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FXXLF and WXXLF Sequences Mediate the NH_2-terminal Interaction with the Ligand Binding Domain of the Androgen Receptor*

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The nuclear receptor superfamily members of eukaryotic transcriptional regulators contain a highly conserved activation function 2 (AF2) in the hormone binding carboxyl-terminal domain and, for some, an additional activation function 1 in the NH_2-terminal region which is not conserved. Recent biochemical and crystallographic studies revealed the molecular basis of AF2 is hormone-dependent recruitment of LXXLL motif-containing coactivators, including the p160 family, to a hydrophobic cleft in the ligand binding domain. Our previous studies demonstrated that AF2 in the androgen receptor (AR) binds only weakly to LXXLL motif-containing coactivators and instead mediates an androgen-dependent interaction with the AR NH_2-terminal domain required for its physiological function. Here we demonstrate in a mammalian two-hybrid assay, glutathione *S*-transferase fusion protein binding studies, and functional assays that two predicted α-helical regions that are similar, but functionally distinct from the p160 coactivator interaction sequence, mediate the androgen-dependent, NH_2- and carboxyl-terminal interaction. FXXLF in the AR NH_2-terminal domain with the sequence ^{23}FQNLF^{27} mediates interaction with AF2 and is the predominant androgen-dependent interaction site. This FXXLF sequence and a second NH_2-terminal WXXLF sequence ^{433}WHTLF^{437} interact with different regions of the ligand binding domain to stabilize the hormone-receptor complex and may compete with AF2 recruitment of LXXLL motif-containing coactivators. The results suggest a unique mechanism for AR-mediated transcriptional activation.

Nuclear receptors facilitate ligand-dependent increases of gene transcription by direct interactions with nuclear coactivators. p160 coactivators have histone acetyltransferase activity (1) and interact with nuclear receptors through their ligand binding and NH_2-terminal regions (2–4). Additional acetyltransferases p300/cAMP response element binding protein and p300/cAMP response element binding protein associated factor interact with the p160 coactivators (5–7). Nuclear receptors also interact with multiprotein complexes referred to as thyroid hormone receptor-associated proteins (8), activator-recruited cofactor (9), or vitamin D receptor-interacting protein complex (10). The p160 coactivators and at least one of the thyroid hormone receptor-associated proteins/activator-recruited cofactor/vitamin D receptor-interacting protein subunit 205 interact in a ligand-dependent manner with activation function 2 (AF2)[1] in the ligand binding domain (LBD) of nuclear receptors through the consensus sequence LXXLL, where L is leucine and *X* is any amino acid (11–16). Crystal structures of nuclear receptor LBDs have shown that a hydrophobic cleft within a multilayered α-helical structure serves as the LXXLL coactivator binding surface AF2 (17, 18). In the estrogen receptor, agonist binding positions helix 12 over the binding cavity to complete the AF2 surface (19, 20), whereas binding of an antagonist such as 4-hydroxytamoxifen displaces helix 12 (21) causing an LXXLL-like sequence in helix 12 to mimic and thereby block coactivator binding (22).

It has become apparent that the AF2 region overlaps with regions that serve as the binding site for a variety of LXXLL-related sequences as recently shown for corepressor binding (23–25). Furthermore, we demonstrated that the AR AF2 region mediates an androgen-dependent NH_2-terminal/carboxyl-terminal (N/C) interaction (26). Therefore we investigated the possibility that AF2 in the AR LBD interacts with an LXXLL-related sequence in the NH_2-terminal domain. In this report, evidence is presented that sequences similar to but distinct from the LXXLL core sequence mediate a direct interaction between the NH_2-terminal and carboxyl-terminal regions of AR. The FXXLF core sequence ^{23}FQNLF^{27} in the AR NH_2-terminal domain binds AF2 in the carboxyl-terminal region in an androgen-dependent manner. In addition, a second motif in the NH_2-terminal region WXXLF with the sequence ^{433}WHTLF^{437} binds to a region of the LBD outside of AF2. Interaction of these NH_2-terminal sequences with the LBD slows the dissociation rate of bound androgen. Sequence specificity was indicated since FXXLF could not be functionally replaced by an LXXLL core sequence.

EXPERIMENTAL PROCEDURES

Transcription Assays and Plasmids—Mammalian two-hybrid N/C interaction assays were performed in Chinese hamster ovary (CHO)

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[1] The abbreviations used are: AF2, activation function 2; AR, androgen receptor; LBD, ligand binding domain; N/C, NH_2-terminal and carboxyl-terminal; CHO, Chinese hamster ovary; MMTV, mouse mammary tumor virus; PCR, polymerase chain reaction; DHT, dihydrotestosterone; GST, glutathione *S*-transferase; TIF2, transcriptional mediator/intermediary factor 2.

cells as described previously (27, 28). VPAR-(1–660) codes for the herpes simplex virus VP16 protein transactivation domain residues 411–456 expressed as a fusion protein with AR NH_2-terminal and DNA binding domain residues 1–660. GALAR-(624–919) codes for a fusion protein of the *Saccharomyces cerevisiae* GAL4 DNA binding domain residues 1–147 and AR LBD residues 624–919. Deletions within VPAR-(1–660) (Δ179–199, Δ394–405, and Δ429–439) were created by two polymerase chain reactions (PCR) using oligonucleotide primers with appropriate deletions. The reporter vector G5E1bLuc contained five GAL4 DNA-binding sites and the luciferase reporter coding region (29). CHO cells were plated at 0.425×10^6/6-cm dish and transfected using DEAE-dextran (27, 28). Cells were incubated for 24 h in the absence and presence of increasing concentrations of dihydrotestosterone (DHT) as indicated and harvested in lysis buffer (Ligand Pharmaceuticals). Luciferase activity was determined using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego).

For the assessment of AR transcriptional activity, monkey kidney CV1 cells maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.), 20 mM Hepes, pH 7.2 (DMEM-H), and antibiotics were transiently transfected by plating 0.425×10^6 cells/6-cm dish. Wild-type and mutant pCMVhAR DNA (10–50 ng/dish) were precipitated with 5 μg of mouse mammary tumor virus promoter region (MMTV)-luciferase reporter vector (5 μg) using calcium phosphate (30). Cells were incubated for 24 or 48 h with the indicated concentrations of hormones and harvested and assayed in lysis buffer as described above. VPAR-(1–660) containing Δ329–381, Δ382–429, Δ430–499, or Δ429–439 was digested with *Afl*II and *Kpn*I, and the resulting inserts were ligated into pCMVhARL26A/F27A digested with the same enzymes. PCMVhARΔ339–499, Δ9–28 (27, 31), and AR-(507–919) (32) were previously described. PCR mutagenesis was used to create single and double amino acid mutations in the ^{23}F*XX*LF^{27} and ^{433}W*XX*LF^{437} domains. L26A/F27AΔ339–499 was prepared by ligating pCMVhARΔ339–499 digested with *Bgl*II/*Afl*II with the insert from pCMVhARL26A/F27A using the same enzymes.

Androgen Binding and Dissociation Assays—Apparent equilibrium binding affinity was determined in whole cell binding assays at 37 °C by plating monkey kidney COS1 cells (0.2×10^6/well of 12-well tissue culture plates) and transfecting 0.1 μg of pCMVhAR wild-type and mutant DNA using DEAE-dextran (26). Twenty four h after transfection, cells were incubated with increasing concentrations of [^{3}H]R1881 from 0.1 to 5 nM in the presence and absence of a 100-fold excess unlabeled R1881. Cells were incubated at 37 °C for 2 h, washed, and harvested in 0.5 ml of 2% SDS, 10% glycerol, and 10 mM Tris, pH 6.8. Radioactivity was determined by scintillation counting. For determination of dissociation rates of [^{3}H]R1881, COS cells were plated at 0.4×10^6 cells/well in 6-well plates and transfected with 2 μg of pCMVhAR DNA/well using DEAE-dextran. Cells were incubated for 2 h at 37 °C with 5 nM [^{3}H]R1881 in the presence and absence of a 100-fold excess unlabeled R1881. Dissociation was started by the addition of 50 μM unlabeled R1881, and the cells were incubated for increasing times at 37 °C up to 3 h, washed once, and harvested in SDS buffer as described above, with radioactivity determined by scintillation counting.

In Vitro Protein Binding Assays—Glutathione *S*-transferase (GST) fusion vectors GSTAR-(1–660) and GSTAR-(1–565) were prepared as described previously (26). pGEX5X-1AR-(1–660) was digested with *Xho*I (blunt) and *Sma*I and religated to make GSTAR-(1–36). pGEX5X-1AR-(1–660) was digested with *Afl*II/*Xho*I, blunt-ended, and religated to make GSTAR-(1–173). pGEX5X-1AR-(1–660) was digested with *Sac*I/*Xho*I, blunt-ended, and religated to make GSTAR-(1–333). pGEX-3XAR-(1–566) was digested with *Bam*HI/*Afl*II, blunt-ended, and religated to make GSTAR-(174–566). GALAR-(1–660)-L26A/F27A was digested with *Bam*HI/*Afl*II, and the insert was ligated into pGEX-5X-1AR-(1–660) digested with *Bam*HI/*Afl*II to make GSTAR-(1–660)-L26A/F27A. GSTAR-(1–660)-L26A/F27A was digested with *Kpn*I/*Afl*II and ligated with the insert from VPAR-(1–660)-Δ429–439 digested with *Kpn*I/*Afl*II to make GSTAR-(1–660)-L26A/F27A-(Δ429–439). AR NH_2-terminal residues 344–566 were PCR-amplified from VPAR-(1–660) with the appropriate deletions, digested at *Bam*HI/*Xho*I primer sites, and ligated into pGEX-4T-1 to make GSTAR-(344–566), GSTAR-(344–566)-Δ339–381, GSTAR-(344–566)-Δ382–429, GSTAR-(344–566)-Δ430–499, and GSTAR-(344–566)-Δ429–439. GSTTIF2 expressing the central TIF2 amino acid residues 624–1141 containing three L*XX*LL motifs was described previously (26). pcDNA3-AR-(624–919)-E897K, V716R, K720A, and V889M were prepared by digesting GALD-H containing the appropriate mutation with *Bam*HI/*Xba*I and ligating the fragment into pcDNA3HA digested with *Bam*HI/*Xba*I.

GST fusion protein binding studies were performed essentially as described previously (26). GST fusion proteins with different regions of the human AR NH_2-terminal region were expressed from XL1-Blue *Escherichia coli* cells treated with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h during log phase growth. Glutathione-agarose beads (Amersham Pharmacia Biotech) were incubated for 1 h at 4 °C with sonicated bacterial supernatants containing the GST-AR fusion proteins. Beads were washed with 0.5% Nonidet P-40, 1 mM EDTA, 0.1 M NaCl, 0.02 M Tris, pH 8.0, and combined with 25 μCi of [^{35}S]methionine (NEN Life Science Products)-labeled human AR LBD (AR amino acid residues 624–919) using TNT T7 Quick-coupled Transcription/Translation System (Promega) and incubated for 2 h at 4 °C in the absence or presence of 0.2 μM DHT. Beads were washed, eluted with SDS, and analyzed on 12% acrylamide gels containing SDS.

RESULTS

FXXLF in the AR NH_2-terminal Region—The androgen-dependent interaction between the AR NH_2-terminal and carboxyl-terminal (N/C) domains occurs in the regions of predicted α-helices 3, 4, and 12 that comprise AF2 of the LBD, overlapping with the binding site for p160 coactivators (26). Because L*XX*LL motifs mediate the interaction of p160 coactivators with AF2 of nuclear receptors (11–16), it raised the possibility that an L*XX*LL-like motif (13–15) in the AR NH_2-terminal region has a similar function to mediate the N/C interaction. Sequence analysis of the AR NH_2-terminal regions previously implicated in the N/C interaction (27) revealed four predicted amphipathic α-helices that resemble L*XX*LL core sequences at residues 21–34, 351–359, 395–405, and 432–434 with another predicted outside these regions (33) at residues 177–201. We tested wild-type, deletion, and single amino acid mutations of these α-helical regions in a variety of assays. These included the mammalian two-hybrid N/C interaction assay performed in CHO cells and functional assays that included the effects of the mutations on [^{3}H]androgen dissociation rate and transcriptional activation. *In vitro* domain interactions were also directly tested using *E. coli*-expressed GST fusion proteins. For the mammalian two-hybrid assay, wild-type and mutant VPAR-(1–660) coding for the AR NH_2-terminal and DNA binding domains were cotransfected with GALAR-(624–919) expressing the LBD (Fig. 1). Similar expression levels of wild-type and mutant VPAR-(1–660) vectors were verified by immunoblot analysis using AR52 antibody (data not shown). Also all of the VPAR-(1–660) constructs, when cotransfected with GALAR-(1–503) coding for just the NH_2-terminal domain, resulted in 2.2–3.2-fold induction of luciferase activity indicative of the NH_2-terminal/NH_2-terminal AR interaction previously reported (27), thus confirming similar expression levels of the VPAR-(1–660) vectors.

Wild-type and mutant VPAR-(1–660) coding for the AR NH_2-terminal and DNA binding regions were coexpressed in the mammalian two-hybrid assay with GALAR-(624–919) coding for the AR LBD. The VPAR-(1–660) fragment with wild-type AR sequence induced 38 ± 19-fold luciferase activity relative to the no hormone control (Fig. 1). Deletion of NH_2-terminal residues 179–199, 394–405, or 429–439 had no significant effect on the interaction (Fig. 1). In contrast, Δ9–28 reduced the interaction to only a 1.5-fold increase over the no hormone control and Δ339–499 to 10-fold relative to the control (Fig. 1) (27). These results suggested interactions between the LBD and residues 9–28 and 339–499. Because ^{23}FQNLF^{27} lies within the 9–28 region and resembles the L*XX*LL core sequence, it was investigated further by mutagenesis. Changing phenylalanine 23 to alanine (F23A) or leucine 26 and phenylalanine 27 to alanine (L26A/F27A) reduced the N/C interaction to 1.6 ± 0.2-fold over background levels, whereas a flanking mutation of glutamine 28 to alanine (Q28A) resulted in an interaction similar to that of wild-type AR (Fig. 1). Changing both phenylalanine residues in ^{23}FQNLF^{27} to leucine (F23L/F27L) to recreate a consensus L*XX*LL sequence resulted in only a 2-fold induction of luciferase activity (Fig. 1). These results indicated that an F*XX*LF motif was required in the N/C inter-

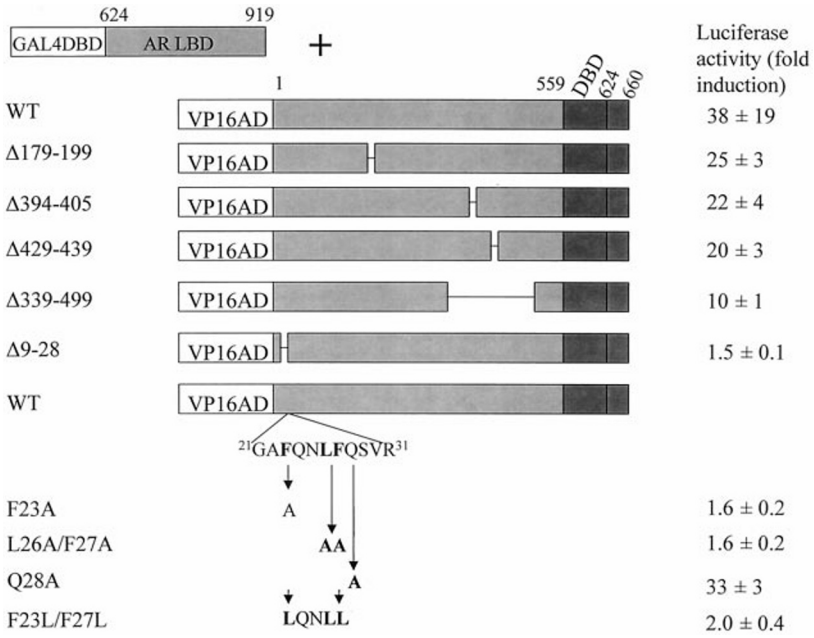

FIG. 1. **Identification of the ^{23}F*XX*LF^{27} motif.** Shown are schematic diagrams and two-hybrid interaction results between the AR LBD and wild-type and AR NH_2-terminal mutants used to define the ^{23}F*XX*LF^{27} motif in the AR NH_2-terminal domain. Mutants were constructed in VPAR-(1–660) coding for the VP16 activation domain (*VP16AD*) expressed as a fusion protein with the AR NH_2-terminal and DNA binding domain (*DBD*) residues 1–660. Wild-type (*WT*) and mutant VPAR-(1–660) were cotransfected with GALAR-(624–919) expressing the AR LBD residues 624–919 (*AR LBD*) as a fusion protein with the GAL4 DNA binding domain (*GAL4DBD*) and analyzed in CHO cells in the mammalian two-hybrid N/C interaction assay using the G5E1bLuc reporter vector as described under "Experimental Procedures." Shown is the mean fold induction and error of luciferase activity from at least three independent experiments determined in the absence and presence of 10 nM DHT. AR amino acid residue numbers are indicated at the *top*. Changes in individual amino acid residues in the NH_2-terminal region are indicated at the *bottom* by *arrows*.

action. Specificity of the ^{23}F*XX*LF^{27} interaction with the AR LBD was indicated by the greatly reduced interaction when F*XX*LF was substituted by L*XX*LL.

The requirement for the F*XX*LF motif in the N/C interaction was also investigated using AR NH_2- and carboxyl-terminal fragments in transient transfection assays using the MMTV-luciferase reporter. As previously reported, the AR DNA binding and ligand binding domain fragment AR-(507–919) had negligible transcriptional activity in the presence of androgen (Fig. 2) indicative of the lack of AF2 transcriptional activity (32) and weak recruitment of p160 coactivators by the AR AF2 region (26). In cotransfection studies, wild-type NH_2-terminal domain residues AR-(1–503) interacted with AR-(507–919) to stimulate an 18–28-fold increase in luciferase activity (Fig. 2). In contrast, NH_2-terminal fragment AR-(1–503) with residues 14–150 deleted (Δ14–150) or with the mutated sequence ^{22}FQNAA^{27} (L26A/F27A) failed to interact with AR-(507–919), supporting an important role for ^{23}FQNLF^{27} in the N/C interaction. Androgen-induced luciferase activity was 6–12-fold with deletion of NH_2-terminal residues 142–337 comprising the AR transactivation domain (Δ142–337) which could have resulted from reduced transactivation by AR rather than a decrease in the N/C interaction as previously suggested (27). Δ339–499 also reduced the interaction but less effectively than did the L26A/F27A mutation (Fig. 2). The results support the requirement for ^{23}FQNLF^{27} in the N/C interaction and the presence of a second interaction site between residues 339 and 499. Similar results using fusion proteins of the AR NH_2-terminal region linked to the VP16 transactivation domain supported the role of these two regions in the N/C interaction (Fig. 2).

Effect of the FXXLF Motif on Androgen Dissociation Kinetics—To establish a functional effect of the NH_2-terminal mutations on AR activity and to obtain additional evidence for the putative second interacting site, we measured the androgen

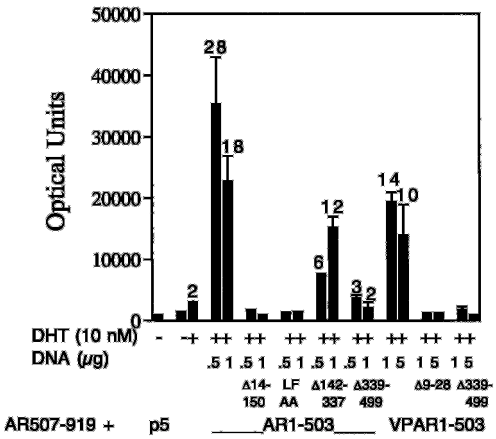

FIG. 2. **N/C interaction using AR fragments and the MMTV-luciferase reporter vector.** PCMVhAR-(507–919) (*AR507–919*, 50 ng/plate) coding for the AR DNA binding domain and LBD was coexpressed in CV1 cells without or with 0.5 or 1 μg of pCMVhAR-(1–503) (*AR1–503*) coding for the AR NH_2-terminal domain with wild-type sequence or the indicated mutant sequence or with 1 or 5 μg of the VP16 activation domain-AR NH_2-terminal domain fusion protein VPAR-(1–503) with wild-type sequence or with the indicated deletions. Assays were performed in CV1 cells using the MMTV-luciferase reporter (5 μg) in the presence and absence of 10 nM DHT. Parent expression vector control pCMV5 (p5) lacked AR sequence. LFAA indicates the L26A/F27A mutation (^{23}FQNAA^{27}). Fold induction of luciferase activity determined relative to the activity in the absence of DHT is indicated above the *bars* and is representative of at least three independent experiments.

dissociation rate using the synthetic radiolabeled androgen [^{3}H]R1881. These studies were based on previous studies that certain mutations in AF2 of the AR LBD cause androgen insensitivity by disrupting the N/C interaction. Although the equilibrium androgen binding affinity was unaffected by these mutations, the dissociation rate of bound androgen increased

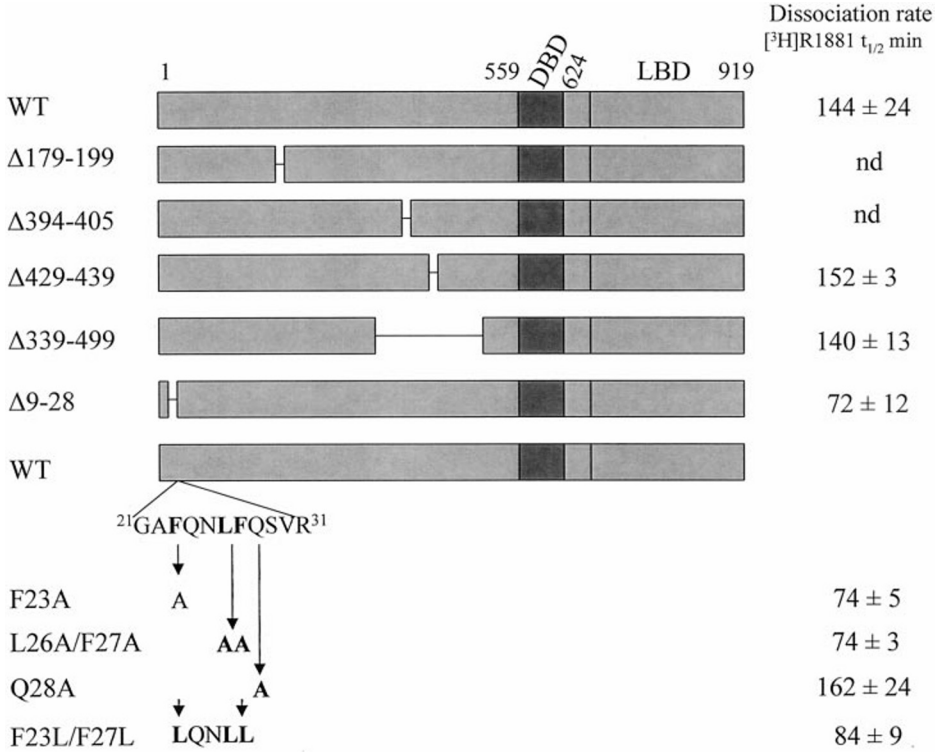

FIG. 3. **Effect of AR NH_2-terminal motif ^{23}F*XX*LF^{27} on androgen dissociation kinetics.** Wild-type (*WT*) and AR mutants shown schematically were constructed in full-length pCMVhAR. Dissociation rates of [^{3}H]R1881 were determined at 37 °C in transiently transfected COS cells as described under "Experimental Procedures." Mean dissociation half-times ($t_{½}$ in min at 37 °C) and errors of at least three independent experiments are shown. The major AR domains are indicated by DNA binding domain (*DBD*) and ligand binding domain (*LBD*) and by amino acid residue number. Individual amino acid changes are indicated with *arrows* at the *bottom*.

suggesting a corresponding increase in association rate (26, 28). The results supported a role for the N/C interaction in slowing the androgen dissociation rate in wild-type AR (31).

Additional evidence that the N/C interaction influences AR ligand binding kinetics is that coexpression of the DNA binding domain and LBD fragment AR-(507–919) with NH_2-terminal fragment AR-(1–660) slows the dissociation of [^{3}H]R1881 by 2-fold (28). In contrast, no effect was seen by coexpression of the nuclear receptor coactivators transcriptional mediator/intermediary factor 2 (TIF2), vitamin D receptor-interacting protein 205, amplified in breast cancer-1 (AIB1) or protein inhibitor of activated signal transducer and activator of transcription-1 (PIAS1), each of which contains multiple consensus L*XX*LL motifs (10, 34–36). Dissociation of [^{3}H]R1881 from the carboxyl-terminal AR-(507–919) fragment (half-time of dissociation $t_{½}$ of 43 ± 3 min, see Fig. 4) was unchanged with $t_{½}$ of 42 ± 4 min at 37 °C when coexpressed with each of these coactivators (data not shown). The results are consistent with a weak interaction of these coactivators with the AR AF2 region compared with the interaction with the AR NH_2-terminal domain (26) and suggest a correspondingly higher apparent binding activity of the ^{23}F*XX*LF^{27} core sequence for AF2 compared with the L*XX*LL motif.

The functional significance of the NH_2-terminal F*XX*LF core sequence was also evident by increased dissociation rates of bound androgen with the introduction of mutations in the F*XX*LF region in full-length AR. Δ9–28, F23A, or L26A/F27A increased the dissociation rate of [^{3}H]R1881 at 37 °C to $t_{½}$ of 72–74 min compared with $t_{½}$ of 144 ± 24 min for wild-type AR (Fig. 3). In contrast, mutation of the flanking carboxyl glutamine (Q28A) had no effect on dissociation rate ($t_{½}$ of 162 ± 24 min) (Fig. 3) or N/C interaction (Fig. 1). Furthermore, changing ^{23}FQNLF^{27} to the consensus L*XX*LL sequence (F23L/F27L) increased the dissociation rate from $t_{½}$ of 144 ± 24 min for wild-type AR to $t_{½}$ of 84 ± 9 min (Fig. 3), which was similar to $t_{½}$ 74 ± 5 min for F23A and L26A/F27A, supporting that an L*XX*LL motif is much less effective than F*XX*LF in mediating the N/C interaction. None of these mutations or those described below changed significantly the apparent equilibrium binding affinity of [^{3}H]R1881 that ranged from 0.3 to 0.8 nM (data not shown), suggesting a corresponding increase in association rate when the dissociation rate increased. The results indicate that the AR NH_2-terminal F*XX*LF motif has an important role in the N/C interaction which results in a reduced rate of androgen dissociation.

WXXLF, the Second N/C Interaction Site—Results from the two-hybrid interaction and androgen kinetic studies provided evidence for a second NH_2-terminal site involved in the N/C interaction. The dissociation rate of [^{3}H]R1881 from AR mutant Δ9–28 (27) or ^{23}FQNAA^{27} (L26A/F27A) of $t_{½}$ of 72–74 min was faster than from wild-type AR ($t_{½}$ of 144 ± 24 min, Fig. 3) but slower than that for AR-(507–919) with $t_{½}$ of 43 ± 3 min which lacks the NH_2-terminal region (Fig. 4). We therefore investigated further the location of a second interacting site that contributed to slowing the androgen dissociation rate. NH_2-terminal deletions Δ429–439 or Δ339–499 alone did not increase the androgen dissociation rate relative to wild-type AR, with dissociation rates $t_{½}$ of 152 ± 3 min and 140 ± 13 min, respectively (Fig. 3). However, when these mutations were combined with the mutation ^{23}FQNAA^{27} (L26A/F27A), the dissociation rate increased to $t_{½}$ of 49 ± 4 min for Δ339–499L26A/F27A and $t_{½}$ of 47 ± 1 min for Δ429–439L26A/F27A (Fig. 4). These rates were similar to that for AR-(507–919) and slightly faster than when L26A/F27A was combined with Δ430–499 ($t_{½}$ of 57 ± 1 min, Fig. 4). On the other hand, no further increases in androgen dissociation rate were observed when L26A/F27A was combined with Δ339–381 or Δ382–429 with $t_{½}$ of 90 ± 9 min (Fig. 4). The results suggest that residues 429–439 contribute to slowing the androgen dissociation rate and thus may be involved of the N/C interaction.

The L*XX*LL-like sequence ^{433}WHTLF^{437} lies within NH_2-terminal residues 429–439. To establish whether this putative W*XX*LF motif contributes to slowing the androgen dissociation rate, several additional mutations were combined with the mutant sequence ^{23}FQNAA^{27} (L26A/F27A). With tryptophan 433 changed to alanine (W433A) or the mutation ^{433}WHTAA^{437}

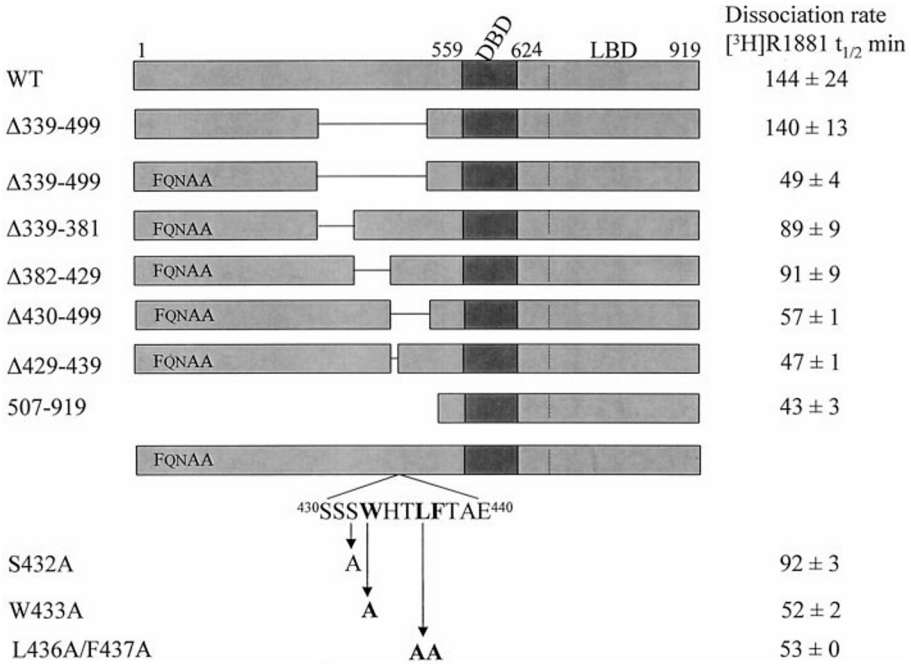

FIG. 4. **Effect of the ^{433}W*XX*LF^{437} motif on androgen dissociation kinetics.** Wild-type (*WT*) AR and AR mutants shown schematically were constructed in full-length pCMVhAR without or with the NH_2-terminal mutation ^{23}FQNAA^{27} as indicated or AR-(507–919) expressing the DNA binding domain and LBD. Mean dissociation half-times ($t_{½}$ in min at 37 °C) and errors of [^{3}H]R1881 were determined from at least three independent experiments as described under "Experimental Procedures." Major AR domains are as described in Fig. 3 legend, and individual amino acid changes are indicated with *arrows*.

(L436A/F437A) was combined with ^{23}FQNAA^{27} (L26A/F27A), the dissociation rate increased to $t_{½}$ of 52 ± 2 min (Fig. 4). However, no increase was observed when the flanking serine 432 was changed to alanine (S432A) and combined with L26A/F27A ($t_{½}$ of 92 ± 3 min) relative to L26A/F27A alone (Fig. 4). When L436A/F437A was combined with E897K, a mutation that interferes with the N/C interaction (26), the androgen dissociation rate increased to 45 ± 2 min (data not shown). Thus NH_2-terminal sequences ^{433}WHTLF^{437} and ^{23}FQNLF^{27} appear to act in concert to slow the androgen dissociation rate.

To test for possible functional equivalence between the F*XX*LF and W*XX*LF core sequences that might have depended on their position within the AR NH_2-terminal sequence rather than the binding motif itself, we replaced phenylalanine 23 with tryptophan to thereby replace ^{23}F*XX*LF^{27} with ^{23}W*XX*LF^{27}. The two-hybrid N/C interaction of this F23W mutant was only 1.9–2.2-fold compared with 35–59-fold for the wild-type control at 1 and 100 nM DHT. W*XX*LF therefore appears to have binding properties distinct from F*XX*LF such that the two motifs are not functionally equivalent. Whereas F*XX*LF appears to be the primary NH_2-terminal interaction site for the AF2 region of the LBD, the core sequence W*XX*LF seems to contribute to the interaction with the AR LBD by further slowing the androgen dissociation rate.

Distinct Binding Regions of the Two NH_2-terminal Motifs—The AR NH_2-terminal sequences that mediate the N/C interaction were investigated further using *E. coli*-expressed GST fusion proteins. AR LBD residues 624–919 were labeled with [^{35}S]methionine and incubated in the presence of 0.2 μM DHT and GST-AR NH_2-terminal fragments of increasing length. Similar expression levels of the GST AR NH_2-terminal domain fusion proteins were confirmed by Coomassie staining of SDS-polyacrylamide gels (data not shown). AR NH_2-terminal fragments 1–36, 1–173, and 1–333 bound to the AR LBD in the presence of 0.2 μM DHT and was reduced to near background levels by the L26A/F27A mutation (Fig. 5*A, lanes 1–7*), providing *in vitro* evidence that ^{23}FQNLF^{27} mediates the N/C interaction. But surprisingly, using the extended NH_2-terminal residues 1–660, the L26A/F27A mutation had only a minimal effect on the *in vitro* binding assay (Fig. 5*A, lanes 8* and *9*). Furthermore, AR NH_2-terminal fragments 174–566 and 334–566 that lacked the ^{23}FQNLF^{27} binding motif showed strong

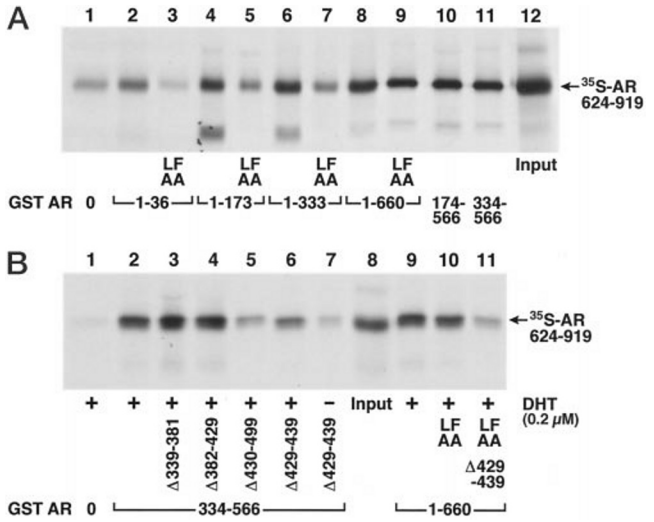

FIG. 5. ***In vitro* GST fusion protein binding studies of AR interacting domains.** GST fusion proteins either lacking AR sequence (*GST AR 0*) or with the indicated AR NH_2-terminal regions with wild-type sequence, or with the L26A/F27A mutation (*LFAA*) (*A*), or the indicated deletions (*B*) were incubated in the presence of 0.2 μM DHT and ^{35}S-labeled AR-(624–919) expressing the AR LBD residues 624–919 with wild-type sequence as described under "Experimental Procedures." 20% of the total radioactivity used in the binding reactions is shown in the *input lanes*.

interaction with the AR LBD (Fig. 5*A, lanes 10* and *11*). The results support the role of the NH_2-terminal F*XX*LF in mediating the N/C interaction and provide additional evidence for a second NH_2-terminal interacting site located between residues 334 and 566.

To determine whether the second site was identical to ^{433}WHTLF^{437} identified in the androgen kinetic studies described above, AR NH_2-terminal deletions Δ339–381, Δ382–429, and Δ430–499 were introduced into GST-AR-(334–556). As shown in Fig. 5*B* (*lane 5*), residues 430–499 were required for interaction of GSTAR-(344–566) with the AR LBD. Indeed, deletion of the predicted short α-helical region containing ^{433}WHTLF^{437} in Δ429–439 greatly decreased the LBD-GSTAR-(344–566) interaction in the presence of DHT (Fig. 5*B, lane 6*). More importantly, introducing the double mutations of

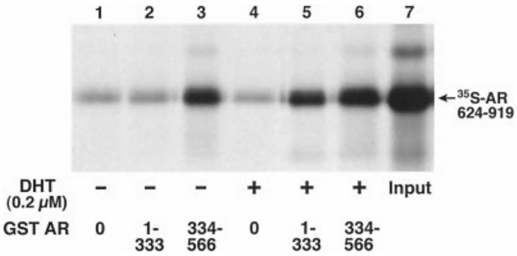

FIG. 6. **Androgen dependence of AR NH_2-terminal fragment interactions with the AR LBD.** Androgen-dependent interactions were tested by incubating ^{35}S-labeled AR-(624–919) expressing the AR LBD containing wild-type sequence together with GST-AR0 (lacking AR sequence), or with AR NH_2-terminal fragments GST-AR-(1–333) or GST-AR-(174–566) in the absence (*lanes 1–3*) or presence (*lanes 4–6*) of 0.2 μM DHT. Incubations were performed as described under "Experimental Procedures." The *input lane* represents 20% of the total ^{35}S-labeled AR-(624–919) used per reaction.

the two putative interacting regions F*XX*LF and W*XX*LF (L26A/F27A and Δ429–439) into AR-(1–660) reduced the N/C interaction to basal levels that were observed in the absence of androgen (Fig. 5*B, lane 11*). The results support the androgen kinetic studies that both ^{23}FQNLF^{27} and ^{433}WHTLF^{437} in the AR NH_2-terminal region mediate the N/C interaction. In addition, they indicate that W*XX*LF interacts with the LBD to a greater extent than was evident in the two-hybrid assay.

The androgen dependence of the interaction of the two NH_2-terminal sites with the AR LBD was further investigated in the GST fusion protein *in vitro* binding assay. Interaction of NH_2-terminal fragment GSTAR-(1–333) containing the F*XX*LF binding motif ^{23}FQNLF^{27} was dependent on the addition of androgen (Fig. 6, *lanes 2* and *5*) which agreed with the androgen dependence of the N/C interaction. However, surprisingly, binding of GSTAR-(334–566) containing the ^{433}W*XX*LF^{437} motif to the ^{35}S-labeled AR-(624–919) LBD fragment was independent of the presence of androgen (Fig. 6, *lanes 3* and *6*). The results raised the possibility that the F*XX*LF and W*XX*LF binding motifs interact with different regions of the AR LBD.

Previously, we showed that certain mutations in the AF2 region of the AR LBD disrupt the N/C interaction without changing the apparent equilibrium binding affinity yet require higher DHT concentrations to induce MMTV-luciferase activity relative to wild-type AR (26). We therefore determined whether one or both of the NH_2-terminal sites were affected by these mutations in the LBD. In GST interaction assays performed in the presence of 0.2 μM DHT, LBD mutations E897K or V716R introduced into ^{35}S-labeled AR-(624–919) greatly reduced the interaction between AR-(1–333) and the LBD (Fig. 7, *lanes 13* and *18*) as well as the interaction with the p160 coactivator fragment, TIF2-M (Fig. 7, *lanes 15* and *20*). However, interaction of these LBD mutants with AR-(174–566) which contained the second interacting motif ^{433}W*XX*LF^{437} was similar to wild-type (Fig. 7, *lanes 14* and *19*). Similar results were observed using V889M (data not shown), a mutation in the AR LBD that causes nearly complete androgen insensitivity (31, 37). On the other hand, ^{35}S-labeled AR-(624–919) containing K720A, a mutation that does not affect the N/C interaction or AR transcriptional activity (26), did not decrease the interaction with AR-(1–333) or AR-(174–566) but eliminated the interaction with TIF2-M (Fig. 7, *lanes 7–10*). The results suggest that glutamic acid 897 and valine 716 in AF2 and valine 889 preceding helix 12 of the LBD interact with ^{23}FQNLF^{27} but not with ^{433}WHTLF^{437}. Because these residues are directly part (glutamic acid 897 and valine 716) or flanking (valine 889) the AF2 region, the results support the interaction of ^{23}FQNLF^{27} with AF2 in the LBD and suggest that ^{433}WHTLF^{437} interacts with another region of the LBD.

Transcriptional Activation—The influence of mutations in the F*XX*LF and W*XX*LF core sequences on AR transcriptional activity by full-length AR was investigated in transient transfection assays using two different luciferase reporter vectors. The ^{23}FQNAA^{27} mutation (L26A/F27A) either alone or combined with the mutation ^{433}WHTAA^{437} (L436A/F437A) reduced the increase in transcriptional activity by at least 50%, whereas Δ429–439 alone had less of an effect (Fig. 8*A*). By using an AR-specific probasin-luciferase reporter (38), mutations in the first or both NH_2-terminal interacting sites also decreased transcriptional activity by about 50% (Fig. 8*B*). The results suggest that the N/C interaction facilitates an optimal transcriptional response.

DISCUSSION

Here we present evidence that the N/C interaction, referred to in the literature also as an interaction between AF1 and the LBD or AF1 and AF2 and initially described for AR (27, 39) and the estrogen receptor (40), is direct, androgen-dependent, and mediated predominantly by the core sequence F*XX*LF in the AR NH_2-terminal region. Both mammalian two-hybrid interaction and GST fusion protein binding studies indicate that NH_2-terminal sequence ^{23}FQNLF^{27} mediates interaction with the AF2 region in the LBD in an androgen-dependent manner. This is substantiated by the observation that single amino acid mutations in either the NH_2-terminal F*XX*LF motif or the LBD AF2 region eliminate the N/C interaction when the apparent androgen binding affinity is not altered. At least one functional consequence of the N/C interaction previously reported (28, 31) and shown here is a decreased dissociation rate of bound androgen. Mutations in ^{23}FQNLF^{27} support this role of the N/C interaction since they increased the androgen dissociation rate.

A surprising finding of the study was that the F*XX*LF motif was not the only L*XX*LL-like motif contributing to the N/C interaction. Even though mutation of ^{23}FQNLF^{27} essentially eliminated the N/C interaction in the two-hybrid assay and the interaction between the AR-(1–333) NH_2-terminal fragment with the AR LBD in GST protein binding studies, mutations in ^{23}FQNLF^{27} had only a marginal effect when the full NH_2-terminal region AR-(1–660) was tested in the GST interaction assay. Further studies located the second interaction site between amino acid residues 429 and 439 in the NH_2-terminal domain. [^{3}H]R1881 dissociation studies implied that ^{433}WHTLF^{437} within this region plays an important role in this interaction. However, although the interaction of ^{23}FQNLF^{27} with the AF2 region was androgen-dependent and largely responsible for the N/C interaction in the two-hybrid assay, interaction with the AR LBD second site ^{433}W*XX*LF^{437} located within the 429–439 region was androgen-independent. Furthermore, using the same AF2 LBD mutations that eliminated interaction with F*XX*LF, it became apparent that the W*XX*LF motif likely interacts with a region of the LBD outside of AF2. To account for these data and the effect of mutations in both the F*XX*LF and W*XX*LF interaction sites on increasing the androgen dissociation rate, it may be that in full-length AR, the second W*XX*LF site becomes available to bind the AR LBD subsequent to the androgen-dependent interaction of F*XX*LF with AF2. GST fusion protein interaction assays using AR NH_2-terminal fragments may circumvent this requirement for androgen binding by releasing structural constraints within the NH_2-terminal region. It suggests further that the binding region of the AR LBD that interacts with the W*XX*LF motif is not significantly altered by androgen binding as is the AF2 region in binding the F*XX*LF motif.

The AR NH_2-terminal motifs F*XX*LF and W*XX*LF resemble the consensus core sequence L*XX*LL that mediates p160 coactivator interaction with AF2 in the LBD of nuclear receptors

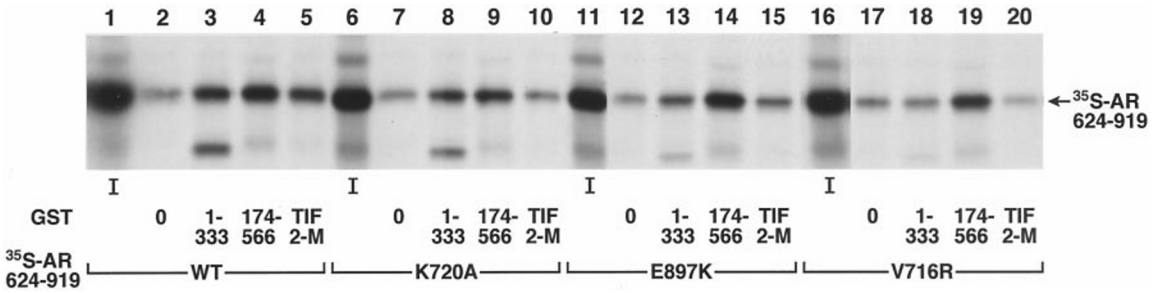

FIG. 7. **LBD amino acids involved in interactions with AR NH_2-terminal fragments.** ^{35}S-Labeled AR-(624–919) with wild-type sequence (*WT*) or with mutations K720A, E897K, or V716R were incubated in the presence of 0.2 μM DHT with GST0 lacking AR sequence, or with AR NH_2-terminal fragments GST-AR-(1–333) or GST-AR-(334–566) as indicated, or with GST-TIF2-M (TIF2 residues 624–1141) as a positive control (26). Input lanes (*I*) represent 20% of the total ^{35}S-labeled AR-(624–919) used per reaction. The major radiolabeled bands represent wild-type or mutant ^{35}S-labeled AR-(624–919).

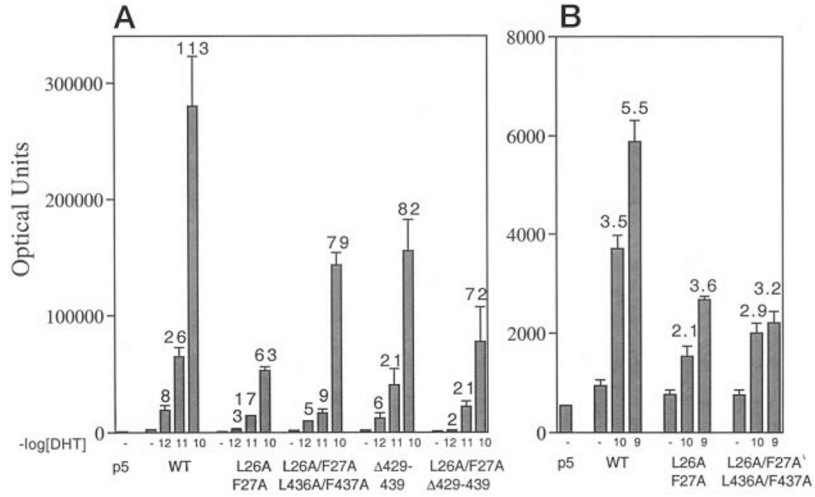

FIG. 8. **Transcriptional activation of wild-type AR and NH_2-terminal domain mutants using luciferase reporter vectors.** Transcriptional activity of full-length wild-type (*WT*) AR or with the indicated AR amino acid or deletion mutations were determined in CV1 cells at increasing DHT concentrations as described under "Experimental Procedures" using MMTV-luciferase (*A*) or probasin-luciferase reporter vectors (*B*). pCMVhAR DNA (25 ng) was transfected with 5 μg of reporter vector. Shown *above* the bars is the fold induction determined relative to the activity in the absence of DHT. The data are representative of at least three independent experiments.

(26). Specificity for the ^{23}F*XX*LF^{27} core sequence for interaction with AF2 was evident because substitution with either L*XX*LL or W*XX*LF essentially abolished the N/C interaction in the two-hybrid assay. The relatively weak AF2 interaction of the L*XX*LL motif when substituted for ^{23}F*XX*LF^{27} is consistent with a weak interaction of the L*XX*LL-containing p160 coactivators with the AR LBD and with the low inherent AF2 activity of the AR LBD. The results reported here as well as previous evidence from GST fusion protein assays (26) support a direct interaction between the NH_2- and carboxyl-terminal regions of AR and contrast with previous reports that the interaction is indirect and bridged (41, 42) or enhanced (43) by p160 coactivators such as steroid receptor coactivator 1.

Several models for the N/C and coactivator interactions of steroid receptors have been proposed that involve parallel *versus* antiparallel orientation of monomers and intra- *versus* intermolecular interactions (12, 27, 28, 40, 44). Binding of the AR NH_2-terminal region to AF2 in the AR monomer or dimer may exclude binding of p160 coactivators as suggested from competition for different coactivator binding (45). The N/C interaction may also influence a proposed temporal sequence of coactivator binding (46) or create a new interacting surface to recruit coactivators (47). p160 coactivators interact with the AR NH_2-terminal region (26) as reported for other nuclear receptors (33, 48). The greater apparent binding affinity of the AR N/C interaction with AF2 compared with p160 coactivator interaction with AF2 is supported by the slower ligand dissociation rate resulting from the N/C interaction but not from p160 coactivator binding. In striking contrast, p160 coactivator binding to the AF2 region slows the ligand dissociation rate from the estrogen receptor (49).

Ligand-dependent N/C interactions have been described for other nuclear receptors including the progesterone receptor, where it facilitates receptor dimerization (44, 50). In peroxisome proliferator-activated receptor-γ, N/C interactions were ligand-independent and reduced ligand binding affinity presumably by modifying conformation of the unliganded receptor (51). The functional significance of the AR N/C interaction is supported by several naturally occurring spontaneous mutations that interfere with the N/C interaction and increase the androgen dissociation rate. These include mutations at valine 889 (28) and isoleucine 898 (26) that cause complete or nearly complete androgen insensitivity and at methionine 886 that causes oligospermic infertility (52). Similarly, the N/C interaction is inhibited by site-directed mutagenesis of LBD residues glutamic acid 893 (53), valine 716, and glutamic acid 897 (26). Whereas some of these mutations also interfere with p160 coactivator binding such as TIF2 to the AF2 region (52, 53), it became clear that a distinct yet overlapping binding site in AR AF2 acts preferentially as the binding site for the AR NH_2-terminal region rather than for p160 coactivators (26). Lysine 720 in human AR corresponds to lysine 366 in the estrogen receptor that was required for AF2 activity (54). Mutations at AR lysine 720, while greatly reducing AR interaction of TIF2, did not decrease AR transcriptional activity significantly (26, 33). On the other hand, I898T, which causes complete androgen insensitivity, did not reduce interaction with TIF2 but greatly reduced the N/C interaction.

Sequence differences in the AF2 region of AR LBD compared with other nuclear receptors likely contribute to the selective binding of F*XX*LF *versus* L*XX*LL motifs (55). The AR AF2 sequence differs from other steroid receptors at several residues that are otherwise highly conserved. Mutating some of these residues to amino acids of other steroid receptors did not

enhance p160 coactivator binding (26). The sequence differences unique to the AR LBD may redirect the function of AF2 toward higher binding affinity for the F*XX*LF binding motif in the AR NH_2-terminal domain rather than for the L*XX*LL motifs in the p160 coactivators. Sequences flanking the L*XX*LL motifs of p160 coactivators (56, 57) apparently contribute to selective binding to AF2 of other nuclear receptors (45). The use of combinatorial phage display screening showed that in some cases sequences flanking the L*XX*LL motifs contribute to specific interactions with nuclear receptors (56). However, our study of ^{23}FQNLF^{27} and ^{433}WHTLF^{437} indicates that of the flanking AR NH_2-terminal sequences tested, none had a predominant role in the N/C interaction. Mutation of glutamine 28 carboxyl-terminal to ^{23}FQNLF^{27} or serine 432 NH_2-terminal to ^{433}WHTLF^{437} did not increase the androgen dissociation rate, an indicator of the N/C interaction.

Evolution of amino acid sequence in the AR NH_2-terminal region and LBD seems to favor conservation of the N/C interaction sites. In the NH_2-terminal region, both ^{23}FQNLF^{27} and ^{433}WHTLF^{437} and their flanking sequence are fully conserved among the primate AR even though other regions of the NH_2-terminal domain are not conserved (58). In the rainbow trout AR α and β forms, which have the strikingly low homology of 19% in the NH_2-terminal amino acid sequence compared with human AR, structural conservation occurs at the androgen-dependent interaction site with the trout sequence ^{22}FQNVF^{26} compared with ^{23}FQNLF^{27} in human AR (59). Furthermore, whereas the primate LBD is fully conserved with respect to human amino acid sequence, rainbow trout AR α and β forms have only 65 and 68% sequence similarity in the LBD, respectively (59), yet residues critical for the N/C interaction are conserved. Natural selection therefore seems to favor residues in AR domains that mediate the N/C interaction supporting their critical role in AR function.

The functional significance of the N/C interaction remains to be fully established. The N/C interaction appears to be required for AR function *in vivo* as suggested by androgen insensitivity mutations. It is, however, apparently not an absolute requirement for AR induction of MMTV reporter gene activity in transient transfection assays. The AR NH_2-terminal and DNA binding domain fragment that lacks the LBD has strong constitutive transcriptional activity with the MMTV-luciferase reporter gene (32, 60), and only a 50% reduction in activity was observed with mutations in full-length AR that disrupt the N/C interaction using either the MMTV- or probasin-luciferase reporter genes. The N/C interaction may nevertheless contribute to androgen-specific gene induction since the AR shares the ability with other steroid receptors to bind simple consensus DNA response elements but has distinctly different physiological effects (61). Furthermore, ligands such as medroxyprogesterone acetate (Provera) that fail to promote the N/C interaction are weak androgens *in vivo* but strong androgen agonists in transient transfection assays (30). These observations support that the N/C interaction is important in mediating AR function *in vivo* and that transient transcriptional assays may not provide a good reflection of this *in vivo* requirement. The N/C interaction may increase the sensitivity of AR to low circulating androgen concentrations by slowing the dissociation rate of bound androgen. This function might be especially important during male sexual development *in utero*.

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