The FXXLF Motif Mediates Androgen Receptor-specific Interactions with Coregulators*

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The androgen receptor (AR) activation function 2 region of the ligand binding domain binds the LXXLL motifs of p160 coactivators weakly, engaging instead in an androgen-dependent, interdomain interaction with an FXXLF motif in the AR NH2 terminus. Here we show that FXXLF motifs are present in previously reported AR coactivators ARA70/RFG, ARA55/Hic-5, and ARA54, which account for their selection in yeast two-hybrid screens. Mammalian two-hybrid assays, ligand dissociation rate studies, and glutathione S-transferase adsorption assays indicate androgen-dependent selective interactions of these FXXLF motifs with the AR ligand binding domain. Mutagenesis of residues within activation function 2 indicates distinct but overlapping binding sites where specificity depends on sequences within and flanking the FXXLF motif. Mutagenesis of the FXXLF motifs eliminated interaction with the ligand binding domain but only modestly reduced AR coactivation in transcription assays. The studies indicate that the FXXLF binding motif is specific for the AR and mediates interactions both within the AR and with coregulatory proteins.

The androgen receptor (AR) belongs to the steroid receptor subfamily of hormone-dependent nuclear receptor transcriptional regulators. Recent studies have established general mechanisms of steroid hormone receptor transcriptional activation. Binding of cognate ligands induces a conformational change in the ligand binding domain which results in formation of a novel hydrophobic interaction surface referred to as activation function 2 (AF2). AF2 recruits LXXLL motif-containing p160 coactivator complexes that have histone acetyltransferase activity (1), resulting in modification of local chromatin structure to facilitate transcription initiation (2). It is believed that interaction between the p160 coactivator LXXLL motifs and the AF2 surface in the ligand binding domain is required for transactivation of nuclear receptors (3–6). We (7) and others (8–14) have reported that the AR and other nuclear receptors also interact with p160 coactivators through their NH2-terminal regions.

The ligand binding domain of some nuclear receptors is also involved in a ligand-dependent, NH2-terminal/carboxyl-terminal (N/C) interaction, shown for the AR (15), estrogen receptor α (ERα) (16), and progesterone receptor (17). For AR, the androgen-induced N/C interaction slows the androgen dissociation rate (7, 18–20), prolongs the AR half-life in the presence of androgen (19, 21), and reduces AF2-mediated transcriptional activity (19). The AR N/C interaction is direct and involves interactions between FXXLF and WXXLF motifs in the NH2-terminal domain with the AF2 binding surface in the ligand binding domain (7, 18, 19). In contrast, the ERα N/C interaction may be indirect, mediated by TIF2 or p300/CBP (22, 23). The crystal structure of the AR ligand binding domain reveals an overall structural arrangement similar to other steroid receptors (24, 25) with subtle changes that seem to favor the N/C interaction. Under normal physiological conditions, AF2 binding of the NH2-terminal FXXLF motif is favored over binding the LXXLL motifs of p160 coactivators, which likely contributes to the weak AR AF2 transcriptional activity in mammalian cells (7, 18, 19). The AR AF2 region nevertheless interacts with the LXXLL motifs of p160 coactivators when these coactivators are overexpressed (7). We recently proposed such a mechanism to account for the recurrent growth of prostate cancer under conditions of androgen deprivation (26). In the present report we investigated the role of the FXXLF motif in androgen-dependent AR interactions with previously reported AR coactivators.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions—**Coding sequences for FXXLF and LXXLL motif-containing peptides were cloned in pGAL0 (15), which expresses the Saccharomyces cerevisiae GALA DNA binding domain amino acid residues 1–147 expressed NH2-terminal to the peptide sequences. GALA peptide fusion plasmids were created using two complementary oligonucleotides that were phosphorylated by T4 polynucleotide kinase, denatured, annealed at room temperature, and cloned at XbaI/NdeI in pGAL0. Oligonucleotides coding for the FXXLF or LXXLL motifs typically contained coding sequence for 7 additional flanking amino acids, with peptides ranging in size from 11 to 21 amino acids.

AR-FXXXA (pCMVhAR-L26AF27A) had 23FQNL27 changed to 23FQNA27, and AR-FXXXA/WXXA (pCMVhAR-L26AF27A/L46A/F437A) had the additional mutation of 433WHTAA437 changed to 433WHTTA437 (18, 19). VPAR507–919, pVP16-ERα-LBD (ERα amino acid residues 312–595), VP16-FRA, and the 5XGAL4Luc3 reporter were gifts from Donald P. McDonnell, Duke University. VPAR507–919 (AR DNA and ligand binding domains) and pVP16-ERα-LBD contained the herpes simplex virus VP16 transactivation domain residues 411–456. pCMVhAR-K720A, pCMVhAR-E897 K, and pCMVhAR-V716R are full-length AR expression vectors with single mutations in
AF2 of the ligand binding domain (7), pCMVhAR-(507–919) codes for the human AR DNA and ligand binding domains (27). pCMVhAR-(424–337) and pCMVhAR-(124–337) were constructed by amplifying the indicated DNA regions using PCR, and the inserts were cloned into pGEX-3X (Amerham Biosciences, Inc.) at EcoRI/BamHI. pcDNA3HA-AR-LBD for in vitro translation expressing human AR ligand binding domain residues 624–919 was created by digesting GAL-AR-(624–919) with BamHI/XbaI and the insert cloned at the same sites in pcDNA3HA. pSG5-HA-AR-(1-244) (clone CS0DI083YK17) and FHL2 (clone CS0DK007YN06) (Invitrogen) had coding insertions cloned at SalI/NotI. pSG5-HA-AR54 was constructed by amplifying the coding region of AR54 pCMV-AR57-AR54 with the insert cloned at BstXI/EcoRI and the media replaced with serum-free medium lacking phenol red. Beads were washed five times with the sonication buffer and incubated for 2 h at 4 °C with and without 1 μM dihydrotestosterone (DHT). In vitro translated proteins were labeled with 25 μCi of [35S]methionine (PerkinElmer Life Sciences) using the TNT T7 Quick Coupled Transcription/Translation System (Promega) in the presence and absence of 10 μM MgCl2. Beads were washed five times with 50 mM Tris, pH 8.0, centrifuged, and the supernatant was incubated with glutathione-agarose beads (Amerham Biosciences, Inc.) for 1 h at 4 °C.

**Mammalian Two-hybrid Assays—**GST fusion proteins were expressed in XL1-Blue Escherichia coli cells treated with 0.5 mM isopropyl β-D-thiogalactopyranoside for 3 h after log phase growth. Bacteria were sonicated in 0.5% Nonident P-40, 1 mM EDTA, 0.1 mM NaCl, 0.02 mM Tris-HCl, pH 8.0, centrifuged, and the supernatant was incubated with glutathione-agarose beads (Amerham Biosciences, Inc.) for 1 h at 4 °C.

**Transcription Assays—**Human hepatocellular carcinoma HeLa cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and 2 mM l-glutamine and penicillin/streptomycin. HeLa cells (3 X 104 cells/well) were transfected using Effectene as described above except using 0.25 μg each of VPAR507–919, GALA-peptide, and 5XGAL4Luc, 150 μl of EC buffer/plate, and 4 μl each of enhancer and Effectene plate in 1 ml of media were added to plates containing 3 ml of fresh media. After 24 h cells were washed in phosphate-buffered saline, and 4 ml of serum-free medium lacking phenol red was added per plate. Cells were incubated for 24 h in the absence or presence of the indicated hormones and assayed for luciferase activity as described above except using 0.5 ml of lysis buffer/plate.

**In Vivo Binding Assays—**GST fusion proteins were expressed in XL1-Blue Escherichia coli cells treated with 0.5 mM isopropyl β-D-thiogalactopyranoside for 3 h after log phase growth. Bacteria were sonicated in 0.5% Nonident P-40, 1 mM EDTA, 0.1 mM NaCl, 0.02 mM Tris-HCl, pH 8.0, centrifuged, and the supernatant was incubated with glutathione-agarose beads (Amerham Biosciences, Inc.) for 1 h at 4 °C.

**Immunoblot Analysis—**Expression levels of the GALA-peptide fusion proteins were determined by immunoblot analysis. COS-1 cells (1.2 X 106 cells/10-cm dish) were transfected using the Effectene kit protocol. On the 2nd day, 8 ml of medium was added to each well, and the DNA was suspended in 0.3 ml of EC buffer (Qiagen). The DNA for each plate was combined with 16 μl of enhancer and 10 μl of Effectene reagent and added to the cells in 1 ml of medium. Peptide detection by immunoblot was increased by the addition of 1 μl MG132 (20 μg/ml, pepstatin A, 24 h prior to cell harvest. Addition of MG132 did not significantly alter the interaction assay results. Nuclear extracts of transfected COS cells were prepared as described previously (30). Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as standard. Proteins (10 μg) were separated on 12% acrylamide gels containing SDS, and the GALA-peptide fusion proteins were detected using the anti-GALA-DNA binding domain monoclonal antibody (Santa Cruz Biotechnology, Inc.).
their selection in two-hybrid screens where the AR ligand binding domain was used as probe. A search of the sequences revealed that ARA70 (31, 32), ARA55 (33, 34), and ARA54 (35) each contain one (ARA70 and ARA54) or two (ARA55) FXXLF motifs. In each case the FXXLF sequences are in regions selected previously by yeast two-hybrid screens. Regions of the recruited fragments (underlined) and the position and sequence of the FXXLF motif regions are shown in Fig. 1.

Interaction of the AR coactivator FXXLF motifs with the AR ligand binding domain was tested in the absence and presence of 10 nM DHT using two-hybrid peptide assays (29) in HeLa or HepG2 cells with GAL4-peptide fusions. Expression of the GAL4-peptide fusions was verified on immunoblots of nuclear extracts from cells treated for 24 h with MG132, a proteosome inhibitor (Fig. 2A). Results are shown for COS cell expression, extracts from cells treated for 24 h with MG132, a proteosome inhibitor. Androgen-dependent interactions were detected which increased luciferase activity by 8- to 31-fold in the two-hybrid assay (Fig. 2B). The results indicate a requirement for the last Ser in QERAS is Gly in the human sequence.

FIG. 1. Schematic diagram of AR and reported AR coregulator proteins and amino acid sequence of the FXXLF motif regions. Shown schematically are full-length AR (amino acid residues 1–919) and previously reported AR coactivators ARA70-(1–614), ARA54-(1–474), and ARA55-(1–444). Shown in the AR diagram is the DNA binding domain (DBD) and ligand binding domain (LBD). The dotted underlined regions highlight fragment regions cloned previously in yeast two-hybrid screens using the AR ligand binding domain as probe (31–35). The dark rectangles indicate the positions of the FXXLF motif sequences. Below are shown amino acid sequences of the FXXLF motif regions for AR and the coregulators. The GAL4-DNA binding domain fusion peptides contained the sequences shown. The AR peptide sequences tested in the two-hybrid assays were AR16–36 with QN at the carboxyl terminus or only residues 20–30. The carboxyl-terminal FXXLF peptide GAL-ARA55-(427–444) included QERAS at the NH2-terminal end. GAL-ARA70-(321–340) had a carboxyl-terminal Trp residue. The last Ser in QERAS is Gly in the human sequence.

FIG. 2. Immunoblot and GAL-FXXLF fusion peptide interactions. Panel A, GAL-FXXLF peptides were expressed in COS cells in the presence of 1 μg MG132 using Effectene reagent as described under "Experimental Procedures." Nuclear extracts (10 μg of protein) were analyzed, and blots were exposed to GAL4 DNA binding domain antibody. Shown are the banding patterns after expressing the empty vector GAL0 and the GAL-peptide fusions indicated. Panel B, GAL-peptide fusions (0.25 μg of DNA) were expressed in HeLa cells with 0.25 μg of VPAR507–919 (AR DNA and ligand binding domain) and 0.25 μg of 5XGAL4Luc3 reporter vector using Effectene as described under "Experimental Procedures." Cells were incubated for 24 h in the absence and presence of 10 nM DHT. Shown is a representative experiment of at least three independent determinations for GAL0 empty vector and GAL-AR-(16–36), GAL-AR-(16–36)-FXAA (ARA16–36AA), GAL-AR-(20–30) (ARA20–30), GAL-ARA54-(447–465) (ARA54), GAL-ARA70-(321–340) (ARA70), GAL-D11-FXXLF (D11Fx), GAL-ARA55-(314–332) (ARA55–1), GAL-ARA55-(427–444) (ARA55–2), GAL-TIPF2 (683–701) (TIPF2–2), and GAL-TIPF2 (738–756) (TIPF2–3). Fold induction relative to the luciferase activity determined in the absence of DHT is shown above the bars. Panel C, GAL-peptide DNA (0.05 μg) was expressed in HepG2 cells in the absence and presence of 10 nM DHT with 0.05 μg of pCMVhAR and 0.1 μg of 5XGAL4Luc3 reporter as described under "Experimental Procedures." The abbreviations are the same as described above. Fold induction relative to activity determined in the absence of DHT is shown above the bars. The data are representative of at least three independent experiments.
with AF2. No interaction was observed in the two-hybrid peptide assay using either of the two GAL-ARA55-FXXLF peptides, providing further evidence that sequences within and flanking the FXXLF motif influence the extent of binding. Similarly, no luciferase activity resulted from any of the GAL-peptides alone because there was no transcriptional activity detected in the absence of androgen (see Figs. 2, B and C, and 3). In a positive control, we observed an 84-fold DHT-dependent interaction with a GAL-D11-FXXLF peptide (D11FX). The D11 peptide contains an LXXLL motif that interacts with AR (29), which we changed to conform to an FXXLF sequence derived from ARA55, ARA54, and ARA70, interaction previously (36). Interaction was also detected between VPAR507–919 and the third LXXLL motif sequence of TIF2 but not with the second LXXLL motif (Fig. 2B).

To investigate further the interactions of the FXXLF motif sequences, the two-hybrid assay was performed in HepG2 cells using full-length AR instead of the DNA and ligand binding domain fragment. Each of the GAL4-FXXLF peptides with sequence derived from AR, ARA70, ARA55, and the D11-FXXLF peptide interacted with the AR, with an increase in luciferase activity of 35–228-fold (Fig. 2C). No interaction or background activity was observed using AR and the empty GAL0 vector control or when the GAL-AR-(164–474)–L457A/F458A vector (AAXAA) showed ACE activity to the level seen with empty vector GAL0 (Fig. 3). There was high background transcriptional activity induced by full-length AR in the HeLa cell two-hybrid protein interaction assay using the 5XGAL4Luc reporter (Fig. 3) which was not detected in HepG2 cells (see Fig. 2C). This result from a cryptic androgen response element because similar background activity was observed in HeLa cells using the parent reporter vector pGL3-basic luciferase (data not shown).

In Vitro Interactions—GST adsorption assays were performed to investigate further the androgen-dependent interactions between the FXXLF motif-containing fragments and the AR ligand binding domain. With each of the GST-FXXLF sequences derived from ARA55, ARA54, and ARA70, interaction with 35S-labeled AR624–919 containing the AR ligand binding domain increased in the presence of androgen (Fig. 4, lanes 3–10). The FXXLF sequence was required for the interaction because mutation to AXXAA eliminated interaction, as shown for ARA70 (Fig. 4, lanes 9–12). Similar results in mammalian two-hybrid assays indicate the requirement for the FXXLF motif in the AR NH2-terminal motif interaction (see Fig. 2, B and C). On the other hand, the FXXLF motif sequence FGSLF in the transcriptional regulators p300 (26–44) (37) and CBP (23–41) (38), and FETL in FHL2 (25–43) (39), failed to interact in mammalian two-hybrid and GST adsorption assays using the AR ligand binding domain or full-length AR (data not shown). The results provide further evidence that sequences within and flanking the FXXLF motifs influence the androgendependent interaction with the AR ligand binding domain. In support of this, mutation of the flanking sequence of the ARA70 FXXLF motif from FKFLLF to APALLF abolished binding to the AR ligand binding domain (32). The LXXLL motif region of TIF2 also interacts with the AR ligand binding domain in GST adsorption assays (7). Two-hybrid results suggest that this is primarily the result of the third LXXLL motif of TIF2 (see Fig. 2, B and C).
diolabeled synthetic androgen, and that this effect is dependent on the NH2-terminal FXXLF motif (18, 20, 21). The dissociation half-time of [3H]R1881 of 111 ± 10 min for wild-type AR determined at 37°C increased to 45 and 32 min, respectively, when the AR NH2-terminal FXXLF motif, or both the FXXLF and WXXLF motifs, were mutated (Fig. 5) as reported previously (18, 19). We created coactivator-AR chimeras in which the AR FXXLF motif region was deleted, and the AR WXXLF motif was mutated to eliminate the inherent AR N/C interaction (see Fig. 5). The effectiveness of this assay in demonstrating domain interactions was shown previously using p160 coactivator-glucocorticoid receptor chimeras containing the LXXLL motif region of TIF2, where the ligand dissociation half-time increased 5–6-fold (19).

When the 171 AR NH2-terminal amino acid region was replaced by ARA54 residues 398–474 containing its FXXLF motif (chimera ARA54-(398–474)/AR-(172–919–919–38)), the half-time of [3H]R1881 dissociation was 84 ± 7 min compared with 111 ± 10 min for wild-type AR and 45 ± 1 and 32 ± 3 min for AR-FXXLF and AR-WXXLF, respectively (Fig. 5). This dissociation half-time provides evidence in support of the an interaction between the ARA54 FXXLF motif region and the AR ligand binding domain. In contrast, chimeras containing the FXXLF regions from ARA70 (ARA70-(320–407)/AR-(172–919–919–38); t1/2 39 ± 1), ARA55 (ARA55-(281–361)/AR-(172–919–919–38) and ARA55-(389–444)/AR-(172–919–919–38); t1/2 37–38 ± 3–5 min), or a construct containing both FXXLF motifs of ARA55 (ARA55-(281–444)/AR-(172–919–919–38); t1/2 35 ± 1 min), were less effective in slowing the androgen dissociation half-time (Fig. 5). Indeed, the multiple LXXLL motif region of TIF2 (TIF2-(632–780)/AR-(172–919–919–38); t1/2 53 ± 2 min) was more effective in slowing the androgen dissociation half-time than were the FXXLF regions of ARA70 or ARA55, but less effective than the FXXLF motif-containing region of ARA54. Inserting the D11-FXXLF peptide sequence at the position of the AR NH2-terminal FXXLF motif resulted in an androgen dissociation half-time of 120 ± 17 min, which is indistinguishable from that of wild-type AR (Fig. 5). The results are consistent with the two-hybrid assay results above for the ARA54-FXXLF sequence and indicate weaker interactions with the corresponding regions of ARA70 and ARA55.

Sequence Requirements of the AR AF2 Binding Site—We tested whether certain amino acid residues in the AR AF2 region of the ligand binding domain were required for interaction with the FXXLF motifs of ARA54, ARA55, and ARA70. The residues tested included lysine 720, whose mutation disrupts AF2 interaction with TIF2, and glutamic acid 897 and valine 716, mutation of which disrupts, in addition, the AR N/C interaction (7). None of these AR AF2 mutations alters the apparent equilibrium binding affinity for [3H]R1881 (7). Wild-type and mutant AR expression vectors were cotransfected with the GAL4-FXXLF and LXXLL peptides in mammalian peptide two-hybrid interaction assays. There was a striking loss of interaction of the ARA54-FXXLF peptide with AR-K720A. In contrast, interaction of K720A remained robust with the AR NH2-terminal FXXLF sequence and with the FXXLF peptides from ARA70 and D11 (Fig. 6A). No interaction was observed between the AR NH2-terminal FXXLF motif and AR-E897K as reported previously (7) and comparatively weaker interactions with FXXLF peptides from ARA54, ARA70, and D11-FXXLF (Fig. 6A). AR-V716R abolished all FXXLF peptide interactions. Thus, glutamic acid 897 and valine 716 are critical in the AF2 binding surface for interaction with a variety of FXXLF motif sequences. The requirement for lysine 720 for the FXXLF sequence from ARA54 and the LXXLL sequence of TIF2, but not the FXXLF sequences of AR or ARA55 or ARA70, suggests subtle differences in the AF2 binding surface for FXXLF and LXXLL motif binding.

Receptor Specificity for FXXLF Motif Binding—We next determined whether the AR ligand binding domain selectively binds the FXXLF motif compared with other steroid receptors. None of the FXXLF peptide sequences derived from AR, ARA54, or ARA70 interacted to a significant extent with the glucocorticoid receptor, the A form of the progesterone receptor (VP16-PR-A), or with the ERα ligand binding domain (VP-ERα-LBD) (Fig. 6B). In striking contrast, the third LXXLL motif of TIF2 between residues 738 and 756 interacted strongly with the glucocorticoid and progesterone receptors, with luciferase activity increasing 92- and 277-fold, respectively, with essentially no interaction detected with the second LXXLL motif of TIF2 (Fig. 6B). For ERα, it was the second LXXLL motif of TIF2 positioned between TIF2 residues 653 and 701 which interacted strongly with the ERα ligand binding domain, increasing luciferase activity 388-fold. A much weaker interaction was detected between the ERα ligand binding domain and the third LXXLL motif of TIF2 (36-fold, Fig. 6B), in agreement with previous reports (13, 41). As evident in Fig. 6B, little or no background transcriptional activity was detected in HepG2 cells for the AR, glucocorticoid receptor, and ERα interaction assays using the 5XGAL4Luc reporter vector and the empty GAL-DNA binding domain vector, GAL0. In contrast, 30–50-fold background transcriptional activity was apparent for VP16PR-A cotransfected with the GAL0 empty vector or the GAL-peptides (Fig. 6B). The results demonstrate a high degree
of selectivity among a group of related FXXLF and LXXLL sequences for steroid receptor binding. The AF2 region of the AR ligand binding domain preferentially binds the FXXLF motif, whereas other steroid receptors interact more strongly with the LXXLL motifs.

**Role of the FXXLF Motifs in Coactivation of AR-mediated Gene Transcription**—Recruitment and transcriptional activation by nuclear receptors and p160 coactivators depend on interactions between AF2 in the ligand binding domain and LXXLL motifs of p160 coactivators (3–6). Using the mouse mammary tumor virus luciferase reporter vector, we investigated the requirement for the FXXLF motifs in coactivator stimulation of AR transcriptional activation. There were only modest increases in AR-mediated transcriptional activity with the coexpression of ARA54, ARA55, or ARA70 when the transcriptional response was compared with controls that lacked the addition of empty vector DNA (Fig. 7A). When equivalent amounts of empty expression vector (pSG5) were added with the AR expression vector and mouse mammary tumor virus luciferase to balance the DNA of the coactivators, there was inhibition of the transcriptional response compared with that determined in the absence of control DNA (Fig. 7A). Transcriptional inhibition caused by empty vector DNA therefore resulted in a greater apparent stimulation of AR-mediated transcriptional activity than was observed when empty vector DNA was omitted in the negative control. Mutating FXXLF to FXXAA in ARA54 and ARA55 had relatively little effect on the transcriptional response (Fig. 7A). The use of two expression vectors for ARA54 (pSG5 for HA-ARA54 and pCMV for ARA54) demonstrated a different overall response, but in each case there was no decrease in luciferase activity detected by introducing a mutation in the FXXLF motif sequence (Fig. 7A). The results suggest that a direct influence of these AR coregulatory proteins on the transcriptional response does not depend on interaction through the FXXLF binding motifs.

We investigated whether the observed decrease in transcriptional response caused by the addition of empty vector DNA was nonspecific. Empty vector and coregulator plasmids were expressed in the presence of the constitutively active luciferase reporters pSV2-Luc, pAR, RSV450-Luc, and pSG5-Luc. We observed inhibition of transcription with the addition of pSG5 empty vector DNA (Fig. 7B) for each constitutively active reporter to an extent similar to the inhibition of AR-mediated activity seen in Fig. 7A. Inhibition was also observed with ARA55, but expression of ARA54, ARA70, or TIF2 had little influence on transcriptional activation. Based on these results,
Fig. 7. Effect of interacting proteins on AR transcriptional activity.

Panel A, AR transactivation was determined in CV1 cells by transfecting 0.1 μg of pCMVhAR without or with 1 μg of pSG5 parent vector DNA or 1 μg of pSG5-expressed coactivators HA-ARA54 (with HA NH2-terminal epitope tag), HA-ARA54-FFXXAA (HA-ARA54AA), ARA54, ARA54-FFXXAA (ARA54AA), ARA55, ARA55-FFXXAA (ARA55AA), ARA55-(FFXXAA)1 (ARA55AA1), ARA55-(FFXXAA)2 (ARA55AA2), ARA55-(FFXXAA/FFXXAA) (ARA55AA), TIF2, TIF2-(LXXAA) (TIF2AA), and ARA70 as described under “Experimental Procedures.” Cells were incubated for 48 h in the absence and presence of 0.1 nM DHT.

Panel B, constitutive transactivation of pSV2-Luc, pA3RSV400Luc, and pSG5-Luc was determined in CV1 cells by transfecting 5 μg of reporter vectors without or with 1 μg of pSG5 empty vector or pSG5-expressed putative coactivators ARA55 (55), HA-ARA54 (H54), ARA55 (54), ARA70 (70), and TIF2 (TIF). Similar transcriptional inhibition was observed with 0.7 μg of pSG5 empty vector DNA rather than 1 μg, where 0.7 μg was the molar DNA equivalent used for the coactivators.

Panel C, transcriptional activity of AR and AR mutants AR507–919 (DNA and ligand binding domains), AR142–337FFXXAA (deletion of AF1 transactivation domain and mutation of the FFXXLF motif), and AR142–337 (deletion of the AF1 NH2-terminal transactivation domain). AR and AR mutant expression vector DNAs (100 ng/6-cm dish) were transfected in the absence and presence of 1 μg of pSG5 empty vector DNA (pS5 or p) or pSG5-expressed TIF2 (TIF or T), ARA54 (54), ARA55 (55), and ARA70 (70). Cells were incubated for 48 h in the absence and presence of 0.1 nM DHT except for AR507–919, which was 10 nM DHT. In panels A–C, data shown are representative of at least three independent experiments with fold induction shown above the bars relative to activity determined in the absence of DHT.
pSG5 empty vector may be a valid control for ARA55, but AR alone without the addition of empty vector DNA appears to be the appropriate control for ARA54, ARA70, and TIF2 expression vectors. The results raise the possibility that the effects of some AR coregulators on AR-mediated transcription have been increased artificially by relating the androgen-induced activity to the empty vector control.

**Effects of Coactivators on AF2 Activity**—We determined the influence of the AR coregulators on AR AF2 activity using several AR deletion mutants. Overexpression of TIF2 strongly stimulates the AF2 activity of AR507–919, an AR DNA and ligand binding domain fragment (Fig. 7C). In striking contrast, ARA70, ARA54, and ARA55 each failed to increase the androgen-dependent AF2 activity of AR507–919 (Fig. 7C) despite the presence of the FXXLF binding sequences in these AR coregulators. TIF2 was the only coactivator tested which increased the transcriptional activity of AR507–919 and ARA142–337-FXXAA. The latter AR mutant lacks the NH2-terminal AF1 residues 142–337 and has the NH2-terminal FXXLF motif mutated to FXXAA (L264A/F27A) (Fig. 7C). Mutation of the FXXLF motif allows access to the AF2 region, which is otherwise occupied in the androgen-induced N/C interaction (19). None of the coregulators, including TIF2, competed for the N/C interaction to increase transactivation by ARA142–337 (Fig. 7C). Thus in contrast to classical coactivator activity, the reported AR coactivators ARA54, ARA55, and ARA70 do not stimulate AR AF2 activity even though they are recruited to the AR ligand binding domain through their FXXLF motifs.

**DISCUSSION**

This report identifies FXXLF motifs in three previously reported AR coactivators, which provides a molecular explanation for their cloning in yeast two-hybrid screens using the AR ligand binding domain as a probe. FXXLF motifs in ARA54, ARA55, and ARA70 interact to different degrees with the AF2 binding surface of the AR ligand binding domain. Interaction of ARA55 was detected in GST affinity assays but only weakly in mammalian two-hybrid assays. Protein interaction and ligand dissociation rate studies indicated that the ARA54 FXXLF motif binds the AR ligand binding domain to an extent somewhat weaker than the FXXLF motif in the AR NH2-terminal region. Interaction of the ARA70 FXXLF motif was intermediate between ARA55 and ARA54. The studies revealed only modest increases in AR transcriptional activity by the AR coregulators, with no transcriptional activity detected through modest increases in AR transcriptional activity by the AR ligand binding domain fragment AR507–919. This contrasted the increase in transcriptional activity induced by TIF2, a p160 coactivator with weaker interacting LXXLL motifs. The results indicate that the AR coregulators ARA55, ARA54, and ARA70 likely function through mechanisms that differ from those of the p160 coactivators.

Biochemical (3–6, 42) and x-ray crystallographic (43–45) evidence established the recruitment of p160 coactivators by steroid receptors as critical for transcription initiation. For most steroid receptors, agonist-induced interactions occur between the AF2 hydrophobic surface in the ligand binding domain and the LXXLL motifs of p160 coactivators such as SRC1 and TIF2. However, the AR AF2 region in the ligand binding domain preferentially binds FXXLF sequences, most notably the 23QNLRF27 sequence in the AR NH2 terminus which mediates the androgen-induced N/C interaction (7, 15, 18, 20). One of the functional consequences of the N/C interaction is to inhibit the interaction of the LXXLL motif regions of the p160 coactivators with the same AF2 region in the AR ligand binding domain (19). The FXXLF motif sequences in this group of previously reported AR coregulators are located in regions that were originally cloned in yeast two-hybrid screens using the AR ligand binding domain as a probe (31–35). GST adorption and peptide mammalian two-hybrid assays indicate that the FXXLF sequences interact with the AR ligand binding domain in an agonist-dependent manner.

Data in this report show that FXXLF motifs interact selectively with the AR ligand binding domain. This is based on observations that none of the FXXLF peptide sequences in the putative AR coactivators or in the AR NH2-terminal region interact with ERα, glucocorticoid, and progesterone receptors. In contrast, the LXXLL sequences of the p160 coactivator TIF2 interact strongly with ERα and the glucocorticoid and progesterone receptors, but only weakly with AR. AR selectivity for the FXXLF motif sequences is supported by a recent report in which only one LXXLL motif sequence (D11) interacted with the AR in a phage display screen of an LXXLL consensus peptide library using the ERα ligand binding domain (29). The FXXLF motif was also favored by the AR ligand binding domain when the D11-LXXLL sequence was mutated to FXXLF. Chapman et al. (36) mutated peptide D11 LMQLL to FMQLF, increasing by almost 4-fold its interaction with the AR ligand binding domain and decreasing its interaction with the glucocorticoid receptor. Similarly, when the carboxyl-terminal LQQL of SRC1 was changed to FQQL, interaction with the AR ligand binding domain increased from 2- to 378-fold, whereas interaction with GR decreased from 450- to 75-fold (36). Together the results provide strong evidence that the FXXLF motif interacts preferentially with the AR ligand binding domain.

Many proteins have sequences that conform to the FXXLF motif. The results of the present report indicate that the sequence determinants for interaction with the AR ligand binding domain lie within and flanking the FXXLF motif. For example, FXXLF sequences present in two members of the basal transcriptional machinery, TAFII250 (214FQNLRF252) and TFIIEc (232FEDLFK439), interacted only weakly with a 4–6-fold increase in luciferase activity in two-hybrid peptide assays with the AR (data not shown). Similarly no interaction was observed between AR and the FXXLF motif peptides derived from the general transcription coactivators CBP (235FGLSLF41) and p300 (29FGSLF44) or from the reported AR coactivator FHLL2 (25FETLF45). Clearly the FXXLF motif alone is not sufficient to predict interaction with the AR ligand binding domain.

Interacting FXXLF motifs likely form amphipathic α-helical structures as reported for the LXXLL motifs of p160 coactivators (3). This is supported by the failure of the CBP and p300 FXXLF motifs to interact with AR even though they are positioned near the coactivator NH2 terminus. The FXXLF motif sequence present in CBP and p300 (FGSLF) contains the α-helix-disrupting amino acid glycine. Interaction of the FXXLF sequence of the putative AR coactivator FHLL2/DRAL, positioned near its NH2 terminus, was also not detected even though a previous report indicated that FHLL2/DRAL increases AR activity in a N/C interaction-dependent manner (46). Thus sequences within and flanking the FXXLF motif contribute to the specificity of interaction with the AR ligand binding domain, where the precise sequence requirements remain to be established. Other coactivators reported to interact with the AR ligand binding domain lack the FXXLF motif. These include Zac-1 (47), hsp40/dnaJ (48), and HBO1 (49), suggesting a different mechanism of interaction. An LXXLL motif sequence is present in Zac-1 but not in hsp40/dnaJ or HBO1. Putative AR coactivators reported to interact with the AR NH2-terminal or DNA binding domains that also lack FXXLF motif sequences include AR4A (50), ARA160 (51), SNURF (52), ANPK (53), Ubc9 (54), ARIP3/PIASxα (55), Rb (56), and Pias1 (57).
The presence of FXXLF interacting motifs in the reported AR coactivators ARA54, ARA55, and ARA70 raises the question of whether these sequences function in vivo in their reported roles as coactivators. We showed previously that interaction of the AR NH2-terminal FXXLF motif with the AF2 region of the ligand binding domain in the androgen-induced N/C interaction suppresses AR interaction with p160 coactivators (19). The weak interacting LXXLL motifs of p160 coactivators apparently did not compete for the AF2 binding surface of the AR ligand binding domain unless the coactivator was overexpressed. In contrast, the relative binding activities of the FXXLF sequences from ARA54 and ARA70 in two-hybrid peptide interaction assays suggest higher affinity interactions that might be sufficient to compete with the AR NH2-terminal FXXLF sequence in the presence of the androgen-induced N/C interaction. Ligand dissociation kinetic studies of the coactivator-AR chimera support the interaction of the ARA54 FXXLF motif sequence but not that of ARA70. However, none of the coactivators, including ARA54, ARA55, and ARA70, contained strong transactivation domains like that of TIF2 as evidenced by their failure to induce transactivation of the AR DNA and ligand binding domain fragment.

The lack of strong transactivation by ARA54, ARA55, and ARA70 raises the possibility that these coregulators function through other mechanisms not directly related to AR transcriptional activity. ARA54 was recently shown to be a RING finger protein with ubiquitin ligase activity, although coexpression of ARA54 did not influence AR degradation (58). Because RING finger proteins transfer ubiquitin to themselves and other proteins (59), it remains to be established whether this activity of ARA54 contributes to AR function. ARA54/His-5 was cloned from a human prostate cDNA library by two-hybrid screening with the LNCap mutant AR (AR-T877A) (33) and from a mouse embryo library using mouse glucocorticoid receptor amino acids 513–562 as probe (34). ARA55 contains three LIM motifs each consisting of a double zinc finger. Mouse ARA55/His-5 localized in focal cell-cell adhesions and nuclear matrix (34) where it may transmit signals from cell attachment sites to regulate transcription factors such as steroid receptors. ARA55/His-5 was also cloned as an hsp27-binding protein (60), consistent with the proposed function of LIM domain proteins as protein interaction molecules. We reported earlier that ARA70 interacts with the AR ligand binding domain and NH2-terminal region (32) where the latter was independent of the FXXLF motif. The mechanism of action of ARA70 remains to be established.

Competition for protein-protein interaction sites is a potential mechanism whereby the AF2 region regulates AR activity because the AF2 site is occupied by the N/C interaction in the presence of androgen. Competition for protein interaction sites in a domain swapping model has been proposed in the activation of Hck, a nonreceptor tyrosine kinase of the Src family (61, 62). Unlike the AR, which undergoes an androgen-dependent interdomain N/C interaction in its active state, Hck kinase is maintained in an inactive state by intramolecular interactions between an NH2-terminal SH3 domain and a linker region, and between an SH2 domain and a carboxy-terminal phosphoryrosine (63). Competing proteins with similar interacting motifs of the Hck kinase are human immunodeficiency virus Nef, a high affinity ligand for Hck which has an SH3 domain, and platelet-derived growth factor receptor, which has a phosphoryrosine. Each competes for the interdomain interactions of Hck to activate the kinase (62, 64). The AR-associated proteins in this study may be part of a larger group of proteins that contain FXXLF motifs with sufficiently high affinity to compete and interact with AF2 by interdomain competition.

Tissue-specific protein expression or altered cell homeostasis may influence the availability of the AR AF2 region to transcriptional activation. A majority of recurrent prostate cancer specimens express levels of SRC1 and TIF2 significantly greater than the levels detected in benign hyperplastic prostate tissue and androgen-dependent prostate cancer (26). This raised the possibility that AR is inappropriately activated through AF2 by overexpressed p160 coactivators in the presence of low circulating androgen in the androgen-deprived prostate cancer patient. In the presence of suppressed testicular androgen, interaction with p160 coactivators remains ligand-dependent, but AF2 is more accessible to activation by overexpressed p160 coactivators because adrenal androgens are less effective in inducing the N/C interaction (19). Specific AR amino acid mutations that occur infrequently in prostate cancer may also contribute to the reactivity of the AR AF2 surface. The AR N/C interaction site overlaps but is not identical to the LXXLL binding site. Lysine 720 is required for interaction with p160 coactivator LXXLL motif binding (7) and for the FXXLF binding motif of ARA54, but not for the N/C interaction. A somatic mutation of lysine 720 to glutamic acid occurred in a bone metastases of hormone refractory prostate cancer (65), which might influence the interaction specificity of the AF2 region. The AR-K720E mutant was reported to retain a normal transcriptional response to androgen (65) typical of several prostate cancer AR mutants (66) but could potentially present an altered interacting surface for additional coactivator binding.

A potentially important observation of the present study is the apparent artificial inhibition of transactivation resulting from cotransfection of empty expression vector DNA. The addition of balancing DNA is pervasive in the steroid receptor field to account for transfected DNA of the putative coactivator under study. Although there is no clear molecular explanation for the apparent transcriptional inhibition, it is important because it renders apparent stimulatory activity to a cotransfected protein which might otherwise not be observed. Inhibition by transfection of expression vector DNA that lacks protein coding sequence could cause squelching of transcription factor activity or inhibit receptor expression levels as suggested recently (40).

The results indicate that the FXXLF motif is a common mediator of androgen-dependent interactions selective for the AR ligand binding domain. The FXXLF motif was originally reported in the AR NH2-terminal domain to mediate the androgen-dependent N/C interaction. Interdomain competition may occur in a temporal sequence of FXXLF motif binding of other proteins. The selectivity of FXXLF motif binding to the AR indicates a role for flanking sequence in establishing specificity. The AR-interacting proteins studied in this report had modest effects on AR transcriptional activation, suggesting that they function through other mechanisms in regulating AR function.

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