Interaction of β-Catenin and TIF2/GRIP1 in Transcriptional Activation by the Androgen Receptor*

Received for publication, April 8, 2005, and in revised form, July 13, 2005 Published, JBC Papers in Press, September 1, 2005, DOI 10.1074/jbc.M503850200

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The multifunctional oncoprotein β-catenin interacts with the activation function-2 domain of androgen receptor (AR) to stimulate androgen receptor transcriptional activity, increase sensitivity, and broaden specificity of ligand interactions. β-Catenin interacts with androgen receptor in close proximity to the binding groove for P160 coactivators such as transcriptional intermediary factor-2 (TIF2)/glucocorticoid receptor interacting protein-1 (GRIP1). β-Catenin can also bind directly to TIF2/GRIP1. Both N- and C-terminal regions of β-catenin are needed for optimal interaction with TIF2/GRIP1. We show that distinct residues of β-catenin are responsible for both binding and functional interactions with androgen receptor and with TCF4, thus allowing the introduction of missense mutations that selectively affect these interactions. β-Catenin and TIF2/GRIP1 are each able to mediate binding between the other and androgen receptor in functional interactions that enhance ligand-dependent transcription. The data strongly imply that AR, β-catenin, and TIF2/GRIP1 bind in a three-way interaction that mediates transcription. Lastly, we observed that a β-catenin C-terminal peptide containing 229 amino acids can bind TIF2/GRIP1 and AR but has a profound dominant inhibitory effect on ligand-dependent transcription. We propose that β-catenin may play an integral role in formation of the androgen-receptor transcriptional complex.

AR2 activity is essential to the growth and progression of prostate cancer at all phases of the disease. A member of the steroid hormone receptor family, AR binds androgen and drives the transcription of androgen-responsive genes in a variety of tissues and cell types. Like the prostatic epithelium from which it is derived, prostate cancer cells are dependent on the presence of androgen when it first presents. Androgen ablation therapy is the first line of treatment for metastatic prostate cancer (1). The vast majority of cases show a clinical response to androgen ablation therapy and results in cleavage of β-catenin near the N terminus to remove the phosphorylation sites and produce a 75-kDa protein. This finding further supports the notion that β-catenin plays an important role in activation of AR signaling in advanced, androgen-independent prostate cancer.

Several studies have suggested interaction of components of the TCF4 transcription factors and androgen receptor transcription complex. A number of reports have described functional and physical interaction between androgen receptor and β-catenin (9, 10, 12), cross-talk between AR-mediated and TCF4-mediated transcription (11, 16), and direct interaction of β-catenin, TIF2/GRIP1, and AR in the region of the AR C-terminal AF-2 domain (11). Moreover, β-catenin can also complex with TIF2/GRIP1 itself, suggesting a three-way interaction with AR and possible regulatory role for β-catenin in androgen action (13).

Here we explore the structural elements of β-catenin that determine interactions with TCF4 and AR and find that missense mutations of critical amino acids affect these two interactions differently. We proceed to show that either TIF2/GRIP1 or β-catenin can each mediate binding of the other to AR and that together they enhance AR-dependent transcriptional activation. The data have implications for regulation of AR action and suggest strategies for intervention of AR signaling in androgen-independent prostate cancer.

MATERIALS AND METHODS

Plasmids and Plasmid Construction—pCMVhAR, pCMVhAR507–919, and GAL4/AR LBD were provided by Elizabeth Wilson, University of North Carolina, Chapel Hill, NC. The construction of pCMVhAR507–919-E893A, pCMVhAR507–919-M894A, pCMVhAR507–919-E897A, pCMVhAR507–919-Q902R, GAL4/AR LBD, GAL4/AR LBD-E893A, GAL4/AR LBD-M894A, GAL4/AR LBD-E897A, and...
AR, TIF2, and β-Catenin Interaction

GAL4/AR LBD-Q902R has been described before (11). GAL4/β-catenin, GAL4/β-catenin-ARM, and VP-16/β-catenin have been reported earlier (11). GAL4/β-cateninΔN and GAL4/β-cateninΔC were provided by Salimuddin Shah and Stephen Byers, Georgetown University. VP-16/β-catenin (S33A), VP-16/β-catenin(Δ15–552) (S33A), and VP-16/β-catenin(Δ 576–781) (S33A), were kindly provided by Andreas Hecht (Max Planck Institute, Freiberg, Germany) and have been described previously. pGEX-KT-β-catenin was kindly provided by David L. Rimm (Yale University, New Haven, CT). K312A, K312A/R386A, R386A, K435A, R469A, and H470A mutations in pcDNA-β-catenin, pGEX-KT-β-catenin, and VP-16/β-catenin were made with the QuikChange site-directed mutagenesis kit as described before. All mutations were confirmed by nucleotide sequencing. TIF2 and TIF2 m123 have been described. TIF2, TIF2 5–1121, and VP-16/TIF2 were provided by Michael Stallcup (University of Southern California, Los Angeles). SubTIF2 was provided by Myles Brown (Dana-Farber Cancer Institute, Boston, MA). pFR-LUC, pM, pVP-16, pSG5, pBSK+, pcDNA3.1(+)+, the Renilla null luciferase reporter, and MMTV-luc have been described before (11). TCF4 plasmid and OT/OF reporter vectors were provided by Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD).

Cell Culture and Transfection—Unless otherwise stated in the figure legends, all transfections were performed in CV-1 cells. Briefly, 24 h before transfection, cells were plated in 24-well microtiter plates (Falcon) at a cell density of 2–4 × 10^5 cells per well. Transfection was performed with Lipofectamine Plus reagent (Invitrogen) with 10 ng of receptor-containing plasmid, 100 ng of reporter, 10 ng of Renilla null luciferase reporter, and the indicated amounts of coregulators. Unless otherwise stated, transfection was routinely done with the total transfected DNA brought up to 300 ng/well with pBSK+ DNA. In experiments with different coregulator cDNA plasmids in different vectors, equimolar amounts of the corresponding plasmid vectors were cotransfected to control for artifacts of the vector DNA. After incubation for 16 h, the cells were washed and phenol red-free medium supplemented with 5% dextran charcoal-stripped fetal calf serum containing hormones or vehicle was added. The final concentration of vehicle ethanol was 0.1%. After a further 24 h, cells were lysed in 100 µl/well 1× passive lysis buffer (Promega, Madison, WI), and 30 µl of the cell lysates was used to assay for luciferase activity with the dual-luciferase assay system from Promega. The data were then normalized for the cotransfected Renilla activity. All samples were in triplicate, and each experiment described was repeated at least two times.

GST Pull-down Assays—293T cells were transfected with pCMV-HAR, wild-type β-catenin, and TCF4, or TIF2 alone with Lipofectamine. Cells were treated with or without 1 nM R1881 or ethanol. 48 h after transfection, cells were collected, washed with ice-cold phosphate-buffered saline, and lysed by lysis buffer (20 mM Tris-HCl, pH 7.4, 120 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM Na3VO4, supplemented with protease inhibitor mixture, Roche Molecular Biochemicals). The lysates were cleared by centrifugation (14,000 g, 10 min at 4 °C). The total protein concentration of the extracts was measured with the DC protein assay (Bio-Rad). GST fusion proteins harboring wild-type β-catenin, β-catenin with point mutations (K312A, R386A, K435A, R469A, and H470A), or double mutation K312A/R386A were expressed in Escherichia coli BL21(DE3) (Stratagene) following induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 1.5 h at 30 °C. Cells were sonicated, and proteins were clarified following the manufacturer’s instruction (Amersham Biosciences). GST fusion proteins were purified on glutathione-Sepharose beads. For binding assays, GST/β-catenin protein, or GST alone bound to glutathione-Sepharose beads was incubated with 500 µg of the 293T cell lysates for 2 h at 4 °C. After extensive washing, the retained proteins were eluted with sample buffer and resolved on a precast 4 to 20% Tris-glycine gel (Invitrogen). Western blotting was performed using antibodies against AR (N-20, Santa Cruz Biotechnology), β-catenin (BD Biosciences Transduction Laboratories), TCF4 (clone 6H5–3, Upstate Biotechnology, Lake Placid, NY), TIF2 (BD Biosciences Transduction Laboratories), and GST (Santa Cruz Biotechnology).

Western Blotting—For detection of the expression of pcDNA3-β-catenin and VP-16/β-catenin mutants, SKBR3 cells were plated over-night in six-well plates. Cells were transiently cotransfected with 1 µg each of wild-type pcDNA3-β-catenin, VP-16/β-catenin, or different mutants, together with 1 µg of GFP vector with a Lipofectamine method according to the manufacturer’s protocol. 48 h after transfection, cell lysates were prepared as described in the previous paragraph. Aliquots of 30 µg of total proteins were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane (Bio-Rad), and probed with β-catenin antibody (BD Biosciences Transduction Laboratories, 1:2000) and anti-GFP antibody (Clontech, 1:5000), followed by a secondary anti-mouse (β-catenin) or anti-rabbit (GFP) immunoglobulin-horseradish peroxidase antibody (1:5000). Signal detection was performed with Supersignal West Pico chemiluminescent substrate (Pierce).

Coimmunoprecipitation—LNCaP cells were cultured in modified Iscove’s minimal essential medium supplemented with 5% charcoal-stripped serum for 48 h. Cells were then treated with 10 nM R1881 or ethanol. Cells were harvested at 24 h, washed with phosphate-buffered saline, and lysed by resuspension in 10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 0.1% Triton X-100, and protease inhibitor mixture (Roche Molecular Biochemicals, Nutley, NJ). Nuclei were isolated by centrifugation (800 g, 10 min at 4 °C) and resuspended in 2 volumes of 20 mM Hepes, pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 mM β-glycerophosphate, and protease inhibitors, incubated for 30 min at 4 °C on a rocker and collected for 30 min at 4 °C by microcentrifuge. Coimmunoprecipitation was done using Catch and Release version 2.0 reversible immunoprecipitation system (Upstate, Charlottesville, VA). Briefly, 500 µg of cell lysate was incubated with 2 µg of antibodies and 10 µl of affinity ligand at room temperature for 30 min in spin columns. Antibodies used for coimmunoprecipitation were rabbit immunoglobulin-purified anti-AR (PG-21, Upstate), monoclonal anti-TIF2 (BD Transduction Laboratories), and monoclonal anti-β-catenin (BD Transduction Laboratories). Columns were washed three times with wash buffer, and proteins bound to the columns were eluted with 70 µl of denaturing elution buffer. Samples were separated on 4–20% Tris-glycine gels, and Western blot analysis was performed as described with either monoclonal antibodies against AR (441, Santa Cruz Biotechnology), TIF2 (BD Transduction Laboratories), and β-catenin (BD Transduction Laboratories) or AR antiserum (PG-21), GRIP1 (M-343, Santa Cruz Biotechnology), and β-catenin (H-102, Santa Cruz Biotechnology). The AR PG-21 antiserum or monoclonal antibody 441 were used at 1:5000 dilution and all other the antibodies at 1:1000 dilution.

To perform coimmunoprecipitation with wild-type AR, mutant β-catenin, and GRIP1, 293 cells were plated onto 100-mm2 dishes and transfected with 3 µg each of wild-type AR, VP16/β-catenin, or VP16/β-catenin(K312A), together with or without wild-type GRIP1 or truncated GRIP1(5–1121). Cells were treated with R1881 for 24 h prior to coimmunoprecipitation. Polyclonal anti-AR (PG-21) was used for coimmunoprecipitation and monoclonal antibodies against AR, TIF2,

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and VP16 (14–5, Santa Cruz Biotechnology) were used for Western blot analysis.

**Statistical Analysis**—Unless otherwise noted, values shown represent mean ± S.D. The differences between groups were analyzed for statistical significance by the two-tailed Student’s t test using the program GraphPad Prism software version 4.02 (GraphPad Software, San Diego, CA). p < 0.05 was considered significant. In figures the following symbols are used to represent p values: *, p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001.

**RESULTS**

**β-Catenin Interactions with AR and TCF4**—We showed previously that ligand-bound AR inhibited the transcriptional interaction between β-catenin and a TCF-responsive promoter (11). Because the interaction of β-catenin with both AR and TCF4 was blocked by inhibitor of β-catenin and TCF (17, 18), we predicted that both AR and TCF4 would interact with the same region of β-catenin (11). To test this hypothesis we generated missense mutations in β-catenin to determine their effect on interactions with AR and TCF4. The location of these mutations is shown in the map in Fig. 1A. K312A and K435A disrupt each of the two lysine residue “charge buttons” that anchor the interaction with TCF proteins (19) and E-cadherin (20). The R386A mutation was chosen because Arg-386 is involved in binding to APC (21) and is also involved in the association with the Phe-21 residue of TCF4 (20). Arginine 469 and histidine 470 resides are required for the binding of TCF4/LEF1 by the formation of charge buttons with residues Lys-435, Asn-426, Lys-508, and Lys-312 (19, 20).

In a mammalian two-hybrid assay, the AR ligand R1881 induced an interaction between wild-type VP-16/β-catenin and GAL4/AR LBD as we previously showed (11). Missense mutant proteins VP-16/β-catenin(K312A), VP-16/β-catenin(R386A), and the double mutant VP-16/β-catenin(K312A/K386A) did not interact with GAL4/AR LBD in the presence of R1881. However, three other missense mutations, K435A, R469A, and H470A did not affect the interaction with GAL4/AR LBD to as great a degree (Fig. 2A). These results suggest that Lys-312 and Arg-386 are required for the interaction between β-catenin and ligand-bound AR. To make sure that the variation of the binding activity of different VP-16/β-catenin mutants was not due to the changes in protein expression levels, we did a parallel Western blot assay and showed that the expression levels of different constructs were not substantially different (Fig. 2A).

The mammalian two-hybrid binding data correlated with transcriptional activation of an AR-responsive reporter construct. In a transient transactivation assay we examined the effect of different β-catenin mutant constructs on the ligand-dependent activation of an N-terminal truncated AR-(507–919). We used the truncated AR-(507–919) instead of a full-length construct, because the truncated receptor is essentially inactive in the absence of coactivators such as TIF2/GRIP1 or β-catenin, and therefore provides a qualitative read-out of the transcriptional interaction between AR and β-catenin constructs. As shown in Fig. 2B, wild-type β-catenin can effectively activate the truncated AR in the presence of R1881. In contrast, β-catenin point mutants K312A, R386A, and the double mutant K312A/R386A could not activate the receptor. The other three β-catenin point mutants, K435A, R469A, and H470A, could activate the receptor to the wild-type level. Western blot showed that the expression of different VP-16/β-catenin mutants were at the comparable levels. These results are consistent with the notion binding of β-catenin to AR is required for coactivation as we have previously suggested.

The β-catenin missense mutations affecting the “charge buttons” had different effects on TCF4-mediated transactivation of the OT-luciferase reporter than they had on AR transactivation. The mutations that produced the greatest disruption of the interaction with AR affected TCF4-mediated transcription to lesser degrees. β-Catenin point mutants K312A and R386A reduced OT-luciferase activities to 45 and 75% of wild-type activity, respectively. The K312A/R386A double mutant induced 32% of wild-type activity (Fig. 2C). In contrast, the mutant β-catenin constructs K435A, R469A, and H470A that retained the ability to interact with AR induced <20% of wild-type TCF4 transactivation activity. These results with the TCF4-responsive reporter assays are similar to those reported by others in LEF-1-mediated transactivation assays (21).

AR and TCF4 can compete for binding of β-catenin, and each has an inhibitory effect on the transcriptional activity of the other in transfection experiments (11). β-Catenin(K435A) had lost nearly all inter-
action with TCF4 and retained interaction with AR, whereas β-catenin(K312A) had the opposite effect. We therefore compared the effects of K312A and K435A with wild-type β-catenin on the ligand dose dependence of AR-mediated transcription. We observed that at 10 pM DHT, which did not activate AR alone, there was activation in the presence of either wild-type or K435A mutant β-catenin. This result underscores the finding that β-catenin can support AR transcriptional activity at castrate levels of DHT. β-Catenin(K435A) had a more potent effect on AR activation than wild type β-catenin, consistent with the notion that the absence of competitive binding to TCF family proteins decreased competition for β-catenin (Fig. 2D). β-Catenin(K312A) had very little activity in the same experiment and was not differentiable from the control plasmid at DHT concentrations <100 pM and not significantly different at all concentrations.
We also examined the competition for β-catenin between AR and TCF4 by a physical association assay. 293T cells were cotransfected with both AR and TCF4 expression vectors, and cells were cultured with or without R1881 before isolation of lysates (Fig. 3). Equal amounts of cell lysates were incubated with agarose beads bound with either GST or GST/β-catenin fusion protein. AR and TCF4 retained on glutathione beads were assayed by Western blotting. In agreement with the mammalian two-hybrid data in Fig. 2A, we observed ligand-dependent binding of AR to GST/β-catenin and GST/β-catenin(K435A), GST/β-catenin(R469A), and GST/β-catenin(H470A) (Fig. 3). In contrast much less binding was observed with GST, GST/β-catenin(K312A), GST/β-catenin(R386A), and GST/β-catenin(K312A/R386A) (Fig. 3). In contrast, binding of TCF4 to the β-catenin fusion constructs was seen best in the absence of R1881. TCF4 bound strongly to GST/β-catenin and less well to GST/β-catenin(K312A), GST/β-catenin(R386A), and GST/β-catenin(K312A/R386A) (Fig. 3). We observed essentially no binding of TCF4 to GST/β-catenin(K312A), GST/β-catenin(H470A), and the double mutant GST/β-catenin(K312A/R386A). The absence of binding to the double mutant in the GST-pull-down assay was in contrast to the result of the transactivation assay where OT-Luc transcriptional activity was 32% of wild type, which may have been due to interaction with endogenous wild type β-catenin expressed in CV-1 cells (Fig. 1C).

The presence of R1881 and exogenous AR expression markedly reduced availability of TCF4 to bind GST/β-catenin fusion proteins. This observation is best explained by the ligand-dependent interaction of AR and TCF4, which decreased the availability of TCF4 for binding to the GST/β-catenin fusion constructs (22). The amounts of GST fusion proteins used in the pull-down assays are shown at the bottom of Fig. 3 and were comparable among the different lysates. Thus differences in AR or TCF4 binding to GST/β-catenin fusion proteins was not due to the differences in fusion proteins bound to the glutathione-agarose beads.

Interaction of β-Catenin with TIF2/GRIP1—We previously showed that β-catenin and TIF2/GRIP1 synergistically activated AR-mediated transcription. Li et al. (13) obtained complementary results and further showed that the AD2 domain of GRIP1 mediated a physical interaction with β-catenin and was necessary for synergism with β-catenin in activating AR. However, the region of β-catenin required for the interaction with TIF2/GRIP1 has not been determined. We performed mammalian two-hybrid assays with GAL4 fusion proteins harboring various regions of β-catenin and VP-16/GRIP1. A GAL4/β-catenin fusion protein bound strongly to the VP-16/GRIP1 fusion protein. GAL4 fusion proteins with either N- or C-terminal β-catenin deletions also bound to VP-16/GRIP1. However, a GAL4/β-catenin fusion construct with the armadillo repeat domain (141–668) showed no detectable binding (Fig. 4A). To control for differences in the basal transcriptional activities of the GAL4/β-catenin constructs we plotted the fold induction of reporter activity induced by the interactions with VP-16/GRIP1 relative to VP-16 alone. Transcription induced by interactions of VP-16/GRIP1 with GAL4/β-catenin-(1–668) and GAL4/β-catenin-(141–781) was equal or greater than transcription induction with GAL4/β-catenin (Fig. 4A, inset). VP-16/GRIP1 did not induce any increase in reporter activity mediated by GAL4/β-catenin-(141–668) that contained the armadillo repeat region alone. The data suggest that the primary sites of interaction between β-catenin and TIF2/GRIP1 resided in the C and N termini of β-catenin. We next confirmed the result of Li et al. (13) that the AD2 domain of GRIP1 is required for the direct interaction with β-catenin (Fig. 4B). They had shown that the region of GRIP1 from 1122–1462 mediated nearly complete binding to β-catenin. We used a slightly shorter construct and found that the interaction with β-catenin was attenuated ~50% compared with wild-type GRIP1 (see constructs in Fig. 1B).

To confirm the physical interaction between β-catenin and TIF2/GRIP1, we performed a set of GST-pull-down assays with GST fusion proteins harboring full-length β-catenin. Five single missense mutations and one double mutation were introduced into the GST fusion constructs to determine whether missense mutations that affected β-catenin interaction with either AR or TCF4 had any effect on interaction with TIF2/GRIP1. As shown in Fig. 4C, TIF2 bound very strongly to most of the GST/β-catenin fusion proteins. GST/β-catenin(H470A) that bound TIF2 weakly. Exposure of the filter to an anti-GST antibody showed that the amounts of GST fusion proteins used in each interaction were comparable (Fig. 4C). Because the armadillo repeat domain is not required for the interaction between β-catenin and TIF2, it is not
surprising that most of the point mutants in the armadillo repeat domain did not interfere with binding of β-catenin to TIF2 in a GST-pull-down assay. The effect of the missense H470A mutation on TIF2 binding is not as yet explained. Importantly, the regions of β-catenin that interacted with AR were not the same that interacted with TIF2/GRIP1. The data were consistent with our proposed model that the three proteins interact by direct three-way binding during AR-mediated transcription.

We also performed a competition experiment in which binding in a mammalian two-hybrid interaction was challenged with wild-type or mutant β-catenin expression. If wild-type or mutant β-catenin competed with GAL4/β-catenin for GRIP1 binding, the reporter activity was expected to decrease. As shown in Fig. 4D, cotransfection of five of the β-catenin mutants induced a significant decrease in the GAL4-responsive reporter activity, suggesting that these β-catenin mutants competed with GAL4/β-catenin for GRIP1 binding. β-Catenin(R469A) induced borderline significant decrease in reporter activity and β-catenin(H470A) was inactive in this assay. These results correlate with the results of the GST-pull-down assay in Fig. 4C. Thus, the sequence requirements for the binding of β-catenin to TIF2 differ from those of TCF/LEF-family transcription factors, APC, conductin, E-cadherin, and β-actin.

**TIF2/GRIP1 and β-Catenin Cooperate in AR Binding and Transactivation—**β-Catenin, TIF2/GRIP1, and AR can form a complex in the presence of androgen and β-catenin and TIF2/GRIP1 can synergize as coactivators of AR-mediated transcription (11, 13). Therefore changes in intracellular levels of both TIF2/GRIP1 and β-catenin can affect the activity of AR that is modulated by the presence of the other molecule. To demonstrate that either TIF2 or β-catenin could mediate the binding of the other to AR, we employed β-catenin missense mutations that were shown in Figs. 1 and 2 to affect either association with AR or interaction with TCF4. We carried out mammalian two-hybrid assays with VP-16 fusion proteins containing full-length, wild-type VP-16/β-catenin, VP-16/β-catenin(K312A), an AR interaction mutant, VP-16/GRIP1, and GAL4/β-catenin(K312A, R366A) or GAL4/β-catenin(K423A, R469A, H470A).
or VP-16/β-catenin(K435A), a TCF4 interaction mutant. We found that wild-type VP-16/β-catenin, VP-16/β-catenin(K435A), and TIF2 all induced reporter activity, indicating these proteins interacted with GAL4/AR LBD in a ligand-dependent manner, whereas VP-16/β-catenin(K312A) did not interact with GAL4/AR LBD due to the disruption of interaction with AR LBD by the K312A mutation (Fig. 5A, top panel). Cotransfection of TIF2 with either wild-type VP-16/β-catenin or VP-16/β-catenin(K435A), enhanced reporter activity to a greater degree than with either coactivator alone. Even though VP-16/β-catenin(K312A) is deficient in AR binding, TIF2 plus VP-16/β-catenin(K312A) induced a greater degree of GAL4 reporter induction than TIF2 alone (Fig. 5A, top panel). TIF2m123 is a mutant of
TIF2 deficient in AR interaction (24). TIF2m123 and VP-16/β-catenin(K312A) together had no effect on GAL4/AR LBD, because both have inactivation of AR binding. TIF2m123 reduced the binding interactions of both VP-16/β-catenin and VP-16/β-catenin(K435A) by approximately one-third to one-half, suggesting that binding of TIF2 to the AF-2 groove is critical for the synergism with VP-16/β-catenin for activation of GAL4/AR LBD. In each case TIF2 enhanced the interaction of the VP-16/β-catenin fusion construct and TIF2m123 inhibited the interaction (Fig. 5A, top panel).

An assay for AR-dependent transcription was also done to determine if the binding interactions seen in the top of Fig. 5A correlated with effects on AR activity. We performed parallel reporter assays with AR-(507–919) instead of GAL4/AR LBD and used the MMTV-Luc reporter for readout of AR transcriptional activation. Whereas the β-catenin constructs had a stronger binding interaction with AR than TIF2, there was much less difference between VP-16/β-catenin and VP-16/β-catenin(K435A) and TIF2 in an AR transcriptional interaction (Fig. 5A, bottom panel). The TIF2m123 construct reduced AR-mediated transcriptional activation by VP-16/β-catenin by 68%, and by VP-16/β-catenin(K435A), but did not abrogate either interaction. This suggests that TIF2m123 had a dominant negative effect by complexing with other members of the transcriptional complex or by interfering with the AR-VP-16/β-catenin complex. Because TIF2m123 has an intact AD2 domain and thus binds VP-16/β-catenin, TIF2m123 likely competed with AR for β-catenin binding (Fig. 5A, bottom panel). However, TIF2 synergized with both VP-16/β-catenin and VP-16/β-catenin(Lys-435) to enhance AR-dependent transcription. TIF2m123 and β-catenin(K312A) together had no effect on MMTV reporter activity due to the disruption of AR interactions of both proteins. On the other hand, TIF2 had a significant synergistic effect with VP-16/β-catenin(K435A). In contrast, VP-16/β-catenin(K312A) had very little effect on TIF2 interaction with AR. Even though TIF2 was able to mediate binding between GAL4/AR LBD and VP-16β-catenin(K312A) (Fig. 5A), the effect on AR-mediated transcription was much less, suggesting further that a three-way interaction of TIF2, VP-16/β-catenin, and AR-(507–919) was important for maximal enhancement of AR transcriptional activity. The data are consistent with an interaction model that requires TIF2/GRIP1 occupancy of the AF-2 that is enhanced by the presence of β-catenin bound either to AR and TIF2/GRIP1 or to TIF2/GRIP1 alone.

The interaction of TIF2 and β-catenin was further examined with a coactivator mutant subGRIP1, in which the three NR boxes have been changed to CoRNR nuclear receptor corepressor binding motifs (24). The subGRIP1 construct alone showed no binding interaction with GAL4/AR LBD and induced 90% inhibition of binding between GAL4/AR LBD and VP-16/β-catenin demonstrating that subGRIP1 was able to block binding by complexing with β-catenin, because subGRIP1 cannot bind to AR LBD (24) (Fig. 5B, top panel). In contrast subGRIP1 reduced by half, but did not abrogate, the transcriptional interaction between AR-(507–919) and VP-16/β-catenin. subGRIP1 had no effect on AR-dependent transcription but was not only unable to synergize the interaction of AR and VP-16/β-catenin but inhibited transcription either by competing for β-catenin or by inhibiting AR LBD directly (Fig. 5B, bottom panel). This result was also consistent with the notion that optimal AR-mediated transcriptional activity required binding of both β-catenin and TIF2/GRIP1 to the AR.

Both TIF2m123 and subGRIP1 retained the ability to bind β-catenin as shown by a modified mammalian two-hybrid assay in which we cotransfected cells with GAL4/β-catenin, the FR-Luc reporter plasmid, and the TIF2/GRIP1 mutants. As shown in Fig. 5C, wild-type GRIP1, subGRIP1, and TIF2m123 induced significant increases in reporter activity as compared with control vector, indicating that both subGRIP1 and TIF2m123 bound β-catenin.

These data thus far suggested that β-catenin and TIF2/GRIP1 could each mediate the binding of the other to a complex with AR, but that interaction of both with AR was required for a synergistic effect on AR-dependent transcription. To explore this interaction further we examined the activity of a GRIP1 construct that did not interact with β-catenin (13). We performed a modified mammalian two-hybrid assay with GAL4/AR LBD, VP-16/β-catenin, and full-length GRIP1 or GRIP1-(5–1121) that deleted the second activating domain that is required for interaction with β-catenin (see Fig. 1B) (13). When cells were cotransfected with VP-16/β-catenin and wild-type full-length GRIP1, the reporter activity was 8.5- and 11.4-fold higher than the activities induced individually by VP-16/β-catenin and TIF2, respectively, indicating a strong synergistic binding of these two factors to agonist bound AR (Fig. 6A). When cells were cotransfected with VP-16/β-catenin and GRIP1-(5–1121) the reporter activity was 4.3- and 2.5-fold of that seen with transfection of the individual VP-16/β-catenin and GRIP1-(5–1121) constructs, respectively (Fig. 6A).

Loss of the second GRIP1 activation domain diminished the coactivation of AR (Fig. 6B). In this assay VP16/β-catenin enhanced the effects of both GRIP1 and GRIP1-(5–1121) to nearly identical degrees (1.17 to 1.18-fold) showing that the attenuation of AR binding by loss of the AD2 domain did not affect interaction with AR-mediated transactivation.

When the binding assay of Fig. 6A was repeated with the mutant VP-16/β-catenin(K312A) that abrogated the direct β-catenin-AR interaction, in place of the wild type β-catenin construct, we observed a result qualitatively very similar (Fig. 6C). This implied that binding of VP-16/β-catenin(K312A) to the transcription complex was mediated entirely by either GRIP1 or GRIP1-(5–1121). The data thus far suggest that TIF2/GRIP1 interaction directly with AR can facilitate the effect of β-catenin on AR-mediated transactivation, and that either TIF2/GRIP1 or β-catenin mediated binding between AR and the other protein.

To further characterize the interactions between AR, TIF2/GRIP1, and β-catenin in transcription we explored four AR missense mutations, E8983A, M894A, E897V, and Q902R, known to affect the interaction of the AR AF2 domain with either TIF2/GRIP1 or β-catenin (11). AR(E893A) was generated in the laboratory and found to selectively disrupt AR binding to β-catenin and attenuate AR interaction with TIF2/GRIP1, AR(M894A) and AR(E897V), also laboratory-derived, and AR(Q902R), a prostate cancer mutation (25), all have substantial defects in TIF2/GRIP1 binding and varying degrees of altered β-catenin interactions. A Q902K mutation was found to be associated with partial androgen insensitivity in one family and was found to decrease sensitivity to R1881 and to decrease interaction with TIF2 (25). It is not clear how the Q902R mutation was selected in a patient with advanced prostate cancer, however, the patient with the Q902R mutation experienced disease progression just 1 month after orchiectomy for a recurrent Gleason score 8 stage C tumor (25). It is therefore possible that the Q902R mutation was not selected by prostate cancer treatment. The relative activities of the AR LBD mutants with β-catenin and TIF2/GRIP1 in mammalian two-hybrid and AR transcriptional assays are shown graphically in Fig. 7A based on data we obtained previously (11).

In mammalian two-hybrid assays GRIP1 alone did not significantly affect reporter activation by GAL4/AR LBD(M894A), E897V, or Q902R. VP-16/β-catenin, on the other hand, interacted with these three constructs to varying degrees (Fig. 7B). VP16/β-catenin was able to engage GRIP1 in the transcriptional complex with these AR-interacting mutants as indicated by the augmentation of the VP-16/β-catenin interaction with the addition of GRIP1 (Fig. 7B). The enhancing effect of
In the case of the TIF2/GRIP1 interaction mutant AR-(507–919)(M894A) GRIP1 induced activation at a level 13% of wild type AR-(507–919) and VP-16/β-catenin at a level of 45% (Fig. 7C). There was no cooperation of GRIP1 and VP-16/β-catenin with AR-(507–919)(M894A). This implies that, despite the cooperative effect observed in the binding assay, the M894A mutation decreased affinity of the binding groove for β-catenin and TIF2/GRIP1 to a degree that prevented a cooperative effect on AR-mediated transcription. The MMTV-luciferase assays with the AR-(507–919)(E893A) mutant suggest that there was insufficient binding of TIF2/GRIP1 to recruit VP-16/β-catenin to a three-way complex and enhance AR-mediated transcription. On the other hand, the AR mutants E897V and Q902R had marked attenuation of transcriptional activation by GRIP1, but with both there was cooperative interaction of VP-16/β-catenin and GRIP1 in AR-mediated transcription (Fig. 7C). In the instance of these two mutations the data suggest that there was sufficient affinity of the AF-2 groove to allow VP-16/β-catenin to recruit and retain TIF2/GRIP1 in a binding conformation with AR.

To confirm further the notion of a three-way interaction between AR, β-catenin, and TIF2/GRIP1, the GAL4/AR LBD(E897V) mutant that did not bind TIF2/GRIP1 but had the most robust interaction with a GRIP1-VP-16/β-catenin complex was used in mammalian two-hybrid experiments that tested the effects of β-catenin mutants with compromised AR and TIF2/GRIP1 binding. As expected GRIP1 and GRIP1-(5–1121) had minimal interaction with GAL4/AR LBD(E897V) (Fig. 7D). VP-16/β-catenin bound to the mutant fusion protein receptor in the presence of hormone and recruited both GRIP1 and GRIP1-(5–1121) to enhance binding and AR-mediated transcription (Fig. 7, D and E). The mutant VP-16/β-catenin(K312A) that has minimal interaction with wild-type AR did not bind to GAL4/AR LBD(E897V). VP-16/β-catenin(K312A) had no interaction with GRIP1-(5–1121), and a low degree of interaction with GAL4/AR LBD(E897V) when GRIP1 was added. The weak effect of GRIP1 and VP-16/β-catenin(K312A) was most likely due to the residual binding of GRIP1 to the mutant AR constructs. These data further show that the binding of VP-16/β-catenin to AR was critical for the enhancement of the interaction with mutant GRIP1.

When AR was not able to interact efficiently with both β-catenin and TIF2/GRIP1 then either of the two cofactors could compete with AR for binding of the other and, paradoxically, inhibit AR-mediated transcription. This is demonstrated in Fig. 7D where VP-16/β-catenin was able to compete GRIP1 off AR and inhibit transcription from AR-(507–919) (E893) in a dose-dependent manner. In a similar manner, GRIP1, whose binding to AR is compromised by the M894A mutation, was able to compete for VP-16/β-catenin and reduce AR-mediated transcription in a dose-dependent manner.

**β-Catenin Domains That Interact with TIF2/GRIP1 and AR**—We wanted to determine the regions of β-catenin required for the two-way interaction with AR and TIF2/GRIP1. Loss of the C-terminal domain of β-catenin reduces the interaction with AR by ~40%, and deletion of armadillo repeats 5 and 6 completely disrupts binding of β-catenin to AR (12). We speculate that optimal interaction with AR requires both the charged surface of the armadillo repeat helical backbone and the C-terminal domain similar to the interaction of β-catenin with TATA binding protein (27). We performed mammalian two-hybrid assays with GAL4/AR LBD, GRIP1, and VP-16 fusion proteins harboring the full length, N-terminal deleted β-catenin(Δ15–552), or C-terminal deleted β-catenin (1–574) (constructs shown in Fig. 1A). The two β-catenin truncation mutants do not interact with AR in the presence of androgen (11). Both GRIP1 and β-catenin(S33A) interacted with

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**FIGURE 6.** TIF2/GRIP1 and β-catenin can each mediate binding of the other to AR. Mammalian two-hybrid (A) and AR-mediated transcription (B) from MMTV-Luc reporter assays with the indicated constructs. C, mammalian two-hybrid assay as in panel A with VP-16/β-catenin(K312A). *p* values for comparisons are shown as indicated.
FIGURE 7. TIF2/GRIP1 and β-catenin interactions with AR AF-2 domain mutants. A, graphic representation of relative mammalian two-hybrid interactions ("binding") and MMTV-luciferase activation for four AR AF-2 domain mutants with β-catenin and TIF2/GRIP1 as previously published (11). B, mammalian two-hybrid assays of AR AF-2 domain mutants with VP-16/β-catenin and GRIP1. p values are shown for significant differences of comparisons at the ends of the brackets. C, MMTV-Luc assays with constructs shown in panel B. p values are shown for significant differences of comparisons at the ends of the brackets. Mammalian two-hybrid (D) and MMTV-Luc reporter (E) assays of AR LBD(E897V) with GRIP1 and β-catenin mutant constructs. In D and E significant differences are indicated between the value at the far left of each bracket and the values below the asterisks. F, MMTV-Luc reporter assay demonstrating competition for binding VP-16/β-catenin between two AR AF-2 mutant constructs and GRIP1. The numbers under the histograms indicate the nanograms of DNA in the transient transfection. The brackets show significant differences compared with the values at the far left end of the respective brackets.
cells exposed or not to R1881. First we demonstrated that three monoclonal antibodies against AR, TIF2, and β-catenin could be used simultaneously to detect their respective antigens (Fig. 9A). We saw no nonspecific bands, even with long Western blot exposures. Because the level of AR expression in LNCaP cells is very high, we used 1:5000 dilutions of AR antibodies in all the Western blot analysis. In the Western blot of β-catenin, we detected the 75-kDa β-catenin cleavage product induced by calpain as previously described (28). The relative densities of the input proteins are shown in the top panel of Fig. 9B.

After immunoprecipitated with polyclonal AR antiserum both TIF2 and β-catenin could be detected by Western blot independent of prior exposure to R1881 (Fig. 9B). This is in agreement with the fact that p160 coactivators can interact directly with the AR N terminus in a ligand-independent manner via a conserved glutamine-rich region (29). We have shown that β-catenin binding to AR is ligand-dependent, but we cannot say whether the ligand-independent immunoprecipitation of AR with β-catenin is mediated by an intervening TIF2/GRIP1 molecule. Densitometric analysis showed that more TIF2 and β-catenin were pulled down in the presence of R1881 than in its absence. Similarly, AR and β-catenin were pulled down by TIF2 antibody, and there was no substantial difference between the R1881 treated and untreated samples. When β-catenin antibody was used to isolate the immunoprecipitate, AR and TIF2 could be pulled down and more AR was detected in the presence of R1881. However, the amount of TIF2/GRIP1 pulled down by β-catenin antibody was comparable in R1881-treated and untreated groups, suggesting the association of TIF2 and β-catenin is independent of ligand binding to AR.

The formation of a three-way physical complex between AR, β-catenin, and TIF2/GRIP1 was also examined with exogenous mutant proteins expressed in 293T cells. Here we used AR, GRIP1, or GRIP1-(5–1121) that has attenuated β-catenin interaction, and VP-16/β-catenin or VP-16/β-catenin(K312A) that has reduced AR interaction. Both full-length, wild-type GRIP1, and truncated GRIP1-(5–1121) could be pulled down by AR antibody in the presence of R1881 (Fig. 10A). When cells were cotransfected with both GRIP1 and VP16/β-catenin, much more GRIP1 could be pulled down (Fig. 10A, lanes 1 and 3). This observation is in agreement with our mammalian two-hybrid and transactivation assay results showing that GRIP1 and VP-16/β-catenin could synergistically coactivate AR-mediated reporter activity (Fig. 5A). More interestingly, when VP-16/β-catenin(K312A), a mutant that cannot bind AR, was used, similar results were obtained (Fig. 10A, lanes 1 and 4), suggesting that β-catenin could be recruited to the GRIP1-AR complex by association with GRIP1 independently of AR. When GRIP1-(5–1121) was used, VP-16/β-catenin and VP-16/β-catenin(K312A) could still enhance the association of GRIP1 with AR to a limited degree (Fig. 10A, lanes 2, 5, and 6). This result is also in agreement with our mammalian two-hybrid and transactivation assays (Fig. 6C) and suggests that even though AD2 of TIF2/GRIP1 is the major region required for the direct association with β-catenin other regions in the N terminus of TIF2/GRIP1 may also be involved in the interaction (13).

We also asked whether GRIP1 could mediate the interaction of AR with β-catenin. To this end, VP-16/β-catenin(K312A) was transfected into 293T cells with or without GRIP1. After R1881 treatment, whole cell lysates were precipitated with polyclonal AR antibody (PG-21). As a positive control, wild-type VP-16/β-catenin was pulled down by AR antibody (Fig. 10B, lane 1). In contrast, VP-6/β-catenin(K312A) could not be pulled down by AR antibody under identical conditions (Fig. 10B, lane 2). However, when VP-16/β-catenin(K312A) was cotransfected with GRIP1, VP-16/β-catenin(K312A) could be pulled down efficiently

GAL4/AR LBD to induce GAL4-dependent transcription (Fig. 8A). Together, VP-16/β-catenin(S33A) and GRIP1 synergized to interact with GAL4/AR in a ligand-dependent manner. When cells were cotransfected with N- or C-terminal β-catenin deletion mutants together with GRIP1 a much stronger reporter activity was seen, suggesting that GRIP1 was able to recruit β-catenin to the transcriptional complex on the androgen-responsive promoter. The reporter activity in the presence of the N-terminal deletion mutant VP-16/β-catenin(S33A)(Δ15–552) and GRIP1 was indistinguishable from the effect of VP-16/β-catenin(S33A) and GRIP1. On the other hand, the effect of VP-16/β-catenin(S33A)-(1–574) and GRIP1 was much less, consistent with the notion that the C terminus of β-catenin is important for the physical interaction with GRIP1.

In an AR-responsive MMTV-Luc reporter assay the deletion mutants VP-16/β-catenin(Δ15–552) and VP-16/β-catenin-(1–574) had no effect on AR-responsive reporter activity (Fig. 8B). Although VP-16/β-catenin(Δ15–552) interacted with GRIP1 in a binding assay, the interaction had a negative effect on AR-mediated transcription compared with the interaction of GRIP1 and AR alone. VP-16/β-catenin-(1–574), which showed minimal binding interaction with GRIP1, had minimal to no effect on AR-responsive transcription in the presence of GRIP1 (Fig. 8B).

The formation of a trimeric complex between AR, TIF2/GRIP1, and β-catenin was strongly suggested by the mammalian two-hybrid data, particularly by the experiments where binding between any two of the constituents was inactivated by missense mutation. Further confirmation of the formation of a trimer was obtained by commmunoprecipitation of endogenous proteins from LNCaP cell nuclear extracts from

**FIGURE 8. Effect of β-catenin truncations on AR and GRIP1 interaction.** Mammalian two-hybrid (A) and MMTV-Luc reporter (B) assays of AR, GRIP1, and subgenomic β-catenin constructs.

AR, TIF2, and β-Catenin Interaction
FIGURE 9. Coimmunoprecipitation of AR, TIF2/GRIP1, and β-catenin. A, simultaneous detection of endogenous AR, TIF2/GRIP1, and β-catenin from LNCaP cells. B, immunoprecipitation and Western blotting of the indicated proteins. The histograms show relative densities in each experiment normalized to the input material in the top panel.
Our findings demonstrate that β-catenin interacts with AR via different amino acid residues than it interacts with TCF4, but we confirm that the two interactions compete for β-catenin binding. Based on our results and others’ there appears to be a complex interaction between TCF4 and AR transcription factors. Indeed, the following binding interactions have been demonstrated: AR–β-catenin (8–12); AR–TCF4 (22); β-catenin–TIF2 (11, 13) in addition to the well-established interactions of AR with p160 coactivators like TIF2 and the obligate interaction of β-catenin and TCF4 in the initiation of TCF-4-dependent transcription (15). Our data also show, through targeted mutational analysis, that the three-way complex of AR, β-catenin, and TIF2 supports a higher level of transcriptional activation than the complex of AR with either of the two alone. Furthermore, either the two coactivators can tether the other to AR resulting, in most instances, in enhanced transcriptional activation. Overexpression of coactivators is expected to enhance AR action, although some have found that expression of p160 steroid receptor coactivator family members is enhanced in advanced prostate cancer. Overexpression of coactivators is expected to enhance AR action, broaden ligand specificity, and mediate AR agonism by the anti-androgen hydroxyflutamide (36). However, in other cohorts different coactivators have been implicated (37). Moreover, to our knowledge genetic change of p160 coactivator genes has not been found. β-Catenin is therefore one of the few AR coactivators that is implicated in prostate cancer development and progression, one might expect that the molecular pathology of prostate cancer might include genetic alterations of AR coactivators. Thus far this has not been demonstrated, although some have found that expression of p160 steroid receptor coactivator family members is enhanced in advanced prostate cancer. Overexpression of coactivators is expected to enhance AR action, broaden ligand specificity, and mediate AR agonism by the anti-androgen hydroxyflutamide (36). However, in other cohorts different coactivators have been implicated (37). Moreover, to our knowledge genetic change of p160 coactivator genes has not been found. β-Catenin is therefore one of the few AR coactivators that is implicated in prostate cancer pathogenesis both by mutation and by post-translational modification (7, 28, 32).

Lastly, we encountered a surprising inhibition of AR-mediated transcription by the C-terminal domain of β-catenin. The precise mechanism of this inhibitory effect was not elucidated, but the data imply that the C-terminal domain fragment of β-catenin has a profound inhibitory interaction with AR. The inhibitory effect was not reversed to a significant degree by supplementation with either CBP or p300, two components of the transcriptional complex that bind to the β-catenin C terminus. If the mechanism of inhibition is due to the binding of the β-catenin C terminus to AR, as implied by the data in Fig. 8, the binding...
must have quite high affinity, as it was incompletely competed by excess full-length β-catenin. The competition by full-length β-catenin did reflect a dose response. On the other hand, the effect of the β-catenin C terminus on AR-mediated transcription was only partially reversed by TIF2/GRIP1 in a manner that suggested a noncompetitive interaction (Fig. 9, A and C). Further experiments will identify the minimal inhibitory region of β-catenin and will elucidate the mechanism of this profound AR inhibition. The mechanism of this inhibition may have relevance in instructing the design of a new class of anti-androgens.

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