Androgen receptor (AR) is a transcriptional factor that belongs to the nuclear receptor superfamily (1–5). After androgen binding 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 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 α-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 acetylation 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 γ (PPARγ), two consecutive LXXLL motifs of the coregulator fragment bridge the peroxisome proliferator-activated receptor γ dimer with each LXXLL motif binding to one peroxisome proliferator-activated receptor γ 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 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 α (ERα), estradiol-ERβ, and antagonist-bound ER-associated peptides (33–36). Rat AR (rAR) amino acid sequence has 100% homology with human AR (hAR) in the

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.

**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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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 testosteron, 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 Plasminoids—**5a-Dihydrotestosterone (5a-DHT), testosterone, 1,25(OH)2D3, and estradiol were obtained from Sigma. Ph.D.

12TM 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 (lacking the initial 37 amino acids) was constructed for mammalian two-hybrid assay (37). The pCMX-VP16-hERα, pCMX-VP16-VDR, and pCMX-GAL4-XXR constructs were generated by PCR of the full-length human ERα, VDR, and RXRα cDNA with primers containing EcoRI and BamHI sites for hERα and VDR and EcoRI and NheI sites for RXRα 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-ARA54 constructs were generated by PCR of pSG5-ARA70, pSG5-ARA54, and pSG5-ARA70C 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-SP90 (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-DBD vector via EcoRI site and purified as described by the manufacturer (Amersham Biosciences). The purified proteins were suspended in 100 μl of interaction buffer (20 mM HEPES, pH 7.9, 150 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 0.1% (w/v) bovine serum albumin, 1 mM phenylmethylsulfonil fluoride, and 10% glycerol), mixed with 5 μl of 108-S-labeled Tnt-expressed hAR full-length proteins (Tnt-coupled hAR GST vector, Promega) in the presence or absence of 1 μM 5α-DHT, and incubated at 4°C for 2 h. After several washes with NENT buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl2, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.5% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonil 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 1012 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 (FW)XXL/F(W) or FXXXL 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 (FW)XXL/F(W) or FXXXL 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 ERα 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 FXXXL, may be able to bind specifically to AR.

**Identification of FXXXL Signature Motif in AR and AR Coregulators**

ARA70, ARA55, ARA54, and FHL2, WXXXL Motif in AR and FXXXL Motif in FHL2—We then surveyed the amino
acid sequence within AR and AR coregulators identified previously, and we found that AR had the (F/W)XXL motif, and FHL2 showed 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 FXXL in AR was important in AR-HSP90 interaction, and signature motifs, FXXL and FXXYL, 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 FXXL 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 5α-DHT can induce AR transactivation to 8-fold (lanes 1 versus 2). Addition of wt pSG5-ARA70N-FXXL 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 FXXL motif and one FXXYL motif, we mutated both of these motifs to AXXAA and tested their influence on the AR transactivation. As expected, we found the mt peptide with FXXYL motif (1) showed much less induction effect than the wt peptide (2).

**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 FXXL motif and peptide 4-9 with the FLY motif, and we performed single mutations each time. We changed the first amino acid Phe of peptide 3-18 to Trp (m1), or we changed the second amino acid Phe to Trp (m2), or we changed the second amino acid Phe to Tyr (m3), or we changed the first amino acid Trp to Phe (m4), or we changed the first amino acid Trp to Tyr (m5), or we changed the first amino acid Tyr to Phe (m6), or we changed the first amino acid Tyr to Trp (m7), or we changed the first amino acid Tyr to Tyr (m8), or we changed the second amino acid Trp to Phe (m9), or we changed the second amino acid Trp to Trp (m10), or we changed the second amino acid Trp to Tyr (m11), or we changed the second amino acid Tyr to Phe (m12), or we changed the second amino acid Tyr to Trp (m13), or we changed the second amino acid Tyr to Tyr (m14). The wt and mt 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 to Trp or Tyr (m2). The changing of Trp to Phe can further enhance the peptide interaction with AR by 20-fold. The changing of Phe to Trp (m2) or to Tyr (m3) or to Tyr (m4) can further enhance the peptide interaction with AR by 20-fold. The changing of Trp to Phe (m2) or to Tyr (m3) or to Tyr (m4) can further enhance the peptide interaction with AR by 20-fold.

Taken together, the data in Fig. 4 demonstrated that these three specific amino acids, Phe, Trp, and Tyr, within the newly identified motifs (LIM) domain-containing proteins, were located in the linkers or just next to the LIMs.
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 /H11002 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 /H11001 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 /H11001 position of the motifs, 4-1 (FXXLF), 4-9 (WXXLF), 3-18 (FXFLYF), 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 /H11002 position of the FXXLF motif, and ARA54 had one Tyr in the /H11001 position of the FXXLF motif. We experimental procedures were done as in B except using wt [35S]methionine-labeled hAR only and substituting GST-HSP90 with wt or mt, full-length, or fragment of GST-AR coregulators.

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-AXXXA, wt pSG5-FHL2-FXXLF/FY), or mt pSG5-FHL2-AXXXA(AA), 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 5α-DHT were added for another 16 h. Relative LUC activity was calculated by the dual luciferase system.

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 FXXLF(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 AXAA 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) [35S]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 μM 5α-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 FXXLF motif will cause weaker AR-AR interaction protein interactions. Similar experimental procedures were done as in B except using wt [35S]methionine-labeled hAR only and substituting GST-HSP90 with wt or mt, full-length, or fragment of GST-AR coregulators.
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 co-regulator 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 FQQLF and WHTLF 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)XXLF, we were interested to know if our newly identified AR-interacting peptides with (F/W)XXLF 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 FXXLF motif-containing peptides may suppress AR N-C interaction, 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)XXLF in AR N terminus and FXXLY motif-containing coactivators (43, 44, 47, 48). Although the
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), which 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)XXLF 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 third 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)XXLF motif 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, and 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 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)XXLF motif-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-γ pathway (29), and was activated in invasive ovarian cancer (53). ARA70 had two lysines in −1 and +2 position of its FXXLF motif (127KFKLLF332), 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 FXFLL motif (148,149), 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 FXFLL 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 β-adrenergic stimulation (55, 56). FHL2 has been linked to the integrin pathway (57), the Rho signal pathway (58), and 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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