

## The Use of Phage Display Technique for the Isolation of Androgen Receptor Interacting Peptides with (F/W)XXL(F/W) and FXXLY New Signature Motifs\*

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Early studies suggested that the signature motif, LXXLL, within steroid hormone receptor p160 coregulators may play important roles for the mediation of receptor-coregulator interaction. Interestingly, several androgen receptor (AR) coregulators, such as ARA70 and ARA55, may not use such a unique motif to mediate their coregulator activity. Here we apply the phage display technique to identify some new signature motifs, (F/W)XXL(F/W) and FXXLY (where F is phenylalanine, W is tryptophan, L is leucine, Y is tyrosine, and X is any amino acid) that can influence the interaction between AR and AR coregulators. Sequence analyses found that several AR coregulators, such as ARA70, ARA55, ARA54, and FHL2, contain FXXL(F/Y) motifs. Both glutathione S-transferase pull-down assays and transient transfection reporter assays demonstrate that these AR coregulators may use the FXXL(F/Y) motif to interact with AR and exert their AR coregulator activity. Exchanging the amino acid of Phe, Trp, or Tyr in this newly identified signature motif cluster may influence these peptides to interact with AR. The motif-containing peptides, as well as ARA70 or ARA54, may require selective flanking sequences for the better interaction with AR. In addition to influencing the AR transactivation, these motifs in AR-interacting peptides/proteins were also able to influence the AR N-/C-terminal interaction. Together, our data suggest that AR interacting peptides and/or AR coregulators may utilize the (F/W)XXL(F/W) and FXXLY motifs to mediate their interaction with AR and exert their influences on the AR transactivation.

Androgen receptor (AR)<sup>1</sup> is a transcriptional factor that belongs to the nuclear receptor superfamily (1–5). After androgens bind to the AR ligand-binding domain (LBD), the receptor undergoes conformational changes. Such changes cause AR to

dissociate from chaperone proteins, become phosphorylated, move into the nucleus, and act as a dimer to bind to target gene-response elements. AR may also recruit some coregulators to bridge between the AR dimer and preinitiation transcriptional complex and enhance target gene transcription (6, 7). Crystal structures of LBD from several nuclear receptors show the ligand almost buried in a hydrophobic pocket, which is formed by conserved core  $\alpha$ -helices 3, 7, and 10 (8–14). This causes the ligand-bound LBD conformational change that may recruit some specific coregulator complex either to enhance or to suppress transcription (15, 16). Before agonist binding, many steroid hormone receptors (SRs) bind to a corepressor, which frequently has histone deacetylase activity, and after agonist binding, these SRs may recruit coactivators, which may have histone acetylase activity (17–23).

The precise repertoire between coregulator complex and SRs is still not clear. The most well studied coregulators are those of the p160 family, which are common SR coregulators. By aligning their amino acid sequences, these p160 coregulators are shown to share a common sequence containing the core consensus LXXLL motif (L is leucine and X is any amino acid) (24–26). Through site-directed mutagenesis, this motif is shown to be essential for the function of coregulators in a ligand-dependent manner, and their flanking sequences could help to differentiate the specificity of their preferred receptor. In addition, some LXXLL motifs are essential to bind to common coregulators such as CBP/p300 (27). In co-crystal structure studies between NCoA-1/SRC-1 nuclear receptor interaction domain and liganded peroxisome proliferator-activated receptor  $\gamma$ , two consecutive LXXLL motifs of the coregulator fragment bridge the peroxisome proliferator-activated receptor  $\gamma$  dimer with each LXXLL motif binding to one peroxisome proliferator-activated receptor  $\gamma$  LBD (9). Because the LBDs of SRs share a highly conserved structure, many known coregulators have this LXXLL motif and use it for interaction with SRs (26–28). However, we found that the LXXLL motif in some AR coregulators, such as ARA70, may not play important roles for the mediation of the AR-AR coregulator interaction (29, 30). We therefore hypothesize that other signature motifs may exist that can play important roles for the AR-AR coregulator interaction.

The phage display screening system is a selection system in which a peptide or protein is fused to the coat protein of a bacteriophage (31, 32). By making a random or partial fixed peptide library and interacting with specific target proteins, this method has been successful in differentiating estradiol-estrogen receptor  $\alpha$  (ER $\alpha$ ), estradiol-ER $\beta$ , and antagonist-bound ER-associated peptides (33–36). Rat AR (rAR) amino acid sequence has 100% homology with human AR (hAR) in the

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<sup>1</sup> The abbreviations used are: AR, androgen receptor; hAR, human androgen receptor; rAR, rat androgen receptor; ARA, AR-associated protein; DBD, DNA-binding domain; DHT, dihydrotestosterone; ER, estrogen receptor; GST, glutathione S-transferase; HSP90, heat shock protein 90; LBD, ligand-binding domain; LIM, Lin11, Isl-1, and Mec-3; MMTV, mouse mammary tumor virus; mt, mutant; N-C, N-/C-terminal; SR, steroid hormone receptor; wt, wild type; VDR, vitamin D receptor.

DNA-binding domain (DBD) and LBD and 85% homology in the hinge region (2), but the rAR-DBD-LBD expressed from *Escherichia coli* was more stable than hAR-DBD-LBD. Through the phage display method and using *E. coli*-expressed rAR-DBD-LBD protein in the presence of testosterone, we fished out some AR-interacting peptides. Further classification allowed us to find some new motif clusters that might play important roles for the mediation of the interaction between AR and AR-interacting peptides.

#### EXPERIMENTAL PROCEDURES

**Materials and Plasmids**—5 $\alpha$ -Dihydrotestosterone (5 $\alpha$ -DHT), testosterone, 1,25(OH)<sub>2</sub>D<sub>3</sub>, and estradiol were obtained from Sigma. Phd-12<sup>TM</sup> peptide library was purchased from New England Biolabs. pSG5-AR and pSG5-ARA70N (ARA70 N terminus, amino acids 1–401) were constructed as described previously (37). FHL2 (full-length and fragments) was cloned from cDNA library of immortal human prostate epithelial cell line HPR-1 (a gift from Dr. Franky Chan, Chinese University of Hong Kong) and was constructed into pSG5 and pGEX-GST vector. pCMX-VP16-AR (AR lacking the initial 37 amino acids) was constructed for mammalian two-hybrid assay (37). The pCMX-VP16-hER $\alpha$ , pCMX-VP16-VDR, and pCMX-GAL4-RXR $\alpha$  constructs were generated by PCR of the full-length human ER $\alpha$ , VDR, and RXR $\alpha$  cDNA with primers containing *EcoRI* and *BamHI* sites for hER $\alpha$  and VDR and *EcoRI* and *NheI* sites for RXR $\alpha$  flanking the 5' and 3' ends, and the resulting PCR products were subcloned into the *EcoRI* and *BamHI*/*NheI* sites of pCMX-VP16 or pCMX-GAL4-DBD vectors.

pGEX-GST-ARA70N, pGEX-GST-ARA55, and pGEX-GST-ARA54 constructs were generated by PCR of pSG5-ARA70, pSG5-ARA55, and pSG5-ARA54 with one primer containing *BamHI* site flanking the 5' end, and the other primer containing *EcoRI* site flanking the 3' end, and the resulting PCR products were subcloned into the *BamHI* and *EcoRI* sites of the pGEX-GST vector. pGEX-GST-HSP90 (heat shock protein 90) construct was generated by PCR of the full-length human HSP90 cDNA with primers containing *BglII* site flanking the 5' and 3' ends, and the PCR product was inserted into pGEX-GST vector via *BamHI* site. Site-directed mutagenesis kit (Stratagene) was used to create single and double amino acid mutations in FXXL(FY) domain of ARA70-N, FHL2, and ARA54-C (amino acids 361–474). The target peptides sequences for wild type (wt) and mutants (mts) were synthesized by two 35-mer oligonucleotides with 15 central complementary nucleotides (IDTDNA) filled in by Klenow enzyme with dNTP and constructed into pCMX-GAL4 vector between *EcoRI* and *BamHI* sites. Peptide D30 from a sequence published previously (38) was synthesized by two complementary oligonucleotides (IDTDNA) and was constructed into pCMX-GAL4 vector between *EcoRI* and *BamHI* sites. PCR was used to construct AR-N (amino acids 1–501) via *BamHI/KpnI* to pCDNA3-FLAG vector and AR-C (amino acids 556–919) via *BamHI/XbaI* into pCDNA3 vector.

**Cell Cultures**—COS-1, CV-1 cell lines (both are green monkey kidney cell lines), and human prostate cancer cell line PC-3 were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum, penicillin (25 units/ml), and streptomycin (25  $\mu$ g/ml). All cell lines were from ATCC.

**Cloning, Expression, and Purification of the rAR-DBD-LBD**—The rAR-DBD-LBD cDNA, from amino acids 538–902 (rat numbering), was cloned from a rat prostate cDNA library (Clontech) by PCR. The rAR-DBD-LBD was expressed as a fusion protein driven by the T7 promoter of pET28c vector (Novagen) to include an N-terminal polyhistidine tag and a thrombin cleavage site. Testosterone was included in the *E. coli* (BL21-DE3) fermentation medium at a concentration of 1  $\mu$ M. Induction with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside was allowed to proceed for 16 h at 18  $^{\circ}$ C in NZCYM media, and pellets were stored at  $-70^{\circ}$ C. With a total of  $\sim$ 80% purity, 4–5 mg of recombinant rAR-DBD-LBD protein was isolated from a 15-g cell pellet after freezing/thawing for four cycles, sonication, chromatography on a nickel-chelate resin, and elution by 0.25 M imidazole.

**Phage Display Procedure**—Detailed procedures for panning and sequencing of peptide have been described previously (31, 32). Briefly, 150  $\mu$ l of 100  $\mu$ g/ml streptavidin in NaHCO<sub>3</sub> solution, pH 8.6, was coated on the wells of a 96-well enzyme-linked immunosorbent assay plate (Corning Glass) at 4  $^{\circ}$ C for 18 h, blocked with 0.1 M NaHCO<sub>3</sub>, pH 8.6, 5 mg/ml bovine serum albumin for 1 h; we then added 30 pmol of biotin-labeled C3(1)-ARE (Biotin-C3(1)ARE1, 5'-Bio-GATC CAG AGT ACG TGA TGT TCT CAG; Biotin-C3(1)ARE2, 5'-Bio-GATC CTG AGA ACA TCA CGT ACT ATG) in TBST solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl,

0.05% Tween 20) to the coated well for 1 h, then added 20 pmol of rAR-DBD-LBD protein in TBST solution, and incubated for 1 h. Between each step, wells were washed with TBST 10 times. Finally, we added  $1.5 \times 10^{11}$  phage in 100  $\mu$ l of TBST with 1  $\mu$ M testosterone to the coated well, incubated for another 1 h, and then washed out unbound phage. We used 0.2 M glycine HCl, pH 2.2, to elute the bound phage and neutralized with 1 M Tris-HCl, pH 9.1. Eluted phage was amplified in *E. coli* (ER2738). The whole procedure was repeated for four rounds, and the phage peptides from the third and fourth rounds were sequenced.

**Mammalian Two-hybrid Assay**—For the luciferase assay, 300 ng of pG5-LUC reporter gene plasmid, 0.5 ng of SV40-*Renilla* luciferase internal control plasmid, 350 ng of wt or mt coregulator or peptide in pCMX-GAL4 vector, and 350 ng of VP16-AR were transfected into PC-3 cells with Superfect kit (Qiagen). After 16 h, ethanol, 1 nM 5 $\alpha$ -DHT, or 10 nM testosterone were added to the wells for another 16 h. The dual luciferase reporter assay system (Promega) was employed to measure luciferase activity.

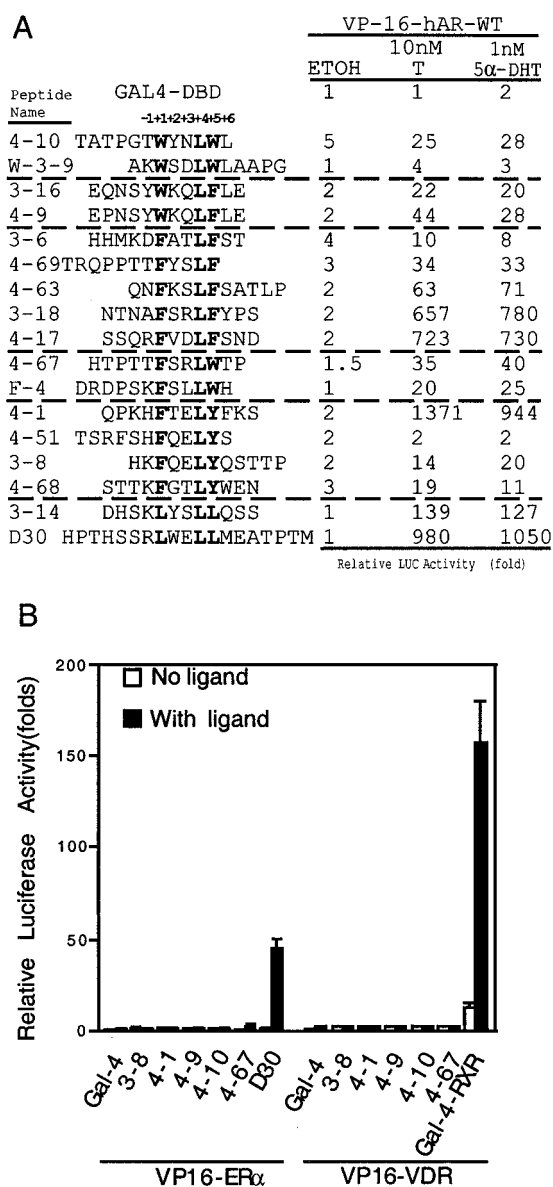
**Transfection and Reporter Gene Assay**—COS-1 and CV-1 cell lines and human prostate cancer cell line PC-3 were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum. For transfection, the cells were plated in 24-well dishes, and plasmids were transfected by Superfect kit as described previously. After 16 h of incubation, the cells were treated with ethanol or steroid hormones for another 16 h and then harvested for the dual luciferase assay. The mouse mammary tumor virus (MMTV)-luciferase reporter gene was used to measure AR transcriptional activity, and an SV40-*Renilla* luciferase plasmid (Promega) was used as an internal control. The dual luciferase reporter assay system (Promega) was employed to measure the luciferase activity.

**Glutathione S-Transferase (GST) Pull-down Assay**—GST-ARA70-N and GST-FHL2 fusion proteins were expressed in *E. coli* strain BL21 and purified as described by the manufacturer (Amersham Biosciences). The purified proteins were suspended in 100  $\mu$ l of interaction buffer (20 mM HEPES, pH 7.9, 150 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 0.1% (w/v) bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol), mixed with 5  $\mu$ l of <sup>35</sup>S-labeled TNT-expressed hAR full-length proteins (TNT-coupled reticulocyte lysate system, Promega) in the presence or absence of 1  $\mu$ M 5 $\alpha$ -DHT, and incubated at 4  $^{\circ}$ C for 2 h. After several washes with NENT buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl<sub>2</sub>, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.5% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 8% glycerol), the bound proteins were separated on an SDS-polyacrylamide gel and visualized by PhosphorImager (Molecular Dynamics).

#### RESULTS

**Screening of the Unique Motif That Interacts with AR**—To identify whether any new signature motif can bind and enhance AR transactivation, we applied phage display library containing 10<sup>12</sup> random 12-amino acid peptides to screen its ability to bind to AR. By using *E. coli*-expressed rAR-DBD-LBD protein with testosterone as bait to screen four times with 12-mers random peptide library, we were able to identify a set of AR-interacting peptides containing a motif cluster with sequences of either (F/W)XXL(F/W) or FXXLY that can interact with rAR. Structure analysis found these three amino acids, Phe, Trp, and Tyr, all belong to the same functional group with bulky, hydrophobic, neutral, and aromatic ring-containing side chains. We also found one peptide containing the classic LXXLL motif in our screening. The synthetic DNA encoding these individual peptides were subcloned into expression vectors to test if they can still interact with hAR in the mammalian two-hybrid assay. As shown in Fig. 1A, at least 12 peptides containing either (F/W)XXL(F/W) or FXXLY motif can interact with hAR (with at least 5-fold induction in the presence of androgen). Mammalian two-hybrid assay further demonstrates these peptides can bind specifically to AR but not ER $\alpha$  or vitamin D receptor (VDR) (Fig. 1B). Together, these data suggest that in addition to the classic LXXLL motif, a new motif, such as (F/W)XXL(F/W) or FXXLY, may be able to bind specifically to AR.

**Identification of FXXLF Signature Motif in AR and AR Coregulators ARA70, ARA55, ARA54, and FHL2, WXXLF Motif in AR and FXXLY Motif in FHL2**—We then surveyed the amino



**FIG. 1. (F/W)XXL(F/W) and FXXLY signature motif clusters exist in screened peptides, and these peptides interact with AR in a ligand-dependent manner and are relatively AR-specific.** **A**, by using *E. coli*-expressed rAR-DBD-LBD protein with testosterone as bait to fish out random 12-mer peptide library displayed on M13 bacteriophage coat, a total of 15 (F/W)XXL(F/W) and FXXLY-signature motif peptides and one LXXLL motif-containing peptide were found from the third and fourth round of screening. 350 ng of VP16-hAR, 300 ng of reporter plasmid pG5-LUC, 0.5 ng of SV40-*Renilla* luciferase plasmid, and 350 ng of GAL4-DBD or GAL4-DBD constructed with the screened peptides were transfected into PC-3 cell line in 24-well plates. After 16 h, ethanol, 10 nM Testosterone, or 1 nM 5 $\alpha$ -DHT was added for another 16 h. A dual luciferase assay system was used. The peptide D30 (35) was added as a positive control. **B**, GAL4-DBD and five GAL4-DBD peptides with different motifs from **A** were chosen to study their interactions with human ER $\alpha$  and human VDR. The same ratio plasmid combination and study procedure were done in PC-3 cell line as in **A** except for substitution of VP16-hAR with VR16-hER $\alpha$  or VP16-VDR. Ethanol, 1  $\mu$ M estradiol, or 10 nM 1,25(OH) $_2$ D $_3$  were added for treatment. A D30 and a full-length human retinoid X receptor- $\alpha$  constructed in GAL4-DBD were used as positive controls.

acid sequence within AR and AR coregulators identified previously, and we found that AR had the (F/W)XXLF motifs; ARA70 (37), ARA55 (39), and ARA54 (40) contained the FXXLF motif, and FHL2 (41) contained both the FXXL(F/Y) motifs (Fig. 2A). After aligning these motifs, we found that the motifs in both ARA55 and FHL2, which were *Lin11*, *Isl-1*, and *Mec-3*

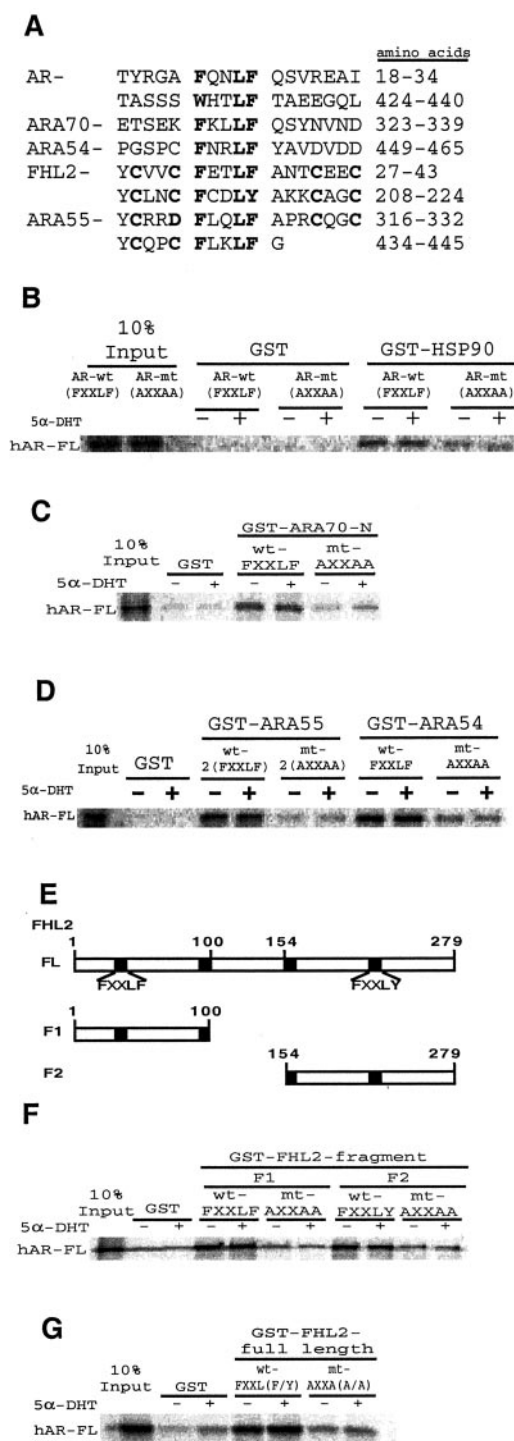
(LIM) domain-containing proteins, were located in the linkers or just next to the LIMs.

(F/W)XXLF in AR has been shown to be important for mediating AR N-C interaction (43, 44). Mutation or deletion of the FXXLF motif in the AR N terminus could not influence [ $^3$ H]R1881 binding to AR significantly ( $K_d$ : 0.3–0.8 nM) (43) or AR binding to specific or nonspecific response element (49). We first tested whether this FXXLF motif had any effect on AR-HSP90 interaction. As shown in Fig. 2B, the data from GST pull-down assay show the mutation of the FXXLF in AR N terminus will weaken the AR-HSP90 interaction.

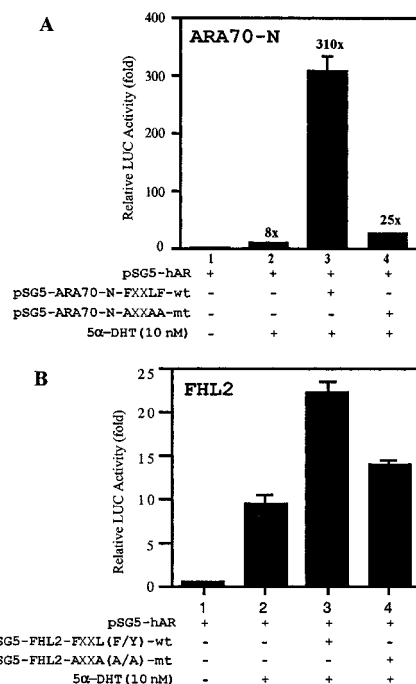
Among AR coregulators, we first mutated the FXXLF motif in ARA70 to AXXAA (where A is alanine) and the FXXL(F/Y) motifs in FHL2 to AXXA(A/A), and we tested their influence on the binding to AR. Interestingly, results from GST pull-down assay show that wt ARA70N-FXXLF can interact well with AR. In contrast, mt ARA70N-AXXAA has little capacity to interact with AR (Fig. 2C). Similar results also occurred in ARA55, ARA54, and FHL2 showing only wt but not mt AR coregulators can interact with AR (Fig. 2, D–G). Together, results from Fig. 2 clearly demonstrated that the signature motif FXXLF in AR was important in AR-HSP90 interaction, and signature motifs, FXXLF and FXXLY, identified from phage display screening may represent new motifs among the AR coregulators, such as ARA70, ARA55, ARA54, and FHL2.

**FXXL(F/Y) Signature Motifs in AR Coregulators Influence AR Transactivation**—The coregulator-ARA70N containing wt FXXLF and mt AXXAA were then ligated to pSG5 expression vector and tested for their influence on the AR transactivation. As shown in Fig. 3A, in COS-1 cells, 10 nM 5 $\alpha$ -DHT can induce AR transactivation to 8-fold (*lanes 1 versus 2*). Addition of wt pSG5-ARA70N-FXXLF further enhances AR transactivation from 8- to 310-fold (*lanes 2 versus 3*). In contrast, addition of mt pSG5-ARA70N-AXXAA only shows much less induction effect (from 8- to 25-fold) for AR transactivation (*lanes 2 versus 4*). Because FHL2 has one FXXLF motif and one FXXLY motif, we mutated both of these motifs to AXXAA and tested their influence on the AR transactivation. As expected, we found the mt FHL2-AXXA(A/A) has much less induction effect on AR transactivation (Fig. 3B). Together, Fig. 3 demonstrates that a newly identified FXXL(F/Y) motif not only influences the interaction between AR and AR coregulator but may also influence the AR coregulator activity.

**Interchanging of Phe, Trp, and Tyr in the Motifs May Influence the Peptide to Interact with AR**—Because the three specific amino acids, Phe, Trp, and Tyr have similar bulky, hydrophobic, neutral, and aromatic ring-containing side chains, we hypothesized that these three amino acids may be exchangeable in some peptides. Among the identified peptides, we picked up peptide 3-18 with the FXXLF motif and peptide 4-9 with the WXXLF motif, and we performed single mutations each time. We changed the first amino acid Phe of peptide 3-18 to Trp (m1) or to Tyr (m2), or we changed the second amino acid Phe to Trp (m3) or to Tyr (m4). We also changed the first amino acid Trp of peptide 4-9 to Phe (m1), or we changed the first amino acid Phe to Trp (m2) or to Tyr (m3). These mt and wt peptides were then tested for their interactions with AR in mammalian two-hybrid assay. In peptide 3-18, the first amino acid Phe is not exchangeable, but the second amino acid Phe can be changed with Tyr although its interaction with AR is significantly reduced (Fig. 4). To our surprise, in the mt peptide 4-9 (m1), the changing of Trp to Phe can further enhance the peptide interaction with AR by 20-fold. The changing of Phe to Trp (m2) in peptide 4-9 had similar interaction with AR. Taken together, the data in Fig. 4 demonstrated that these three specific amino acids, Phe, Trp, and Tyr, within the newly identified motifs



**FIG. 2. Effect of FXXLF motif in AR-HSP90 interaction and effects of FXXL(F/Y) motifs in AR-FXXL(F/Y) motifs-containing AR interaction protein interactions.** *A*, FXXLF motif exists in AR, ARA70, ARA55, ARA54, and FHL2. WXXLF motif exists in AR. FXXLY motif was found in FHL2. The FXXL(F/Y) motifs in LIMs proteins ARA55 and FHL2 were located in the linkers or close to the LIMs. *B*, mutation of the FXXLF motif to AXXAA in AR N terminus reduced AR interaction with HSP90 in GST pull-down assay. The GST-HSP90 and GST control proteins were purified as instructed by the manufacturer (Amersham Biosciences). Five microliters of *in vitro* translated wild type (*wt*) or mutant type (*mt*) [<sup>35</sup>S]methionine-labeled hAR interacted with GST-HSP90 protein or GST control bound to glutathione-Sepharose beads in a pull-down assay as described in the presence or absence of 1  $\mu$ M 5 $\alpha$ -DHT. After extensive washing, the pull-down complex was loaded onto an 8 or 10% polyacrylamide gel and visualized by autoradiography. The input represents 10% of the amount of labeled protein used in the pull-down assay. *C–G*, mutations in these FXXL(F/Y) motifs will cause weaker AR-AR interaction protein interactions. Similar ex-

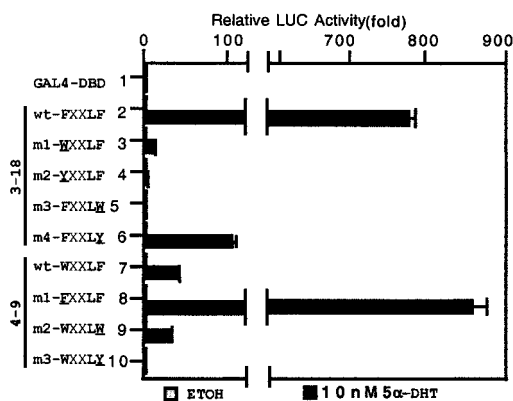


**FIG. 3. Effects of wt and mt FXXL(F/Y) motifs-containing AR interaction proteins on AR transactivation.** Both ARA70-N (*A*) and FHL2 (*B*) were transfected in COS-1 cells. In COS-1 cells, 100 ng of pSG5-hAR, 600 ng of wt pSG5-ARA70-N-FXXLF, mt pSG5-ARA70-N-AXXAA, wt pSG5-FHL2-FXXL(F/Y), or mt pSG5-FHL2-AXXA(A/A), 300 ng of MMTV-LUC reporter plasmid, and 0.5 ng of SV40-Renilla luciferase plasmid were transfected in 24-well plates. After 16 h, ethanol or 10 nM 5 $\alpha$ -DHT were added for another 16 h. Relative LUC activity was calculated by the dual luciferase system.

could not always be interchangeable, and the interchange of Phe, Trp, and Tyr in the motifs may influence the peptide to interact with AR, although the change of the first Phe to Trp did not influence the interaction between mutant peptide and AR in peptide 4-9, and exchanging the first Trp to Phe can further enhance mutant peptide 4-9-AR interaction.

*The Flanking Sequence of the Motifs May Also Influence Peptide and Protein Interaction with AR*—From the screened peptides, some amino acids seemed to appear frequently in some flanking sequence. In motif -1 flanking position (Fig. 1), the positively charged amino acids Arg, Lys, and His were found in almost half of the peptides (7 of 15). We picked up three peptides, 4-17 (RFXXLF), F-4 (KFXXLF), and 4-1 (HFXXLY), from different motifs with relatively strong interaction with AR, and we mutated these positively charged amino acids to Ala; we then tested their interactions with AR. As shown in Fig. 5A, the Arg and Lys, but not His, were important for these peptides to interact with AR. In the motif flanking +6 position, we found hydrophobic amino acids Phe, Trp, Tyr, and Leu in half of the screened peptides (7 of 14). We chose four peptides with these four amino acids in flanking +6 position of the motifs, 4-1 (FXXLYF), 4-9 (WXXLFL), 3-18 (FXXLYF), and 4-68 (FXXLYW), mutated them to Ala, and then tested their interactions with AR. As expected, we found their mutated peptides lost most interaction ability with AR. Due to these convincing data, we also checked the AR coregulators to see whether these rules can apply. We found the ARA70 had one Lys in the -1 position of the FXXLF motif, and ARA54 had one Tyr in the +6 position of the FXXLF motif. We

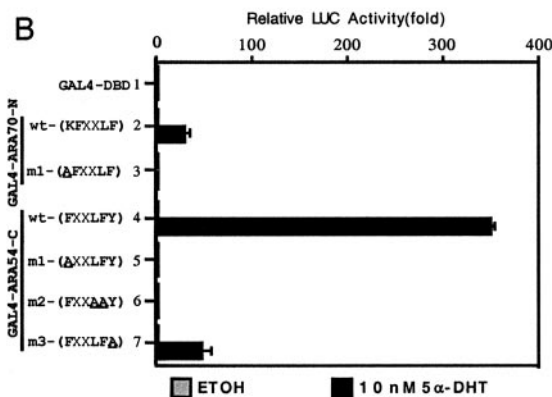
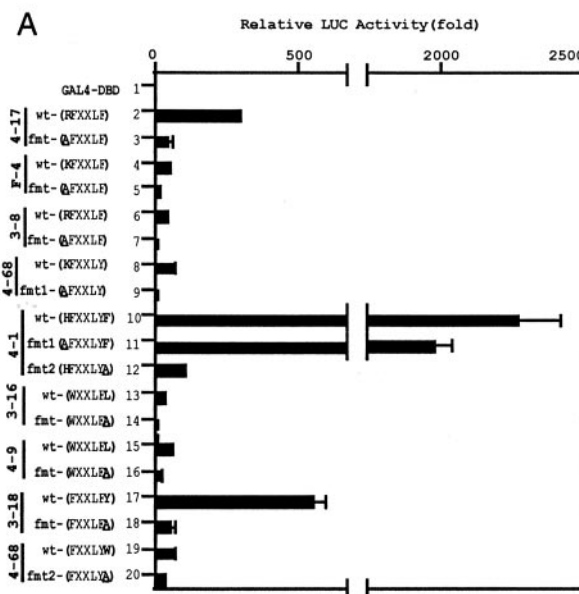
perimental procedures were done as in *B* except using wt [<sup>35</sup>S]methionine-labeled hAR only and substituting GST-HSP90 with wt or mt, full-length, or fragment of GST-AR coregulators.



**FIG. 4. Interchanging of Phe, Trp, and Tyr in the motifs may influence the peptide interaction with AR.** FXXLF-containing peptide 3-18 and WXXLF-containing peptide 4-9 were chosen. In peptide 3-18, the first amino acid Phe was changed to Trp (m1) or Tyr (m2), or the second amino acid Phe was mutated to Trp (m3) or Tyr (m4). In peptide 4-9, the first amino acid Trp was mutated to Phe (m1), or the first amino acid Phe was changed to Trp (m2) or Tyr (m3). In 24-well plates, 350 ng of VP16-hAR and 350 ng of GAL4-DBD or GAL4-DBD constructed with wt or mt peptides, 300 ng of reporter plasmid pG5-LUC, and 0.5 ng of SV40-*Renilla* luciferase plasmid were transfected into the PC-3 cell line. After 16 h, ethanol or 10 nM 5 $\alpha$ -DHT was added for another 16 h. A dual luciferase assay system was used.

therefore expect the FXXLF motif plus its flanking sequence within ARA70 and ARA54 may play important roles for the mediation of their coregulator activity. As shown in Fig. 5B, mutation of the Lys in the -1 position of the FXXLF motif in ARA70 will weaken interaction between AR and ARA70 N terminus. Interestingly, using different approaches, Zhou *et al.* (42) also found that lysines around the FXXLF motif (-1 and +2 position of FXXLF) in ARA70 are important for ARA70 to interact with AR, which correlated well with our observations. To prove that the Tyr in +6 position of the FXXLF motif in ARA54 may also play important roles for the interaction between ARA54 and AR, we mutated the Tyr in ARA54 C terminus to Ala and tested for interaction with AR in the mammalian two-hybrid assay. As shown in Fig. 5B, the mt ARA54-C (m3) poorly interacted with AR compared with the wt. Taken together, results from Fig. 5 suggests that the newly identified motif may prefer positively charged amino acids, either Lys or Arg, but not His, in the -1 position, and hydrophobic amino acids Phe, Trp, Tyr, and Leu in the +6 position that may influence how this motif mediates the interaction between coregulator and AR. This conclusion was further confirmed by using mutation studies of this motif within two previously identified AR coregulators, ARA70 and ARA54.

**FXXLY Motif-containing Peptides May Block AR Transactivation through Interference with AR N-C Interaction**—Early studies suggested that <sup>23</sup>FQNL<sup>F27</sup> and <sup>429</sup>WHTLF<sup>F433</sup> sequence within AR N terminus may play important roles for the AR N-C interaction (43). As these sequences fit into our newly identified motif sequence, (F/W)XXL(F/W), we were interested to know if our newly identified AR-interacting peptides with (F/W)XXL(F/W) and FXXLY motif have any influence on the AR N-C interaction. By using pCDNA3-FLAG-hAR-N (amino acid 1-501) and pCDNA3-hAR-C (amino acid 556-919) in MMTV-LUC reporter assays (38), we found the identified AR-interacting peptides 3-18, 4-1, and 4-67 could suppress the AR N-C interaction (Fig. 6). We then tested if these AR interacting peptides could also influence the AR transactivation. As shown in Fig. 7, peptide 4-1 but not peptide 3-18 suppressed AR transactivation. Together, these data suggest the FXXL(F/W/Y) motif-containing peptides may suppress AR N-C interac-

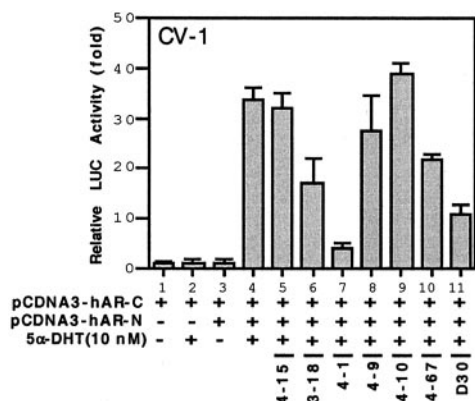


**FIG. 5. The flanking sequence of the motifs can influence peptide and protein interaction with the AR.** A, amino acids with side chains containing a basic group, such as Arg, Lys, but not His, in the -1 position of the motifs and hydrophobic amino acids Phe, Trp, Tyr, and Leu in the +6 position of the motifs will influence the peptide interaction with AR. The peptides with motifs were aligned to choose eight peptides, and all the candidate amino acids were changed to Ala. B, the basic group containing amino acid Lys in the -1 position of the FXXLF motif in ARA70 and hydrophobic amino acid Tyr in the +6 position of the FXXLF motif in ARA54 was important for N terminus of ARA70 and C terminus of ARA54 to interact with AR. ARA70 amino acids 1-401 and ARA54 amino acids 361-474 were constructed into GAL4-DBD as described previously (40). Mutations of ARA70-N and ARA54-C in GAL4-DBD were created by site-directed mutagenesis kit. In PC-3 cell lines, 350 ng of VP16-hAR, 350 ng of wt or mt GAL4-DBD-peptide, wt or mt GAL4-DBD-ARA70-N, wt or mt GAL4-DBD-ARA54-C, or vector only, 300 ng of pG5-LUC reporter, and 0.5 ng of SV40-*Renilla* luciferase plasmid were transfected into 24-well plates. After 16 h, ethanol or 10 nM 5 $\alpha$ -DHT was added for another 16 h. A dual luciferase assay system was used.

tion, and the suppression of AR N-C interaction may not always result in the suppression of AR transactivation.

#### DISCUSSION

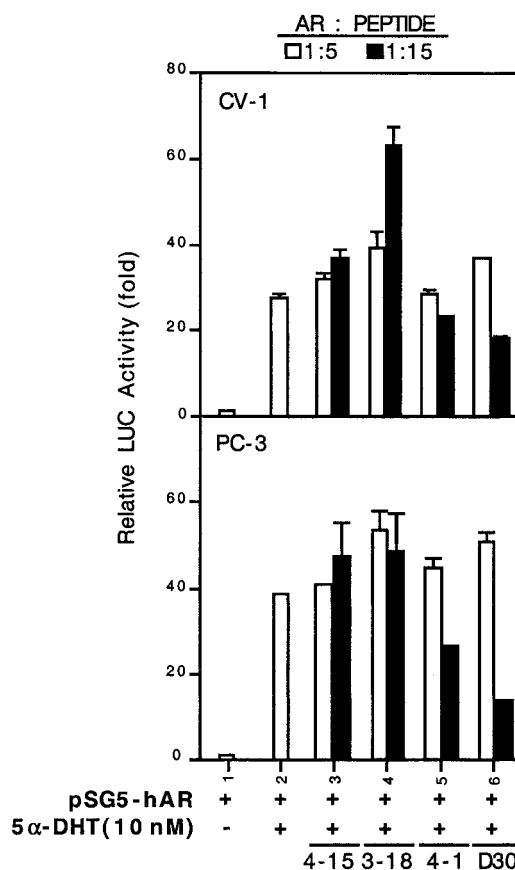
Previous reports demonstrated that the (F/W)XXL(F/W) in AR N terminus (<sup>23</sup>FQNL<sup>F27</sup> and <sup>429</sup>WHTLF<sup>F433</sup>) might play important roles in AR N-C interaction, could slow down ligand-AR dissociation rate, might selectively activate genes, and might compete with activation function 2 recruitment of LXXLL motif-containing coactivators (43, 44, 47, 48). Although the



**FIG. 6. Some peptides can block AR N-C interaction.** 70 ng of pCDNA3-hAR-C (amino acids 1–501) alone or combined with 70 ng of pCDNA3-FLAG-hAR-N (amino acids 556–919), 250 ng of MMTV-LUC, 0.5 ng of SV40-*Renilla* luciferase, and 350 ng of GAL4-DBD-peptide plasmids were transfected into CV-1 cell line in 24-well plates, plus GAL4-DBD to individual wells to make equal amounts of plasmid in every well. After 16 h, ethanol or 10 nM 5 $\alpha$ -DHT was added for another 16 h. A dual luciferase assay was used. One LXXLL-containing peptide, D30, screened by estrogen-ER (35, 38), which can interact with AR and can block AR N-C interaction, served as a positive control. Peptide 4-15, which could not interact with AR in mammalian two-hybrid assay, served as a negative control.

FXXLF in AR will not influence ligand binding to the AR or the AR binding to specific or nonspecific DNA-response elements, this motif will influence the AR interacting with HSP90 (Fig. 2B) (43, 49). Later studies found the FXXLF motif was also present in the AR coregulators, such as ARA70, ARA55, and ARA54 (37, 39, 40), was AR-specific, and might play important roles in the interaction between AR and AR coregulators (30, 42, 45, 46). By using phage display techniques, we successfully isolated several AR-interacting peptides, and we found that these peptides containing (F/W)XXL(F/W) or FXXLY motif were AR-specific (Fig. 1B) and might play important roles to mediate the interaction between AR and AR-interacting peptides. Although one LXXLL-containing peptide was found in the 3rd round of 50 sequenced clones, this peptide did not show up again in the fourth round of 50 sequenced clones after increasing the screening stringency. This result may support the previous observation that the FXXLF motif in AR N terminus may compete with LXXLL motif-containing coactivators to bind to activation function 2 (43). The previous findings, plus our ability to identify the (F/W)XXL(F/W) and FXXLY motifs from phage display techniques, suggest *in vitro* screening using phage display technique is a feasible approach to isolate AR-interacting peptides.

Compared with the FXXLF motif, although the WXXLF motif was also found in the AR N terminus and could mediate AR N-C interaction and selective gene activation, the liganded AR-associated coregulators, such as ARA70, ARA55, ARA54, and FHL2 contain the FXXLF motif but not the WXXLF motif (37, 39–41, 43, 48). Because Phe, Trp, and Tyr belong to the same functional group of amino acids with bulky, hydrophobic, neutral, and aromatic ring-containing side chains, we tested whether they were exchangeable in these motif clusters. Data in this report show that the second amino acid Phe in the peptide 3-18 containing FXXLF can be changed to Tyr, although the interaction with AR drops to 20% compared with the wt peptide. The first amino acid Trp in the peptide 4-9 containing WXXLF can be changed to Phe with interaction with AR dramatically increased to 20-fold, and the first Phe can be substituted with Trp without an obvious change of interaction with AR. Although these phenomena were observed, most of the time the interchange of Phe, Trp, and Tyr in the motifs



**FIG. 7. Some peptides can partially block AR transactivation.** 70 ng of pSG5-hAR, 250 ng of MMTV-LUC reporter plasmid, and 0.5 ng of SV40-*Renilla* luciferase plasmid were transfected into CV-1 and PC-3 cell lines in 24-well plates, with addition of 350 or 1050 ng of GAL4-DBD-peptide to individual wells, and GAL4-DBD was added to make an equal amount of plasmid in each well. After 16 h, ethanol or 10 nM 5 $\alpha$ -DHT was added for another 16 h. Peptide 4-15 served as a negative control. A dual luciferase assay was used.

may influence the peptide interaction with AR. In the presence of the other nine fixed amino acids, most of the time an individual peptide had its own preferred motif but seemed to prefer Phe in the +1 position compared with Trp. The evidence supporting this concept, other than the mutants study in Fig. 4, was that nine peptides had Phe but only four had Trp in the +1 position of the motif among the screened out peptides. These results may support the finding that most of the liganded AR-associated coregulators contain the FXXLF motif instead of the WXXLF motif (37, 39–41).

These (F/W)XXL(F/W)- and FXXLY-containing peptides may require selective flanking sequences for the better interaction with liganded AR. ARA70 was first found to be involved in the activation of the *RET* proto-oncogene in a thyroid neoplasm and has recently been shown to be a ligand-dependent transcriptional coregulator for AR (37, 50). The interaction domain between ARA70 and AR has been defined (42). ARA70 could enhance estradiol binding to AR (51), was linked to the peroxisome proliferator-activated receptor- $\gamma$  pathway (29), and was activated in invasive ovarian cancer (53). ARA70 had two lysines in -1 and +2 position of its FXXLF motif (<sup>327</sup>KFKLLF<sup>332</sup>), and mutation of these two lysines to alanines decreased ARA70 interaction with AR and ARA70 coregulator function (42). Our data showed the -1 position of the motifs positively charged amino acids, Arg and Lys but not His, is important for peptides and ARA70 N terminus to interact with AR (Fig. 5). At the location of +6 position of the motifs, we also found hydrophobic amino acids, Phe, Trp, Tyr, and Leu, ap-

peared in half of these cluster peptides and were important for peptides to interact with AR. This rule also could be demonstrated in ARA54. ARA54 is a RING finger protein, initially cloned as an AR coactivator (40), that possibly has a ubiquitin-protein isopeptide E3 ligase effect (54). ARA54 has one Tyr in the +6 position of the FXXLF motif (<sup>454</sup>FNRLFY<sup>459</sup>), and mutation of this Tyr will decrease the fragment of ARA54 (amino acid 361–474) interaction with AR in mammalian two-hybrid assay.

Both ARA55 and FHL2 contain the LIM motif and FXXLF motif and were screened from yeast two-hybrid system by using AR-LBD-containing bait with ligand (39, 41). FHL2 expresses in cardiac muscle, prostate, and testes (41). In mice studies, the FHL2-knockout mouse does not influence cardiac development but does modify the hypertrophic response to  $\beta$ -adrenergic stimulation (55, 56). FHL2 has been linked to the integrin pathway (57), the Rho signal pathway (58), and the  $\beta$ -catenin pathway (59, 60). ARA55 has been linked to proline-rich tyrosine kinase 2 (Pyk2) (61), could bind to HSP27 (62), has been implicated in cellular senescence, differentiation, and involvement in myogenic differentiation (63), and reduced cell spreading on fibronectin (52). Previous studies showed the fragment of ARA55, but not the fragment of FHL2, could interact with AR LBD (45). After we discovered the FXXLY motif from phage display screening, we found one FXXLY motif existing in the C terminus of FHL2. By aligning the FXXL(F/Y) motifs, we found the motifs were all located in the linkers of LIMs or next to the LIMs (Fig. 2A). These data strongly suggest that these motifs may have similar functions. Our results showed that these two motif-containing linkers with two nearby LIMs could interact with AR in GST pull-down assay. Furthermore, these two motifs also play important roles to mediate the interaction between FHL2 and AR. Because these peptide motifs are located in the conserved positions of the LIMs proteins, we predict some LIM domain-containing proteins with these FXXL(F/Y) motifs, such as paxillin and leupaxin, may be able to interact with AR, and these motifs may play important roles to mediate the interaction between LIM proteins and AR.

(F/W)XXLF motifs has been proven important for AR N-C interaction (43, 44), and our screened peptides containing similar functional motifs also can interrupt AR N-C interaction. Although the screened out peptides share similar interaction motifs, the differences in the motif and in the surrounding sequences lead to the functional property differences of the peptides. We observed that the Phe in the +1 position of the motifs seems to have higher fidelity compared with Trp, the positively charged amino acids Lys and Arg, but not His, in the –1 position, and hydrophobic amino acids Phe, Trp, Tyr, and Leu in the +6 position of the motifs were preferred, but this still cannot predict the function of these peptides. The blocking of AR N-C interaction by the peptide seems to correlate with the peptide binding affinity to AR, as peptides 3-18, 4-1, 4-67, and D30 can bind more firmly to AR and had better blocking ability on AR N-C interaction. The blocking abilities of these peptides, however, may not be able to reflect their ability to block the AR transactivation. Recently, our group (52) demonstrated that supervillin, an AR coregulator, can block AR N-C interaction but can enhance AR transactivation. This proved that AR N-C interaction is important but not essential for enhancement of AR transactivation. Chang and McDonnell (38) also observed that overexpression of the AF2-binding peptides can block the AR N-C interaction but will not influence AR transactivation. They proposed that both AR N terminus and AR-AF2-binding peptides in the presence of agonist could hold the AR-LBD in active conformation, stabilize the whole AR structure, and allow the AR N terminus to interact with

proper coregulators. The other possibility is the peptide-AR interaction surface was limited in restricted areas compared with coregulator-AR interaction, which has multiple contact sites, and the peptides may not have a high affinity to compete with coregulator binding to AR.

In conclusion, by using phage display techniques to screen peptides that may interact and modulate AR function, we found new (F/W)XXL(F/W) and FXXLY signature motifs. The functional similarity of this motif cluster combined with flanking sequence effects extended the importance of this motif cluster in AR-related protein-protein interactions.

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#### REFERENCES

- Chang, C. S., Kokontis, J., and Liao, S. T. (1988) *Science* **240**, 324–326
- Chang, C. S., Kokontis, J., and Liao, S. T. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7211–7215
- Lubahn, D. B., Joseph, D. R., Sullivan, P. M., Willard, H. F., French, F. S., and Wilson, E. M. (1988) *Science* **240**, 327–330
- Trapman, J., Klaassen, P., Kuiper, G. G., van der Korput, J. A., Faber, P. W., van Rooij, H. C., Geurts van Kessel, A., Voorhorst, M. M., Mulder, E., and Brinkmann, A. O. (1988) *Biochem. Biophys. Res. Commun.* **153**, 241–248
- Tilley, W. D., Marcelli, M., Wilson, J. D., and McPhaul, M. J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 327–331
- Keller, E. T., Ershler, W. B., and Chang, C. (1996) *Front. Biosci.* **1**, 59–71
- Roy, A. K., Lavrovsky, Y., Song, C. S., Chen, S., Jung, M. H., Velu, N. K., Bi, B. Y., and Chatterjee, B. (1999) *Vitam. Horm.* **55**, 309–352
- Williams, S. P., and Sigler, P. B. (1998) *Nature* **393**, 392–396
- Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998) *Nature* **395**, 137–143
- Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature* **389**, 753–758
- Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **375**, 377–382
- Bourguet, W., Germain, P., and Gronemeyer, H. (2000) *Trends Pharmacol. Sci.* **21**, 381–388
- Matias, P. M., Donner, P., Coelho, R., Thomaz, M., Peixoto, C., Macedo, S., Otto, N., Jochko, S., Scholz, P., Wegg, A., Basler, S., Schafer, M., Egner, U., and Carrondo, M. A. (2000) *J. Biol. Chem.* **275**, 26164–26171
- Sack, J. S., Kish, K. F., Wang, C., Attar, R. M., Kiefer, S. E., An, Y., Wu, G. Y., Scheffler, J. E., Salvati, M. E., Krystek, S. R., Jr., Weinmann, R., and Einspahr, H. M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 4904–4909
- Fritsch, M., Leary, C. M., Furlow, J. D., Ahrens, H., Schuh, T. J., Mueller, G. C., and Gorski, J. (1992) *Biochemistry* **31**, 5303–5311
- Beekman, J. M., Allan, G. F., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1993) *Mol. Endocrinol.* **7**, 1266–1274
- Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995) *Nature* **377**, 397–404
- Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) *Cell* **87**, 953–959
- Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R. A. (1997) *Nature* **387**, 49–55
- Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387**, 43–48
- Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997) *Cell* **89**, 373–380
- Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) *Nature* **389**, 194–198
- Wong, J., Shi, Y. B., and Wolffe, A. P. (1997) *EMBO J.* **16**, 3158–3171
- Le Douarin, B., Nielsen, A. L., Garnier, J. M., Ichinose, H., Jeanmougin, F., Losson, R., and Chambon, P. (1996) *EMBO J.* **15**, 6701–6715
- Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) *Nature* **387**, 733–736
- Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387**, 677–684
- McInerney, E. M., Rose, D. W., Flynn, S. E., Westin, S., Mullen, T. M., Krones, A., Inostroza, J., Torchia, J., Nolte, R. T., Assa-Munt, N., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1998) *Genes Dev.* **12**, 3357–3368
- Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1995) *Science* **270**, 1354–1357
- Heinlein, C. A., Ting, H. J., Yeh, S., and Chang, C. (1999) *J. Biol. Chem.* **274**, 16147–16152
- Heinlein, C. A., and Chang, C. (2002) *Endocr. Rev.* **23**, 175–200
- Parmley, S. F., and Smith, G. P. (1989) *Adv. Exp. Med. Biol.* **251**, 215–218
- Scott, J. K., and Smith, G. P. (1990) *Science* **249**, 386–390
- Paige, L. A., Christensen, D. J., Gron, H., Norris, J. D., Gottlin, E. B., Padilla, K. M., Chang, C. Y., Ballas, L. M., Hamilton, P. T., McDonnell, D. P., and

- Fowlkes, D. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3999–4004
34. Norris, J. D., Paige, L. A., Christensen, D. J., Chang, C. Y., Huacani, M. R., Fan, D., Hamilton, P. T., Fowlkes, D. M., and McDonnell, D. P. (1999) *Science* **285**, 744–746
35. Chang, C., Norris, J. D., Gron, H., Paige, L. A., Hamilton, P. T., Kenan, D. J., Fowlkes, D., and McDonnell, D. P. (1999) *Mol. Cell. Biol.* **19**, 8226–8239
36. Hall, J. M., Chang, C. Y., and McDonnell, D. P. (2000) *Mol. Endocrinol.* **14**, 2010–2023
37. Yeh, S., and Chang, C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5517–5521
38. Chang, C. Y., and McDonnell, D. P. (2002) *Mol. Endocrinol.* **16**, 647–660
39. Fujimoto, N., Yeh, S., Kang, H. Y., Inui, S., Chang, H. C., Mizokami, A., and Chang, C. (1999) *J. Biol. Chem.* **274**, 8316–8321
40. Kang, H. Y., Yeh, S., Fujimoto, N., and Chang, C. (1999) *J. Biol. Chem.* **274**, 8570–8576
41. Muller, J. M., Isele, U., Metzger, E., Rempel, A., Moser, M., Pscherer, A., Breyer, T., Holubarsch, C., Buettner, R., and Schule, R. (2000) *EMBO J.* **19**, 359–369
42. Zhou, Z. X., He, B., Hall, S. H., Wilson, E. M., and French, F. S. (2002) *Mol. Endocrinol.* **16**, 287–300
43. He, B., Kemppainen, J. A., and Wilson, E. M. (2000) *J. Biol. Chem.* **275**, 22986–22994
44. He, B., Bowen, N. T., Minges, J. T., and Wilson, E. M. (2001) *J. Biol. Chem.* **276**, 42293–42301
45. He, B., Minges, J. T., Lee, L. W., and Wilson, E. M. (2002) *J. Biol. Chem.* **277**, 10226–10235
46. Hsu, C., Yeh, S., and Chang, C. (2002) *84th Endocrine Society Annual Meeting*, San Francisco, June 19–22, p. 527, Abstr. P3–144, The Endocrine Society Press, Bethesda, MD
47. Gregory, C. W., He, B., and Wilson, E. M. (2001) *J. Mol. Endocrinol.* **27**, 309–319
48. He, B., Lee, L. W., Minges, J. T., and Wilson, E. M. (2002) *J. Biol. Chem.* **277**, 25631–25639
49. Callewaert, L., Verrijdt, G., Christiaens, V., Haelens, A., and Claessens, F. (2003) *J. Biol. Chem.* **278**, 8212–8218
50. Santoro, M., Dathan, N. A., Berlingieri, M. T., Bongarzone, I., Paulin, C., Grieco, M., Pierotti, M. A., Vecchio, G., and Fusco, A. (1994) *Oncogene* **9**, 509–516
51. Yeh, S., Miyamoto, H., Shima, H., and Chang, C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5527–5532
52. Ting, H. J., Yeh, S., Nishimura, K., and Chang, C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 661–666
53. Shaw, P. A., Rittenberg, P. V., and Brown, T. J. (2001) *Gynecol. Oncol.* **80**, 132–138
54. Ito, K., Adachi, S., Iwakami, R., Yasuda, H., Muto, Y., Seki, N., and Okano, Y. (2001) *Eur. J. Biochem.* **268**, 2725–2732
55. Chu, P. H., Bardwell, W. M., Gu, Y., Ross, J., Jr., and Chen, J. (2000) *Mol. Cell. Biol.* **20**, 7460–7462
56. Kong, Y., Shelton, J. M., Rothermel, B., Li, X., Richardson, J. A., Bassel-Duby, R., and Williams, R. S. (2001) *Circulation* **103**, 2731–2738
57. Wixler, V., Geerts, D., Laplantine, E., Westhoff, D., Smyth, N., Aumailley, M., Sonnenberg, A., and Paulsson, M. (2000) *J. Biol. Chem.* **275**, 33669–33678
58. Martin, B., Schneider, R., Janetzky, S., Waibler, Z., Pandur, P., Kuhl, M., Behrens, J., von der Mark, K., Starzinski-Powitz, A., and Wixler, V. (2002) *J. Cell Biol.* **159**, 113–122
59. Wei, Y., Renard, C. A., Labalette, C., Wu, Y., Levy, L., Neuveut, C., Prieur, X., Flajolet, M., Prigent, S., and Buendia, M. A. (2003) *J. Biol. Chem.* **278**, 5188–5194
60. Wang, X., Yang, Y., Guo, X., Sampson, E. R., Hsu, C. L., Tsai, M. Y., Yeh, S., Wu, G., Guo, Y., and Chang, C. (2002) *J. Biol. Chem.* **277**, 15426–15431
61. Jia, Y., Ransom, R. F., Shibamura, M., Liu, C., Welsh, M. J., and Smoyer, W. E. (2001) *J. Biol. Chem.* **276**, 39911–39918
62. Shibamura, M., Iwabuchi, Y., and Nose, K. (2002) *Cell Struct. Funct.* **27**, 21–27
63. Nishiya, N., Tachibana, K., Shibamura, M., Mashimo, J. I., and Nose, K. (2001) *Mol. Cell. Biol.* **21**, 5332–5345