Liganded Androgen Receptor Interaction with β-Catenin

NUCLEAR CO-LOCALIZATION AND MODULATION OF TRANSCRIPTIONAL ACTIVITY IN NEURONAL CELLS*

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A yeast two-hybrid assay was employed to identify androgen receptor (AR) protein partners in gonadotropin-releasing hormone neuronal cells. By using an AR deletion construct (AR-(Δ371–485)) as a bait, β-catenin was identified as an AR-interacting protein from a gonadotropin-releasing hormone neuronal cell library. Immunolocalization of co-transfected AR and FLAG-β-catenin demonstrated that FLAG-β-catenin was predominantly cytoplasmic in the absence of androgen. In the presence of 5α-dihydrotestosterone, FLAG-β-catenin completely co-localized to the nucleus with AR. This effect was specific to AR because liganded progesterone, glucocorticoid, or estrogen α receptors did not translocate FLAG-β-catenin to the nucleus. Agonist-bound AR was required because the AR antagonists casodex and hydroxyflutamide failed to translocate β-catenin. Time course experiments demonstrated that co-translocation occurred with similar kinetics. Nuclear co-localization was independent of the glycogen synthase kinase-3β, p42/44 ERK mitogen-activated protein kinase, and phosphatidylinositol 3-kinase pathways because inhibitors of these pathways had no effect. Transcription assays demonstrated that liganded AR repressed β-catenin/T cell factor-responsive reporter gene activity. Conversely, co-expression of β-catenin/T cell factor repressed AR stimulation of AR-responsive reporter gene activity. Our data suggest that liganded AR shuttles β-catenin to the nucleus and that nuclear interaction of AR with β-catenin may modulate transcriptional activity in androgen target tissues.

Androgen receptor (AR)[1] is a member of the nuclear steroid receptor superfamily and is vital for normal sexual development in males (reviewed in Ref. 1). In androgen target cells, AR is predominantly localized in the cytoplasmic fraction in the absence of ligand and translocates to the nucleus in the presence of the endogenous androgens, testosterone and 5α-dihydrotestosterone (5α-DHT), where it activates transcription of AR-responsive genes (2, 3). In addition to the genomic actions of AR, nongenomic signaling mechanisms of androgens have also been described in several cell systems. Through activation of the Src/Shc/ERK signaling pathway, AR was shown to be anti-apoptotic in osteoblasts, osteocytes, embryonic fibroblasts, and HeLa cells (4). Activation of the ERK mitogen-activated protein kinase pathway by AR has also been demonstrated in prostate cancer cell lines (5). In bone, androgens have been shown to increase intracellular calcium, diacylglycerol, and inositol 1,4,5-trisphosphate formation (6).

The genomic actions of AR are dependent upon its nuclear translocation where it then binds to androgen-response elements, some of which conform to the consensus sequence 5′-GG(A/T)ACANNNTGTTCT-3′ (7), and stimulates the transcription of target genes. Several androgen-response elements are also recognized by the glucocorticoid receptor, and it is believed that protein-protein interactions play a role to discriminate AR- *versus* GR-mediated effects at these sites (8). Other nuclear factors that modulate AR transcriptional activity include cAMP-response element-binding protein, AP-1, and members of the POU family of homeodomain transcription factors (8–11). Protein-protein interactions are also involved in the role of AR as a transcriptional repressor independent of DNA binding. Recently, the mechanism of AR repression of the α-glycoprotein hormone subunit gene in the αT3-1 pituitary gonadotrope cell line was found to be through interactions with c-Jun and ATF-2 (12). Repression of the luteinizing hormone β subunit gene involved protein-protein interactions between AR and SF-1 (13). In a separate study, AR-mediated repression of the luteinizing hormone β subunit gene was also found to occur through protein-protein interactions with Sp1 and to a lesser degree Egr-1 (14).

The yeast two-hybrid system has been used successfully to identify protein partners of AR. Both co-activators and transcriptional repressors have been found. For example, Chang and colleagues (15–18) have identified the androgen receptor co-activators ARA70, ARA55, ARA54, and ARA267 from yeast two-hybrid screens of prostate cell libraries. Additionally, TIP60, a co-activator of human immunodeficiency virus Tat protein, and TRAM-1, a thyroid receptor co-activator, have been isolated from yeast two-hybrid screens using AR as a bait (19, 20). The androgen receptor-specific co-activator FHL2 was also identified by a yeast two-hybrid screen (21). The amino-terminal enhancer of split was identified as an AR repressor using a yeast two-hybrid assay (22).

In this study, we performed a yeast two-hybrid screen using a GT1-7 cell cDNA library to identify potential protein partners

[1] The abbreviations used are: AR, androgen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; ERα, estrogen receptor α; GSK-3β, glycogen synthase kinase-3β; GnRH, gonadotropin-releasing hormone; TCF, T cell-factor; 5α-DHT, 5α-dihydrotestosterone; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; RT, room temperature; MMTV, murine mammary tumor virus; FITC, fluorescein isothiocyanate; rAR, rat AR; hAR, human AR; N-C, amino- and carboxyl-terminal.

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of AR in GT1-7 GnRH neuronal cells and found β-catenin as an AR-interacting protein. β-Catenin was originally identified at adherins junctions involved in the binding of cadherins with the actin cytoskeleton (23) and therefore is important in the regulation of cell adhesion. However, a role for β-catenin in development and signal transduction was proposed because of sequence similarity with the *Drosophila* Armadillo protein that is a component of the *wingless* signaling pathway (24). In the absence of signaling mechanisms that stabilize cytoplasmic β-catenin, its levels in the cytoplasm are tightly regulated because of phosphorylation by glycogen synthase kinase-3β (GSK-3β) and subsequent proteosomal degradation (25, 26). During development, Wnt signaling pathways inhibit β-catenin degradation and cause accumulation of cytoplasmic β-catenin (27). Subsequently, β-catenin translocates to the nucleus and forms a complex with the T cell factor/lymphoid enhancer factor (TCF/Lef) family of transcription factors and stimulates transcription of target genes. Because it lacks a nuclear localization signal, the mechanisms for β-catenin nuclear translocation are not well understood. In our studies, we found that agonist bound AR but not progesterone receptor (PR), glucocorticoid receptor (GR), or estrogen receptor α (ERα)-induced nuclear translocation of β-catenin. Furthermore, liganded AR repressed β-catenin/TCF activation of β-catenin/TCF-responsive promoter activity. Conversely, β-catenin/TCF expression repressed AR-responsive promoter activity. Our results suggest that interactions between AR and β-catenin may modulate the nuclear localization of β-catenin and transcriptional activity in AR-responsive target tissues.

EXPERIMENTAL PROCEDURES

Reagents—AR (PA1–111A), ERα (PA1–308), GR (PA1–511A), and polyclonal FLAG (PA1–984) antibodies were obtained from Affinity Bioreagents (Golden, CO). Anti-PR (LS434) was a kind gift from Dean Edwards (University of Colorado Health Sciences Center). Anti-Myc (3F10), anti-GSK-3β (clone 7), and monoclonal anti-β-catenin (C19220) were purchased from Transduction Laboratories (Lexington, KY). Polyclonal anti-β-catenin was from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-GSK-3β antibody (9336) was purchased from Cell Signaling Technology (Beverly, MA). Secondary anti-mouse and anti-rabbit Texas Red or FITC-conjugated antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). The yeast Matchmaker 3 system was purchased from CLONTECH (Palo Alto, CA). 5α-DHT was purchased from Steraloids (Wilton, NH). Casodex and hydroxyflutamide were obtained from Zeneca Pharmaceuticals (Wilmington, DE) and Schering Corp. (Kenilworth, NJ), respectively. All other reagents unless otherwise noted were obtained from Sigma.

Cell Culture—Cell lines were grown in Dulbecco's modified Eagle's medium supplemented with either 5 or 10% fetal calf serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 0.25 μg/ml of amphotericin B at 37 °C in humidified 5% CO_2, 95% air. GT1-7 cells are immortalized murine GnRH neuronal cells derived from an SV40 large T antigen-targeted hypothalamic GnRH-producing tumor that displays neuronal specific markers and produce GnRH (28). NLT cells are also immortalized murine GnRH neuronal cells that produce GnRH but were derived from an olfactory tumor, thus representing early migrating GnRH neuronal cells (29). COS-7 cells were obtained from the Cancer Center Cell Culture Core facility, University of Colorado Health Sciences Center.

Plasmids—pGBKT7-AR-(Δ371–485) was generated by ligating PCR products corresponding to nucleotides 1–1110 of rat AR (rAR) (amino acids 1–370) to nucleotides 1458–2709 (amino acids 486–903) of rAR into pGBKT7. Briefly, the forward 5′-atgggtcgacatggaggtgcagtta-3′ and reverse 5′-tttatgcatcggcccggacagagc-3′ primers were used to generate the 1–1110 fragment. The forward 5′-ggcatgcattatcctggtggagttgtg-3′ and reverse 5′-tataggctgcagtcactgtgtgtggaa-3′ primers were used to generate the 1458–2709 fragment. Both fragments were ligated after restriction enzyme digestion with *Nsi*I. The ligation product was further digested with *Pst*I and then subcloned into pGBKT7 that had been cut with *Sma*I and *Pst*I. pCMV-rAR, pCMV-human AR (hAR), pCMV-AR-(1–503), pCMV-AR-(1–660), pCMV-AR-R617K618,632,633M, pCMV-AR-K720A, pCMV-AR-E897K and pCMV-AR-V716R were made as described (2, 30–34). pCMV-myc-AR was generated by subcloning

hAR into the *Sfi*I and *Not*I sites of pCMV-myc (CLONTECH). β-Catenin, TCF-4, and pGL3-OT plasmid constructs were kind gifts of Bert Vogelstein and Ken Kinzler (The Johns Hopkins University, Baltimore, MD). pGL3-OT contains a trimerized TCF optimal promoter sequence upstream of the luciferase gene and is a modified version of the TOP-FLASH vector (35). FLAG-β-catenin was a kind gift of E. R. Fearon (University of Michigan, Ann Arbor, MI). MMTV-luciferase contains sequences −1161 to +102 of the MMTV-long terminal repeat coupled to a luciferase reporter gene (36). The human glucocorticoid receptor (GR) construct RSHGRα was a kind gift from Ron Evans, and human PR (pSG5hPR) and ERα were obtained from Pierre Chambon.

Yeast Two-hybrid Assay—Yeast two-hybrid analysis was performed using the CLONTECH Matchmaker 3 system. A cDNA library generated from GT1-7 neuronal cell mRNA was subcloned into the Gal4 activation domain vector pGAD10. Rat AR-(Δ371–485) was subcloned into the bait plasmid pGBKT7 that contains the Gal4 DNA binding domain to generate pGBKT7-AR-(Δ371–485). Yeast AH109 cells were sequentially transformed with the library vector and pGBKT7-AR-(Δ371–485). Approximately 4×10^6 independent clones were plated on synthetic drop-out media (CLONTECH) without histidine, leucine, or tryptophan in the presence of 30 mM 3-aminotriazole and 200 nM 5α-DHT. Positive clones were confirmed by replating and selecting clones that grew in the presence, but not absence, of 5α-DHT and by liquid β-galactosidase assays.

For the β-galactosidase assays, pGBKT7-AR-(Δ371–485) and potential positive interacting clones were transformed into Y187 cells. Cells were grown to stationary phase in appropriate selection media and then diluted 1:4 into 8 ml of YPD media. Cells were grown an additional 5 h in the absence or presence of 10 nM 5α-DHT. Cells were harvested and lysed by three freeze/thaw cycles in buffer containing 100 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$. To 0.1 ml of cell lysate was added 0.7 ml of lysis buffer containing 40 mM β-mercaptoethanol, followed by 0.16 ml of lysis buffer containing 4 mg/ml of *o*-nitrophenyl β-D-galactopyranoside. Samples were incubated at 37 °C, and absorbance values were measured at 420 nm.

Immunoprecipitation and Immunoblot Analysis—COS-7 cells were co-transfected with pCMV-myc-AR and FLAG-β-catenin in the presence of 10 nM 5α-DHT. Cells were rinsed with cold PBS and lysed in buffer containing 50 mM HEPES, pH 7.5; 0.5% Triton X-100; 5 mM EDTA; 50 mM NaCl; 10 mM sodium pyrophosphate; 50 mM NaF; 1 mM Na_3VO_4; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml each aprotinin, leupeptin, and pepstatin. For immunoprecipitation assays, 300 μg of lysates were incubated with 1 μg anti-Myc antibody for 2 h at 4 °C. Immune complexes were precipitated for 1 h using protein A + G (Oncogene Sciences) and washed three times in lysis buffer prior to analysis by SDS-PAGE and immunoblotting. Proteins were separated using 8% SDS-PAGE, transferred to HyBond-P membranes (Amersham Biosciences), and probed with either anti-Myc or anti-FLAG antibodies (1 μg/ml each). For immunoblot analysis of cell extracts, 20–40 μg of whole cell lysates were separated using 8% SDS-PAGE, transferred to HyBond-P, and incubated with either anti-Myc, anti-FLAG, or β-catenin antibodies. Immunoreactive proteins were visualized using ECL (Amersham Biosciences) following the manufacturer's directions.

Immunocytochemistry—NLT GnRH neuronal cells were cultured in Dulbecco's modified Eagle's medium containing 5% charcoal-stripped fetal bovine serum for 36–48 prior to transfection. For transfections, ~15,000 NLT cells were plated on glass coverslips in 24-well plates and allowed to attach overnight. Cells were transfected using 320 ng of FLAG-β-catenin and 80 ng of steroid receptor constructs using LipofectAMINE plus (Invitrogen) and incubated for 16 h. For immunocytochemistry, cells were rinsed with 1 ml PBS at RT and then fixed in 4% paraformaldehyde/PBS for 30 min at RT. Cells were rinsed twice with 1 ml of PBS and then permeabilized with 0.5 ml of 5% bovine serum albumin, 0.2% Triton X-100 in PBS for 90 min at RT. For double label experiments, cells were incubated with either anti-AR (2 μg/ml), anti-PR (1:1000), or anti-GR (2 μg/ml) and either monoclonal anti-FLAG (M2 Sigma; 2 μg/ml for AR and GR) or polyclonal anti-FLAG (2 μg/ml for PR) antibodies overnight at 4 °C in 3% bovine serum albumin in PBS. Cells were then rinsed three times with PBS and incubated with anti-rabbit or mouse secondary antibodies conjugated to either Texas Red or FITC (1:1000) in 3% bovine serum albumin in PBS for 90 min at RT. For immunocytochemistry of endogenous β-catenin, cells were fixed and incubated with polyclonal anti-β-catenin (Santa Cruz Biotechnology) followed by anti-rabbit FITC conjugate (1:1000). Coverslips were coated with 1 mg/ml *p*-phenylenediamine in 75% glycerol, 0.25× PBS prior to mounting and visualization. Images were viewed using a Zeiss Axioskop II microscope.

Luciferase Reporter Assays—NLT cells were grown in 5% charcoal-

stripped fetal bovine serum in Opti-MEM for 36–48 h prior to transfection. Cells were transfected using LipofectAMINE Plus (Invitrogen) with 1 μg of the luciferase reporter construct (MMTV-LUC or pGL3-OT), 0.5 μg of β-catenin, 0.3 μg of TCF-4, and 0.5 μg of either pCMV-AR or empty control pCMV vector. Cells were also transfected with 0.1 μg of RSVβgal to control for transfection efficiency. Cells were harvested 16–18 h post-transfection and assayed for luciferase and β-galactosidase activities as described previously (37).

Statistical Analysis—Comparisons between ethanol control, and 5α-DHT-treated samples were analyzed using unpaired *t* test using GraphPad InStat version 3.02 for Windows, GraphPad Software, San Diego, CA.

<div align="center">RESULTS</div>

The Armadillo Repeat of β-Catenin Binds AR in the Yeast Two-hybrid Assay—To identify neuronal protein partners of AR, a cDNA library was prepared from the GT1-7 GnRH cell line. This library was used in a yeast two-hybrid screen using an AR deletion mutant (AR-(Δ371–485)) that contained a truncation of amino acids 371–485 (Fig. 1*A*). This construct was designed to possess lower transactivation activity in the yeast two-hybrid system than the wild-type AR, yet still contain AR domains essential for potential protein-protein interactions, *i.e.* N-C domains and DNA-binding domain. From ~4 × 10^6 clones screened in the presence of 5α-DHT, one of the positive clones encoded an open reading frame of 236 amino acids. BLAST analysis against the NCBI protein sequence data base demonstrated that this open reading frame consisted of amino acids 187–423 of murine β-catenin. This region of β-catenin spans the Armadillo repeat region beginning at repeat 2 and ending in repeat 7 (Fig. 1*A*). These Armadillo repeats are also necessary for β-catenin binding to cadherins, APC, axin, and TCF transcription factors (38).

The dependence of ligand for the interaction between AR-(Δ371–485) and β-catenin-(187–423) was confirmed using the yeast two-hybrid assay. Constructs were co-transformed into the yeast strain Y187 which allowed for quantitative analysis of the interaction by measuring β-galactosidase reporter activity (Fig. 1*B*). No measurable β-galactosidase activity was detected in the absence of 5α-DHT with the bait plasmid alone or together with β-catenin-(187–423). In the presence of 5α-DHT, β-galactosidase activity was detected using AR-(Δ371–485) alone because of the ability of this construct to transactivate the β-galactosidase reporter gene. β-Galactosidase activity was increased ~5-fold, however, when the interaction between AR-(Δ371–485) and β-catenin-(187–423) was assayed in the presence of 5α-DHT, confirming the ligand dependence of this interaction.

AR Interacts with β-Catenin in Mammalian Cells—Because the yeast two-hybrid assay detected an interaction between an AR deletion mutant and the Armadillo repeat region of β-catenin, COS-7 cells were co-transfected with full-length FLAG β-catenin and myc-AR to determine whether AR could interact with β-catenin in mammalian cells. Protein complexes were immunoprecipitated with anti-Myc and immunoblotted with anti-FLAG. Fig. 1*C* demonstrates that FLAG-β-catenin was co-immunoprecipitated with AR in cells transfected with both constructs but not from cells transfected with FLAG-β-catenin and control pCMV-myc vector.

To confirm the presence of β-catenin in GnRH neuronal cells, endogenous β-catenin was visualized by immunoblot analysis and immunocytochemistry. By immunoblot analysis, β-catenin was present in both GT1-7 and NLT cell lines, with slightly higher expression in NLT cells (Fig. 1*D*). Immunocytochemical analysis demonstrated that endogenous β-catenin was predominantly localized to cell membranes, consistent with its cytoskeletal role. Additionally, low levels of diffuse cytoplasmic staining was apparent in both cell lines (not shown). These
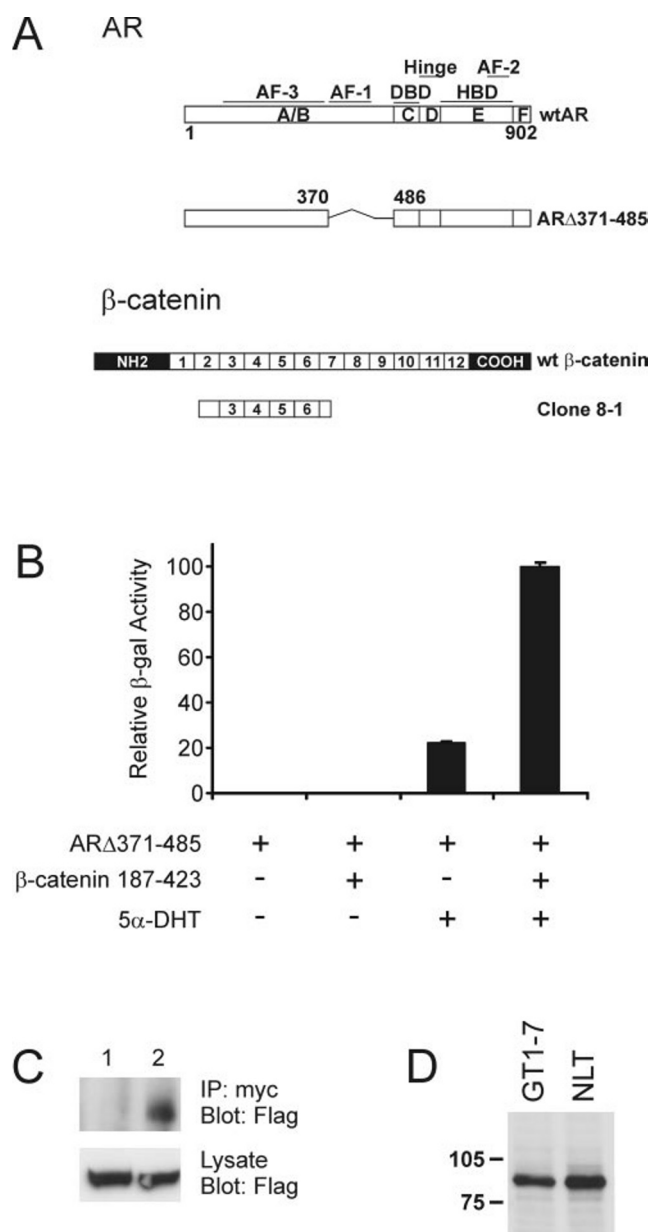

FIG. 1. **Androgen receptor interacts with β-catenin.** *A*, the rat AR construct (AR-(Δ371–485)) used in the yeast two-hybrid screen contains a deletion of the activation domain 1 spanning amino acids 371–485. Alignment of the β-catenin fragment isolated from the yeast two-hybrid screen with full-length β-catenin demonstrated that clone 8 spans Armadillo repeat regions 2–7. *B*, β-galactosidase activity in yeast Y187 cells transformed with AR-(Δ371–485) and β-catenin-(187–423) is ligand-dependent. Data are expressed as mean ± S.E., *n* = 4. *C*, COS-7 cells were transfected with FLAG-β-catenin and either pCMV1 (*lane 1*) or pCMV-myc-AR (*lane 2*). Cell lysates were immunoprecipitated (*IP*) using anti-Myc antibody. Immunoprecipitated samples and total cell lysates were immunoblotted with anti-FLAG (M2) to detect β-catenin. *D*, immunoblot analysis of cell lysates from GT1-7 and NLT cells demonstrate the presence of endogenous β-catenin. 20 μg of total cell lysates were analyzed by SDS-PAGE and immunoblot analysis using monoclonal β-catenin antibodies and visualized by ECL.

results confirmed the presence of β-catenin in GT1-7 and NLT GnRH neuronal cell lines and are in agreement with previous studies (39) that investigated the presence and distribution of β-catenin in GT1–1 cells during neurite outgrowth.

β-Catenin Co-localizes to the Nucleus with AR—The mechanism of nuclear translocation of β-catenin is not completely understood. To determine whether liganded AR could facilitate nuclear translocation of β-catenin, initial studies were per-

formed using GT1-7 cells that were co-transfected with rat AR and FLAG-β-catenin. To discriminate nuclear and cytoplasmic β-catenin from cytoskeletal components, cells were transfected with FLAG-β-catenin. Co-localization of AR and FLAG-β-catenin in the nucleus was observed in the presence of 5α-DHT (not shown); however, GT1-7 cells adhered poorly to the slides required for immunocytochemical analysis. Therefore, further experiments were performed in the NLT GnRH neuronal cell line. In co-transfected cells, AR and FLAG-β-catenin were distributed between the cytoplasmic and nuclear fractions in the absence of 5α-DHT. In the presence of 5α-DHT, however, both AR and FLAG-β-catenin were exclusively nuclear, suggesting that cytoplasmic β-catenin translocated to the nucleus with liganded AR (Fig. 2*A*). In the absence of AR, 5α-DHT alone had no effect on β-catenin localization (data not shown).

The time course for nuclear translocation of AR in response to 5α-DHT has been shown previously to be rapid, within 30 min (3). In the presence of 5α-DHT, AR translocated to the nucleus in NLT cells within 15–30 min (Fig. 2*B*). FLAG-β catenin also localized to the nucleus within 15–30 min in the presence of AR and 5α-DHT (Fig. 2*B*), suggesting that a direct interaction between AR and β-catenin was necessary for co-localization.

Anti-androgens Do Not Translocate β-Catenin to the Nucleus—The anti-androgenic compounds casodex and hydroxyflutamide have been shown previously to target AR to the nucleus; however, they do not activate transcription by AR and antagonize the physiological effects of androgens (40). To determine whether these anti-androgens could also induce the co-localization of FLAG-β-catenin with AR, NLT cells were co-transfected with AR and FLAG-β-catenin and treated with either 100 nM casodex or hydroxyflutamide. Although these anti-androgens induced nuclear localization of AR, they failed to result in β-catenin translocation (Fig. 2*C*). Flutamide, which binds AR with very low affinity (40), did not induce nuclear translocation of AR or FLAG-β-catenin (data not shown). The fact that casodex and hydroxyflutamide induced nuclear translocation of AR but not β-catenin suggests that translocation of AR alone is not sufficient to induce complete nuclear localization of β-catenin and that agonist, but not antagonist, -bound AR was required.

Because transfected β-catenin is distributed between the nucleus and cytoplasm in the absence of 5α-DHT, an alternative explanation for the nuclear co-localization of AR with β-catenin might be that 5α-DHT treatment induced cytoplasmic β-catenin degradation, thus leaving only the nuclear fraction. The balance between cytoplasmic and nuclear β-catenin during development and Wnt signaling is due to the regulation of degradation and stabilization of cytoplasmic β-catenin (27). Cytoplasmic β-catenin is rapidly degraded through a proteosomal pathway following phosphorylation by GSK-3β (25, 26). To exclude the possibility that liganded AR was activating GSK-3β and thus enhancing the cytoplasmic degradation of β-catenin, immunocytochemical localization experiments were performed in the presence of 20 mM LiCl, an inhibitor of the GSK-3β pathway (31). No changes were observed in the pattern of nuclear co-localization in the absence or presence of LiCl (data not shown). Furthermore, when NLT cells were transfected with AR and treated with 5α-DHT, there was no change in the activity of GSK-3β as determined by anti-phospho-GSK-3β immunoblot analysis (Fig. 2*D*). Because GSK-3β activation is downstream of the phosphatidylinositol 3-kinase pathway, we also tested the phosphatidylinositol 3-kinase inhibitor wortmannin in the nuclear translocation assay, and it had no effect (data not shown). These results confirm that the GSK-3β is not influential in the shuttling of cytoplasmic β-catenin to the nucleus.
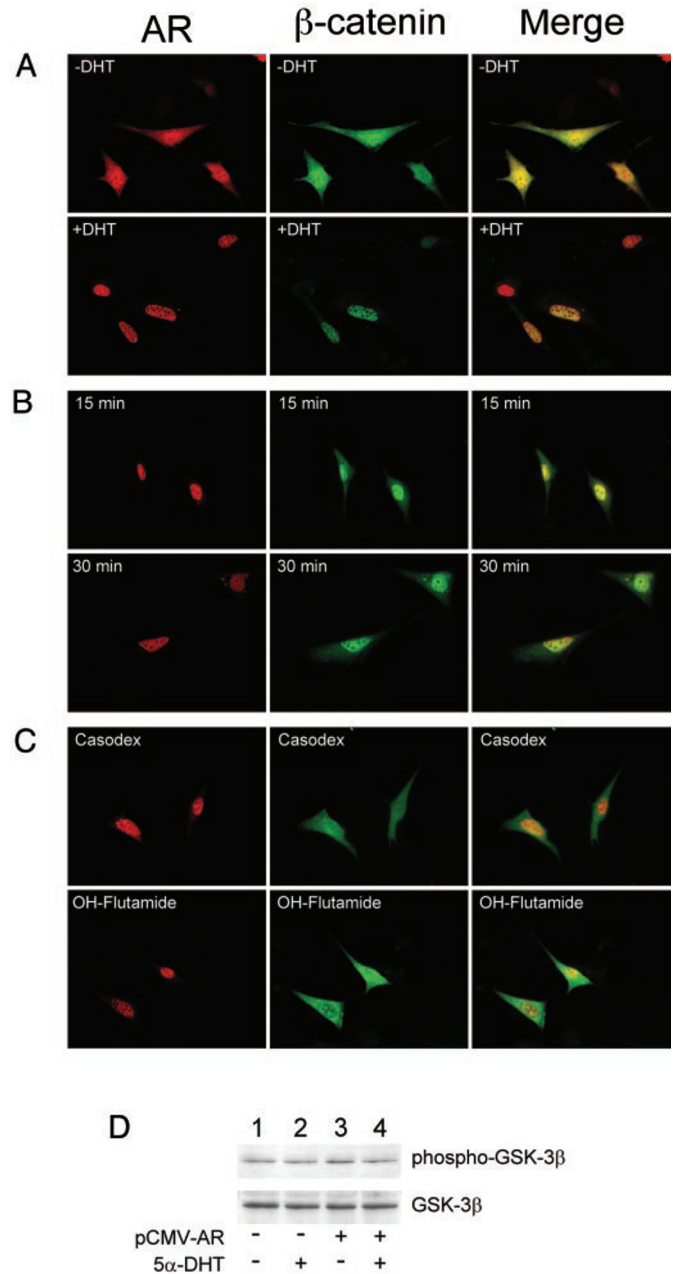

FIG. 2. **Androgen receptor induces nuclear localization of β-catenin.** *A*, NLT cells were transfected with rat AR and FLAG-β-catenin and were either untreated (*upper panel*) or treated with 10 nM 5α-DHT for 18 h (*lower panel*). Cells were fixed and visualized for AR using anti-AR (PA1–111A) followed by a Texas Red conjugated secondary anti-rabbit antibody. β-Catenin was visualized using anti-FLAG (M2) followed by a FITC-conjugated secondary anti-mouse antibody. *B*, NLT cells were transfected with rat AR and FLAG-β-catenin and treated with 10 nM 5α-DHT for either 15 (*upper panel*) or 30 min (*lower panel*). AR and β-catenin were visualized as in *A*. *C*, NLT cells were transfected with rat AR and FLAG-β-catenin and treated for 18 h with either 100 nM casodex (*upper panel*) or 100 nM OH-flutamide (*lower panel*). AR and β-catenin were visualized as in *A*. (Magnification, ×400.) *D*, liganded AR does not activate GSK-3β. NLT cells were either untransfected (*lanes 1* and *2*) or transfected with pCMV-rAR (*lanes 3* and *4*) and either untreated (*lanes 1* and *3*) or treated with 10 nM 5α-DHT (*lanes 2* and *4*). Phospho-GSK-3β was determined by SDS-PAGE and immunoblot analysis using a phospho GSK-3β antibody (Cell Signaling). Total GSK-3β was determined using GSK-3β antibody (Transduction Laboratories). Proteins were visualized by ECL.

In addition to the effects of AR as a transcription factor, liganded AR is known to activate the ERK mitogen-activated protein kinase pathway through a non-genomic mechanism (4,
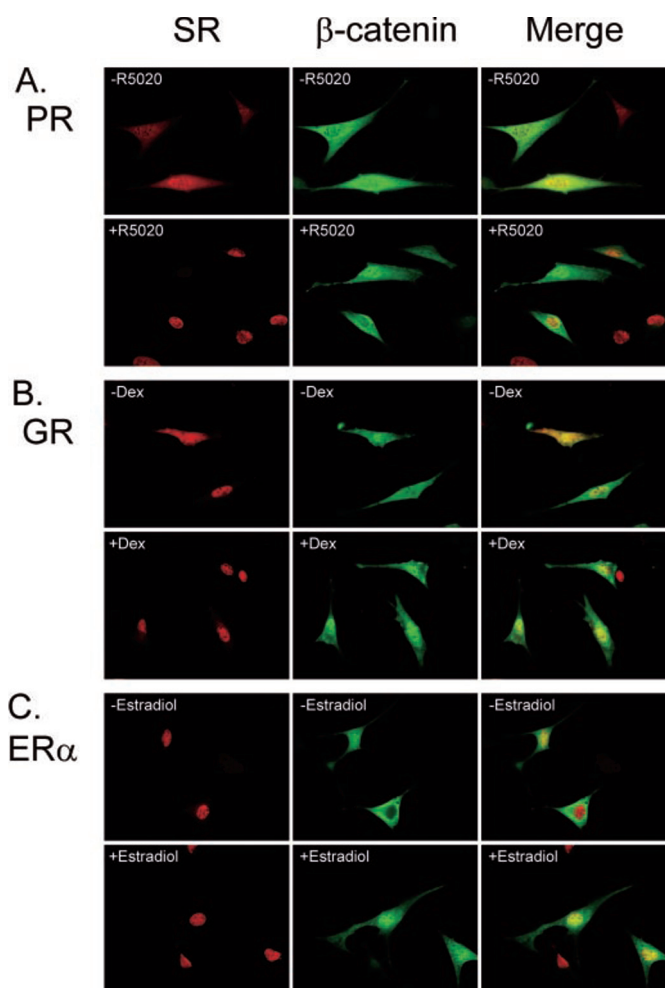
FIG. 3. **β-Catenin nuclear translocation is not induced by PR, GR, or ER**α. NLT cells were transfected with FLAG-β-catenin and the indicated human steroid receptors (*SR*), either PR (*A*), GR (*B*), or ERα (*C*). Transfected cells were either untreated or treated with 10 nM R5020 for PR, 100 nM dexamethasone (*Dex*) for GR, and 50 nM 17β-estradiol for ERα for 18 h. Cells were fixed and visualized as described under "Experimental Procedures." In each case, the steroid receptors were visualized using a Texas Red secondary conjugated antibody, and β-catenin was visualized using a FITC secondary conjugated antibody. (Magnification, ×400.)

FIG. 4. **β-Catenin nuclear translocation is not induced by AR-(1–503) or AR-(1–660).** NLT cells were transfected with FLAG-β-catenin and either human AR-(1–503) (*A*) or AR-(1–660) (*B*) and either untreated (−*DHT*) or treated with 10 nM 5α-DHT (+*DHT*) for 18 h. The AR constructs and β-catenin were visualized as in Fig. 2. (Magnification, ×400.)

5). We tested the ability of the MEK1/2 inhibitor PD98059 on AR-mediated translocation of β-catenin, and this compound also had no effect on nuclear translocation (data not shown). Taken together, these data suggest that a direct interaction between AR and β-catenin occurs, and co-nuclear translocation is responsible for the nuclear accumulation of β-catenin in the presence of 5α-DHT.

PR, GR, and ERα Do Not Translocate β-Catenin to the Nucleus—To determine the specificity of other steroid hormone receptors for translocation of β-catenin, NLT neuronal cells were co-transfected with FLAG-β-catenin and either PR, GR, or ERα. PR was both cytoplasmic and nuclear in the absence of the PR ligand, R5020, but shifted to a completely nuclear localization in the presence of ligand. FLAG-β-catenin, however, remained diffusely distributed in the cell either in the absence or presence of R5020 (Fig. 3*A*). Similarly, GR in the presence of dexamethasone completely translocated to the nucleus, whereas β-catenin staining remained cytoplasmic (Fig. 3*B*). ERα localized to the nucleus in the absence or presence of 17β-estradiol. However, β-catenin failed to co-localize with ERα to the nucleus (Fig. 3*C*). Together, these results demonstrate that liganded AR is unique among these steroid recep-
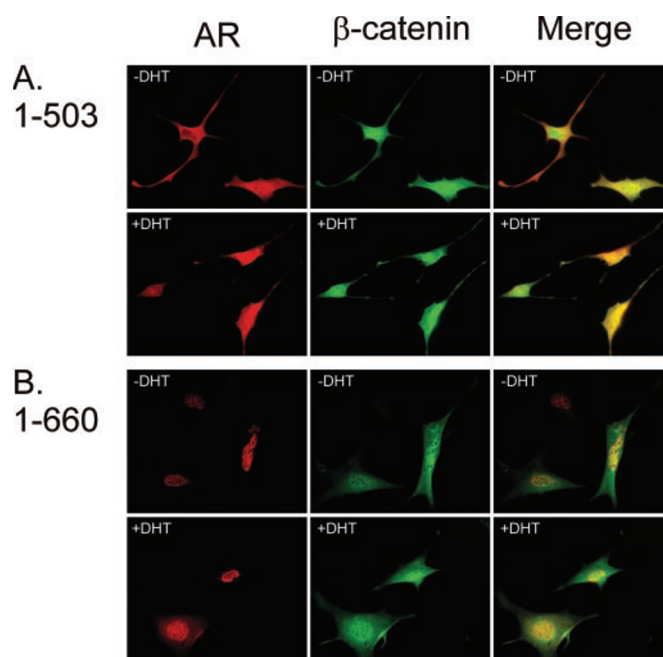
tors in its ability to co-localize β-catenin to the cell nucleus in GnRH neuronal cells.

The Carboxyl Terminus of AR Is Required to Translocate Cytoplasmic β-Catenin to the Nucleus—AR-dependent transcriptional activity is dependent on interactions between the amino and carboxyl termini of the receptor (30, 41). To test for androgen receptor domains that mediate nuclear co-localization with β-catenin, NLT cells were co-transfected with FLAG-β-catenin and several mutant forms of the androgen receptor. AR-(1–503) is a truncated AR lacking the DNA and ligand binding domains of the receptor (2). This truncated receptor lacks the nuclear localization signal of AR and remained cytoplasmic in the absence or presence of 5α-DHT (Fig. 4*A*). Expression of AR-(1–503) failed to induce nuclear translocation of the β-catenin (Fig. 4*A*). AR-(1–660) is truncated after the DNA binding domain but prior to the ligand binding domain (2). This construct is active toward AR-responsive promoters in the absence or presence of androgen (2). Expression of AR-(1–660) was constitutively nuclear in neuronal cells in the absence or presence of 5α-DHT. Nuclear β-catenin was more prominent in the AR-(1–660) transfected cells when compared with the full-length receptor in the absence of 5α-DHT (Fig. 2*A*) but did not completely translocate β-catenin to the nucleus (Fig. 4*B*) when compared with the full-length receptor in the presence of 5α-DHT (Fig. 2*A*).

One mechanism by which anti-androgens are thought to act is by disruption of N-C interactions of AR and thereby inhibition of co-activator binding (42). Because anti-androgens induced AR nuclear translocation, but not that of β-catenin, we investigated the co-translocation of β-catenin with mutant ARs that are defective in either N-C interaction or p160 co-activator binding (32). The results of these experiments are summarized in Table I. AR K720A, which is functional for N-C interactions but is defective for co-activator binding, and AR E897K and V716R, which are defective for N-C interactions, were each effective in translocating β-catenin to the nucleus. Additionally, as would be expected, the yeast two-hybrid bait construct

TABLE I

Steroid receptor and β-catenin nuclear co-localization

Steroid receptor constructs were co-transfected into NLT GnRH neuronal cells with FLAG-β-catenin. Intracellular localization of the steroid receptors and FLAG-β-catenin was determined by immunocytochemistry as described under "Experimental Procedures." The respective single amino acid mutations of the human AR are indicated for AR K720A, E897K, and V716R; R617K618,632,633M contains four methionine substitutions at the indicated basic amino acids; AR-(1–503) and AR-(1–660) are AR truncation deletion mutants of the human AR; PR, GR, and ERα are human progesterone, glucocorticoid, and estrogen receptor α, respectively. ++ indicates complete nuclear translocation; + indicates partial nuclear translocation; and − indicates no nuclear translocation.

Steroid receptor	Steroid receptor nuclear translocation	β-Catenin nuclear translocation
rAR	++	++
hAR	++	++
AR K720A	++	++
AR E897K	++	++
AR V716R	++	++
AR R617K618,632,633M	−	−
AR-(Δ371–485)	++	++
AR-(1–503)	−	−
AR-(1–660)	++	+
PR	++	−
GR	++	−
ERα	++	−

AR-(Δ371–485) which interacted with β-catenin also induced β-catenin translocation. Taken together, these data suggest that the carboxyl-terminal hormone binding domain of AR is required for complete β-catenin translocation; however, functional N-C interactions or p160 co-activator binding are not required.

Nuclear Translocation of AR Is Required for Nuclear Translocation of β-Catenin—Our results with the deletion mutants AR-(1–503) and AR-(1–660) suggested that the carboxyl terminus as well as nuclear localization of AR were required to translocate β-catenin to the nucleus. To determine whether nuclear localization of β-catenin was dependent upon nuclear translocation of AR, β-catenin translocation was tested using the AR construct R617K618,632,633M, which is defective for nuclear localization in the presence of androgen (31). This mutated AR contains methionine substitutions for the basic amino acids at positions 617, 618, 632, and 633 located within the nuclear localization signal of AR. In GnRH neuronal cells, AR R617K618,632,633M was defective for nuclear localization in the absence or presence of 5α-DHT and did not translocate β-catenin to the nucleus (Table I). These data demonstrate that nuclear translocation of agonist-bound AR was required for β-catenin nuclear localization.

Ligand-dependent Repression of AR and β-Catenin/TCF-mediated Transcription—Korinek et al. (43) have recently demonstrated the stimulation of a TCF promoter-luciferase reporter construct in cell lines transfected with β-catenin and TCF-4. To determine whether the interaction between AR and β-catenin could modulate β-catenin-dependent transcriptional activity of a β-catenin/TCF-responsive promoter, NLT GnRH neuronal cells were transfected with AR, TCF-4, and β-catenin and the reporter plasmid pGL3-OT. The luciferase reporter plasmid pGL3-OT contains a trimerized TCF optimal promoter sequence upstream of the luciferase gene (35). In agreement with previous studies (43), minimal reporter activity was observed in the absence of transfected TCF-4 and β-catenin in GnRH neuronal cells. This activity was unaffected by AR in the absence of 5α-DHT but was repressed by 27% in the presence of AR plus 5α-DHT (Fig. 5A). Co-expression of TCF-4 and β-catenin resulted in a luciferase reporter activity increase of 35-fold. In the absence of 5α-DHT, AR did not modulate this activity.
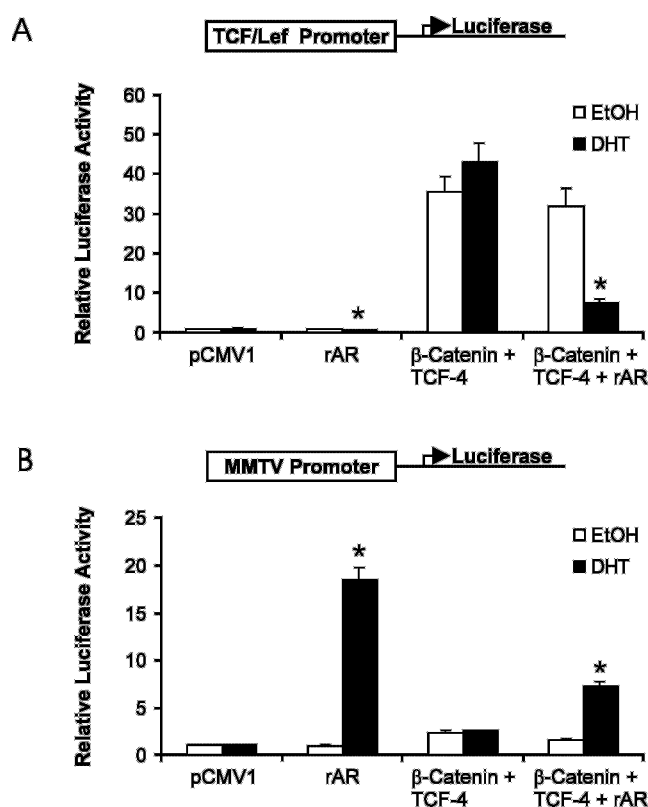

FIG. 5. **AR and β-catenin/TCF-mediated transcriptional activity are suppressed in GnRH neuronal cells.** NLT cells were transfected with rat AR, β-catenin, and TCF-4 and assayed for either β-catenin/TCF-4 transcriptional activity using a TCF/Lef-luciferase (pGL3-OT) reporter construct (*A*), or AR transcriptional activity using MMTV-luciferase as a reporter system (*B*). Cells were either untreated (EtOH vehicle control) or treated with 10 nM 5α-DHT. Data are expressed as mean ± S.E. from three independent experiments as relative activity normalized to pCMV1. *, $p < 0.05$ compared with EtOH vehicle control.

However, in the presence of 5α-DHT, luciferase activity was blunted to only 8-fold, a repression of ~75% (Fig. 5A).

Because ligand-bound AR modulated the transcriptional activity of the β-catenin-TCF complex, we investigated the ability of the β-catenin-TCF complex to modulate AR transcriptional activity. NLT cells were transfected with the AR-responsive reporter construct MMTV-LUC and either AR alone or in combination with β-catenin/TCF-4 (Fig. 5B). In the presence of 5α-DHT, AR stimulated MMTV-luciferase activity ~18-fold. In the absence of AR, β-catenin/TCF-4 had a modest 2-fold stimulatory activity on MMTV-luciferase in the absence or presence of 5α-DHT. However, in the presence of 5α-DHT and AR, β-catenin/TCF-4 repressed AR stimulation of MMTV-luciferase to only 7-fold, a 61% repression. Together, these data suggest a functional interaction between liganded AR and β-catenin/TCF that effectively represses both AR and β-catenin/TCF-mediated activation of gene transcription in neuronal cells.

DISCUSSION

Transcriptional activity of steroid hormone receptors requires accessory proteins to act as either co-activators or co-repressors. We used a yeast two-hybrid screen to identify AR-binding proteins in a neuronal cell cDNA library. We identified β-catenin as a novel AR-interacting protein. Furthermore, liganded AR enhanced the nuclear translocation of β-catenin and modulated β-catenin/TCF-dependent transcriptional activity.

The mechanisms of nuclear translocation of β-catenin are not completely understood. Cytoplasmic levels of β-catenin are tightly regulated by GSK-3β-dependent phosphorylation and

β-Catenin Nuclear Co-translocation with AR

subsequent proteosomal degradation. Accumulation of cytoplasmic β-catenin through either activation of Wnt signaling or conversely in cancers expressing mutated and degradation-resistant β-catenin results in nuclear translocation. Proteins larger than 40 kDa are generally excluded from the nucleus unless they contain a nuclear localization signal (NLS) (reviewed in Ref. 44). Because β-catenin is ~95 kDa, it is unlikely to enter the nucleus in a passive manner. However, β-catenin does not contain a nuclear localization signal, and its import into the nucleus is independent of the importin/karyopherin and Ran pathways (45, 46). Previous studies (47–49) have shown that co-expression of TCF proteins enhanced β-catenin nuclear localization. Additionally, the adenomatous polyposis coli tumor suppressor protein can shuttle β-catenin to the nucleus (50). Together, these results suggest that accessory proteins are required to chaperone β-catenin into the nucleus. In addition to these proteins, our results demonstrated that agonist bound AR but not GR, PR, or ERα translocated β-catenin to the nucleus, supporting the hypothesis that in androgen target tissues liganded AR may be a nuclear shuttling protein for β-catenin.

The mechanism by which AR translocates β-catenin to the nucleus appears to be due to the direct protein-protein interaction and not through secondary signaling events. AR did not activate GSK-3β activity in neuronal cells; therefore, the accumulation of nuclear β-catenin could not be attributed to a loss of cytoplasmic β-catenin. The time course for the nuclear translocation of β-catenin also paralleled that of AR. However, although AR was completely nuclear and the majority of β-catenin was nuclear after 15–30 min, some cytoplasmic staining of β-catenin was still observed. After 18 h however, complete AR and β-catenin nuclear localization was seen. The residual cytoplasmic β-catenin seen 15–30 min after treatment with 5α-DHT may be due to a pool of β-catenin that was not in direct proximity with AR. Because we have observed nuclear β-catenin in transfected cells in the absence of AR and 5α-DHT, some β-catenin enters the cell nucleus in the absence of AR. It is possible that following treatment with 5α-DHT in AR-transfected cells, residual cytoplasmic β-catenin could subsequently enter the nucleus in an AR-independent manner. Once in the nucleus, this pool of β-catenin could be sequestered by agonist-bound AR, thus demonstrating complete nuclear localization of β-catenin. Because the majority of β-catenin enters the cell nucleus with the same kinetics as AR, these results support a role for direct protein-protein interactions between AR and β-catenin in the process of nuclear co-localization.

To investigate whether functional AR was required for the translocation of β-catenin, AR mutants were tested for their ability to translocate β-catenin. By using the truncated AR-(1–503) and the nuclear translocation-deficient mutant R617K618,632,633M, neither the mutated receptors nor β-catenin was translocated to the nucleus. The truncation mutant AR-(1–660) was constitutively nuclear in the absence or presence of ligand, yet failed to completely translocate β-catenin like the full-length receptor. However, enhanced nuclear localization of β-catenin was observed with AR-(1–660) when compared with full-length AR in the absence of 5α-DHT or β-catenin alone, suggesting that AR-(1–660) could interact with β-catenin. The mechanism for the inability of AR-(1–660) to completely translocate β-catenin to the nucleus is not clear. It is possible that the AR carboxyl terminus, containing the hormone binding domain, participates in the interaction with β-catenin. Alternatively, without the hormone binding domain, AR-(1–660) is constitutively nuclear and does not occupy the same cytoplasmic compartment with β-catenin and therefore

cannot interact with most of the cytoplasmic β-catenin. Although AR-(1–660) may enhance the translocation of β-catenin to the nucleus, these data demonstrate that the carboxyl terminus of AR is required for complete β-catenin nuclear co-translocation.

Because the carboxyl terminus of AR interacts with the amino terminus to recruit p160 family of co-activators for AR-mediated transcription, our results with wild-type, AR-(Δ371–485), and AR-(1–660) suggested that functional N-C interactions and ability of AR to recruit co-activators might be required for AR and β-catenin co-translocation. To test this hypothesis, we used the mutant AR constructs K720A, E897K, and V716R which are deficient for recruitment of p160 co-activators. Additionally, AR V716R and E897K do not form functional N-C interactions (32). However, all of these constructs effectively co-translocated β-catenin to the nucleus (Table I), demonstrating that neither functional N-C interactions nor p160 co-activator binding were essential for β-catenin co-translocation. However, our results with the AR antagonists suggest that nuclear translocation alone of full-length AR is not sufficient to translocate β-catenin to the nucleus. In several laboratories, casodex and hydroxyflutamide have previously been shown to translocate AR to the nucleus, but did not induce DNA binding (24, 30). In neuronal cells, these anti-androgens induced nuclear localization of AR but not β-catenin. Together, our results suggest that for complete nuclear translocation of β-catenin the carboxyl terminus of AR is important and that liganded AR must be in a correct conformation to allow DNA binding. However, N-C interactions and p160 co-activator recruitment do not appear to be essential.

The role of β-catenin/TCF signaling in normal physiology of cells is not well understood. Downstream target genes of the β-catenin/TCF pathway include Myc, Fra, and Jun (35, 51), cyclin D-1 (52) and matrix metalloproteinase-7 (53). These genes are involved in normal growth and development as well in tumorigenesis. Mutated forms of β-catenin have been identified in tumors including colon, prostate, ovaries, and thyroid (54–60). Transforming mutations in β-catenin are concentrated at the amino terminus, which contains consensus phosphorylation sites for GSK-3β. These mutations inhibit the phosphorylation of β-catenin and its subsequent degradation, thereby increasing both the cytoplasmic and nuclear fractions of cellular β-catenin (reviewed in Ref. 61). Unregulated nuclear accumulation of β-catenin leads to a transformed phenotype through up-regulation of β-catenin/TCF-responsive genes. The interaction of AR with β-catenin suggests that AR may play a role in either the physiologic or pathophysiologic functions of β-catenin/TCF.

Our findings suggest that AR attenuates transcriptional activity of the β-catenin-TCF complex in GnRH neuronal cells. Endogenous β-catenin and TCF activity are present in GnRH neuronal cells; however, in the absence of co-transfected β-catenin and TCF-4, low TCF-luciferase reporter activity was observed. This activity was decreased by 27% by agonist-bound AR, demonstrating that AR repressed endogenous β-catenin/TCF signaling. Consistent with the results of Korinek *et al.* (43), in which activation of TCF-luciferase reporter in the B-cell line IIA1.6 required co-transfection with β-catenin and TCF-4, co-transfection of β-catenin and TCF-4 was required to maximally stimulate TCF-luciferase reporter activity in GnRH neuronal cells. The low level of activation of TCF-luciferase reporter activity in untransfected cells may be due to low expression of endogenous TCF proteins or to the localization of endogenous β-catenin. In the absence of mutated β-catenin or Wnt signaling, β-catenin is primarily extra-nuclear, and therefore low tran-

scriptional activity of the β-catenin/TCF pathway is observed. When transfected with a β-catenin expression plasmid, the distribution of β-catenin is both cytoplasmic and nuclear. Because overexpressed β-catenin is partially nuclear, this effect may mimic the nuclear localization observed during Wnt signaling or by degradation-resistant forms of mutated β-catenin. These results raise the possibility that agonist-bound AR may modulate aberrant β-catenin/TCF signaling in tumors of androgen-dependent tissues that contain mutated forms of β-catenin. For example, Truica *et al.* (62) recently showed that AR could interact with both wild-type and a phosphorylation-defective S33F β-catenin mutant in the LnCAP prostate cancer cell line. In contrast to our studies in which cells were co-transfected with AR, β-catenin, and TCF-4, the LnCAP experiments involved transfection with β-catenin alone. In our studies, β-catenin/TCF attenuated AR-stimulated MMTV-luciferase activity, whereas in LnCAP cells β-catenin enhanced AR target gene reporter activity up to 4-fold and enhanced the sensitivity of AR to weak AR agonists including androstenedione, dehydroepiandrosterone, and 17β-estradiol. Together with these results, our data suggest that the functional consequences of the interaction of AR with β-catenin may also be dependent upon the levels of nuclear TCF proteins.

In addition to interactions between AR and β-catenin, other nuclear receptor pathways have recently been shown to modulate β-catenin/TCF-mediated transcription. The retinoic acid receptor can also interact with β-catenin and cross-talks with the β-catenin/TCF pathway (63). In those studies, 9-*cis*-retinoic acid inhibited β-catenin/TCF reporter signaling in several tumor cell lines, and mutated β-catenin augmented retinoic acid receptor-mediated gene expression in MCF-7 cells. In addition, estrogen receptors were also shown to modulate β-catenin/TCF reporter gene activity (64). Rather than interacting with β-catenin, these studies demonstrated that the interaction between ER occurred with TCF family members. Curiously, TCF-4 antagonized but TCF-1 stimulated the transcriptional activity of ER. These data support cross-talk of β-catenin and liganded steroid receptors that may be cell type- or promoter-specific and may be dependent upon the complement of TCF transcription factors present in the cells.

In summary, we have identified β-catenin as a liganded AR-interacting protein in neuronal cells. In addition, agonist but not antagonist-bound AR shuttles β-catenin to the nucleus, and this effect was specific to AR but not PR, GR, or ERα. Within the nucleus, AR and β-catenin/TCF antagonize each other's effects on promoter activity in neuronal cells. Together these experiments and other emerging data suggest that steroid receptors may cross-talk with the β-catenin/TCF at several levels and may modulate the actions of the β-catenin/TCF pathway in normal development as well as pathophysiologic conditions.

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