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Activation Function 2 in the Human Androgen Receptor Ligand Binding Domain Mediates Interdomain Communication with the NH_2-terminal Domain*

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Activation function 2 in the ligand binding domain of nuclear receptors forms a hydrophobic cleft that binds the L*XX*LL motif of p160 transcriptional coactivators. Here we provide evidence that activation function 2 in the androgen receptor serves as the contact site for the androgen dependent NH_2- and carboxyl-terminal interaction of the androgen receptor and only weakly interacts with p160 coactivators in an L*XX*LL-dependent manner. Mutagenesis studies indicate that it is the NH_2-/carboxyl-terminal interaction that is required by activation function 2 to stabilize helix 12 and slow androgen dissociation critical for androgen receptor activity *in vivo*. The androgen receptor recruits p160 coactivators through its NH_2-terminal and DNA binding domains in an L*XX*LL motif-independent manner. The results suggest a novel function for activation function 2 and a unique mechanism of nuclear receptor transactivation.

Steroid receptors interact with coactivators during the recruitment of active transcription initiation complexes required for hormone-regulated gene transcription (1). Transcriptional activation domains in the steroid receptors that may mediate these interactions include activation function 1 in the NH_2-terminal domain and activation function 2 (AF2)[1] in the ligand binding domain (LBD). Recent studies have focused on a family of p160 coactivators that interact with the AF2 region that include steroid receptor coactivator 1 (SRC1) (2) and the human transcriptional intermediary factor 2 (TIF2) (3). SRC1 and TIF2 contain distinct nuclear receptor interaction domains in the central and/or carboxyl-terminal regions (3, 4). Mutagenesis studies demonstrated a functional link between AF2 activity in the LBD and the binding of p160 coactivators (5, 6). The p160 coactivators interact with the AF2 hydrophobic surface of the LBD through conserved L*XX*LL motifs that form amphipathic α helices (7, 8). Recent co-crystal structures of nuclear receptor LBDs and L*XX*LL motif fragments confirm that AF2 recruits TIF2 and SRC1 through their L*XX*LL motifs (6, 9–11). A multistep mechanism for transcriptional activation by nuclear receptors involves hormone-dependent recruitment and association through these L*XX*LL binding motifs of histone acetyltransferase activity associated with the p160 coactivator family, CREB-binding protein/p300, and p300/CREB-binding protein-associated factor, resulting in chromatin remodeling (12, 13) and the formation of a transcriptionally competent Srb/mediator coactivator complex (thyroid hormone receptor-associated protein/vitamin D receptor-interacting protein) complex (14).

However, androgen receptor (AR) AF2 activity is not detected in a variety of mammalian cell lines (15–18) despite homology of the region with other nuclear receptors. We therefore investigated the mechanism whereby AR recruits p160 coactivators and the role of AF2 in AR function. It is demonstrated that weak interactions between the AR LBD and SRC1 and TIF2 correspond with weak AR AF2 activity. The AF2 surface in the AR LBD instead functions as a strong interaction site for the AR NH_2-terminal domain that is required for AR activity *in vivo*. SRC1 and TIF2 interact with the AR NH_2-terminal and DNA binding domain (DBD) regions in an L*XX*LL motif-independent manner mediated by the carboxyl-terminal region of SRC1 and the carboxyl-terminal and central regions of TIF2.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—TIF2 constructs were as described previously (3). pCR3.1SRC1a was provided by Ming-Jer Tsai and Bert O'Malley (2, 19). The GALSRC1 constructs were prepared as follows: GALSRC1–1441 by using the pCR3.1hSRC1a *Bsp*HI(blunt)/*Xba*I fragment cloned into pGALO (*Sma*I/*Xba*I); GALSRC1–780 by digesting GALSRC1–1441with *Bam*HI/*Xba*I and religating; GALSRC568–1441 by cloning the *Eco*RI(blunt)/*Xba*I pCR3.1hSRC1a fragment into pGALO (*Sal*I(blunt)/*Xba*I); GALSRC564–800 and GALSRC564–1138 using the polymerase chain reaction (PCR) amplification using 5′-*Nde*I and 3′-*Xba*I primers and the fragment cloned into pGALO (*Nde*I/*Xba*I); GALSRC568–954 by digesting GALSRC568–1441 with *Sac*I/*Xba*I and

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[1] The abbreviations used are: AF2, activation function 2; AR, androgen receptor; LBD, ligand binding domain; TIF2, transcriptional intermediary factor 2; SRC1, steroid receptor coactivator 1; PCR, polymerase chain reaction; N/C, NH_2-terminal and carboxyl-terminal; DHT, dihydrotestosterone; DBD, DNA binding domain; GST, glutathione *S*-transferase; MMTV, mouse mammary tumor virus; CHO, Chinese hamster ovary.

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religating; GALSRC1139–1441 by cloning the *Hin*dIII(blunt)/*Xba*I pCR3.1hSRC1a fragment into pGALO (*Sal*I(blunt)/*Xba*I); GALSRC1139–1437 by cloning the PCR-amplified fragment of pCR3.1hSRC1a using 5′-*Bam*HI and 3′-*Xba*I primers into pGALO (*Bam*HI/*Xba*I). VPAR (human AR residues 1–919) and VPAR1–660 (AR NH_2-terminal and DBD residues 1–660) contained the VP16 transactivation domain (16, 20). VPSRC1 constructs were created as follows: VPSRC1–1441 by excising full-length SRC1a from pCR3.1hSRC1a using *Bsp*HI (blunt)/*Xba*I and cloned into pNLVP16 (*Xho*I(blunt)/*Xba*I); VPSRC568–1441 by cloning the *Eco*RI(blunt)/*Xba*I pCR3.1hSRC1a fragment into pNLVP16 (*Sal*I(blunt)/*Xba*I). VPSRC564–800 by amplification of pCR3.1hSRC1a using a 5′ *Nde*I and 3′ *Xba*I primers into pNLVP16 (*Nde*I/*Xba*I); VPSRC1139–1441 by cloning the *Hin*dIII(blunt)/*Xba*I fragment of pCR3.1hSRC1a into pNLVP16 (*Sal*I (blunt)/*Xba*I). Single base mutations in the AR LBD were created by PCR mutagenesis, and all constructs were verified by DNA sequencing.

Mammalian Two Hybrid Assay—The NH_2-terminal and carboxyl-terminal (N/C) interaction assay between the AR NH_2- and carboxyl-terminal regions was determined using GALAR624–919, a fusion protein with *Saccharomyces cerevisiae* GAL4 DBD residues 1–147 and AR LBD residues 624–919 in pGALO (16, 20) with VPAR1–660 (AR NH_2-terminal and DBD residues 1–660) containing the herpes simplex virus VP16 transactivation domain residues 411–456 (16, 20). CHO cells were transfected using DEAE-dextran (16, 20) with 1 μg of GAL and VP16 fusion vectors and 5 μg of G5E1b-luciferase reporter. Activity was determined as indicated or in the presence or absence of 1 μM dihydrotestosterone (DHT). Fold induction relative to the no hormone control is indicated above the bars. For interactions between TIF2 and SRC1, GALAR624–919 was cotransfected with VPTIF2 or VPSRC1 fusion constructs in the CHO two hybrid assay. VPAR and VPAR1–660 were expressed with GALTIF2 or GALSRC1 mutants containing the GAL4 DBD. Control interactions were with pNLVP16 (VP16).

In Vitro Binding Assays—GST fusion proteins were expressed in XL1-Blue *Escherichia coli* cells treated with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h after log phase growth. Bacteria were sonicated and centrifuged, and the supernatant was incubated with glutathione-agarose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C. Beads are washed five times with 0.5% Nonidet P-40, 1 mM EDTA, 0.1 M NaCl, 0.02 M Tris-HCl, pH 8.0, and incubated for 2 h at 4 °C with and without 0.2 μM DHT, and *in vitro* translated proteins were labeled with 25 μCi of [^{35}S]methionine (NEN Life Science Products) using the TNT T7 quick coupled transcription/translation system (Promega) in the presence and absence of 0.2 μM DHT. Beads were centrifuged, washed five times, and boiled in SDS. Input lanes contain approximately 20% that used for the binding reactions. GSTAR1–660 was prepared by excising AR1–660 coding for AR NH_2-terminal and DBD residues 1–660 from GALAR using *Tth*III(blunt)/*Bam*HI and cloned into pGEX-5X-1 (Amersham Pharmacia Biotech) at *Sma*I/*Bam*HI. GSTTIF2M (TIF2 624–1141) and GSTTIF2C (TIF2 1144–1464) were PCR amplified, and fragments were cloned in pGEX-2T (*Eco*RI/*Bam*HI). TIF2 carboxyl-terminal residues 1143–1464 were amplified from pSG5TIF2 by PCR and cloned into pcDNA3HA (provided by Yue Xiong) at the *Bam*HI/*Xba*I sites to prepare ^{35}S-labeled TIF2-C. pGEMhAR (provided by Jiann-an Tan and Frank S. French) coded for full-length human AR residues 1–919 and was used to prepare ^{35}S-AR. GSTAR1–565 was prepared by digesting GALAR1–919 with *Hin*dIII(blunt)/*Bam*HI and cloned into pGEX-3X at *Eco*RI(blunt)/*Bam*HI. pcDNA3HA-AR-LBD expressing the human AR LBD residues 624–919 was digested from GALAR624–919 with *Bam*HI/*Xba*I and cloned in the same sites in pcDNA3HA for *in vitro* translation.

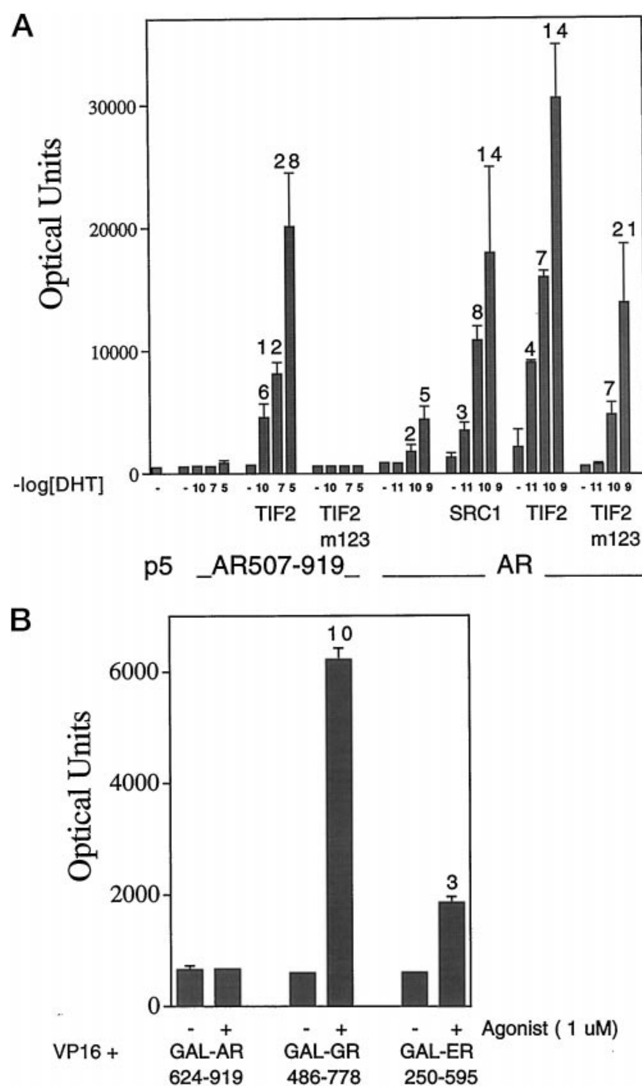

FIG. 1. **Transcriptional activation by AR.** *A*, effect of overexpression of p160 coactivators. The expression vector pCMVhAR507–919 coding for the AR DBD and LBD (AR507–919, 50 ng) was cotransfected without or with 2 μg of pSG5TIF2 or pSG5TIF2 m123 and 5 μg of mouse mammary tumor virus (MMTV) luciferase reporter. Full-length human AR expression vector pCMVhAR (AR, 20 ng) was cotransfected without or with 6 μg of pSG5SRC1, pSG5TIF2, or pSG5TIF2 m123 together with 5 μg of the MMTV luciferase reporter. The parent vector pCMV5 (*p5*, 50 ng) was cotransfected with 5 μg of the luciferase reporter. Monkey kidney CV-1 cells were transfected using calcium phosphate (34). The last two leucine residues in each of three L*XX*LL motifs were mutated to alanine in pSG5TIF2 m123 (3). *B*, transcriptional activity of AR, glucocorticoid receptor (*GR*), and estrogen receptor (*ER*) LBDs expressed as fusion proteins with the GAL4 DBD. CHO cells were cotransfected with 1 μg of pNLVP16 parent vector (*VP16*) together with 1 μg of GALAR624–919, GALGR486–778, or GALER250–595, and 5 μg of G5E1b-luciferase reporter (16, 20). Cells were incubated 24 h with or without 1 μM DHT, dexamethasone, or 17β-estradiol with the cognate receptor fragment.

RESULTS AND DISCUSSION

Expression of the AR DBD and LBD fragment AR507–919 (Fig. 1*A*) or AR LBD residues 624–919 fused with the GAL4 DBD (GALAR624–919, Fig. 1*B*) shows little or no induction of transcriptional activity indicating the absence of AF2 activity. In contrast, agonist-dependent AF2 activity of the GAL4-glucocorticoid or estrogen receptors LBD fusion proteins were 16 ± 6-fold and 3.6 ± 0.3-fold (Fig. 1*B*). Lack of AF2 activity by the AR LBD might result from failure to recruit p160 coactivators. Moreover, in transient cotransfection assays, expression of SRC1 or TIF2 increased full-length AR transcriptional activity about 3–6-fold, which surprisingly was only partially diminished by mutation of the three L*XX*LL motifs in TIF2 (TIF2 m123, Fig. 1*A*) and SRC1 (21), suggesting that p160 coactivators can increase AR transactivation in an L*XX*LL motif-independent manner. We therefore investigated the interaction of AR with SRC1 and TIF2.

Of several fragments tested in a mammalian two hybrid assay, only TIF624–1287 and SRC568–1441 each with three (3) and four L*XX*LL motifs, respectively, interacted 2–3-fold with the AR LBD (GAL-AR624–919, Fig. 2), which was less than 10% the activity observed in the N/C interaction (see below and Fig. 4*A*), suggesting weak coactivator binding affinity compared with the interaction between the NH_2- and carboxyl-terminal AR domains. Although results are shown at

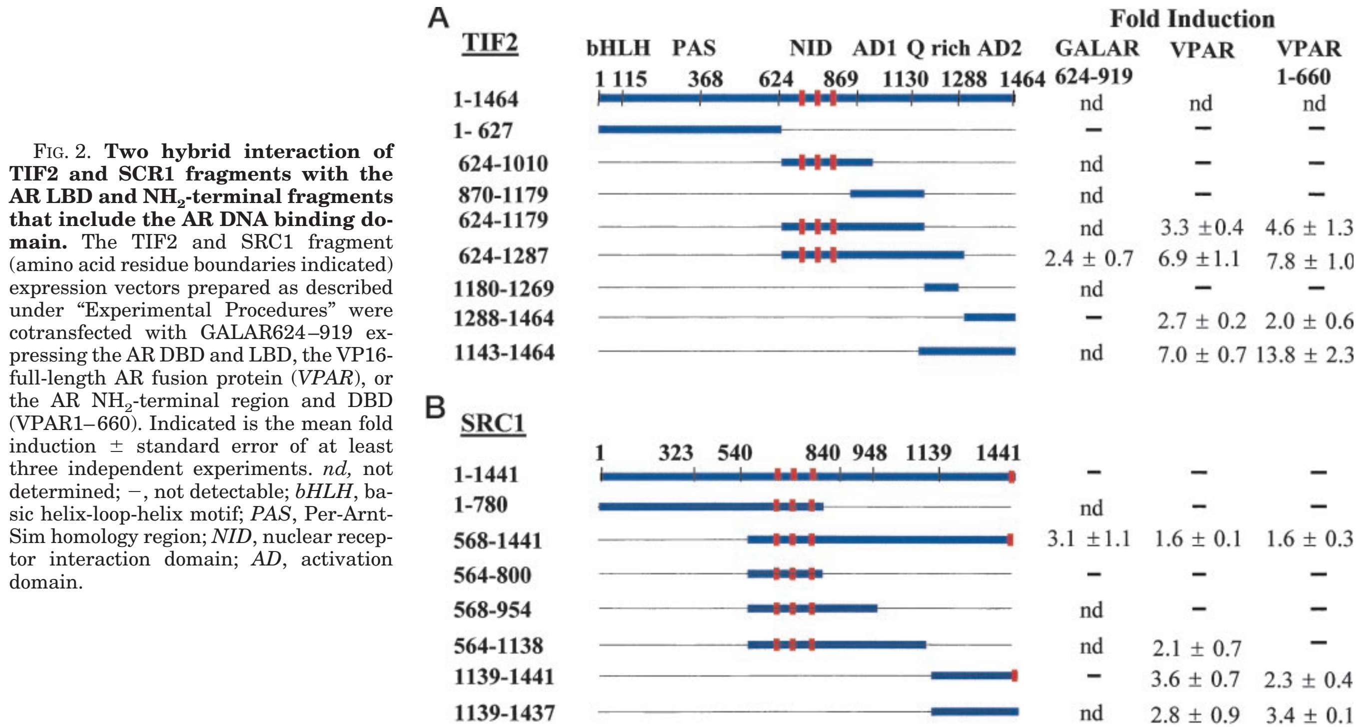

FIG. 2. **Two hybrid interaction of TIF2 and SCR1 fragments with the AR LBD and NH_2-terminal fragments that include the AR DNA binding domain.** The TIF2 and SRC1 fragment (amino acid residue boundaries indicated) expression vectors prepared as described under "Experimental Procedures" were cotransfected with GALAR624–919 expressing the AR DBD and LBD, the VP16-full-length AR fusion protein (*VPAR*), or the AR NH_2-terminal region and DBD (VPAR1–660). Indicated is the mean fold induction ± standard error of at least three independent experiments. *nd,* not determined; −, not detectable; *bHLH*, basic helix-loop-helix motif; *PAS*, Per-Arnt-Sim homology region; *NID*, nuclear receptor interaction domain; *AD*, activation domain.

TABLE I
Summary of AR LBD mutants

Apparent equilibrium binding affinity and dissociation half-times were determined in COS cells at 37 °C using wild-type or mutant pCMVhAR full-length AR or AR507–919 coding for the DBD and LBD (20, 33). DHT concentration for at least 10-fold transcriptional activity (MMTV-Luc) was determined in CV-1 cells using pCMVhAR full-length wild-type and mutant. TIF2 two hybrid interaction was determined using VPTIF624–1287 and GALAR624–919 with wild-type or mutant sequence in CHO cells at 1 μM DHT, shown as fold induction relative to activity determined in the absence of hormone. The AR-TIF2 interaction was also determined by cotransfecting pCMVhAR507–919 and pSG5TIF2 with the MMTV-luciferase reporter in CV-1 cells assayed at 10 nM DHT. The N/C interaction (16, 20) shows the DHT concentration for at least 3-fold induction using VPAR1–660 and GALAR624–919 with wild-type or mutant sequence determined in CHO cells. The N/C interaction was also determined by cotransfecting pCMVhAR1–660 and pCMVhAR1–503 and MMTV-Luc in CV-1 cells at 10 nM DHT. Androgen insensitivity syndrome (AIS) stage is on a scale where 1 is normal and 7 is complete (44). AF2, helix, and loop regions were based on crystal structures of estrogen and progesterone receptor LBDs (29, 30). Signature sequence is amino acid residues 718–741 in human AR (31). PC indicates somatic prostate cancer mutation; ++, activity equivalent to wild-type; +, greatly reduced but detectable activity; −, not detectable; nd, not determined.

Mutation	Binding affinity	Dissociation half-time		MMTV-Luc	TIF2 interaction		N/C interaction		AIS mutation, stage	Helix
		AR1–919	AR507–919		Two hybrid fold	AR507–919 + TIF2	Two hybrid	AR507–919 + AR1–503		
	nM	*min*		*nM DHT*			*nM DHT*			
Wild-type	0.48 ± 0.25	149 ± 32	44 ± 4	0.001	2.1 ± 0.5	++	0.1	++		1
AF2 domain										
V716R	0.40 ± 0.04	28 ± 3	20 ± 2	0.1	1.0 ± 0	−	−	−		3
K720A	0.54 ± 0.15	134 ± 11	26 ± 4	0.001	1.2 ± 0.2	+	1	++	K720E, PC	3
I737T	0.44 ± 0.20	147 ± 38	42 ± 2	0.1	1.1 ± 0.04	+	100	++	3	4
E897K	0.52 ± 0.18	67 ± 11	38 ± 2	1	1.0 ± 0	−	−	−		12
I898T	0.34 ± 0.02	42 ± 5	7 ± 1	0.1	1.7 ± 0.2	++	−	+	6,7	12
Signature sequence										
L722A	0.58 ± 0.13	102 ± 24	nd	1	1.9 ± 0.2	nd	10		L722F, 6–7	3-4 loop
F725L	0.82 ± 0.32	127 ± 39	38 ± 5	0.1	1.2 ± 0.1	++	100	++	3,4	3-4 loop
L728A	0.56 ± 0.17	82 ± 9	nd	0.1	1.4 ± 0.03	nd	100	nd	L728S, 3–5	3-4 loop
Y739A	0.65 ± 0.16	34 ± 3	8 ± 1	0.1	2.7 ± 0.4	++	10	++	Y739R, 6–7	4
W741A	0.67 ± 0.31	33 ± 5	7 ± 1	0.1	1.9 ± 0.4	++	100	+	W741R, 6–7	5
Other domains										
H729A	0.70 ± 0.30	132 ± 32	nd	0.01	3.3 ± 1.0	nd	0.1	nd		3-4 loop
A735T/V736L	0.42 ± 0.02	216 ± 56	47 ± 6	0.001	2.7 ± 0.5	++	0.1	++		4
Q798A	0.48 ± 0.19	148 ± 27	37 ± 5	0.001	1.2 ± 0.3	++	0.1	++	Q798E, 5	7–8 loop
Q867H/P868D	0.52 ± 0.19	272 ± 57	50 ± 7	0.001	2.9 ± 0.6	++	0.1	++		11-12 loop
V889M	0.46 ± 0.25	18 ± 2	3 ± 0	1	1.5 ± 0.3	++	100	+	7	11-12 loop

saturating DHT concentrations (1 μM, Fig. 2, Table I), interactions between the p160 coactivators and the AR LBD in the two hybrid assay were detected at 0.01 nM DHT. The LBD regions of the glucocorticoid (486–778) and estrogen (250–595) receptors interacted with these fragments 69 ± 4-fold and 5.9 ± 1.2-fold, and 7.5 ± 1.7 and 8.2 ± 1.7, respectively (data not shown). However, overexpressed TIF2, but not a mutant with three mutated L*XX*LL motifs, increased activation by the AR LBD (AR507–919, Fig. 1*A*), indicating that exogenously expressed coactivators can rescue L*XX*LL motif-dependent AF2 activity in the AR LBD, which as shown below was blocked by site-directed mutations in AF2 (see Fig. 4*B*). The results suggest that the apparent lack of AR AF2 activity results from inefficient L*XX*LL motif-dependent recruitment of endogenous coactivators. Recovery of AF2 by overexpression of p160 coactivators suggests overall retention of nuclear receptor AF2

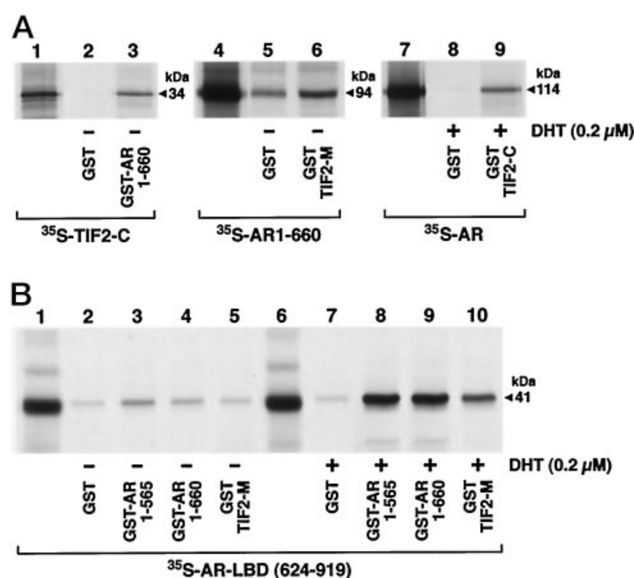

FIG. 3. ***In vitro* interactions between AR, AR fragments, and TIF2 by GST adsorption.** GST adsorption experiments were performed as described under "Experimental Procedures." *A*, the *in vitro* translated ^{35}S-labeled carboxyl-terminal TIF2-C fragment (amino acid residues 1143–1464), the AR NH_2-terminal region and DBD (^{35}S-AR1–660), and full-length AR (^{35}S-AR) were incubated with GST or the indicated GST fusion resins including GST-AR NH_2-terminal region and DBD (GST-AR1–660), the central TIF2 fragment GSTTIF2M (amino acid residues 624–1141), and the carboxyl-terminal TIF2 fragment GSTTIF2-C (residues 1143–1464). *B*, ^{35}S-AR-LBD (624–919) coding for LBD residues 624–919 was reacted with GST or GST fusion protein resins containing the AR NH_2-terminal region (GST-AR1–565), the AR NH_2-terminal region, and DBD (GST-AR1–660) or GSTTIF2M. Incubations were performed in the presence and absence of 0.2 μM DHT.

structure (6, 22, 23).

The role of the AR NH_2-terminal and DBD regions in p160 coactivator recruitment was also investigated using the two hybrid assay. A 2–5-fold interaction between TIF624–1179 or TIF1288–1464 with full-length AR (VPAR, Fig. 2*A*) or the constitutively active NH_2-terminal and DBD fragment AR1–660 (VPAR1–660, Fig. 2*A*) indicates interaction of AR with two regions of TIF2. This interaction increases to 7–14-fold by including the TIF2 glutamine-rich region in TIF624–1287 and TIF1143–1464 (Fig. 2*A*). The results of GST adsorption assays confirm that both the central and carboxyl-terminal domains of TIF2 interact with the AR NH_2-terminal and DBD fragment (Fig. 3*A*). Deletion mapping of SRC1 indicates that mainly its carboxyl-terminal region interacts with AR or the AR NH_2-terminal fragment, and deletion of the SRC1 carboxyl-terminal L*XX*LL motif did not diminish this interaction (Fig. 2*B*). Deletions of AR NH_2-terminal residues 339–499, but not Δ14–150 or Δ142–337, decreased the SRC1 interaction by 50% suggesting this region of the NH_2 terminus contributes to the L*XX*LL-independent interaction with TIF2 and SRC1 (data not shown). We concluded that AR can recruit p160 coactivators through its NH_2-terminal and DBD regions independent of the L*XX*LL motifs by interacting with the carboxyl-terminal region of SRC1 or the carboxyl and central regions of TIF2. Whereas the role of nuclear receptor NH_2-terminal domains in recruiting 160 coactivators has been controversial (4, 24–28), this interaction clearly contributes to the L*XX*LL motif-independent activation of AR.

The function of the AF2 region in AR-mediated gene activation was further investigated by site-directed mutagenesis. Sequence alignments based on steroid receptor LBD crystal structure predictions (29–31) place several androgen insensitive and site-directed mutations within AF2 helices 3, 4, and 12 and a highly conserved nuclear receptor signature sequence (31). Sites for mutagenesis were based on an association with the androgen insensitivity syndrome and with retention of high affinity androgen binding (Table I). All of the AR LBD mutants expressed at similar levels based on binding capacity and retained high affinity binding of the synthetic androgen [^{3}H]R1881 (K_d 0.3–0.7 nM) (Table I) indicating conservation of the ligand binding pocket. However, mutations at V889M, Y739A, W741A, E897K, I898T, and V716R increased the dissociation rate of androgen bound to full-length AR by 2–5-fold (Table I) suggesting an increased association rate and perturbation of the hormone binding region. V889M lies between helices 11 and 12 and causes nearly complete androgen insensitivity (32), increases the androgen dissociation rate (33), and interferes with the androgen-dependent interaction between the AR NH_2- and carboxyl-terminal regions (16, 20). The N/C interaction facilitates AR transcriptional activity at physiological androgen concentrations (34) but, unlike peroxisome proliferator-activated receptor γ (35), is not required for high affinity androgen binding (16).

When expressed in full-length AR, all AF2/signature sequence mutants, with the exception of K720A (see below), required 100–1000-fold higher DHT concentrations to activate an androgen responsive reporter (Table I) indicating greatly reduced function by the mutant ARs. Almost all of the AF2/signature mutants had reduced to undetectable interaction with TIF2 (Table I), SRC1 (data not shown), and the AR NH_2-terminal domain (Table I), whereas most mutants outside this region had wild-type activity. Transcriptional activity at 0.1–1 nM DHT in the absence of an N/C interaction for V716R and E897K (Table I) shows that AR function can be compensated *in vitro* by elevated androgen levels (34), whereas *in vivo*, decreased N/C interaction is associated with partial (I737T, F725L) or complete (I898T, V889M) androgen insensitivity (Fig. 4*A*). Transcriptional activity of the AR DBD/LBD fragment AR507–919 coexpressed with TIF2 or with the AR NH_2-terminal fragment AR1–503 lacking the AR DBD was also decreased by several of the mutations (Fig. 4*B*, Table I). Thus many of the same residues in the AF2/signature sequence serve as both a weak binding site for p160 coactivators and for the AR NH_2-terminal domain. However, the binding sites are not identical, because AR mutant I898T greatly decreased the N/C interaction but retained strong p160 coactivator binding, and K720A retained the N/C interaction but essentially lost p160 coactivator binding (Fig. 4, *A* and *B*).

The functional significance of the AR AF2 region was therefore distinguished by these mutations, K720A and I898T. Lys-720 lies within helix 3 of the AF2 hydrophobic surface in a region highly conserved among nuclear receptors. Lys-720 corresponds to Lys-366 in mouse estrogen receptor, whose mutation eliminates estrogen receptor transcriptional activity (22), and to Lys-301 in peroxisome proliferator-activated receptor γ, where it forms part of an L*XX*LL motif charge clamp (9). K720A retains the transcriptional activity of wild-type AR (Table I) (36), even though the p160 coactivator binding by the LBD is low to undetectable (Fig. 4, *A* and *B*, Table I). Retention of wild-type AR transcriptional activity by K720A correlates with the 21-fold N/C interaction (Fig. 4*A*), but not with the L*XX*LL motif-dependent p160 coactivator recruitment by the AR LBD (Fig. 4, *A* and *B*). An AR somatic mutation at this same site (K720E) in a bone metastases of hormone refractory prostate cancer also retained a normal transcriptional response (37, 38) typical of most prostate cancer AR mutations (39). A mutation at the corresponding Lys-366 in the estrogen receptor distinguished the binding of SRC1 and RIP140, coactivators that interact through L*XX*LL motifs at the same hydrophobic cleft

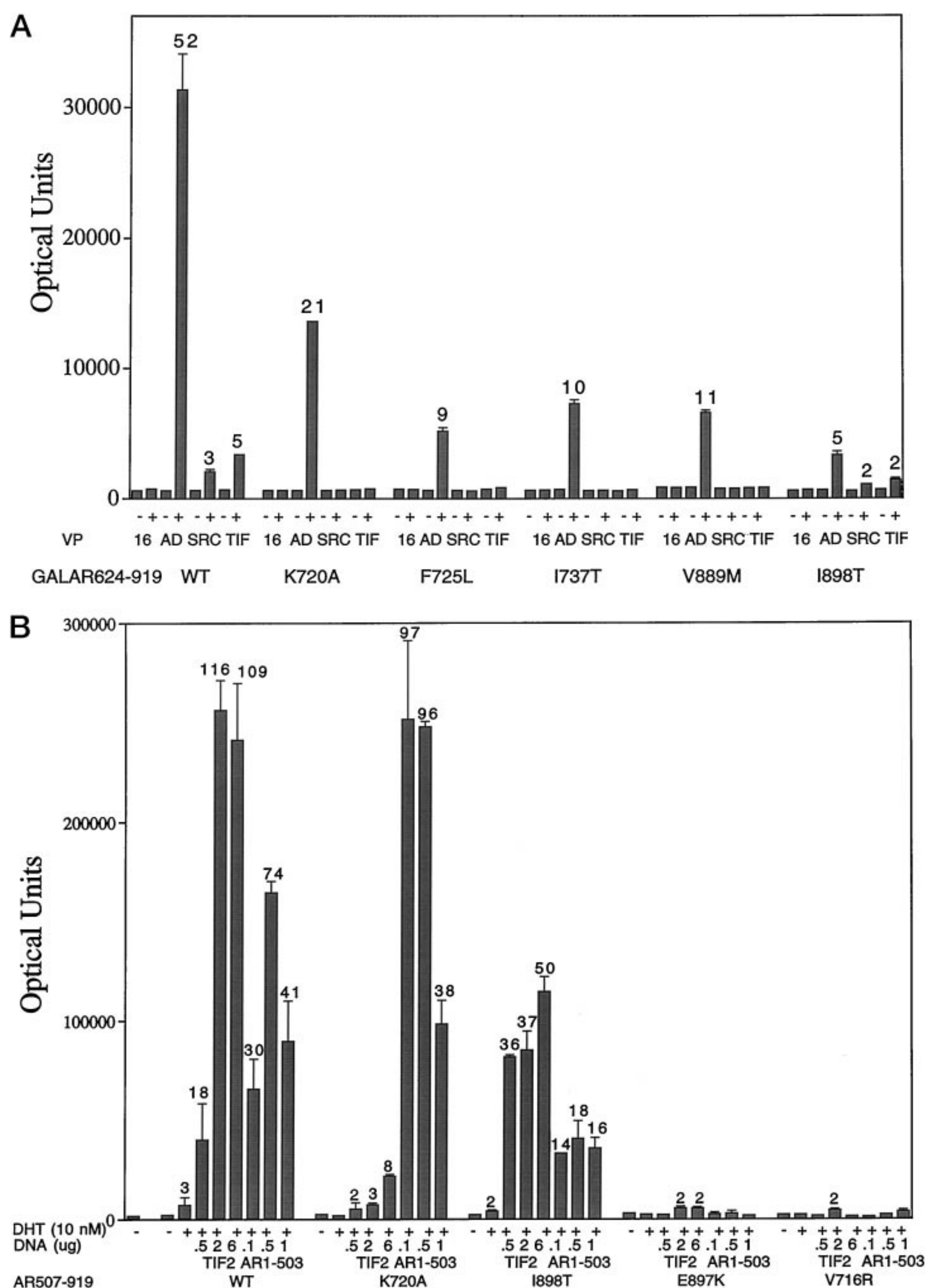

FIG. 4. **AR mutations that distinguish coactivator binding and the N/C interaction.** *A,* two hybrid interaction assay between the AR LBD mutants and the AR NH_2-terminal domain, TIF2 and SRC1. GALAR624–919 coding for the AR LBD residues 624–919 with wild-type sequence (*WT*) or the indicated mutations were tested in the CHO cell two hybrid assay as described under "Experimental Procedures" using pNLVP16 (*VP16*), VPAR1–660 (*AD*) coding for the NH_2-terminal region and DBD, or the VP16 fusion proteins with full-length SRC1 (*SRC*) and TIF2 (*TIF*). The experiment shown is representative of at least three independent experiments where fold induction is shown above the *bars*. *B,* transcriptional activation by the AR LBD in the presence of TIF2 and the AR NH_2-terminal region. Transient cotransfection experiments were performed in CV-1 cells using the MMTV-luciferase reporter vector as described under "Experimental Procedures" in the absence (−) or presence (+) of 10 nM DHT. AR507–919 with wild-type (*WT*) or mutant sequence as indicated were coexpressed with 0.5, 2, and 6 μg of pSG5-TIF2 or 0.1, 0.5, or 1 μg of pCMVhAR1–503 coding for the AR NH_2-terminal region but lacking the AR DBD. The experiment shown is representative of at least three experiments, and the fold induction is shown above the *bars*.

(22), suggesting this residue contributes to multiple overlapping interaction sites. I898T, on the other hand, retains strong coactivator binding to AF2 but has a greatly reduced N/C interaction (Fig. 4, *A* and *B*) and is associated with complete androgen insensitivity (Table I). Thus a decline in the N/C interaction at AF2, but to a much less extent coactivator interaction at AF2, is associated with androgen insensitivity and thus loss of AR function *in vivo*.

Although p160 coactivators may contribute to the N/C interaction (4, 40, 41), several lines of evidence, including recent studies with the progesterone receptor (42), support a direct N/C interaction. 1) In our studies, overexpression of TIF2 or SRC1 has no effect on the AR N/C interaction in the mammalian two hybrid assay (data not shown). 2) The AR N/C interaction is detected in both mammalian and yeast two hybrid assays. 3) AR GST adsorption experiments where the GST-AR LBD fusion protein interacts in an androgen-dependent manner with the AR NH_2-terminal domain (Fig. 3*B*) are consistent with a direct N/C interaction. 4) The N/C interaction site in the AR LBD overlaps, but is not identical to, the p160 coactivator L*XX*LL motif binding site. 5) The AR LBD appears to bind the NH_2-terminal domain with higher affinity than it does the L*XX*LL motif. The data predict that AF2 mutations that disrupt p160 coactivator binding alter male phenotypic expression only if they interfere with the overlapping N/C interaction site.

Most AF2 and signature sequence mutations that increase the androgen dissociation rate and cause severe androgen insensitivity (Table I) (43) are associated with helix 12 (29). Androgen dissociation rates from the DBD/LBD AR507–919 fragment increased 7-fold from $t_{1/2}$ 44 min to $t_{1/2}$ 3–8 min at 37 °C by W741A, I898T, Y739A, and V889M (Table I). Trp-741 in helix 5 is predicted to contact Ile-898 in helix 12, Tyr-739 in helix 4 contacts Val-911 in helix 12, and Val-889 lies between helices 11 and 12 (Fig. 5). Trp-741 corresponds to Trp-755 in the progesterone receptor, which directly interacts with bound agonist (29), so a mutation at this site could directly increase

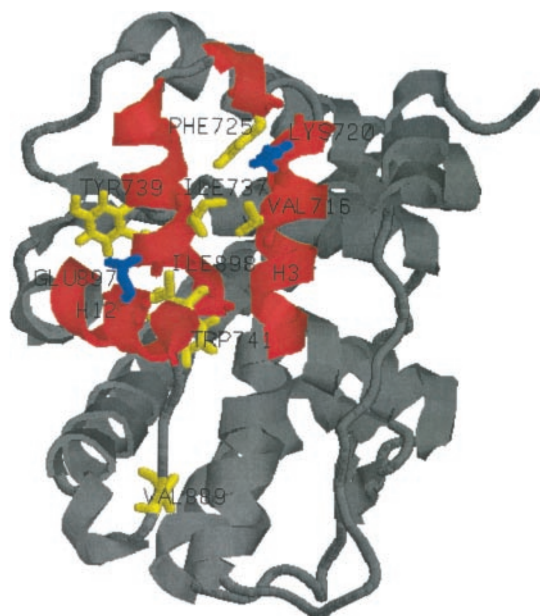

FIG. 5. **Model of the AR LBD.** The predicted model of the AR LBD is shown based on the LBD structure of the progesterone and other nuclear receptors (29, 30, 46). All indicated residues are conserved with the progesterone receptor except Ile-898 in AR is substituted for Val-912. Part of helices 3, 4, 12, and a short 3 residue helix between helices 3–4 that comprise AF2 are shown in *red*. AR AF2 and the signature sequence include charged residues Glu-897 and Lys-720 in *blue* and hydrophobic residues in *yellow*.

the ligand dissociation rate. On the other hand, I737T in helix 4 and F725L between helices 3 and 4 cause only partial androgen insensitivity (44) and are not predicted to contact helix 12. Nor do they influence the androgen dissociation rate or completely disrupt the N/C interaction (Table I). These and other mutations not associated with helix 12 (V716R, K720A, Q867H/P868D) retained the wild-type androgen dissociation rate. Thus helix 12 appears to stabilize androgen in the binding pocket.

The N/C interaction appears to further stabilize helix 12 and bound androgen. As we showed previously, deletion of the NH_2-terminal domain increases the androgen dissociation rate by 4–5-fold (20, 33). Furthermore, E897K in helix 12 eliminates the N/C interaction and increases the androgen dissociation rate 2-fold (Table I). Glu-897 is equivalent to Glu-471 in helix 12 in peroxisome proliferator-activated receptor γ, which forms part of the L*XX*LL charge clamp of AF2 (9), supporting overlapping coactivator and AR NH_2-terminal binding sites. V716R, though not positioned near helix 12, eliminates the N/C interaction, and the androgen dissociation rate increased 5-fold (Table I). Mutations at Gln-867 and Pro-868 in the loop between helices 10–11 to the conserved residues HD of the progesterone and estrogen receptors (Q867H/P868D, Table I), where Gln-867 juxtaposes Tyr-915 in helix 12, increased the N/C interaction 2-fold (data not shown) and slowed the androgen dissociation rate to a similar extent (Table I).

Thus the N/C interaction and the AF2/signature sequence residues appear to contribute to the positioning of helix 12, which results in slowing the dissociation rate of bound androgen. SRC1 slowed estrogen receptor ligand dissociation (45); however, overexpression of TIF2 had no effect on androgen dissociation from full-length AR (data not shown). The data are consistent with overlapping LBD AF2 binding sites for TIF2 and the AR NH_2-terminal domain, which in the presence of androgen agonist participates in the N/C interaction. For the AR, p160 coactivator recruitment appears to be mediated primarily by the AR NH_2-terminal and DBD regions. As illustrated in Fig. 5, the data suggest that AF2 in the AR LBD serves predominantly as an N/C interaction site, which upon agonist binding contributes to stabilization of helix 12 to slow androgen dissociation necessary for AR functional activity at physiological androgen concentrations.

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