Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH$_2$-terminal domain

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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) 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 LXXLL motifs that form amphipathic $\alpha$ helices (7, 8). Recent co-crystal structures of nuclear receptor LBDs and LXXLL motif fragments confirm that AF2 recruits TIF2 and SRC1 through their LXXLL motifs (6, 9–11). A multistep mechanism for transcriptional activation by nuclear receptors involves hormone-dependent recruitment and association through these LXXLL 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 Srb1/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 LXXLL motif-independent manner mediated by the carboxyl-terminal region of SRC1 and the carboxyl-terminal and central regions of TIF2.

**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 BspHI ( blunt)/XbaI fragment cloned into pGALO ( SmaI/XbaI); GALSRC1–780 by digesting GALSRC1–1441 with BamHI/XbaI and religating; GALSRC568–1441 by cloning the EcoRI ( blunt)/XbaI pCR3.1hSRC1a fragment into pGALO ( SalI/ blunt/XbaI); GALSRC564–800 and GALSRC564–1138 using the polymerase chain reaction (PCR) amplification using 5’- NdeI and 3’- XbaI primers and the fragment cloned into pGALO ( NdeI/XbaI); GALSRC568–954 by digesting GALSRC568–1441 with SacI/XbaI and

**EXPERIMENTAL PROCEDURES**
religating; GALSRC1139–1414 by cloning the HindIII (blunt)/XhoI pCR3.1hSRC1a fragment into pGALO (SalI/blunt/XbaI); GALSRC1139–1437 by cloning the PCR-amplified fragment of pCR3.1hSRC1a using 5′-BamHI and 3′-XbaI primers into pGALO (BamHI/XbaI). VPAR (human AR residues 1–919) and VPAR1–660 (AR NH2-terminal and DBD residues 1–660) containing the VP16 transactivation domain (16, 20). VPSRC1 constructs were created as follows: VPSRC1–1441 by excising full-length SRC1a from pCR3.1hSRC1a using BspHI (blunt)/XbaI and cloned into pNLVP16 (Xhol/blunt/XbaI); VPSRC568–1441 by cloning the EcoRI (blunt)/XbaI pCR3.1hSRC1a fragment into pNLVP16 (SalI/blunt/XbaI). VPSRC564–800 by amplification of VPSRC1139–1441 using a 5′ NdeI and 3′ XhoI primers into pNLVP16 (Ndel/XbaI). VPSRC1139–1441 by cloning the HindIII (blunt)/XbaI fragment of pCR3.1hSRC1a into pNLVP16 (SalI/blunt)/XbaI). 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 NH2-terminal and carboxy-terminal (N/C) interaction assay between the AR NH2- and carboxy-terminal regions was determined using GALBAR624–919, a fusion protein with *Streptomyces* sp. GAL4 DBD residues 1–147 and AR LBD residues 624–919 in pGALO (16, 20) with VPAR1–660 (AR NH2-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 VPTF12 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 Vivo Binding Assays**—GST fusion proteins were expressed in XL1-Blue *Escherichia coli* cells treated with 0.5 μM isopropl-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 were 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 [35S]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 residues 1–660 coding for AR NH2-terminal and DBD residues 1–660 from GALAR using TslI (blunt)/BamHI and cloned into pGEX-5X-1 (Amersham Pharmacia Biotech) at Smal/BamHI. GSTTIF2M (TIF2 624–1141) and GSTTIF2C (TIF2 1144–1464) were PCR amplified, and fragments were cloned in pGEX-2T (EcoRI/BamHI). TIF2 carboxy-terminal residues 1143–1464 were amplified from pSG5TIF2 by PCR and cloned into pcDNA3HA (provided by Yue Xiong) at the BamHI/XhoI sites to prepare 35S-labeled TIF2-C. pGEMAR (provided by Jean-an Tan and Frank S. French) coded for full-length human AR residues 1–919 and was used to prepare 35S-AR. GSTAR1–565 was prepared by digesting GALAR1–919 with HindIII (blunt)/BamHI and cloned into pGEX-3X at EcoRI (blunt)/BamHI. pcDNA3HA-AR-LBD expressed the human AR LBD residues 624–919 was digested from GALBAR624–919 with BamHI/XhoI and cloned in the same sites in pcDNA3HA for in vitro translation.

**RESULTS AND DISCUSSION**

Expression of the AR DBD and LBD fragment AR507–919 (Fig. 1A) or AR LBD residues 624–919 fused with the GAL4 DBD (GALAR624–919, Fig. 1B) shows little or no induction of transactivation 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- to 6-fold and 3.6- to 0.3-fold (Fig. 1B). 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 transactivation activity about 3–6-fold, which surprisingly was only partially diminished by mutation of the three LXXLL motifs in TIF2 (TIF2 m123, Fig. 1A) and SRC1 (21), suggesting that p160 coactivators can increase AR transactivation in an LXXLL 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 LXXLL motifs, respectively, interacted 2–3-fold with the AR LBD (GALAR624–919, Fig. 2), which was less than 10% the activity observed in the N/C interaction (see below and Fig. 4A), suggesting weak coactivator binding affinity compared with the interaction between the NHR and carboxy-terminal AR domains. Although results are shown at
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**A**

| TIF2 | bHLH | PAS | NID | AD1 | Q rich AD2 | GALAR | VPAR | VPARD |
|------|------|-----|-----|-----|-----------|-------|-------|-------|
| 1-1464 | 115 | 368 | 624 | 869 | 130 | 1288 | 1464 | nd | nd |
| 1-627 | 624-1010 | nd | nd | nd | nd | nd | nd | nd |
| 870-1179 | 2.4 ± 0.7 | 6.9 ± 1.1 | 7.8 ± 1.0 | nd | nd | nd | nd |
| 624-1287 | 4.6 ± 1.3 | nd | nd | nd | nd | nd | nd |
| 1180-1269 | 2.7 ± 0.2 | 2.0 ± 0.6 | nd | nd | nd | nd | nd |
| 1288-1464 | 7.0 ± 0.7 | 13.8 ± 2.3 | nd | nd | nd | nd | nd |
| 1434-1464 | nd | nd | nd | nd | nd | nd | nd |

**Table I**

Summary of AR LBD mutants

| Mutation | Binding affinity | Dissociation half-time | MMTV-Luc | TIF2 interaction | N/C interaction | AIS mutation, stage | Helix |
|----------|------------------|------------------------|----------|------------------|-----------------|---------------------|-------|
|          | AR1–919 | AR507–919 | AR1–919 | AR507–919 | AR1–919 and TIF2 | AR507–919 and AR1–503 |        |
| Wild-type | 0.48 ± 0.25 | 149 ± 32 | 44 ± 4 | 0.001 | 2.1 ± 0.5 | ++ | 11-12 loop |
| AF2 domain |       |        |        |        |        |        |        |
| V716R | 0.40 ± 0.04 | 28 ± 3 | 20 ± 2 | 0.1 | 1.0 ± 0 | – | – |
| K720A | 0.54 ± 0.15 | 134 ± 11 | 28 ± 4 | 0.001 | 1.2 ± 0.2 | + | 1 |
| I737T | 0.44 ± 0.20 | 147 ± 38 | 42 ± 2 | 0.1 | 1.1 ± 0.04 | + | 100 |
| E897K | 0.52 ± 0.18 | 67 ± 11 | 38 ± 2 | 1 | 1.0 ± 0 | – | – |
| I885T | 0.34 ± 0.02 | 42 ± 5 | 7 ± 1 | 0.1 | 1.7 ± 0.2 | + | – |
| Signature sequence |       |        |        |        |        |        |        |
| L722A | 0.58 ± 0.13 | 102 ± 24 | nd | 1 | 1.9 ± 0.2 | nd | 10 |
| F725L | 0.82 ± 0.32 | 127 ± 39 | 38 ± 5 | 0.1 | 1.2 ± 0.1 | + + | L722F, 6–7 |
| L728A | 0.56 ± 0.17 | 82 ± 9 | 11 ± 1 | 0.1 | 1.4 ± 0.03 | nd | 100 |
| Y739A | 0.65 ± 0.16 | 34 ± 3 | 8 ± 1 | 0.1 | 2.7 ± 0.4 | ++ | ++ |
| W741A | 0.67 ± 0.31 | 33 ± 5 | 7 ± 1 | 0.1 | 1.9 ± 0.4 | ++ | ++ |
| Other domains |       |        |        |        |        |        |        |
| H729A | 0.70 ± 0.30 | 132 ± 32 | nd | 0.01 | 3.3 ± 1.0 | nd | 1 |
| A735T/V736L | 0.42 ± 0.02 | 216 ± 56 | 47 ± 6 | 0.001 | 2.7 ± 0.5 | + | – |
| Q798A | 0.48 ± 0.19 | 148 ± 27 | 37 ± 5 | 0.001 | 1.2 ± 0.3 | ++ | Q798E, 5 |
| Q867H/P868D | 0.52 ± 0.19 | 272 ± 57 | 50 ± 7 | 0.001 | 2.9 ± 0.6 | + | ++ |
| V889M | 0.46 ± 0.25 | 18 ± 2 | 3 ± 0 | 1 | 1.5 ± 0.3 | ++ | 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 LXXLL motifs, increased activation by the AR LBD (AR507–919, Fig. 1A), indicating that exogenously expressed coactivators can rescue LXXLL motif-dependent AF2 activity in the AR LBD, which as shown below was blocked by site-directed mutations in AF2 (see Fig. 4B). The results suggest that the apparent lack of AR AF2 activity results from inefficient LXXLL motif-dependent recruitment of endogenous coactivators. Recovery of AF2 by overexpression of p160 coactivators suggests overall retention of nuclear receptor AF2...
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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. 2A) or the constitutively active NH$_2$-terminal and DBD fragment AR1–660 (VPAR1–660, Fig. 2A) 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. 2A). The results of GST adorption assays confirm that both the central and carboxyl-terminal domains of TIF2 interact with the AR NH$_2$-terminal and DBD fragment (Fig. 3A). 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 domain (SRC1 1–565) coding for LBD residues 624–919 was reacted with GST or GST fusion protein containing 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.

In vitro interactions between AR, AR fragments, and TIF2 by GST adorption. GST adorption experiments were performed as described under “Experimental Procedures.” A, the in vitro translated 35S-labeled carboxyl-terminal TIF2-C fragment (amino acid residues 1143–1464), the AR NH$_2$-terminal region and DBD (35S-AR1–660), and full-length AR (35S-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, 35S-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.

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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 LXXLL 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). Retention of wild-type AR transcriptional activity by K720A correlates with the 21-fold N/C interaction (Fig. 4A), but not with the LXXLL motif-dependent p160 coactivator binding 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 LXXLL motifs at the same hydrophobic cleft 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 [3H]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, V899M) androgen insensitivity (Fig. 4A). 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. 4B, 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).
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 (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 NH2-terminal domain (Fig. 3B) 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 LXXLL motif binding site. 5) The AR LBD appears to bind the NH2-terminal domain with higher affinity than it does the LXXLL 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...
AR, p160 coactivator recruitment appears to be mediated primarily by the AR NH2-terminal and DBD regions. As illustrated in Figure 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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