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The F*XX*LF 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 L*XX*LL motifs of p160 coactivators weakly, engaging instead in an androgen-dependent, interdomain interaction with an F*XX*LF motif in the AR NH_2 terminus. Here we show that F*XX*LF 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 F*XX*LF 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 F*XX*LF motif. Mutagenesis of the F*XX*LF motifs eliminated interaction with the ligand binding domain but only modestly reduced AR coactivation in transcription assays. The studies indicate that the F*XX*LF binding motif is specific for the AR and mediates interactions both within the AR and with coregulatory proteins.

The androgen receptor (AR)[1] 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 L*XX*LL 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 L*XX*LL 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 NH_2-terminal regions.

The ligand binding domain of some nuclear receptors is also involved in a ligand-dependent, NH_2-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 F*XX*LF and W*XX*LF motifs in the NH_2-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 NH_2-terminal F*XX*LF motif is favored over binding the L*XX*LL 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 L*XX*LL 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 F*XX*LF motif in androgen-dependent AR interactions with previously reported AR coactivators.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Coding sequences for F*XX*LF and L*XX*LL motif-containing peptides were cloned in pGAL0 (15), which expresses the *Saccharomyces cerevisiae* GAL4 DNA binding domain amino acid residues 1–147 expressed NH_2-terminal to the peptide sequences. GAL4 peptide fusion plasmids were created using two complementary oligonucleotides that were phosphorylated by T4 polynucleotide kinase, denatured, annealed at room temperature, and cloned at *Nde*I/*Xba*I in pGAL0. Oligonucleotides coding for the F*XX*LF or L*XX*LL motifs typically contained coding sequence for 7 additional flanking amino acids, with peptides ranging in size from 11 to 21 amino acids.

AR-F*XX*AA (pCMVhAR-L26A/F27A) had ^{23}FQNLF^{27} changed to ^{23}FQNAA^{27}, and AR-F*XX*AA/W*XX*AA (pCMVhAR-L26A/F27A/L436A/F437A) had the additional mutation of ^{433}WHTLF^{437} changed to ^{433}WHTAA^{437} (18, 19). VPAR507–919, pVP16-ERα-LBD (ERα amino acid residues 312–595), VP16-PR-A, and the 5XGAL4Luc3 reporter were gifts from Donald P. McDonnell, Duke University. VPAR-(507–919) (AR DNA and ligand binding domains) and pVP16-ERα-LBD contained the *herpes simplex* virus VP16 transactivation domain residues 411–456. pCMVhAR-K720A, pCMVhAR-E897K, and pCMVhAR-V716R are full-length AR expression vectors with single mutations in

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[1] The abbreviations used are: AR, androgen receptor; AF2, activation function 2; N/C, NH_2-terminal/carboxyl-terminal; ERα, estrogen receptor α; CBP, cAMP response element binding protein-binding protein; GST, glutathione *S*-transferase; Luc, luciferase; DHT, dihydrotestosterone; R1881, methyltrienolone.

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AF2 of the ligand binding domain (7). pCMVhAR-(507–919) codes for the human AR DNA and ligand binding domains (27). pCMVhARΔ142–337 and pCMVhARΔ142–337L26A/F27A (ARΔ142–337F*XX*AA) have the AF1 region deleted in the NH_2-terminal domain (19, 28).

Glutathione *S*-transferase (GST)-ARA54-(432–474), GST-ARA70-(320–407), GST-ARA55-(405–444), and GST-ARA55-(301–340) were constructed by amplifying the indicated DNA regions using PCR, and the inserts were cloned into pGEX-3X (Amersham Biosciences, Inc.) at *Eco*RI/*Bam*HI. 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 *Bam*HI/*Xba*I and the insert cloned at the same sites in pcDNA3HA.

pSG5-HAmHic5 (pSG5-HA-ARA55) was a gift from Michael R. Stallcup, University of Southern California, Los Angeles. pCMV-sport6-ARA54 (clone CS0DI083YK17) and FHL2 (clone CS0DK007YN06) (Invitrogen) had coding inserts cloned at *Sal*I/*Not*I. pSG5-HA-ARA54 was constructed by amplifying the coding region of ARA54 from pCMV-sport6-ARA54 using PCR, with the 1.4-kb insert cloned in pSG5-HA at *Eco*RI/*Xho*I. The 5′-primer had an *Eco*RI site, and the 3′-primer had an *Xho*I site. DNA amplification using PCR was used to create pSG5-HA-ARA54-L457A/F458A with the insert cloned at *Hin*dIII/*Bam*HI and pSG5-HA-ARA55-L442A/F443A with the insert cloned at *Bst*EII/*Xho*I. The mutagenesis strategy was a two-step PCR to create pSG5-HA-ARA55-L325A/F326A with the insert cloned at *Bst*EII/*Xho*I. pSG5-HA-ARA55-L325A/F326A/L442A/F443A was created by amplifying pSG5-HA-ARA55-L325A/F326A with the insert cloned in pSG5-HA-ARA55-L442A/F443A at *Bst*EII/*Xho*I. ARA70-(320–407)-AR-(172–919)-A*XX*AA (where A*XX*AA is human AR mutation W433A/L436A/F437A), ARA54-(398–474)-AR-(172–919)-A*XX*AA, ARA55-(281–361)-AR-(172–919)-A*XX*AA, ARA55-(269–444)-AR-(172–919)-A*XX*AA, and ARA55-(281–444)-AR-(172–919)-A*XX*AA were constructed by DNA amplification using PCR of the indicated regions and cloning the inserts at *Bgl*II/*Afl*II of AR-W433A/L436A/F437A (19). Constitutively active luciferase reporter vectors included pSG5-Luc (from Kurt Hoffman and Walter Heyns, Catholic University of Leuven, Belgium), pSV2-Luc (from P. Kay Lund, University of North Carolina at Chapel Hill), and $pA_3RSV_{400}Luc$ (from Arthur Gutierrez-Hartman, University of Colorado, Denver). In D11-F*XX*LF-AR, a D11 peptide sequence (29) was modified to replace the L*XX*LL motif with F*XX*LF (see Fig. 1) and constructed to replace AR amino acid residues 16–34 by DNA amplification of pCMVhAR using a 5′-*Bgl*II-D11 oligonucleotide and a 3′-oligonucleotide at the AR *Afl*II site. The fragment was inserted into pCMVhAR digested with *Bgl*II/*Afl*II and reamplified using a 5′-oligonucleotide containing a *Bgl*II site and coding sequence for AR NH_2-terminal 1–15 amino acids and part of the D11 peptide coding sequence and the 3′-*Afl*II oligonucleotide. The resulting fragment was reinserted into the *Bgl*II/*Afl*II site of pCMVhAR. GAL-ARA70-(321–499), GAL-ARA70-(321–499)-F328A/L331A/F332A (GAL-ARA70-(321–499)-A*XX*AA), GAL-ARA54-(361–474), and GAL-ARA54-(361–474)-L457A/F458A (GAL-ARA54-(361–474)-F*XX*AA) were constructed by DNA amplification of the indicated regions using PCR and the inserts cloned into GAL0 at *Nde*I/*Sac*I. GAL-ARA55-(251–444) and GAL-ARA55-(251–444)-L325A/F326A/L442A/F443A (GAL-ARA55-(251–444)$(F\mathit{XX}AA)_2$) were constructed by amplifying the indicated regions using PCR, and the inserts were cloned into GAL0 at *Nde*I/*Xba*I. Amplification of plasmid DNA by PCR was done using Vent-polymerase, and all amplified regions were verified by DNA sequencing.

Mammalian Two-hybrid Assays—Human hepatocellular carcinoma HepG2 cells (American Type Culture Collection) were maintained in 5% CO_2 at 37 °C in Eagle's minimum essential medium (Invitrogen) containing 10% fetal bovine serum (Hyclone), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and penicillin/streptomycin. For peptide interaction assays, HepG2 cells were plated at 2×10^5 cells/well of 12-multiwell tissue culture plates. The next day medium was exchanged with 0.8 ml of fresh culture medium. DNA for transfection was prepared for 4 wells each following the Effectene protocol (Qiagen). Wild-type or mutant pCMVhAR, pCMVhGR, VP16PRA, or VP16-ERα-LBD (0.05 μg) or VPAR507–919 (0.15 μg) was combined with 0.05 μg of GAL4-peptide DNA/well and 0.1 μg of 5XGAL4Luc3/well in 75 μl of EC buffer/well, 1 μl of enhancer/well, 1 μl of Effectene/well, and 0.4 ml of media/well, and 400 μl was added to the plates. The next day cells were washed in phosphate-buffered saline and the media replaced with serum-free medium lacking phenol red. Hormones were added as indicated, and cells were incubated overnight at 37 °C. The next day cells were washed with phosphate-buffered saline and harvested in 220 μl of lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100), and 0.1 ml was analyzed using a Monolight 2010 (Analytical Luminescence Laboratories) or LumiStar Galaxy (BMG Labtechnologies) luminometer.

Human epithelioid cervical 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 (3.5×10^5 cells/6-cm dish) were transfected using Effectene as described above except using 0.25 μg each of VPAR507–919, GAL4-peptide, and 5XGAL4Luc3, 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 Vitro 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% Nonidet P-40, 1 mM EDTA, 0.1 M NaCl, 0.02 M Tris-HCl, pH 8.0, centrifuged, and the supernatant was incubated with glutathione-agarose beads (Amersham Biosciences, Inc.) for 1 h at 4 °C (7). 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 [^{35}S]methionine (PerkinElmer Life Sciences) using the TNT T7 Quick Coupled Transcription/Translation System (Promega) in the presence and absence of 1 μM DHT. Beads were centrifuged, washed five times, and boiled in SDS. Input lanes contained ~10% of that used for the binding reactions.

Transcription Assays—Transient transcriptional activity of wild-type and mutant AR was determined in the presence and absence of cotransfecting expression vectors for the putative AR coactivators. Monkey kidney CV1 cells were transiently transfected using calcium phosphate precipitation (19). Wild-type and mutant pCMVhAR DNA (100 ng/6-cm dish) were precipitated with 5 μg of mouse mammary tumor virus luciferase reporter vector DNA. Cells were incubated for 48 h in the absence and presence of the indicated hormones, harvested, and assayed for luciferase activity as described above.

Ligand Dissociation Assays—Dissociation half-times of [^{3}H]R1881 (methyltrienolone, 70–87 Ci/mmol, PerkinElmer Life Sciences) from AR were determined at 37 °C in monkey kidney COS-1 cells using 4×10^5 cells/well in 6-well plates transfected with 2 μg of wild-type or mutant pCMVhAR DNA/well using DEAE-dextran (19). 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. Radiolabeled ligand dissociation was started by the addition of 50 μM unlabeled R1881 and the cells incubated for increasing times at 37 °C up to 3 h, washed once, and harvested in 0.5 ml of 10 mM Tris, pH 6.8, 2% SDS, and 10% glycerol, with radioactivity determined by scintillation counting. In previous studies we reported overall slower ligand dissociation half-times for AR (18–21) caused by the actual temperature of the incubator being 35 °C rather than the 37 °C incubation during the dissociation phase of the experiments.

Immunoblot Analysis—Relative expression levels of the GAL4-peptide fusion proteins were determined by immunoblot analysis. COS-1 cells (1.2×10^6 cells/10-cm dish) were transfected using the Effectene kit protocol. On the 2nd day, 8 ml of medium was added to each plate, 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 μM MG132 (Sigma), a proteosome inhibitor, 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 GAL4-peptide fusion proteins were detected using the anti-GAL4-DNA binding domain monoclonal antibody (Santa Cruz Biotechnology, Inc.).

RESULTS

FXXLF Interacting Motifs in AR Coactivators—Previous studies in our laboratory showed that the AF2 region of the AR ligand binding domain binds preferentially in the presence of androgen an AR NH_2-terminal F*XX*LF motif (^{23}FQNLF^{27}) compared with the L*XX*LL motifs of the p160 coactivators (18, 19). We have now investigated whether a group of reported AR coactivators contain F*XX*LF motifs, which might account for

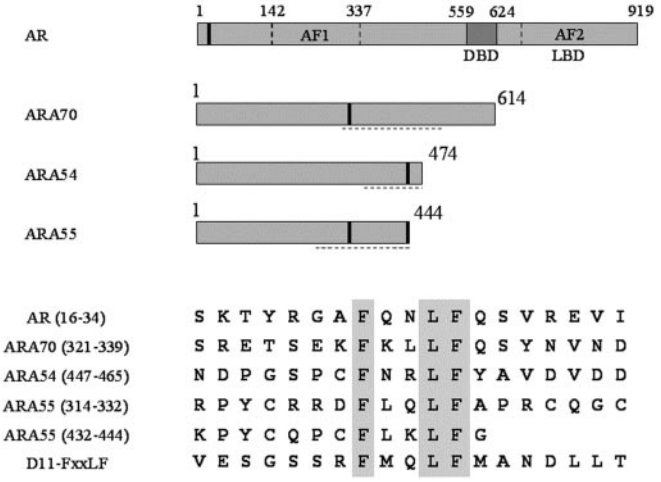

FIG. 1. **Schematic diagram of AR and reported AR coregulatory proteins and amino acid sequence of the F*XX*LF motif regions.** Shown schematically are full-length AR (amino acid residues 1–919) and previously reported AR coactivators ARA70-(1–614), ARA54-(1–474), and ARA55-(1–444). Shown in the AR diagram 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 F*XX*LF motif sequences. Below are shown amino acid sequences of the F*XX*LF 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 F*XX*LF peptide GAL-ARA55-(427–444) included QERAS at the NH_2-terminal end. GAL-ARA70-(321–340) had a carboxyl-terminal Trp residue. The last Ser in QERAS is Gly in the human sequence.

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) F*XX*LF motifs. In each case the F*XX*LF sequences are in regions selected previously by yeast two-hybrid screens. Regions of the recruited fragments (*underlined*) and the position and sequence of the F*XX*LF motif regions are shown in Fig. 1.

Interaction of the AR coactivator F*XX*LF 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. 2*A*). Results are shown for COS cell expression, but similar results were obtained using HeLa cells. Peptide detection in both cases required the addition of MG132, but treatment with MG132 did not alter the interaction results (data not shown). Androgen-dependent interactions were detected which increased luciferase activity by 8–48-fold in two-hybrid assays between VPAR507–919 (AR DNA and ligand binding domain) and the GAL4-F*XX*LF fusion peptides with sequences from AR (GAL-AR-(16–36)), ARA54 (GAL-ARA54-(447–465)), and ARA70 (GAL-ARA70-(321–340)) (Fig. 2*B*). Mutation of F*XX*LF to F*XX*AA in GAL-AR-(16–36) eliminated the interaction based on loss of luciferase activity in the two-hybrid assay (Fig. 2*B*). The results indicate a requirement for the F*XX*LF binding motif in the AR-interacting peptide. Decreasing the size of the AR F*XX*LF peptide sequence from residues 16–36 to 20–30 increased the magnitude of the interaction of the GAL4-AR-F*XX*LF peptide, with luciferase activity increasing from 8- to 31-fold in the two-hybrid assay (Fig. 2*B*). This result provided the first indication that amino acid residues flanking the AR F*XX*LF sequence influence the interaction

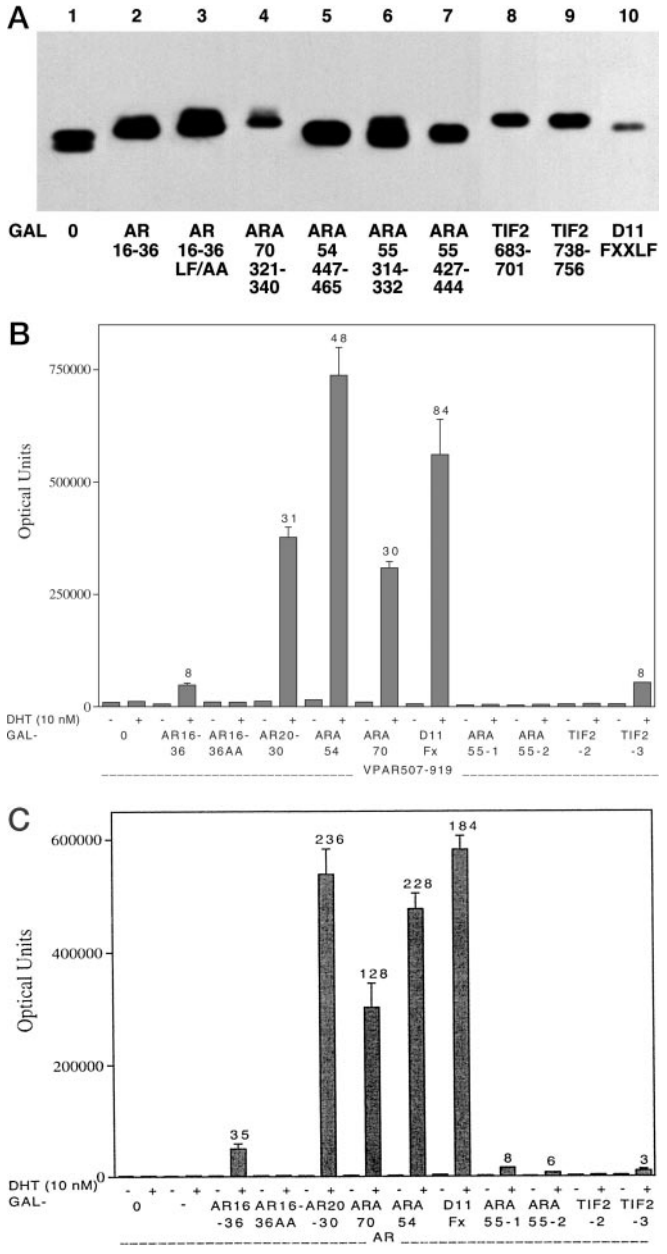

FIG. 2. **Immunoblot and GAL-F*XX*LF fusion peptide interactions.** *Panel A*, GAL-F*XX*LF peptides were expressed in COS cells in the presence of 1 μM MG132 using Effectene reagent as described under "Experimental Procedures." Nuclear extracts (10 μg of protein) were analyzed, and blots were exposed 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)-F*XX*AA (AR16–36AA), GAL-AR-(20–30) (AR20–30), GAL-ARA54-(447–465) (ARA54), GAL-ARA70-(321–340) (ARA70), GAL-D11-F*XX*LF (D11Fx), GAL-ARA55-(314–332) (ARA55–1), GAL-ARA55-(427–444) (ARA55–2), GAL-TIF2-(683–701) (TIF2–2), and GAL-TIF2-(738–756) (TIF2–3). 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.

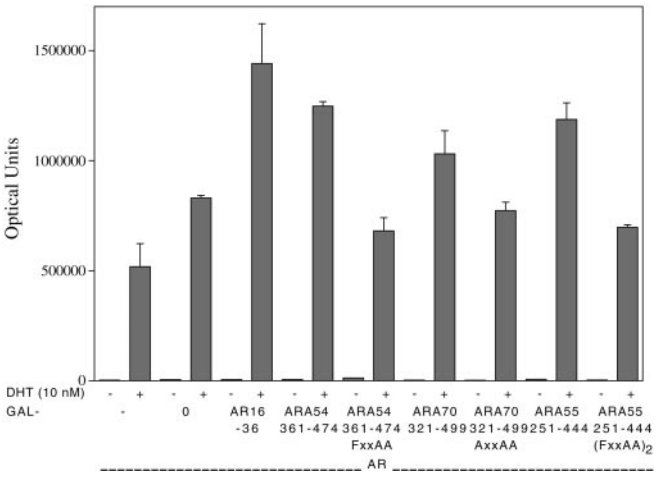

FIG. 3. **Interaction of coactivator fragments with AR.** HeLa cells were transfected using Effectene as described under "Experimental Procedures" with 0.15 μg of pCMVhAR, 0.25 μg of 5XGAL4Luc3 reporter vector either with no further addition (−) or with 0.15 μg of GAL0 empty vector or GAL-AR-(16–36), GAL-ARA54-(361–474), GAL-ARA54-(361–474)-L457A/F458A(F*XX*AA), GAL-ARA70-(321–499), GAL-ARA70-(321–499)-F328A/L331A/F332A (A*XX*AA), GAL-ARA55-(251–444), or GAL-ARA55-(251–444-L325A/F326A/L442A/F443A) (F*XX*AA$_2$). Cells were incubated in the absence and presence of 10 nM DHT. Data shown are representative of three independent experiments.

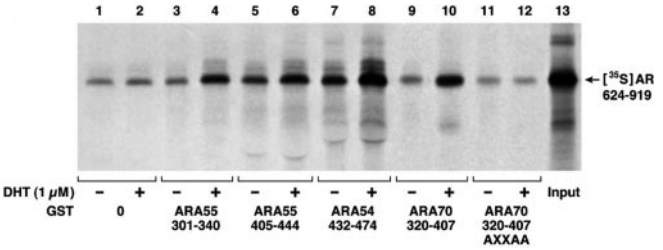

FIG. 4. **GST-peptide adsorption assays with ^{35}S-labeled AR ligand binding domain.** The GST-0 empty parent vector pGEX-3X or the GST fusion peptides indicated were expressed in *E. coli* as described under "Experimental Procedures" in the absence and presence of 1 μM DHT and ^{35}S-labeled AR-(624–919) expressing ligand binding domain residues 624–919. The input lane represents 10% of the total ^{35}S-labeled AR-(624–919) used in each sample.

with AF2. No interaction was observed in the two-hybrid peptide assay using either of the two GAL-ARA55-F*XX*LF peptides, providing further evidence that sequences within and flanking the F*XX*LF 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-F*XX*LF peptide (D11FX). The D11 peptide contains an L*XX*LL motif that interacts with AR (29), which we changed to conform to an F*XX*LF motif as described previously (36). Interaction was also detected between VPAR507–919 and the third L*XX*LL motif sequence of TIF2 but not with the second L*XX*LL motif (Fig. 2*B*).

To investigate further the interactions of the F*XX*LF 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-F*XX*LF peptides with sequence derived from AR, ARA70, ARA54, and the D11-F*XX*LF peptide interacted with the AR, with an increase in luciferase activity of 35–228-fold (Fig. 2*C*). No interaction or background activity was observed using AR and the empty GAL0 vector control or when the GAL-AR-(16–36)-F*XX*LF sequence was mutated to F*XX*AA (Fig. 2*C*). In this assay there were weaker interactions detected with the GAL-ARA55 fusion peptides containing the first or second F*XX*LF sequence. The third L*XX*LL motif of TIF2 in residues 738–756 increased luciferase activity in the two-hybrid assay by 3-fold, whereas again, no interaction was detected with the second L*XX*LL motif of TIF2 between residues 683 and 701 (Fig. 2*C*). The reporter vector used in these assays has five GAL4 binding sites in the promoter region. Although two-hybrid assays provide an indirect measurement of protein interactions, previous GST adsorption studies using mutants of the AF2 region in the AR ligand binding domain indicated a direct interaction with the F*XX*LF motif (19). The increase in luciferase activity thus depended on the recruitment of full-length AR by the GAL4-peptide fusions. The results raised the possibility that F*XX*LF sequences in ARA54 and ARA70, but probably not ARA55 or the L*XX*LL motifs of TIF2, interact sufficiently to compete for the androgen-dependent and specific N/C interaction (7, 15, 18–21). Alternatively, interactions may occur with the androgen-bound AR monomer, which may or may not undergo an intramolecular N/C interaction (20).

To demonstrate that the F*XX*LF motif sequences in the AR-interacting proteins accounted for their selection in previous yeast two-hybrid screens using the AR ligand binding domain as probe (31–35), the fragments shown *underlined* in Fig. 1 were expressed as fusion proteins with the GAL4 DNA binding domain in HeLa cells with full-length AR. Mutagenesis of the F*XX*LF motifs in the GAL fusion proteins of ARA54-(361–474), ARA70-(321–499), and ARA55-(251–444) reduced the overall luciferase 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. 2*C*). This resulted 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 F*XX*LF motif-containing fragments and the AR ligand binding domain. With each of the GST-F*XX*LF sequences derived from ARA55, ARA54, and ARA70, interaction with ^{35}S-labeled AR624–919 containing the AR ligand binding domain increased in the presence of androgen (Fig. 4, *lanes 3–10*). The F*XX*LF sequence was required for the interaction because mutation to A*XX*AA eliminated interaction, as shown for ARA70 (Fig. 4, *lanes 9–12*). Similar results in mammalian two-hybrid assays indicated the requirement for the F*XX*LF motif in the AR NH$_2$-terminal motif interaction (see Fig. 2, *B* and *C*). On the other hand, the F*XX*LF motif sequence FGSLF in the transcriptional regulators p300-(26–44) (37) and CBP-(23–41) (38), and FETLF 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 F*XX*LF motifs influence the androgen-dependent interaction with the AR ligand binding domain. In support of this, mutation of the flanking sequence of the ARA70 F*XX*LF motif from KFKLLF to AFALLF abolished binding to the AR ligand binding domain (32). The L*XX*LL 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 L*XX*LL motif of TIF2 (see Fig. 2, *B* and *C*).

Effects of Coactivator FXXLF Sequences on Androgen Dissociation Rate—Previously we showed that the AR N/C interaction slows the dissociation kinetics of bound [^{3}H]R1881, a ra-

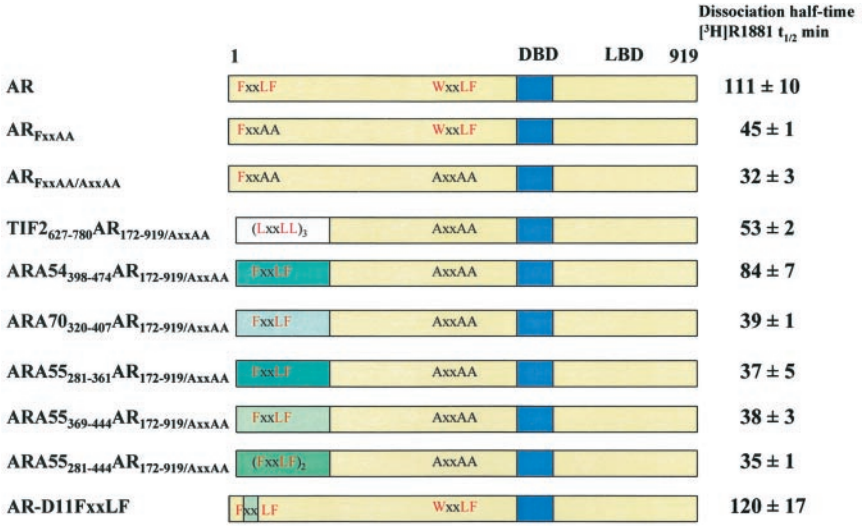

FIG. 5. **Ligand dissociation half-times for AR and chimeras.** Shown schematically are the AR, AR N/C interaction mutants AR-F*XX*AA, and AR-F*XX*AA/A*XX*AA described previously (19) and AR chimeras containing the indicated regions of the putative coactivators expressed as fusion proteins. Construction of the mutant expression vectors and dissociation half-times were determined as described under "Experimental Procedures" using [^{3}H]R1881. Shown are the dissociation half-times in min determined at 37 °C ± S.E. of three independent experiments each determined in duplicate. The positions of the DNA binding domain (*DBD*) and ligand binding domain (*LBD*) are indicated.

diolabeled synthetic androgen, and that this effect is dependent on the NH_2-terminal F*XX*LF motif (18, 20, 21). The dissociation half-time of [^{3}H]R1881 of 111 ± 10 min for wild-type AR determined at 37 °C increased to 45 and 32 min, respectively, when the AR NH_2-terminal F*XX*LF motif, or both the F*XX*LF and W*XX*LF motifs, were mutated (Fig. 5) as reported previously (18, 19). We created coactivator-AR chimeras in which the AR F*XX*LF motif region was deleted, and the AR W*XX*LF 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 L*XX*LL motif region of TIF2, where the ligand dissociation half-time increased 5–6-fold (19).

When the 171 AR NH_2-terminal amino acid region was replaced by ARA54 residues 398–474 containing its F*XX*LF motif (chimera ARA54-(398–474)AR-(172–919)-A*XX*AA), the half-time of [^{3}H]R1881 dissociation was 84 ± 7 min compared with 111 ± 10 min for wild-type AR and 45 ± 1 and 32 ± 3 min for AR-F*XX*AA and AR-F*XX*AA/A*XX*AA, respectively (Fig. 5). This dissociation half-time provides evidence in support of the an interaction between the ARA54 F*XX*LF sequence and the AR ligand binding domain. In contrast, chimeras containing the F*XX*LF regions from ARA70 (ARA70-(320–407)-AR-(172–919)-A*XX*AA; $t_{1/2}$ 39 ± 1), ARA55 (ARA55-(281–361)-AR-(172–919)-A*XX*AA and ARA55-(369–444)-AR-(172–919)-A*XX*AA; $t_{1/2}$ 37–38 ± 3–5 min), or a construct containing both F*XX*LF motifs of ARA55 (ARA55-(281–444)-AR-(172–919)-A*XX*AA, $t_{1/2}$ 35 ± 1 min), were less effective in slowing the androgen dissociation half-time (Fig. 5). Indeed, the multiple L*XX*LL motif region of TIF2 (TIF2-(627–780)-AR-(172–919)-A*XX*AA; $t_{1/2}$ 53 ± 2 min) was more effective in slowing the androgen dissociation half-time than were the F*XX*LF regions of ARA70 or ARA55, but less effective than the F*XX*LF motif-containing region of ARA54. Inserting the D11-F*XX*LF peptide sequence at the position of the AR NH_2-terminal F*XX*LF 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-F*XX*LF 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 F*XX*LF 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 [^{3}H]R1881 (7). Wild-type and mutant AR expression vectors were cotransfected with the GAL4-F*XX*LF and -L*XX*LL peptides in mammalian peptide two-hybrid interaction assays. There was a striking loss of interaction of the ARA54-F*XX*LF peptide with AR-K720A. In contrast, interaction of K720A remained robust with the AR NH_2-terminal F*XX*LF sequence and with the F*XX*LF peptides from ARA70 and D11 (Fig. 6*A*). No interaction was observed between the AR NH_2-terminal F*XX*LF motif and AR-E897K as reported previously (7) and comparatively weaker interactions with F*XX*LF peptides from ARA54, ARA70, and D11-F*XX*LF (Fig. 6*A*). AR-V716R abolished all F*XX*LF peptide interactions. Thus, glutamic acid 897 and valine 716 are critical in the AF2 binding surface for interaction with a variety of F*XX*LF motif sequences. The requirement for lysine 720 for the F*XX*LF sequence from ARA54 and the L*XX*LL sequence of TIF2, but not the F*XX*LF sequences of AR or ARA55 or ARA70, suggests subtle differences in the AF2 binding surface for F*XX*LF and L*XX*LL motif binding.

Receptor Specificity for FXXLF Motif Binding—We next determined whether the AR ligand binding domain selectively binds the F*XX*LF motif compared with other steroid receptors. None of the F*XX*LF 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. 6*B*). In striking contrast, the third L*XX*LL 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 L*XX*LL motif of TIF2 (Fig. 6*B*). For ERα, it was the second L*XX*LL motif of TIF2 positioned between TIF2 residues 683 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 L*XX*LL motif of TIF2 (36-fold, Fig. 6*B*), in agreement with previous reports (13, 41). As evident in Fig. 6*B*, little or no background transcriptional activity was detected in HepG2 cells for the AR, glucocorticoid receptor, and ERα in 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. 6*B*). The results demonstrate a high degree

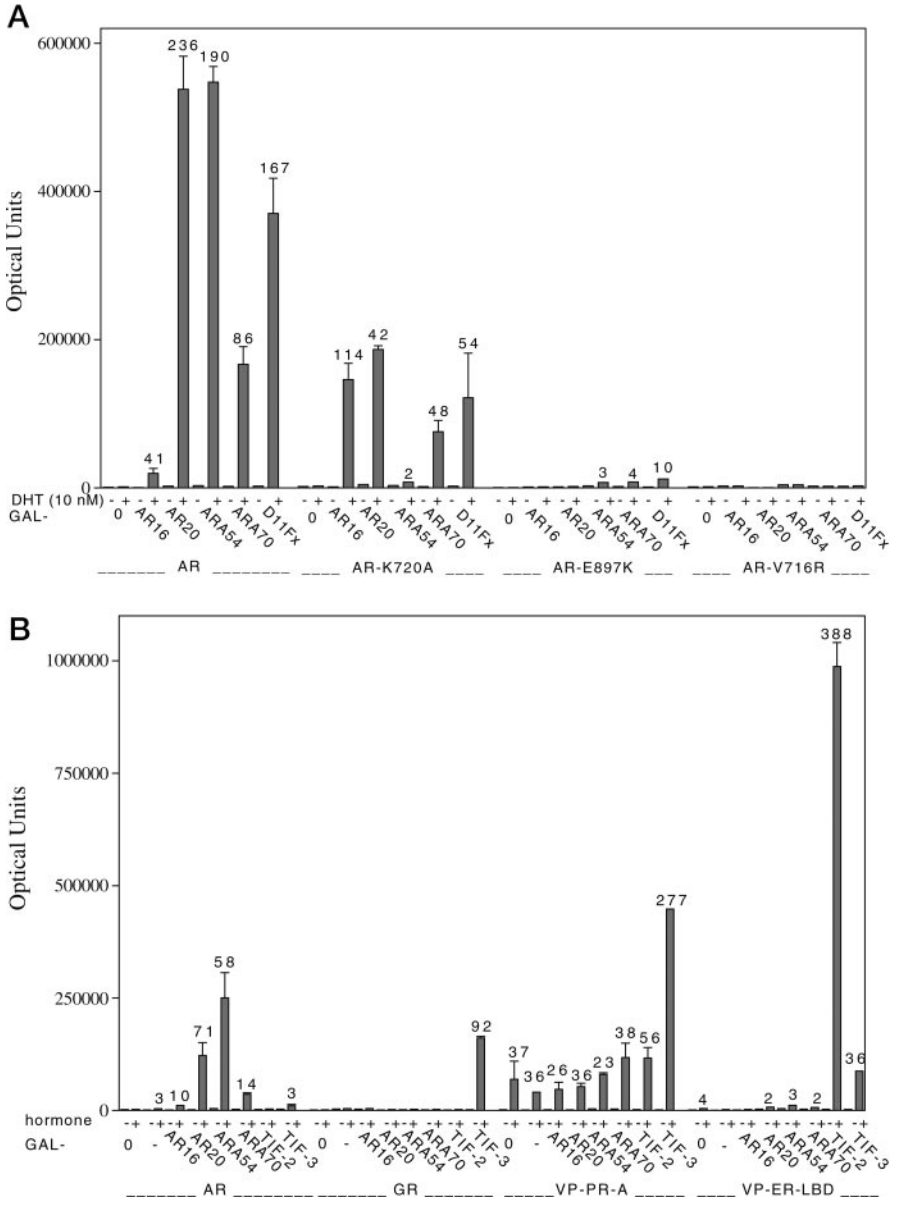

FIG. 6. **AF2 amino acid sequence requirements and steroid receptor specificity of F*XX*LF motif interactions.** *Panel A*, GAL-peptide DNA (0.05 μg) was cotransfected with pCMVhAR expressing full-length wild-type (AR) or with mutant sequences K720A, E897K, and V716R (0.05 μg) and 0.1 μg of 5XGAL4Luc3 in HepG2 cells as described under "Experimental Procedures." Cells were incubated in the absence and presence of 10 nM DHT. The GAL-peptides included the empty vector GAL0, GAL-AR-(16–36) (AR16), GAL-AR-(20–30) (AR20), GAL-ARA54-(447–465) (ARA54), GAL-ARA70-(321–340) (ARA70), and GAL-D11-F*XX*LF (D11Fx). Shown is a representative experiment of at least three determinations with the fold induction relative to activity determined in the absence of DHT shown above the *bars*. *Panel B*, steroid receptor specificity of F*XX*LF sequence interactions was determined in HepG2 cells using the GAL-peptides (0.05 μg) and pCMVhAR (AR), pCMVhGR (GR), VP-PR-A coding for the VP16 transactivation domain and PR-A lacking 164 amino acid residues of full-length PR-B, and VP-ERα-LBD coding for the VP16 transactivation domain and ERα ligand binding domain residues 312–595 (0.05 μg) with 0.1 μg of 5XGAL4Luc3 as described under "Experimental Procedures." Cells were incubated at hormone concentrations determined to be optimal: 10 nM DHT for AR, 10 nM dexamethasone for GR, 10 nM R5020 for PR, and 1 μM estradiol for ERα. Abbreviations are as indicated in *panel A*. In addition, results are shown for the GAL-TIF2 peptides containing the second (GAL-TIF2-(683–701) (TIF-2)) and third (GAL-TIF2-(738–756) (TIF-3)) L*XX*LL motif sequences of TIF2. Data shown are representative of three independent experiments, and fold induction is indicated above the *bars*.

of selectivity among a group of related F*XX*LF and L*XX*LL sequences for steroid receptor binding. The AF2 region of the AR ligand binding domain preferentially binds the F*XX*LF motif, whereas other steroid receptors interact more strongly with the L*XX*LL 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 L*XX*LL motifs of p160 coactivators (3–6). Using the mouse mammary tumor virus luciferase reporter vector, we investigated the requirement for the F*XX*LF 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. 7*A*). 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. 7*A*). 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 F*XX*LF to F*XX*AA in ARA54 and ARA55 had relatively little effect on the transcriptional response (Fig. 7*A*). 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 F*XX*LF motif sequence (Fig. 7*A*). The results suggest that a direct influence of these AR coregulatory proteins on the transcriptional response does not depend on interaction through the F*XX*LF 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, $pA_3RSV_{400}Luc$, and pSG5-Luc. We observed inhibition of transcription with the addition of pSG5 empty vector DNA (Fig. 7*B*) for each constitutively active reporter to an extent similar to the inhibition of AR-mediated activity seen in Fig. 7*A*. Inhibition was also observed with ARA55, but expression of ARA54, ARA70, or TIF2 had little influence on transcriptional activation. Based on these results,

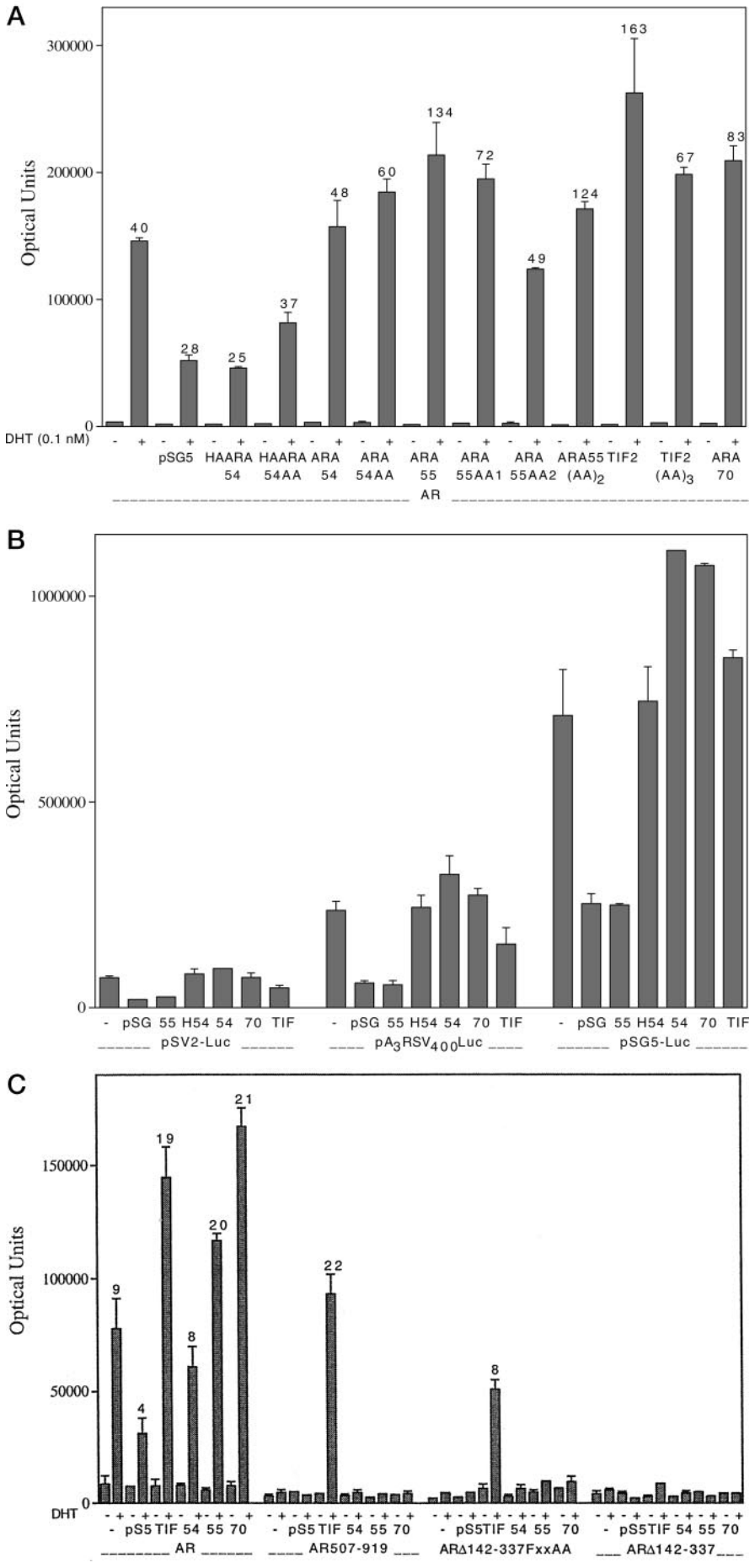

FIG. 7. **Effect of interacting proteins on AR transcriptional activity.** *Panel A*, AR transactivation was determined in CV1 cells by transfecting 0.1 μg of pCMVhAR without or with 1 μg of pSG5 parent vector DNA or 1 μg of pSG5-expressed coactivators HA-ARA54 (with HA NH_2-terminal epitope tag), HA-ARA54-F*XX*AA (HA-ARA54AA), ARA54, ARA54-F*XX*AA (ARA54AA), ARA55, ARA55-(F*XX*AA)$_1$ (ARA55AA1), ARA55-(F*XX*AA)$_2$ (ARA55AA2), ARA55-(F*XX*AA/F*XX*AA) (ARA55(AA)$_2$), TIF2, TIF2-(L*XX*AA)$_3$ (TIF2(AA)$_3$, 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, pA$_3$RSV$_{400}$Luc, 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*), ARA54 (*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), ARΔ142–337F*XX*AA (deletion of AF1 transactivation domain and mutation of the ^{23}F*XX*LF^{27} motif), and ARΔ142–337 (deletion of the AF1 NH_2-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. 7*C*). In striking contrast, ARA70, ARA54, and ARA55 each failed to increase the androgen-dependent AF2 activity of AR507–919 (Fig. 7*C*) despite the presence of the F*XX*LF binding sequences in these AR coregulators. TIF2 was the only coactivator tested which increased the transcriptional activity of AR507–919 and ARΔ142–337-F*XX*AA. The latter AR mutant lacks the NH_2-terminal AF1 residues 142–337 and has the NH_2-terminal F*XX*LF motif mutated to F*XX*AA (L26A/F27A) (Fig. 7*C*). Mutation of the F*XX*LF 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 ARΔ142–337 (Fig. 7*C*). 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 F*XX*LF motifs.

DISCUSSION

This report identifies F*XX*LF 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 probe. F*XX*LF 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 F*XX*LF motif binds the AR ligand binding domain to an extent somewhat weaker than the F*XX*LF motif in the AR NH_2-terminal region. Interaction of the ARA70 F*XX*LF 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 their interaction with a ligand binding domain fragment AR507–919. This contrasted the increase in transcriptional activity induced by TIF2, a p160 coactivator with weaker interacting L*XX*LL 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 L*XX*LL motifs of p160 coactivators such as SRC1 and TIF2. However, the AR AF2 region in the ligand binding domain preferentially binds F*XX*LF sequences, most notably the ^{23}FQNLF^{27} sequence in the AR NH_2 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 L*XX*LL motif regions of the p160 coactivators with the same AF2 region in the AR ligand binding domain (19). The F*XX*LF 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 adsorption and peptide mammalian two-hybrid assays indicate that the F*XX*LF sequences interact with the AR ligand binding domain in an agonist-dependent manner.

Data in this report show that F*XX*LF motifs interact selectively with the AR ligand binding domain. This is based on observations that none of the F*XX*LF peptide sequences in the putative AR coactivators or in the AR NH_2-terminal region interact with ERα, glucocorticoid, and progesterone receptors. In contrast, the L*XX*LL 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 F*XX*LF motif sequences is supported by a recent report in which only one L*XX*LL motif sequence (D11) interacted with the AR in a phage display screen of an L*XX*LL consensus peptide library using the ERα ligand binding domain (29). The F*XX*LF motif was also favored by the AR ligand binding domain when the D11-L*XX*LL sequence was mutated to F*XX*LF. 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 LQQLL of SRC1 was changed to FQQLF, 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 F*XX*LF motif interacts preferentially with the AR ligand binding domain.

Many proteins have sequences that conform to the F*XX*LF 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 F*XX*LF motif. For example, F*XX*LF sequences present in two members of the basal transcriptional machinery, TAFII250 (^{234}FLRLF^{252}) and TFIIEα (^{422}FEDLF^{439}), 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 F*XX*LF motif peptides derived from the general transcription coactivators CBP (^{23}FGSLF^{41}) and p300 (^{26}FGSLF^{44}) or from the reported AR coactivator FHL2 (^{25}FETLF^{43}). Clearly the F*XX*LF motif alone is not sufficient to predict interaction with the AR ligand binding domain.

Interacting F*XX*LF motifs likely form amphipathic α-helical structures as reported for the L*XX*LL motifs of p160 coactivators (3). This is supported by the failure of the CBP and p300 F*XX*LF motifs to interact with AR even though they are positioned near the coactivator NH_2 terminus. The F*XX*LF motif sequence present in CBP and p300 (FGSLF) contains the α-helix-disrupting amino acid glycine. Interaction of the F*XX*LF sequence of the putative AR coactivator FHL2/DRAL, positioned near its NH_2 terminus, was also not detected even though a previous report indicated that FHL2/DRAL increases AR activity in a N/C interaction-dependent manner (46). Thus sequences within and flanking the F*XX*LF 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 F*XX*LF motif. These include Zac-1 (47), hsp40/dnaJ (48), and HBO1 (49), suggesting a different mechanism of interaction. An L*XX*LL motif sequence is present in Zac-1 but not in hsp40/dnaJ or HBO1. Putative AR coactivators reported to interact with the AR NH_2-terminal or DNA binding domains that also lack F*XX*LF motif sequences include ARA24 (50), ARA160 (51), SNURF (52), ANPK (53), Ubc9 (54), ARIP3/PIASxα (55), Rb (56), and PIAS1 (57).

The presence of F*XX*LF 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 NH_2-terminal F*XX*LF 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 L*XX*LL 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 F*XX*LF sequences from ARA54 and ARA70 in two-hybrid peptide interaction assays suggest higher affinity interactions that might be sufficient to compete with the AR NH_2-terminal F*XX*LF sequence in the presence of the androgen-induced N/C interaction. Ligand dissociation kinetic studies of the coactivator-AR chimeras support the interaction of the ARA54 F*XX*LF 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. ARA55/Hic-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/Hic-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/Hic-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 NH_2-terminal region (32) where the latter was independent of the F*XX*LF 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 NH_2-terminal SH3 domain and a linker region, and between an SH2 domain and a carboxyl-terminal phosphotyrosine (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 phosphotyrosine. 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 F*XX*LF 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 L*XX*LL binding site. Lysine 720 is required for interaction with p160 coactivator L*XX*LL motif binding (7) and for the F*XX*LF 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 F*XX*LF motif is a common mediator of androgen-dependent interactions selective for the AR ligand binding domain. The F*XX*LF motif was originally reported in the AR NH_2-terminal domain to mediate the androgen-dependent N/C interaction. Interdomain competition may occur in a temporal sequence of F*XX*LF motif binding of other proteins. The selectivity of F*XX*LF 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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