Androgen-induced NH2- and COOH-terminal Interaction Inhibits p160 Coactivator Recruitment by Activation Function 2*

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The androgen receptor undergoes an androgen-specific NH2- and COOH-terminal interaction between NH2-terminal motif FXXXLF and activation function 2 in the ligand binding domain. We demonstrated previously that activation function 2 forms overlapping binding sites for the androgen receptor FXXXLF motif and the LXXLL motifs of p160 coactivators. Here we investigate the influence of the NH2- and COOH-terminal interaction on androgen receptor function. Specificity and relative potency of the motif interactions were evaluated by ligand dissociation rate and the stability of chimeras of transcriptional intermediary factor 2 with full-length and truncated androgen or glucocorticoid receptor. The results indicate that the androgen receptor activation function 2 interacts specifically and with greater avidity with the single FXXXLF motif than with the LXXLL motif region of p160 coactivators, whereas this region of the glucocorticoid receptor interacts preferentially with the LXXLL motifs. Expression of the LXXLL motifs as a fusion protein with the glucocorticoid receptor resulted in loss of agonist-induced receptor destabilization and increased half-time of ligand dissociation. The NH2- and COOH-terminal interaction inhibited binding and activation by transcriptional intermediary factor 2. We conclude that the androgen receptor NH2- and COOH-terminal interaction reduces the dissociation rate of bound androgen, stabilizes the receptor, and inhibits p160 coactivator recruitment by activation function 2.

The androgen receptor (AR)1 is a member of the steroid receptor family of nuclear receptors that act as ligand-dependent transcriptional regulators. The AR shares with other steroid receptors an overall structural arrangement that includes a COOH-terminal ligand binding domain, central DNA binding region, and a less well conserved NH2-terminal region (Fig. 1). Within these domains are two major transactivation regions, activation function 1 in the NH2-terminal region and activation function 2 (AF2) in the ligand binding domain. The NH2-terminal activation function 1 region, although not well defined, appears to be critical for AR-mediated gene activation. The AF2 region in the ligand binding domain forms a putative hydrophobic binding site for the LXXLL motifs of p160 coactivators (1–6), as recently revealed in the crystal structure of the AR ligand binding domain (7, 8). The p160 group of transcriptional coregulators includes steroid receptor coactivator 1 (SRC1), transcriptional intermediary factor 2 (TIF2, SRC2), and the SRC3/TRAM1/AIB1/pCIP/ACTR/RAC3 group of activators (9, 10), which are associated with histone acetyltransferase activity and can recruit CREB-binding protein, pCAF, and other coactivators required for chromatin modification (11).

The contribution of the AF2 region to AR-mediated transcriptional activity is unclear. Androgen-dependent transcriptional activity of an AR DNA and ligand binding domain fragment (AR-(507–919)) was only observed in cells that overexpressed TIF2 or SRC1 (12), which suggests that the AR AF2 insufficiently recruits p160 coactivators. We also showed recently that AF2 in the AR ligand binding domain can function in addition as a binding site for the AR NH2-terminal region. Mutagenesis studies indicated that the androgen-induced interaction between the AR NH2- and COOH-terminal (N/C) domains is mediated by two LXXLL-related sequences in the AR NH2-terminal region (see Fig. 1). These are FQNLF (FXXXLF motif) at residues 23–27 and WHTLF (WXXLL motif) at residues 433–437 (13). In the presence of androgen, the FXXXLF motif interacts with the AR AF2 in the ligand binding domain, whereas interaction of the WXXLF motif remains to be characterized (12, 13). Most importantly, the N/C interaction is selectively induced by ligands that have AR agonist activity in vivo, such as the high affinity, biologically active androgens testosterone and dihydrotestosterone and the lower affinity anabolic steroids. In striking contrast, the N/C interaction is not induced by ligands that bind the AR and cause its nuclear transport but fail to induce AR-mediated gene activation in vivo (14). The N/C interaction therefore appears to be critical for AR function in vivo as further evidenced by the association of the androgen insensitivity syndrome with single amino acid mutations that disrupt the N/C interaction (12, 15).

In the present study we made use of two strategies to test the effects of the N/C interaction on AR function. We investigated to what extent the AR AF2 recruits p160 coactivators in the presence and absence of the N/C interaction in wild-type and mutant AR. Second, we took advantage of the observation that the agonist-induced N/C interaction (16), which was also reported for estrogen receptor α (17) and the progesterone recep-
tor (18), does not occur in the glucocorticoid receptor (GR) (19). Chimeras were created in which the three LXXL motifs of TIF-2 was fused to the NH2-terminal region of AR and GR. TIF-2 was shown previously to increase the transcriptional activity of nuclear receptors through interaction of its LXXL motifs with the AF2 region of nuclear receptors (3, 5, 12, 13, 20). The effects of an imposed N/C interaction in the TIF2(LXXL), glucocorticoid receptor chimeras were determined by measuring rates of ligand dissociation and protein degradation. The results indicate that two functional effects of the N/C interaction are agonist-induced receptor stabilization and inhibition of p160 coactivator recruitment.

EXPERIMENTAL PROCEDURES

Preparation of AR and GR Expression Vectors—pCMVhAR126A/F27A (AR-FXXAA) is the full-length AR expression vector with the coding region for 433W, changed to 433WHITAAe377 as described previously (13). (AR-507-919) codes for the AR DNA binding domain and ligand binding domain residues 507–919 (21). AR-E897K, AR-I898T, and AR-V716R have single amino acid mutations in the AF2 region and were previously described (12). pCMVhAR-W433A/L436A/F437A (AR-AXXXA) was constructed by digesting glutathione S-transferase-AR (334–566), 3A433/L436A/F437A with the TIF2/LXXL fragment, and the fragment was subcloned in similarly digested pCMVhAR. (AR-1–503)-L26A/F27A (AR-1–503)-XXXA, (AR-1–503)-W433A/L436A/F437A, and AR-1–503–L26A/F27A/W433A/L436A/F437A (AR-1–503-XXXA) were constructed by digesting AR-FXXAA, AR-AXXXA, and AR-FXXAA/XXXA, respectively, with KpnI/BamHI and religating the vectors. pCMVhAR1412–337L26A/F27A (AR1412–377XXXAA) was created by double PCR mutagenesis by amplifying AR-FXXAA (13), digesting with BglII/KpnI, and subcloning into pCMVhAR1412–337 digested with the same enzymes. GALAR-(172–503)-L26A/F27A/W433A/L436A/F437A (AR-(172–503))-XXXA), which has the 433WHITAAe377 motif mutated to 433XXXAAe377, was constructed by PCR-amplifying the coding region for residues 363–933 in human progesterone receptor B and subcloning the fragment into pGAL0 (16).

TIF2(LXXL), (AR-172–919) and TIF2(LXXLAA), (AR-172–919) were constructed by PCR-amplifying the 627–780 amino acid region of pSGSTIF2 p123, whereas the latter has the NH2-terminal 172 amino acid residues of human AR and places the TIF2 sequences NH2-terminal and in-frame. TIF2(LXXL) (AR-172–919) was constructed by PCR-amplifying the 627–780 amino acid region of pSGSTIF2, the fragment was digested as above and subcloned in AR-AXXXA, which has the 433WHITAAe377 motif mutated to 433XXXAAe377, SRC1(LXXL)AR-(172–919) was constructed by PCR-amplifying the 611–780 amino acid region of SRC1a (22, 23), digesting with BglII/AflI, and subcloning into pCMVhAR digested with the same enzymes, which removes the NH2-terminal 171 residues of AR. A1B1(LXXL)AR-(172–919) was constructed by PCR-amplifying the 600–770 amino acid region of A1B1, digesting with BglII/AflI, and subcloning into pCMVhAR digested with the same enzymes, which removes the first 171 human AR residues.

TIF2(LXXL), (GR-132–777) and TIF2(LXXLAA), (GR-132–777) were constructed by PCR-amplifying the 627–780 residue region of pSGSTIF2 or pSGSTIF2 p123 as above. The fragments were digested with KpnI/SalI and subcloned into pCMVhGR digested with the same enzymes. This removes 131 human GR amino acid residues of human GR. TIF2(LXXL) (GR) and TIF2(LXXLAA), (GR) were constructed by PCR-amplifying the 2–132 amino acid region of pCMVhGR and subcloning the SalI fragment into TIF2(LXXL) (GR-132–777) and TIF2(LXXLAA) (GR-132–777). This reinserts the NH2-terminal 2–132 residues of GR. The sequence of all PCR-amplified regions was verified by sequence analysis.

Transcriptional Assays—Cell lines and transfection methods were selected to optimize transcriptional activity (CV-1 or HeLa cells) or expression levels (COS cells). Among these different cell lines we did not observe qualitative differences in response. Monkey kidney CV1 cells were plated at 4.2 × 104 cells/8-cm dish in 5% bovine calf serum in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM Hepes, pH 7.2, penicillin and streptomycin, and 2 mM L-glutamine in a 5% CO2 incubator at 37 °C. The next day 0.21 ml of H2O/plate and 30 μl of freshly prepared 2 M CaCl2 were added to the DNA solutions by 0.24 ml 2× Hepes-buffered saline/plate (0.28 mM NaCl, 1.5 mM NaH2PO4, 0.05 mM Hepes, pH 7.2) while vortexing. After 30 min at room temperature to allow for calcium phosphate precipitation, the mixture was briefly vortexed, and 0.475 ml was added to each plate containing 4 ml of 5% fetal bovine serum. The cells were incubated for 4 h, the media were aspirated, and the cells were incubated for 3 min with 1.5 ml of 15% glycerol in DMEM containing 5% bovine calf serum by a 4 ml phosphate-buffered saline wash. Cells were placed in 4 ml of serum-free, phenol red-free DMEM with and without hormones and incubated overnight. The following day, serum-free media with and without hormone were replaced, and the cells were incubated 24 h. The next day cells were washed with 4 ml of phosphate-buffered saline and aspirated dry, 0.5 ml of lysis buffer (25 mM Tris (Tris base) phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100) was added, and 0.1 ml analyzed for luciferase activity using a Monolight luminometer.

To determine transcriptional activity induced by the GAL4 DNA binding domain and receptor ligand binding domain fusion proteins, COS cells were transfected with 0.25 μg each/plate of the GAL4-based medium (MEM) containing 10% fetal bovine serum, penicillin and streptomycin, and 2 mM L-glutamine in a 5% CO2 incubator at 37 °C. Cells were transfected with 0.25 μg each/plate of the GAL4-AR ligand binding domain, GAL4-GR ligand binding domain, and GAL4-progesterone receptor ligand binding domain vectors described above, pSGSTIF2 and pG5Elb-luciferase reporter, which contain 5 tandem GAL4 binding sites. The day after plating, medium was replaced with fresh MEM containing 10% fetal bovine serum. DNA was combined with 0.15 ml of EC buffer/plate (Qiagen) and 4 μl of enhancer/plate, vortexed, and incubated for 5 min at room temperature. Effectene reagent (Qiagen, 4 μl/plate) was added, vortexed for 10 s, and incubated for 10 min. MEM containing 10% serum was added (1 ml/plate) and mixed, and 1 ml of the DNA solution was added to each plate. After incubation overnight at 37 °C, cells were washed with 4 ml of phosphat e-buffered saline, and 4 ml of serum-free, phenol red-free MEM with and without hormones was added per plate as indicated. The next day cells were washed with phosphate-buffered saline and harvested in 0.5 ml of lysis buffer described above and analyzed for luciferase activity.

Ligand Dissociation Rate Studies—Monkey kidney COS cells were plated at 0.4 × 104 cells/well in 6-well plates in 3 ml of 10% bovine calf serum in DMEM containing 20 mM Hepes, pH 7.2, penicillin and streptomycin, and 2 mM L-glutamine in a 5% CO2 incubator at 37 °C. Cells were transfected with 2 μg/well pCMVhAR or pCMVhGR wild-type or mutant DNA using 0.95 μl/well of 1.08 × TBS (TBS: 0.14 mM NaCl, 3 mM KCl, 1 mM CaCl2, 0.65 mM MgCl2, 0.9 mM Na2HPO4, and 25 mM Tris, pH 7.5) for 4 h. Cells were washed with 4 ml of phosphate-buffered saline, and 4 ml of serum-free, phenol red-free MEM with and without hormones was added per plate as indicated. The next day cells were washed with phosphate-buffered saline and harvested in 0.5 ml of lysis buffer described above and analyzed for luciferase activity.
with the AR ligand binding domain is weaker compared with that of the single AR FXXLF motif. Specificity of the interaction in TIF2(LXXL)AR was assessed in two control experiments. Mutation of the 3 LXXL motifs in TIF2 to LXXAA (Fig. 2B, TIFLX3AR and Fig. 3) and of the second N/C AR interaction domain WXXLF to AXXAA similarly decreased the dissociation half-time of TIF2(LXXL)AR from 97 min to ~60 min, which was ~15 min longer than the \( t_{1/2} \) of 44 min for AR-(507–919) (Fig. 2B and Fig. 3). The results support a limited interaction between the three LXXL motifs of TIF2 and the AR ligand binding domain compared with that observed with the single AR NH\(_2\)-terminal FXXLF sequence.

The relevance of ligand dissociation studies with the TIF2/AR chimeras was further characterized by creating TIF2/GR chimeras. We chose the GR because we had found previously that deletion of the GR NH\(_2\)-terminal region (residues 1–398) did not change the rapid dissociation rate of [\(^3\)H]dexamethasone (19), supporting the absence of an N/C interaction in GR. Replacing NH\(_2\)-terminal GR amino acid residues 1–131 with the same (LXXL)\(_3\)-containing region of TIF2 dramatically slowed the dissociation half-time of [\(^3\)H]dexamethasone from GR to 31 to 168 min (Fig. 2C, TIFLXL3GR and Fig. 3). The effectiveness of the LXXL motifs to slow ligand dissociation from GR in the TIF2-GR chimera contrasted the relative inability of this region to slow the dissociation rate of [\(^3\)H]R1881 from TIF2(LXXL)GR-(172–919)/AXXAA (\( t_{1/2} \) 64 min) when compared with AR-FXXAA/WXXAA (\( t_{1/2} \) 43 min) and AR-(507–919) (\( t_{1/2} \) 44 min). The results suggest a much more effective interaction of the p160 coactivator LXXL motifs with the AR ligand binding domain compared with that with the TIF2-GR chimera. Remarkably, with the NH\(_2\)-terminal insertion of the TIF2 LXXL motif region, the dissociation half-time of [\(^3\)H]dexamethasone from GR decreased to the same slow dissociation half-time as observed for [\(^3\)H]R1881 from AR caused by the N/C interaction with the naturally occurring FXXLF motif. When the TIF2 LXXL motifs were mutated in the TIF2-GR chimera to TIF2(LXXL)GR-(132–777), the ligand dissociation half-time was indistinguishable from that of wild-type GR (\( t_{1/2} \) 28 min, Fig. 2C, TIFLXL3AR and Fig. 3). These results demonstrate that it was the LXXL motifs in the TIF2 fragment that simulated an N/C interaction in GR, causing a dramatic reduction in dissociation half-time of [\(^3\)H]dexamethasone. The same dependence on the LXXL motifs was obtained using fusion proteins with the TIF2(LXXL) motif region expressed at the NH\(_2\) terminus of full-length GR (Fig. 3).

We compared the relative effects of the (LXXL)\(_3\) region of TIF2 with those of two other members of the p160 coactivator family. Replacement of the NH\(_2\)-terminal 171 amino acid residues of AR with the (LXXL)\(_3\) motif regions of SRC1 or AIB1 indicated that these regions of SRC1 (\( t_{1/2} \) 58 min) and AIB1 (\( t_{1/2} \) 51 min) in the chimeras each slowed the ligand dissociation rate to less of an extent than the same region of TIF2 (\( t_{1/2} \) 97 min) and considerably less than the reduction induced by AR FXXLF (\( t_{1/2} \) 158 min, Fig. 3). Taken together, the data of Figs. 1–3 indicate that none of the LXXL motifs in the 3 p160 coactivators tested was as effective as the single FXXLF motif in AR in slowing the dissociation rate of bound androgen. The results suggest that the AF2 region of AR interacts preferentially with the FXXLF motif. The results raise the question of whether these LXXL motifs of the p160 coactivators can compete for the AR interdomain N/C interaction and activate the AR through the AF2 region of the ligand binding domain. We tested this in cotransfection assays with increasing amounts of TIF2 DNA.

**Influence of the N/C Interaction on TIF2 Activation of AF2**—The ability of the N/C interaction to influence AR activation by
the p160 coactivators was assessed by measuring transcriptional activation of AR and AR mutants and, in control experiments, of the TIF2/GR chimeras that mimicked the N/C interaction of the AR. In initial studies, we compared the intrinsic AF2 activities of the ligand binding domains of AR, the progesterone receptor, and GR in GAL4-DNA binding domain fusion proteins and their relative activation by TIF2 in the absence of hormone (Fig. 4). TIF2 overexpression resulted in only a 23-fold activation of GAL-AR ligand binding domain compared with the 197- and 193-fold induction of GAL-progesterone receptor ligand binding domain and GALGR-ligand binding domain, respectively, in the presence of hormone (Fig. 4). The results indicate that compared with the progesterone receptor and GR ligand binding domain, the AR ligand binding domain has inherently weak AF2 activity that could be overcome to some extent by TIF2 overexpression.

The high transcriptional activity of the AR NH2-terminal activation function 1 region (amino acid residues 142–337, Fig. 1) (26) makes it difficult to measure AF2 activity in the presence of activation function 1. A deletion mutant (AR-(142–337)) was therefore used in which the NH2-terminal transactivation domain residues 142–337 were deleted, but the N/C interaction remained intact, as indicated by two hybrid assays (12) and a ligand dissociation rate equivalent to wild-type AR (19). Increasing the amount of transfected pSG5TIF2 expression vector DNA from 0.2 to 5 μg was relatively ineffective in activating AR-(142–337), with only a 1.2-fold increase in transcriptional activation (Fig. 4). The results indicate that compared with the progesterone receptor and GR ligand binding domain, the AR ligand binding domain has inherently weak AF2 activity that could be overcome to some extent by TIF2 overexpression.
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The results of Figs. 5, 433 WHTLF 437 sequence in AR

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mated in the absence of hormone is shown above the

bars.

fold activation detected with 5 μg TIF2 DNA (Fig. 5A). In striking contrast, with a construct in which the N/C interac-

tion was weakened by changing the 23FXXLF 

27 motif to 23FXXAA 

27 (AR142–337FXXAA, Fig. 5A), TIF2 was about 100 times more effective in increasing AR-mediated transac-

tion based on the amount of transfected TIF2 DNA. A similar activation of 12–13-fold was observed using 5 μg of TIF2 with AR142–337 or 0.05 μg of TIF2 with AR142–337FXXAA. However, TIF2 activation of AR142–337FXXAA was less than that observed when the entire NH2-terminal region was deleted (Fig. 5A). The weaker activation of TIF2 from AR142–337FXXAA compared with that with AR-(507–919) likely resulted from the presence of the 438 WHTLF 

137 sequence in AR142–337FXXAA, as this WXXLF motif contributes to the N/C interaction (13) and therefore may partially inhibit TIF2 recruitment by AF2.

We also tested transcriptional coactivation of AF2 using a TIF2 mutant in which all three LXXL motifs were changed to LXXAA. TIF2(LXXAA)3 did not coactivate with AR142–337, AR142–337FXXAA, or AR-(507–919) above the low intrinsic levels observed with the mutant AR alone (Fig. 5B). Especially striking was the decrease in transcriptional activation with AR142–337FXXAA and AR-(507–919) from 179- and 290-fold with TIF2 to near background levels with the TIF2(LXXAA)3 mutant. The results of Figs. 5, A and B, suggest that the androgen-induced AR N/C interaction mediated by the FXXLF and WXXLF motifs inhibits p160 coactivator interaction with AF2 in the ligand binding domain. Mutations in the FXXLF region were required to significantly overcome the androgen-induced inhibition imposed by the N/C interaction on p160 coactivator recruitment by AF2.

Similar dose-response studies were performed using TIF2-GR chimeras. Introducing the putative N/C interaction in TIF2-(LXXL)3GR-(132–777) resulted in a reduced response to TIF2 activation compared with that observed with the TIF2(LXXAA)3GR-(132–777) mutant (Fig. 5C). A 10-fold higher amount of TIF2 was required to activate TIF2(LXXL)3GR-(132–777) (0.5 μg of TIF2, 160-fold) above background levels compared with 0.05 μg, the lowest level of TIF2 tested with the LXXAA mutant (142-fold, Fig. 5C). Thus, in agreement with results with AR, the N/C interaction imposed in GR by insertion of the NH2-terminal LXXL motifs attenuated activation of the receptor by TIF2. It is nevertheless noteworthy that increased TIF2 expression (5 μg of pSG5TIF2 DNA) was effective in overcoming the inhibition created by the artificially induced N/C interaction in GR, suggesting that a coregulatory protein with a binding region of similar or greater affinity for AF2 can compete more efficiently for the N/C interaction if it is expressed at sufficiently high levels.

**Effect of the FXXLF, WXXLF, and LXXL Motifs on Receptor Stabilization**—An unusual property of the AR is its dramatic stabilization by agonist binding (27), which previous data suggested is mediated by the N/C interaction (15). In contrast, most steroid receptors including the estrogen receptor α (28, 29), thyroid hormone receptor (30), GR (31), and progesterone receptor (32) undergo agonist-induced decreases in receptor levels. To further investigate the contribution of the N/C interaction to androgen-induced AR stabilization, we determined the effects of mutations in the NH2-terminal FXXLF and WXXLF interaction motifs on AR levels by immunoblot analysis. The addition of 0.5 μM DHT to the growth media resulted in a dramatic increase in AR protein (Fig. 6A, lanes 2 and 3), indicating androgen-induced receptor stabilization. In contrast, the FXXAA as well as the FXXAA/AXXXA double mutant AR proteins were detected at similar levels in the absence and presence of DHT (Fig. 6A, lanes 4–7). We also noted that in the absence of androgen there was a reproducible increase in the levels of these AR mutants relative to wild-type AR. The results support a role of the FXXLF and WXXLF-mediated N/C interaction in ligand-induced AR stabilization.

We further investigated the influence of the FXXLF and WXXLF-mediated N/C interaction on AR stabilization by coex-

pression of the COOH-terminal fragment AR-(507–919) that contains the DNA and ligand binding domains together with wild-type AR NH2-terminal fragment AR-(1–503) and AR-(1–503) fragments containing the FXXAA and AXXXA mutations. Coexpression of AR-(507–919) with wild-type AR-(1–503) resulted in a modest increase in AR-(507–919) levels assayed in the presence of 0.5 μM DHT (Fig. 6B, lanes 1 and 2). Mutations in the FXXLF, WXXLF, or both motifs in AR-(1–503), which decrease the N/C interaction between AR-(1–503) and AR-(507–919), result in reduced protein levels of AR-(507–919), although surprisingly, no major changes in protein levels of the AR-(1–503) fragments were observed (Fig. 6B). The data further support an NH2-terminal FXXLF- and WXXLF-motif role in androgen-induced AR stabilization.

We made use of the TIF2-GR chimeras to substantiate the role of the N/C interaction in receptor stabilization. Full-length GR undergoes a striking agonist-induced decrease in receptor levels with the addition of 1 μM dexamethasone (Fig. 6C, lanes 1 and 2). In contrast, the TIF2-GR chimera TIF2(LXXL)3GR-(132–777), which was shown above to dramatically slow the dissociation half-time of [3H]dexamethasone (see Fig. 2C and Fig. 3), exhibited loss of dexamethasone-induced GR de-

stabilization (Fig. 6C, lanes 3 and 4). When the last two leucine residues in each of the three LXXL motifs were mutated to alanine in TIF2(LXXAA)3GR-(132–777), which was shown above to reverse the ligand dissociation half-time to that of wild-type GR, degradation of the TIF2-GR chimera was indistinguishable from that of wild-type GR (Fig. 6C, lanes 5 and 6). Similar results were observed with the TIF2(LXXL)3GR chimeras in which the TIF2 fragment was expressed as a fusion.
protein with full-length GR, although the extent of stabilization was less pronounced (Fig. 6C, lanes 7–10). Taken together, the results indicate that the agonist-induced N/C interaction increases the half-time of ligand dissociation and allows for agonist-induced receptor stabilization and the absence of agonist-induced destabilization that is characteristic of wild-type AR but not GR.

Degradation rates of AR and several AR mutants were determined using [35S]methionine pulse-chase labeling. As summarized in Table I, mutation of the NH2-terminal FXXLF and WXXLF motifs resulted in degradation rates intermediate between those of full-length AR and AR-(507–919), as determined in COS cells at 35 °C. Increased AR degradation in the presence of 5 nM DHT compared with that of wild-type AR was also observed for AR AF2 mutants E897K, I898T, and V716R. These mutations were shown previously to disrupt the N/C interaction (12). The results support a critical role for the N/C interaction in androgen-induced AR stabilization.

**DISCUSSION**

Several lines of evidence indicate that the agonist-induced N/C interaction between the LXXLL-like sequences 23FQNLF27 and 433WHTLF437 in the AR NH2-terminal region and the AF2 region in the AR ligand binding domain is specific for AR and required for functional activity. The N/C interaction is critical to AR function, because AF2 mutations that disrupt the N/C interaction without affecting the equilibrium ligand binding affinity cause the androgen insensitivity syndrome, whereas mutations that disrupt p160 coactivator binding without affecting the N/C interaction have wild-type activity (12, 15). The N/C interaction slows ligand dissociation and increases AR stability yet interferes with p160 coactivator recruitment. Like the LXXLL motifs of p160 coactivators (1, 5), the AR FXXLF motif forms an amphipathic α-helix that interfaces within the hydrophobic groove of AF2.

Results of experiments with chimeric receptors indicate the AR N/C interaction has greater specificity and potency compared with the interaction of AF2 with the LXXLL motifs of...
p160 coactivators. In previous studies, AR/GR chimeras only slightly increased ligand dissociation half-times (19), suggesting that the FXXLF motif interacts less well with the GR AF2 than this region interacts with the TIF2-derived LXXLL motifs. In unpublished studies, 2 glutathione S-transferase affinity matrix assays showed that TIF2 LXXLL motif-containing fragments interact with the estrogen receptor α ligand binding domain, whereas the AR FXXLF fragment does not.

The AR AF2 region only weakly recruits p160 coactivators compared with the AF2 region of the progesterone or glucocorticoid receptor. This relatively weak interaction is further hindered by the N/C interaction. The hinge region of AR (residues 628–646) was reported to contribute to the low transcriptional activity of AR AF2 (34). However, in our unpublished studies, deletion of hinge residues 624–647 only minimally increased AR AF2 transcriptional activity of a GAL4 fusion protein with the AR ligand binding domain expressed in HeLa cells and resulted in a similar increase in the N/C interaction. The lower transcriptional activity of the AR AF2 region relative to other nuclear receptors more likely results from sequence divergence-induced structural differences and by the N/C interaction.

One functional consequence of the agonist-induced AR N/C
interaction may be to present a novel surface to attract AR-specific coactivators. A LIM domain, heart-specific protein FHL2 (35) is a reported AR coactivator that interacts with full-length AR but not with the NH2-terminal region (36), suggesting it recognizes an N/C interaction-induced conformation. The AR N/C interaction may contribute to the recognition of weaker androgen response elements whose regulation is androgen-specific (37).

Most steroid receptors undergo ligand-induced down-regulation resulting from ubiquitin-mediated proteolysis by the proteosome. Rates of receptor degradation have been correlated with activation potency (38), and activation domains and degradation signals can overlap (39). Proteosome-mediated degradation of estrogen receptor α (29, 40) was linked with coactivator recruitment and transcriptional potency. Mutations in the estrogen receptor α AF2 region at residues critical for coactivator recruitment stabilized the receptor (41), raising the possibility that p160 coactivator interaction with ligand-bound receptors is required for receptor degradation. The thyroid hormone receptor is also rapidly degraded by the proteosome (30), but ligand-dependent degradation of retinoid X receptor did not require transcriptional activity or interaction with p160 coactivators (42).

In our unpublished studies, AR degradation is mediated by the proteosome; however, in contrast to most nuclear receptors, AR and the vitamin D receptors (43) undergo agonist-induced stabilization. For the vitamin D receptor, inhibition of ubiquitin-proteosome-mediated degradation amplified the transcriptional response (43–45). For AR, it remains to be established whether in vivo transcriptional activity at certain androgen response elements requires the N/C interaction or the resulting agonist-induced increase in AR stability. The 5-fold reduced dissociation half-time of bound dexamethasone and reversal of the dexamethasone-induced decrease in GR levels in the TIP2-GR chimeras dramatically demonstrated the influence of the N/C interaction on receptor stabilization. This artificial N/C interaction in GR resulted in ligand dissociation and stability properties similar to those of wild-type AR.

### Table I

| Degradation half-times determined in the presence of 5 nM DHT | h |
|-------------------------------------------------------------|---|
| AR                                                         | 12.0 ± 3.0 |
| AR-(507–919)                                               | 2.4 ± 0.2 |
| NH-terminal mutations                                       | |
| FXXAA                                                     | 5.6 ± 0.6 |
| FXXAA/WXXAA                                               | 7.4 ± 0.8 |
| AP2 mutations                                              | |
| E897K                                                     | 4.5 ± 1.8 |
| I898T                                                     | 6.2 ± 0.4 |
| V716R                                                     | 5.8 ± 0.2 |

2 B. He and E. M. Wilson, unpublished material.
Dose-response transcription assays where TIF2 is transiently overexpressed as shown here indicate that the AR N/C interaction blocks AF2 recruitment of p160 coactivators. Transcriptional inhibition is predicted for other p160 coactivators such as SRC1 that interact with AF2 through LXXLL motifs. It is not known, however, to what extent inhibition of p160 coactivator recruitment by the N/C interaction limits the activity of these coactivators in vivo. Neither is it known how the interaction of other coactivators such as p300/CREB-binding protein or ARA70 with the AR is affected by the N/C interaction. Previous studies indicated that mutation of lysine 720 reduced or ARA70 with the AR is affected by the N/C interaction.

action of other coactivators such as p300/CREB-binding protein and COOH-terminal domains.

action of other coactivators such as p300/CREB-binding protein and COOH-terminal domains. 

action interferes with the recruitment of p160 coactivators. 

ing proteins. Pharmaceutical ligands that bind AR with moderate or high affinity may promote interactions with related peptide sequences present in coregulatory proteins. Whether the FXXLF motif or related sequences occur in AR-specific coactivators that have sufficient affinity to compete for the agonist-induced N/C interaction or are induced to interact by other ligands remains to be established. In the presence of such ligands, coregulatory proteins might inhibit or compete for the androgen-induced N/C interaction to regulate tissue-selective AR-mediated gene activation.

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