**Dependence of Selective Gene Activation on the Androgen Receptor NH$_2$- and COOH-terminal Interaction**

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The agonist-induced androgen receptor NH$_2$- and COOH-terminal (N/C) interaction is mediated by the FXXLF and WXXLF NH$_2$-terminal motifs. Here we demonstrate that agonist-dependent transactivation of prostate-specific antigen (PSA) and probasin enhancer/promoter regions requires the N/C interaction, whereas the sex-limited protein gene and mouse mammary tumor virus long terminal repeat do not. Transactivation of PSA and probasin response regions also depends on activation function 1 (AF1) in the NH$_2$-terminal region but can be increased by binding an overexpressed p160 coactivator to activation function 2 (AF2) in the ligand binding domain. The dependence of the PSA and probasin enhancer/promoters on the N/C interaction allowed us to demonstrate that in the presence of androgen, the WXXLF motif with the sequence **433WHITLF437** contributes as an inhibitor to AR transactivation. We further show that like the FXXLF and LXXLL motifs, the WXXLF motif interacts in the presence of androgen with AF2 in the ligand binding domain. Sequence comparisons among species indicate greater conservation of the FXXLF motif compared with the WXXLF motif, paralleling the functional significance of these binding motifs. The data provide evidence for promoter-specific differences in the requirement for the androgen receptor N/C interaction and in the contributions of AF1 and AF2 in androgen-induced gene regulation.

Steroid receptors are ligand-activated transcription factors that regulate gene activation through a series of events triggered by high affinity hormone binding and mediated by receptor binding to response element DNA and coactivators. At least two domains have been identified that mediate nuclear receptor interactions with coregulators. These are activation function 1 (AF1) in the NH$_2$-terminal region and activation function 2 (AF2) in the ligand binding domain. The AF2 binding surface in the ligand binding domain is comprised of helices 3, 4, and 12 and forms after hormone binding. For many nuclear receptors, transactivation depends on AF2 recruitment of p160 coactivator complexes that have histone acetyl transferase activity to modify chromatin structure (1). The p160 coactivators are a group of proteins that include steroid receptor coactivator 1 (SRC1), transcriptional intermediary protein 2 (TIF2, GRIP1 or SRC2), and the steroid receptor coactivator 3 subfamily (SRC3). Interaction with AF2 is mediated by the p160 coactivator LXXLL motif that forms an amphipathic $\alpha$-helix and binds the AF2 hydrophobic binding surface in the nuclear receptor ligand binding domain (2–5). For the androgen receptor (AR), the functional importance of AF2 recruitment of p160 coactivators is unclear, with data implicating the AR NH$_2$-terminal AF1 region in AR-mediated gene activation.

The AF2 binding site in the AR ligand binding domain was shown to mediate the agonist-induced NH$_2$- and COOH-terminal (N/C) interaction (6–10). Agonist-induced N/C interdomain interactions are also reported for the estrogen (11) and progesterone receptors (12), but not for the glucocorticoid receptor (GR) (7, 13). Two AR NH$_2$-terminal LXXLL-like motifs that interact with the AR ligand binding domain are the FXXLF and WXXLF motifs (14). Mutagenesis studies and mammalian two-hybrid and GST affinity matrix assays demonstrated that the FXXLF motif interacts in the presence of androgen with AF2 (14). However the site of interaction of the WXXLF motif was not determined, nor was it clear whether interaction of the WXXLF motif depends on androgen binding. In addition, previous studies made use of the MMTV luciferase reporter vector, which may direct transcription through mechanisms that differ from other androgen responsive enhancer/promoters.

Here we show the functional importance of the AR N/C interaction using androgen responsive enhancer/promoter regions derived from the prostate specific antigen (PSA) and probasin genes. Use of these responsive regions allowed us to demonstrate, in addition, that the WXXLF motif inhibits recruitment of TIF2 by the AF2 region but less than does the FXXLF motif. Using a shorter NH$_2$-terminal fragment than previously described (14), we show that the WXXLF motif interacts in the presence of androgen with the AF2 region of the ligand binding domain. The data provide evidence that the AR N/C interaction is required for AR-mediated regulation of two androgen-dependent genes. The relatively high sequence conservation of the FXXLF and WXXLF motifs among species further supports the functional importance of the AR N/C interaction.

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1 The abbreviations used are: AF1, activation function 1; AR, androgen receptor; N/C, NH$_2$-terminal and COOH-terminal; AF2, activation function 2; TIF2, transcriptional intermediary factor 2; GST, glutathione S-transferase; GR, glucocorticoid receptor; Luc, luciferase; PSA, prostate-specific antigen; MMTV, mouse mammary tumor virus; DHT, dihydrotestosterone.
EXPERIMENTAL PROCEDURES

Plasmid Construction—GAL-peptide fusion proteins were constructed as described (15) and contain GAL4 DNA binding domain residues 1–147 and the peptide sequences indicated. pCMVhAR-A4E962–919 with wild-type AR sequence or with mutations K720A, V716R, and E897K for in vitro translation were described (14). GST-AR-(412–460) was created by amplifying the coding sequence in pCMVhAR, digesting the fragment with BglII and cloning the fragment into pGEX3X digested with the same enzymes. GST-AR-(412–460)XWXXA, where 437WHFL437 is changed to WHTAA, was created in the same manner except by amplifying using PCR the corresponding region of the mutant pCMVhAR vector. AR-FXXAA, where 438QNFL438 is changed to FQNAA, and AR-FXXAA/XAXXA, which in addition has 438WHFL438 changed to HAA/A, were described (13, 15). AR-AAXXA (AR–AA/AHAA/AHAA/AHAA/AHAA/AHAA/AHAA/AHAA) was created by amplifying using PCR the coding sequence in pCMVhAR between XAIIE and HindIII using a 5′_mutant oligonucleotide primer. The fragment was inserted into pCMVhAR digested with the same enzymes. AR-FXXAA/XAXXA-K720A and AR-FXXAA/XAXXA-E897K were created by digesting pCMVhAR-K720A and pCMVhAR-E897K (10) with HindIII/XhoI and cloning the ligand binding domain fragments into AR-FXXAA/XAXXA digested with the same enzymes. DNA amplification by PCR was performed using Vent-polymerase enzyme (New England Biolabs). All regions of DNA that were amplified using PCR were sequenced to verify the absence of random errors. The human GR vectors GR(L90)_5 and GR(L90)_5 were obtained from Hinrich Gronemeyer, University of Louis Pasteur, Strasbourg, France. Mouse mammary tumor virus (MMTV)-Luc was provided by Ronald M. Evans, the Salk Institute for Biological Studies. The pB5S mammalian expression vector for TIF2 was provided by Hinrich Gronemeyer, University of Louis Pasteur, Strasbourg, France. 5XGAL4Luc3 was provided by Donald P. McDonald, Duke University.

Transient Transfection Assay—Monkey kidney CV1 cells were maintained in Dulbecco’s modified Eagle’s medium containing 20 mM Hepes, pH 7.2, penicillin/streptomycin, and 2 mM l-glutamine. Cells were transfected with wild-type and mutant AR expression vectors and luciferase reporter vectors using the calcium phosphate DNA precipitation method (13). The pCMVhAR (AR–AA/AHAA/AHAA/AHAA/AHAA/AHAA/AHAA/AHAA) was created using a double PCR mutagenesis strategy. The AR NH2-terminal region of AR-FXXAA/XAXXA was amplified using a 5′_primer preceding the BglII site, internal primers flanking the deleted region, and a primer 3′_of the BstEII site. The amplified fragments were digested with BglII/BstEII and cloned into AR-FXXAA/XAXXA digested with the same enzymes. DNA amplification by PCR was performed using Vent-polymerase enzyme (New England Biolabs). All regions of DNA that were amplified using PCR were sequenced to verify the absence of random errors. The human GR vectors GR(L90)_5 and GR(L90)_5 were obtained from Hinrich Gronemeyer, University of Louis Pasteur, Strasbourg, France. Mouse mammary tumor virus (MMTV)-Luc was provided by Ronald M. Evans, the Salk Institute for Biological Studies. The pB5S mammalian expression vector for TIF2 was provided by Hinrich Gronemeyer, University of Louis Pasteur, Strasbourg, France. 5XGAL4Luc3 was provided by Donald P. McDonald, Duke University.

Two-hybrid Peptide Interaction Assay—Human epithelioid cervical carcinoma HeLa cells were maintained in Eagle’s medium containing 20 mM Hepes, pH 7.2, penicillin/streptomycin, and 2 mM l-glutamine. Cells were transfected with wild-type and mutant AR expression vectors and luciferase reporter vectors using the calcium phosphate DNA precipitation method (13). The pCMVhAR (AR–AA/AHAA/AHAA/AHAA/AHAA/AHAA/AHAA/AHAA) was created using a double PCR mutagenesis strategy. The AR NH2-terminal region of AR-FXXAA/XAXXA was amplified using a 5′_primer preceding the BglII site, internal primers flanking the deleted region, and a primer 3′_of the BstEII site. The amplified fragments were digested with BglII/BstEII and cloned into AR-FXXAA/XAXXA digested with the same enzymes. DNA amplification by PCR was performed using Vent-polymerase enzyme (New England Biolabs). All regions of DNA that were amplified using PCR were sequenced to verify the absence of random errors. The human GR vectors GR(L90)_5 and GR(L90)_5 were obtained from Hinrich Gronemeyer, University of Louis Pasteur, Strasbourg, France. Mouse mammary tumor virus (MMTV)-Luc was provided by Ronald M. Evans, the Salk Institute for Biological Studies. The pB5S mammalian expression vector for TIF2 was provided by Hinrich Gronemeyer, University of Louis Pasteur, Strasbourg, France. 5XGAL4Luc3 was provided by Donald P. McDonald, Duke University.

In Vitro Protein Interaction Assay—GST fusion proteins were expressed in XLI-Blue Escherichia coli cells treated with 0.5 mM isopropyl β-D-thiogalactopyranoside and extracted and incubated with glutathione-agarose beads (Amersham Biosciences) as described (14). In vitro translated proteins were labeled in the presence of 25 μCi of [35S]methionine (PerkinElmer Life Sciences) using the Tnt T7 Quick Coupled Transcription/Translation System (Promega) in the presence and absence of 1 μM dihydrotestosterone (DHT). Washed beads were boiled in SDS buffer and input lanes contained ~10% of the binding reactions.

RESULTS

Response Element Specificity of the WXXLF and FXXLF Motif—Mediated N/C Interaction and Coactivation by TIF2—We investigated whether androgen response regions derived from different enhancer/promoter regions have a similar requirement for the androgen-induced N/C interaction in AR-mediated gene regulation. Luciferase reporter vectors were tested that contain enhancer/promoter regions from the androgen-regulated genes, PSA (16, 17), probasin (18), sex-limited protein (pGCΔ9) (19, 20), and MMTV (21–23). The role of the WXXLF and FXXLF motifs and effects of TIF2 coactivation on luciferase activity were determined using wild-type AR (AR-FXXLF/WXXLF) or AR in which FXXLF was changed to XAXXA, WXXLF was changed to AXXAA, or both mutations were created in the same protein. AR transactivation of the PSA and probasin enhancer/promoter regions (Fig. 1A) and the pGCΔ9 and MMTV promoters (Fig. 1B) was increased 2–3-fold by TIF2 coexpression. Mutation of the FXXLF motif (AR-FXXAA/WXXLF) decreased to low levels transactivation of the PSA and probasin luciferase reporters in the absence of TIF2 coexpression (Fig. 1A). This decrease in activity was recovered by coexpression of TIF2. In contrast, mutation of the FXXLF motif had no major effect on transactivation of the MMTV-Luc and pGCΔ9-Luc enhancer/promoters in the absence of TIF2 coexpression (Fig. 1B). Surprisingly, mutation of the WXXLF motif alone (AR-FXXLF/AAXXA) increased the response of the PSA and probasin enhancer/promoters, which was increased further by TIF2 coexpression (Fig. 1A). In contrast, mutation of WXXLF had relatively little effect on the response of MMTV-Luc and pGCΔ9-Luc (Fig. 1B). Mutating both binding motifs (AR-FXXAA/AAXXA) decreased transactivation of the PSA and probasin enhancer/promoters, which was rescued by coexpression of TIF2 (Fig. 1A), again with relatively little effect on the pGCΔ9 and MMTV promoters.

The results indicate that in contrast to the pGCΔ9 and MMTV enhancer/promoters, androgen regulation of the PSA and probasin enhancer/promoter regions depends on the AR N/C interaction mediated by the FXXLF and WXXLF motifs in the presence of androgen. The increase in transactivation by mutating WXXLF alone suggests an inhibitory role of this motif in AR activity, whereas a decrease in transactivation by the FXXAA and AXXAA/AAXXA mutants indicates a strong requirement for the N/C interaction. The results are in agreement with the FXXLF motif primarily mediating the N/C interaction, whereas mutation of the WXXLF motif alone does not abolish the N/C interaction (14). The detrimental effect on transactivation of losing the N/C interaction was recovered by TIF2 overexpression. The results support previous evidence from androgen insensitivity syndrome mutations (9) where the N/C interaction is critical for AR-mediated transactivation of androgen-dependent genes in vivo.

We investigated further the inhibitory effect of the WXXLF motif in AR-mediated transactivation of the PSA enhancer/promoter by mutating the WXXLF and FXXLF binding motifs in an AR mutant in which the NH2-terminal AF1 residues 142–327 were deleted. Deletion of AF1 resulted in nearly background levels of activity in the absence or presence of TIF2 coexpression (Fig. 2), indicating an important role for AF1 in
AR-mediated transactivation. Introducing mutations into the FXXLF (AR-FXXAA\textDelta142\textendash337) and FXXLF plus WXXLF motifs (AR-FXXAA/AXXAA\textDelta142\textendash337) increased AR-mediated transactivation of the PSA61-Luc reporter in the absence and presence of TIF2 coexpression. CV1 cells were transfected using calcium phosphate DNA precipitation as described under "Experimental Procedures" with (per 6-cm dish) 50 ng of pCMVhAR plus 2 μg of pGCA9-Luc or 5 μg of MMTV-Luc, or 100 ng of pCMVhAR with 5 μg of PSA61-Luc or 5 μg of probasin-Luc. pCMVhAR had the wild-type sequence (AR-FXXLF/WXXLF) or had mutations in the FXXLF (AR-FXXAA/WXXLF), WXXLF (AR-FXXLF/AXXAA), or both motifs (AR-FXXAA/AXXXA) and was assayed in the absence and presence of 5 μg of pSG5-TIF2. Cells were incubated for 40 h in the absence and presence of 1 nM DHT, and luciferase activity was determined. The data are representative of at least three independent experiments.

We conclude that in the absence of AF1, both FXXLF and WXXLF motifs have a small inhibitory effect on AR transactivation in the absence of TIF2 coexpression. There was a synergistic inhibitory effect by both motifs, possibly reflecting inhibition of endogenous TIF2 coactivation through AF2 in the ligand binding domain. The greater level of TIF2-stimulated luciferase activity observed with the FXXLF mutant compared with the WXXLF mutant indicates that FXXLF more effectively inhibits AR-mediated gene activation by TIF2 than does
Enhancer/Promoter Requirements for the N/C Interaction
Using a Receptor Chimera—To evaluate further the requirement for the N/C interaction in transactivation of the PSA enhancer/promoter region versus the MMTV promoter, we made use of a previously described GR chimera GR/LXXLL, in which an artificial N/C interaction was introduced (13). GR/LXXLL, contains the 3 LXXLL motif region of TIF2 that interacts in the presence of dexamethasone with the GR ligand binding domain AF2 region and results in a 5-fold slower dissociation half-time of [3H]dexamethasone compared with wild-type GR. In the presence of 1 or 10 nM dexamethasone, GR stimulated the PSA (Fig. 3A) and probasin reporters (data not shown), and the MMTV (Fig. 3B) and pGCA9 luciferase reporters (data not shown), with the weakest response from pGCA9. GR/LXXLL, caused greater ligand-induced transactivation of the PSA61-Luc reporter than did GR, or GR/LXXAA, in which the 3 LXXLL motifs were mutated to LXXXA (Fig. 3A). Similar increases in gene activation by GR/LXXLL, were observed using the probasin-Luc and pGCA9-Luc reporters (data not shown). In contrast, response of the MMTV-luciferase reporter was similar for GR and the GR chimeras (Fig. 3B). The results support observations above that transactivation of the androgen responsive enhancer/promoters from PSA and probasin depend on an agonist-induced N/C interaction for optimal transactivation compared with the MMTV enhancer/promoter. The increased response of pGCA9-Luc to GR/LXXLL, suggests that it shares some properties of PSA and probasin response elements that are sensitive to the effects of an N/C interaction.

TIF2 Coactivation of AR-mediated Transactivation of the PSA Reporter Requires Recruitment by AF2—We next investigated whether the increase in AR transactivation of PSA61-Luc (Fig. 4A) and probasin-Luc by TIF2 required TIF2 binding to the AF2 region of the ligand binding domain. In contrast to TIF2, TIF2m123, a mutant in which the 3 LXXLL motifs were changed to LXXAA, was unable to coactivate AR or the AR N/C interaction mutants using the PSA61-Luc reporter (Fig. 4A). Transactivation in the presence of TIF2m123 was less than the control without TIF2 coexpression. Similar results were observed using the probasin and MMTV-Luc reporters (data not shown).

Fig. 2. Role of the WXXLF and FXXLF motifs in inhibiting TIF2 coactivation of the PSA enhancer/promoter by an AR AF1 deletion mutant. Androgen induction of the PSA61-Luc reporter vector was determined by transfecting CV1 cells with pCMVhARΔ142–337 lacking the AF1 transactivation residues 142–337, which had the wild-type binding motif sequence (AR-FXXLF/WXXLFΔ142–337) or the FXXLF (AR-FXXA/WXXLFΔ142–337), WXXLF (AR-FXXLF/AAXXAΔ142–337), or both motifs were mutated (AR-FXXA/AAXXAΔ142–337). Incubations were performed in the absence and presence of coexpression of pSG5-TIF2 (5 μg) and 1 nM DHT as indicated. The data are representative of at least three independent experiments.

Fig. 3. Requirement for the N/C interaction in transactivation by different enhancer/promoters using a TIF2-GR chimera. Dexamethasone-induced activation of PSA61-Luc (A) and MMTV-Luc (B) was determined in CV1 cells as described under “Experimental Procedures.” Cells were transfected with 100 ng (for PSA61-Luc) and 50 ng (for MMTV-Luc) with pCMVhGR (GR) or the TIF2-pCMVhGR chimera TIF2-627–780-GR-9–777 containing wild-type LXXLL ((LXXLL),GR) or LXXAA mutant sequence ((LXXAA),GR) in the absence and presence of 1 and 10 nM dexamethasone (DEX). The 627–780 region of TIF2 contains 3 LXXLL motifs. Luciferase activity shown is representative of at least three independent experiments.
shown). The data suggest that coactivation by TIF2 of each of the enhance/promoters requires interaction of the TIF2 LXXLL motifs with the AR AF2 region and that TIF2m123 acts to a limited extent as a dominant negative inhibitor of endogenous TIF2. The lack of coactivation by TIF2m123 suggests, in addition, that an interaction between other regions of TIF2 and the AR NH$_2$-terminal region (10, 30, 31) were not sufficient to mediate increased transactivation.

A specific role for AF2 in AR transactivation of the PSA61-Luc reporter by TIF2 was investigated using two AR AF2 mutants. Lysine 720 in helix 3 of the ligand binding domain is critical for TIF2 coactivator LXXLL motif binding, but not for the binding of the AR NH$_2$-terminal FXXLF motif (14). Glutamic acid 897 in helix 12 of the ligand binding domain is required for both TIF2 LXXLL motif binding and the N/C interactions (14). AR-FXXLF/WXXLF-K720A did not decrease AR-mediated transactivation of the PSA61-Luc reporter in the absence of TIF2 expression but blocked coactivation by TIF2 (Fig. 4B). AR-FXXLF/WXXLF-E897K reduced inherent AR activity and blocked coactivation by TIF2. Furthermore, AR transactivation was not stimulated by TIF2 when AR had either of these AF2 mutations and where the N/C interaction was interrupted by mutations in the FXXLF and WXXLF motifs. The results indicate that TIF2 coactivation of AR requires binding to AF2.

Androgen Dependence of the WXXLF Motif Interaction with AF2—Previously we used a GST affinity matrix assay to determine the effect of androgen on the interaction of the FXXLF and WXXLF motifs with the ligand binding domain. We demonstrated an androgen-dependent interaction of the FXXLF motif with AF2, but results with the WXXLF motif were inconclusive (14). Extensive binding in the absence of androgen of a 233-amino acid AR-GST fusion peptide containing the WXXLF motif raised the possibility that some of the binding was nonspecific (14). We therefore prepared a fusion peptide with a shorter sequence containing the WXXLF motif. GST-AR-(412-460) bound 3-fold higher levels of $^{35}$S-labeled AR ligand binding domain residues 624–919 in the presence than in the absence of DHT (Fig. 5A, lanes 4 and 5). In addition, mutation of residues LF to AA in the WXXLF motif in GST-AR-(412-460)/WXXAA eliminated the androgen-dependent interaction (Fig. 5A, lanes 6 and 7). The results indicate that the WXXLF motif binds the AR ligand binding domain in an androgen-dependent manner.

The predicted amphipathic $\alpha$-helical structure of the WXXLF sequence and the hormone dependence of its interaction with the ligand binding domain suggested that the WXXLF motif interacts with the AF2 binding surface. We tested a series of AF2 mutants that were shown previously to decrease or eliminate TIF2 LXXLL motif binding (4, 5, 10) and the binding of the AR FXXLF motif in the N/C interaction (10, 14). AR AF2 mutations E897K, V716R and K720A introduced into the $^{35}$S-labeled ligand binding domain each reduced or eliminated...
androgen-dependent binding of the WXXLF motif to the AF2 region of the ligand binding domain using GST affinity matrix binding and mammalian two-hybrid peptide interaction assays. A, the GST fusion protein (GST-AR) lacking the AR sequence (0) or GST-AR-(412–460) with wild-type WXXLF or WXXAA mutant sequence, were incubated as described under “Experimental Procedures” with 35S-labeled AR ligand binding domain residues 624–919 in the presence and absence of 1 μM DHT. 35S-labeled AR-(624–919) had wild-type sequence (WT) or contained AF2 mutations E897K, V716R, or K720A as indicated. 10% of the input radioactivity used in the binding reactions is shown in lanes 1, 8, 13, and 18 (I). Migration of 35S-labeled AR-(624–919) is indicated by an arrow. B, peptide interaction assays were determined in HeLa cells transfected as described under “Experimental Procedures” without the GAL4 vector (-), or with GAL-0 lacking AR sequence (0), GAL-AR-(16–36) containing AR amino acid residues 16–36 with the FXXLF motif, or GAL-AR-(426–444) containing AR amino acid residues 426–444 with the WXXLF motif. Cells were cotransfected with pCMvAR that had mutations in the FXXLF and WXXLF motifs (AR-FXXAA/AXXXAA) in the absence or presence of AF2 mutations K720A or E897K as indicated. Incubations were performed for 24 h in the absence and presence of 10 nM DHT. Luciferase activity is representative of at least three independent experiments.

To confirm that AF2 in the AR ligand binding domain is the binding site for the WXXLF motif, we used a peptide two-hybrid interaction assay previously described in HeLa cells (15). GAL4-DNA binding domain fusion protein GAL-AR-(426–444) was constructed to contain the GAL4 DNA binding domain and 19 amino acids of the WXXLF region. GAL-AR-(426–444) was coexpressed with AR-FXXAA/AXXXAA, an AR mutant in which LF in both NH2-terminal binding motifs and Trp in the second motif were changed to Ala to eliminate the FXXLF- and WXXLF-mediated interdomain N/C interaction. GAL-AR-(426–444) bound AR-FXXAA/AXXXAA in the presence of androgen, with an increase in luciferase activity greater than that observed with AR-FXXAA/AXXXAA alone (Fig. 5B). The extent of interaction of the GAL-AR-(426–444) WXXLF peptide was less than that observed with GAL-AR-(16–36), a fusion protein containing the 21-amino acid region of the FXXLF motif (Fig. 5B).

To further substantiate the androgen-dependent binding of the WXXLF motif to AF2, we used a peptide two-hybrid interaction assay previously described in HeLa cells (15). GAL4-DNA binding domain fusion protein GAL-AR-(426–444) was constructed to contain the GAL4 DNA binding domain and 19 amino acids of the WXXLF region. GAL-AR-(426–444) was coexpressed with AR-FXXAA/AXXXAA, an AR mutant in which LF in both NH2-terminal binding motifs and Trp in the second motif were changed to Ala to eliminate the FXXLF- and WXXLF-mediated interdomain N/C interaction. GAL-AR-(426–444) bound AR-FXXAA/AXXXAA in the presence of androgen, with an increase in luciferase activity greater than that observed with AR-FXXAA/AXXXAA alone (Fig. 5B). The extent of interaction of the GAL-AR-(426–444) WXXLF peptide was less than that observed with GAL-AR-(16–36), a fusion protein containing the 21-amino acid region of the FXXLF motif (Fig. 5B).

To confirm that AF2 in the AR ligand binding domain is the binding site for the WXXLF motif, the two AF2 mutants described above, AR-FXXAA/AXXXAA-K720A and AR-FXXAA/
AXXAA-E897K, were tested by peptide interaction. Binding of the two peptides containing the WXXLF (GAL-AR-(426–444)) or FXXLF (GAL-AR-(16–36)) motifs was compared. AR-FXXAA/AXXXA-K720A showed little interaction with GAL-AR-(426–444) above background levels in the presence of DHT (Fig. 5B). In contrast, GAL-AR-(16–36) containing the FXXLF motif bound AR-FXXAA/AXXXA-K720A as indicated by the increase in luciferase activity. Interaction with AR-FXXAA/AXXXA-E897K was near background levels for both the FXXLF and WXXLF fusion peptides (Fig. 5B). In studies not shown, GAL-AR-(426–444) containing the WXXLF motif interacted as well with wild-type AR as it did with AR-FXXAA/AXXXA. However, it did not interact with AR-V716R, an additional AF2 mutant in which the NH₂-terminal binding motifs were not modified. None of the AF2 mutations caused a change in the apparent equilibrium androgen binding affinity (10).

The results indicate androgen-dependent binding of the WXXLF motif to the AF2 region of the ligand binding domain that is weaker than that observed with the FXXLF motif. The greater dependence of the FXXLF sequence on lysine 720 suggests slight differences in the AF2 binding surface for the FXXLF and WXXLF sequences, where the binding site for WXXLF more closely resembles the binding site requirements for the TIF2 LXXLF motif (10).

**DISCUSSION**

Here we report that enhancer/promoter regions derived from the PSA and probasin genes require the androgen-induced N/C interaction for effective AR-mediated transactivation. This contrasts the enhancer/promoter region of MMTV that was relatively nonselective with regard to the presence of the N/C interaction for transactivation by AR. The dependence of the enhancer/promoter regions of the PSA and probasin genes on the N/C interaction allowed us to demonstrate, in addition, that in the absence of androgen, the WXXLF motif has a significant but more minor role than the FXXLF motif in mediating the N/C interaction and in inhibiting the recruitment of TIF2 to AF2. Unlike the FXXLF motif, WXXLF was not required for the androgen-induced N/C interaction, consistent with earlier results that mutating WXXLF alone did not decrease the ligand dissociation half-time (14). The WXXLF motif is weaker in its apparent affinity for AF2 and in its ability to inhibit TIF2 binding. The predicted amphipathic α-helix and androgen dependence of the WXXLF motif interaction with the ligand binding domain suggested its interaction with AF2. GST affinity matrix and two-hybrid peptide interaction assays confirmed this. The role of the WXXLF motif in inhibiting AR transactivation was evident when mutations in WXXLF increased AR transactivation of the PSA and probasin enhancer/promoters. However, in a deletion mutant lacking the NH₂-terminal AF1 region, the WXXLF mutant did not overcome the predominant inhibitory effect of the FXXLF motif on TIF2 recruitment by AF2.

Homology comparisons support the relative functional importance of the FXXLF and WXXLF binding motifs. FQNL/F is highly conserved among mammals (24–28) (Fig. 6), paralleling its predominant role in the N/C interaction and in modulating AF2 accessibility to p160 coactivators. In Japanese eel ARα and goldfish AR, tyrosine (Y) replaces phenylalanine (F) in position 1 of the motif, and in these and rainbow trout AR α and β, valine (V) replaces leucine (L). These conservative substitutions preserve the predicted amphipathic α-helical structure that characterizes a binding motif for AF2. It is not known, however, whether an N/C interaction occurs in the AR of these fish species. In agreement with its less prominent role in the N/C interaction, WHTLF is conserved in mammals and Xenopus, but in fish is less conserved than FQNL/F (Fig. 6). Tryptophan (W) is conserved throughout but there is significant sequence deviation at other positions such that an amphipathic α-helix is not predicted for the WXXLF motif region in fish AR.

Our previous studies of ligand dissociation rate, two-hybrid interactions and GST affinity matrix binding suggest an androgen-induced N/C interaction selectively induced by biologically active androgens (8–10). Moreover, we observed that certain mutations that cause the androgen insensitivity syndrome disrupt the N/C interaction without significantly altering androgen binding affinity (9, 10). Nevertheless we lacked evidence for the functional importance of the N/C interaction in that the MMTV-luciferase reporter was influenced by but did not require the N/C interaction for activation (10, 14). The critical role for the N/C interaction in AR transactivation of the androgen responsive PSA and probasin enhancer/promoters reported here shows a greater dependence on the N/C interac-
tion than was required for transactivation of the MMTV and pGL\(\Delta\phi\) promoters. The increase in transactivation mediated by a GR chimera with an artificially introduced N/C interaction demonstrated further the striking stimulatory effect of an N/C interaction on transactivation of the PSA and probasin enhancer/promoter regions.

Under normal physiological conditions, AR transactivation appears to be mediated by the NH\(_2\)-terminal AF1 region between amino acid residues 142–337. Deletion of AF1 results in essentially complete loss of AR transactivation of the androgen responsive enhancer/promoters tested in this study. The increase in transactivation that results from mutating WXXLF in the absence or presence of TIF2 coexpression requires AF1 and is also inhibited by mutations in AF2. The results suggest that the increased transactivation by WXXLF mutants reflects a synergistic effect between AF1 and AF2 brought about by exposing AF2 to activation by endogenous coactivators such as TIF2. In agreement with this, the WXXLF motif binds AF2 and inhibits AF2 mediated transactivation by blocking the binding of TIF2, albeit to a less extent than does the FXXLF motif. The data support that the N/C interaction that is mediated by binding of both the FXXLF and WXXLF motifs to AF2 reduces p160 coactivator interaction at this site. Inhibition of AF2 coactivation by the androgen-induced N/C interaction renders AF1 the predominant activation domain in AR unless coactivator expression levels exceed the capacity of the N/C interaction to block coactivator binding.

Several lines of evidence support the predominant role of AF1 in AR transactivation. The AF2 mutant AR-K720A that blocks TIF2 binding to AF2 (10) does not decrease AR-mediated transactivation except in response to TIF2 overexpression. The agonist-induced N/C interaction mediated by the FXXLF and WXXLF motifs results in competitive inhibition of p160 coactivator binding at AF2. AF2 preferentially binds the FXXLF sequence compared with the LXXXL sequences of the p160 coactivators (15). Under normal physiological conditions, p160 coactivator expression was relatively low in benign adult human prostate when compared with prostate cancer (29). It is clear that AR transactivation can be increased by TIF2 overexpression, which enables TIF2 LXXXL motifs to compete successfully for interaction with AF2. Thus in situations of high TIF2 expression such as in prostate cancer (29), AR transactivation may be mediated by both AF1 and AF2. We (10) and others (30, 31) have reported that TIF2 and other p160 coactivators interact with the AR NH\(_2\)-terminal domain. In the present study using the PSA and probasin enhancer/promoters, mutations in AF2 abolished TIF2 stimulation of AR transactivation. Thus interaction of overexpressed TIF2 with the AR NH\(_2\)-terminal domain was secondary to TIF2 binding to AF2. In support of this, TIF2m123, which has mutations in the LXXXL motifs, was inactive in the presence and absence of the N/C interaction mediated by the PSA, probasin, MMTV, and pGC\(\Delta\phi\)-Luc reporters, indicating that TIF2 interaction with AF2 is required for AR coactivation. The results with TIF2 contrast previous reports that GRIP1 and SRC1e interactions with the AR NH\(_2\)-terminal domain are sufficient for coactivation of AR transactivation (30, 31). The differences suggest that p160 coactivators may use different mechanisms to increase AR-mediated gene activation.

It is not clear what features of the enhancer/promoter regions require the N/C interaction in AR transactivation. A viral promoter such as MMTV lacks receptor specificity and is activated by the AR, GR, and the progesterone receptor (32, 33). Other enhancer/promoters show varying degrees of specificity for AR transactivation. In our studies, the MMTV, PSA, and probasin luciferase reporters were activated by AR and GR, whereas there was little GR transactivation of pGL\(\Delta\phi\). It is well established that GR stimulates the MMTV promoter (21–23), whereas the probasin (34, 35) and pGL\(\Delta\phi\) (20) enhancer/promoters are reported to be selectively activated by AR. It was the PSA, probasin and pGL\(\Delta\phi\) enhancer/promoters that showed increased transactivation by the GR chimera with an imposed N/C interaction. At this time there are no evident predictive features that differentiate these enhancer/promoter androgen response elements in terms of their sensitivity to the N/C interaction. We expect that the differences in requiring a receptor N/C interaction relate to the sequence or organization of the response elements. Enhancer/promoters of androgen-regulated genes such as PSA and probasin that have not been attributed to ancient viral insertions tend to have more widely spaced response elements compared with viral-derived promoters. An antiparallel AR dimer mediated by the N/C interaction in the AR dimer could promote folding of genomic DNA in a way that brings response elements into close proximity and provide synergy between response elements through this and mechanisms that remain to be established.

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References

1. Spencer, T. E., Jenat xr, G., Barcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) Nature 389, 194–198
2. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) Nature 387, 733–736
3. Darmont, B. D., Wagner, R. L., Apriyetti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) Genes Dev. 12, 3343–3356
4. Noble, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Wilson, T. M., Glass, C. K., and Milburn, M. V. (1998) Nature 395, 137–143
5. Shiao, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) Cell 95, 927–937
6. Wang, C. I., Zhou, Z. X., Sar, M., and Wilson, E. M. (1993) J. Biol. Chem. 268, 19004–19012
7. Zhou, Z. X., Lane, M. V., Kempainen, J. A., French, P. S., and Wilson, E. M. (1995) J. Biol. Chem. 270, 29893–29990
8. Langley, E., Zhou, Z. X., and Wilson, E. M. (1995) J. Biol. Chem. 267, 29893–29990
9. Langley, E., Kempainen, J. A., and Wilson, E. M. (1998) J. Biol. Chem. 273, 92–101
10. He, B., Kempainen, J. A., Voege, J. J., Gronemeyer, H., and Wilson, E. M. (1999) J. Biol. Chem. 274, 37219–37225
11. Kraus, W. L., McNerney, E. M., and Katzenellenbogen, B. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12314–12318
12. Tetel, M. J., Giangrande, P. H., Leonhardt, S. A., McDonnell, D. P., and Edwards, D. P. (1999) Mol. Endocrinol. 13, 910–924
13. He, B., Bowen, N. T., Minges, J. T., and Wilson, E. M. (2001) J. Biol. Chem. 276, 42293–42301
14. He, B., Kempainen, J. A., and Wilson, E. M. (2000) J. Biol. Chem. 275, 22896–22904
15. He, B., Minges, J. T., Lee, L. W., and Wilson, E. M. (2002) J. Biol. Chem. 277, 10226–10235
16. Cleutjens, K. B. J. M., van Eekelen, C. C. E. M., van der Korput, H. A. G. M., Brinkmann, A. O., and Trapman, J. (1996) J. Biol. Chem. 271, 6379–6388
17. Cleutjens, K. B. J. M., van der Korput, H. A. G. M., Ehren-van Eekelen, C. C., Sikors, R. A., Fasancia, C., Chang, L. W., and Trapman, J. (1997) Mol. Endocrinol. 11, 1259–1265
18. Rennie, P. S., Bruchovsky, N., Leco, K. J., Sheppard, P. C., McQueen, S. A., Cheng, H., Snook, R., Hamel, A., Bock, M. E., MacDonald, B. S., Nickel, B. E., Chang, C., Liao, S., Cattini, P. A., and Matusiak, R. J. (1995) Mol. Endocrinol. 7, 23–36
19. Lorenzi, P., Stavenhagen, J., Kalff, M., and Robins, D. M. (1988) Mol. Cell. Biochem. 8, 235–2360
20. Scheller, A., Scheinman, R. I., Thompson, E., Scarlett, C. O., and Robins, D. M. (1996) Mol. Cell. Endocrinol. 121, 75–86
21. Chalepakis, G., Postma, J. P. M., and Beato, M. (1988) Nucleic Acids Res. 16, 10237–10247
22. Guarna, W. H., and Salmons, B. (1992) Biochem. J. 283, 625–632
23. Cordingley, M. G., Riegel, A. T., and Hager, G. L. (1987) Cell 48, 261–270
24. Chouson, C. S., Kempainen, J. A., and Wilson, E. M. (1998) J. Mol. Endocrinol. 20, 334–342
25. Tan, J. A., Joseph, D. R., Quarmby, V. E., Lubahn, D. B., Sar, M., French, P. S., and Wilson, E. M. (1988) Mol. Endocrinol. 2, 1276–1285
26. Chang, C. S., Kolomits, J., and Liao, S. T. (1998) Proc. Natl. Acad. Sci. U. S. A.
27. Charest, N. J., Zhou, Z. X., Lubahn, D. B., Olsen, K. L., Wilson, E. M., and French, F. S. (1991) Mol. Endocrinol. 5, 573–581
28. He, W. W., Fischer, L. M., Sun, S., Bilhartz, D. L., Zhu, X. P., Young, C. Y., Kelley, D. B., and Tindall, D. J. (1990) Biochem. Biophys. Res. Commun. 171, 697–704
29. Gregory, C. W., He, B., Johnson, R. T., Ford, O. H., Mohler, J. L., French, F. S., and Wilson, E. M. (2001) Cancer Res. 61, 4315–4319
30. Alen, P., Claessens, F., Verhoeven, G., Rombauts, W., and Peeters, B. (1999) Mol. Cell. Biol. 19, 6085–6097
31. Bevan, C. L., Hoare, S., Claessens, F., Heery, D. M., and Parker, M. G. (1999) Mol. Cell. Biol. 19, 8383–8392
32. Cato, A. C., Henderson, D., and Ponta, H. (1987) EMBO J. 6, 363–368
33. Otten, A. D., Sanders, M. M., and McKnight, G. S. (1988) Mol. Endocrinol. 2, 143–147
34. Greenberg, N. M., DeMayo, F. J., Sheppard, P. C., Barrios, R., Lebovitz, M., Finegold, M., Angelopoulos, R., Dodd, J. G., Duckworth, M. L., Rosen, J. M., and Matusik, R. J. (1994) Mol. Endocrinol. 8, 230–239
35. Kasper, S., Rennie, P. S., Bruchovsky, N., Sheppard, P. C., Cheng, H., Lin, L., Shiu, R. P. C., Snoek, R., and Matusik, R. J. (1994) J. Biol. Chem. 269, 31763–31769
36. Corpet, F. (1988) Nucleic Acids Res. 16, 10881–10890
37. Lubahn, D. B., Joseph, D. R., Sar, M., Tan, J. A., Higgs, H. N., Larson, R. E., French, P. S., and Wilson, E. M. (1988) Mol. Endocrinol. 2, 1265–1275
38. Fischer, L. M., Catz, D., and Kelley, D. B. (1995) Dev. Biol. 170, 115–126
39. Taken, J., and Yamashita, S. (1999) J. Biol. Chem. 274, 5674–5680
40. Todo, T., Ikeuchi, T., Kobayashi, T., and Nagahama, Y. (1999) Biochem. Biophys. Res. Commun. 254, 378–383
41. Touhata, K., Kinoshita, M., Tokuda, Y., Toyohara, H., Sakaguchi, M., Yokoyama, Y., and Yamashita, S. (1999) Biochim. Biophys. Acta 1450, 481–485