing rat c-fos driven by the RSV-LTR promoter (37) were kind gifts of Dr. R. Tjian (University of California, Berkeley, CA). pSG5-TIF2 and pSG5-mTIF2, encoding full-length TIF2 and mutant TIF2 (in which all three LXXLL nuclear-receptor-interacting motifs were mutated to LXXAA), respectively, were provided by Dr. H. Grone- meyer (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg) (25). The reporter vectors pARE-TATA-Luc and pGALA- TATA-Luc, contained five GAL4 DNA-binding sites and two tandem copies of the ARE from the aminotransferase gene, respectively, driving the luciferase reporter gene (17). All constructs were sequenced to confirm the fidelity of enzymatic manipulations.

**Transient Transfections**—HeLa cells were maintained in RPMI 1640 medium and suspended in Dulbecco's modified Eagle's medium. 1.5–1.8 × 10⁶ cells were seeded into each well of 24-well plates 20 h prior to transfections. For Western blotting, COS-7 cells were seeded on P60 Petri dishes 29 h before transfection. All transient transfections were performed using LipofectAMINE (38), and appropriate amounts of empty parent vector were added to the replicates, if indicated, to normalize the amount of total DNA in each well to prevent general scotching.

**Immunoblotting**—Immunoblotting was performed as previously described (39). Transfected COS-7 cells were lysed, and 10 μg of protein from the cell lysate was resolved on 8% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto Hybond-C nitrocellulose membranes, and AR protein was detected using the rabbit polyclonal antibody, NH27, which recognizes amino acids 360–564 of the AR (gift of A. Mizokami, Kitakyushu, Japan).

**Confocal Immunofluorescence Microscopy**—COS-7 cells were seeded on 15-mm diameter coverslips on 12-well plates and transfected with LipofectAMINE. Five hours after transfection, the cells received fresh medium with 10% charcoal-treated fetal bovine serum and were cultured for an additional 20 h in the presence or absence of increasing doses of DHT. Cells were fixed in 4% paraformaldehyde in phosphate buffer saline and permeabilized with 1% Brij. AR protein was detected with the antibody NH27 (1:50 dilution). Fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody was used for visualization of the receptor protein under a confocal laser scanning biological microscope (Olympus Fluoview IX70, Tokyo, Japan).

**RESULTS**

**Effect of Δ628–646 on Full-length AR Activity**—To test the consequences of deleting amino acids 628–646 from full-length AR, WT AR or mutant AR(Δ628–646) was expressed in the HeLa cells and transactivation activity measured with a multimeric ARE reporter linked to a luciferase reporter gene (Fig. 2). WT AR activity at 0.001 nM DHT was 2-fold higher than replicates not exposed to hormone and reached a maximum of 1600-fold increase in activity at 10 nM (Fig. 2A). Further increases in androgen dose did not raise AR activity above this maximum, indicating that saturating doses of hormone had been reached. However, the transactivation response of mutant...
AR was biphasic and differed from WT. Low doses of androgen (0.001–0.01 nM) did not increase mutant AR activity significantly, resulting in WT AR activity 10- to 44-fold higher than mutant AR. Surprisingly, this pattern was reversed for doses of androgen of 0.1 nM. The AR mutant, despite having its hinge region and the associated NLS deleted, exhibited AR activity that was more than double that of the WT AR for doses of DHT and mibolerone between 0.1 and 1000 nM (Fig. 2). These differences in transactivation function were not due to changes in protein expression, because both WT AR and deletion mutants were present in approximately equal amounts in the cells, as indicated by immunoblotting (Fig. 2C).

**Effect of Hinge Deletion on Activation Function of the LBD**—To measure activation function of the ARLBD, a chimeric construct, comprising the GAL4DBD fused in-frame to the ARLBD, was coexpressed with the GAL4-TATA-Luc reporter gene (Fig. 3). WT ARLBD chimeric protein did not demonstrate significant transactivation activity with, or without androgen, consistent with previous studies indicating that very little transactivation function resides in the AR LBD (5, 15, 19). Unexpectedly, deletion of residues 628–646 from the ARLBD increased androgen-dependent transactivation activity markedly. The ARLBD(Δ628–646) fragment was about 30–40 times more active than the intact WT LBD. Whereas no androgen-dependent increase in AF2 activity was observed for the WT ARLBD, dose-dependent augmentation of ARLBD(Δ628–646) AF2 activity was observed for doses of DHT and mibolerone from 0.01 to 1000 nM. This suggests that amino acids 628–646 may serve to inhibit the transactivation potential of AF2 of the ARLBD.

**Cellular Localization of AR Protein**—A short stretch of basic amino acids located within amino acids 628–646 of the hinge region forms part of a bipartite NLS, the other portion located 10 residues upstream in the DBD (31). To determine whether nuclear localization of the AR is affected by the hinge deletion, immunofluorescence confocal microscopy was performed (Fig. 4). In the absence of androgen, transfected AR was located in both cytoplasm and the nucleus (Fig. 4, top panels). With low doses of DHT (0.01 nM), WT AR was observed increasingly in the nucleus. At doses of DHT >1 nM, WT AR protein was localized mainly in the nucleus, and at 100 nM the WT AR signal was observed almost exclusively in the nucleus (Fig. 4, bottom panels). The deletion mutant surprisingly behaved in a similar manner, with most of the AR being located in the nucleus in the presence of androgen doses >1 nM, except that the nuclear signals were marginally less intense. Thus deletion of the hinge portion of the bipartite NLS in the mutant AR did not prevent its localization to the nucleus, suggesting that the intact portion located in the DBD was sufficient for this purpose (32).

**Effect of TIF2 on AF2 Function**—The effects of coactivator on mutant AR were next investigated, because AF2 is dependent on their efficient recruitment to the ARLBD. Of the three
steroid coactivators identified to date, TIF2 interacts the strongest with AR (39). Consistent with this observation, TIF2 augmented full-length WT AR activity by over 100% (Fig. 5). Remarkably, the AR(D628–646) mutant, was observed to display even greater TIF2-augmented activity, being 70% higher than similarly treated WT AR. Thus the synergistic activity of TIF2 on full-length AR function was present when the hinge region was deleted. To further define this effect, a chimeric construct consisting of the GAL4DBD linked in-frame to ARLBD was coexpressed with TIF2, and transactivation activity was measured with a GAL4 reporter gene (Fig. 6A). In the absence of TIF2, no AF2 function of the WT LBD was observed, whereas deletion of the 628–646 region resulted in significant ARLBD AF2 activity. The presence of TIF2 augmented the AF2 function of the WT LBD by more than 80-fold. Strikingly, the activity of LBD(D628–646) fusion protein with TIF2 was 8-fold higher than that of the corresponding TIF2-stimulated WT LBD fragment, and 40-fold higher than that observed with mutant LBD alone. In contrast, a TIF2 mutant, with three LXXLL nuclear-receptor-interacting motifs mutated, was not able to synergize with either WT LBD or LBD(D628–646) transactivation function, indicating that residues 628–646 exert their repressive actions via LXXLL motifs of steroid receptor coactivators. To further delineate the sites of action of TIF2, LBD mutants incorporating two substitutions, N727K and M886V, were constructed. The N727K,M886V mutations have previously been demonstrated to have partially defective interactions with TIF2 (17, 18), resulting in minimal androgen insensitivity and male infertility. The WT ARLBD chimeric protein did not have any activity in the absence of hormone, but displayed strong androgen-dependent activity in the presence of TIF2 (Fig. 6B). As expected, the LBD(N727K,M886V) fusion protein was partially defective in the presence of TIF2, having only half the activity of the WT. Nevertheless, deletion of residues 628–646 resulted in TIF2-dependent augmentation of mutant AR activity, such that triply mutated LBD(D628–646,N727K,M886V) was more than 3-fold stronger than the doubly mutated LBD(N727K,M886V) fragment. Thus mutations in AR residues 727 and 886, unlike TIF2 with mutated LXXLL motifs, did not abrogate the stimulatory action of the coactivator on LBD(D628–646). Overall, the data indicate that codons 628–646 harbor a functional element that directly, or indirectly, represses the activity of TIF2, and deletion of these codons enables maximal androgen-dependent coactivator-induced AF2 function to be expressed.

Effect of c-Jun and c-Fos on AF2 Function—AP-1 is a transcription factor whose components are the nuclear proteins encoded by c-Fos and c-Jun proto-oncogenes (36). Expression of these proto-oncoproteins can have a negative effect on steroid receptor function (40). Because the sites of interaction of c-Jun with AR include the DBD and the adjacent hinge region (41–43), the effects of these proto-oncoproteins on AR activity were examined. The presence of either c-Jun or c-Fos exerted a profound inhibitory effect on both WT AR and AR(D628–646).
activity (Fig. 5). The presence of c-Jun/c-Fos together resulted in hormone-independent stimulation of reporter gene activity but inhibition of DHT-stimulated AR(Δ628–646) activity. The equal inhibitory effect on both WT and deletion AR indicates that residues 628–646 are not likely to be the binding site for either proto-oncoproteins. The effect of cotransfecting c-Jun and c-Fos on the transactivation function of the chimeric ARLBD was examined (Fig. 7A). Although c-Jun and c-Fos stimulated WT ARLBD activity slightly in a hormone-independent manner, the presence of c-Jun reduced activity of DHT-stimulated LBD(Δ628–646) action by 70%. Surprisingly c-Fos had a strong hormone-dependent stimulatory effect on WT LBD activity, increasing reporter gene activity by 100-fold compared with LBD not exposed to c-Fos. Deletion of the hinge region enhanced this stimulatory effect of c-Fos on LBD activation function. The presence of both c-Jun/c-Fos resulted in hormone-independent activation of reporter gene. It is important to note that, even in the absence of AR, the activity of ARE and GAL4-driven reporter genes was increased 10-fold by the Jun/Fos heterodimer (data not shown). In contrast, Jun or Fos alone did not have this stimulatory effect on the reporter genes. Although the actions of c-Jun, c-Fos, and their heterodimers are complex and dependent on the particular cellular systems examined (40), deleted codons 628–646 did not appear to have a critical role in their actions, because almost equivalent effects were observed for both WT and deletion AR.

**DISCUSSION**

The main finding in this report is that a strong AF2 domain in the ARLBD can be demonstrated in mammalian cells and that this AF2 activity is normally inhibited, directly or indirectly, by residues 628–646 in the hinge region. For a wide range of androgen doses, deletion of residues 628–646 did not appear to have a critical role in their actions, because almost equivalent effects were observed for both WT and deletion AR.

Binding of NcoR Fragment to ARLBD—Because NcoR can interact with the hinge regions of the THR (3) and PPARα (2) (Fig. 1), we investigated whether this corepressor mediates the effects of the AR hinge region. To this end we made a VP16 construct fused in-frame to the NcoRC9 fragment, which incorporated both C-terminal steroid receptor-binding domains (3, 35), but we excluded the repressor regions of NCoR. This VP16-NcoRC9 chimera was coexpressed with the LBD fragment in the mammalian two-hybrid assay (Fig. 7B). Neither the ARLBD nor the NcoRC9 chimera alone stimulated reporter gene activity. Expression of WT ARLBD and NcoRC9 together increased reporter gene activity slightly, indicating that normal binding of NcoRC9 to the ARLBD was weak. As observed previously, the LBD(Δ628–646) chimera exhibited marked AF2 activity, which was increased in an additive manner in the presence of the NcoRC9, indicating that NcoR was unlikely to bind to the deleted region.

![Fig. 6. Effect of TIF2 on LBD activation function. A, fusion proteins consisting of GAL4DBD fused to WT ARLBD or ARLBD(Δ628–646) were coexpressed with 100 ng of full-length TIF2 or mTIF2 (wherein the last two leucines in all three LXXLL motifs were mutated to alanine) with or without 0.1 nM DHT. B, chimeric proteins consisting of GAL4DBD fused to WT LBD, or LBD mutants containing the double substitutions N727K,M886V with, or without the Δ628–646 deletion were coexpressed with TIF2 and exposed to 0.1 nM of MB. Transactivation activity was measured with GAL4-TATA-Luc reporter vector, and luciferase activity in relative light units (RLU) was mean (± S.E.) of at least three replicates. The horizontal bar in A denotes a change in scale, and differences in scale between panels are due to adjustments in luminometer sensitivity.](https://example.com/fig6)

![Fig. 7. Effect of the corepressors, c-Jun, c-Fos, and NcoR on transactivation function of ARLBD fragment. A, fusion proteins consisting of GAL4DBD fused to WT ARLBD or ARLBD(Δ628–646) were coexpressed with 100 ng of c-Jun, c-Fos, or c-Jun/c-Fos combination, with or without 0.1 nM of DHT, as indicated. B, the mammalian two-hybrid assay was used to measure interactions between WT ARLBD or ARLBD(Δ628–646) fusion proteins, and a NcoR fragment, containing both receptor-interacting regions, fused to the VP16AD. Transactivation activity was measured with GAL4-TATA-Luc reporter vector, and luciferase activity in relative light units (RLU) was mean (± S.E.) of at least three replicates.](https://example.com/fig7)
646 in the hinge region resulted in a mutant receptor with more than double the activity of the WT AR. This repressive effect was even more evident when the LBD was expressed as a fusion protein, because the deletion mutant was capable of raising transactivation activity 30-fold higher than the WT. It is of interest to note that, although GAL4DBD-ARLBD(Δ628–646) was transcriptionally more active than the WT LBD, the overall activity of the chimeric LBD constructs was a fraction of the full-length AR activity in terms of relative light units, reflecting synergistic interactions between activation regions of TAD and LBD in the intact receptor (14, 15). Our study provides a basis for understanding why AF2 function of the AR, unlike other steroid receptors, is weak and cannot be readily elicited. Intriguingly, the activation function of full-length AR(Δ628–646) was biphasic. At low doses of androgen (≤0.01 nM), the activity of full-length mutant AR was lower than the WT, but this was reversed at higher doses. This biphasic pattern could reflect partially defective AR nuclear transport at low androgen levels, due to deletion of the hinge portion of the bipartite NLS (32). At higher androgen doses, the intact DBD portion of the NLS could permit sufficient nuclear transport for the stronger intrinsic transactivation activity of the deletion mutant to manifest. The observation that this biphasic response was not observed with the chimeric mutant protein supports this hypothesis, because a strong NLS is present in the GAL4DBD moiety.

Mutagenesis studies indicate a functional link between AF2 activity in the LBD and the binding of p160 coactivators, such as SRC1 and TIF2, which interact specifically with the AF2 activity in the LBD and the binding of p160 coactivators, such as SRC1 and TIF2, which interact specifically with the AF2 activity in the LBD and the adjacent hinge region (41–43). Although the actions of c-Jun, c-Fos, and c-Jun/c-Fos heterodimers on AR activity are complex and may be either inhibitory or stimulatory depending on the receptor, cell, or promoter contexts (40), the question arises whether the repressor element in AR residues 628–646, exerts its effects through the AP-1 pathway. The presence of c-Jun, c-Fos, or c-Jun/c-Fos together profoundly inhibited both WT and Δ628–646 AR activity. In contrast to some studies suggesting that c-Jun is stimulatory (41), the strongest inhibition was associated with c-Jun in our system. The prominent AF2 activity of ARLBD(Δ628–646) fusion protein was reduced by two-thirds in the presence of c-Jun. In sharp contradistinction, c-Fos stimulated AF2 function in an androgen-dependent manner. The AF2 activity of WT LBD increased by two orders of magnitude in the presence of c-Fos, reminiscent of the activity of the coactivator TIF2. The AF2 activity of AR(Δ628–646) in the presence of c-Fos was even higher than WT, indicating that c-Fos was able to exert its effects even in the absence of residues 624–648. Although we observed diverse effects of the AP-1 system on AR activity, none of these appear to be affected by the absence or presence of residues 624–648, suggesting that the repressive effects of the hinge region were not predominantly mediated through these proto-oncoproteins.

Nuclear receptor corepressors, like NcoR, are thought to silence transcription by promoting a closed chromatin configuration through histone deacetylation. In the THR, binding of NcoR to the hinge region decreases transcription, and defective release of corepressor by hinge mutants are found in patients with resistance to thyroid hormone (45). NcoR also preferentially associates with antagonist-occupied PR (46) and may mediate the inhibitory effects of ER antagonists (4). A fusion of NcoR repressor domain to the ER-LBD strongly inhibits estrogen-dependent responses in breast cancer cells (47). In our study, the C-terminal fragment of NcoR, NcoRC9, containing two independent receptor-interacting regions demonstrated very little interaction with WT ARLBD or AR(Δ628–646). Thus it is unlikely that residues 628–646 are a critical binding site for NCoRC9, because interactions with WT and mutant LBD were equally weak.

Although the action of coactivators like those of the p160 and p300 family of proteins are well established (1), factors causing repression of steroid receptor action, although less well understood, are increasingly being reported. Thus NCoR and SMRT are found to be key regulators of ligand-dependent transcriptional activity of the human PR (46). Opposing effects of coactivators and corepressor determine agonists and antagonists activity of glucocorticoid-regulated gene expression (48–50). A 37-kDa REA protein potentiates the activities of dominant negative ERs and anti-estrogen-ligated ER (51). Binding of the orphan nuclear receptors TR4 to the AR (52) and SHP to ER (53) can result in heterodimers that down-regulate target gene expression. Although a negative modulation domain in the amino-terminal region of the PR has been described (54), this is the first report of an inhibitory element in the AR. Our data indicate that relatively strong AF2 function exists in the AR LBD and that it is normally inhibited by an element centered on residues 628–646 of the hinge region. Despite deletion of a portion of NLS and slightly reduced nuclear translocation, the deletion mutant exhibited significantly stronger basal and TIF2-augmented activity than the WT AR. Our results predict that the mutations in the AR hinge region might result in a release of a normal inhibitory function, leading to excessive AR activity and possibly unrestricted growth of androgen-regu-
lated tissues, making it imperative to determine the precise mechanisms whereby this region inhibits the AF2 domain.

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