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Intermolecular NH_2-/Carboxyl-terminal Interactions in Androgen Receptor Dimerization Revealed by Mutations That Cause Androgen Insensitivity*

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Structural alignment of the human androgen receptor dimer was investigated by introducing steroid binding domain mutations that cause partial or complete androgen insensitivity into fusion proteins containing the full-length androgen receptor or the steroid binding domain. Most of the mutants had unchanged apparent equilibrium androgen binding affinity and increased dissociation rates of [^{3}H]methyltrienolone and required increased dihydrotestosterone concentrations for transcriptional activation. In a 2-hybrid protein interaction assay in mammalian cells, the steroid binding domain interacts with an NH_2-terminal-DNA binding domain fragment and with the full-length androgen receptor at physiological androgen concentrations in a dose-dependent manner. However, mutations at Val-889 and Arg-752 disrupt the NH_2-/carboxyl-terminal interaction when introduced into the steroid binding domain fragment but not when present in the full-length androgen receptor. The N-C bimolecular interaction reduces the dissociation rate of bound androgen and slows the degradation rate of the carboxyl-terminal steroid binding domain fragment. The results suggest that steroid binding domain residues Val-889 and Arg-752 are critical to the NH_2-/carboxyl-terminal interaction and that an intermolecular N-C interaction occurs during receptor dimerization that results in an antiparallel arrangement of androgen receptor monomers.

Steroid hormone receptor dimerization required for optimal DNA binding (1–5) is mediated by the second zinc finger region in the DNA binding domain (6) and by a carboxyl-terminal region in the steroid binding domain (7–10). Some steroid receptors do not require steroid binding for dimerization (11–13) or dimerize independent of DNA binding (5, 14–16). For the androgen receptor (AR),[1] binding to androgen response element DNA requires androgen-dependent AR dimerization (17). Recent evidence using a 2-hybrid protein interaction assay in mammalian cells indicates that AR dimerization involves an androgen-dependent interaction between the NH_2-terminal and carboxyl-terminal steroid binding domains (N-C interaction) that is of sufficiently high affinity to occur bimolecularly, independent of the AR DNA binding domain (18). Similar N-C interactions in the estrogen receptor (ER) led to a parallel dimerization model (19). An unresolved issue in the steroid receptor field, therefore, is whether receptor dimerization has a parallel or antiparallel orientation (20). In support of the parallel model, evidence was reported for ER dimerization through direct hydrophobic interactions between steroid binding domains (21–23). An Src homology 2 domain-type interaction with phosphorylated tyrosine 537 in the ER steroid binding domain may be involved in ER dimerization although dimer orientation was not addressed (24). Another report suggested dimerization occurs in a head-to-toe, antiparallel arrangement (25). In support of the antiparallel model, the glucocorticoid receptor NH_2-terminal domain was implicated in dimerization (26). For the thyroid hormone and vitamin D receptors, the DNA binding domain has rotational flexibility relative to the dimerization interface in the steroid binding domain, allowing a 180° change in conformation (27). The vitamin D receptor may form a symmetrical, head-to-tail, antiparallel dimer through the DNA binding domain but is influenced by the steroid binding domain dimerization interface (28).

Several lines of evidence argue against a parallel dimer for androgen-activated AR. First, no interaction is observed between the AR steroid binding domains in the 2-hybrid protein interaction assay in the presence or absence of androgen (18). Second, the N-C interaction is of sufficiently high affinity to promote a bimolecular interaction. Third, the steroid specificity of N-C-mediated dimerization parallels that of full-length AR. Fourth, antiparallel interactions between N-C regions of AR NH_2- and carboxyl-terminal fragments promote high affinity DNA binding. In the present report, evidence for an intermolecular N-C interaction in an antiparallel AR dimer was obtained by the introduction of single base mutations into the AR steroid binding domain that cause partial or complete androgen insensitivity without altering the apparent equilibrium androgen binding constant and by examining the influence of the NH_2-terminal domain on androgen dissociation rates and protein turnover.

EXPERIMENTAL PROCEDURES

Expression Plasmids—Eukaryotic expression vectors pGALO, containing the DNA binding domain of the *Saccharomyces cerevisiae* GAL4 protein (amino acid residues 1–147), and pNLVP, coding for the transcriptional activation domain of the herpes simplex virus VP16 protein

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[1] The abbreviations used are: AR, androgen receptor; ER, estrogen receptor; DHT, dihydrotestosterone; R1881, methyltrienolone; N-C, NH_2-terminal and carboxyl-terminal; CHO, Chinese hamster ovary; C-C, carboxyl-terminal and carboxyl-terminal; V889M, valine 889 to methionine AR mutation; R752Q, arginine 752 to glutamine AR mutation; Y763C, tyrosine 763 to cysteine AR mutation; Y763H, tyrosine 763 to histidine AR mutation; V866M, valine 866 to methionine AR mutation; MMTV, mouse mammary tumor virus; PCR, polymerase chain reaction; Tfm, testicular feminized.

TABLE I
Equilibrium dissociation constant, K_d (nM)

Apparent equilibrium binding constants and standard deviations were determined in COS cells using increasing [^{3}H]R1881 as described under "Experimental Procedures."

	Full-length	AR507–919	GALD-H	AIS grade[a]
Wildtype	0.64 ± 0.32	0.25 ± 0.06	0.12 ± 0.04	—
V889M	0.79 ± 0.35	0.41 ± 0.26	0.19 ± 0.06	6
R752Q	1.2 ± 0.32	1.0 ± 0.70	0.55 ± 0.11	7
Y763C	0.84 ± 0.34	0.54 ± 0.27	0.29 ± 0.30	2/3[b]
Y763H	1.1 ± 0.45	0.23 ± 0.03	0.32 ± 0.13	7
V866M	2.9 ± 0.65	1.1 ± 0.50	0.35 ± 0.10	6

[a] AIS grades are as previously reported (35).
[b] AIS grade 2/3 was associated with shortening of the Gln repeat in the NH_2-terminal region to 12 residues (38) (wild-type 23 Gln ± 2).

TABLE II
Dissociation half-time of [^{3}H]R1881 (min)

Dissociation half-times were determined at 37 °C as described under "Experimental Procedures" with averages and standard deviation from at least three independent experiments.

	Full-length AR	AR507–919	GALD-H
Wildtype	150 ± 34	39 ± 2	30 ± 6
V889M	18 ± 2	4 ± 1	6 ± 3
R752Q	12 ± 2	6 ± 2	13 ± 3
Y763C	52 ± 5	21 ± 6	14 ± 1
Y763H	35 ± 4	ND[a]	10 ± 4
V866M	36 ± 5	20 ± 2	20 ± 4

[a] ND, not determined.

(residues 411–456), were previously described (29) and kindly provided by Gordon Tomaselli, Johns Hopkins University. These vectors were used to construct the full-length AR vector pNLVP-hAR (designated here as pVPAR) and the carboxyl terminal AR fragment vector pGALD-H (containing AR amino acid residues 624–919) (18). pVPAR1–660 contains the VP16 transactivation domain 5′ of human AR residues 1–660, including the NH_2-terminal, DNA binding, and part of the hinge regions, and was constructed from the full-length vector by deleting the carboxyl terminal portion using *Tth*III/*Xba*I followed by ligation of the filled ends. Androgen insensitivity mutations V889M and V866M were introduced into pGALD-H and pVPAR by polymerase chain reaction (PCR) amplification of pCMVhAR-V889M (30, 31) and pCMVhAR-V866M (32). Amplified fragments were cloned into pGALO at *Nde*I/*Xba*I, in the case of the D-H fragment, or into VPAR using *CspXI*/*Xba*I. Bi-directional PCR mutagenesis was used to construct pGALD-H-R752Q, which was used to construct pCMVhAR-R752Q at the *TthIII*/*Xba*I restriction sites and pVPAR-R752Q at *Bst*EII/*Xba*I. pCMVhAR-Y763C was constructed by PCR mutagenesis changing TAC codon 763 to TGC. PCR fragments were digested with *Hinc*II/*Xba*I, and a triple ligation reaction was performed with the *Nde*I/*Hinc*II fragment of pVPAR or pGALD-H. pCMVhAR-Y763C was constructed in the *Hin*dIII/*Xba*I fragment from pGALD-H-Y763C. The sequence of all PCR-amplified regions was verified.

Cell Culture, DNA Transfections, Binding Assays, and Immunoblots—Chinese hamster ovary (CHO) cells were maintained in alpha minimum essential medium containing 10% bovine calf serum, 20 mM Hepes, pH 7.2, penicillin, and streptomycin, plated at 4.5×10^5 cells/6-cm dish, and transfected with 1–2 μg of expression vector DNA and 5 μg of G5E1bLuc reporter vector using DEAE-dextran as described (18). After addition of increasing concentrations of DHT and incubation for 24 h, cells were harvested and assayed for luciferase activity as described (18).

Apparent equilibrium binding affinity was determined in whole cell binding assays as described (33). Monkey kidney COS-1 cells were plated at 3.5×10^5 cells/well of 6-well culture plates and transfected using DEAE-dextran with 3 μg of DNA for AR fragments containing the steroid binding domain and 1–2 μg for full-length AR. After 48 h, cells were incubated with 0.25–5 nM [^{3}H]R1881 for 2 h at 37 °C. Dissociation rate constants were determined by labeling with 5 nM [^{3}H]R1881 for 2 h at 37 °C followed by the addition of a 10,000 molar excess of unlabeled R1881. Cells were washed twice with phosphate-buffered saline and harvested in 0.5 ml of 2% SDS, 10% glycerol, and 10 mM Tris, pH 6.8. Specific binding was determined from the difference in radioactivity in the presence and absence of a 100-fold molar excess unlabeled R1881 as described previously (31).

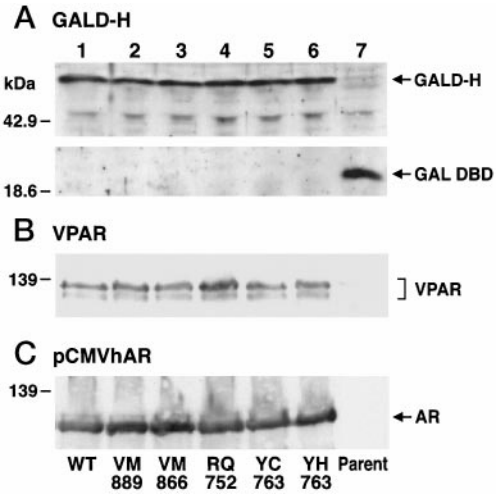

FIG. 1. **Immunoblots of partial and full-length AR with wild-type or androgen insensitivity mutation sequence expressed alone or as fusion proteins.** COS-1 cells were transfected using DEAE-dextran as described under "Experimental Procedures" with 10 μg of DNA/1.2×10^6 cells/10-cm dish. Forty-eight h after transfection and in the absence of androgen, COS cells were harvested in SDS containing buffer and analyzed in 12% (*A*) or 9% (*B*, *C*) acrylamide gels. *A*, GALD-H expression vectors contain the *S. cerevisiae* GAL4 DNA binding domain residues 1–147 (*GAL DBD*) and AR steroid binding domain residues 624–919 (coded by exons D-H) with the indicated androgen insensitivity mutations. Expressed GALD-H fusion proteins (50 kDa apparent M_r; 49 kDa calculated; 50 μl) were detected using a rabbit anti-GAL DNA binding domain antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The GAL4 DNA binding domain (19 kDa apparent M_r; 16.3 kDa calculated) is evident in *lane 7* from the expressed parent vector, pGALO, that lacks AR sequence. *B*, VPAR expression vectors contain the herpes simplex virus VP16 transactivation domain residues 411–456 and full-length AR residues 1–919 with the indicated mutations. VPAR fusion proteins (120 and 126 kDa apparent M_r; 112 kDa calculated; 50 μl) were detected using antipeptide AR52 IgG previously described (34). Expression of the parent vector pVP16 without AR sequence is shown in *lane 7*. *C*, pCMVhAR expression vectors contain full-length AR residues 1–919 with the indicated mutations. Full-length AR (113 kDa apparent M_r; 102 kDa calculated) was detected using AR52 IgG. Expression of the parent vector pCMV5 lacking AR sequence is shown in *lane 7*. Shown are extracts (40 μl) from cells expressing parent vectors with wild-type AR sequence (*lane 1, WT*) or with the following androgen insensitivity AR mutations: V889M (*lane 2, VM889*), V866M (*lane 3, VM866*), R752Q (*lane 4, RQ752*), Y763C (*lane 5, YC763*), Y763H (*lane 6, YH763*), and the parent vectors lacking AR sequence (*lane 7*).

Protein expression levels were compared by immunoblot analysis of wild-type and mutant full-length AR and partial AR fusion proteins expressed in COS-1 cells and analyzed in 9 or 12% acrylamide SDS gels using AR52 antipeptide antibody (34) or the GAL4 DNA binding domain antibody (Santa Cruz Biotechnology). Secondary antibody was anti-rabbit IgG (Promega).

Degradation rates were determined in COS-1 cells (1×10^6/10-cm dish) transiently transfected using DEAE-dextran with 4 μg of AR507–919 plus either 8 μg of parent expression plasmid pCMV5 or 8 μg of AR1–660. Cells were maintained in 10% serum and, after 48 h, were incubated in serum-free, methionine-free media as described previously (31) except with 100 μCi [^{35}S]L-methionine/L-cysteine PRO-MIX *in vitro* cell labeling mix (1000 Ci/mmol) (Amersham Life Science, Inc.). After 30 min of labeling, cells were washed, placed in serum-free media with or without 100 nM DHT at 37 °C, and harvested at time intervals up to 7 h. Cells were harvested in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.15 M NaCl, 5 mM EDTA, and 50 mM Tris, pH 7.4), and cell extracts were incubated with AR52 anti-peptide antibody and Pansorbin and processed for polyacrylamide gel analysis as described previously (31).

RESULTS

Steroid Binding Domain Amino Acid Residues Involved in the N-C Interaction and Polarity of AR Dimerization—The polarity of AR dimerization was investigated by introducing naturally occurring mutations that cause partial or severe forms of

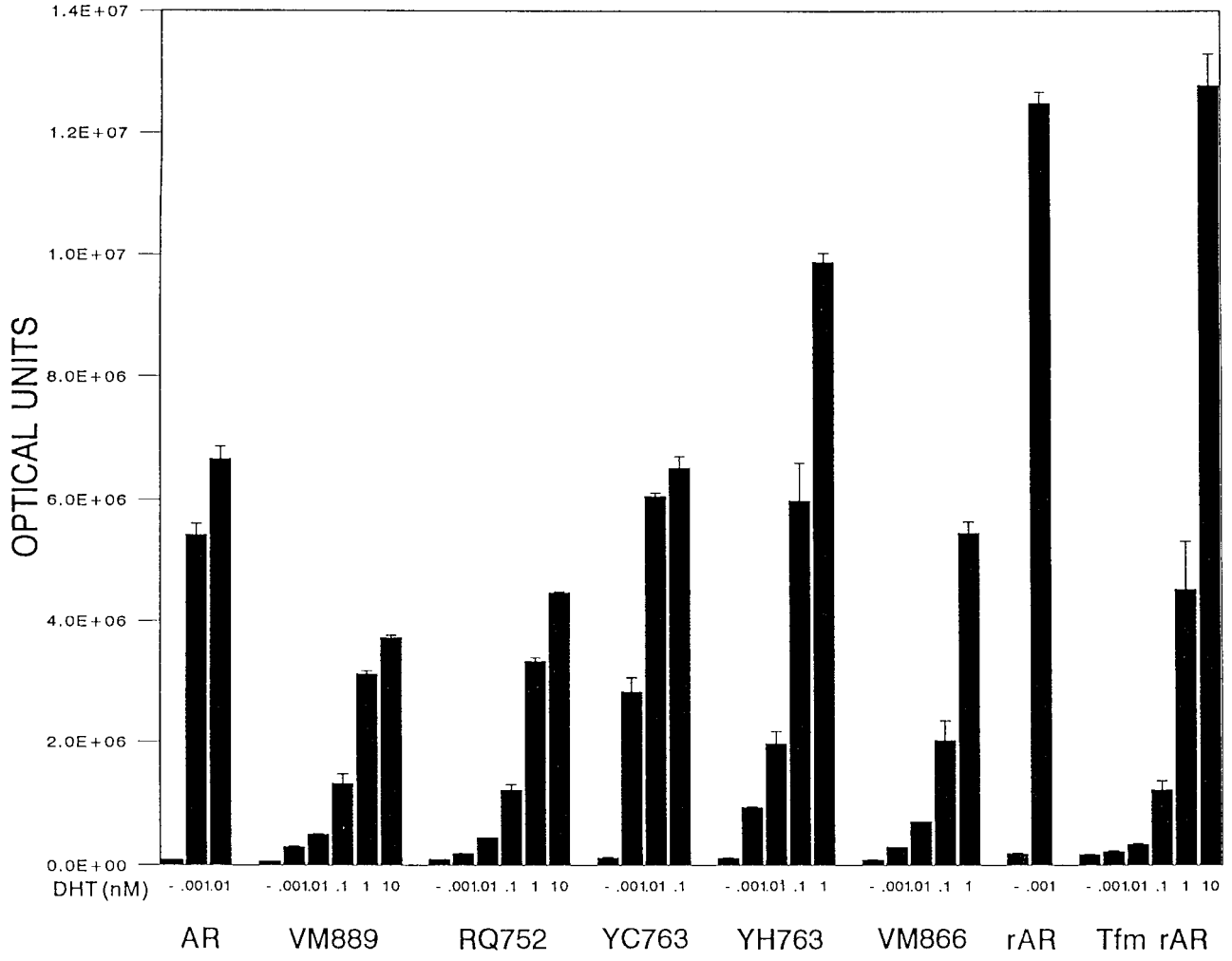

FIG. 2. **Androgen-induced transcriptional activation of the MMTV promoter-luciferase reporter vector by full-length wild-type AR and mutant ARs that cause partial or complete androgen insensitivity.** Monkey kidney CV1 cells (3.5×10^5/6-cm dish) were transiently transfected with 100 ng of pCMVhAR with wild-type sequence (AR) or with the indicated human androgen insensitivity mutations and the testicular feminized androgen-resistant rat mutation (R734Q) in pCMVrAR and 5 μg of MMTV-luciferase reporter vector using calcium phosphate precipitation as described previously (47). Cells were incubated for 30 h with increasing concentrations of DHT from 0.001 to 100 nM as indicated, and optical light units were determined using an automatic luminometer. Shown is a representative experiment of at least three independent determinations.

androgen insensitivity. The mutations were constructed by mutagenesis of GALD-H, which contains human AR steroid binding domain residues 624–919 (coded by exons D-H) fused to the carboxyl-terminal end of GAL4 DNA binding domain residues 1–147, or into VPAR, which contains full-length human AR residues 1–919 fused to the carboxyl-terminal end of VP16 transactivation domain residues 411–456. AR mutations that result in partial or complete androgen insensitivity either do not significantly alter (V889M, R752Q, Y763C, Y763H) or decrease (V866M) the apparent equilibrium binding affinity for the synthetic androgen, [^{3}H]R1881 (Table I). Equilibrium androgen binding affinities of the full-length AR mutants shift toward higher affinities when the NH_2-terminal region is deleted in AR507–919 and in the GALD-H fusion proteins (Table I). However all of the full-length androgen insensitivity mutants have increased rates of [^{3}H]R1881 dissociation (Table II) relative to wild-type AR, suggesting correspondingly increased association rates in mutants with equilibrium binding constants similar to that of wild-type AR. Deletion of the NH_2-terminal domain results in increased dissociation rates of [^{3}H]R1881 (Table II) for all mutants as previously reported for wild-type AR (18). Expression levels of the wild-type and mutants are similar for the partial AR fusion proteins in GALD-H (Fig. 1*A*), for full-length ARs expressed as fusion proteins with the VP16 transactivation domain (Fig. 1*B*), or full-length AR (Fig. 1*C*) as determined from immunoblots of extracts from transiently transfected COS-1 cells. However, protein expression from pCMV5 is at least 2-fold higher than from pVP16. When introduced into the mammalian expression vector pCMVhAR and assayed with the MMTV-luciferase reporter vector, most of the androgen insensitivity mutants require DHT concentrations above the physiological range to promote full transcriptional activation (Fig. 2).

GALD-H interacts with VPAR1–660 (AR NH_2-terminal, and DNA binding domain residues 1–660 fused carboxyl terminal to the VP16 transactivation domain) in the presence of DHT in a concentration-dependent manner. Luciferase activity of 3–16 fold becomes detectable at 0.01 nM DHT, with full activation between 22–31 fold at 1–10 nM DHT (Figs. 3*A*, 4*A*, 5*A*, and 6*A*, left). VP-full-length AR (VPAR) interacts with GALD-H in a similar concentration dependent manner with DHT, with up to an 11-fold increase in luciferase activity at 0.01 nM DHT and full activation of 12–34 fold at 1–5 nM DHT (Figs. 3*B*, 4*B*, 5*B*, and 6*B*, *left*). These results indicate that the N-C interaction occurs as a bimolecular reaction when one monomer is either full-length AR or the NH_2-terminal and DNA binding regions

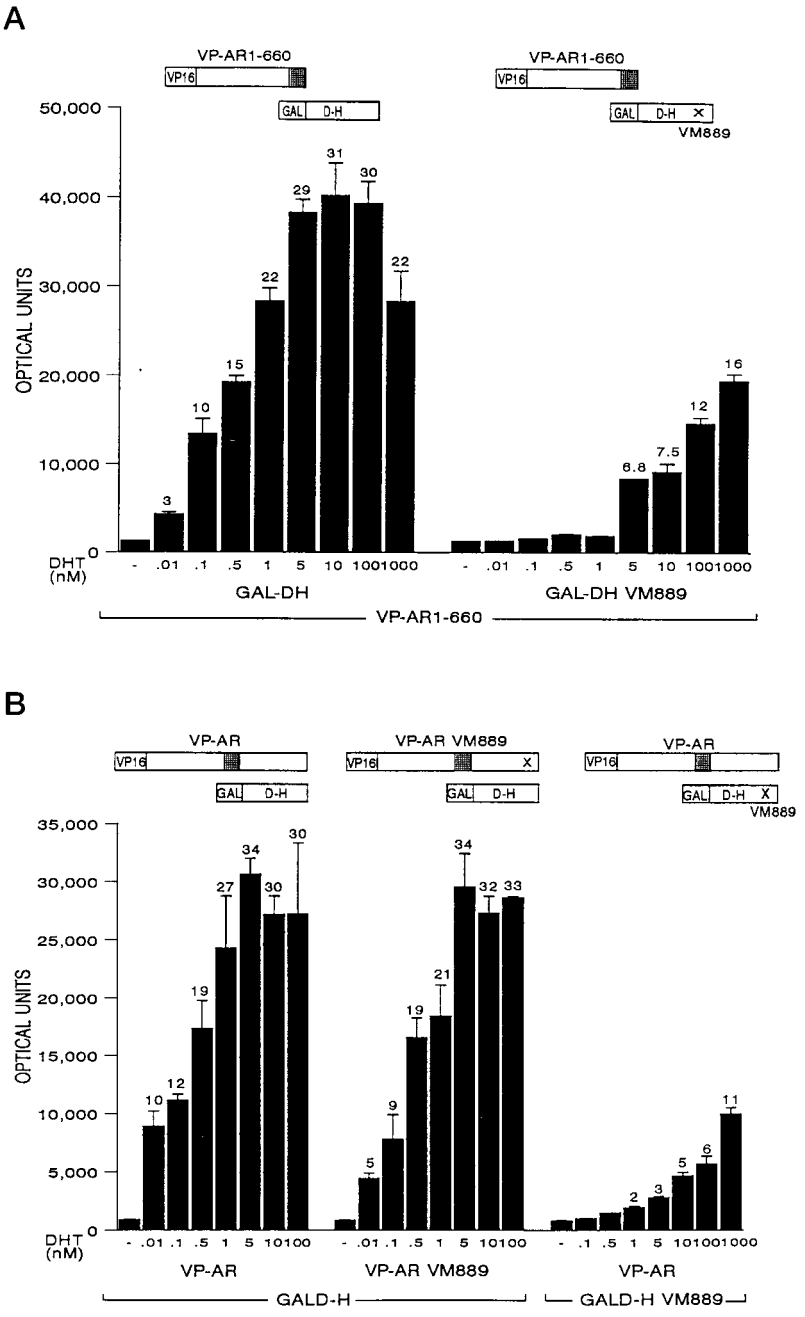

FIG. 3. **2-hybrid protein interaction assay between AR NH_2- and carboxyl-terminal domains.** The 2-hybrid assay was performed in CHO cells as described previously (18). *A*, VPAR1–660, coding for AR NH_2-terminal residues 1–660 and the VP16 activation domain, was cotransfected with GALD-H, coding for wild-type AR steroid binding domain residues 624–919 (*left*) or with GALD-H-V889M (*right*) (codon 889 GTG→ATG). *B*, VPAR, coding for wild-type full-length AR residues 1–919 (*left*) or VPAR-V889M (*middle*) expressed as fusion proteins with the VP16 activation domain were transfected with GALD-H with wild-type sequence, or VPAR was transfected with GALD-H-V889M (*right*). DNA (1 μg of each expression plasmid) was transfected with 5 μg of G5E1bLuc reporter plasmid using DEAE-dextran and incubated for 30 h with 0.01 nM to 1 μM DHT as indicated. Luciferase activity is shown as optical light units and is representative of at least three independent experiments. Schematic diagrams of the constructs are shown where the *shaded area* represents the AR DNA binding domain, and × represents the V889M mutation. Fold induction relative to luciferase activity in the absence of DHT is indicated *above the bars*.

and the other monomer contains only the AR steroid binding domain. The similar dose-dependent N-C interaction between the steroid binding domain and either full-length AR or the NH_2-terminal fragment supports the antiparallel dimer model. Self dimerization of VPAR apparently does not interfere with its interaction with GALD-H.

V889M—V889M results from a GTG → ATG mutation in exon H that causes grade 6 androgen insensitivity (female external phenotype with pubic hair in a 46XY genetic male) (30, 35) without altering equilibrium androgen binding (30) but increasing the dissociation rate of [^{3}H]R1881 (31) from the full-length mutant AR (Table II). The dissociation rate of [^{3}H]R1881 increases about 4-fold further with deletion of the NH_2-terminal region in AR507–919 (DNA and steroid binding domains) (Table II), suggesting that, at the high androgen concentration (50 μM unlabeled R1881) used in the dissociation experiment, the NH_2-terminal region slows androgen dissociation from V889M to an extent similar to wild-type AR. The 5–10-fold faster androgen dissociation rate from AR507–919-V889M and GALD-H-V889M relative to these wild-type AR fragments suggests, in addition, that increased androgen dissociation is an inherent defect of the V889M mutation in the steroid binding domain (Table II).

The interaction between GALD-H-V889M and VPAR1–660 occurs only at DHT concentrations of 5 nM or greater, about 500 times the concentration required for the wild-type GALD-H and VPAR1–660 interaction, and increases to only 12–16-fold, up to 1 μM DHT (Fig. 3*A*, *right*). On the other hand, reactivity of VPAR-V889M with GALD-H was similar to wild-type VPAR (Fig. 3*B*, *middle*), and little interaction is observed between VPAR and GALD-H-V889M except at high DHT concentrations (Fig. 3*B*, *right*). The results support an intermolecular N-C interaction where GALD-H interacts with the NH_2-terminal region of VPAR-V889M, which is unaffected by the V889M steroid binding mutation in VPAR-V889M. The similar dose response in interaction between GALD-H with VPAR or VPAR-

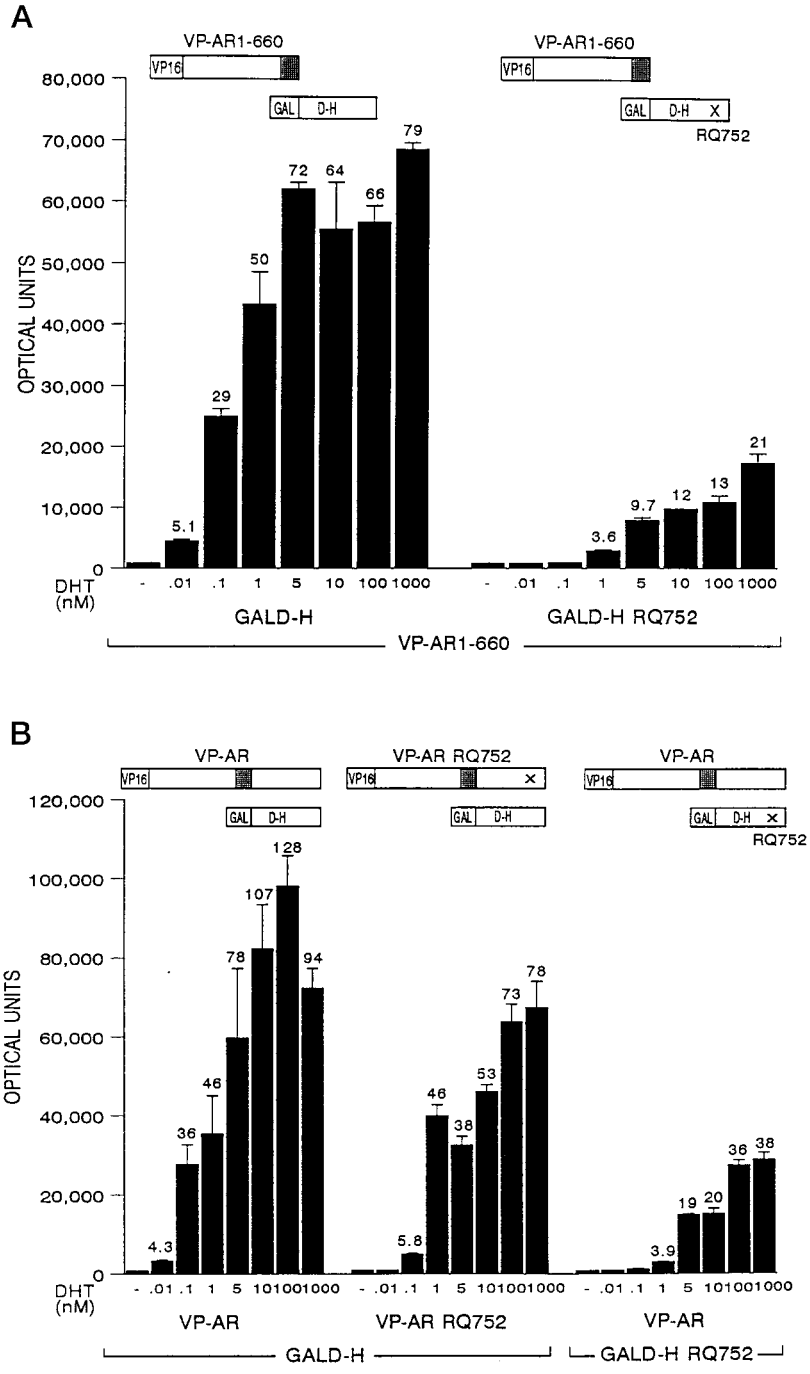

FIG. 4. **2-hybrid protein interaction assay of R752Q.** The 2-hybrid assay was performed as described (18). The R734Q AR mutation of the testicular feminized rat (33) was introduced into pCMVhAR at codon 752 CGA→CAA of human AR. *A*, VPAR1–660 coding for AR NH_2-terminal residues 1–660 fused to the VP16 transactivation domain was transfected with GALD-H (*left*) or GALD-H-R752Q (*right*). *B*, VPAR (*left*) or VPAR-R752Q (*middle*) was transfected with GALD-H, or VPAR was transfected with GALD-H-R752Q (*right*). DNA (1 μg of each plasmid) was cotransfected with 5 μg of G5E1bLuc reporter plasmid using DEAE-dextran and incubated for 30 h with 0.01 nM to 1 μM DHT as indicated. Luciferase activity shown as optical light units is representative of at least three independent experiments. Schematic diagrams of the constructs are shown where the *shaded area* represents the AR DNA binding domain, and × represents the R752Q mutation. Fold induction relative to luciferase activity without DHT is indicated *above the bars*.

V889M suggests that low physiological DHT concentrations are sufficient to promote this bimolecular reaction and may reflect the retained high androgen binding affinity of V889M (Table I).

R752Q—Androgen insensitivity in the testicular feminized (Tfm) rat results from a single base mutation CGG → CAG in exon E of the AR steroid binding domain changing Arg 734 to Gln (33). This mutation lowers androgen binding capacity with only slightly lower apparent equilibrium binding affinity (Table I) (33). The Tfm rat AR mutation occurs in human AR (Arg 752 to Gln, CGA → CAA) in two unrelated families with complete androgen insensitivity; however, in these cases, androgen binding activity was not reported (36, 37). The R752Q human AR mutant and the R734Q Tfm rat mutant (33) require at least 10,000-fold higher DHT concentrations than wild-type AR for transcriptional activation of a mouse mammary tumor virus reporter vector (Fig. 2). Like V889M, the apparent binding affinity of R752Q for [^{3}H]R1881 is similar to that of wild-type AR (Table I), and dissociation of [^{3}H]R1881 is at least 11 times faster in full-length AR and increases or is similar in the truncated fragments containing the DNA and steroid binding domains (AR507–919) or the steroid binding domain fusion protein in GALD-H (Table II). These results suggest that the R752Q binding defect is inherent to the steroid binding domain. This previously unrecognized rapid androgen dissociation kinetics from human AR R752Q and Tfm rat AR R734Q, combined with the low binding capacity, likely account for the inability of other laboratories (36, 37) to detect androgen binding using conventional radiolabeling binding assays of endogenous AR in fibroblasts from affected individuals with the R752Q mutation and for the 10% binding levels relative to wild-type siblings resulting from the synonymous R734Q mutation in the Tfm rat (33).

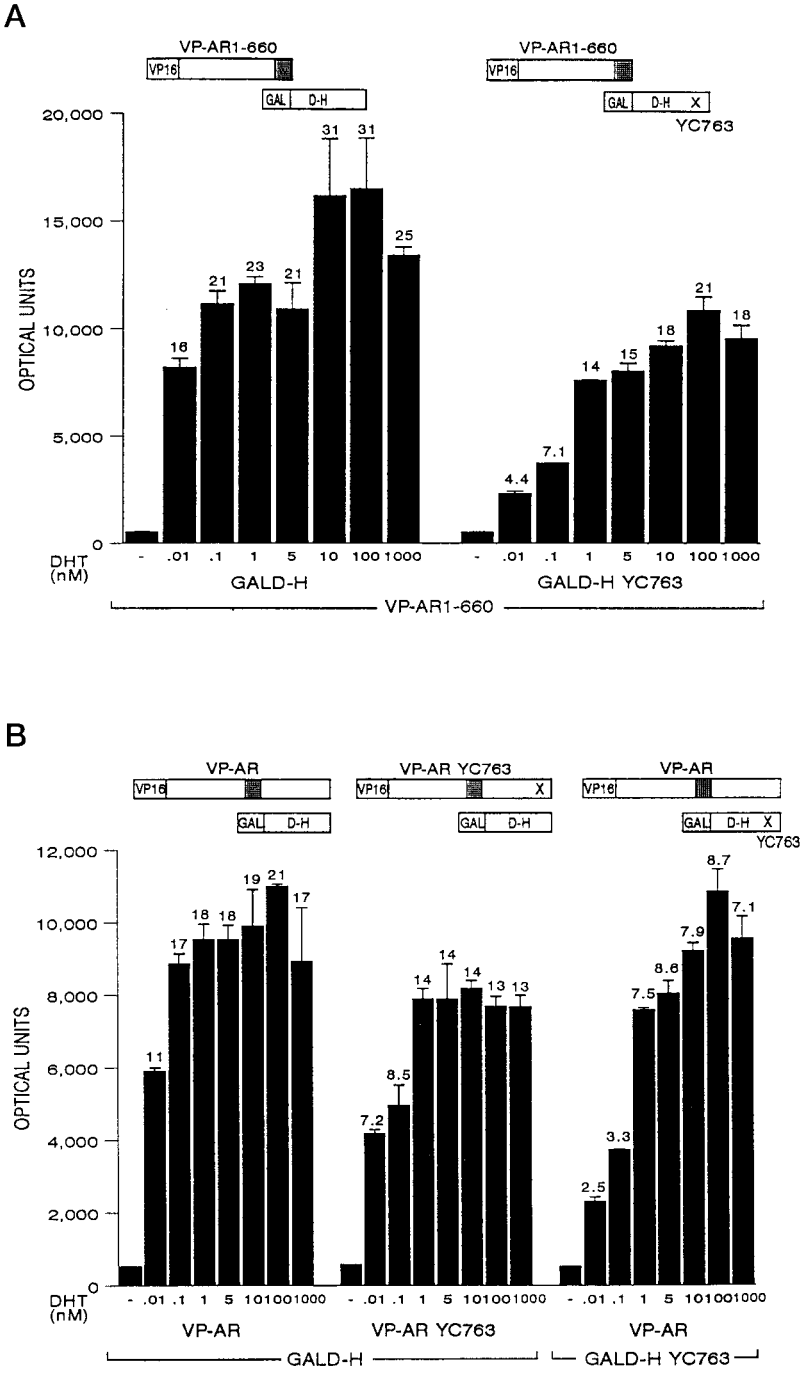

FIG. 5. **2-hybrid protein interaction assay of Y763C.** *A*, VPAR1–660 cotransfected with GALD-H (*left*) or GALD-H-Y763C (TAC→TGC) (*right*). *B*, VPAR (*left*) or VPAR-Y763C (*middle*) was cotransfected with GALD-H, or VPAR was cotransfected with GALD-H-Y763C (*right*). DNA (1 μg) was cotransfected with 5 μg of G5E1bLuc reporter plasmid using DEAE-dextran and incubated for 30 h with DHT as indicated. Luciferase activity shown as optical light units is representative of three independent experiments. Schematic diagrams of the constructs are shown where the *shaded area* represents the AR DNA binding domain, and × represents the Y763C mutation. Fold induction relative to luciferase activity without DHT is indicated *above the bars*.

In the 2-hybrid protein interaction assay, R752Q disrupts the N-C interaction between VPAR1–660 and GALD-H-R752Q (Fig. 4*A*, *right*) to an extent similar to that observed for V889M (Fig. 3*A*), suggesting that Val-889 and Arg-752 are both critical residues in the steroid binding domain for the N-C interaction. Also like V889M, the interaction between VPAR-R752Q and GALD-H is similar to that of wild-type VPAR (Fig. 4*B*, *left* and *middle*), and GALD-H-R752Q fails to interact with VPAR at physiological androgen concentrations (Fig. 4*B*, *right*), supporting the antiparallel N-C dimer orientation. In both cases, retention of high affinity binding with rapid binding and dissociation kinetics suggests that perturbations around the androgen binding pocket are critical to the N-C interaction.

Y763C—Tyr at 763 in exon E changes to Cys (TAC → TGC) in a family with partial androgen insensitivity (grade 2/3, male phenotype with hypospadias and virilization at puberty) (38) and to His (TAC → CAC) in an unrelated family with complete androgen insensitivity (35). The Y763C mutation (reported previously as Y761C) occurred together with shortening of the NH_2-terminal Gln repeat from an average length of 22 residues to 12 residues (38). Y763C is reported to increase the androgen dissociation rate 2- to 3-fold with a slight increase in apparent equilibrium binding affinity (K_d) (38). When recreated in full-length AR, we observe a similar increase in the dissociation rate of [^{3}H]R1881 from Y763C with the apparent equilibrium binding affinity indistinguishable from wild-type AR (Tables I and II). The 2-hybrid protein interaction assay results indicate only a slight decrease in transcriptional activity (Fig. 5), suggesting that Tyr-763 is not critical for the N-C interaction. This conclusion is supported by 2-hybrid results with Y763H (data not shown). Y763H had similar equilibrium binding but 4-fold faster dissociation kinetics of [^{3}H]R1881 relative to wild-type

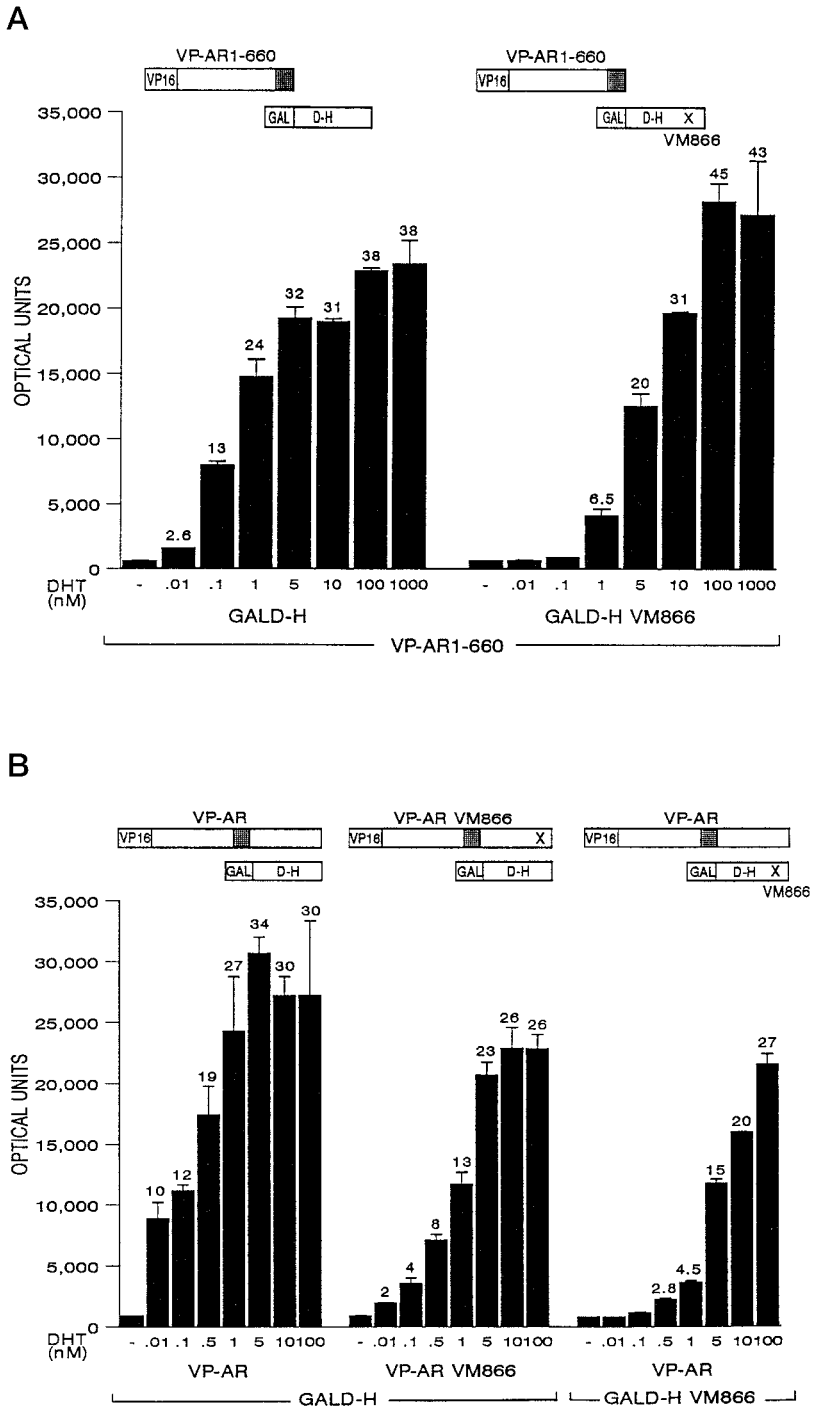

FIG. 6. **2-hybrid protein interaction assay of V866M.** The V866M mutation GTG→ATG was introduced into VPAR and GALD-H as described under "Experimental Procedures." *A*,VPAR1–660 was transfected with GALD-H (*left*) or GALD-H-V866M (*right*). *B*, VPAR (*left*) or VPAR-V866M (*middle*) was transfected with GALD-H, or VPAR was transfected with GALD-H-V866M (*right*). DNA (1 μg each) was cotransfected with 5 μg of G5E1bLuc reporter plasmid using DEAE-dextran and incubated for 30 h with DHT as indicated. Luciferase activity shown as optical light units is representative of at least three independent experiments. Schematic diagrams are shown, where the *shaded area* represents the AR DNA binding domain, and × represents the V866M mutation. Fold induction relative to luciferase activity without DHT is indicated *above the bars*.

AR (Tables I and II) and causes more severe androgen insensitivity.

V866M—Because the V889M and R752Q mutations shorten the dissociation half-time of bound androgen 7–12-fold and Y763C and Y763H only 2–4 fold, and whereas only the former two were implicated in the N-C interaction, we investigated androgen insensitivity mutant V866M to determine whether increased androgen dissociation kinetics is associated with disruption of the N-C interaction. This GTG → ATG mutation in exon G of the steroid binding domain causes complete androgen insensitivity with a reported 4-fold reduction in androgen binding affinity (32). We also observe a 4–5-fold reduction in apparent equilibrium binding affinity of [^{3}H]R1881 and a 3–4-fold increase in the dissociation rate of [^{3}H]R1881 (Tables I and II). GALD-H-V866M reacted with VPAR1–660 in a dose-dependent manner with the dose response shifted to about 5-fold higher DHT concentrations (Fig. 6*A*), a shift that can be accounted for by the decreased apparent equilibrium androgen binding affinity (Table I). Decreased sensitivity to DHT is also observed in the interaction between GALD-H with VPAR-V866M and between GALD-H-V866M and VPAR (Fig. 6*B*). The parallel shift in dose response by the V866M mutant proteins with the reduced binding affinity suggests that increased androgen dissociation kinetics greater than 4-fold is associated with disruption of the N-C interaction and that Val 866 is not a critical residue for the N-C interaction.

Effect of the N-C Interaction on Androgen Dissociation—It was shown previously that deletion of AR NH_2-terminal residues 1–507 causes a 3-fold increase in the dissociation rate of [^{3}H]R1881 from the steroid binding domain with the apparent

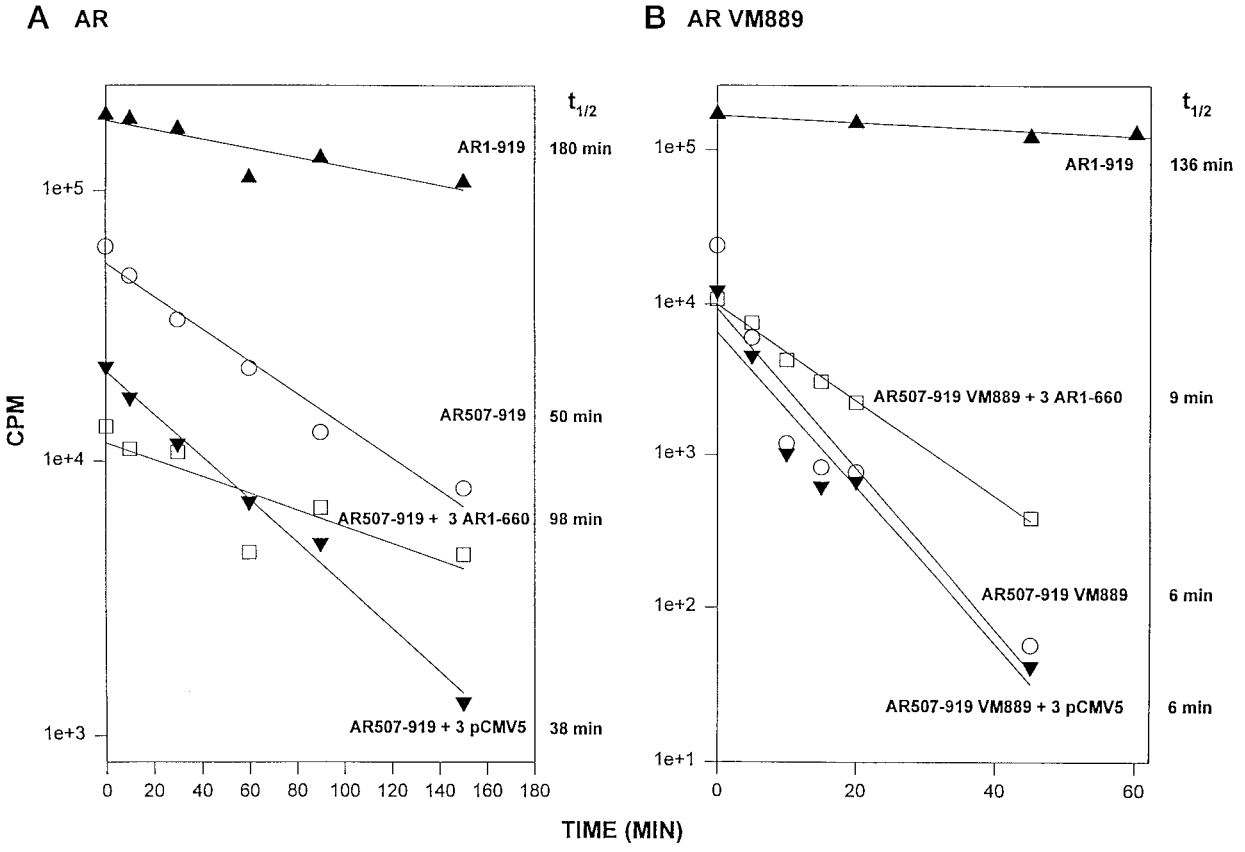

FIG. 7. **Effect of the bimolecular N-C dimerization on [^{3}H]R1881 dissociation.** AR507–919 (2 μg) (44) containing the coding region for the AR DNA and steroid binding domains with wild-type (*A*) or V889M (*B*) sequence in pCMV5 was transiently transfected in COS-1 cells alone (0) or with a 3-fold molar excess (6 μg) pCMV5 either lacking AR sequence (▼) or with AR1–660 in pCMV5 (□). AR1–660 contains the AR NH_2-terminal and DNA binding domain residues 1–660 (44). Full-length pCMVhAR (AR1–919) was included as a control (▲). COS cells (3.5 × 10^5/6 cm dish) transfected using DEAE-dextran were labeled with 5 nM [^{3}H]R1881 for 2 h at 37 °C. Dissociation was initiated by the addition of 10,000-fold molar excess of unlabeled R1881. Cells were washed twice with phosphate-buffered saline and harvested in SDS sample buffer, and radioactivity was determined by scintillation counting. Nonspecific binding was determined by the addition of 100-fold excess unlabeled R1881 to parallel samples at the initiation of the binding reaction.

equilibrium binding affinity unchanged (31), indicating that the association rate increases in parallel, and the NH_2-terminal region slows androgen association and dissociation kinetics. It was not clear, however, whether slow androgen dissociation necessary for AR stabilization and transcriptional activity (31, 39) is caused by an intramolecular N-C interaction in the AR monomer or as part of the intermolecular N-C interaction in an antiparallel dimer. Coexpression of the DNA and steroid binding domain fragment AR507–919 with a 3-fold molar excess of transfected AR NH_2-terminal fragment AR1–660 DNA slowed the dissociation rate of [^{3}H]R1881 from AR507–919 about 2-fold (Fig. 7*A*). The dissociation rate of [^{3}H]R1881 from AR507–919 with the V889M mutation also decreases by coexpression of AR1–660 in the presence of a high concentration of unlabeled androgen in the dissociation experiments (50 μM DHT). The dissociation rate of V889M remained, however, about 10-fold faster than wild-type AR (Fig. 7). In this bimolecular dimerization reaction, therefore, androgen dissociation rates slow toward that observed for wild-type AR, suggesting that the N-C intermolecular interaction associated with dimerization reduces the kinetics of androgen binding and dissociation in the steroid binding domain pocket.

Effect of the N-C Interaction on AR Degradation—To investigate whether the antiparallel N-C interaction also mimics the decrease in AR turnover observed with the transcriptionally active full-length AR, the carboxyl-terminal AR507–919 vector was coexpressed with a 2-fold molar excess of the NH_2-terminal AR1–660 expression vector or with the parent vector as a control. Degradation half-time of the [^{35}S]methionine/cysteine-labeled AR507–919 was 2 h at 37 °C in the presence or absence of 100 nM DHT, or in the presence of the NH_2-terminal fragment in the absence of DHT (Fig. 8). The degradation half-time slowed to 4 h, however, in the presence of the NH_2-terminal fragment and 100 nM DHT (Fig. 8). The results suggest that the antiparallel structural orientation in the bimolecular N-C interaction of the AR fragments recapitulates the reduced androgen dissociation and receptor turnover that occur in the transcriptionally active full-length AR.

DISCUSSION

The N-C interaction observed for AR (18) and ER (19) has raised at least two dimerization models for steroid receptors. The parallel dimer model proposed for ER (19) implicates an *intra*molecular N-C interaction within the ER monomer that potentiates ER dimerization through conformational changes and subsequent intermolecular interactions between the steroid binding domains. The antiparallel dimer model proposed for AR implicates an *inter*molecular N-C interaction between monomers (18) in androgen-dependent AR dimerization and specific DNA binding (17). Natural mutations in the AR steroid binding domain that cause partial or complete androgen insensitivity were introduced into AR fusion proteins to identify steroid binding domain residues critical for the N-C interaction and to investigate further the molecular alignment of the AR dimer. Because naturally occurring mutations in the steroid binding domain often decrease the apparent equilibrium an-

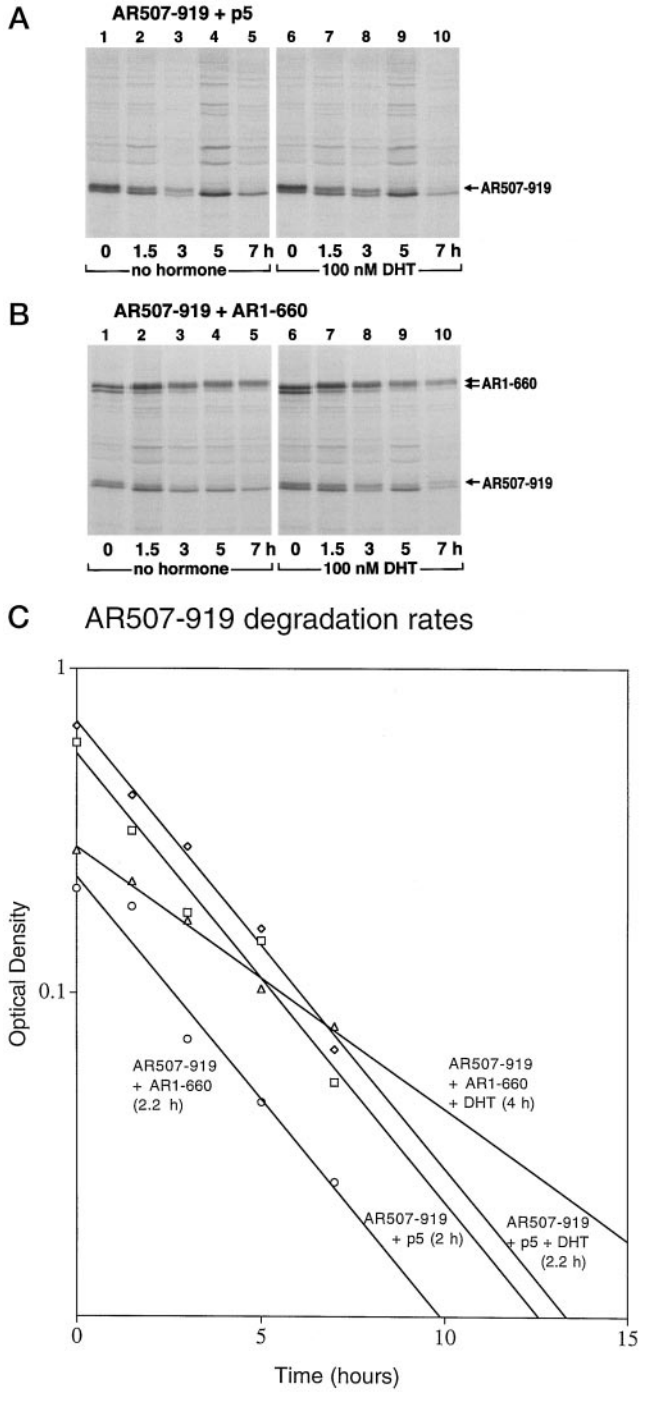

FIG. 8. **Influence of the AR NH_2-terminal and DNA binding domain fragment on degradation of the steroid binding domain fragment.** Transient transfections in COS-1 cells were performed using DEAE-dextran and 4 μg of AR507–919 plus 8 μg of the parent vector pCMV5 (p5) or 8 μg AR1–660 coding for the NH_2-terminal and DNA binding regions. After 48 h, cells were incubated for 30 min with 100 μCi of ^{35}S-labeled cysteine-methionine as described under "Experimental Procedures" followed by a chase period. AR fragments were harvested at increasing time intervals, immunoprecipitated using AR52 anti-peptide antibody, and analyzed on a 9% acrylamide gel containing SDS (*A* and *B*). *C*, shown are degradation rate data for AR 507–919 + pCMV5 (p5) in the absence of androgen (□), AR507–919 + p5 + 100 nM DHT (◇), AR507–919 + AR1–660 in the absence of androgen (○), and AR507–919 + AR1–660 + 100 nM DHT (△). Intensities of the AR507–919 bands shown in *A* and *B* were determined on an LKB laser densitometer and are shown on a semi-log scale.

drogen binding affinity, it can be difficult to assess their role in AR dimerization and DNA binding. More than 70 missense mutations that cause partial or complete androgen insensitivity are located throughout the AR steroid binding domain (35). The few that do not change the apparent androgen binding affinity yet are associated with partial or complete androgen insensitivity are particularly helpful in revealing additional mechanisms involving the steroid binding domain.

A series of AR gene mutations that cause androgen insensitivity without altering the apparent equilibrium androgen binding affinity were introduced into full-length AR expressed as a fusion protein with the VP16 transactivation domain or into the AR steroid binding domain expressed as a fusion protein with the GAL4 DNA binding domain to investigate dimerization polarity. Two residues, Val-889 and Arg-752, were identified as steroid binding domain residues critical for the N-C interaction. Both V889M and R752Q mutations disrupt the N-C interaction, suggesting that loss of function by these mutations in individuals with androgen insensitivity relates to ineffective N-C interaction at physiological androgen concentrations. AR with either mutation is transcriptionally active but requires androgen concentrations above the physiological range to overcome the defect.

Steroid binding domain mutation V889M causes grade 6 (35) androgen insensitivity (30). Its unaltered apparent equilibrium binding constant with 8-fold increased dissociation and presumably increased association rates suggest that Val-889 is not directly part of the steroid binding pocket but forms a structural barrier to androgen binding and dissociation. Val-889 is situated 30 residues from the AR carboxyl terminus in a region with sequence similarity to the region between helices 11 and 12 near the steroid binding pocket of the thyroid hormone β receptor based on crystal structure analysis (40). Homo- and heterodimerization interfaces of the thyroid hormone β receptor (41) include helical regions 10 and 11 (40) corresponding to human AR residues 855–883 preceding the V889M mutation. The proximity of Val-889 to the steroid binding pocket suggests this region undergoes conformational changes with androgen binding. From the crystal structure of the human RXR-α ligand binding domain, it was proposed that the region of helix 11 changes dramatically upon ligand binding (42). A region between residues 389–429 in the predicted helices 9 and 10 has a role in establishing the specificity of RXR homodimerization and heterodimerization with RAR and thyroid hormone receptor and subsequent DNA binding (13). Ligand binding to thyroid hormone receptor and RAR exposes a region different from the ninth heptad repeat to promote interactions with RXR (43).

Val-889 precedes by four amino acids the AF2 activation core region predicted for AR based on sequence similarity among the family of steroid hormone receptors (44). Unlike other steroid receptors, however, a transcriptional activation function in the AR steroid binding domain has remained elusive (18, 45, 46). It is conceivable that this region contributes to transcriptional activation through its interaction with the NH_2-terminal region. Val-889 follows by two amino acids a region in the steroid binding domain implicated in ER dimerization and high affinity DNA binding (9). A preponderance of indirect evidence implicates, therefore, the region of Val-889 not only in major conformational changes upon androgen binding that could alter the availability of the steroid binding pocket but in association with AR dimerization and transcriptional activation. We postulate that one of the underlying mechanisms in these processes involves the intermolecular N-C interaction that occurs during androgen-induced AR dimerization.

Mutations at both residues implicated in the N/C interaction, Val-889 and Arg-752, cause an 8–12-fold increase in androgen dissociation rate, whereas dissociation rates of the other mutations were only 3–4-fold increased. This result suggests that

very rapid androgen dissociation either promotes or results from disruption of the N-C interaction. The further increase in dissociation rate with deletion of the NH_2-terminal domain suggests stabilization of bound androgen by the NH_2-terminal domain. In the case of V889M and R752Q, some degree of stabilization of bound androgen in the full-length mutants likely resulted from the high, pharmacological androgen levels used in the dissociation experiments, concentrations at which these mutants are transcriptionally active. The NH_2-terminal region not only stabilizes androgen in the steroid binding pocket but also slows the turnover rate of the steroid binding domain in the bimolecular N-C interaction with wild-type AR fragments, both of which parallel characteristics of the full-length transcriptionally active AR. Whether the rapid androgen dissociation kinetics of V889M and R752Q is an inherent property of the steroid binding domain or results from the disrupted N-C interaction is unclear. Both Val-889 and Arg-752 are not likely directly part of the androgen binding pocket but could influence dissociation and association rates without significantly changing the apparent equilibrium binding constant while also having a role in the N-C interaction. Based on sequence similarity with the thyroid hormone β receptor, the crystal structure places Val-889 on the same surface as Arg-752 across the steroid binding pocket adjacent to a mixed β sheet region (40). Val-866, on the other hand, not implicated in the N-C interaction, is on a different surface of the steroid binding domain.

None of the AR mutations introduced into the full-length AR-VP16 fusion protein significantly alter the N-C interaction with the wild-type GAL4 DNA binding domain-AR steroid binding domain fusion protein. However, some of the same mutations introduced into the GALD-H steroid binding domain fragment eliminate the interaction with full-length AR at physiological androgen concentrations. The failure of full-length VPAR to interact with V889M-GALD-H or R752Q-GALD-H except at high androgen concentration, whereas GALD-H interacts with the mutant VPARs, indicates that the AR N-C interaction is intermolecular and that any resulting conformation changes do not impose a C-C interaction between the steroid binding domains. The lack of C-C interactions between human AR monomers agrees with previous results in the 2-hybrid protein interaction assay (18).

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