The Glucocorticoid Receptor Represses Cyclin D1 by Targeting the Tcf-β-Catenin Complex

Received for publication, March 10, 2006, and in revised form, April 27, 2006. Published, JBC Papers in Press, April 27, 2006, DOI 10.1074/jbc.M602290200

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The ability of glucocorticoids (GCs) to regulate cell proliferation plays an important role in their therapeutic use. The canonical Wnt pathway, which promotes the proliferation of many cancers and differentiated tissues, is an emerging target for the actions of GCs, albeit existing links between these signaling pathways are indirect. By screening known Wnt target genes for their ability to respond differently to GCs in cells whose proliferation is either positively or negatively regulated by GCs, we identified c-myc, c-jun, and cyclin D1, which encode rate-limiting factors for G1 progression of the cell cycle. Here we show that in U2OS/GR cells, which are growth-arrested by GCs, the glucocorticoid receptor (GR) represses cyclin D1 via Tcf-β-catenin, the transcriptional effector of the canonical Wnt pathway. We demonstrate that GR can bind β-catenin in vitro, suggesting that GC and Wnt signaling pathways are linked directly through their effectors. Down-regulation of β-catenin by RNA interference impeded the expression of cyclin D1 but not of c-myc or c-jun and had no significant effect on the proliferation of U2OS/GR cells. Although these results revealed that β-catenin and cyclin D1 are not essential for the regulation of U2OS/GR cell proliferation, considering the importance of the Wnt pathway for proliferation and differentiation of other cells, the repression of Tcf-β-catenin activity by GR could open new possibilities for tissue-selective GC therapies.

Glucocorticoids and other steroid hormones regulate survival, differentiation, and proliferation of a large variety of cells. Due to their ability to suppress the immune system and induce cell cycle arrest, GCs are commonly used in the treatment of inflammatory diseases and cancer. Whereas the induction of cell cycle arrest and apoptosis by GCs in cancer cells is therapeutically desirable, a similar action of GCs in bones and cancer cells is therapeutically desirable, a similar action of GCs in bones is therapeutically desirable, a similar action of GCs in bones is therapeutically desirable. The key effector of the classical Wnt pathway is glycogen synthase kinase-3 (GSK-3), which phosphorylates free β-catenin and marks it for proteasomal destruction (11). Activation of Wnt signaling inhibits GSK-3β activity, leading to accumulation and nuclear import of β-catenin. Nuclear β-catenin interacts with members of the Tcf/Lef (T cell factor/lymphoid enhancing factor) family and activates the expression of Wnt target genes (12).

Connections between Wnt and steroid hormone signaling have, to date, been best characterized for the androgen receptor (AR), which in the presence of androgens binds β-catenin and transports it into the nucleus (13–16). Nuclear β-catenin augments the transcriptional activity of AR, whereas AR suppresses Tcf-β-catenin activity (13–19). Recruitment of β-catenin by AR correlates with the hormone-dependent growth of prostate cancer cells (20). Although among the SRs only AR promotes nuclear import of β-catenin (13), the estrogen receptor (ER) has also been found to interact with β-catenin, both physically and functionally (21). In contrast to AR and ER, evidence linking GR to Wnt signaling is thus far indirect. In Con8 rat mammary epithelial cells, GCs interfere with Ras and tumor necrosis factor-α signaling, resulting in increased expression of β-catenin and translocation of β-catenin from the nucleus to the plasma membrane (22). In osteoblasts, GCs induce the expression of the Wnt inhibitor Dickkopf 1 (23) and the Tcf-β-catenin corepressor HDAC1 (24) and activate GSK-3β via the phos-
that GC and Wnt pathways communicate directly through their regulatory proteins. Two checkpoints at the G1/S and G2/M boundaries of the cell cycle enable growth factors and other intra- or extracellular cues to control the cell cycle. Cells that are arrested by GCs often have lower RNA and protein levels of D-type cyclins and of growth factor-activated transcription factors that are rate-limiting for progression through G1 phase, including c-Myc and c-Jun (25, 27–35). The expression of several of these regulators is also controlled by Wnt (36–38).

The goal of the present study was to investigate whether Wnt-dependent activation and GR-mediated repression of cell cycle regulator genes are functionally coupled. Here we demonstrate that GR represses the expression of cyclin D1 in human U2OS/GR osteosarcoma cells by inhibiting the activity of Tcf-β-catenin without changing the levels or nuclear localization of β-catenin. GR binds β-catenin in vitro, indicating that GC and Wnt pathways communicate directly through their effectors.

**Materials and Methods**

**Cell Lines**—Monkey CV1 kidney fibroblasts (American Tissues and Clones Collection) were maintained in Dulbecco's modified Eagle's medium (4.5 g/liter glucose) and 5% fetal bovine serum. Construction and maintenance of the human U2OS osteosarcoma cells expressing rat GR (U2OS/GR) are described in Ref. 27. As shown previously, GR is expressed in this cell line at physiological levels (~50,000 receptors/cell) (27).

**Microarray Analysis**—The microarray analysis of U2OS/GR and A549 cells used in this study has been previously described (39).

**RNA Isolation and Quantitative Reverse Transcription PCR (RT-PCR) of cDNA**—Isolation of total RNA and quantitative RT-PCR were performed as described in Ref. 39, except that PCRs were carried out in a MyiQ thermocycler (Bio-Rad) in a total volume of 40 µl for 35 cycles. Primers used were as follows: c-myc (forward, 5'-GAACACACAAACGTCTTTGAG-3'; reverse, 5'-GGACAGGATGTATGCTGTTG-3'); c-jun (forward, 5'-CATCCTGCTCCTCACAATTGC-3'; reverse, 5'-CACCCTGTTCTCCAGCATTG-3'); cyclin D1 (forward, 5'-GCAATGGAAGGTCTGCTCCG-3'; reverse, 5'-CAGGAGTGTGGTATGACG-3'); cyclin E (forward, 5'-ATGACATTGCTTCCGGGTCC-3'; reverse, 5'-CTTTCAGGACGCTACTT-3'); β-actin (forward, 5'-CCATGACCTTTTGTATCAG-3'; reverse, 5'-TTGCTCAGGGAGATATC-3'); β-catenin (forward, 5'-CATCCTGCTCCTCACAATTGC-3'; reverse, 5'-CACCCTGTTCTCCAGCATTG-3'). The generation of specific PCR products was confirmed by melting curve analysis and gel electrophoresis. β-A ctin was used as an internal standard to correct for differences in cDNA levels between samples. Induction of cDNA species were calculated as described in Ref. 40.

**RNA Interference**—β-Catenin siRNA (identification number 42816; Ambion) and nonsilencing control siRNA (catalog number 1022076; Qiagen) were transfected into U2OS/GR cells to a final concentration of 10 nM, using the HiPerFect Transfection Reagent (5 µl/ml culture; Qiagen). Transfected cells were incubated for at least 16 h before incubation with hormone (see figure legends to Figs. 2 and 5). Isolation of total RNA and quantitative RT-PCR was performed as described above.

**Nucleocytoplasmic Cell Fractionation**—Nuclear and cytoplasmic extracts from ~5 × 10⁶ U2OS/GR cells were prepared by using NE-PER nuclear and cytoplasmic extraction reagents (Pierce), as described by the manufacturer. Proteins were separated by SDS-PAGE and probed by immunoblotting. Separation of cytoplasmic and nuclear proteins was evaluated by analyzing the fractions for the presence of the mitohon-
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The β-galactosidase activity was used to correct for differences in transfection efficiency.

**Protein Expression and Purification**—The construction of the GR expression plasmids has been described above. 35S-Labeled rat GR and derivatives were expressed using the TNT *in vitro* expression kit (Promega). *In vitro* synthesis of GR was performed in the presence of 10 μM CORT or ethanol (vehicle). β-Catenin glutathione S-transferase (GST) fusion proteins were constructed by cloning PCR-amplified β-catenin fragments (aa 1–781, 90–781, 356–781, 443–781, 1–356, and 150–356 of human β-catenin) into a pGEX4T1 derivative that contains a previously engineered C-terminal His tag. GST fusion proteins were expressed in BL21DE3 at 37°C for 3 h (GST-β-catenin 1–781 and 356–781), at 16°C for 6 h (GST-β-catenin 1–356 and 150–356), or at 13°C for 9 h (GST-β-catenin 90–781 and 443–781). Expression was induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside at an *A*<sub>600</sub> = 0.7–0.9. Cell pellets were resuspended in sonication buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, 10% glycerol, protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of leupeptin, pepstatin, and aprotinin), incubated with 0.1 mg/ml lysozyme (10 min, 0°C), and lysed by sonication. Lysates were cleared by centrifugation (100,000 g, 4°C). The filtered supernatants were loaded on Talon resin (Clontech) that had been equilibrated with sonication buffer containing 6 mM imidazole. After washing with several column volumes of the same buffer, proteins were eluted with an imidazole gradient (6–300 mM). Eluted proteins were 90–95% pure as judged by Coomassie Blue staining. Resultant GST fusion proteins were 90–95% concentrated using a Bio-Rad protein assay. Resultant GST fusion proteins were 90–95% pure as judged by Coomassie Blue staining.

GST Pull-down Assays—Talon-purified GST fusion proteins (2–4 μM final concentration) bound to glutathione-agarose beads (40% final concentration) were incubated with 2.5 μL of 35S-labeled receptors (1–10 nM final concentration) and 10 μM CORT, RU486 or vehicle in 50 μL of binding buffer (150 mM KOAc, pH 7.4, 4 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM dithiothreitol, 25 μg/ml bovine serum albumin, 0.01% Nonidet P-40, and protease inhibitors (listed above) at 4°C for 2 h under rotation. Beads were collected by centrifugation (1,000 × g, 1 min, 4°C) and washed five times with 200 μL of binding buffer at 4°C before proteins were eluted by boiling in 20 μL of SDS loading buffer and analyzed by SDS-PAGE. The fraction of bound receptor was quantitated using an Amersham Biosciences PhosphoImager.

**Cell Count and Flow Cytometry Analysis**—U2OS/GR cells (6–9 × 10<sup>6</sup>) were transfected with either control or β-catenin siRNA and incubated for 24 h. Untransfected and transfected cells (1.0 × 10<sup>6</sup> each) were seeded into 6-well plates and cultured in the presence of 10 nM DEX or ethanol vehicle for 1–4 days. Cells were trypsinized, resuspended in Dulbecco's modified Eagle's medium, stained with trypan blue, and counted using a hemocytometer.

To assess cell cycle distribution, untransfected and transfected cells were seeded into 10-cm plates and cultured in the presence of 10 nM DEX or ethanol vehicle for 2 or 4 days. Cells were trypsinized, pelleted, resuspended in PBS (2.0 × 10<sup>5</sup> cells/ml) containing 1% fetal bovine serum, fixed by adding 10 ml of 80% cold ethanol (-20°C) per 1 ml of cell suspension under continuous mixing, and nuted overnight at 4°C. Cells were pelleted, washed with PBS, and resuspended in PBS containing 0.5% fetal bovine serum in a density of 1.5 × 10<sup>6</sup> cells/ml. DNA was stained by adding a 4X propidium iodide solution and RNase A to a final concentration of 50 and 100 μg/ml, respectively. After 15 min of incubation at room temperature, nuclear emitted fluorescence was measured in 30,000 total events/sample, using a BD LSRII FACScan flow cytometer with Diva software (488-nm excitation, 610/20 band pass with 545 LP, fluorescence threshold, sample rate 200–300 events/s).

**RESULTS**

**Identification of GC-regulated Wnt Target Genes**—Human U2OS/GR osteosarcoma cells are well characterized with respect to GC target genes previously identified by microarray analysis (39). Using the results of these microarray studies and a database of 84 genes that are directly controlled by Tcf-β-catenin (available on the World Wide Web at www.stanford.edu/~rnusses/pathways/targets.html), we identified 20 Wnt target genes that are expressed in U2OS/GR cells. Genes in this group are involved in cell cycle regulation or signaling (c-myc, c-jun, fra1, cyclin D1, id2, FGF 18, VEGF-C, follistatin, MMP7, axin2, dickkopf1, and frizzled7), cell adhesion (claudin 1, connexin 43, E-cadherin, fibronectin, and N-ras-CAM), and transcriptional regulation (PPARβ, pitx 2, and engrailed 2).

By analyzing the response of these genes to GCs, we found that the expression of most of these genes did not significantly change upon a short term (2-h) exposure to DEX in the presence of the protein synthesis inhibitor cycloheximide (Fig. 1A). Only two genes (engrailed 2 and frizzled 7) were up-regulated, and four genes (c-myc, c-jun, fra1, and cyclin D1) were down-regulated by more than 30% in the presence of DEX (Fig. 1, A and B).

To identify genes that might be involved in the GC-dependent regulation of cell proliferation, we also analyzed the expression of these Wnt target genes in human A549 lung carcinoma cells, which were included as a control group in the U2OS/GR microarray studies (39). Whereas GCs arrest the growth of U2OS/GR cells, they promote the proliferation of A549 cells. With the exception of engrailed 2, frizzled 7, FGF18, and the cell cycle regulator genes c-myc, c-jun, and cyclin D1, most Wnt target genes responded similarly to DEX in U2OS/GR and in A549 cells (Fig. 1, A and B). Consistent with a role of c-myc, c-jun, and cyclin D1 in the GC-dependent regulation of cell proliferation, these cell cycle regulator genes were down-regulated by DEX in U2OS/GR cells but not, or to a much lesser extent, in A549 cells. Notably, in U2OS/GR and A549 cells, DEX did not increase the expression of the Wnt inhibitor Dickkopf.
that are negatively regulated by GCs.

These hormones but not the antagonist RU486 induce the expression of these genes (Fig. 1C). Demonstrating that DEX and CORT can activate GC-responsive genes under our conditions, we showed that these hormones but not the antagonist RU486 induce the expression of hIAP2, a known positively regulated GC target gene (39) (Fig. 1C). These results suggest that cyclin D1, c-myc, and c-jun are Wnt target genes that are negatively regulated by GCs.

In U2OS/GR Cells, the Expression of Cyclin D1 Is Dependent on β-Catenin—The expression of cyclin D1, c-myc, and c-jun is regulated by several transcription factors whose relative contributions to the transcriptional activation of these genes vary in a cell-dependent manner. Therefore, although cyclin D1, c-myc, and c-jun are known Wnt target genes, in U2OS/GR cells their expression is not necessarily dependent on Tcf-β-catenin.

Cell fractionation experiments revealed that U2OS/GR cells contain cytoplasmic and nuclear β-catenin and that a 2-h exposure to DEX (the condition under which the previous microarray analysis was performed) had no detectable effect on the expression levels or nuclear localization of β-catenin (Fig. 2A). This result indicates that the Wnt pathway is constitutively active in U2OS/GR cells.

To determine whether expression of cyclin D1, c-myc, and c-jun in U2OS/GR cells is dependent on Tcf-β-catenin, we down-regulated the expression of β-catenin using RNA interference. Transfection of U2OS/GR cells with β-catenin siRNA lowered the transcript levels of β-catenin and cyclin D1 by more than 80% but did not affect the levels of c-myc, c-jun, or a negative control (hp70), whose expression is not regulated by Tcf-β-catenin (Fig. 2B). In contrast, transfection of a non-silencing control siRNA did not reduce the transcript levels of any of these genes (Fig. 2B). The observed changes in β-catenin and cyclin D1 transcript levels corresponded to changes in β-catenin and cyclin D1 protein levels (Fig. 2C). These results demonstrated that in U2OS/GR cells, expression of cyclin D1, but not of c-myc and c-jun, is controlled by Tcf-β-catenin.

FIGURE 2. Analysis of β-catenin expression and function in U2OS/GR cells. A, expression and nuclear-cytoplasmic localization of β-catenin in U2OS/GR cells that were either untreated (U), or treated with vehicle (−H) or 100 nM DEX (+H) for 2 h. Successful separation of nuclear (20 μg/lane) and cytoplasmic (100 μg/lane) proteins was confirmed for by monitoring the localization of the mitochondrial CVd. Equal loading and transfer was monitored by Ponceau staining of the blot (loading control [LC], selected Ponceau-stained band). B, RT-PCR analysis of hp70, β-catenin (β-cat), cyclin D1 (cyclD1), c-myc, and c-jun RNA levels in U2OS/GR cells transfected with either a nonsilencing control or β-catenin siRNA, as indicated. After transfection, cells were incubated for 24 h and harvested. Data were transformed and normalized as described in the legend to Fig. 1C. Shown are the averages and S.D. values of three experiments. 

FIGURE 3. Regulation of Tcf-β-catenin activity by GR. A, composition of wild type (WT) and mutant cyclin D1 promoter constructs (used in C) based on Ref. 36. The black bar represents the PCR fragment used in the following CHIP assay, B, CHIP analysis monitoring the interaction of β-catenin with the promoters of cyclin D1 and hp70 (negative control) in U2OS/GR cells treated for 2 h with either vehicle (−H) or 100 nM DEX (+H). β-Catenin was immunoprecipitated using a rabbit β-catenin antibody (C 2206, Sigma). cyclin D1 and hp70 promoter sequences in the immunoprecipitate were quantified by RT-PCR. Data were transformed by the standard ΔΔCt method, with amplification of a ribosomal Rpl19 promoter fragment as internal control. Shown are the averages and S.D. values of four experiments normalized to the amount of hp70 sequences present in the immunoprecipitates of vehicle-treated cells. The significance of the hormone-dependent reduction of immunoprecipitated cyclin D1 promoter fragments was analyzed using a paired Student’s t test (*, p < 0.01). C, relative activities of wild type and mutant cyclin D1 luciferase reporters (shown in A) transiently co-transfected into CV1 cells with β-catenin S37A and either empty or GR-containing expression vector (50 ng each). Cells were treated with vehicle (−H) or 100 nM CORT (+H). Relative luminescence units (RLU) were normalized with respect to the reporter activity in the absence of GR. Shown are the averages and S.D. values of three or more independent experiments performed in triplicate. *p = 0.0025. D, activity of the TOPFLASH luciferase reporter transiently co-transfected into CV1 cells with β-catenin S37A and either an empty expression vector (−GR) or a vector encoding wild type or mutant GR (GR; 50 ng each). The GR mutants used in this experiment were ΔAF1 (horizontal stripes), ΔAF1/ΔTRE (narrow diagonal stripes), and ΔBD/LBD (wide diagonal stripes). Cells were treated with 10 μM CORT. Relative luminescence units were derived as described in C. The significance of the difference in the repression of TOPFLASH by wild type GR and ΔAF1/ΔTRE mutants (p = 0.001) was analyzed using a paired Student’s t test. E, immunoblot of transiently expressed proteins from D (boxed samples, 100 μg of total protein/lane) using blotting for the mitochondrial protein CVd as a loading control. F, inhibition of TOP FLASH activation by GR in the presence of 10 μM CORT or RU486. Conditions were similar to those in D.

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silencing control siRNA did not reduce the transcript levels of any of these genes (Fig. 2B). The observed changes in β-catenin and cyclin D1 transcript levels corresponded to changes in β-catenin and cyclin D1 protein levels (Fig. 2C). These results demonstrated that in U2OS/GR cells, expression of cyclin D1, but not of c-myc and c-jun, is controlled by Tcf-β-catenin.

GCs Change the Recruitment or Accessibility of β-Catenin at the Cyclin D1 Promoter in U2OS/GR Cells—In addition to five Tcf binding sites, the regulatory region of cyclin D1 contains binding sites for AP-1, EtsA, -B, and -C, and CREB (36) (Fig. 3A). In principle, each of these elements could be involved in the inhibition of cyclin D1 by GCs. To determine whether GCs inhibit cyclin D1 via Tcf-β-catenin, we monitored whether DEX alters the interaction of β-catenin with the cyclin D1 promoter using chromatin immunoprecipitation (ChIP) assays.
Incubation of cross-linked protein-DNA complexes with a β-catenin antibody efficiently precipitated a cyclin D1 promoter fragment that contains the four more proximal Tcf binding sites of the cyclin D1 promoter (Fig. 3, A and B). An hsp70 promoter fragment that does not contain Tcf binding sites co-immunoprecipitated with β-catenin with 10-fold lower efficiency than did the cyclin D1 promoter fragment, which demonstrates that in these ChIP experiments, immunoprecipitation of DNA sequences is dependent on β-catenin (Fig. 3B). A 2-h exposure of U2OS/GR cells to DEX reduced the amount of cyclin D1 promoter sequences that co-immunoprecipitated with β-catenin by 45% without significantly affecting the precipitation of the hsp70 promoter fragment (p = 0.01) (Fig. 3B). These results demonstrate that DEX reduces either the presence or the accessibility of β-catenin at the cyclin D1 promoter.

GR Represses Cyclin D1 via Tcf-β-Catenin—The observed DEX-dependent effects on β-catenin at the cyclin D1 promoter suggest that GR might compete either with Tcf for binding to β-catenin or with β-catenin for binding to Tcf, as has been shown in case of AR (19). Alternatively, GR might interact with other transcription factors at the cyclin D1 promoter and reduce the accessibility of β-catenin indirectly by recruiting chromatin remodeling factors and other coregulators. To determine whether repression of cyclin D1 by GCs depends on Tcf-β-catenin, we analyzed the ability of GR to inhibit the expression of mutant cyclin D1 promoters that lack individual response elements. As shown in Fig. 3A and C, we found that deletion of individual AP1, Ets, and CREB binding sites in the cyclin D1 promoter had no effect on GR-mediated repression; only the deletion of the −75 Tcf binding site very slightly, but significantly (p < 0.0025), reduced the inhibitory effect of GR by about 20%. Notably, although GR is known to transrepress AP1, the GR-mediated repression of the cyclin D1 promoter is not dependent on the AP1 binding site.

In addition to the AP1, Ets, and CREB binding sites, the cyclin D1 ΔTcf promoter mutant still contains four Tcf binding sites, which is probably the reason for the relatively small effect the deletion of the −75 Tcf binding site had on GR-mediated repression. Therefore, next, we used the Tcf-β-catenin-regulated luciferase reporter TOPFLASH to monitor the effects of GR on Tcf binding sites directly. As shown in Fig. 3D, GR strongly inhibited the activity of the TOPFLASH reporter, which confirms the ability of GR to repress the activity of Tcf-β-catenin. These luciferase reporter assays were performed in transiently transfected monkey CV1 kidney cells using the β-catenin S37A mutant, which cannot be phosphorylated by GSK-3β and hence evades destruction by the proteasome (45). As expected, hormone treatment did not alter the expression of β-catenin (Fig. 3E), which confirms our previous conclusion that repression of Tcf-β-catenin by GR is not caused by the down-regulation of β-catenin.

Repression of Tcf-β-Catenin Is Independent of GR AF1 but Responds to Changes in AF2—To map the GR surfaces responsible for the repression of Tcf-β-catenin, we investigated the ability of GR mutants to inhibit the activity of the TOPFLASH reporter. Most GR transcriptional coregulators interact with one or both GR activation surfaces, the AF1 (activation function 1) in the N-terminal domain of GR and the AF2 in the GR LBD. Whereas deletion of AF1 by itself did not affect the repression of Tcf-β-catenin activity by GR, deletion of the entire N-terminal domain or a mutation (E773R) in AF2 in addition to the AF1 deletion reduced the ability of GR to repress Tcf-β-catenin by 20% (p = 0.02) or 50% (p = 0.001), respectively (Fig. 3D). As shown in Fig. 3E, all GR deletion constructs are expressed at comparable levels. The GR residue Glu773 is located in α-helix 12 of the GR LBD. This helix is a ligand-dependent switch, which in the presence of an agonist adopts a conformation that enables Glu773 to stabilize the interaction of coregulators with AF2 (46). In the presence of an antagonist (RU486), α-helix 12 blocks the interaction of coregulators with the AF2 (46). As shown in Fig. 3F, RU486 impairs the GR-dependent inhibition of TCF-β-catenin to a similar extent as the E773R mutation. These results demonstrate that repression of Tcf-β-catenin by GR is independent of AF1 but appears to involve the ability of AF2 to interact with coregulators.

GR Interacts with β-Catenin in Vitro—Results by others have demonstrated that ER and AR interact with β-catenin (13–19, 21). Using GST pull-down experiments, we found that full-length GR also interacted robustly with β-catenin, both in the absence and in the presence of CORT or RU486 (Fig. 4A). Mapping studies revealed that, contrary to AR, GR interacts with β-catenin primarily through the DBD and not the LBD (Fig. 4B). Deletion of AF1 slightly increased binding of GR to β-catenin, whereas the AF2 mutation E773R did not affect this interaction (Fig. 4B). Binding experiments mapping the GR binding sites on β-catenin showed that the β-catenin N-terminal domain and the first five repeats of the armadillo domain (aa 1–356) are sufficient for GR binding (Fig. 4C), whereas a similar β-catenin fragment (aa 1–350) is not able to interact with AR (15).

GR and AR Repress Tcf-β-Catenin by Different Mechanisms—It has been shown that AR represses Tcf-β-catenin by competing with Tcf for binding to β-catenin (19). Since the β-catenin fragment that binds GR also contains part of the Tcf binding site (47), it is possible that GR represses Tcf-β-catenin using a similar mechanism. In this case, we would expect that ligands and mutations within GR have similar effects on the ability of GR to bind β-catenin and to inhibit the activity of Tcf-β-catenin. However, whereas RU486 and the AF2 mutation E773R did not affect the interaction of GR with β-catenin (Fig. 4B), they did reduce the GR-dependent inhibition of Tcf-β-catenin activity by 40–60% (Fig. 3, D and F). Therefore, unless in vivo binding of GR and β-catenin is modified, simple competition of GR and Tcf for binding to β-catenin is not sufficient to explain the inhibitory effect of GR on Tcf-β-catenin activity. These results suggest that AR and GR might use different mechanisms to interact with β-catenin and to repress the activity of Tcf-β-catenin.

Down-regulation of β-Catenin and Cyclin D1 in U2OS/GR Cells Does Not Induce Cell Cycle Arrest—D-type cyclins and c-Myc are often down-regulated in GC-arrested cells; however, their relative contributions to GC-induced cell cycle arrest vary in a cell type-specific manner (31, 48). The ability of β-catenin to regulate the expression of cyclin D1, but not c-myc and c-jun, enabled us to assess the contribution of β-catenin and cyclin D1 to the GC-induced arrest of U2OS/GR cells. To this end, we transfected U2OS/GR cells with either control or β-catenin siRNA and monitored the effects of hormone on the expression of β-catenin, cyclin D1, and c-myc and on the ability of these cells to proliferate. To avoid transfection-induced variability, the different treatment groups were generated by splitting a pool of siRNA-transfected cells into aliquots that were treated with hormone or vehicle for 6 h to 4 days.

As shown in Fig. 5, A and B, the presence of β-catenin siRNA lowered β-catenin RNA and protein levels by 75%. This siRNA-mediated effect was partially relieved over time, but even after 2 days of hormone treatment, β-catenin RNA and protein levels were still reduced by 60%. Whereas the treatment of U2OS/GR cells with hormone did not change β-catenin transcript levels significantly, DEX treatments for 2 days reduced β-catenin protein levels in all samples by ~50%. This result indicates that prolonged incubation in DEX induces a mechanism that controls the protein level of β-catenin.
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The effects of the β-catenin siRNA on cyclin D1 expression were similar to those described for the expression of β-catenin. However, contrary to β-catenin, DEX reduced cyclin D1 RNA and protein levels (Fig. 5, A and B). Samples that were treated with both β-catenin siRNA and DEX had the lowest amount of cyclin D1 RNA and protein, suggesting that the down-regulation of cyclin D1 by either β-catenin siRNA or DEX treatment alone is only partial.

In contrast to β-catenin and cyclin D1, c-myc transcript levels were not changed by the β-catenin siRNA (Fig. 5A). A 6-h treatment of U2OS/GR cells with DEX strongly down-regulated c-myc RNA levels. However, after incubation with DEX for 2 days, c-myc RNA levels were largely restored, indicating that either the expression or the RNA stability of c-myc is dynamically regulated.

In the absence of hormone, the β-catenin siRNA-induced reduction in β-catenin and cyclin D1 levels had no detectable effect on the ability of U2OS/GR cells to proliferate, whereas all hormone-treated cells were growth-arrested (Fig. 5C). To increase the resolution of these experiments, we also analyzed the cell cycle distribution of these cells after 2 and 4 days of hormone treatment. As shown in Table 1, treatment of U2OS/GR cells with DEX for 2 days effectively blocked progression through the G₁ phase. In contrast, the presence of the β-catenin siRNA did not alter the cell cycle distribution of these cells. These results argue against a pivotal role of β-catenin and cyclin D1 for the proliferation of U2OS/GR cells.

**DISCUSSION**

In contrast to steroid hormones that are usually distributed by blood and act on many tissues simultaneously, Wnts act locally. Both types of signals control the ability of cells to proliferate, differentiate, or survive, suggesting that cells must possess mechanisms to integrate these signals into a coherent response. Several mechanisms have been suggested by which steroid hormone and Wnt signaling pathways might cooperate in regulating the proliferation of a variety of untransformed and transformed cells (7). In contrast to ER and AR, which have been shown to interact with β-catenin, thus far the proposed connections between GR and Wnt pathways have been indirect and appear to rely on other signal transduction cascades, including those of Ras, transforming growth factor-α, and phosphatidylinositol 3-kinase (22, 24, 25). In this study, we show that GR interacts with β-catenin and represses the activity of TCF-β-catenin, suggesting that GC and Wnt signaling are linked directly at the level of transcriptional regulatory complexes. Due to the high structural conservation of NRs, proteins that interact
with a particular NR often also interact with other members of the NR family. Although it had been originally suggested that β-catenin specifically interacts with AR (13), this conclusion has required revision because of the finding that ER can physically and functionally interact with β-catenin (21). In this study, we present evidence that GR also interacts with β-catenin.

Like that of AR and ER, the interaction of GR with β-catenin does not require hormone but responds to the presence of hormone. Whereas the interaction of AR and β-catenin depends on the AR LBD and the β-catenin armadillo motifs 5 and 6 (15, 16), binding of GR to β-catenin appears to require the GR DBD and an N-terminal β-catenin fragment (aa 1–356). A similar β-catenin fragment (aa 1–350) fails to bind AR (15). At present, little is known about the surfaces that mediate the interaction between ER and β-catenin, other than that the ER LBD is not necessary for β-catenin binding (21). The observed differences in the binding of AR, ER, and GR to β-catenin are likely to be at least partially responsible for the different functional consequences of these interactions on steroid hormone and Wnt signaling. For example, hormone-bound AR, but not ER or GR, enables the nuclear transport of β-catenin, which disrupts the control of the GSK-3β over β-catenin (13, 14). Moreover, β-catenin acts as an AR coactivator by facilitating the recruitment of coactivators to the AR LBD (16). There is no evidence thus far that β-catenin augments the transcriptional activity of GR.3

AR and some class II nuclear receptors, such as the thyroid hormone receptor, have been shown to inhibit the activity of Tcf-β-catenin (13–19, 49). Several mechanisms have been proposed to explain the inhibition of Tcf-β-catenin activity by NRs, including competition of NRs and Tcf for binding to β-catenin (19), competition of NRs and β-catenin for binding to Tcf (50), or competition of NRs and Tcf for binding to common chromatin remodeling factors, such as p300/CREB-binding protein, SWI/SNF, and RSC (12, 51). Each of these mechanisms would be compatible with the GC-dependent changes in the interaction of β-catenin with the cyclin D1 promoter observed in our ChIP assays. Although the results of our interaction studies alone are consistent with the potential competition of GR and Tcf for binding to β-catenin, the differences in β-catenin binding versus Tcf-β-catenin inhibition by the RU486-bound GR or by the GR AF2 mutant E773R argue against a simple competition mechanism.

Our observation that inhibition of Tcf-β-catenin by GR requires AF2 implies that this activity involves the interaction of GR with other coregulators. Based on these results, we propose a model whereby GR interacts with Tcf-β-catenin in a hormone-independent manner but represses the activity of Tcf-β-catenin in the presence of hormone by recruiting negatively acting coregulators through its AF2. Cell-dependent changes in the expression or activation of these coregulators could explain the observed differences in the response of cyclin D1 to DEX in U2OS/GR and A549 cells.

Testing this model would require determining whether GR interacts with TCF-β-catenin at the cyclin D1 promoter. Since this promoter contains five closely spaced TCF-β-catenin binding sites as well as an AP1 enhancer, which might recruit GR without contributing to GR-mediated repression, the interpretation of such binding experiments will be difficult. DEX treatment of U2OS/GR cells resulted in a 45% reduction of β-catenin-cyclin D1 promoter complexes, suggesting that not all TCF-β-catenin complexes at the cyclin D1 promoter are inhibited by GR. Clearly, further studies are necessary to determine the precise mechanism by which GR represses the activity of TCF-β-catenin. It has been suggested that GCS impair the activity of Tcf-β-catenin by up-regulating the expression of the histone deacetylase HDAC1 (24). However, this mechanism is unlikely, because DEX inhibited cyclin D1 expression in the presence of the protein synthesis inhibitor cycloheximide. Moreover, based on the microarray studies, DEX does not up-regulate HDAC1 expression in U2OS/GR or A549 cells.

Despite extensive research effort, there is no consensus on the mechanisms that enable GCs to cause cell cycle arrest. Evidence has been gathered for several mechanisms that appear to operate in a cell type-specific manner. These mechanisms include 1) The GR-dependent activation of cyclin-dependent protein kinase inhibitors; 2) the GR-dependent transcriptional repression of mitogenic factors (e.g. cyclin-dependent kinases, type D and type E cyclins, and E2Fs); and 3) the GC-dependent activation of GSK-3β, resulting in the proteolytic degradation of c-Myc and cyclin D1. It has been shown that in U2OS/GR cells, GCS down-regulate D-type cyclins and the cyclin-dependent kinases 4 and 6, leading to hypophosphorylation of the Rb protein, inactivation of the transcription factor E2F-1, and reduced expression of the E2F-1 target c-myc (27). Here we show that although c-myc transcript levels are strongly reduced by exposure of U2OS/GR cells to DEX for 6 h, they recover upon prolonged exposure to DEX (Fig. 5A). This result argues against an essential role of transcriptional repression of c-myc in the GC-mediated cell cycle arrest of U2OS/GR cells.

C-Myc, β-catenin, and cyclin D1 are known substrates of GSK-3β, which marks these proteins for proteolytic degradation (11, 25, 52). We show that hormone treatment of U2OS/GR cells for 2 days reduced β-catenin protein but not RNA levels (Fig. 5). This result is consistent with a hormone-dependent increase in GSK-3β activity, as seen in differentiating osteoblasts (25). In contrast, in U2OS/GR cells, regulation of cyclin D1 expression by DEX occurs at least partially at the RNA level. In our experiments, a 60% reduction in the protein levels of β-catenin

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3. S. Takayama, unpublished data.

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**TABLE 1**

Hormone-induced changes in cell cycle distribution of untransfected (U), control siRNA-transfected (C), and β-catenin siRNA-transfected (β) U2OS/GR cells

| Treatment | Group | 2-Day treatments | 4-Day treatments |
|-----------|-------|-----------------|-----------------|
|           |       | G0/G1 | S          | G0/G1 | S          | G0/G1 | S          |
| No treatment | U    | 46.8  | 36.1      | 17.1  | 46.4  | 36.8      | 16.8  |
|           | C    | 46.7  | 34.5      | 18.9  | 43.4  | 38.5      | 18.1  |
| −H        | β    | 42.3  | 37.7      | 20.0  | 39.7  | 37.4      | 23.0  |
| +H        | U    | 46.1  | 35.9      | 18.1  | 43.1  | 37.8      | 19.2  |
|           | C    | 46.8  | 33.8      | 19.4  | 45.2  | 37.1      | 17.7  |
|           | β    | 45.8  | 35.1      | 20.0  | 41.8  | 35.6      | 22.6  |

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**TABLE 2**

Percentage fluorescence at cell cycle phase

| Treatment | Group | 2-Day treatments | 4-Day treatments |
|-----------|-------|-----------------|-----------------|
|           |       | G0/G1 | S          | G0/G1 | S          | G0/G1 | S          |
| No treatment | U    | 46.8  | 36.1      | 17.1  | 46.4  | 36.8      | 16.8  |
|           | C    | 46.7  | 34.5      | 18.9  | 43.4  | 38.5      | 18.1  |
| −H        | β    | 42.3  | 37.7      | 20.0  | 39.7  | 37.4      | 23.0  |
| +H        | U    | 46.1  | 35.9      | 18.1  | 43.1  | 37.8      | 19.2  |
|           | C    | 46.8  | 33.8      | 19.4  | 45.2  | 37.1      | 17.7  |
|           | β    | 45.8  | 35.1      | 20.0  | 41.8  | 35.6      | 22.6  |
and cyclin D1 had no effect on cell proliferation (Fig. 5, B and C, Table 1). Since cells are usually very responsive to changes in the expression of these regulators, these results suggest that in U2OS/GR cells, β-catenin and cyclin D1 are not essential for G1 progression.

In this study, we show that GR represses cyclin D1 by targeting Tcf-β-catenin and provide evidence for a direct link between GC and Wnt signaling pathways. Based on the importance of Wnt for the proliferation of many cancers and osteoblasts, this mechanism could be of considerable interest for the treatment of cancer and the prevention of GC-induced osteoporosis.

Acknowledgments—We acknowledge Dr. M. K. Derynck (University of California, San Francisco) and D. Nonaka (University of California, San Francisco) for the original microarray data collection, Drs. Y. Chinenov (Hospital of Special Surgery) and C. Kentros (University of Oregon) for assistance with real time PCR, and Dr. C. Doe (University of Oregon) as well as Jolene Bradford and Dr. Bill Godfrey (Molecular Probes) for help with FACScan flow cytometry. We thank Drs. P. von Hippel (University of Washington), K. Yamamoto (University of California, San Francisco), and members of the Darimont laboratory for critical comments on the manuscript.

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